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OPTIMIZATION OF ESTROGEN ANALYSES AND QUANTIFICATION OF ENVIRONMENTAL SAMPLES FROM PENNSYLVANIA WATER SYSTEMS

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by

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ABSTRACT

The increase in the presence of pharmaceuticals and endocrine disrupting hormones (EDCs) in the environment has had a direct impact on aquatic organisms in the environment. Estrogen hormones, one class of EDCs, have been quantified in natural soil and water systems at concentrations as low as parts per trillion (ng L^{-1}). The presence of three estrogen compounds in particular, estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2), have been linked to human and animal waste disposal methods including land application of manure, wastewater irrigation, leaking septic systems, and direct stream discharge of wastewater effluent. To better understand estrogen behavior in the environment, numerous laboratory studies have focused on estrogen interactions with solid and solution matrices.

The goals of this dissertation were to (1) determine an optimal estrogen extraction solvent that yielded the largest E1, E2, and EE2 recovery percentages, (2) optimize separation of ten estrogen and estrogenic compounds using different stationary and mobile phase combinations and high performance liquid chromatography (HPLC), (3) characterize E1, E2, and EE2 sorption in soils varying in organic carbon content and in sorption systems where pure water was used to equilibrate soils versus effluent, and (4) evaluate the possible presence of E1, E2, and EE2 in two different geographic regions within Pennsylvania: the Sinnemahoning Creek Watershed and the Penn State Living Filter Wastewater Irrigation Site

For the first objective, four organic solvents and three solid phase extraction (SPE) loading ratios were evaluated in order to determine an optimal method to extract estrogens from an agricultural soil (1.09 %TOC). Solvents used to extract the soil included methanol (MeOH), dichloromethane (DCM), ethyl acetate (EA) and 50:50 MeOH:DCM. The three SPE loading ratios tested were 0:100, 5:95, and 10:90 MeOH:water. This study also tested different soil

sterilization methods to see if sterilization had any effect on estrogen recoveries from soil: sodium azide (NaN_3) at varying concentrations (100, 250 and 350 mg L^{-1}) and autoclaving. Extraction results indicated that either a MeOH extraction combined with an SPE procedure that uses a 10:90 MeOH:water loading ratio or a DCM extraction would be the optimal method. These methods yielded extraction recoveries ranging from 25.76 to 39.54%. Results from the microbial inhibition study indicated that out of the two inhibition methods tested, autoclaving the soil is the optimal soil sterilization method; autoclaving yielded the highest estrogen recoveries.

For the second objective, standard solutions containing ten estrogenic compounds were separated using six different stationary phase and mobile phase combinations. Three stationary phases were evaluated, C_{18}, biphenyl, and RP-Amide, and two mobile phase organic modifiers were evaluated, ACN and MeOH. Results indicated that the combination of C_{18} and ACN provided the greatest separation. When C_{18} and ACN were used to analyze the combined standard there were no issues associated with peak co-elution.

For the third objective, results indicated that sorption varied between E1, E2, and EE2 and between the three soils studied. Partition coefficient values, K_d and K_f, increased with an increase in soil organic carbon concentration and vice versa, and for the most part, the organic carbon normalized K_{oc} values also indicated that partitioning to organic carbon in the soil was the key sorption mechanism. The rank in sorption from highest to lowest for all three soils was E1 > E2 > EE2. When wastewater was used to equilibrate the cropped B soil, estrogen sorption did decrease as was expected. Extraction recovery of each estrogen decreased when the soil organic carbon content increased.

Finally, E1, E2, and EE2 baseline data was established for the Penn State Living Filter wastewater irrigation site, and the Sinnemahoning Creek Watershed. At the Living Filter, all

three compounds were detected in groundwater well samples. However, all three estrogens were not quantified. E1 was quantified in all fourteen wells, E2 was only quantified in one well on site, and in all fourteen wells, concentrations of EE2 were either not detected (ND) or were quantified in trace amounts (T). In the Sinnemahoning Creek Watershed, both E1 and E2 were detected in the water samples. However, while E1 was quantified in all but one location (ranging from 9.86 to 37.11 ng L^{-1}); E2 was quantified in only two of the twenty locations (23.02 and 32.47 ng L^{-1}). The synthetic estrogen, EE2, was below instrument detection limits (ND) in all samples.

Findings from this dissertation can be used to improve upon the current methodology used to extract and analyze environmental samples for estrogen hormones, increase our understanding of how estrogens interact with soil and water environments, and better predict transport of estrogen compounds in the environment. Overall, this information can be used to ensure that the sustainability of soil and water environments is maintained, especially in areas where waste sources are being used to fertilize and irrigate crops and recharge groundwater supplies.

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Chapter 1

Introduction

Humans and animals excrete synthetic and naturally produced estrogenic hormones (Johnson and Williams, 2004; Hanselman et al., 2003; Erb et al., 1977), and current waste disposal methods are unable to prevent low levels of these hormones from entering the environment (Baronti et al., 2000). Estrogen presence in the environment has been linked to the following waste management practices: direct stream discharge, effluent irrigation, septic systems that are leaking, combined sewer overflow events, land application of biosolids and manure spreading (Langdon et al., 2014; Woodward et al., 2014; Phillips et al., 2012; Shappell et al., 2010; Wilcox et al., 2009; Barel-Cohen et al., 2006). Literature studies that collected soil and water samples from areas adjacent to a known waste source quantified estrogens in the ng L^{-1} concentration range (Arnon et al., 2008; Finlay-Moore et al., 2000). From a regulatory standpoint these low levels appear insignificant. However, even at these low levels, estrogens are a concern. Environmental exposure to estrogens at low levels can alter sexual differentiation and reproductive functions in vertebrates, and possibly humans (Dickerson and Gore, 2007; Kidd et al., 2007; Jobling et al., 1998; Purdom et al., 1994). Since estrogen hormones have a clear effect on environmental organisms, understanding their behavior in the environment is critical.

Unfortunately, the process of quantifying estrogen concentrations in soil and water samples is challenging. The majority of this dissertation focused on three estrogen hormones: two naturally produced estrogens, estrone (E1) and 17β-estradiol (E2), and the synthetic estrogen 17α-ethynylestradiol (EE2). These three estrogens are neutral, hydrophobic compounds with low water solubilities (Table 1-1). They adsorb to soils, especially those with high organic carbon contents. This high adsorption affinity makes it difficult to extract them from soils. The methods

used to extract estrogens from soil vary among literature sources, and even with efficient methods, recovery percentages can be low (Arnon et al., 2008; Hutchins et al., 2007; USEPA, 2007; Lee et al., 2003; Colucci et al., 2001). Therefore, Chapter 2 of this dissertation addressed extraction challenges and optimized three extraction parameters: the organic solvent used to extract soil, the methanol:water loading ratio used during a solid phase extraction (SPE) cleanup procedure, and the method used to inhibit microbial activity in the soil.

Even after environment samples are extracted, analysis of these samples poses an additional challenge. Environmental concentrations are low (ng L^{-1}), and quantification at these levels requires a sensitive analysis technique. To quantify lab and environmental samples, a majority of studies used high-performance liquid chromatography (HPLC) coupled with one of the following instruments: a single mass spectrometer (MS), tandem mass spectrometers (MS-MS), or an ultraviolet visible (UV) detector (Tomŝíková et al.; 2012; Labadie and Hill, 2007; Beck et al., 2005; Hu et al., 2005; Beausse, 2004; Benijts et al., 2004). The goal of Chapter 3 was optimize the separation of ten estrogen and estrogenic compounds using different stationary and mobile phase combinations and high performance liquid chromatography (HPLC): five natural estrogens: 17β-estradiol, 17α-estradiol, estrone, estriol, and progesterone; three synthetic estrogens: 17α-ethynylestradiol, mestranol, and diethylstilbestrol; and two phtyoestrogens: biochanin A and genistein. Three stationary bonded phases were tested with individual standards, a combined standard, and a combined standard that also included soil matrix components: a traditional C_{18} bonded phase, a biphenyl bonded phase, and an RP-Amide bonded phase. All three phases were tested once using an acetonitrile (ACN) and water mobile phase and then retested using a methanol (MeOH) and water mobile phase.

In order to model environmental data and predict the fate and transport of estrogens in the environment, certain parameters must be defined. One such parameter is the sorption partition coefficient (K_d). The goal of Chapter 4 was to determine estrogen specific K_d values using batch sorption studies and the optimal extraction methodology from Chapter 2. Batch sorption studies focused on the hormones E1, E2 and EE2 are common in the literature (Hildebrand et al., 2006; Ying and Kookana, 2005; Das et al., 2004; Lee et al., 2003). Yet, the design of this sorption study differs from what has been done and provides new information to this field. This study was conducted using a multi-sorbate system, all equilibrating solutions contained E1, E2 and EE2. For two different scenarios, K_d values were determined: when equilibration solutions were made in pure water and when equilibration solutions were made in filtered wastewater. Another factor that sets this research apart from a majority of the literature studies is that this study extracted the soil and analyzed the sorbed phase instead of using a difference method.

The final goal of this dissertation was to collect and analyze environmental water samples from two locations in Pennsylvania: the Living Filter Wastewater Irrigation Site and the Sinnemahoning Creek Watershed. A number of literature studies have been conducted related to quantification of hormones in ground and surface water environments and were used to guide the two projects conducted in Chapter 5 (Bartelt-Hunt et al., 2011; Karnjanapiboonwong et al., 2011; Loos et al., 2010; Barel-Cohen et al., 2006; Shappell, 2006; Beck et al., 2005; Kolpin et al, 2002) The initial reconnaissance data in Chapter 5 will help establish general E1, E2, and EE2 baseline data for waters within the Living Filter and the Sinnemahoning Creek Watershed. Beyond research, the Sinnemahoning Creek Watershed study contained an outreach effort. Working with county extension educators and the Cameron County Conservation District, this study engaged local high school students in the sampling and lab processes.

Chapter 6 summarizes the overall conclusions from this dissertation and provides recommendations for future environmental estrogen research.

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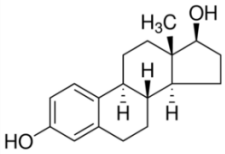
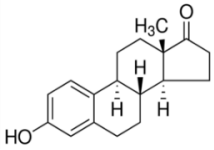
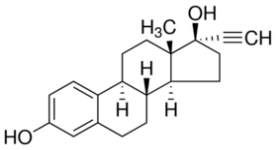
Table 1-1. Estrogen chemical properties.

Compound	Structure	Molecular Weight ($g\ mol^{-1}$)	Water Solubility ($mg\ L^{-1}$)	log K_{ow}	pka
17β-estradiol (E2)		272.4	13[a]	3.94[a]	10.23[b]
Estrone (E1)		270.4	13[a]	3.43[a]	10.4[c]
17α-ethynylestradiol (EE2)		296.4	4.8[a]	4.15[a]	10.21[b]

Images and molecular weights are from *Sigma-Aldrich*
[a] Cited by Lai et al. (2000)
[b] Perrin et al. (1977)
[c] Nghiem and Schafer (2002)

Chapter 2

Improvements to Estrogen Extraction Methods in Soils

Abstract

Endocrine disrupting compounds, including the estrogen hormones estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) have been quantified in natural soil and water systems. Extracting estrogen hormones from soil matrices is challenging, and extraction parameters vary between studies which exist in the literature. This study tested four organic solvents and three solid phase extraction (SPE) loading ratios in order to determine an optimal method to extract estrogens from an agricultural soil (1.09 %TOC). Solvents used to extract the soil included methanol (MeOH), dichloromethane (DCM), ethyl acetate (EA) and 50:50 MeOH:DCM. These four solvents cover a range of polarity and hydrophobicity. Therefore, it was expected that they would yield a range in estrogen extraction recoveries from soil. The three SPE loading ratios tested were 0:100, 5:95, and 10:90 MeOH:water. This study also considered different soil sterilization methods to see if sterilization had any effect on estrogen recoveries from soil: sodium azide (NaN_3) at varying concentrations (100, 250 and 350 mg L^{-1}) and autoclaving. Extraction results indicated that in single and multi-sorbate systems, depending on the estrogen hormone, either a MeOH extraction combined with an SPE procedure that uses a 10:90 MeOH:water loading ratio or a DCM extraction would be the optimal method. These methods yielded extraction recoveries ranging from 25.76 to 39.54%. Results from the microbial inhibition study indicated that autoclaving the soil is the optimal soil sterilization method, as autoclaving yielded the highest estrogen recoveries.

Introduction

Emerging contaminants, pharmaceuticals and endocrine disrupting compounds (EDCs), are present in the environment. A key study by Kolpin et al. (2002) quantified emerging contaminants in 139 surface streams across the United States. Estrogen hormones are one class of EDCs that were quantified by Kolpin et al. (2002) in a number of U.S. streams and they are the focus of this work. A number of studies have quantified estrogen hormones in soil systems and have linked them to the following processes and practices: land application of manure (Shappell et al., 2010), land application of biosolids (Langdon et al., 2014), effluent irrigation of crops (Woodward et al., 2014) and leaching from agricultural storage lagoons (Arnon et al., 2008). Research has shown that an increase in environmental estrogen exposure can affect endocrine and reproductive processes in fish and other vertebrates (Purdom et al., 1994; Dickerson and Gore, 2007).

From the literature, log K_{ow} values for estrogens range from 3.10-3.94 (Lai et al., 2000). Therefore, they adsorb to soils and sediments in the environment, especially those with high organic carbon contents, with log K_{oc} values ranging from 2.99 to 3.34 (Lee et al., 2003). This high adsorption affinity makes it difficult to extract estrogen hormones from solid environmental matrices. Extraction parameters that can be adjusted in order to improve method recoveries include the number of extractions, soil-to-solvent ratio, extraction time, organic solvent and sterilization method. Of these parameters, both the organic solvent used to extract and sterilization method vary greatly within the literature.

Solvents used to extract have included ethyl acetate (EA) (Zhang et al., 2015; Arnon et al., 2008), dichloromethane (DCM) (Sarmah et al., 2008; Lee et al., 2003), acetonitrile (ACN) (USEPA, 2007), acetone (Xuan et al., 2008), methanol (MeOH) (Woodward et al., 2014), and

50:50 MeOH:acetone (Hutchins et al., 2007). A number of studies extracted the solid phase multiple times and varied the organic solvent between extractions: i.e. EA then acetone (Jacobsen et al., 2005; Colucci et al., 2001) or MeOH then acetone (Langdon et al., 2014; Ternes et al., 2002).

Common sterilization methods used to inhibit microbial activity in the literature include sodium azide (NaN_3) (Hildebrand et al., 2006; Yu and Huang, 2005; Yu et al., 2004), mercuric chloride ($HgCl_2$) (Bera et al., 2011; Bonin and Simpson, 2007; Hildebrand et al., 2006), and autoclaving (Xuan et al., 2008; Hildebrand et al., 2006; Jacobsen et al., 2005; Colucci and Topp, 2001; Colucci et al., 2001). Microbial activity is the major degradation mechanism for estrogen hormones in soils (Xuan et al., 2008; Colucci and Topp, 2001; Colucci et al., 2001). It is essential that lab studies incorporate a method for limiting microbial activity in order to limit estrogen dissipation and conversion. If dissipation and conversion are not accounted for, quantified concentration values can be misleading.

One goal of this study was to optimize the extraction of two natural estrogens, 17β-estradiol (E2) and its metabolite estrone (E1), and one synthetic estrogen, 17α-ethynylestradiol (EE2), from an agricultural soil by testing the extractability of estrogens using DCM, MeOH, EA and a 50:50 MeOH:DCM mixture. Based on the chemistry of MeOH, when it is used to extract soil, a solid phase extraction (SPE) process is required for cleanup. Therefore, this study also examined three MeOH:water loading ratios used during an SPE procedure to determine if the loading ratio had any effect on the estrogen extraction recovery. A second goal of this study was to determine if different soil sterilization methods affect estrogen recoveries from soil: NaN_3 at varying concentrations (100, 250 and 350 mg L^{-1}) and autoclaving. The same experimental

conditions were used to test both sterilization methods, and the overall objective was to determine if one method was associated with higher estrogen extraction recoveries.

Materials and Methods

Soils

A Hagerstown silt loam soil, A horizon, sampled from The Russell E. Larson Agricultural Research Farm (located approximately 16 km southwest of University Park), was used. Standard soil analysis methods were used to characterize the soil for cation exchange capacity (CEC), pH, texture, percent total organic carbon (%TOC) and total nitrogen (%TN) (Klute, 1986; Sparks, 1996) (Table 2-1).

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma- Aldrich, MO: E1 (≥99%), E2 (≥98%), EE2 (≥98%), LC-MS grade MeOH, ACN and EA, HPLC grade methyl tert-butyl ether (MTBE), DCM, ammonium hydroxide (NH_4OH), NaN_3 (≥99.5%), and calcium chloride ($CaCl_2$ >99%). All water used was ultra-pure deionized (18MΩ), unless otherwise indicated.

Solvent Extraction

Three pure solvents and one solvent mixture were evaluated for extraction efficiency: EA, DCM, MeOH and 50:50 MeOH:DCM. A 10 mg L^{-1} working stock that included E1, E2 and EE2 was made in MeOH. To prepare the 10 µg L^{-1} equilibrating solution, 1 mL of the working stock was mixed with 0.735 g $CaCl_2$ and 0.025 g NaN_3 in 1 L of water (<0.1% MeOH). Each solvent study was conducted in triplicate. For each sample, 50 g of soil and 100 mL of equilibrating solution were added to a glass centrifuge bottle (1:2 soil:solution), and each bottle, covered in foil, was mixed for 24 hours on a rotary shaker. Following this, each bottle was centrifuged for 1 hour at 1500 rpm (366 RCF). The supernatant was decanted from each bottle

and an aliquot (30 mL) of the supernatant was filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and run through the SPE procedure described below. Weights were recorded before the equilibration solution was added and after it was decanted to determine the mass of equilibration solution remaining in the soil. After decanting, 100 mL of solvent extractant was added to the soil and shaken for 1 hour on a rotary shaker, then centrifuged for 1 hour at 1500 rpm. Thirty milliliters of supernatant was removed from each bottle and filtered. After filtration, the extracts were evaporated to dryness under a gentle stream of nitrogen gas. Samples were brought up to a 1 mL volume with 10:90 MeOH:water. One milliliter samples were analyzed using the chromatography conditions described below.

SPE Loading Ratios

In order to evaluate the importance of MeOH:water ratios when using an SPE process for cleanup and concentration, three SPE MeOH:water loading ratios, 0:100, 5:95 and 10:90, were considered. Pure MeOH was first used to extract the soil as described above, creating nine additional MeOH extraction samples. After the samples were evaporated to dryness, instead of being brought up to a 1mL volume with a 10:90 MeOH:water ratio for analysis, as is normally done, triplicates were brought up to a 30 mL volume in one of the three ratios described and were processed through the SPE procedure described below.

Microbial Inhibition Study

Data were analyzed from the solvent extraction portion of this study before the microbial inhibition methods were evaluated. Therefore, the organic solvent and SPE loading ratio results from the solvent extraction study were employed in this study: MeOH solvent and 10:90 SPE loading ratio.

Sodium Azide Effects

Two NaN_3 equilibrating solutions were prepared and tested. The first 10 µg L^{-1} equilibrating solution was prepared by mixing 1 mL of the 10 mg L^{-1} working stock with 0.292 g $CaCl_2$ and 0.100 g NaN_3 in 1 L of water (<0.1% MeOH). The second 10 µg L^{-1} equilibrating solution was prepared by mixing 1 mL of the 10 mg L^{-1} working stock with 1.022 g $CaCl_2$ and 0.350 g NaN_3 in 1 L of water (<0.1% MeOH). All work was conducted in triplicate. Each centrifuge bottle contained 50 g of the Hagerstown silt loam soil. In three of the six bottles, 100 mL of the 100 mg L^{-1} NaN_3 equilibrating solution was added, and in the other three bottles, 100 mL of 350 mg L^{-1} NaN_3 equilibrating solution was added (1:2 soil:solution). Each bottle was mixed for 24 hours on a rotary shaker. Following this, each bottle was centrifuged for 1 hour at 1500 rpm. The supernatant was decanted from each bottle and an aliquot (30 mL) of the supernatant was filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and run through the SPE procedure described below. Weights were recorded before the equilibration solution was added and after it was decanted to determine the mass of equilibration solution remaining in the soil. After decanting, one hundred 100 mL of MeOH was added to the soil and shaken for 1 hour on the rotary shaker, then centrifuged for 1 hour at 1500 rpm. Thirty milliliters of supernatant was removed from each bottle and filtered. After filtration, the extracts were evaporated to dryness under a gentle stream of nitrogen gas. Once evaporated, samples were brought to a 30 mL volume with a 10:90 MeOH:water ratio. These diluted samples were processed through the SPE procedure described below before analysis.

Autoclaving

For this study a single 10 µg L^{-1} equilibrating solution was prepared by mixing 1mL of the 10 mg L^{-1} working stock into 1 L of water (<0.1% MeOH). As with previous studies, all

work was done in triplicate. Each centrifuge bottle contained 50 g of autoclaved Hagerstown silt loam soil. The soil was autoclaved under a vacuum setting using the standard method outlined in Sparks (1996). One hundred milliliters of equilibrating solution was added to each centrifuge bottle (1:2 soil:solution), and each bottle was mixed for 24 hours on a rotary shaker. Following this, each bottle was centrifuged for 1 hour at 1500 rpm. The supernatant was decanted from each bottle and an aliquot (30 mL) of the supernatant was filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and run through the SPE procedure described below. Weights were recorded before the equilibration solution was added and after it was decanted to determine the mass of equilibration solution remaining in the soil. After decanting, 100 mL of MeOH was added to the soil and shaken for 1 hour on the rotary shaker, then centrifuged for 1 hour at 1500 rpm. Thirty milliliters of supernatant was removed from each bottle and filtered. After filtration, the extracts were evaporated to dryness under a gentle stream of nitrogen gas. Once evaporated, samples were brought to a 30 mL volume with a 10:90 MeOH:water ratio. These diluted samples were processed through the SPE procedure described below prior to analysis.

Solid Phase Extraction (SPE) Procedure

An SPE procedure was used to cleanup and concentrate each sample. The SPE method used was a modified version of the procedure provided in the Waters Application Notebook (Waters, 2008). An Oasis HLB Plus (Waters, MA) cartridge was pre-conditioned with 5 mL of MTBE, 5 mL of MeOH and 5 mL of water, followed by a loading phase of 30 mL of either the equilibration solution or the diluted extraction solution. The cartridge was washed with 5 mL of a 40:60 MeOH:water solution and 5 mL of water. Finally, the cartridge was eluted with 6 mL of a 90:10 MTBE:MeOH solution. The 6 mL of eluent was evaporated under a gentle stream of

nitrogen gas, and each evaporated vial was brought to a 1 mL volume with 10:90 MeOH:water. This 1 mL sample was analyzed using the chromatography conditions described below.

Chromatography Conditions

The 1 mL equilibration solution samples and extraction samples were analyzed for estrogens using an LC-MS-MS. The column was an XTerra MS C18 Column, 3.5 µm, 2.1 x 100 mm (Waters, MA). The Tandem MS used for quantification was a MicroMass Quattro micro API (Waters, MA). The mobile phase solvents were 0.6% NH_4OH in ACN (solvent A) and 0.6% NH_4OH in water (solvent B), and these two solvents were run in gradient mode. The initial mobile phase started with 10% solvent A and 90% solvent B, and increased linearly for five minutes to 90% solvent A and 10% solvent B. This was held for two minutes. From seven minutes to the end of the run (10 min), the gradient was dropped to 10% solvent A and 90% solvent B. The operating mode was ESI-. Flow rate was 0.25 mL min^{-1}, and the injection volume was 20 µl. External 6 to 8 point standard calibration curves were generated (1 to 1000 µg L^{-1}). Peak area outputs from samples were used against calibration curves to estimate sample concentrations. The measured limit of detection (LOD) was 0.48 µg L^{-1}, 2.16 µg L^{-1} and 1.78 µg L^{-1} for estrone, 17α-ethynylestradiol and 17β-estradiol respectively.

Statistical Analyses

All statistics were conducted using SAS 9.3 software (SAS Institute, NC). Before data were analyzed, they were tested for normality. In this study, all data were normal. A one-way analysis of variance (ANOVA) test was used to evaluate the differences in means between the seven independent groups (seven extractants). A *proc anova* model was used in SAS. If a difference in means was observed, a Tukey test was conducted using a significance level of $\alpha = 0.05$.

Results and Discussion

Solvent Extraction and SPE Loading Ratios

Recovery percentages and Tukey groupings for each solvent and SPE loading ratio are listed in Table 2-2. Even though the estrogen extraction recoveries are low, they are in agreement with literature reported values. Low recoveries could be the result of non-extractability and/or transformation (Colucci et al., 2001; Lee et al., 2003).

For E1, there was no statistically significant difference between recovery percentages using the first four solvents (Table 2-2). However, of the four solvents, MeOH had the highest recovery percentage, 26.12%. For E2, both DCM (25.76%) and MeOH (11.61%) had significantly higher recovery percentages than EA (3.31%) and MeOH:DCM (1.39%). For EE2, there was no significant difference between the four solvents, but of the four solvents, DCM yielded the highest recovery (20.82%). The recoveries represent the ability of each solvent to extract estrogens from the soil.

The three MeOH:water loading ratios show that for E1, both the 5:95 (37.47%) and 10:90 (39.54%) MeOH:water loading ratios yielded significantly higher recovery percentages than the 0:100 loading ratio (26.13%) (Table 2-2). For E2, there was no significant difference between the three loading ratios. Finally, for EE2, there was no significant difference between the three loading ratios, but out of the three ratios, the 10:90 ratio had a somewhat higher recovery percentage (27.22%). These results represent the ability of MeOH to extract the soil and the affect SPE loading ratios can have on estrogen recoveries.

Overall, the results indicated that for E1, a MeOH extractant combined with either a 5:95 or 10:90 SPE loading ratio yielded the highest recovery. There is no significant difference between these two loading ratios, but both are significantly different from the rest of the

treatments represented in Table 2-2. For E2, DCM yielded the highest recovery percent (25.76%). For EE2, there was no significant difference between the treatments. However, a MeOH extractant combined with a 10:90 SPE loading ratio did yield the highest recovery (27.22%).

Microbial Inhibition Methods

A second goal of this study was to determine if different soil sterilization methods affected E1, E2, and EE2 recoveries from soil: NaN_3 at varying concentrations (100, 250 and 350 mg L^{-1}) and autoclaving. For E1 and EE2, there was no statistically significant difference between the four sterilization methods (Table 2-3). In general, for E1, the soil sterilized with 100 mg L^{-1} of NaN_3 did yield the highest recovery (61.04%), and for EE2 the soil that was autoclaved yielded the highest recovery (46.15%). For E2, there was a statistically significant difference in extraction recovery between the soils sterilized with NaN_3 and the soils sterilized using an autoclave. The autoclaved soil yielded the highest recovery of E2, 59.89% (Table 2-3).

The results in Table 2-3 suggest that autoclaving soil may be the optimal microbial inhibition method. Statistically, autoclaved soil yielded the highest E2 recovery, and in general, it also yielded the highest EE2 recovery, and the second highest E1 recovery (55.55%). These results are not unexpected. Several studies have quantified estrogen degradation in autoclaved soils versus non-autoclaved soils, and results from these studies have shown that there is limited degradation of estrogens in autoclaved soils (Xuan et al., 2008; Hildebrand et al., 2006; Jacobsen et al. 2005; Colucci and Topp, 2001; Colucci et al., 2001). There are concerns that autoclaving soil could enhance E2 sorption, but Hildebrand et al. (2006) showed that the microbial inhibition level achieved by autoclaving far outweighs the slight E2 sorption enhancement. Hildebrand et al. (2006) also showed that conversion of E2 to E1 was inhibited only slightly by NaN_3. Finally,

work conducted by Wolf et al. (1989) determined that NaN_3 does not inhibit all microbial activity, but autoclaving the soil twice does. As a result of this, a portion of the E1 recovery seen in Table 2-3 could be the result of E2 conversion. Overall, results indicated that future laboratory studies, such as sorption studies, should be done using soil that is sterilized with an autoclave instead of soil that is sterilized with NaN_3. Field studies could be carried out using any of the three NaN_3 concentrations, each having an established recovery value, or using an additional inhibition method that has an established estrogen recovery value.

Summary and Conclusion

The two goals of this study were to determine the optimal solvent extraction and (when used) an SPE loading ratio for estrogen recovery from an agricultural soil, and to evaluate different soil sterilization methods to see if sterilization had any effect on estrogen recoveries from soil. Overall, extraction results indicated that in single and multi-sorbate systems, depending on the estrogen hormone, either a MeOH extraction combined with an SPE procedure that uses a 10:90 MeOH:water loading ratio or a DCM extraction would be the optimal method. The soil sterilization results, NaN_3 at varying concentrations and autoclaving, indicated that autoclaving the soil is the optimal soil sterilization method. Autoclaving yielded the highest estrogen recoveries.

Results from this study can be used to improve upon current soil extraction methodology. It is essential that we know how to extract these compounds from soils, that we have some measure for recovery percentages from soil matrices, and that we can account for microbial degradation. A sound extraction method is needed to quantify estrogen hormones in environmental samples and to ensure that estrogen hormones are not accumulating in the soil profile or transporting to ground water sources. This is especially critical for areas where drought

is forcing the use of wastewater to irrigate crops and in areas where biosolids and manures are being used to amend agricultural landscapes. Future studies could expand upon the soil extraction method described in this chapter by including different soils with different organic carbon contents.

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Table 2-1. Hagerstown silt loam soil characteristics, A horizon.

Texture	(%)
Sand	25.3
Silt	44.6
Clay	30.1
pH (1:1 H_2O)	5.6
TOC (%)	1.09
TN (%)	0.16
CEC (meq/100g)	24.5

Table 2-2. Average extraction recovery (%) for each estrogen hormone (±SD) and each extractant, N=3.

Extractant	SPE loading ratio MeOH:water	E1	E2	EE2
EA	-	21.68 (±5.54) [b]	3.31 (±0.72) [b]	12.29 (±3.61) [a ,b]
DCM	-	17.12 (±2.97) [b]	25.76 (±7.85) [a]	20.82 (±13.24) [a, b]
MeOH	-	26.12 (±3.47) [b]	11.61 (±11.86) [a, b]	10.80 (±2.36) [a, b]
MeOH:DCM	-	15.29 (±4.16) [b]	1.39 (±0.41) [b]	6.86 (±1.24) [b]
MeOH	0:100	26.13 (±5.80) [b]	3.66 (±2.24) [b]	16.04 (±4.27) [a, b]
MeOH	5:95	37.47 (±2.03) [a]	5.60 (±0.41) [b]	20.51 (±1.57) [a, b]
MeOH	10:90	39.54 (±2.54) [a]	5.64 (±0.55) [b]	27.22 (±5.79) [a]

[a,b] Tukey groupings after running a one-way ANOVA. Values in the same column followed by the same letter indicate that there is no statistically significant difference.

Table 2-3. Average extraction recovery (%) for each estrogen hormone (±SD) and each sterilization method, N=3.

Sterilization Method	E1	E2	EE2
NaN_3 (100 mg L^{-1})	61.04 (±14.8) [a]	7.39 (±2.2) [b]	29.58 (±6.9) [a]
NaN_3 (250 mg L^{-1})	39.54 (±2.54) [a]	5.64 (±0.55) [b]	27.22 (±5.79) [a]
NaN_3 (350 mg L^{-1})	49.64 (±3.85) [a]	7.21 (± 2.00) [b]	31.19 (± 0.84) [a]
Autoclave	55.55 (± 12.02) [a]	59.89 (± 9.53) [a]	46.15 (± 13.32) [a]

[a,b] Tukey groupings after running a one-way ANOVA. Values in the same column followed by the same letter indicate that there is no statistically significant difference.

Chapter 3

Optimizing the Stationary Phase and the Mobile Phase in Liquid-Chromatography in order to Separate Multi-Sorbate Estrogenic Solutions

Abstract

Estrogenic compounds have been detected in environmental samples including waters, soils and sediments. The environmental concentrations are typically low, parts per trillion (ppt), and in order to analyze environmental samples a sensitive analysis technique is required. High performance liquid chromatography (HPLC) coupled to a single mass spectrometer or tandem mass spectrometer is often used to analyze environmental samples. On the separation end, most studies use a C_{18} stationary phase (column) and acetonitrile (ACN) as the organic modifier in the mobile phase. The goal of this study was to develop a method for a target compound separation using a reference standard solution that contained 10 estrogenic compounds. The compound separation was evaluated using six different stationary phase and mobile phase combinations. Three stationary phases were tested, C_{18}, biphenyl, and RP-Amide. The bonding forces and interactions between estrogens and the three stationary phases vary. The C_{18} column will promote dispersive, van der Walls forces; the biphenyl column will promote dispersive, van der Walls forces and pi-pi(star) interactions; and the RP-Amide column will promote dispersive, van der Walls forces and possible hydrogen bonding with the amide group. Two mobile phase organic modifiers were tested, ACN and methanol (MeOH). Another goal of this study was to analyze a standard solution that contained all ten compounds and soil matrix in order to determine whether or not the presence of soil matrix had any effect on the estrogen separations. Results indicated that the combination of C_{18} and ACN provided the greatest separation. When the optimized separation using C_{18} and ACN was used to analyze the combined standard, co-

elution did not occur. The C_{18} and ACN combined standard matrix results indicated that there was no effect on separation when soil matrix compounds remained in the sample after solid phase extraction (SPE).

Introduction

Humans and animals excrete synthetic and naturally produced estrogen hormones (Johnson and Williams, 2004; Hanselman et al., 2003; Erb et al., 1977). Current waste disposal methods are unable to prevent low levels of estrogen hormones from entering the environment (Anderson et al., 2003; Baronti et al., 2000). The presence of estrogen hormones in the environment has been linked to direct stream discharge, effluent irrigation, leaky septic systems, combined sewer overflow events, land application of biosolids and manure spreading (Langdon et al., 2014; Woodward et al., 2014; Phillips et al., 2012; Shappell et al., 2010; Wilcox et al., 2009; Barel-Cohen et al., 2006). Detected environmental concentrations in soil and water samples are low (ng L^{-1}) (Arnon et al., 2008; Boyd et al., 2003; Kolpin et al., 2002; Finlay-Moore et al., 2000). However, even at these levels, exposure to environmental estrogenic compounds can alter sexual differentiation and reproductive functions in vertebrates (Dickerson and Gore, 2007; Kidd et al., 2007; Matthews et al., 2000; Purdom et al., 1994).

Quantification in the parts per trillion (ng L^{-1}) concentration range requires a sensitive analysis technique. The two separation techniques that were frequently cited in the literature for quantification of estrogenic compounds in environmental samples were gas chromatography (GC) (Tomŝíková et al., 2012; Beck et al., 2008; Liu et al., 2004; Quintana et al., 2004; Jeannot et al., 2002) and high-performance liquid chromatography (HPLC) (Tomŝíková et al., 2012; Labadie and Hill, 2007; Havlíková et al, 2006; Beck et al., 2005; Hu et al., 2005; Mitani et al., 2005; Benijts et al., 2004; Vanderford et al., 2003; López de Alda and Barceló, 2001). To increase instrument sensitivity, these two separation techniques were often coupled with either a single mass spectrometer (MS) or tandem mass spectrometers (MS-MS). Overall, HPLC is used more often than GC. While both methods achieve the same level of sensitivity, HPLC does not

require a derivatization step prior to analysis (Tomŝíková et al., 2012; Gomes et al., 2004; Kuster et al., 2004). However, there is at least one drawback to using HPLC coupled to mass spectrometry; matrix effects can lead to ion suppression, although there are a number of ways to counter ion suppression during sample preparation and analysis (Benijts et al., 2004).

The HPLC methods used to quantify estrogenic compounds in environmental samples vary within the literature. The stationary phase chemistry (column) and the mobile phase organic modifier were the two factors that varied the most between methods (Tomŝíková et al., 2012). The C_{18} column was the most common stationary phase used (Labadie and Hill, 2007; Havlíková et al, 2006; Beck et al., 2005; Hu et al., 2005; López de Alda and Barceló, 2001), followed by C_8 (Castiglioni et al., 2005; Mitani et al., 2005; Benijts et al., 2004), C_{12} (Vanderford et al., 2003) and phenyl (Hu et al., 2005). Acetonitrile (ACN) and methanol (MeOH) were the two mobile phase organic modifiers cited in most papers (Tomŝíková et al., 2012).

The main goal of this research study was to separate standard estrogenic solutions using different stationary and mobile phase combinations in order to identify an optimal combination for environmental estrogen analyses. Three stationary phases were tested: C_{18}, the most common phase reported in the literature, biphenyl, and RP-Amide. The latter two chemistries were chosen because they are orthogonal chemistries to C_{18}, and they deviate from the chemistries that have already been evaluated for estrogen separations in the literature. Each stationary phase was tested twice, once with ACN as the organic phase modifier and once with MeOH as the organic phase modifier (six total combinations). A multi-sorbate estrogenic solution was run on each combination, and it contained five natural estrogens, three synthetic estrogens and two plant estrogens: 17β-estradiol, 17α-estradiol, estrone, estriol, progesterone, 17α-ethynylestradiol, diethylstilbestrol (DES), mestranol, genistein, and biochanin A. These compounds were selected

based on their presence together in the environment, their ability to elicit estrogenic responses in vertebrates, their structures, and their molecular weights (Kolpin et al., 2010; Liu et al., 2010; Peng et al., 2006; Beausse, 2004; Colborn et al., 1993). It was important to choose estrogen isomeric compounds that would exhibit similar chemical behavior in the environment and similar chemical selectivity during analysis because these are the compounds that could interfere with quantification during estrogen analyses. A second solution, containing the exact same estrogenic compounds, was also run on each stationary and mobile phase combination. However, this second solution also contained soil matrix components. Soil water extracts were processed through a solid phase extraction procedure (SPE), and the soil matrix components remaining after SPE were added to the second combined standard. Throughout this study, this matrix solution is referred to as the combined standard with matrix. This was done to determine whether or not the presence of soil matrix colloidal material had any effect on target compound separation. Overall, the goal was to optimize the separation of multi-sorbate estrogenic solutions, which is especially vital for researchers that have limited access to MS systems.

Materials and Methods

Chemicals and Reagents

Estrone (≥99%), estriol (≥97%), 17β-estradiol (≥98%), 17α-estradiol (≥98%), 17α-ethynylestradiol (≥98%), DES (≥99%), mestranol (≥99%), progesterone (≥99%) genistein (≥98%), biochanin A, LC grade MeOH, HPLC grade methyl tert-butyl ether (MTBE) and formic acid, CH_2O_2 (≥95%) were also purchased from Sigma-Aldrich (St. Louis, MO). ACN and Water-0.1% Formic Acid were purchased from J.T. Baker (Philipsburg, NJ). Additional chemical information for each estrogen hormone and estrogenic compound is located in Table 3-1.

Soil

A Hagerstown silt loam soil, A horizon, sampled from The Russell E. Larson Agricultural Research Farm (located approximately 16 km southwest of University Park), was used to prepare the combined standard with soil matrix material.

Preparation of Combined Standard with Matrix

Environmental samples, especially soil extracts, contain compounds that can interfere with the analyte(s) of interest during analysis. These compounds collectively are termed a sample's matrix. To create a standard that contained matrix, 50 g of the control soil and 100 mL of water were added to a glass centrifuge bottle (1:2 soil:solution). Each bottle was shaken for 24 hours on a rotary shaker. Following this, each bottle was centrifuged for 1 hour at 1500 rpm (366 RCF). Thirty (30) mL of supernatant was removed from the centrifuge bottle, filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and processed through the SPE procedure described below.

Solid Phase Extraction (SPE) Procedure

The SPE method used was a modified version of the procedure provided in the Waters Application Notebook (Waters, 2008). An Oasis HLB Plus (Part# 186000132) (Waters, MA) cartridge was pre-conditioned with 5 mL of MTBE, 5 mL of MeOH and 5 mL of water, followed by a loading phase of the 30 mL filtered soil solution. The cartridge was washed with 5 mL of a 40:60 MeOH:water solution and 5 mL of water. Finally, the cartridge was eluted with 6 mL of a 90:10 MTBE:MeOH solution. The 6 mL of eluent was evaporated under a gentle stream of nitrogen gas, and the combined standard with matrix was prepared in this dried vial as described in the following section. It is assumed that this dried vial contains some soil matrix components.

Standard Preparation

In order to test the separating ability of each column, high concentration standards were prepared and analyzed. Working stock solutions were made for each of the 10 compounds. Working stocks were prepared by mixing 50 mg of compound into 50 mL of MeOH (1 mg mL^{-1}). To make single compound standards that stayed within the solubility limits for each compound a two-step process was used. First, 1 mL of the working stock (1 mg mL^{-1}) was added to 9 mL of MeOH to create a second set of working stocks (0.1 mg mL^{-1}). Then, 1 mL of the second working stock (0.1 mg mL^{-1}) was added to 9 mL of water to create a 0.01 mg mL^{-1} standard with a 10:90 solvent to water ratio. The 10:90 solvent to water ratio matches the initial HPLC mobile phase ratio used in this study. This process was carried out for each of the ten compounds. The combined standard without matrix was created by mixing together 1 mL from each of the 10 individual standards. The combined standard (10 mL at a concentration of 0.001 mg mL^{-1}) also had a 10:90 solvent to water ratio.

To create the combined standard with matrix, 1 mL of each individual standard (0.01 mg mL^{-1}) was added to the dried sample vial described in the SPE process above that contained soil matrix. The combined standard with matrix (10 mL at a concentration of 0.001 mg mL^{-1}) also had a 10:90 solvent to water ratio. Prior to analysis and standard preparation, it was determined that all standard concentrations were great enough to be detected by the instrument. Anything above 0.02 µg per injection could be easily detected. All standards were stored in the dark at 4 ˚C until analysis.

Chromatography Conditions

HPLC, coupled to a photodiode array UV-VIS (PDA) detector was used to analyze the above standards. For this study a Shimadzu UFLCxR equipped with an LC-20AD XR Liquid

Chromatograph and SPD-M 20A diode array detector were used. Three columns with the same dimensions (5µm, 150mm x 4.6 mm) but different stationary phase chemistries were tested in this study: a Restek Ultra C18, a Restek Ultra Biphenyl, (Bellefonte, PA) and a Supelco Ascentis RP-Amide column (St. Louis, MO). Each column was tested twice, once with a 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B) mobile phase and a second time with a 0.1% formic acid in water (A) and 0.1% formic acid in MeOH (B) mobile phase. A gradient mode was used for each mobile phase at a flow rate of 1.2 mL min $^{-1}$, except for when the biphenyl column evaluated with MeOH (Table 3-2). The sample injection volume was 50 µL and the column oven temperature was set to 28 ˚C.

Results and Discussion

Data Analysis

In order to quantify the separation quality, the following parameters were calculated for each LC analysis: resolution (Rs), retention factor (k'), selectivity (α), asymmetry (A_s) and tailing factor (t_f). There are defined target values for each parameter. Together, these parameters can be used to describe an HPLC run and determine whether or not an optimal separation was achieved. Resolution describes the degree of overlap between two peaks in a chromatogram (Poole, 2003). For this research study the following equation was used to calculate resolution:

$$Rs = \frac{2(tr_2 - tr_1)}{w_1 + w_2} \qquad 3.1$$

where tr_2 and tr_1 are the retention times of the first and second eluting peak respectively, w_1 is the peak width of the first peak, and w_2 is the peak width of the second peak. For both w_1 and w_2 the peak width at 50% height was used. An optimal separation is achieved when Rs ≥ 1.5.

The retention factor (or capacity factor) incorporates two times: the time a substance spends in the stationary phase and the time a substance spends in the mobile phase (Poole, 2003). Mathematically, it is defined as the ratio of these two times. The numerator represents the time in the stationary phase and the denominator represents the time in the mobile phase is. The following equation described the retention factor:

$$k' = \frac{(t_r - t_0)}{t_0} \qquad 3.2$$

where t_r is the retention time of the peak and t_0 is the void time. When it comes to the quality of a separation, the preferred k' range is 2-10, and an optimal k' range is generally considered to be 4-6. One way to achieve optimal k' values is to adjust the mobile phase gradient.

Selectivity (selectivity factor or separation factor) describes the relative retention of two peaks (Poole, 2003). In general, it is used to determine how well two solutes separate. Selectivity is defined by the following equation:

$$\alpha = \frac{k'_i}{k'_j} \qquad 3.3$$

where k'_i is the k' value of the second or later eluting compound, and k'_j is the k' value of the first or earlier eluting compound. A selectivity value of 1 indicates that the two compounds are co-eluting. The most effective way to modify selectivity, which encompasses each solute's retention factor, is to change the stationary phase chemistry.

The final parameter used to describe each LC run was symmetry. Symmetry refers to the shape of the peak. In an ideal chromatographic system all peaks would be Gaussian peaks (Poole, 2003). However, perfect Gaussian peaks are rare. Symmetry is defined using the following equation:

$$S = \frac{b}{f} \qquad 3.4$$

where b is the area of the back half of the peak and f is the area of the front half of the peak. When peaks are asymmetrical (A_s), the more common case, a similar equation is used:

$$A_s = \frac{b_{0.1}}{f_{0.1}} \qquad 3.5$$

where $b_{0.1}$ is the area of the back half of the peak and $f_{0.1}$ is the area of the front half of the peak, both measured at 10% of the peak height. A symmetrical peak will have an S value of 1. For asymmetrical peaks, A_s values close to 1 are optimal and anything under 2 is acceptable. Some software systems provide a tailing factor (t_f) value, instead of S or A_s, to describe symmetry. The tailing factor is defined using the following equation:

$$t_f = \frac{w_{0.05}}{2f_{0.05}} \qquad 3.6$$

where $w_{0.05}$ is the peak width and $f_{0.05}$ is the width of the front half of the peak, both measured at 5% of the peak height. The more symmetrical a peak, the closer the tailing factor is to 1.

Parameter Results for each Mobile Phase and Stationary Phase Combination

Before combined standards were analyzed, each individual standard was analyzed on all 6 stationary and mobile phase combinations to determine individual compound retention times and optimal detection wavelengths. This information was then used to identify each compound in the combined standard.

C_{18} and ACN

Calculated separation parameters for the C_{18} and ACN analysis are reported in Table 3-3, and Figures 3-1 and 3-2 are the associated chromatograms. In this analysis, the compound mestranol was not detected in either the individual standard or the combined standard. When the

standards were prepared, there with issues with mestranol precipitation and it is believed that the compound precipitated out of solution between standard preparation and analysis. The remaining nine compounds in the mixture were separated. Only three of the compounds had k' values outside of the preferred range (2-10): biochanin A, DES, and progesterone. Resolution values for the combined standard were all ≥ 1.5. The selectivity values were close to 1. However, the chromatograms and other separation parameters do not indicate co-elution. Both the tailing factor and asymmetry values were within the accepted range. The tailing factor is close to 1, and the asymmetry value is below 2. The compound progesterone (17.9) was detected in the chromatogram at 200 nm (Figure 3-1). However, the peak shape and height were more pronounced in the chromatogram at 254 nm (Figure 3-2).

RP-Amide and ACN

Results for the RP-Amide and ACN analysis are listed in Table 3-4. All 10 compounds in the mixture were detected, but not all 10 compounds were separated completely. All 10 compounds had k' values inside the preferred range (2-10). Two of the compounds had resolution values below 1.5: 17α-estradiol and estrone. Looking at Figure 3-3, these low resolution values correlate with the three peaks at retention times 16.22, 16.34 and 16.47. These three peaks do overlap which is supported by the resolution results. Almost all of the selectivity values were close to 1, but co-elution did not occur in the chromatogram. For the most part, the tailing factor and asymmetry values were within the accepted range, except for the three peaks that overlapped. Since these three peaks are not separated completely, a tailing factor and asymmetry value could not be measured.

Biphenyl and ACN

Results for the Biphenyl and ACN analysis are listed in Table 3-5. Eight of the ten compounds were separated successfully. Two of the compounds had k' values outside of the preferred range (2-10): mestranol and progesterone. There was only one case, estrone, where the resolution value for the combined standard was less than 1.5. The two compounds DES and estrone co-eluted (14.6). This is indicated by the selectivity value of 1, the low resolution value between the two compounds, and the chromatogram in Figure 3-4. For all ten compounds, the tailing factor and asymmetry values were within the accepted range.

C_{18} and MeOH

Results for this analysis are listed in Table 3-6. As seen above with C_{18} and ACN, the compound mestranol was not detected in either the individual standard or the combined standard. Again, it is believed that the compound precipitated out of solution between standard preparation and analysis. Of the remaining nine compounds in the mixture, only six were separated. Eight of the nine compounds had k' values outside of the preferred range (2-10). The only compound with a k' within the preferred range was estriol. Three of the resolution values for the combined standard were less than 1.5. Three compounds co-eluted at the 18.88 retention time: 17β-estradiol, estrone and DES. This is supported by their selectivity values which were either 1 or close to 1 and the chromatogram in Figure 3-5. The peak at the 18.88 retention time overlapped with the 17α-ethynylestradiol peak at 18.76. As a result of co-elution and overlap, 5 of the tailing factors and 4 of the asymmetry values could not be calculated. The remaining tailing factor and asymmetry values in Table 3-6 were within the accepted range. The compound progesterone was not present in Figure 3-5 because it was not detected at 200 nm. It was only detectable (21.2) when the wavelength was changed to 254 nm (Figure 3-6).

RP-Amide and MeOH

Results for this analysis are listed in Table 3-7. With this combination, only five of the 10 compounds were separated: estriol (16.89), mestranol (18.98), genistein (19.53), estrone (19.86) and progesterone (20.41). Not one of the 10 compounds had a k' value within the preferred range (2-10). Four of the resolution values for the combined standard were less than 1.5. Three compound peaks overlapped (Figure 3-7): 17α-estradiol (20.31), 17α-ethynylestradiol (20.48) and 17β-estradiol (20.62). This peak overlap explained the low resolution values. The compounds progesterone and 17α-ethynylestradiol were difficult to separate initially. Therefore, the individual standard areas and peak heights were evaluated for both progesterone and 17α-ethynylestradiol at different wavelengths. After evaluating each compound at different wavelengths, it was clear that at 200 nm, the 20.48 peak in the combined standard was 17α-ethynylestradiol, and at 254 nm, the 20.41 peak in the combined standard was progesterone (Figure 3-8). This was one case where the detector was able to capture spectral differences between compounds and these spectral differences could be used to separate the two compounds. Two other compounds were also detected at the 254 nm wavelength: DES and biochanin A. These two compounds co-eluted at 21.44 (Figure 3-8). Co-elution explained the selectivity value of 1 and the resolution value of 0. As a result of co-elution and peak overlap, three tailing factors and three asymmetry values could not be calculated. All of the remaining tailing factors and asymmetry values were within the accepted range. Finally, the peak at 21.109 in Figure 3-7 was likely interference from sample preparation or from the solvents used in the study. A peak at this retention time was detected in a blank 10:90 MeOH:water sample that was analyzed using the RP-Amide column and MeOH as the organic modifier.

Biphenyl and MeOH

Results for this analysis are listed in Table 3-8. Seven of the 10 compounds were separated. Only three of the 10 compounds had k' values within the preferred range (2-10). Two of the resolution values for the combined standard were less than 1.5. Two compounds co-eluted and had a selectivity value of 1: 17β-estradiol and 17α-estradiol (18.44). The 18.44 peak overlapped with the 17α-ethynylestradiol peak at 18.32 (Figure 3-9), which led to the low resolution values. Three compounds exhibited better response at the 254 nm: genistein (16.7), biochanin A (20), and progesterone (24.2) (Figure 3-10). As a result of co-elution, four of the tailing factors and three of the asymmetry values were not calculated. The remaining tailing factor and asymmetry values were within the accepted range.

Soil Matrix Effects

Results from the combined standard with matrix analyses indicated that the presence of soil matrix had no effect on separations. Table 3-9 is one combined standard with matrix example, and Figures 3-11 and 3-12 are the associated chromatograms. This combined standard with matrix example was analyzed on the C_{18} column with ACN as the organic modifier. The compound progesterone was detected in the chromatogram at 200 nm (Figure 3-11). However, the peak shape and height were more pronounced in the chromatogram at 254 nm (Figure 3-12). Comparisons were made between the C_{18} and ACN analysis without matrix (Table 3-3) and the C_{18} and ACN analysis with matrix (Table 3-9). The retention times for each compound nearly matched between the two analyses. By having similar retention times, the two different analyses also had similar k', resolution, selectivity, and symmetry values. For all of the remaining five stationary and mobile phase combinations, the same results were seen when the combined standard and combined standard with matrix were compared. Retention times and separation

parameters did not vary between the two standards. Overall, results indicated that after SPE, if soil matrix components remained in the sample, they did not affect the LC separation.

Mobile Phase Comparisons

For estrogen analyses, ACN and MeOH are the common mobile phase organic modifiers used in the literature. There are pros and cons to each, and they range from practical matters such as price and toxicity to actual analysis parameters such as total run time, peak shape and interaction with the stationary phase. Results from the above analyses highlighted three key points. When ACN was used as the mobile phase organic modifier, the total time it took for all of the compounds to elute from the column was less than the total time it took for all of the compounds to elute when MeOH was used. The biphenyl and ACN analysis provided a more optimal separation than the biphenyl and MeOH analysis. This was not expected. The bonding mechanisms for estrogens onto the biphenyl column are dispersive forces and pi-pi(star) interactions. Acetonitrile limits pi-pi(star) interactions. Therefore, it was expected that since MeOH should have promoted more of the overall bonding mechanisms it should have produced a better separation. That was not observed. These results instead suggested that dispersive forces were driving estrogen selectivity to the biphenyl column not pi-pi(star) interactions. When the same stationary phase was tested twice, once with ACN in the mobile phase and once with MeOH in the mobile phase, the elution order of the 10 compounds from the column changed between the two tests. This change in elution order occurred on all three columns when the organic modifier was switched from ACN to MeOH. The organic modifier was altering the selectivity of the system, a process usually attributed more to the stationary phase.

Summary and Conclusions

The main goal of this study was to determine an optimal stationary and mobile phase combination that could separate a multi-sorbate estrogenic solution. Results from six different stationary and mobile phase pairings indicated that the combination of C_{18} and ACN provided the greatest separation of the 10 compounds in the combined standard. When C_{18} and ACN were used to separate the combined standard peak overlap and co-elution did not occur. This combination is the one used most in the literature for estrogen analyses (Tomŝíková et al., 2012; Labadie and Hill, 2007; Havlíková et al, 2006), and these results provided further support for their use. From best to worst in their ability to separate the combined standards, the stationary and mobile phase combinations were ranked: C_{18} and ACN , RP-Amide and ACN, Biphenyl and ACN, Biphenyl and MeOH, C_{18} and MeOH, RP-Amide and MeOH. It is important to note, that this ranking is based on the separation of all 10 compounds in the combined standard. This ranking changes depending on the compounds of interest. These ten compounds were chosen because they are isomeric in either structure or weight, and they are often found together in environmental samples and waste sources known to contain estrogenic compounds.

Another goal of this study was to determine whether or not the presence of soil matrix had any effect on estrogen separations. Results from the C_{18} and ACN combined standard with matrix analysis indicated that there was no effect on separation when matrix components remained in the sample after SPE. While not shown, these same results were also seen when the combined standard with matrix was analyzed on the remaining five stationary and mobile phase combinations. In addition, results indicated that for the same stationary phase, the elution order for the 10 compounds in the combined standard changed when the organic modifier was

switched from ACN to MeOH. This suggested that the organic modifier had a strong influence on estrogen selectivity.

Only a limited number of studies have evaluated multiple stationary phase and mobile phase combinations, and even fewer have analyzed multi-sorbate standards that have contained estrogens and isomeric target compounds. These results can be used to guide future analyses of estrogen hormones and estrogenic compounds in complex environmental samples.

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Table 3-1. Chemical structures and characteristics for the ten compounds used to create the combined standards.

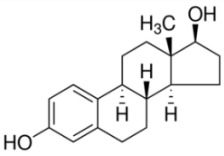
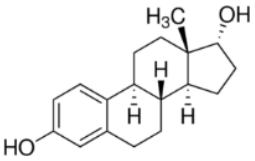
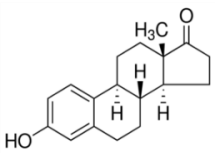
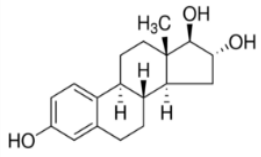
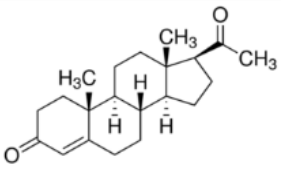
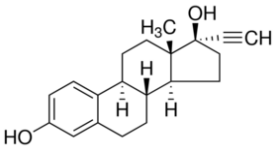
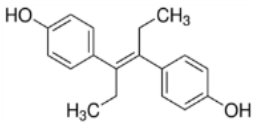
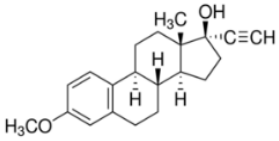
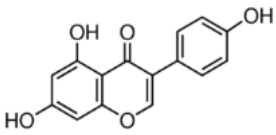
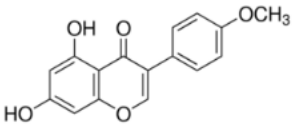
Compound	Structure	Molecular Weight ($g\ mol^{-1}$)	Water Solubility ($mg\ L^{-1}$)	log K_{ow}
17β-estradiol		272.4	13	3.94
17α-estradiol		272.4	13	-
Estrone		270.4	13	3.43
Estriol		288.4	27.3	2.45
Progesterone		314.5	8.81	3.87

Table 3-1 Continued.

Compound	Structure	Molecular Weight (g mol^{-1})	Water Solubility (mg L^{-1})	log K_{ow}
17α-ethynylestradiol		296.4	4.8	4.15
Diethylstilbestrol		268.3	12	5.07
Mestranol		310.4	1.13	4.61
Genistein		270.2	0.81	3.04
Biochanin A		284.3	1000	3.41

Images and molecular weights are from *Sigma-Aldrich*
Chemical data is from Bolton et al. (*pubchem.ncbi.nlm.nih.gov*)

Table 3-2. High performance liquid chromatography (HPLC) mobile phase gradient. All runs were 30 minutes long. B represents the percent organic modifier, acetonitrile (ACN) or methanol (MeOH), and A represents the percent water. The gradient that ramps up to 90% B was used for all run combinations except for the Biphenyl/MeOH run which ramped up to 100% B.

			Biphenyl/MeOH	
Time	**A**	**B**	**A**	**B**
(min.)	---------- percent % ----------			
0	90	10	90	10
1	90	10	90	10
21	10	90	0	100
26	10	90	0	100
27	90	10	90	10
30	90	10	90	10

Table 3-3. Separation parameters for each compound in the combined standard, analyzed using a C_{18} stationary phase and acetonitrile (ACN) mobile phase.

Compound	**tr**	**k'**	**α**	**Rs**	**tf**	**As**	**Wavelength (nm)**
Estriol	8.7	5.6	-	-	1.2	1.4	200
Genistein	11.2	7.5	1.32	37.4	1.2	1.4	200
17β-Estradiol	13.0	8.9	1.19	26.6	1.2	1.3	200
17α-Estradiol	13.6	9.3	1.05	8.1	1.1	1.3	200
17α-Ethynylestradiol	13.9	9.6	1.03	4.2	1.2	1.3	200
Estrone	14.4	9.9	1.04	6.1	1.1	1.3	200
Biochanin A	14.7	10.1	1.02	3.8	1.1	1.2	200
Diethylstilbestrol	14.9	10.3	1.02	2.9	1.2	1.3	200
Progesterone	17.9	12.6	1.22	36.8	1.1	1.2	254
Mestranol	-	-	-	-	-	-	-

tr: retention time; k': retention factor; α: selectivity; Rs: resolution; tf: tailing factor; As: asymmetry.

Table 3-4. Separation parameters for each compound in the combined standard, analyzed using an RP-Amide stationary phase and acetonitrile (ACN) mobile phase.

Compound	tr	k'	α	Rs	tf	As	Wavelength (nm)
Estriol	10.8	4.8	-	-	1.2	1.4	200
Genistein	15.5	7.3	1.53	54.1	1.1	1.3	200
17β-Estradiol	16.2	7.7	1.05	6.7*	-	-	200
17α-Estradiol	16.3	7.8	1.01	1.2*	-	-	200
Estrone	16.5	7.8	1.01	1.4*	-	-	200
17α-Ethynylestradiol	16.8	8.0	1.02	3.7	1.2	1.3	200
Progesterone	17.5	8.4	1.05	7.9	1.1	1.2	200
Biochanin A	17.8	8.5	1.02	2.7	1.2	1.3	200
Diethylstilbestrol	19.0	9.2	1.08	12.5	1.1	1.2	200
Mestranol	19.2	9.3	1.01	2.6	1.3	1.5	200

tr: retention time; k': retention factor; α: selectivity; Rs: resolution; tf: tailing factor; As: asymmetry.
* $w_{0.5}$ used to calculate Rs was estimated using peak variance

Table 3-5. Separation parameters for each compound in the combined standard, analyzed using a Biphenyl stationary phase and acetonitrile (ACN) mobile phase.

Compound	**tr**	**k'**	**α**	**Rs**	**tf**	**As**	**Wavelength (nm)**
Estriol	9.1	5.2	-	-	1.1	1.3	200
Genistein	11.6	6.9	1.33	34.9	1.2	1.3	200
17β-Estradiol	12.8	7.7	1.12	16.2	1.1	1.2	200
17α-Estradiol	13.2	7.9	1.03	4.4	1.1	1.2	200
17α-Ethynylestradiol	13.8	8.3	1.05	7.6	1.1	1.2	200
Diethylstilbestrol	14.6	8.9	1.07	10.7	1.1	1.2	200
Estrone	14.6	8.9	1.00	0.0	1.1	1.2	200
Biochanin A	14.8	9.0	1.02	2.7	1.1	1.2	200
Mestranol	17.2	10.7	1.18	29.4	1.3	1.5	200
Progesterone	18.3	11.4	1.07	11.2	1.2	1.3	200

tr: retention time; k': retention factor; α: selectivity; Rs: resolution; tf: tailing factor; As: asymmetry.

Table 3-6. Separation parameters for each compound in the combined standard, analyzed using a C_{18} stationary phase and methanol (MeOH) mobile phase.

Compound	**tr**	**k'**	**α**	**Rs**	**tf**	**As**	**Wavelength (nm)**
Estriol	14.5	9.7	-	-	1.1	1.2	205
Genistein	16.1	10.9	1.12	19.0	1.1	1.2	205
17α-Ethynylestradiol	18.8	12.8	1.18	27.2	-	-	205
17β-Estradiol	18.9	12.9	1.01	1.3*	-	-	205
Estrone	18.9	12.9	1.00	0.0*	-	-	205
Diethylstilbestrol	18.9	12.9	1.00	0.0*	-	-	205
17α-Estradiol	19.1	13.0	1.01	2.4	-	1.3	205
Biochanin A	19.4	13.2	1.02	3.3	1.1	1.2	205
Progesterone	21.2	14.6	1.10	23.1	1.2	1.4	254
Mestranol	-	-	-	-	-	-	-

tr: retention time; k': retention factor; α: selectivity; Rs: resolution; tf: tailing factor; As: asymmetry.
* $w_{0.5}$ used to calculate Rs was estimated using peak variance

Table 3-7. Separation parameters for each compound in the combined standard, analyzed using a RP-Amide stationary phase and methanol (MeOH) mobile phase.

Compound	tr	k'	α	Rs	tf	As	Wavelength (nm)
Estriol	16.9	10.6	-	-	1.0	1.1	205
Mestranol	19.0	12.1	1.14	21.7	0.8	0.7	205
Genistein	19.5	12.4	1.03	5.8	1.2	1.3	205
Estrone	19.9	12.7	1.02	3.4	1.0	1.1	205
17α-Estradiol	20.3	13.0	1.02	4.6	-	-	205
Progesterone	20.4	13.0	1.01	1.1	1.2	1.4	254
17α-Ethynylestradiol	20.5	13.1	1.00	0.6	-	-	205
17β-Estradiol	20.6	13.2	1.01	1.3	-	-	205
Diethylstilbestrol	21.4	13.7	1.04	6.9	0.9	0.8	254
Biochanin A	21.4	13.7	1.00	0.0	0.9	0.8	254

tr: retention time; k': retention factor; α: selectivity; Rs: resolution; tf: tailing factor; As: asymmetry.

Table 3-8. Separation parameters for each compound in the combined standard, analyzed using a Biphenyl stationary phase and methanol (MeOH) mobile phase.

Compound	tr	k'	α	Rs	tf	As	Wavelength (nm)
Estriol	14.9	8.1	-	-	1.1	1.1	205
Genistein	16.7	9.3	1.14	19.5	1.1	1.2	254
Diethylstilbestrol	17.6	9.8	1.06	10.4	1.0	1.1	205
17α-Ethynylestradiol	18.3	10.3	1.04	6.9*	-	-	205
17β-Estradiol	18.4	10.3	1.01	1.1*	-	-	205
17α-Estradiol	18.4	10.3	1.00	0.0*	-	-	205
Biochanin A	20.0	11.3	1.09	17.3	1.1	1.3	254
Estrone	20.6	11.7	1.03	6.3	1.1	1.3	205
Mestranol	21.5	12.1	1.04	9.0	1.1	1.1	205
Progesterone	24.2	13.9	1.14	27.8	-	1.7	254

tr: retention time; k': retention factor; α: selectivity; Rs: resolution; tf: tailing factor; As: asymmetry.
* $w_{0.5}$ used to calculate Rs was estimated using peak variance

Table 3-9. Separation parameters for each compound in the combined standard with soil matrix, analyzed using a C_{18} stationary phase and acetonitrile (ACN) mobile phase.

Compound	**tr**	**k'**	**α**	**Rs**	**tf**	**As**	**Wavelength (nm)**
Estriol	8.8	5.6	-	-	1.2	1.4	200
Genistein	11.2	7.5	1.32	37.4	1.2	1.3	200
17β-Estradiol	13.0	8.9	1.19	26.6	1.2	1.3	200
17α-Estradiol	13.7	9.4	1.05	8.1	1.1	1.3	200
17α-Ethynylestradiol	13.9	9.6	1.03	4.2	1.2	1.3	200
Estrone	14.4	9.9	1.04	6.1	1.1	1.2	200
Biochanin A	14.7	10.2	1.02	3.8	1.1	1.3	200
Diethylstilbestrol	14.9	10.3	1.02	2.9	1.2	1.3	200
Progesterone	17.9	12.6	1.22	36.9	1.1	1.2	254
Mestranol	-	-	-	-	-	-	-

tr: retention time; k': retention factor; α: selectivity; Rs: resolution; tf: tailing factor; As: asymmetry.

Figure 3-1. Chromatogram for the combined standard (wavelength= 200 nm) analyzed using a C_{18} stationary phase and acetonitrile (ACN) mobile phase.

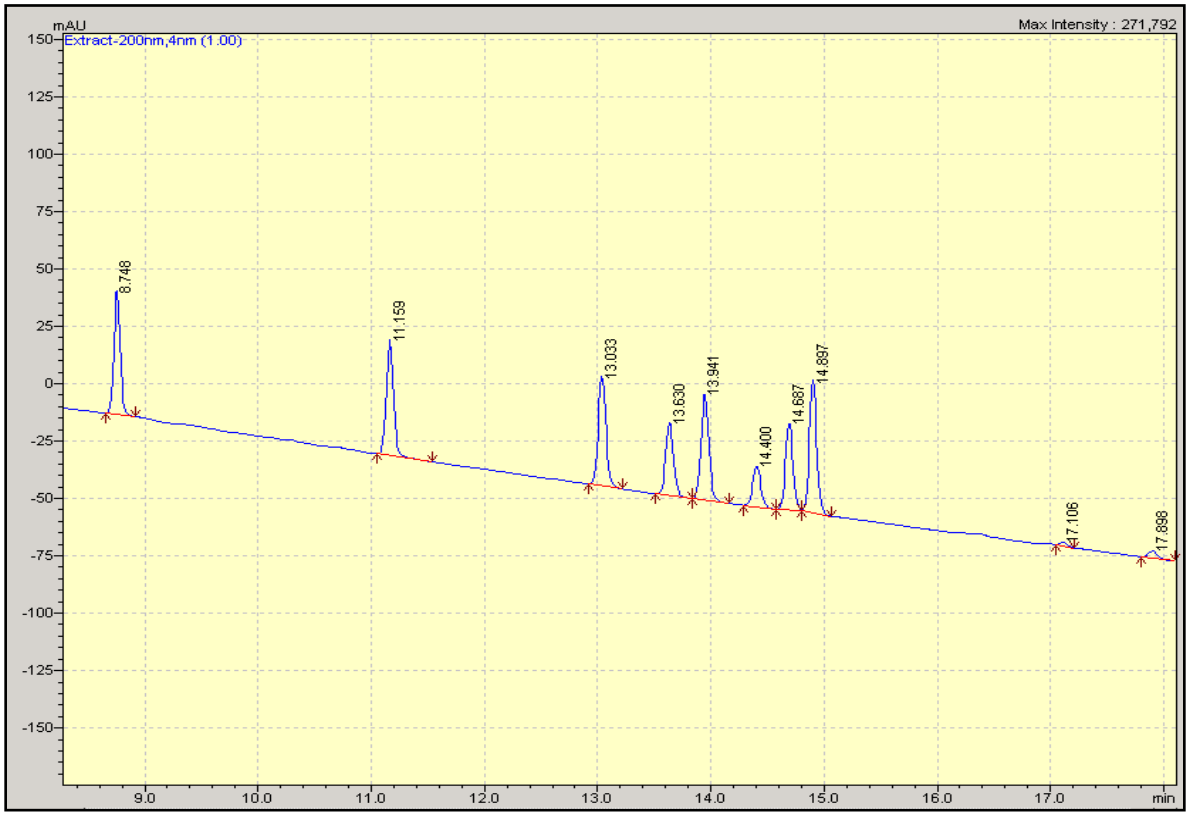


Figure 3-2. Chromatogram for the combined standard (wavelength= 254 nm) analyzed using a C_{18} stationary phase and acetonitrile (ACN) mobile phase. The peak at 17.9 minutes is the compound progesterone.

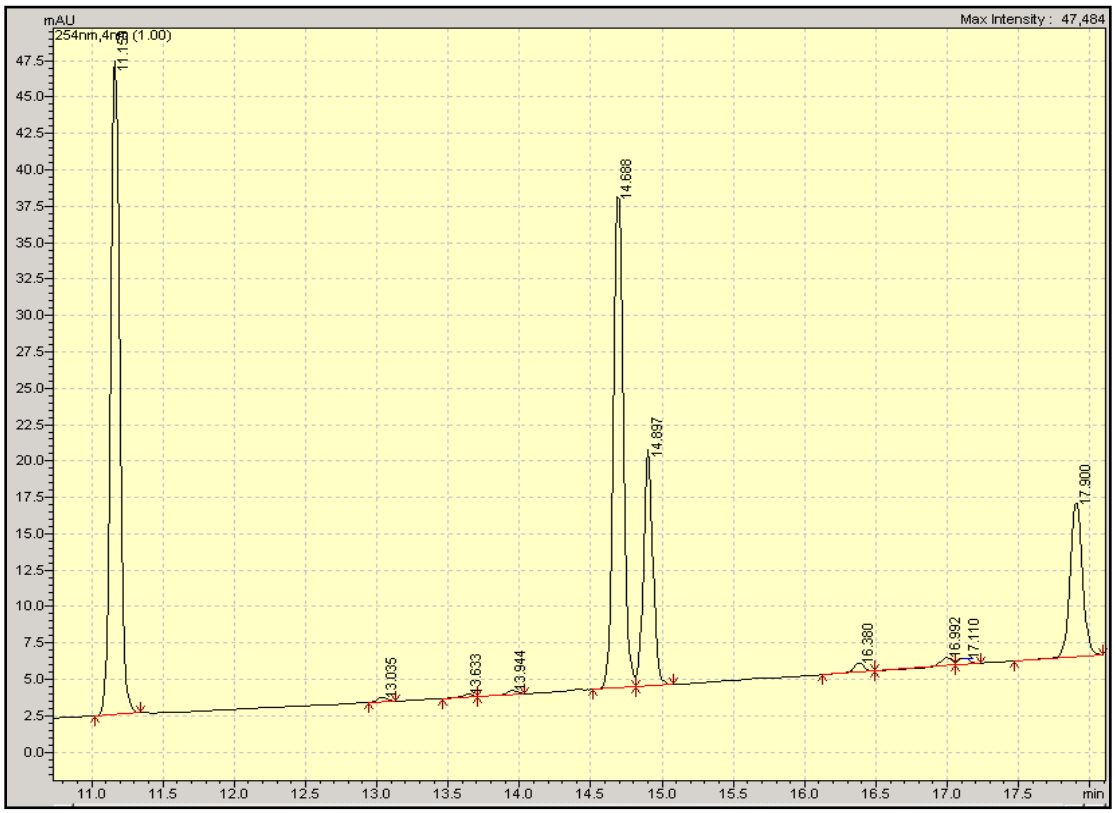


Figure 3-3. Chromatogram for the combined standard (wavelength= 200 nm) analyzed using an RP-Amide stationary phase and acetonitrile (ACN) mobile phase.

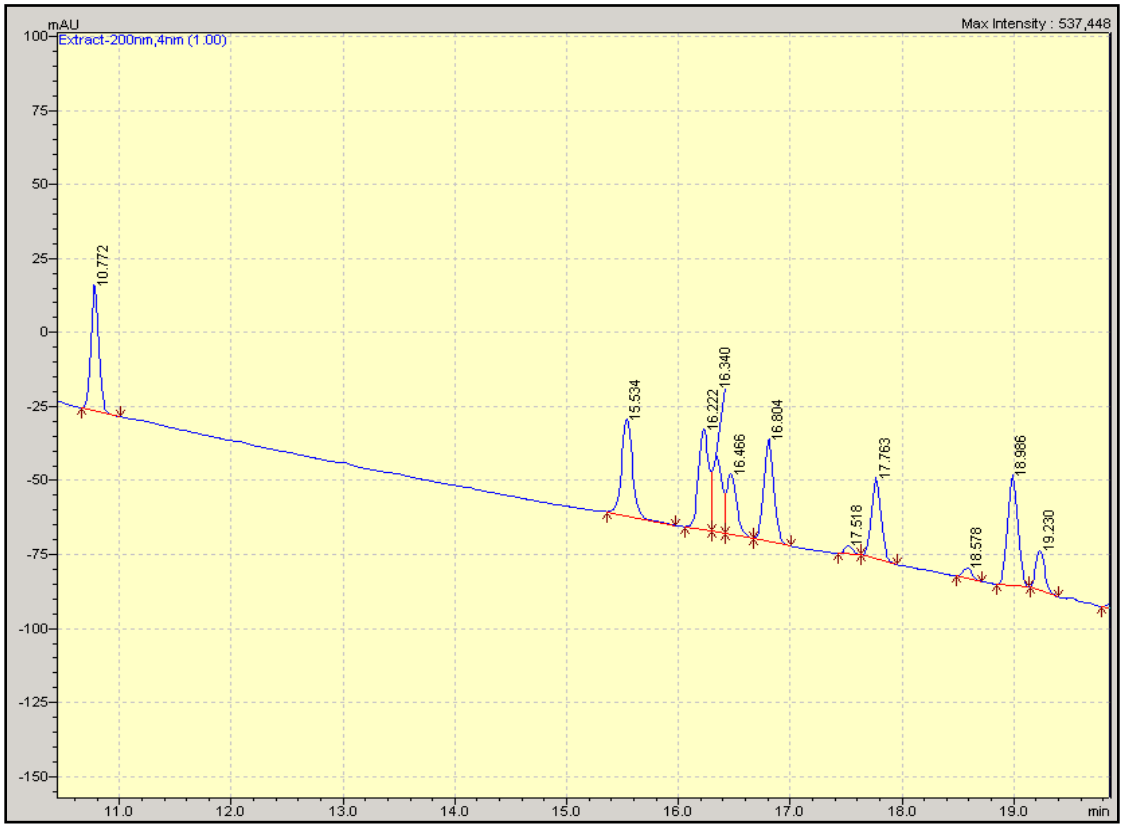


Figure 3-4. Chromatogram for the combined standard (wavelength= 200 nm) analyzed using a Biphenyl stationary phase and acetonitrile (ACN) mobile phase.

Figure 3-5. Chromatogram for the combined standard (wavelength= 205 nm) analyzed using a C_{18} stationary phase and methanol (MeOH) mobile phase.

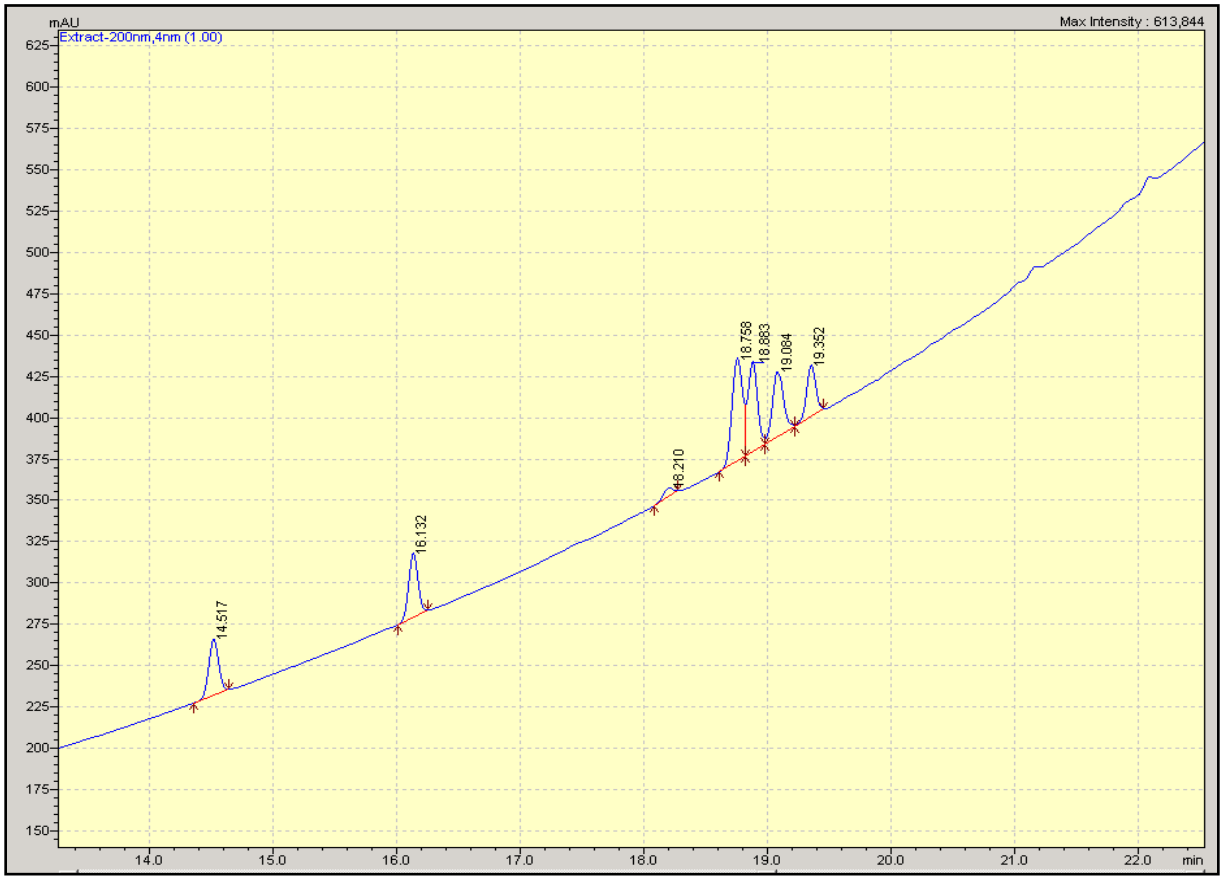


Figure 3-6. Chromatogram for the combined standard (wavelength= 254 nm) analyzed using a C_{18} stationary phase and methanol (MeOH) mobile phase. The peak at 21.2 minutes is the compound progesterone.

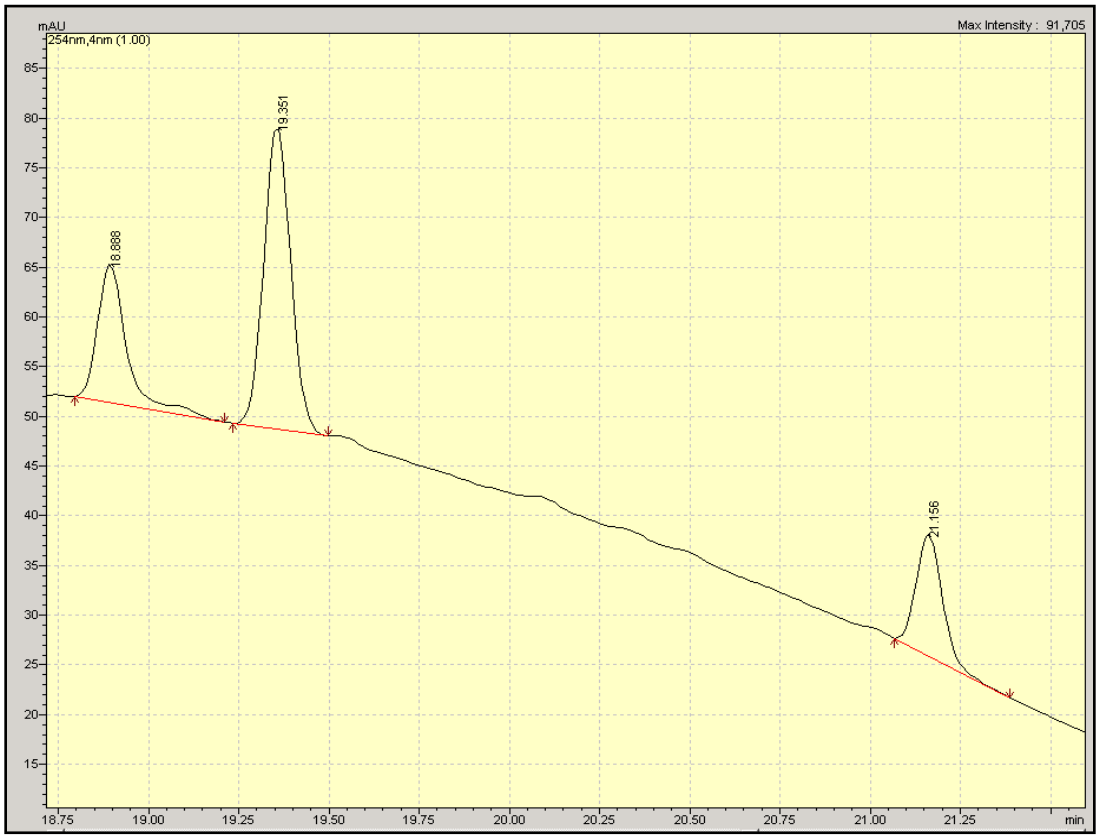


Figure 3-7. Chromatogram for the combined standard (wavelength= 205 nm) analyzed using an RP-Amide stationary phase and methanol (MeOH) mobile phase.

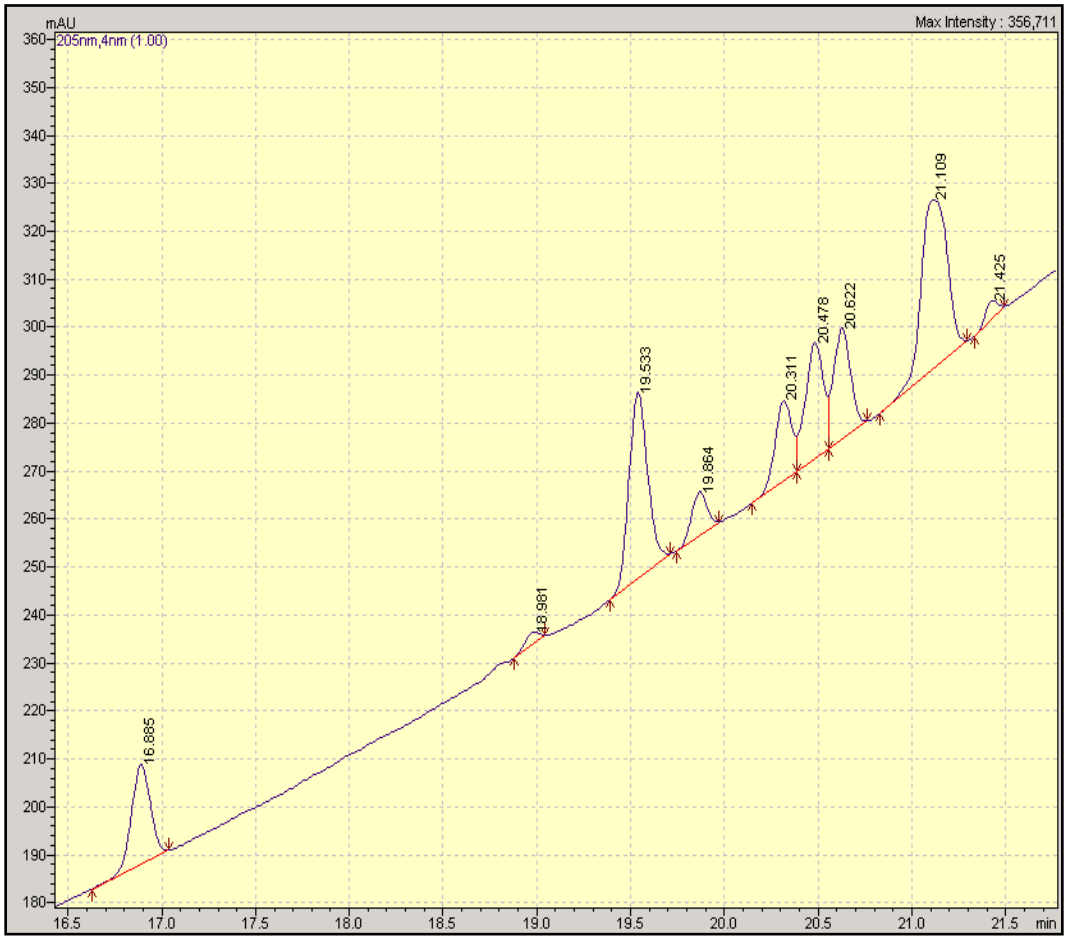


Figure 3-8. Chromatogram for the combined standard (wavelength= 254 nm) analyzed using an RP-Amide stationary phase and methanol (MeOH) mobile phase. The peak at 20.4 minutes is the compounds progesterone, and the compounds DES and biochanin A co-eluted at 21.14 minutes.

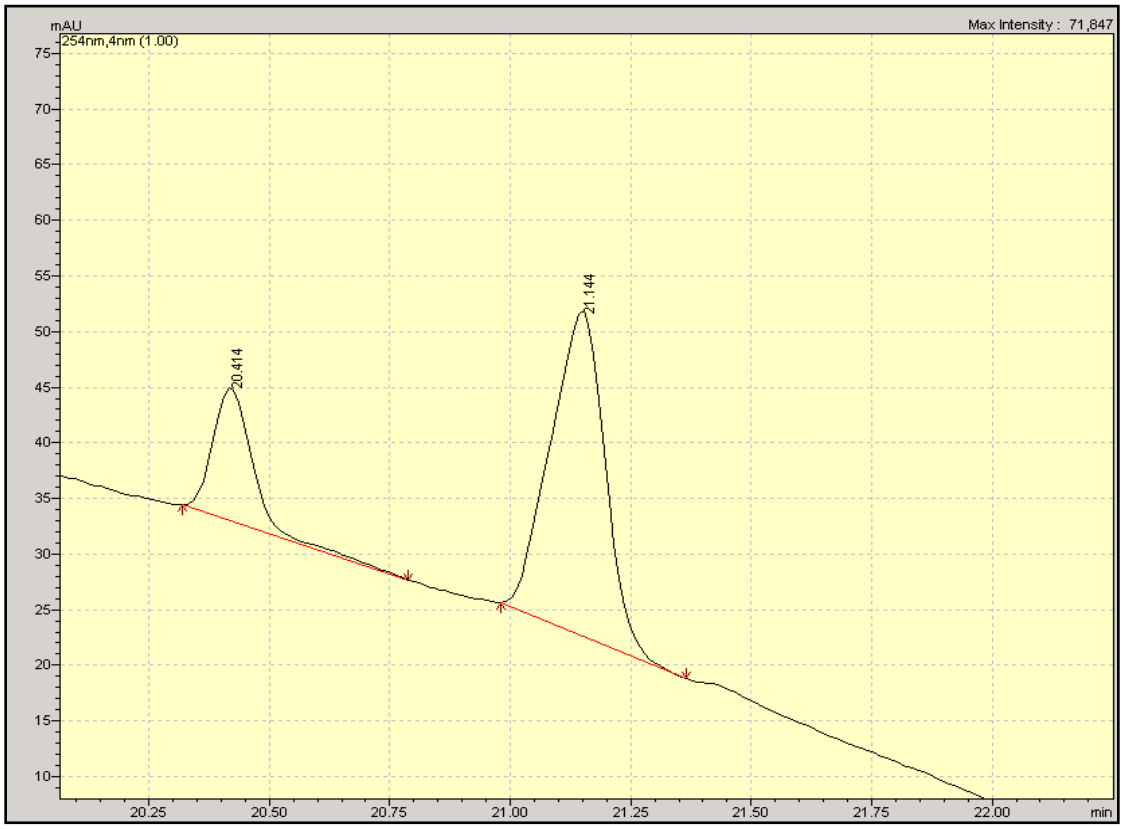


Figure 3-9. Chromatogram for the combined standard (wavelength= 205 nm) analyzed using a Biphenyl stationary phase and methanol (MeOH) mobile phase.

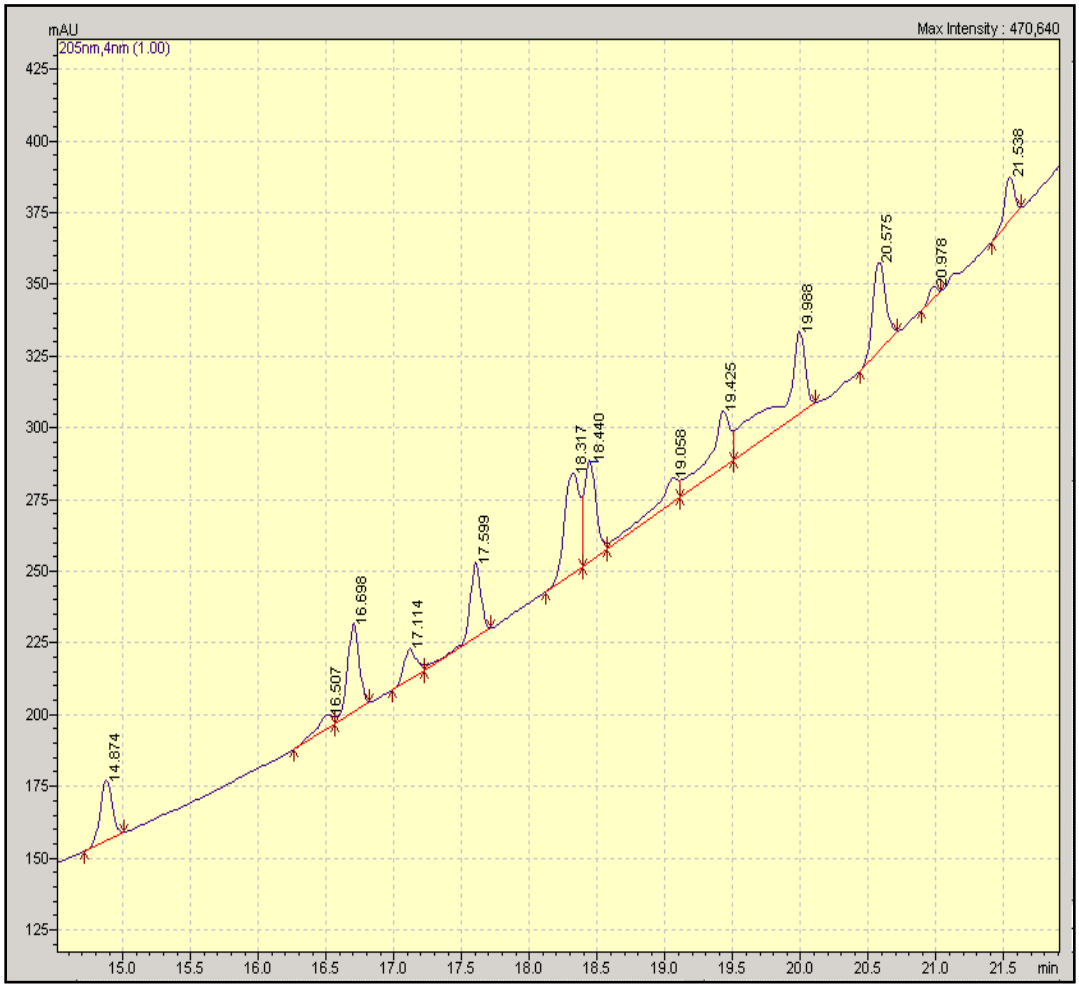


Figure 3-10. Chromatogram for the combined standard (wavelength= 254 nm) analyzed using a Biphenyl stationary phase and methanol (MeOH) mobile phase. The peaks at 16.7, 20.0, and 24.2 minutes are genistein, biochanin A, and progesterone respectively.

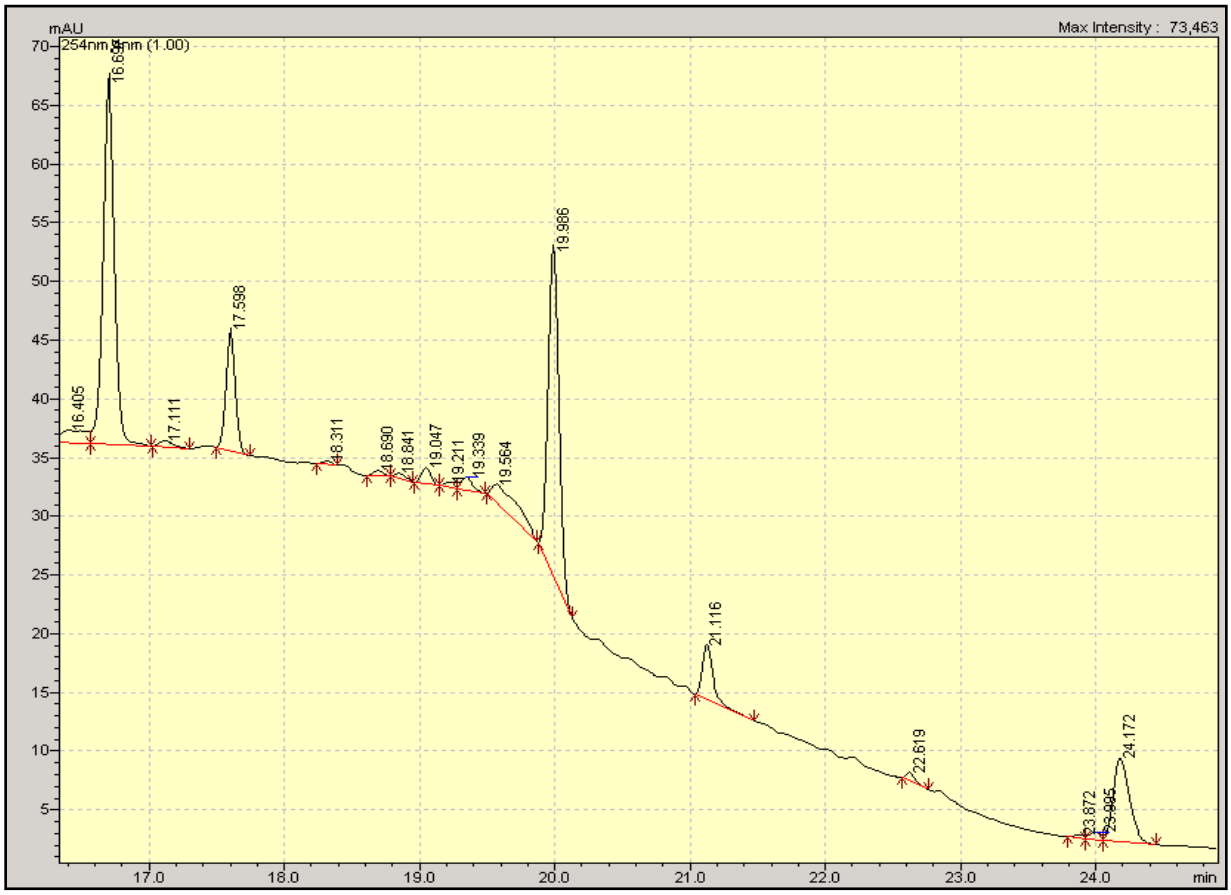


Figure 3-11. Chromatogram for the combined standard with matrix (wavelength= 200 nm) analyzed using a C_{18} stationary phase and acetonitrile (ACN) mobile phase.

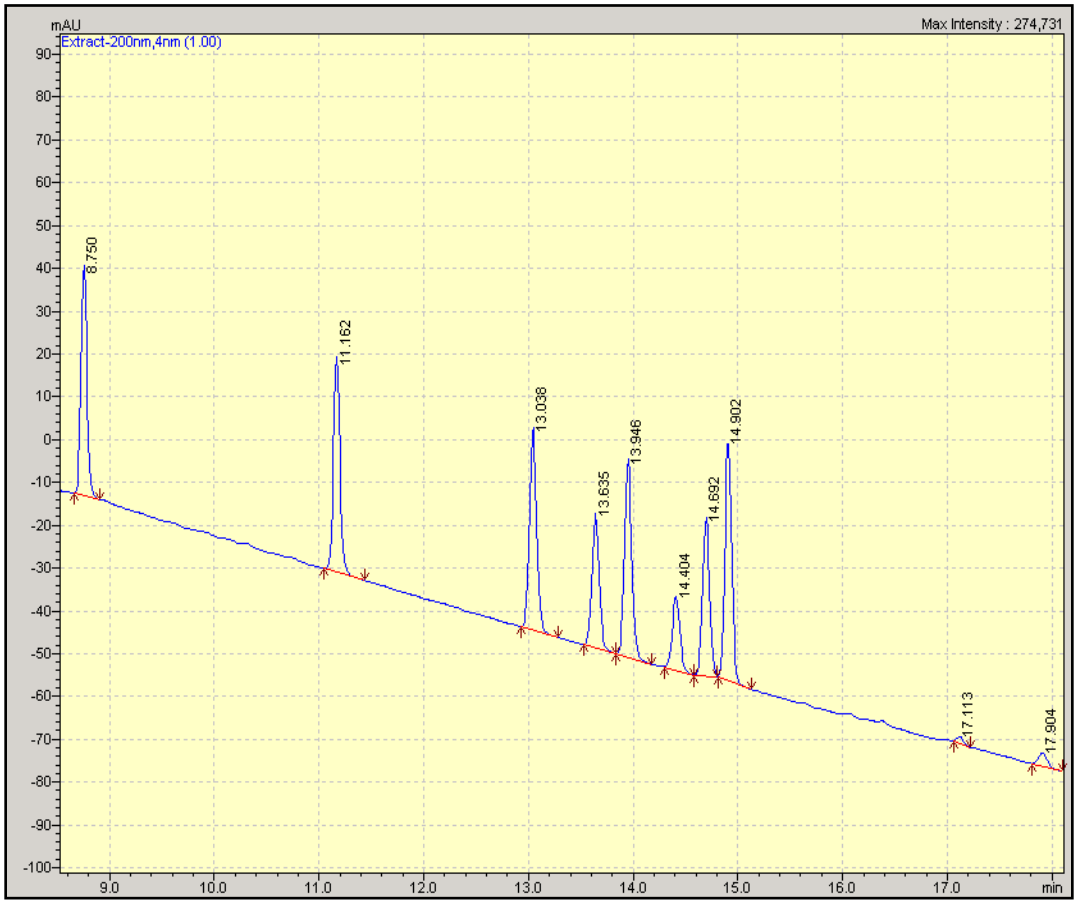
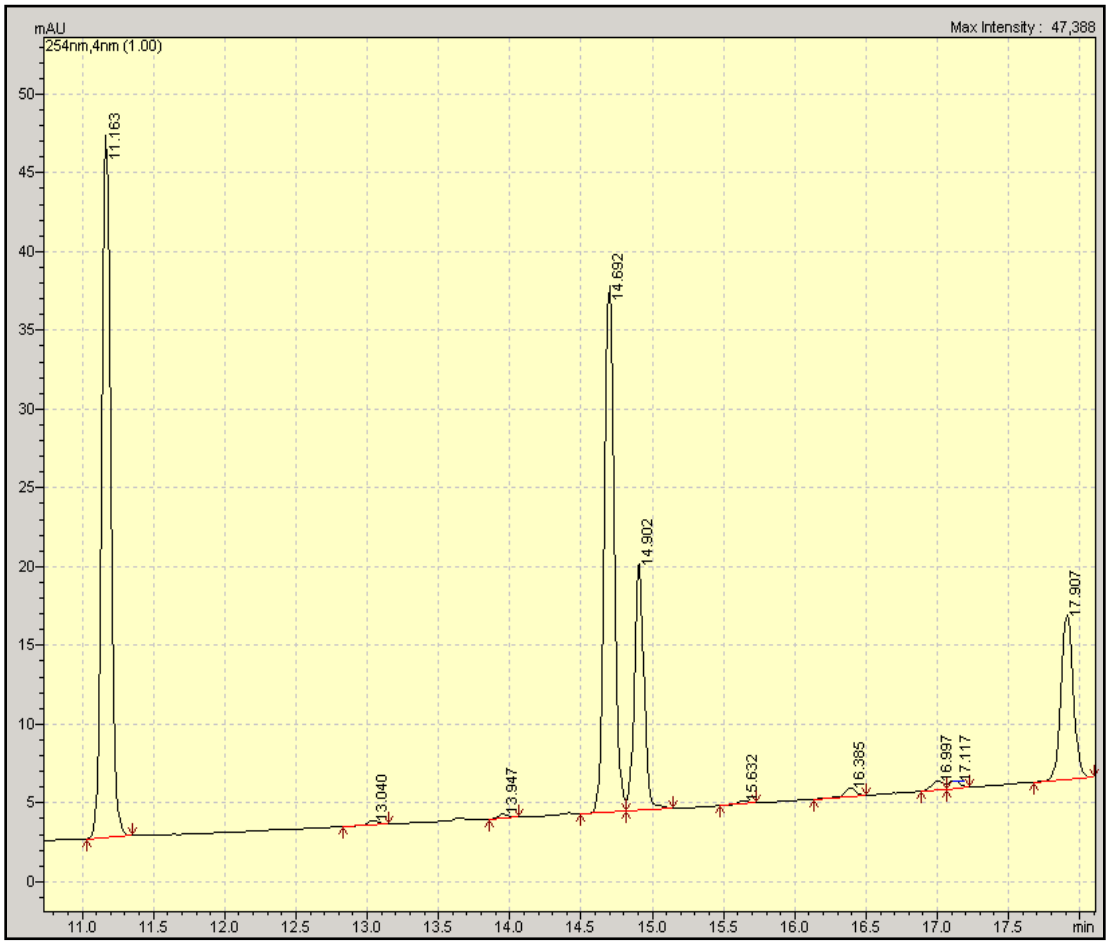


Figure 3-12. Chromatogram for the combined standard with matrix (wavelength= 254 nm) analyzed using a C_{18} stationary phase and acetonitrile (ACN) mobile phase. The peak at 17.9 minutes is the compound progesterone.



Chapter 4

Sorption of Estrogen Hormones in Field Soils

Abstract

The presence of estrogen hormones in soil environments has been well documented. In order to predict the fate and transport of estrogen hormones in soils, a key parameter, the sorption partition coefficient (K_d), must be derived for each estrogen compound. The goals of this study were to evaluate E1, E2, and EE2 sorption in three soils varying in organic carbon content, determine if there was a difference in E1, E2, and EE2 sorption when soils were equilibrated with pure water solutions versus effluent solutions, and to extract the sorbed phase and calculate E1, E2, and EE2 extraction recoveries from each soil. Estrogen sorption data were fit with both a linear and Freundlich model. Results indicated that sorption varied between E1, E2, and EE2 in the soils studied. For the pure water system, in the cropped A soil, K_d values were 41.50 for E1, 40.02 for E2, and 30.88 for EE2. In the cropped B soil, K_d values were 9.85 for E1, 7.24 for E2, and 6.59 for EE2. In the effluent system, in the cropped A soil, K_d values were 48.02 for E1, 43.44 for E2, and 43.76 for EE2. In the cropped B soil, K_d values were 4.72 for E1, 3.32 for E2, and 3.24 for EE2. The cropped A soil had a higher organic carbon content, and it was associated with larger K_d and K_f values. Organic carbon normalized log K_{oc} values indicated that in certain cases sorption differences were potentially driven by organic carbon quality not quantity. The rank in sorption from highest to lowest for all three soils was E1 > E2 > EE2. Estrogen sorption did decrease in the multi-sorbate, wastewater systems when the cropped B soil was used (0.26% TOC). In terms of recovery, E1 had the highest extraction recovery range (36.6 to 97.8%), followed by EE2 (20.7 to 67.1%), and then E2 (13.2 to 40.7%). The extraction recoveries decreased with an increase in soil organic carbon content.

Introduction

Estrogen hormones are present in the natural environment and have been quantified in a variety of water, sediment and soil matrices (Langdon et al., 2014; Thompson et al., 2009; Labadie and Hill, 2007; Shappell et al., 2006; Kolpin et al., 2002; Lai et al., 2000). Their presence in soil has been linked to land application of manure (Shappell et al., 2010; Thompson et al., 2009), land application of biosolids (Langdon et al., 2014), effluent irrigation (Woodward et al., 2014; Karnjanapiboonwong et al., 2011; Mahjoub et al., 2011), and leaching from agricultural storage lagoons (Arnon et al., 2008). In an ideal system, the soil acts as a natural filter, adsorbing and degrading estrogen compounds out of solution before percolating water reaches surface and groundwater sources. Soil filtration is critical because research has shown that environmental estrogen exposure in aquatic environments can affect endocrine and reproductive processes in fish and other vertebrates (Purdom et al., 1994; Dickerson and Gore, 2007).

In order to predict the fate and transport of estrogen hormones in soils, a number of parameters must be defined for the area being studied. One of these parameters is the sorption partition coefficient (K_d). Estrogens are neutral, hydrophobic compounds, log K_{ow} values range from 3.43 to 4.15 (Hildebrand et al., 2006; Lai et al., 2000). They favor adsorption, especially adsorption to soils with high organic carbon contents, log K_{oc} values ranging from 2.99 to 3.34 (Lee et al., 2003). Batch sorption studies focused on estrogen sorption to various environmental sorbents are abundant in the literature. Estrogen sorption has been described for soils (Bonin and Simpson, 2007; Hildebrand et al., 2006; Casey et al., 2005; Ying and Kookana, 2005; Das et al., 2004; Casey et al., 2003; Lee et al., 2003), sediments (Lai et al., 2000), mineral phases (Shareef et al., 2006; Van Emmerik et al., 2003), dissolved organic matter (Yamamoto et al., 2003), and

different soil size fractions (Sun et al., 2012). Even though estrogen sorption has been well characterized, a number of sorption related uncertainties exist. The goal of this study was to fill in some of the existing gaps in the literature.

First, this study was conducted using a multi-sorbate system, all equilibrating solutions contained estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2). A number of studies have characterized E1, E2, and EE2 sorption in the lab using single-sorbate systems, and the results from these studies were used to predict each estrogen's behavior in the environment (Sun et al., 2012; Stumpe and Marschner, 2007; Hildebrand et al., 2006; Casey et al., 2005; Lee et al., 2003; Casey et al., 2003). However, this characterization may not accurately predict estrogen behavior in the environment because estrogens are rarely, if ever introduced into the environment as single-sorbates. They are usually introduced as part of a more complex, multi-sorbate solution that contains E1, E2, EE2, and a number of other organic waste compounds (Wilson and Jones-Lepp, 2013; Karnjanapiboonwong et al., 2011; Swartz et al., 2006). Only a limited number of estrogen sorption studies have been conducted in either bi-sorbate (Bera et al., 2011; Yu and Huang, 2005; Yu et al., 2004; Lai et al., 2000) or multi-sorbate systems (Bonin and Simpson, 2007).

Studies that compared bi-sorbate or multi-sorbate systems to single sorbate-systems documented distinct differences in estrogen sorption characteristics. Yu et al. (2004) showed that in E1 and E2 bi-sorbate systems, sorption partition coefficients decreased. Competitive sorption did occur between E1 and E2. Studies have suggested that competitive sorption is driven by differences in hydrophobicity between compounds and differences in concentration (Bera et al., 2011; Yu and Huang, 2005; Lai et al., 2000). Bonin and Simpson (2007) looked at a tri-sorbate system; E1, E2, and EE2 were in the equilibrating solution. Results from the tri-sorbate batch

study indicated that when E1, E2, and EE2 were equilibrated with mineral samples, soil samples, or high carbon samples, sorption decreased compared to single-sorbate systems for all three estrogens (all K_F values decreased). This study will mimic the tri-sorbate system used by Bonin and Simpson (2007), and results from this study will provide a more realistic proxy for estrogen sorption behavior in the environment.

Second, this study extracted the soil and analyzed the sorbed phase, a step rarely seen in the literature (Lee et al., 2003). Most of the sorption studies in the literature analyzed the aqueous phase (equilibration solution) and then calculated the sorbed phase by subtracting the aqueous phase concentration from the total concentration applied to the soil before equilibration (Sun et al., 2012; Karnjanapiboonwong et al., 2010; Stumpe and Marschner, 2007; Hildebrand et al., 2006; Yu et al., 2004). This method for determining the sorbed phase is referred to as the difference method. As stated in Lee et al. (2003), using the difference method can be problematic since estrogens undergo biologic degradation and possible abiotic degradation on clays and oxides. The difference method might suggest that estrogens are being sorbed, when really they are being degraded and/or converted to another compound.

Finally, this research study looked at how different equilibrating solution matrices could affect estrogen sorption behavior. K_d or K_f values were determined for two batch studies, each using a different equilibrating solution matrix. In one batch study, the equilibrating solutions were prepared by spiking known concentrations of E1, E2, and EE2 into ultra-pure deionized water (18MΩ). Then, the spiked pure-water standards were equilibrated with three soils ranging in organic carbon content, similar to what is seen in the literature (Karnjanapiboonwong et al., 2010; Ying and Kookana, 2005; Casey et al., 2005; Yu et al., 2004; Casey et al., 2003; Lee et al. 2003). In the second batch study, the equilibrating solutions were prepared by spiking known

concentrations of E1, E2, and EE2 into effluent that had undergone primary and secondary treatment at the Penn State Wastewater Treatment Plant. The spiked-effluent standards were then equilibrated with three soils ranging in organic carbon content. The second batch study is trying to determine whether or not the effluent matrix has any effect on estrogen sorption onto soils. Three different mechanisms have been described in the literature, and each mechanism has the potential to reduce E1, E2, and EE2 sorption onto soils: effluent organic matter components limiting accessibility to soil binding sites, competition with other organics in the effluent for soil binding sites, and sorption to dissolved organics in the aqueous phase (Mitchell and Simpson, 2012; Stumpe and Marschner, 2010; Stumpe and Marschner, 2007; Yu and Huang, 2005; Yu et al., 2004; Lai et al., 2000).

Data from a batch study that uses effluent as the equilibrating solution matrix should provide a more realistic proxy for environmental estrogen sorption behavior than data from a batch study that uses pure water as the equilibrating solution matrix. As mentioned above, when these compounds are introduced into the environment, they are introduced via complex waste sources, not pure water sources. To our knowledge, the use of effluent or any equilibrating solution matrix other than pure water has only been done six times in the literature (Bera et al., 2011; Stanford et al., 2010; Stumpe and Marschner, 2010; Lucas and Jones, 2009; Stumpe and Marschner, 2007; Lai et al., 2000) and only 3 times with wastewater (Stanford et al., 2010; Stumpe and Marschner, 2010; Stumpe and Marschner, 2007). Most of the work using wastewater has been done by Stump and Marschner (2010 and 2007). In both studies, they indicated that wastewater as an equilibrating matrix or DOC from wastewater present during equilibration, decreased E1 and E2 sorption onto soils. Stanford et al. (2010) also showed that using wastewater as the equilibration matrix decreased E2 sorption. More specific to wastewater

effects, Stanford et al. (2010) linked decreased E2 sorption to surfactants. The hydrophobic surfactants were out competing estrogens for available binding sites.

Together, these three distinctions make the design of this sorption study different from what has been done previously in the literature. It is predicted that this study will provide new, more accurate sorption information for researchers modeling E1,E2, and EE2 fate and transport in environmental soil systems.

Materials and Methods

Soils

Three soils were used in this study. The A and B horizons of a Hagerstown silt loam soil were sampled from a cropped area at The Russell E. Larson Agricultural Research Farm (located approximately 16 km southwest of University Park). Another Hagerstown silt loam soil (A horizon) was sampled from a forested area at the Penn State Living Filter (located approximately 3.2 km northwest of University Park). After collection, all individual soils were mixed, air-dried, ground, and passed through a 2-mm sieve. Standard soil analysis methods were used to characterize the soil for cation exchange capacity (CEC), pH, texture, percent total organic carbon (%TOC) and total nitrogen (%TN) (Klute, 1986; Sparks, 1996) (Table 4-1).

Effluent

All of the effluent generated at University Park is treated at the University Park Wastewater Treatment Plant (WWTP). After primary and secondary treatment, the effluent is transported via pipelines from the University Park WWTP to the Penn State Living Filter Wastewater Irrigation Site. For this study, wastewater samples were collected at the Penn State Living Filter Wastewater Irrigation Site. At the Living Filter, effluent samples were collected from lateral 10/1 4/4. The following effluent samples were collected: triplicate 1-L samples for

initial estrogen concentrations (ng L^{-1}), triplicate 500 mL samples for dissolved organic carbon (DOC) and total nitrogen (TN), and two 1-L samples for the sorption study. All of the 1-L samples were filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI). The samples used to determine the initial estrogen concentrations were then run through the solid phase extraction (SPE) process described below. The samples needed for the sorption study were placed in a dark refrigerator (4˚C) until they were needed for the sorption study (within 1 month). The triplicate 500 mL samples were transported on ice from each sampling location to the Penn State Institutes of Energy and the Environment (PSIEE) Water Quality Lab for DOC (mg L^{-1}) and TN (mg L^{-1}) analyses (Table 4-2).

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich, MO: E1 (≥99%), E2 (≥98%), EE2 (≥98%), LC-MS grade methanol (MeOH) and acetonitrile (ACN), HPLC grade methyl tert-butyl ether (MTBE), ammonium hydroxide (NH_4OH), sodium azide (NaN_3 ≥99.5%), and calcium chloride ($CaCl_2$ >99%). All water used was ultra-pure deionized (18MΩ).

Method Development

A batch-equilibration method was used to measure E1, E2 and EE2 sorption.

Pure Water Batch Study

Five equilibration solutions, containing E1, E2 and EE2 together, were prepared in water: 0, 25, 50, 75 and 100 µg L^{-1}. The equilibrating concentrations represent the total concentration of each estrogen in solution. In addition to the three estrogens, each equilibration solution also contained 730 mg L^{-1} $CaCl_2$ and 250 mg L^{-1} NaN_3. In this batch study, all work was done in triplicate. Accounting for triplicates, the 5 water equilibrating solutions and 3 soils, there were 45 samples in total. For each sample, 10 g of soil and 20 mL of equilibrating solution (1:2

soil:solution) were added to a glass centrifuge bottle, and each bottle, covered in foil, was mixed for 24 hours on a rotary shaker. Literature studies have reported that estrogen equilibration is reached within 1 to 72 hours (Lee et al., 2003; Lai et al., 2000), with a few specifically citing 24 hours (Bera et al., 2011; Karnjanapiboonwong et al., 2010; Hildebrand et al., 2006; Casey et al., 2005). In addition, this research study is focused on short-term sorption and what happens after initial contact with a wastewater source. For these reasons, only one, 24-h equilibration time was used to generate sorption isotherms. Following the 24 hour equilibration, each bottle was centrifuged for 1 hour at 1500 rpm (366 RCF). The supernatant was decanted from each bottle and an aliquot (10 mL) of the supernatant was filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and run through the SPE procedure described below. Weights were recorded before the equilibrating solution was added and after it was decanted to determine the mass of equilibration solution remaining in the soil. After decanting, 20 mL of MeOH was added to the soil. Covered bottles were shaken for 1 hour on a rotary shaker, then centrifuged for 1 hour at 1500 rpm. Ten milliliters of supernatant were removed from each bottle and filtered. After filtration, the extraction solution (10 mL) was evaporated to dryness under a gentle stream of nitrogen gas. It was then reconstituted with 10 mL of 10:90 MeOH:water. These diluted extraction samples were processed through the SPE procedure described below before analysis.

Living Filter Effluent Batch Study

Five equilibration solutions, containing E1, E2 and EE2 together, were prepared in wastewater that was collected at the Penn State Living Filter site: 0, 25, 50, 75 and 100 µg L^{-1}. In addition to the three estrogens, each equilibration solution also contained 730 mg L^{-1} $CaCl_2$ and 250 mg L^{-1} NaN_3. In this batch study, all work was done in triplicate. Accounting for triplicates, the 5 wastewater equilibrating solutions and 3 soils, there were 45 samples in total.

The wastewater batch study followed the same exact procedure described above for the water batch study. For each sample, 10 g of soil and 20 mL of equilibrating solution (1:2 soil:solution) were added to a glass centrifuge bottle, and each bottle, covered in foil, was mixed for 24 hours on a rotary shaker. Following the 24 hour equilibration, each bottle was centrifuged for 1 hour at 1500 rpm (366 RCF). The supernatant was decanted from each bottle and an aliquot (10 mL) of the supernatant was filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and run through the SPE procedure described below. Weights were recorded before the equilibrating solution was added and after it was decanted to determine the mass of equilibration solution remaining in the soil. After decanting, 20 mL of MeOH was added to the soil. Covered bottles were shaken for 1 hour on a rotary shaker, then centrifuged for 1 hour at 1500 rpm. Ten milliliters of supernatant were removed from each bottle and filtered. After filtration, the extraction solution (10 mL) was evaporated to dryness under a gentle stream of nitrogen gas. It was then reconstituted with 10 mL of 10:90 MeOH:water. These diluted extraction samples were processed through the SPE procedure described below before analysis.

Solid Phase Extraction (SPE) Procedure

An SPE procedure was used to cleanup and concentrate each sample. The SPE method used was a modified version of the procedure provided in the Waters Application Notebook (Waters, 2008). An Oasis HLB Plus (Waters, MA) cartridge was pre-conditioned with 5 mL of MTBE, 5 mL of MeOH and 5 mL of water, followed by a loading phase of 10 mL of either the equilibration solution or the extraction solution. The cartridge was washed with 5 mL of a 40:60 MeOH:water solution and 5 mL of water. Finally, the cartridge was eluted with 6 mL of a 90:10 MTBE:MeOH solution. The 6 mL of eluent was evaporated under a gentle stream of nitrogen

gas, and each evaporated vial was brought to a 1 mL volume with 10:90 MeOH:water. This 1 mL sample was analyzed using the chromatography conditions described below.

Chromatography Conditions

The 1 mL equilibration solution samples and extraction samples were analyzed for estrogens using an LC-MS-MS. The column was an XTerra MS C18 Column, 3.5 µm, 2.1 x 100 mm (Waters, MA). The Tandem MS used for quantification was a MicroMass Quattro micro API (Waters, MA). The mobile phase solvents were 0.6% NH_4OH in ACN (solvent A) and 0.6% NH_4OH in water (solvent B), and these two solvents were run in gradient mode. The initial mobile phase started with 10% solvent A and 90% solvent B, and increased linearly for five minutes to 90% solvent A and 10% solvent B. This was held for two minutes. From seven minutes to the end of the run, the gradient was dropped to 10% solvent A and 90% solvent B. The operating mode was ESI-. Flow rate was 0.25 mL min^{-1}, and the injection volume was 20 µl. External 6 to 8 point standard calibration curves were generated (1 to 1000 µg L^{-1}). Peak area outputs from samples were used against calibration curves to estimate sample concentrations. The measured limit of detection (LOD) was 0.48 µg L^{-1}, 2.16 µg L^{-1} and 1.78 µg L^{-1} for estrone, 17α-ethynylestradiol and 17β-estradiol respectively.

Results

Data Analysis

To analyze the sorption data, a clear method for dealing with samples that fell below the instrumentation's LOD was necessary. In this study, when LC-MS-MS output concentrations were below detection limit (BDL), the LOD/√2 was used (Hewett and Gonser, 2007). The estrogen specific LOD/√2 values used were 0.34 µg L^{-1} for E1, 1.26 µg L^{-1} for E2, and 1.53 µg L^{-1} for EE2.

Statistical Analysis

All statistics were conducted using Microsoft Excel. A t-test was used to evaluate the differences in means between the two groups, either the two soils or the two equilibrating solutions. Statistics were conducted for each estrogen on an individual basis; statistical comparisons were not made between the three compounds. For each t-test, a significance level of $\alpha = 0.05$ was used.

Initial Effluent Concentrations

Average concentrations in the effluent collected from the Living Filter spray field varied for each estrogen (Table 4-2). In the Living Filter effluent, the average E2 concentration was 9.3 (±7) ng L^{-1}, the average E1 concentration was 23.8 (±12.3) ng L^{-1}, and the average EE2 concentration was 4.7 (±1.6) ng L^{-1}. This effluent was used as an equilibration solution matrix during the sorption study.

In order to use the effluent as an equilibration solution and interpret sorption results, the concentration of each estrogen present in the effluent had to be determined prior to its use in the sorption study. Then, the estrogen concentration and the amount of effluent shaken with each soil (20 mL) were used to determine the mass (ng) of each estrogen present in the sorption system. This effluent mass was then incorporated into the total equilibration mass added during the sorption study. Overall, results in Table 4-2 show that the estrogen equilibration concentrations (25 to 100 µg L^{-1}) used during the sorption study far exceeded the estrogen concentrations already present in the effluent.

Linear and Freundlich Sorption Models

Estrogen sorption isotherms can be linear or non-linear. In the case of linearity we use the equation:

$$s = K_d c \qquad 4.1$$

where s (μg kg^{-1}) is the sorbed phase, c (μg L^{-1}) is the solution phase, and K_d (L kg^{-1}) is the distribution partition coefficient. In the case of non-linearity, a Freundlich model was used. The Freundlich equation is:

$$s = K_f c^{\beta} \qquad 4.2$$

where s (μg kg^{-1}) is the sorbed phase, c (μg L^{-1}) is the solution phase, K_f (L kg^{-1}) is the Freundlich distribution coefficient, and β is a linearity parameter. Estrogen sorption onto organic carbon is cited as the dominant sorption mechanism for estrogens in soils (Lai et al., 2000; Lee et al., 2003), and for that reason both K_d and K_f values should be normalized to organic carbon. This involves an additional factor, the fraction of organic carbon in the soil, f_{oc}. The relationship between the distribution partition coefficient and the organic carbon partition coefficient is represented by the following equation:

$$K_d = K_{oc} f_{oc} \qquad 4.3$$

where K_{oc} (L kg^{-1}) is the organic carbon distribution partition coefficient.

The two models described above are most often used to fit estrogen sorption behavior, therefore it was important to consider both when fitting the data from this study. In Tables 4-3 and 4-4 the fitting parameters are shown for the pure water and LF effluent sorption systems. All data were fit with both a linear and Freundlich model.

Estrogen Sorption using Pure Water Equilibration Solution

The linear and Freundlich fitting parameters for the pure water system are listed in Table 4-3. For all three estrogens, the Freundlich model was a better fit. The R^2 values for the Freundlich model were slightly higher (0.80 to 0.98) than the R^2 values for the linear model

(0.66 to 0.97). The beta values for the Freundlich model were all either less than 1 or slightly higher than 1.

Model fitting parameters for E1, E2, and EE2 in the forested A soil have been omitted from Table 4-3. Normally, in an ideal linear fit system, as the spiking concentration increases, the concentrations of each estrogen in the equilibration phase and the extraction phase also increase. In a Freundlich fit system, as the spiking concentration increases, the concentrations of each estrogen in the equilibration phase and the extraction phase also increase until sorption saturation is reached. For E1, E2, and EE2, when the forested A soil was used, as the spiking concentration increased, the concentrations in the equilibration and extraction phases did not always increase (Appendix A, Tables A-1, A-2, and A-3). It is hypothesized that the concentration in the equilibration phase did not increase as the spiking concentration increased because of the high %TOC (6.55%) in the forested A soil. There were enough sorption sites to accommodate the increased concentration of estrogens in the spiking solution. As a result, both the linear and Freundlich fits were poor and indicated a combination of negative K_d values, extremely high K_f values, negative β values, and/or low R^2 values. Variability between samples was also high for E1, E2, and EE2 when the forested A soil was used. A K_d or K_f value for these systems could not be determined with confidence.

The data in Table 4-3 were used to calculate the average K_d value and the average K_f value for each estrogen compound and soil combination (Tables 4-5 to 4-8). In the cropped A soil, K_d values were 41.50 for E1 (Table 4-5), 40.02 for E2 (Table 4-6), and 30.88 for EE2 (Table 4-7). In the cropped B soil, K_d values were 9.85 for E1 (Table 4-5), 7.24 for E2 (Table 4-6), and 6.59 for EE2 (Table 4-7). The K_f values for the cropped A soil were 60.70 for E1, 53.51 for E2, and 57.97 for EE2. In the cropped B soil, K_f values were 5.53 for E1, 6.13 for E2, and

4.60 for EE2 (Table 4-8). The K_d values were normalized to organic carbon using the f_{oc} for each soil. The f_{oc} values were derived from the %TOC values listed in Table 4-1. The normalized K_{oc} values for each estrogen and soil combination are shown in Tables 4-9 to 4-11. The average E1 K_{oc} value was 4,029 in the cropped A soil and 3,788 in the cropped B soil (Table 4-9). The average E2 K_{oc} value was 3,885 in the cropped A soil and 2,784 in the cropped B soil (Table 4-10). Finally, the average EE2 K_{oc} value was 2,997 in the cropped A soil and 2,535 in the cropped B soil (Table 4-11). The range of the log K_{oc} values (3.40 to 3.61) is in agreement with those reported by other literature studies (Sarmah et al., 2008; Sangsupan et al., 2006; Lee et al., 2003). Lee et al. (2003) reported log K_{oc} values of 3.22 for E1, 3.46 for E2, and 2.91 for EE2. Sangsupan et al. (2006) reported log K_{oc} values ranging from 3.06 to 3.37 for E2, and Sarmah et al. (2008) reported a log K_{oc} value of 3.55 for EE2.

Estrogen Sorption using Living Filter Effluent Equilibration Solution

The linear and Freundlich fitting parameters for the Living Filter effluent system are listed in Table 4-4. Except for the case of E2 in the cropped A soil, the Freundlich model was a better fit. The R^2 values for the Freundlich model were slightly higher (0.52 to 0.99) than the R^2 values for the linear model (0.64 to 1.00). The beta values for the Freundlich model were all either less than 1 or slightly higher than 1. The lowest R^2 values were seen when E2 was fit with the Freundlich model using the cropped A soil (1.03 %TOC). The R^2 values ranged from 0.54 to 0.96.

As reported in the previous section, model fitting parameters for E1, E2, and EE2 in the forested A soil have been left out of Table 4-4. Again, for E1, E2, and EE2, as the spiking concentration increased, the concentrations in the equilibration and extraction phases varied (Appendix A, Tables A-1, A-2, and A-3). As a result, both the linear and Freundlich fits were

poor and indicated a combination of negative K_d values, extremely high K_f values, negative β values, and/or low R^2 values. A K_d or K_f value could not be determined with confidence.

The data in Table 4-3 were used to calculate the average K_d value and the average K_f value for each estrogen compound and soil combination (Tables 4-5 to 4-8). In the cropped A soil, K_d values were 48.02 for E1 (Table 4-5), 43.44 for E2 (Table 4-6), and 43.76 for EE2 (Table 4-7). In the cropped B soil, K_d values were 4.72 for E1 (Table 4-5), 3.32 for E2 (Table 4-6), and 3.24 for EE2 (Table 4-7). The K_f values for the cropped A soil were 59.44 for E1, 48.76 for E2, and 46.03 for EE2. In the cropped B soil, K_f values were 9.04 for E1, 10.66 for E2, and 8.01 for EE2 (Table 4-8). The K_d values were normalized to organic carbon using the f_{oc} for each soil. The f_{oc} values were derived from the %TOC values listed in Table 4-1. The normalized K_{oc} values for each estrogen and soil combination are shown in Tables 4-9 to 4-11. The average E1 K_{oc} value was 4,662 in the cropped A soil and 1,815 in the cropped B soil (Table 4-9). The average E2 K_{oc} value was 4,217 in the cropped A soil and 1,275 in the cropped B soil (Table 4-10). Finally, the average EE2 K_{oc} value was 4,248 in the cropped A soil and 1,247 in the cropped B soil (Table 4-11). The range of the log K_{oc} values (3.10 to 3.67) is in agreement with those reported by other literature studies (Sarmah et al., 2008; Sangsupan et al., 2006; Lee et al., 2003).

Estrogen Recoveries

Extraction recoveries (%) for E1, E2, and EE2 for all three soils and each equilibration solution are reported in Table 4-12. For all soils and equilibration solutions, E1 had the highest extraction recoveries, 36.6 to 97.8%. EE2 recoveries ranged from 20.7 to 67.1%. E2 had the lowest extraction recoveries, 13.2 to 40.7%. For each estrogen, the percent recovery was lowest in the forested A soil. This soil has the highest organic carbon content. Estrogen recoveries decreased with an increase in soil organic carbon content, a trend also seen in Lee et al. (2003).

Overall, these recoveries are in agreement with literature reported estrogen extraction recovery values (Sarmah et al., 2008; Lee et al., 2003; Colucci et al., 2001). Additional estrogen recoveries for each equilibration solution, each soil, and each individual equilibration concentration are shown in Appendix A (Tables A-4 and A-5).

Discussion

Sorption Differences between Soils

E1, E2, and EE2 sorption varied between the soils studied. Looking at the K_d and K_f values in Tables 4-5 through 4-8, for all three estrogens, sorption was highest in the cropped A soil, followed by the cropped B soil. The %TOC for the cropped A soil is 1.03%, and the %TOC for the cropped B soil is 0.26%. Except for the case of EE2 in the pure water system, there was a statistically significant difference between all of the cropped A K_d values and the cropped B K_d values (Tables 4-5 to 4-7). The K_d and K_f values were higher in the cropped A soil than the K_d and K_f values in the cropped B soil. This relationship between estrogen sorption and organic carbon concentration in soils has been well documented (Stumpe and Marschner, 2007; Hildebrand et al., 2006; Lee et al., 2003).

The data were normalized for organic carbon quantity (Tables 4-9 to 4-11). For all three estrogens, there was no statistically significant difference in K_{oc} values between the cropped A soil and cropped B soil in the pure water system. However, in the effluent system, there was a statistically significant difference in K_{oc} values between the cropped A soil and the cropped B soils in two of the three cases. For E1 and E2, K_{oc} values were higher in the cropped A soil than the cropped B soil (Table 4-9 and 4-10). Since the data were normalized, the K_{oc} differences seen between the two soils could not be attributed to organic carbon quantity. Instead, the data suggest

that for E1 and E2 in the effluent system, organic carbon quality or another soil mineralogy property could be driving.

The K_d results in Tables 4-5 to 4-7 were used to rank estrogen sorption for each soil. For the pure water equilibration system, the sorption rank from highest to lowest in the cropped A soil was E1 > E2 > EE2. In the cropped B soil, the rank was also E1 > E2 > EE2. Ranks could not be established for the forested A soil since the data for E1, E2, and EE2 could not be fit with confidence. In the effluent equilibration system, the sorption rank from highest to lowest in the cropped A soil was E1 > EE2 > E2. In the cropped B soil the rank was E1 > E2 > EE2. The estrogen sorption ranks within each soil are somewhat surprising. The log K_{ow} values cited in this study were 3.43 for E1, 3.94 for E2, and 4.15 for EE2. Based on these log K_{ow} values, EE2 should exhibit the most sorption, followed by E2 and then E1 (Hildebrand et al., 2006). Results from this study did not exhibit a sorption trend with estrogen specific log K_{ow} values cited above.

Sorption Differences between Equilibration Solutions

Sorption differences between equilibrating solutions were only seen when the cropped B soil was used. For all three estrogens, there was a statistically significant difference in sorption between the pure water equilibration solution and the effluent equilibration solution. The effluent equilibration solutions had lower K_d and K_{oc} values (Table 4-5 to 4-7 and Tables 4-9 to 4-11). It was hypothesized that for all of the soils and estrogen compounds, sorption would decrease when the effluent equilibration solution was used. A study by Stumpe and Marschner (2007) documented a decrease in E2 sorption onto varying soils in the presence of a wastewater equilibration matrix. They predicted that the decrease in E2 sorption was the result of soluble organics present in the wastewater. Stanford et al. (2010) also observed a decrease in E2 sorption when a wastewater matrix was used. Yu et al. (2004) suggested that sorption of E1, E2, and EE2

onto different soils and sediments would decrease as a result of competitive sorption. The cropped B soil results agree with the limited studies that have been published on estrogen sorption in multi-sorbate systems with wastewater. A decrease in sorption between the two matrices was seen. There was no statistically significant difference between the equilibrating solutions when the cropped A soil was used (Tables 4-9 to 4-11). It is hypothesized that because the cropped A soil had a higher %TOC there were enough sorption sites and a high enough sorption capacity that potential sorption effects from the effluent matrix would have been negligible. While in the cropped B soil, because of the lower %TOC, competition with organics in the effluent and sorption to dissolved organics in the effluent would have been more pronounced.

Summary and Conclusions

The goal of this study was to evaluate E1, E2, and EE2 sorption in three soils varying in organic carbon content. Results from this study indicated that organic carbon content is a key mechanism for E1, E2, and EE2 sorption in soils, a mechanism that has been seen in other studies (Stumpe and Marschner, 2007; Hildebrand et al., 2006; Lee et al., 2003). The K_d and K_f values for each estrogen were larger in the soil with the higher %TOC and vice versa. Results from this study did not show a trend with estrogen specific log K_{ow} values. The rank in sorption from highest to lowest for all three soils was E1 > E2 > EE2.

Another key goal was to determine if there was a difference in E1, E2, and EE2 sorption when soils were equilibrated with pure water solutions versus effluent solutions. Based on published literature studies, it was hypothesized that for the three soils and three estrogen compounds, sorption would decrease when the effluent equilibration solution was used. As predicted, estrogen sorption did decrease in the multi-sorbate, wastewater systems when the

cropped B soil was used. This decrease was likely the result of competition with organics present in the effluent and possible sorption to dissolved organics in the effluent. There were no significant sorption differences between the two equilibrating solutions when the cropped A soil was used.

Finally, extraction recoveries (%) for E1, E2, and EE2 for all three soils and each equilibration solution were determined. Across the board, E1 had the highest extraction recovery, followed by EE2, and then E2. Extraction recovery values were lowest when the forested A soil (%TOC 6.55) was used. The extraction recoveries decreased with an increase in soil organic carbon content. Studies that extracted the sorbed phase instead of using the difference method achieved recoveries similar to the recoveries achieved in this study.

The K_d, K_f, and recovery values from this study can be used to increase our understanding of E1, E2, and EE2 sorption in soils and in turn better predict transport of these three compounds in the environment.

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Table 4-1. Soil characteristics for the three soils used during the sorption study.

	Hagerstown		
	Cropped		Forested
	A	B	A
Texture (%)			
Sand	26.2	40.6	38.5
Silt	46.0	33.2	44.6
Clay	27.8	26.2	16.9
pH (1:1 H_2O)	6.5	6.6	4.8
TOC (%)	1.03	0.26	6.55
TN (%)	0.13	0.07	0.48
CEC (meq/100g)	24.5	25.7	20.8

Table 4-2. Wastewater characterization of the Living Filter effluent. All values in the table are averages (N=3).

	LF Effluent
Estrogen Conc. (ng L^{-1})	
17β-Estradiol	9.3 (±7.0)
Estrone	23.8 (±12.3)
17α-Ethynylestradiol	4.7 (±1.6)
DOC (mg L^{-1})	3.66 (±0.09)
TN (mg L^{-1})	6.35 (±0.04)

Table 4-3. Linear and Freundlich fit parameters for the cropped A and cropped B soils shaken with the pure water equilibration solution.

	Estrogen- rep	**K_d**	**R^2**	**K_f**	**β**	**R^2**
Cropped A	E1-1	36.08	0.72	66.13	0.74	0.85
	E1-2	52.90	0.91	79.29	0.82	0.96
%TOC = 1.03	E1-3	35.53	0.87	63.68	0.78	0.94
	E2-1	47.68	0.86	54.04	0.95	0.93
	E2-2	37.70	0.71	56.25	0.73	0.85
	E2-3	34.68	0.85	50.23	0.77	0.93
	EE2-1	24.07	0.66	50.10	0.73	0.80
	EE2-2	45.26	0.89	55.68	0.93	0.95
	EE2-3	23.30	0.97	68.14	0.37	0.98
Cropped B	E1-1	9.94	0.87	6.42	1.14	0.90
	E1-2	9.10	0.87	5.90	1.17	0.92
%TOC = 0.26	E1-3	10.52	0.93	4.26	1.36	0.95
	E2-1	7.54	0.91	6.17	1.06	0.93
	E2-2	6.35	0.88	7.44	0.95	0.93
	E2-3	7.84	0.95	4.79	1.20	0.97
	EE2-1	6.62	0.93	5.28	1.06	0.95
	EE2-2	5.84	0.88	5.67	1.02	0.93
	EE2-3	7.32	0.90	2.86	1.35	0.94

Table 4-4. Linear and Freundlich fit parameters for the cropped A and cropped B soils shaken with the Living Filter effluent equilibration solution.

	Estrogen (rep)	**K_d**	**R^2**	**K_f**	**β**	**R^2**
Cropped A	E1-1	44.62	1.00	57.25	0.95	0.99
	E1-2	53.34	0.99	59.14	0.94	0.99
%TOC = 1.03	E1-3	46.11	0.93	61.94	0.80	0.97
	E2-1	43.87	0.74	44.58	0.61	0.54
	E2-2	47.82	0.98	50.40	0.68	0.96
	E2-3	38.64	0.66	51.29	0.94	0.62
	EE2-1	36.26	0.98	41.41	0.89	0.99
	EE2-2	59.31	0.99	41.95	1.28	0.97
	EE2-3	35.70	0.82	54.74	0.62	0.52
Cropped B	E1-1	5.95	0.90	7.37	0.80	0.96
	E1-2	4.01	0.74	9.84	0.80	0.84
%TOC = 0.26	E1-3	4.20	0.82	9.90	0.95	0.88
	E2-1	3.82	0.89	7.99	0.78	0.96
	E2-2	2.64	0.87	15.31	0.51	0.95
	E2-3	3.49	0.87	8.67	0.77	0.91
	EE2-1	3.74	0.81	8.22	0.80	0.92
	EE2-2	2.73	0.64	9.23	0.73	0.75
	EE2-3	3.26	0.79	6.57	0.86	0.85

Table 4-5. Average K_d values from linear fit for estrone (E1).

	A	B
Pure Water	41.50 *	9.85 * +
Effluent	48.02 *	4.72 * +

* : indicates a statistically significant difference between soils A and B. + : indicates a statistically significant difference between the two equilibration solutions, pure water and effluent.

Table 4-6. Average K_d values from linear fit for 17β-estradiol (E2).

	A	B
Pure Water	40.02 *	7.24 * +
Effluent	43.44 *	3.32 * +

* : indicates a statistically significant difference between soils A and B. + : indicates a statistically significant difference between the two equilibration solutions, pure water and effluent.

Table 4-7. Average K_d values from linear fit for 17α-ethynylestradiol (EE2).

	A	B
Pure Water	30.88	6.59 +
Effluent	43.76 *	3.24 * +

* : indicates a statistically significant difference between soils A and B. + : indicates a statistically significant difference between the two equilibration solutions, pure water and effluent.

Table 4-8. Average K_f values from Freundlich fit.

	E1	E2	EE2
Pure Water			
Cropped A	69.70	53.51	57.97
Cropped B	5.53	6.13	4.60
LF Effluent			
Cropped A	59.44	48.76	46.03
Cropped B	9.04	10.66	8.01

Table 4-9. Average K_{oc} values for estrone (E1). K_{oc} values calculated using K_d values and the fraction of organic carbon (f_{oc}) specific to each soil.

	A	B
Pure Water	4,029	3,788 $^{+}$
Effluent	4,662 *	1,815 $^{* +}$

* : indicates a statistically significant difference between soils A and B. + : indicates a statistically significant difference between the two equilibration solutions, pure water and effluent.

Table 4-10. Average K_{oc} values for 17β -estradiol (E2). K_{oc} values calculated using K_d values and the fraction of organic carbon (f_{oc}) specific to each soil.

	A	B
Pure Water	3,885	2,784 $^{+}$
Effluent	4,217 *	1,275 $^{* +}$

* : indicates a statistically significant difference between soils A and B. + : indicates a statistically significant difference between the two equilibration solutions, pure water and effluent.

Table 4-11. Average K_{oc} values for 17α-ethynylestradiol (EE2). K_{oc} values calculated using K_d values and the fraction of organic carbon (f_{oc}) specific to each soil.

	A	B
Pure Water	2,997	2,535 +
Effluent	4,248	1,247 +

* : indicates a statistically significant difference between soils A and B. + : indicates a statistically significant difference between the two equilibration solutions, pure water and effluent.

Table 4-12. Average estrogen extraction recoveries (% ± SD) from each soil and equilibration solution.

	E1	E2	EE2
Pure Water			
Cropped A	94.7 ± 8.5	37.9 ± 4.3	67.1 ± 6.4
Cropped B	49.6 ± 5.7	40.7 ± 5.6	47.2 ± 6.2
Forested A	47.1 ± 7.6	13.2 ± 2.9	28.5 ± 6.4
LF Effluent			
Cropped A	97.8 ± 8.3	26.5 ± 2.8	61.5 ± 6.0
Cropped B	51.9 ± 7.9	39.1 ± 8.2	48.8 ± 8.5
Forested A	36.6 ± 5.4	15.0 ± 8.2	20.7 ± 3.5

Chapter 5

Quantification of Estrogen Hormones in Ground and Surface Waters Sampled from North Central and Central Pennsylvania

Abstract

The presence of endocrine disrupting compounds (EDCs) in groundwater and surface water has been linked to human and animal waste disposal methods including land application of manure, wastewater irrigation, leaking septic systems and direct stream discharge of wastewater effluent. Across Pennsylvania, all of these disposal methods are utilized in some capacity. This study was designed to evaluate the possible presence of estrogen hormones, one class of EDCs, in two different geographic locations in Pennsylvania: the Sinnemahoning Creek Watershed located in north central Pennsylvania and the Penn State Living Filter Wastewater Irrigation Site located in central Pennsylvania. If detected, the goal was to quantify and establish estrogen baseline data for 17β-estradiol (E2) and estrone (E1), two natural estrogens, and 17α-ethynylestradiol (EE2), the synthetic estrogen in oral contraceptives. Fourteen groundwater well locations were sampled at the Living Filter. Triplicate 1-L samples were taken at each location. Twenty locations throughout the Sinnemahoning Creek Watershed were sampled, and these locations represented private water wells, public supply wells and surface waters. A ^{13}C internal standard was added to each sample immediately after collection and was used to track the recovery of the estrogens during processing and analysis. At the Living Filter, all three compounds were detected in groundwater well samples. However, all three estrogens were not quantified. E1 was quantified in all 14 wells, and the concentrations ranged from 2.19 to 18.79 ng L^{-1}. Well W-6 was the only well that had concentrations of E2 above the detection limit and above the limit of quantification, 7.43 ng L^{-1}. In all 14 wells, concentrations of EE2 were either

not detected (ND) or were quantified in trace amounts. In the Sinnemahoning Creek Watershed, both E1 and E2 were detected in the water samples. However, while E1 was quantified in all but one location (ranging from 9.86 to 37.11 ng L^{-1}), E2 was quantified in only two of the twenty locations (23.02 and 32.47 ng L^{-1}). The synthetic estrogen, EE2, was ND in all samples.

Introduction

Natural and synthetic estrogen hormones have been detected and quantified in waters across the United States and the world. Estrogen presence in surface water environments has been well documented (Rief et al., 2012; Barel-Cohen et al., 2006; Shappell, 2006; Beck et al., 2005; Kolpin et al, 2002). Less abundant, are studies that have quantified estrogens in waters from drinking water plants (DWPs) (Benotti et al., 2009; Kim et al., 2007; Rodriguez-Mozaz et al., 2004) and groundwaters (Bartelt-Hunt et al., 2011; Karnjanapiboonwong et al., 2011; Vulliet and Cren-Olivé, 2011; Loos et al., 2010; Hohenblum et al., 2004; Wicks et al., 2004). Both of these sources are linked directly to human consumption. It is critical that consumptive water sources are analyzed for estrogen hormones because studies have shown that environmental estrogen exposure can not only affect wildlife at concentrations as low as 1 ng L^{-1} (Purdom et al., 1994) but could also pose a potential threat to humans (Dickerson and Gore, 2007; Matthews et al., 2000).

Researchers that analyzed DWP waters did not detect estrogens in samples collected throughout the DWP distribution system or in finished water sampled, even though estrogens were quantified in surface waters upstream of the plants (Benotti et al., 2009; Kim et al., 2007; Rodriguez-Mozaz et al., 2004). Benotti et al. (2009) analyzed 19 U.S. DWPs and their source waters for 17β-estradiol (E2), estrone (E1) and 17α-ethynylestradiol (EE2). They quantified all three estrogens in source waters at maximum concentrations of 17, 0.9, and 1.4 ng L^{-1} for E2, E1, and EE2 respectively. However, for all three estrogens, all distribution samples and finished water samples had concentrations below the max reporting level (MRL). Their work also showed that different treatment technologies could enhance estrogen removal: chlorination removed up to 95% and ozone treatment removed up to 99% of estrogens from drinking waters.

Unlike the drinking water studies, estrogens (E1, E2 and EE2) have been detected in groundwaters (Bartelt-Hunt et al., 2011; Karnjanapiboonwong et al., 2011; Vulliet and Cren-Olivé, 2011; Loos et al., 2010; Hohenblum et al., 2004). Estrogen groundwater concentrations were found in the parts per trillion range (ng L^{-1}). Their presence in groundwater has been linked to a number of different sources: septic systems (Phillips et al., 2015; Shaider et al., 2014; Barber et al., 2009; Standley et al., 2008; Swartz et al., 2006), effluent irrigation and recharge basins (Wilson and Jones-Lepp, 2013; Karnjanapiboonwong et al., 2011; Mahjoub et al., 2011), and agricultural practices (Shrestha et al., 2012; Bartelt-Hunt et al., 2011; Fine et al. 2003).

Most of the studies focused on the transport of estrogens from septic systems to groundwater sources have been done on Cape Cod, MA, an area where septic tanks are abundant. Swartz et al. (2006) quantified E1 and E2 in aqueous phase septic tank samples and found that E1 concentrations ranged from below instrument detection limits (BDL) to 120 ng L^{-1}, and E2 concentrations ranged from BDL to 45 ng L^{-1}. They also quantified E1 (23 ng L^{-1}) and E2 (3 ng L^{-1}) in wells down gradient from septic leach fields. As the distance from the septic leach fields increased, breakdown and attenuation of E2 increased, while E1 was found to be more persistent. Standley et al. (2008) quantified E1 in groundwater fed, residential ponds on Cape Cod. They sampled ponds in low density populated areas and high density populated areas. In the high density areas, E1 levels reached 3 ng L^{-1}, and E2 was not detected. Surprisingly, E2 was detected in one of the seven low density ponds (2.2 ng L^{-1}), but this one hit was attributed to animals and humans frequenting the area, not septic input.

Reuse of wastewater effluent to irrigate crops and recharge groundwater is another potential contamination source. These reuse practices are especially prevalent in arid regions where freshwater resources are limited. Karnjanapiboonwong et al. (2011) quantified E1, E2 and

EE2 in groundwater at a wastewater irrigation site located in west Texas. The 6,000 acre site has received 70+ years of effluent irrigation at an average rate of 13 million gallons per day (MGD). Groundwater concentrations ranged from 12 to 147 ng L^{-1} for E2, 62 to 79 ng L^{-1} for E1, and 11 to 230 ng L^{-1} for EE2. At a site in Arizona, effluent is injected into recharge basins. Wilson and Jones-Lepp (2013) quantified E1 in a groundwater monitoring well near the injection site. E1 concentrations ranged from ND to 1.7 ng L^{-1}. E1 was not detected in any of the monitoring wells located further from the injection site, suggesting that as distance from the site increased, the estrogen levels decreased.

Agricultural settings are also a source of estrogen groundwater contamination. Contamination can result from leaking storage lagoons and from land application of manure (Shrestha et al., 2012; Bartelt-Hunt et al., 2011; Thompson et al., 2009; Fin et al., 2003). Shrestha et al. (2012) determined that E2 conjugates can transport from the soil surface to groundwater sources, and concluded that if hydrolyzed during transport, this would equate to a groundwater E2 concentration of 258 ng L^{-1}. Thompson et al. (2009) also looked at manure applied soils and found extensive E2 transport at depth in the soil. This was surprising since E2 is hydrophobic and has a high organic carbon sorption affinity. They attributed the E2 presence at depth to transport via dissolved organic carbon (DOC). Finally, Bartelt-Hunt et al. (2011) quantified E1 in agriculture storage lagoons ranging from 330 to 3600 ng L^{-1}, and they were able to quantify E1 in nearby groundwater wells ranging from 40 to 390 ng L^{-1}.

In a report published by the United States Geological Survey (USGS) in cooperation with the Pennsylvania Department of Environmental Protection (PaDEP), E1, E2 and EE2 were quantified in streams and stream bed sediments throughout Pennsylvania. In the six groundwater wells studied, they quantified four pharmaceuticals and organic compounds, none of which were

E1, E2, or EE2 (Rief et al., 2012). The goal of this study was to quantify E1, E2, and EE2 in groundwaters sampled from two different areas in Pennsylvania: the Living Filter in central PA and the Sinnemahoning Creek Watershed in north central PA. Each site represents at least one potential groundwater contamination source, as described above. The Living Filter is a wastewater irrigation site where 100% of The Pennsylvania State University campus effluent has been irrigated for 25+ years. Previous research has quantified E1, E2, and EE2 in soils at the site (Woodward et al., 2014), but published studies that have quantified estrogen hormones in the groundwater wells on site are limited.

The second sampling area, the Sinnemahoning Creek Watershed, served as a collaborative study with the Cameron County Conservation District. Samples collected in the Sinnemahoning Creek Watershed were diverse: domestic wells, public supply wells, springs, wastewater treatment plant discharge, and DWP waters. These samples have possible inputs from septic tanks and agricultural practices. This portion of the study had three goals: sample wells and surface waters near and around the rural town of Emporium, PA, incorporate high-school aged students in the sampling process, and educate students on the issue of emerging contaminants. Direct comparisons of the results cannot be made between the two sites since the scales, management, geology, soils, and groundwater are so different, but general conclusions can be made about each individual site. Overall, these two case studies provide a general reconnaissance of estrogen presence in north central and central Pennsylvania groundwaters. Results from this study can be used to enhance management of Pennsylvania waters and prevent potential human health risks.

Background Site Descriptions

Living Filter

Penn State's Living Filter is a wastewater spray irrigation site located in Centre County. It is approximately 3.2 km from The Pennsylvania State University campus (University Park, PA) (Figure 5-1). The Living Filter is divided into two sites, the Astronomy site and the Gamelands site. The two sites together encompass over 600 acres, and these acres are a mix of cropped, forested and grassland areas. The crops grown on site rotate mainly between wheat (*Triticum aestivum* L.*)* and corn (*Zea mays* L.), and the forested area consists of mature hardwood trees. Both sites receive wastewater from the Penn State University campus which serves around 46,848 students, 46% female, and 12,839 faculty and staff, 48% female (Penn State University, 2015). Before the wastewater is irrigated at the Living Filter, it undergoes primary and secondary treatment at the University Park Wastewater Treatment Plant. After treatment, the wastewater is pumped 4 km to the two irrigation sites and is irrigated at a weekly rate of 5 cm $acre^{-1}$. In recent years, the average amount applied is around 2.5 MGD.

Geologically, the Living Filter is located in the Ridge and Valley physiographic province of Pennsylvania. This province is dominated by sandstone ridges and carbonate valleys. The Living Filter is located in the Nittany Valley, a predominately carbonate valley. The bedrock at the site is part of the Gatesburg Formation (Cambrian). Figure 5-2 shows that approximately 63% of the site is the Mines member of the Gatesburg Formation, and 37% of the site is the Gatesburg Lower members. Both members are made up of dolomite, limestone, chert, and sandstone in laminated, thick bedded, and massive forms. The rolling topography seen at the site, also known as karst topography, is a product of the geology. Karst forms from the dissolution and/or collapse of carbonate bedrock beneath the surface, forming sinkholes at the surface and

fracture pathways in the bedrock. Sinkholes can act as direct conduits between the surface and groundwater (Ciolkosz et al., 1995).

Groundwater at the site generally flows in the north east direction at the Gamelands site and in the south east direction at the Astronomy site (Figures 5-3 and 5-4). For these flow maps, Surv-CADD was used to generate groundwater flow contours using groundwater elevation values at each well. The disparity in groundwater flow directions between the two neighboring sites can be explained using Figure 5-5. Regional groundwater flow comes into the Gamelands site from the north west direction and turns towards the north east direction in a trough like flow pattern towards the airport and Bellefonte. At the Astronomy Site a groundwater mound near the entrance directs flow radially, towards the trough heading north east and towards the Big Hollow drainage basin in the south west and south east direction.

Soils in the Nittany Valley are predominantly carbonate residuum soils. At the Living Filter, dolomite residuum soils make up the largest percentage of the soils at the site (80%). The site also contains sandstone residuum soils, weathered from thin sandy beds in the Gatesburg Formation. Four soil series make up a majority of the site: Hagerstown (36%), Hublersburg (38%), Morrison (17%), and Opequon (6%) (Figure 5-6).Table 5-1 describes each soil in further detail. The remaining 3% of the site is made up of Clarksburg silt loam and Nolin silt loam soils. Overall, the soils at the site are well drained, and except for the shallow Opequon soil, they are deep soils. The main textures are silt loam, silty clay loam and sandy loam.

Sinnemahoning Creek Watershed

The Sinnemahoning Creek Watershed is located in north central Pennsylvania (Figure 5-7). It includes portions of the counties Cameron, Clearfield, Clinton, Elk, McKean, and Potter. It is the largest tributary to the West Branch of the Susquehanna (Western Pennsylvania

Conservancy, 2010), and its headwaters begin in three tributary streams: Bennett Branch, Driftwood Branch, and First Fork Sinnemahoning Creek. The watershed is primarily rural, and includes four populated areas: Austin, Driftwood, Emporium, and Saint Mary's (Western Pennsylvania Conservancy, 2010). The land contains seven state parks and four state forests and is situation within the Pennsylvania Wilds. The population for the entire watershed is around 9,000 people. The sampling for this study was conducted within a 10 mile radius of downtown Emporium, a borough within Cameron County, PA. According to 2014 census data, the population of Cameron County is around 4,800 people, and the gender split is 49.5% male and 50.5% female (U.S. Census, 2015), and the population of Emporium is around 2,000 people.

The Sinnemahoning Creek Watershed is located in the Appalachian Plateau physiographic province of Pennsylvania. The watershed is within the Pittsburgh Low Plateau section and the Deep Valleys section (Western Pennsylvania Conservancy, 2010). The Pittsburgh Low Plateau is characterized by narrow, shallow valleys and old mine operations, and the Deep Valleys section is characterized by deep, steep sloped, shale valleys and sandstone ridges. The major formations within the watershed are the Huntley Mountain Formation (30%), Pottsville Formation (25%), Catskill Formation (20%), Shenango Formation through Oswayo Formation (12%), Allegheny Formation (7%), Glenshaw Formation (2.5%), Burgoon Sandstone (2%), Lock Haven Formation (1%), and the Casselman Formation (0.5%) (Figure 5-8). The sampling sites were located within four of these Formations: Lock Haven, Catskill, Huntley Mountain, and Shenango through Oswayo. A variety of Mississippian and Devonian aged rocks make up these four Formations: thin conglomerates, sandstones, siltstones, mudstones, gray shale, and red shale.

The groundwater data for this area is limited. Three wells in downtown Emporium are represented on the PaGWIS records through the PA DCNR website (Pennsylvania Department of Natural Resources, 2015). These wells have static water levels that range from 20 to 50 feet. Groundwater elevation values were established for these three points by subtracting the static water levels from the land elevations. The groundwater level in the downtown Emporium area is estimated to be somewhere between 1000 and 1037 feet above sea level. In addition, the USGS has a groundwater monitoring well at Sinnemahoning State Park, Cameron and Potter County. The monitoring well is approximately 30 miles (SE) from downtown Emporium, and it was drilled in the Catskill Formation. The reported groundwater level at the well is 985.8 feet above sea level (measured on April 25, 2014) using the NAVD 1988 reference (U.S. Geological Survey, 2015).

The Sinnemahoning Creek Watershed is 1,050 square miles. There are too many soil series mapped to describe the soils in detail for the entire watershed. However, only six of these series are associated with the sampling locations: Albrights, Atkins, Basher, Buchanan, Leck Kill, and Philo. These six soils are described in more detail in Table 5-2. The parent material varies between these six series. The Atkins, Basher, and Philo series are alluvium, the Albrights and Buchanan series are colluvium, and Leck Kill is residuum. All of the soils are deep, but the drainage varies between series: from poorly drained to well drained. The textures range and include fine and coarse soils: silt loam, loam, gravelly loam, and channery silt loam. Land use includes crops, pastures and mixed hardwood forests.

Materials and Methods

Chemicals and Reagents

Three labeled estrogens were purchased for this study: 17β-Estradiol-13, 14, 15, 16, 17, 18-$^{13}C_6$ (99%) and Estrone-13, 14, 15, 16, 17, 18-$^{13}C_6$ (99%) from Cambridge Isotope Laboratories (Tewksbury, MA) and 17α-Ethynyl-$^{13}C_2$-estradiol (99%) from CDN Isotopes (Pointe-Claire, Quebec). LC-MS grade methanol (MeOH) and acetonitrile (ACN), HPLC grade methyl tert-butyl ether (MTBE) and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich (St. Louis, MO). All water used was ultra-pure deionized (18MΩ).

Sample Collection and Processing

Fourteen groundwater wells located at Penn State's Living Filter were sampled (April 21-29, 2015). The wells were sampled at both the Astronomy and Gamelands sites (Figure 5-9). Well depths, groundwater levels, and soils varied for each well (Table 5-3). Triplicate samples were collected at each well in 1-L amber glass bottles. A 100 µg L^{-1} solution containing all three labeled estrogens was made in MeOH, and each 1-L sample was spiked with 1 mL of this labeled solution (0.1 µg L^{-1} concentration of each labeled standard in each 1-L sample). The 42, 1-L samples were brought back to the Penn State facilities on ice and were stored in a dark refrigerator at 4˚C until they were extracted, within two weeks. All 42 samples were filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and processed through the solid-phase extraction (SPE) procedure described below.

Twenty locations throughout the Sinnemahoning watershed were sampled (April 25, 2014) (Figure 5-10). These sampling locations included both surface and groundwater sources. A number of sites had multiple sampling locations. Characterization of each sampling site is presented in Table 5-4. None of the personal wells sampled received any kind of water treatment.

The water collected provides a realistic proxy of the raw groundwater, with the pumping system being the only possible place for differences to occur between the groundwater and the water analyzed.

Triplicate samples were collected from each of the 20 locations in 1-L amber glass bottles. A 100 µg L^{-1} solution containing all three labeled estrogens was made in MeOH, and each 1-L sample was spiked with 1 mL of this labeled solution (0.1 µg L^{-1} concentration of each labeled standard in each 1-L sample). The 60, 1-L samples were brought back to the Penn State facilities on ice and were stored in a dark refrigerator at 4˚C until they were extracted, within one month. Results from prior degradation studies done in the lab suggested that sample degradation is limited within one month of storage in a dark refrigerator. All 60 samples were filtered through a 0.7 µm glass fiber filter (Pall Corporation, MI) and processed through the SPE procedure described below.

Solid Phase Extraction (SPE) Procedure

An SPE procedure was used to cleanup and concentrate each sample. The SPE method used was a modified version of the procedure provided in the Waters Application Notebook (Waters, 2008). An Oasis HLB Plus (Waters, MA) cartridge was pre-conditioned with 5 mL of MTBE, 5 mL of MeOH and 5 mL of water, followed by a loading phase of the 1-L water sample. The cartridge was washed with 5 mL of a 40:60 MeOH:water solution and 5 mL of water. This washing step removes some of the unwanted compounds in our sample that are not the analyte(s) of interest. Finally, the cartridge was eluted with 6 mL of a 90:10 MTBE:MeOH solution. The 6 mL of eluent was evaporated under a gentle stream of nitrogen gas, and each evaporated vial was brought to a 1 mL volume with 10:90 MeOH:water. All of the 1 mL samples were analyzed

using high performance liquid chromatography tandem mass spectrometry (LC-MS-MS) and the conditions described below.

Chromatography Conditions

The column was an XTerra MS C18 Column, 3.5 µm, 2.1 x 100 mm (Waters, MA). The Tandem MS used for quantification was a MicroMass Quattro micro API (Waters, MA). The mobile phase solvents were 0.6% NH_4OH in ACN (solvent A) and 0.6% NH_4OH in water (solvent B), and these two solvents were run in gradient mode. The initial mobile phase started with 10% solvent A and 90% solvent B, and increased linearly for five minutes to 90% solvent A and 10% solvent B. This was held for two minutes. From seven minutes to the end of the run, the gradient was dropped to 10% solvent A and 90% solvent B. The operating mode was ESI-. Flow rate was 0.25 mL min^{-1}, and the injection volume was 20 µl. External 6 to 8 point standard calibration curves were generated (1 to 1000 µg L^{-1}). Peak area outputs from samples were used against calibration curves to estimate sample concentrations.

Results and Discussion

Data Analysis

In this study, the limit of detection (LOD) refers to the lowest estrogen concentration that is statistically different from a blank (99% confidence) (Ripp, 1996). Each estrogen compound has its own specific LOD value. The measured LOD values for analyzed samples of the three estrogens in this study were 1.78, 2.16, and 0.48 µg L^{-1} for E2, EE2, and E1 respectively. These values were determined using a calibration curve method (Ripp, 1996). Average responses (N=7) for multiple standards ranging from 0 to 10 µg L^{-1} were used to create calibration curves (R^2 values between 97-99 %) for each estrogen. The slope of each curve (b) and the standard

deviation of the responses (S_a) were then used to calculate the LOD specific to each compound using the following equation:

$$LOD = 3 (Sa/b) \qquad 5.1$$

If a sample has a concentration below the LOD that does not mean that the compound concentration in the sample is zero. It only means that the sample concentration is somewhere between zero and the LOD. It is important to note that during the extraction process, field water samples were concentrated by three orders of magnitude in order to be read on the LC-MS-MS. For E2, EE2, and E1, the LOD values measured correspond to the following field concentrations: 1.78, 2.16 and 0.48 ng L^{-1}. In this study, samples with concentrations below the LOD were indicated with the letters ND, not detected.

The limit of quantification (LOQ) refers to the lowest estrogen concentration that can be quantified with a degree of confidence (Ripp, 1996). Just like LOD, each estrogen compound has its own specific LOQ value. The measured LOQ values for the analyzed samples of the three estrogens in this study were 5.94, 7.2, and 1.6 µg L^{-1} for E2, EE2, and E1 respectively. These values were also calculated using the calibration method and the following equation:

$$LOQ = 10 (Sa/b) \qquad 5.2$$

Again, it must be noted that during the extraction process, field water samples were concentrated by three orders of magnitude in order to be read on the LC-MS-MS. Therefore, for E2, EE2 and E1, the LOQ values correspond to the following field concentrations: 5.94, 7.2, and 1.6 ng L^{-1}. In general, if detected values fall between the LOD and the LOQ, the values are referred to as trace amounts and must be taken with caution. They are not quantifiable numbers, and they cannot be used statically in order to draw major conclusions. In this study, detected samples with average concentrations between the LOD and LOQ were indicated by the letter T, trace concentrations.

Finally, it must be noted that although triplicate samples collected at each sampling location were collected at the exact same time, high variability occurred between samples. A statistic used to describe variability is the coefficient of variation (CV). To calculate CV, the standard deviation is divided by the mean (Zar, 2010). CV values are often expressed as percentages; the ratio of the standard deviation to the mean is multiplied by 100. In this study, any set of samples with a CV higher than 50% was considered high and was marked in Tables 5-5 and 5-6 with the symbol †.

^{13}C Recovery

As described in the "Sample Collection and Processing" section, the 1-L samples collected from both sites were spiked with a standard solution that contained ^{13}C labeled E1, E2 and EE2. The labeled material was used to track sample recovery throughout extraction and analysis. All of the samples collected from both sites exhibited 100% recovery of the ^{13}C internal standard except for the set of finished waters collected at the Emporium Drinking Water Plant. This means that no concentration adjustments had to be made for E1, E2, or EE2 to account for sample loss between collection and analysis. It is believed that the inability to recover the internal standard in the DWP finished water samples was related to chlorination treatment within the DWP distribution system. Chlorination is known to remove estrogens during the treatment process (Benotti et al., 2009).

Living Filter

All three estrogen hormones were detected in the Living Filter groundwater wells (Table 5-5), but all three estrogens were not quantified. E1 was quantified in all 14 wells, and the concentrations ranged from 2.19 to 18.79 ng L^{-1}. Only one well had concentrations of E2 above the detection limit and above the LOQ, well W-6. The concentration of E2 in well W-6 was 7.43

ng L^{-1}. In all 14 wells, concentrations of EE2 were either ND or were quantified in trace amounts as T.

The highest concentrations (10 to 20 ng L^{-1}) of E1 were measured in the following wells: W-2, W-6, G-10, and F-3 (Figure 5-11). Of the three wells located on the Gamelands site, wells W-2 and W-6 are located at topographic low spots. Overland flow of spray irrigation onto these well locations could be greater than the other wells. This increase in irrigation runoff could have increased the estrogen levels seen in the wells. Well W-6 is also considered the "worst case scenario" well because all of the groundwater at the Gamelands site is flowing towards that well (Figure 5-5). Well G-10 was drilled in a Morrison sandy loam soil, and the bedrock below the soil has a high permeability. It also sits directly in the center of the groundwater flow path. The combination of the sandy soil texture and the high permeability bedrock would promote faster infiltration and percolation of irrigation water through the profile. As documented by Swartz et al. (2006), E1 is more mobile. These two factors could explain the high estrone levels at G-10. On the Astronomy Site, well F3 could have a high average estrone concentration for two reasons: it is also located at a topographic low point on site and out of all 14 wells sampled; it is the shallowest well (36.5 m). Barnes et al. (2008) found a correlation with concentration of pharmaceuticals and well depth. Shallow wells had higher concentrations of organics. This could explain the high E1 concentration at well F3. It is also believed that overland flow from the neighboring Spearly farm may impact well F3. At the Spearly farm, manure is land applied to crops and a small number of animals are housed.

The only well that had a quantifiable concentration of E2 was well W-6 on the Gamelands site. The concentration of E2 at this well was 7.43 ng L^{-1}. As mentioned above for E1, this well is located in a topographic low spot, and it is located within the groundwater flow

path. A study by Thompson et al. (2009) noted that E2 reached the water table (groundwater depth ranged from 1.65 to 2.11 m) in wells near manure applied soils, and hypothesized that this occurred as a result of transport of DOC. Another study by Wicks et al. (2004) quantified E2 in karst aquifer springs ranging from 13 to 80 ng L^{-1}. These springs were direct conduits between groundwater and surface water, and they were located mainly near forested areas. A few springs were also located near small towns and home septic systems. One could argue that the levels measured by Wicks et al. (2009) represented natural background of E2 in a karst system. The concentration of E2 measured at the Living Filter was below the range reported by Wicks et al. (2009). It could be hypothesized that the concentration of E2 measured at the Living Filter was simply background E2 and not the product of effluent irrigation.

Most of the studies that have analyzed groundwater well samples for E1, E2, and EE2 have detected and quantified E1 (Wilson and Jones-Lepp, 2013; Bartelt-Hunt et al., 2011; Karnjanapiboonwong et al., 2011; Swartz et al., 2006). Only a handful of studies have detected and quantified E2 and EE2 (Hohenblum et al., 2004; Karnjanapiboonwong et al., 2011; Swartz et al., 2006; Wicks et al., 2004). Karnjanapiboonwong et al. (2011) quantified all three estrogens in groundwater wells at a wastewater irrigation site in west Texas. Concentrations ranged from 12 to 147 ng L^{-1} for E2, 62 to 79 ng L^{-1} for E1, and 11 to 230 ng L^{-1} for EE2. At the Living Filter, all three estrogens had concentrations well below the levels reported by Karnjanapiboonwong et al. (2011). Wilson and Jones-Lepp (2013) quantified E1 and E2 in treated wastewater used to recharge groundwater, but they were only able to quantify E1 in one of the monitoring wells close to the injection site E1 (ND to 1.7 ng L^{-1}). At the Living Filter, the quantified E1 concentrations were higher than the concentrations reported by Wilson and Jones-Lepp (2013).

There is no real consensus in the literature regarding background concentrations; variability in E1 and E2 concentrations between studies is high.

Estrogen concentration data for the Living Filter groundwater wells are, for the most part, in agreement with estrogen groundwater concentrations reported in the literature. The only two estrogens quantified in wells at the Living Filter were E1 and E2. These are the two naturally produced estrogens, produced by humans and animals. The site is open to the public and wildlife is abundant at the site. The E1 and E2 levels could be a product of natural inputs. EE2 was not quantified in any of the wells on site. This is encouraging for the site because EE2 is the synthetic estrogen found in oral contraceptive and is the most potent estrogen of the three. The absence of EE2 and the low levels of E1 and E2 in the wells, suggests that the soil at the Living Filter successfully filters out estrogens from the effluent and prevents their transport to groundwater sources.

Sinnemahoning Creek Watershed

In the samples collected from the Sinnemahoning Creek Watershed only two estrogens were detected and quantified: E1 and E2 (Table 5-6). E1 was quantified in waters collected from 19 of the 20 sampling locations, and the concentrations ranged from ND to 37.11 ng L^{-1} (Figure 5-12). Only two locations had concentrations of E2 above the LOD and above the LOQ, samples W08607 and W08622. The concentrations of E2 ranged from ND to 32.47 ng L^{-1}. EE2 was ND in all of the water samples collected. Results were subdivided into four categories: private wells, springs, wastewater treatment plant samples and drinking water supplies.

Private Wells

Twelve private wells were sampled and analyzed for E1, E2 and EE2. Concentrations of E1 ranged from 9.86 to 25.01 ng L^{-1}. Only one of the 12 wells had a quantifiable concentration

of E2 (32.47 ng L^{-1}). None of the 12 wells had detectable levels of EE2. The potential sources of estrogens into these private well supplies were: backyard septic systems, direct input of animal waste (uncovered well), and transport from surface agricultural practices. All of the private wells had backyard septic systems and two of the wells were located in areas with grazing cattle and manure excretions onto the soil surface (W08607 and W08608). Of the two wells located in the vicinity of cattle, one of the wells was poorly-covered and hand-dug (W08607). The Sinnemahoning Creek results are in agreement with work done by Swartz et al. (2006). They quantified E1 (maximum concentration 23 ng L^{-1}) and E2 (maximum concentration 3 ng L^{-1}) in wells down gradient from septic leach fields, and E1 was more persistent. For E2, as the distance from the septic leach fields increased, E2 concentrations decreased, and they attributed this to degradation and attenuation of E2. The persistence of E1was also seen in the Sinnemahoning Creek private well samples, and E1 concentrations were similar to the concentrations reported by Swartz et al. (2006). Unfortunately, information related to the homeowner's septic was unavailable. Therefore, direct linkages to septic cannot be made at this time. The one hit for E2 in well W08607 could be from animal waste or runoff from the nearby, upslope cattle grazing area. Both of these scenarios are likely since this well was poorly-covered and open to natural inputs.

Springs

Two springs were sampled in the watershed, W08609 and W08611. E1 was the only estrogen quantified in the springs at concentrations of 15.72 and 17.64 ng L^{-1}. Spring inputs are not always clear and spring waters can interact with a variety of contaminant sources. Wicks et al. (2004) quantified E2 in Missouri springs and found concentrations between 13 and 80 ng L^{-1}. E1 is the degradation product of E2, and environmental concentrations are usually in the same

order of magnitude for the two estrogens. As mentioned previously, the Wicks et al. (2009) study could be used as a background proxy. If used as background, a rough justification could be made that the E1 spring concentrations measured in the Sinnemahoning Creek Watershed were also a reflection of natural background.

Emporium Sewage Treatment Plant

Three samples were taken from the wastewater treatment plant: upstream of the effluent discharge point, at the effluent discharge point, and downstream of the effluent discharge point. The effluent discharges into the Driftwood Branch of Sinnemahoning Creek, a tributary of Sinnemahoning Creek. The average concentrations of E1 were 22.55 ng L^{-1} for the upstream sampling point, 19.38 ng L^{-1} for the discharge point, and 37.11 ng L^{-1} for the downstream sampling point. E2 was quantified in the upstream samples, 23.02 ng L^{-1}. EE2 was ND in all of the sampling locations. In most studies, the discharge point (W08623) would have had the highest concentration of all three estrogens, and as the discharge effluent mixed with the stream water, the concentration of each hormone would decrease as a result of dilution (Johnson et al., 2000). Johnson et al. (2000) looked at European treatment plants and compiled a list of studies that quantified E1, E2, and EE2 in effluent discharge. The concentrations of E1 in the effluent ranged from ND to 54 ngL^{-1}, E2 ranged from ND to 12 ng L^{-1}, and EE2 ranged from ND to 4.5 ng L^{-1}. The Emporium Sewage Treatment Plant had an E1 effluent discharge concentration within the range reported by Johnson et al. (2000). Esteban et al. (2014) quantified estrogens in surface water samples associated with high WWTP inputs. They measured E1 concentrations in surface streams around 20 ng L^{-1}, and they cited additional studies that quantified E2 in U.S. surface streams at levels ranging from 3.76 to 13 ng L^{-1}. The Sinnemahoning upstream and downstream samples both had E1 concentrations in the same range as those reported by Esteban

et al. (2014). However, the Sinnemahoning upstream sample had a slightly higher E2 concentration than the levels reported by Esteban et al. (2014) for other U.S. streams.

It must be noted that the sampling upstream and downstream of the Emporium Sewage Treatment Plant was done on the bank of the stream, at multiple locations, and by three different supervised students. Ideally, samples should have been collected in the middle of the stream. At the bank of the stream, the likelihood of natural animal inputs is higher than in the middle of the stream. A definitive reason for the higher concentrations of E1 and E2 upstream and downstream of the discharge point cannot be determined, but it is hypothesized that the E1 and E2 concentrations measured were more than likely the result of animal and even human inputs. The possibility of natural inputs and high variability (CV > 50%) exhibited between samples, could explain why the results do not indicate that stream water dilution of the effluent occurred. Overall, the WWTP data suggested that the Emporium Sewage Treatment Plant was not adding to the stream estrogen load beyond what was naturally present in the stream.

Drinking Water Supplies

Three locations associated with drinking water supplies were sampled: a public supply well (W08606), Emporium DWP intake water (W08624), and Emporium DWP finished water (W08625). The public supply well is located in Sizerville State Park near the West Branch Cowley Run stream. The DWP intake water is sourced from the Driftwood Branch of the Sinnemahoning Creek. Technologies within the DWP include chlorine disinfection, coagulation and flocculation, sedimentation, and filtration. The DWP finished water serves the Emporium Borough and parts of Shippen township, total population around 1500.

The only estrogen detected in any of the samples was E1. It was detected in the public supply well at an average concentration of 20.43 ng L^{-1}, and it was detected in the DWP intake

water at an average concentration of 9.94 ng L^{-1}. Benotti et al. (2009) analyzed 19 U.S. DWPs and their source waters for E1, E2, and EE2. They quantified all three estrogens in source waters at maximum concentrations of 17, 0.9, and 1.4 ng L^{-1} for E2, E1, and EE2 respectively. However, for all three estrogens, all distribution samples and finished water samples had concentrations below the max reporting level (MRL). The E1 concentration for the Emporium DWP intake water (source) is higher than what was reported four source waters in Benotti et al. (2009), but it is in agreement with E1 source water values (up to 22 ng L^{-1}) reported by Rodriguez-Mozaz et al. (2004). As a result of poor ^{13}C recovery in the DWP finished water samples, likely from the chlorination process (Benotti et al., 2009), E1, E2, and EE2 concentration values could not be calculated for these samples.

Outreach Effort

An additional goal of the Sinnemahoning Creek Watershed study was to incorporate high school aged students in the sampling process and teach them about water quality science and the issue of emerging contaminants. This study was a collaborative effort with the Cameron County Conservation District and Penn State's Extension Water Resources Program. Prior to participating in field work, students met several times with Jim Clark, a PSU extension water resources educator, to learn about the issue of emerging contaminants and the study that they would be involved in. On the day of sampling, a small group of students from Cameron County High School participated in the afternoon collection (April 25, 2014). After touring the Emporium Sewage Treatment Plant, the students helped collect samples downstream of the effluent discharge point. Students were instructed on sample collection procedures and the use of ^{13}C as an internal standard. Students also got the chance to tour the Emporium Drinking Water Plant, and at the DWP they had another chance to assist in sampling the intake and finished

waters. Two weeks later (May 12, 2014), a subset of the students that helped sample, traveled to University Park to visit two water labs on campus. The students toured the Penn State Agricultural and Analytical Services Lab and the lab of Dr. Jack Watson. Students were shown the estrogen extraction procedure, and they received hands on experience in carrying out an extraction. They were also introduced to a high performance liquid chromatography (HPLC) system. They were walked through how an HPLC works, what an HPLC column is, and how to interpret output data from an HPLC. Students were given PowerPoint slides from Jim Clark and the Watson lab that described the issue of emerging contaminants, the overall goal of the study, how estrogens are extracted and analyzed, and results from this study. At a future community board meeting, Jim Clark and any of the students that have not graduated yet, will present this Power Point to community members in Cameron County. Hopefully the presentation will increase community awareness on the issue of emerging contaminants and encourage future sustainable management of Pennsylvania waters.

Risk Communication

Estrogen hormones are on an Environmental Protection Agency contaminant candidate list. This means that their presence in the environment is well documented and they pose a potential risk to human health, but they are not regulated currently under the Safe Drinking Water Act. In addition to the lack of government regulatory levels, there are few guides on how to interpret and communicate environmental estrogen data. For this study, the I70 method presented in Vulliet and Cren-Olivé (2011) was used to evaluate the potential human health risks for estrogens that were quantified in drinking water sources (Table 5-7).

Results from both study sites, not including stream results, were scanned to identify the maximum estrogen levels quantified: the E1 max was 25.01 ng L^{-1} and the E2 max was 32.47 ng

L^{-1}. For both sites, all of the EE2 levels were either ND or detected at trace levels. To carry out the I70 method, the maximum detected EE2 trace level of 6.96 ng L^{-1} was used. It was assumed that human's consume, on average, 2 L day^{-1} of water. Therefore, the calculated daily water intake (DWI) for each estrogen was 50 ng day^{-1} for E1, 64.94 ng day^{-1} for E2, and 13.92 ng day^{-1} for EE2. Therapeutic dose (TD) levels range for each estrogen. The E2 therapeutic dose levels used in hormone replacement therapies (HRT) range from 0.025 to 2 mg day^{-1} (Johnson and Williams, 2004). For this study, the minimum TD was used, 0.025 mg day^{-1}. The EE2 therapeutic dose levels used in oral contraceptives range from 10 to 35 µg day^{-1}. Again, the minimum TD was used, 10 µg day^{-1}. No therapeutic dose was identified or used for E1 in this study. Using this data, the TD/DWI ratio was calculated (Table 5-7). This ratio equates to the total number of days that it would take for the DWI to equal one TD. Finally, the I70 values, the total amount ingested if drinking the DWI for 70 years, were calculated for each estrogen and are found in Table 5-7.

Some general statements can be drawn from the results in Table 5-7. The amount of EE2 ingested after 70 years of drinking water that contained the maximum EE2 concentration would be equal to the amount of EE2 ingested in 35.6 days of taking the therapeutic dose. The amount of E2 ingested after 70 years of drinking water that contained the maximum E2 concentration would be equal to the amount of E2 ingested in 66.4 days of taking the therapeutic dose. In both cases, the amount ingested daily via the DWI was much less than the daily amount present in a TD. To reach the daily EE2 TD level, a person would have to drink 1,436 L of water that contained the maximum EE2 concentration all in one day. To reach the daily E2 TD level, a person would have to drink 770 L of water that contained the maximum E2 concentration all in one day. Both scenarios are not plausible.

It is important to note that all of the calculations made above, assumed that everything ingested is metabolized by the body. In reality, estrogens move through the body in a few days and only 43% of what is ingested is actually metabolized (Johnson and Williams, 2004). The rest is excreted from the body as either free estrogens or more often as estrogen conjugates. Johnson and Williams (2004) estimated male and female excretion values for E1 and E2. Female excretion values for E1 ranged from 11.7 to 550 μg day^{-1}. Female excretion values for E2 ranged from 3.2 to 393 μg day^{-1}. The two ranges were estimated for menstruating females and pregnant females. Male excretion values of E1 ranged from 2.8-3.9 μg day^{-1}, and the male excretion value for E2 was estimated to be 1.8 μg day^{-1}. In terms of the natural estrogens E1 and E2, what both males and females would ingest based on the DWI (ppt range) listed in Table 5-7, would be much less than what is excreted naturally (ppb range) in one day. In addition, Shore and Shemesh (2003) estimated that 0.1 μg (100 ng) of estrogens, includes both E1 and E2, are consumed daily through food intake, mainly meat and dairy products. This food intake value for E1 and E2 is double what would be ingested based on the DWI.

The I70 method and estrogen estimates from other studies both show that the groundwater levels measured for E1 and E2 are a low potential health risk to humans. The EE2 levels in the groundwater are also a low potential health risk, at least for females taking oral contraceptive. Vulliet and Cren-Olivé (2011) stated that pregnant women should avoid any levels of EE2. Therefore, the EE2 levels quantified in the groundwater could be a potential health risk to pregnant females. No studies could be found that outline potential EE2 health risks for men. However, as mentioned previously, the maximum EE2 value used to conduct the I70 model was a trace concentration, between the LOD and LOQ. It was used as an estimate for the model, but it was not a quantifiable concentration to be taken with confidence. None of the water samples

collected had quantifiable EE2 concentrations, so in reality, the EE2 potential risk is negligible at both sites.

Summary and Conclusions

The goal of this study was to quantify E1, E2, and EE2 in groundwaters sampled from two different areas in Pennsylvania: the Living Filter in central PA and the Sinnemahoning Creek Watershed in north central PA. At the Living Filter, a 25+ year wastewater irrigation site, all three estrogen hormones were detected in the groundwater wells, but all three estrogens were not quantified. E1 was quantified in all 14 wells, and the concentrations ranged from 2.19 to 18.79 ng L^{-1}. Only one well had concentrations of E2 above the LOD and above the LOQ, well W-6. The concentration of E2 in well W-6 was 7.43 ng L^{-1}. In all 14 wells, concentrations of EE2 were either ND or were quantified in trace amounts. A direct link between the concentrations seen and irrigation on site could not be made, especially since the concentrations quantified were in the same range as multiple literature studies that have sampled various surface and groundwater sources. It was hypothesized that the concentrations quantified in the groundwater wells were simply natural background concentrations. Even though the results from this study indicated that the Living Filter was not contributing estrogens to the groundwater beyond natural background, ongoing monitoring would be useful.

In the samples collected from the Sinnemahoning Creek Watershed only two estrogens were detected and quantified: E1 and E2. E1 was quantified in waters collected from 19 of the 20 sampling locations and the concentrations ranged from ND to 37.11 ng L^{-1}. Only two locations had concentrations of E2 above the LOD and above the LOQ. The concentrations of E2 ranged from ND to 32.47 ng L^{-1}. EE2 was ND in all of the water samples collected. Again, direct links between the concentrations measured and sources such as septic systems and agriculture could

not be made. Linking these concentrations to specific practices was outside the scope of this study. As with the Living Filter data, the concentrations quantified in the Sinnemahoning Creek Watershed were in the same range as multiple literature studies, and the concentrations quantified were likely natural background concentrations.

These two case studies served as a reconnaissance of estrogen presence for north central and central Pennsylvania groundwaters. Risk assessment work suggested that the potential human health risks are low for the two sampling areas. State agencies and other researchers could continue sampling throughout the state, to add to this data set, and to create a more robust estrogen database for Pennsylvania waters. This information could be used to enhance management of Pennsylvania waters and ensure that the potential human health risks for future generations remain low.

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Table 5-1. Soil data for the four major soil series found at the Living Filter (Central PA).

Series	Map Unit Symbol	% Site	Parent Material	Drainage	Land Use
Hagerstown	HaB HaC HcB HcC HcD	36	Residuum	WD	Crops and Mixed Hardwood
Hublersburg	HuB HuC	38	Residuum	WD	Pasture and Mixed Hardwood
Morrison	MrB MrC MsB MsD	17	Residuum	WD	Wooded Areas and Crops
Opequon	OhB OhD OxB OxD ORF	6	Residuum	WD	Pasture and Mixed Hardwood

WD: well drained.

Table 5-2. Soil data for the six major soil series associated with sampling locations in the Sinnemahoning Creek Watershed (North Central PA).

Series	Map Unit Symbol	Parent Material	Drainage	Land Use
Albrights	AbD	Colluvium	MW to SPD	Mixed Hardwood and Crops
Atkins	At	Alluvium	PD	Mixed Hardwood
Basher	Bb	Alluvium	MWD	Crops
Buchanan	BuD	Colluvium	MW to SPD	Mixed Hardwood
Leck Kill	LeF LeD	Residuum	WD	Mixed Hardwood and Crops
Philo	Ph	Alluvium	MWD	Pasture

WD: well drained; MW: moderately well drained; PD: poorly drained; SPD: somewhat poorly drained.

Table 5-3. Well information for the 14 wells sampled at the Living Filter (Central PA).

Well	Well depth (ft.)	Grade Elevation (ft.)	Groundwater Elevation (ft.)
W-1	289	1122.4	995.58
W-2	219	1182.6	993.29
W-5	208	1158.7	990.22
W-6	350	1257.2	984.76
W-7	343	1280	991.25
G-10	317	1205.8	989.46
G-12	323	1250	989.37
P-1	175	1007.7	935.5
P-2	175	1036.2	974.34
P-3	275	1159.2	985.74
P-4	200	1077.9	993.19
P-5	344	1211.3	993.19
F-3	120	1064	986.7
UN-14	390	1052.3	942.3

Table 5-4. Characterization of the 20 locations sampled in the Sinnemahoning Creek Watershed (North Central PA).

Site ID	Sample Type
W08606	Public Supply Well
W08607	Personal Well
W08608	Personal Well
W08609	Spring
W08610	Personal Well
W08611	Spring
W08612	Personal Well
W08613	Personal Well
W08614	Personal Well
W08615	Personal Well
W08616	Personal Well
W08617	Personal Well
W08618	Personal Well
W08619	Personal Well
W08620	Personal Well
W08621	Stream Downstream of WWTP
W08622	Stream Upstream of WWTP
W08623	WWTP Discharge Point
W08624	DWP Intake Water
W08625	DWP Finished Water

Table 5-5. Concentrations of estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2) quantified in the 14 groundwater wells at the Living Filter (Central PA).

	Average Estrogen Concentration (ng L^{-1})		
Well	**E1**	**EE2**	**E2**
W-1	8.06†	ND	T*
W-2	16.33†	T*	T*
W-5	2.62†	ND	T
W-6	11.45†	T	7.43†
W-7	6.90	T*	T*
G-10	14.45†	T*	T*
G-12	3.94	T	T*
P-1	7.42†	ND	T*
P-2	5.60	T	T*
P-3	2.87	ND	T
P-4	7.84†	ND	T*
P-5	2.19†	ND	T
F-3	18.79†	ND	T*
UN-14	3.77	T	T*

ND- Concentrations below LOD

T- Trace concentrations between LOD and LOQ

* One of the three samples had a value above the LOQ

† Coefficient of Variation greater than 50 %

Table 5-6. Concentrations of estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2) quantified in the samples taken from the Sinnemahoning Creek Watershed (North Central PA).

		Average Estrogen Concentration (ng L^{-1})		
Site ID	**Location**	**E1**	**EE2**	**E2**
W08606	Public Supply Well	20.43	ND	ND
W08607	Personal Well	21.42†	ND	32.47†
W08608	Personal Well	25.01†	ND	ND
W08609	Spring	17.64	ND	ND
W08610	Personal Well	19.44†	ND	T*
W08611	Spring	15.72	ND	ND
W08612	Personal Well	23.50†	ND	ND
W08613	Personal Well	13.10	ND	ND
W08614	Personal Well	11.63	ND	ND
W08615	Personal Well	19.51†	ND	ND
W08616	Personal Well	19.91†	ND	ND
W08617	Personal Well	11.43	ND	ND
W08618	Personal Well	21.16†‡	ND	ND
W08619	Personal Well	11.67	ND	ND
W08620	Personal Well	9.86	ND	ND
W08621	Stream Downstream of WWTP	37.11	ND	ND
W08622	Stream Upstream of WWTP	22.55†	ND	23.02†
W08623	WWTP Discharge Point	19.38†	ND	ND
W08624	DWP Intake Water	9.94	ND	ND
W08625	DWP Finished Water	ND	ND	ND

ND- Concentrations below LOD

T- Trace concentrations between LOD and LOQ

* One of the three samples had a value above the LOQ

† Coefficient of Variation greater than 50 %

‡ Average of N=2

Table 5-7. Dosage data to assess potential human health risks from exposure to estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2) in drinking water.

Compound	Max ($ng\ L^{-1}$)	DWI ($ng\ day^{-1}$)	TD ($\mu g\ day^{-1}$)	TD/DWI	I70 (μg)
EE2	6.96	13.92	10	718	356
E2	32.47	64.94	25	385	1659
E1	25.01	50.00	-	-	1277

Max: maximum measured value; DWI: Drinking water intake; TD: therapeutic dose; I70: amount of ingestion in 70 years based on DWI.

Figure 5-1. Location of the Living Filter Wastewater Irrigation Site in relation to the Penn State University main campus.

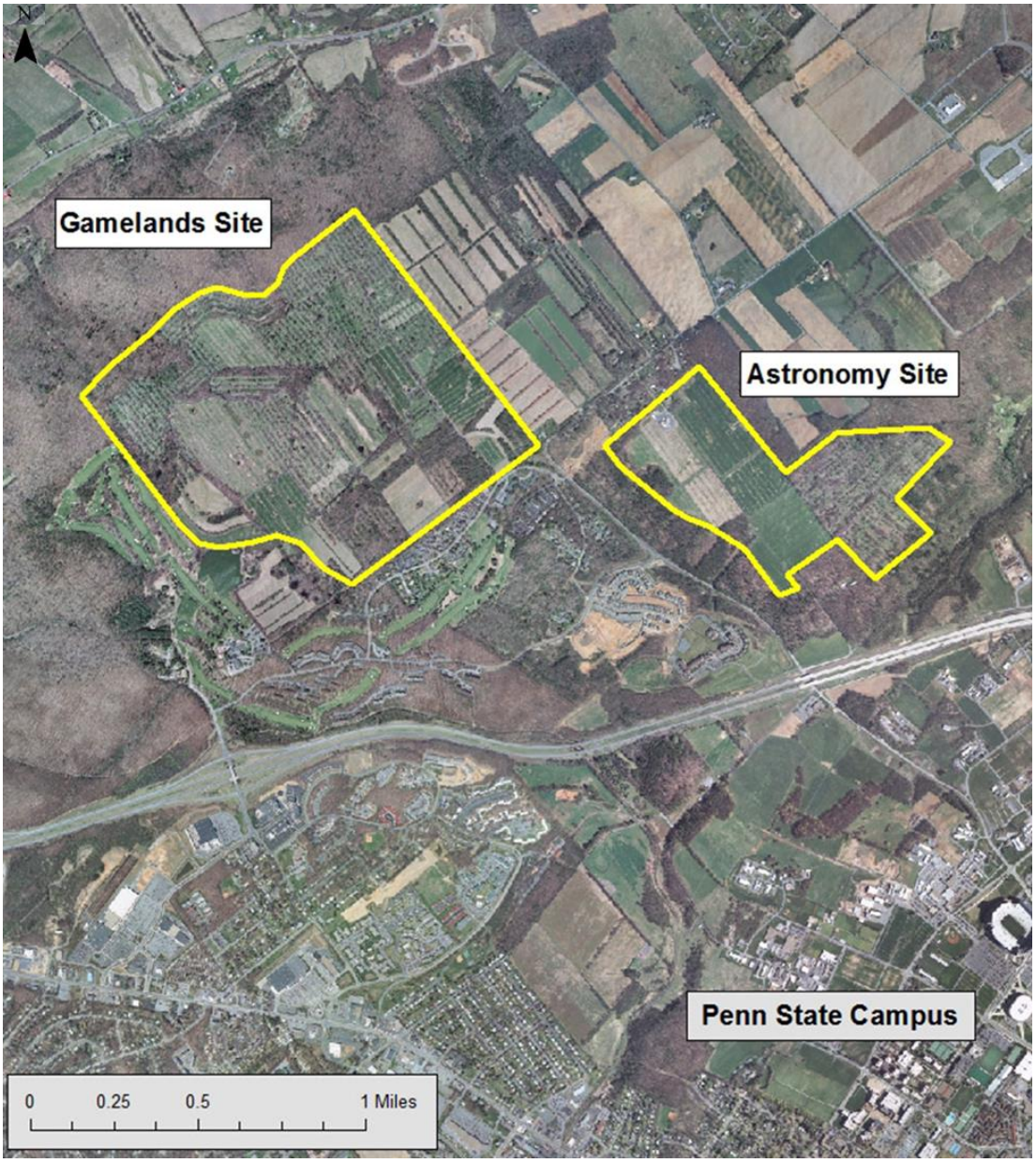


Figure 5-2. Geologic map of the Living Filter. Both members of the Gatersburg Formation are comprised of Cambrian aged dolomites, limestones, sandstones, and cherts.

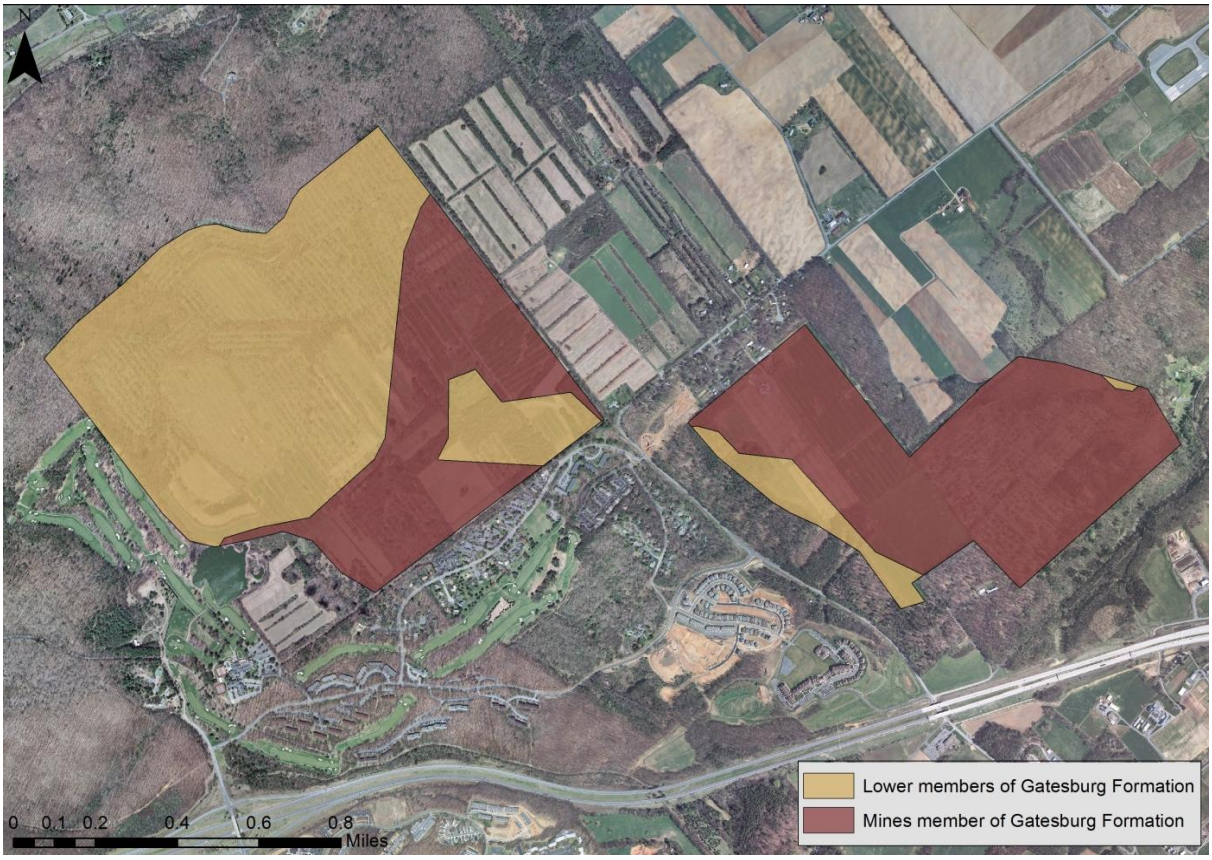
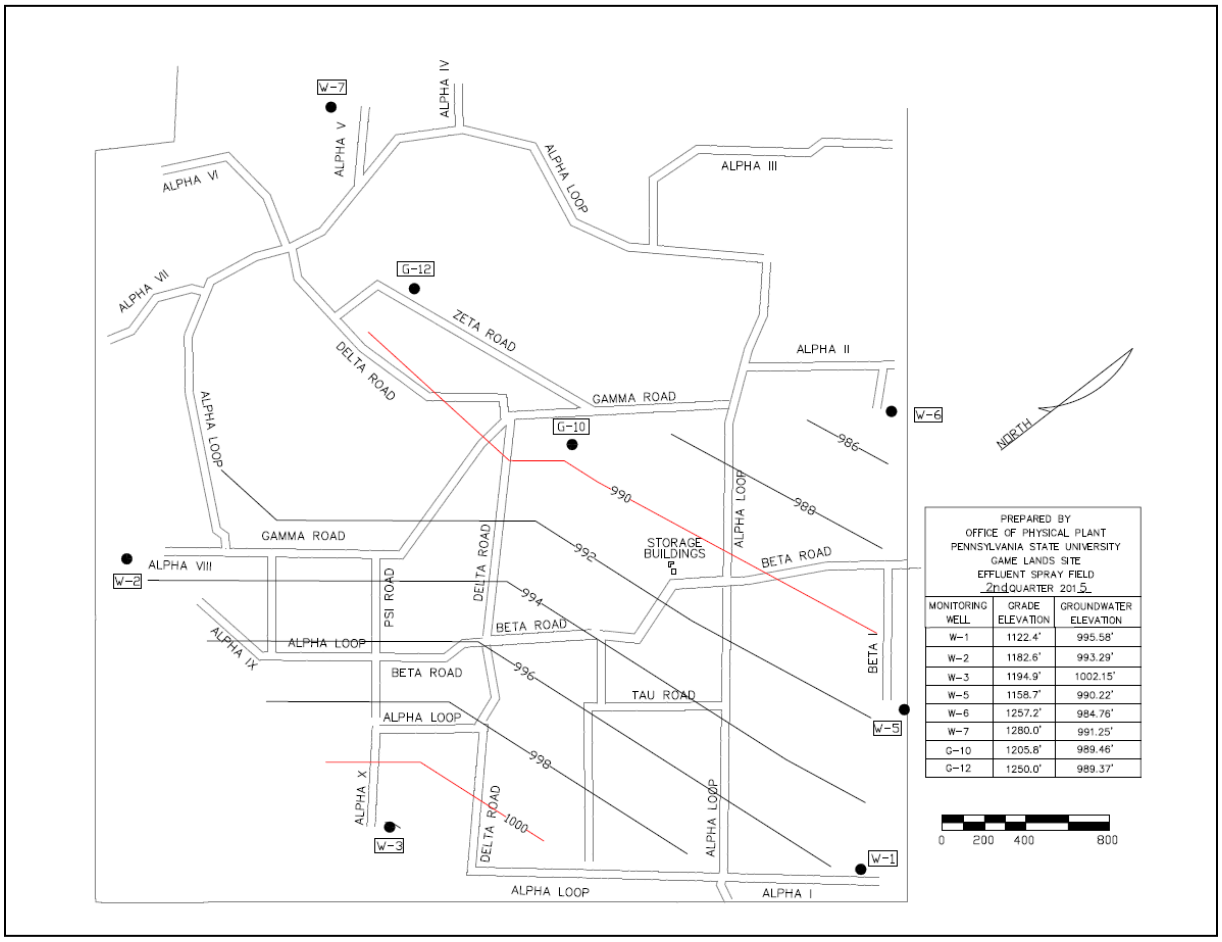


Figure 5-3. Groundwater flow contour map of the Gamelands Site of the Living Filter (Central PA).



MONITORING WELL	GRADE ELEVATION	GROUNDWATER ELEVATION
W-1	1122.4'	995.58'
W-2	1182.6'	993.29'
W-3	1194.9'	1002.15'
W-5	1158.7'	990.22'
W-6	1257.2'	984.76'
W-7	1280.0'	991.25'
G-10	1205.8'	989.46'
G-12	1250.0'	989.37'

Figure 5-4. Groundwater flow contour map of the Astronomy Site of the Living Filter (Central PA).

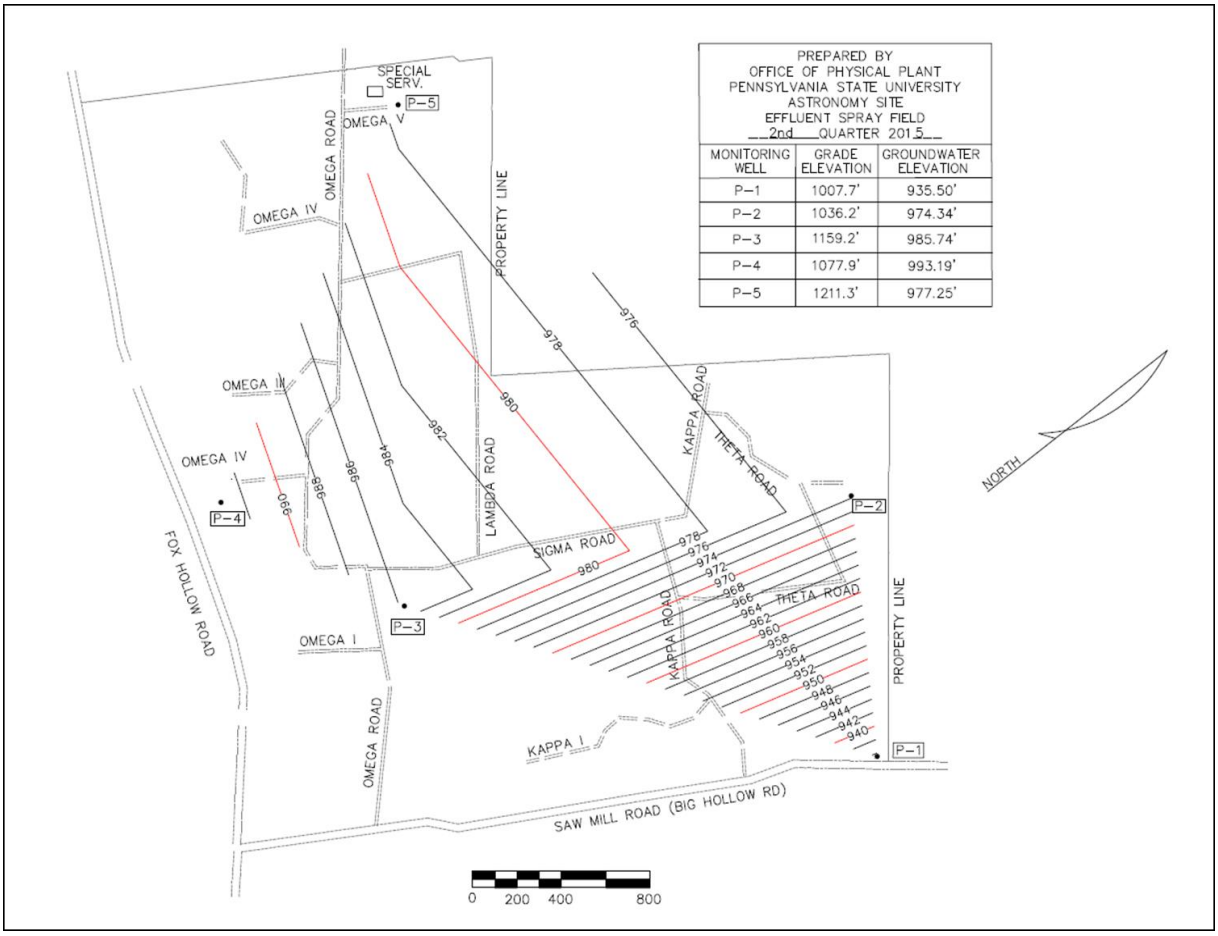


Figure 5-5. Water table contour elevations and flow map for the region surrounding the Gamelands and Astronomy Site, Central PA (From Parizek et al., 1967).

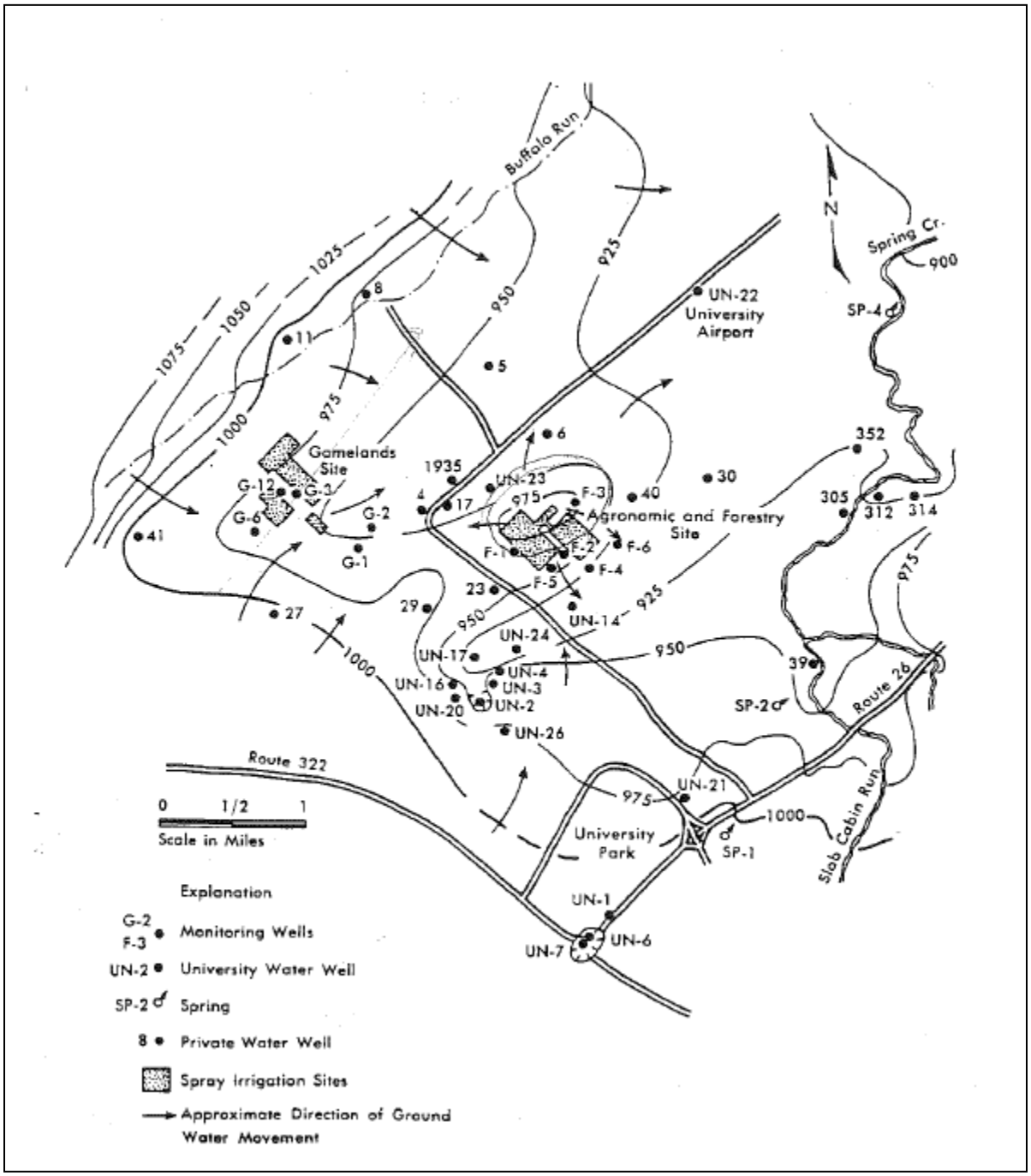


Figure 5-6. Soil series mapped at the Living Filter (Central PA).

Figure 5-7. Location of the Sinnemahoning Creek Watershed within the state of Pennsylvania.

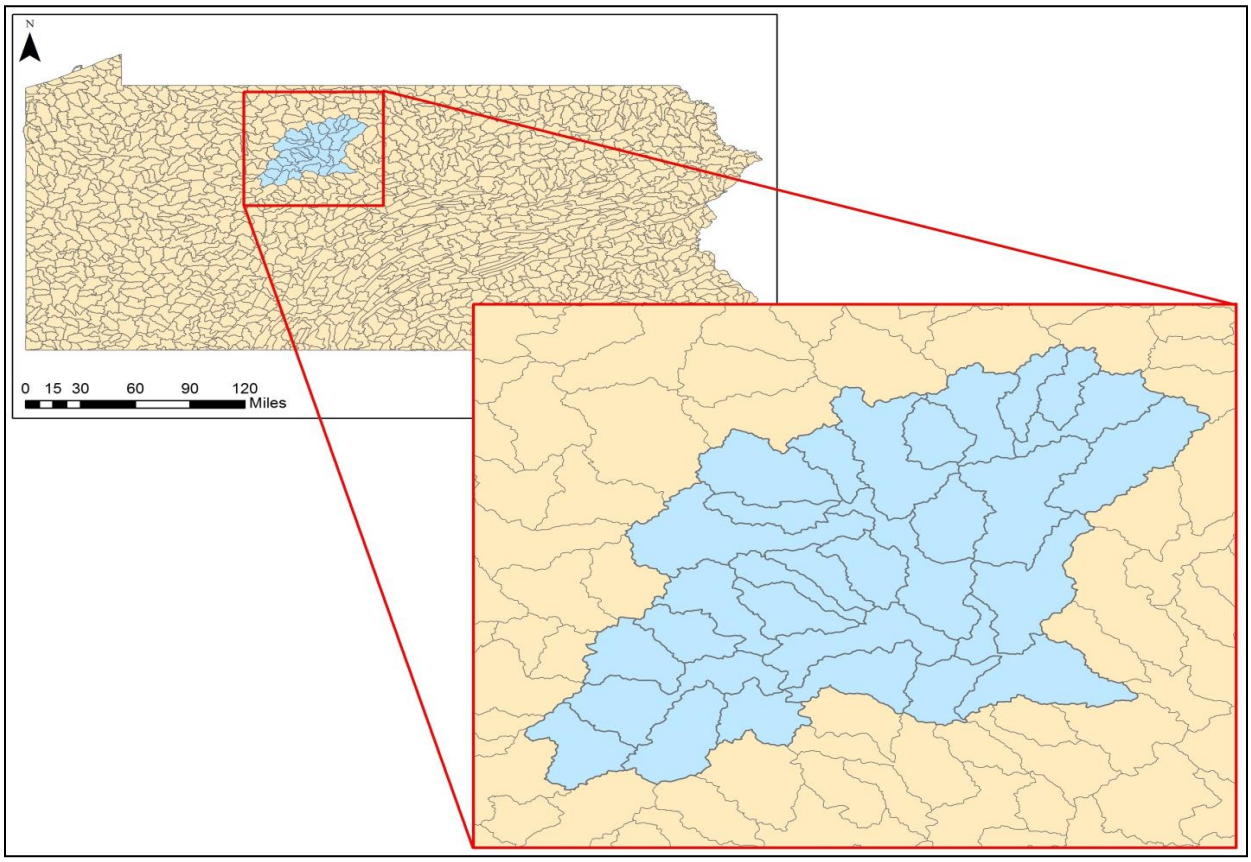


Figure 5-8. Geologic map of the Sinnemahoning Creek Watershed (North Central PA) and the 20 sampling locations.

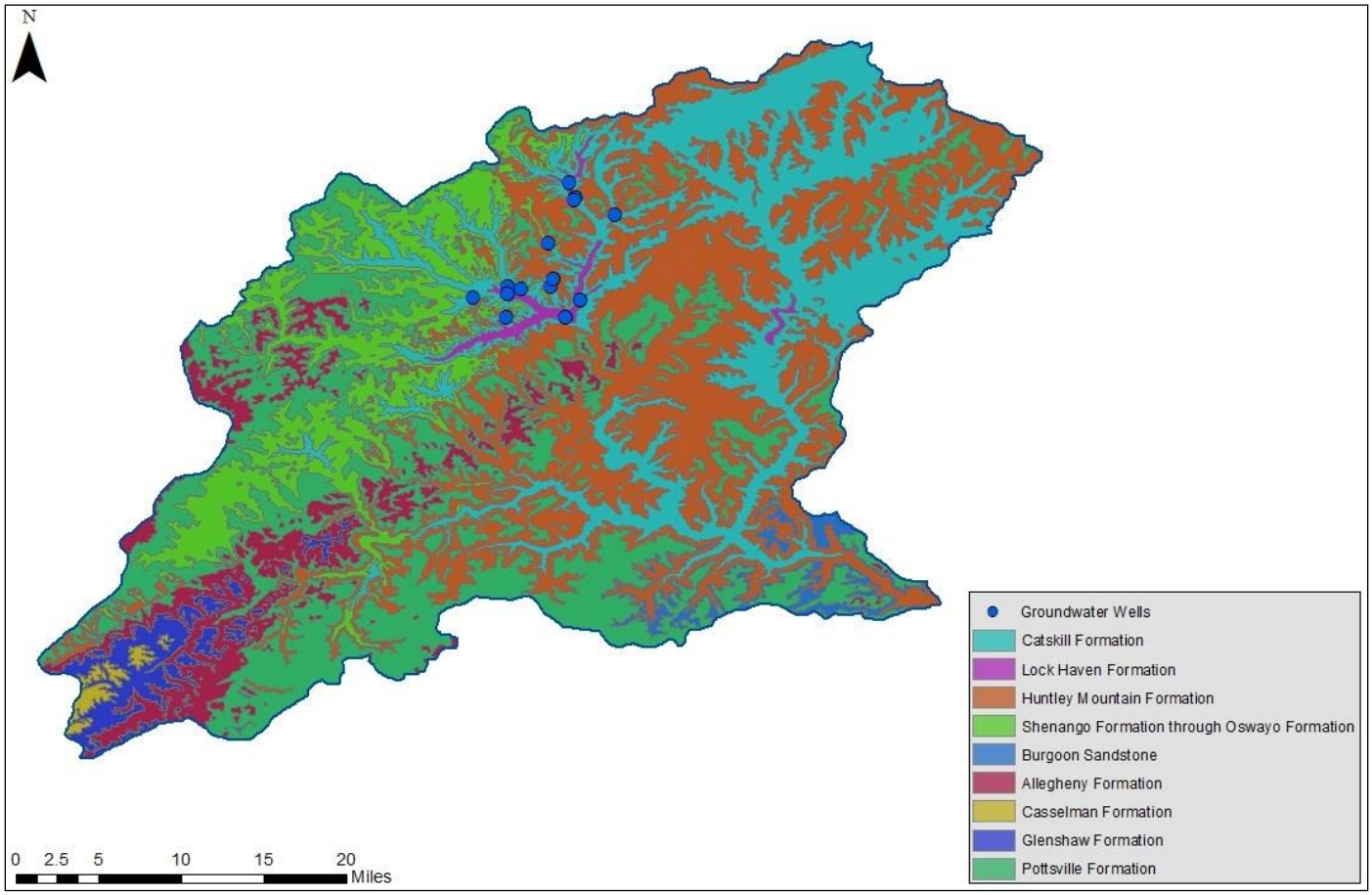


Figure 5-9. Location of the 14 groundwater wells sampled at the Living Filter (Central PA).

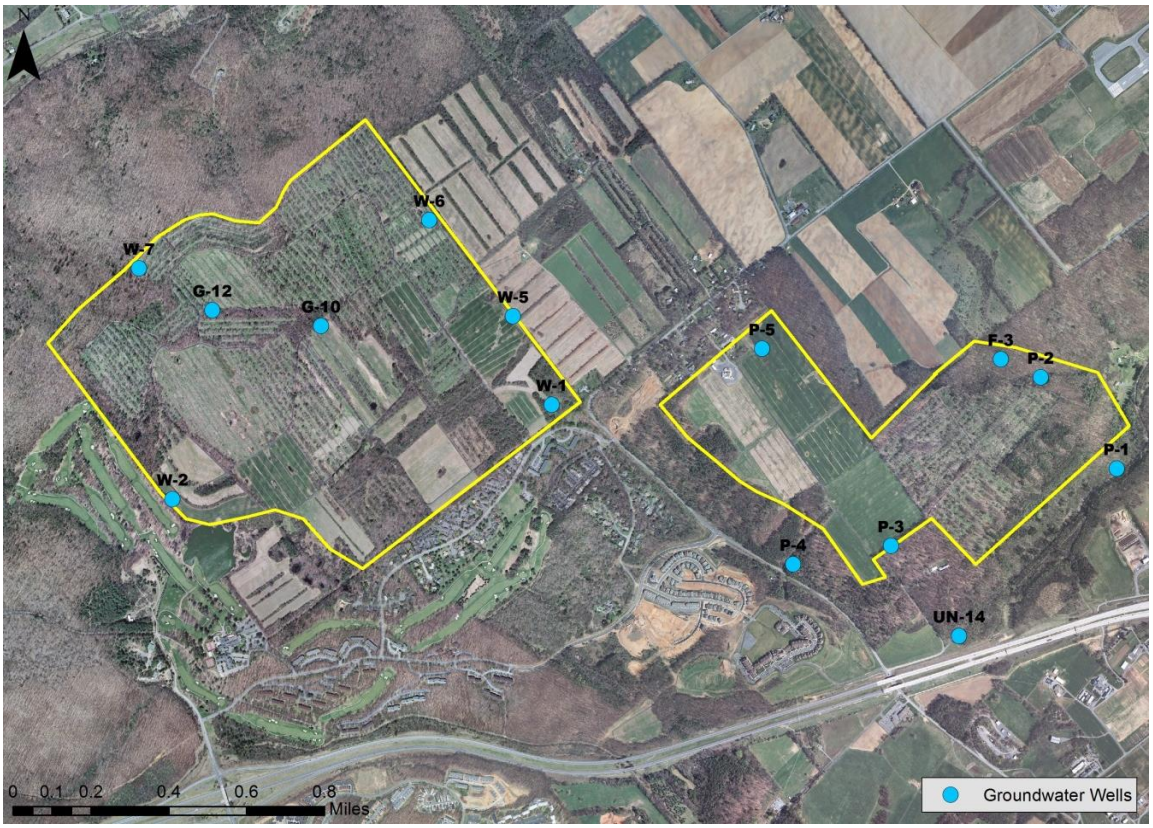


Figure 5-10. Distribution of the twenty locations sampled within the Sinnemahoning Creek Watershed (North Central PA).

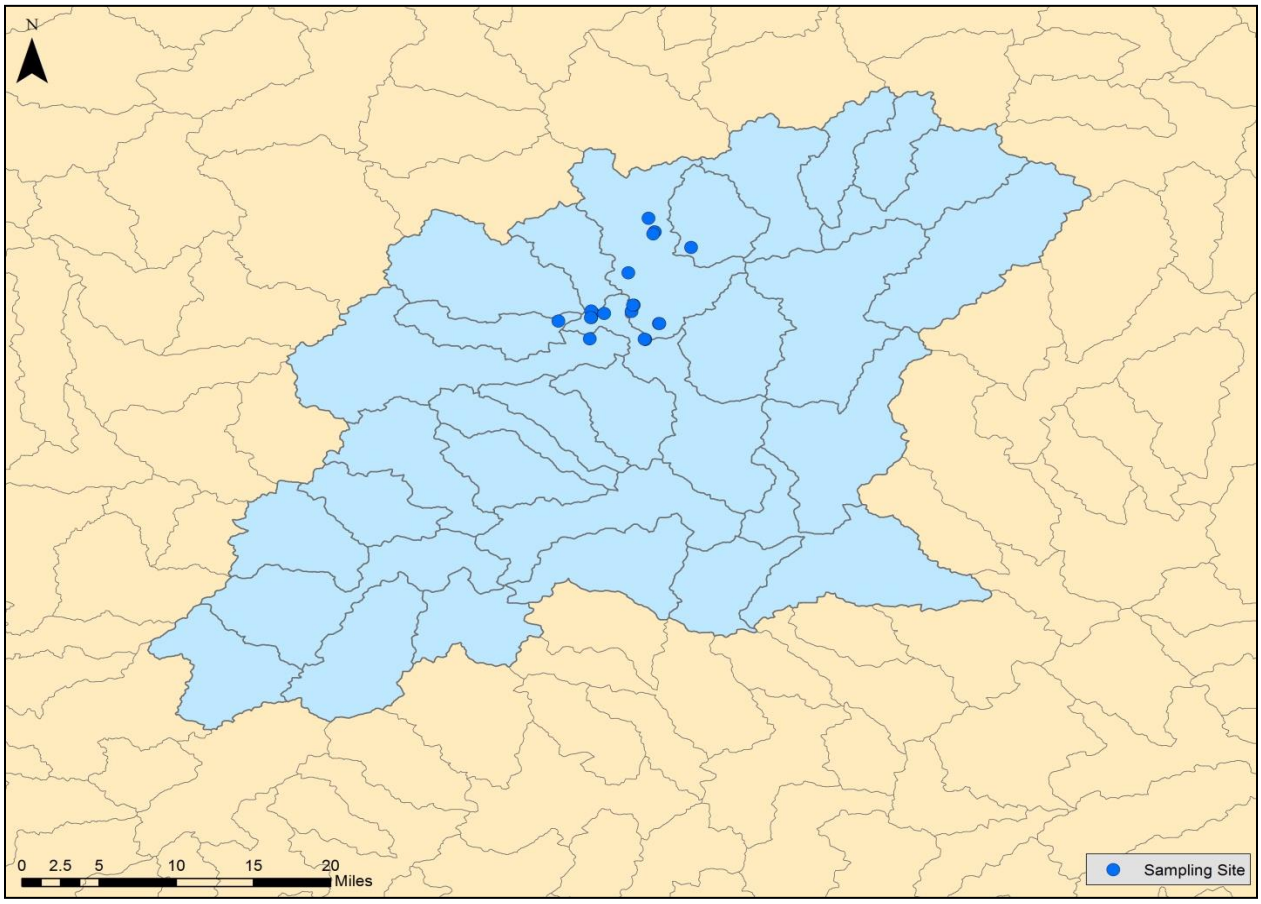


Figure 5-11. Estrone (E1) levels in the 14 wells sampled at the Living Filter (Central PA).

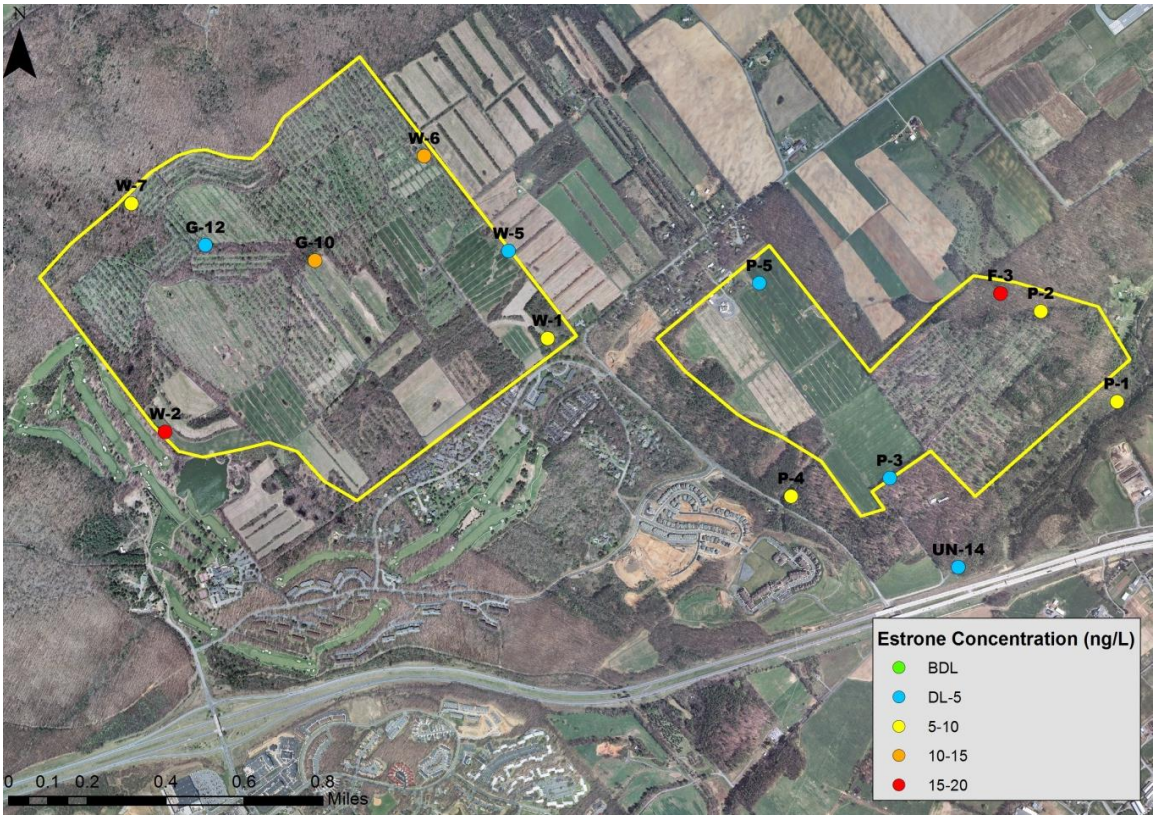
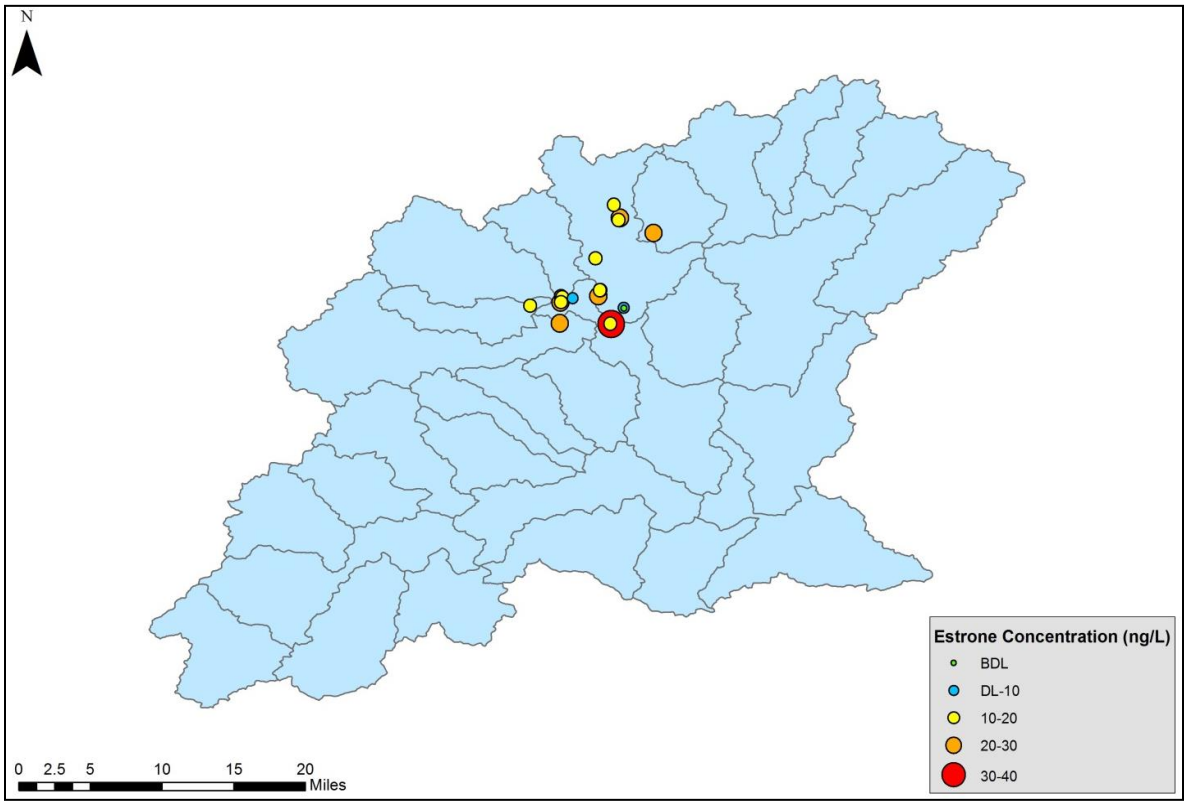


Figure 5-12. Estrone (E1) levels in the 20 locations sampled in the Sinnemahoning Creek Watershed (North Central PA).



Chapter 6

Summary and Future Needs

The main goals of this dissertation were to optimize the extraction and analysis of E1, E2, and EE2 in soils, characterize E1, E2, and EE2 sorption in soils varying in organic carbon content and in sorption systems where pure water was used to equilibrate soils versus effluent, and to establish E1, E2, and EE2 baseline data for two different geographic regions within Pennsylvania: the Sinnemahoning Creek Watershed and the Penn State Living Filter Wastewater Irrigation Site. In Chapter 2, an extraction method was developed to increase estrogen extraction recoveries from soils. An optimal solvent extractant, SPE loading ratio, and microbial inhibition method that increased estrogen recovery from an the extraction method selected depended upon the particular estrogen of interest. Either a MeOH extraction combined with an SPE procedure that uses a 10:90 MeOH:water loading ratio (E1, EE2) or a DCM extraction (E2) would be the optimal method. The soil sterilization results, NaN_3 at varying concentrations versus autoclaving, indicated that autoclaving the soil is the optimal soil sterilization method. Autoclaving yielded the highest estrogen recoveries. The optimized solvent extraction method, not including the sterilization results, was used to carry out the batch sorption study procedures in Chapter 4.

Results from Chapter 3 indicated that the combination of C_{18} and ACN provided the greatest separation of ten estrogenic compounds. This combination is the one used most in the literature for estrogen analyses. From best to worst in their ability to separate the combined estrogen standards, the stationary and mobile phase combinations were ranked: C_{18} and ACN , RP-Amide and ACN, Biphenyl and ACN, Biphenyl and MeOH, C_{18} and MeOH, RP-Amide and MeOH. This stationary phase and mobile phase combination was used to analyze soil and water samples in Chapters 4 and 5.

In Chapter 4, sorption of E1, E2, and EE2 was characterized for soils varying in organic carbon content and two different equilibration matrices. Results from this study indicated that organic carbon content is a key mechanism for E1, E2, and EE2 sorption in soils, a mechanism that has been seen in other studies. High K_d and K_f values for each estrogen were associated with the high organic carbon content soils and vice versa. The rank in sorption from highest to lowest for the three estrogens studied was E1 > E2 > EE2. It was expected that sorption of E1, E2, and EE2 would decrease when effluent was used to equilibrate the soils. Differences in sorption between the two equilibrating matrices were seen when the low organic carbon soil was used (cropped B). Extraction recoveries varied between the three estrogens. E1 had the highest extraction recovery, followed by EE2, and then E2. For each estrogen, the extraction recovery percentage decreased with an increase in soil organic carbon concentration.

In the final chapter, Chapter 5, E1, E2, and EE2 were quantified in ground and surface waters sampled from the Living Filter in central PA and the Sinnemahoning Creek Watershed in north central PA. At the Living Filter, all three estrogen hormones were detected in the groundwater wells, but all three estrogens were not quantified. E1 was quantified in all fourteen wells, and the concentrations ranged from 2.19 to 18.79 ng L^{-1}. Only one well (W-6) had concentrations of E2 above the detection limit and above the limit of quantification (LOQ). The concentration of E2 in well W-6 was 7.43 ng L^{-1}. In all fourteen wells, concentrations of EE2 were either not detected (ND) or were quantified in trace amounts (T). Within the Sinnemahoning Creek Watershed only two estrogens were detected and quantified: E1 and E2. E1 was quantified in waters collected from nineteen of the twenty sampling locations and the concentrations ranged from ND to 37.11 ng L^{-1}. Only two locations had concentrations of E2 above the detection limit and above the limit of quantification (LOQ). The concentrations of E2

ranged from ND to 32.47 ng L^{-1}. EE2 was not detected (ND) in any of the water samples collected. For both sites, the concentrations of E1, E2, and EE2 quantified were in the same range as multiple literature studies, and the concentrations quantified were likely natural background concentrations. Risk assessment work suggested that the potential human health risks from estrogen exposure are low for the two sampling areas.

Method development is an important first step when extracting and analyzing soil and water samples for estrogen compounds. While laboratory studies indicate rapid dissipation of estrogen hormones and a high sorption affinity of estrogens for organic carbon, both mechanisms that would reduce transport and quantity of estrogens in field soils, estrogens are still being detected and quantified in environmental samples. The use of wastewater to irrigate crops, the reuse of wastewater for other fresh water purposes, and the use biosolids and manures to amend agricultural landscapes are all becoming more prevalent. It is essential that we know how to extract these compounds from soil and water samples, that we have some measure for recovery percentages from these samples, that we can analyze these compounds at low concentrations and in complex environmental matrices, and that we can generate the estrogen specific sorption data needed to predict environmental fate and transport. It is especially important that we continue monitoring soil and water systems to ensure that estrogen compounds are not accumulating in the soil profile or transporting to ground water sources and keep potential human health risks low for future generations.

Future laboratory work should continue to characterize estrogens in complex systems: multi-sorbate, in effluent matrices, and a variety of soils. These complex systems provide a more realistic proxy for estrogen behavior in the environment than the traditional pure water, single-sorbate systems. A shift in future field monitoring studies from wastewater treatment plants to

management practices such as effluent irrigation, effluent recharge basins, and biosolid application would be beneficial. Literature field studies on these estrogen sources are limited, and their use across the United States, especially in arid regions, is only going to increase. Future studies should also incorporate more natural background reconnaissance. In order to determine whether land and water management practices are affecting environmental estrogen concentrations, natural background concentrations of estrogen compounds must be established.

APPENDIX A

Sorption Concentration and Recovery Data

Table A-1. Equilibration and extraction concentrations for E1 using the forested A horizon soil.

	Pure Water		LF Effluent	
	E1 (µg L^{-1})	**E1 (µg kg^{-1})**	**E1 (µg L^{-1})**	**E1 (µg kg^{-1})**
0-1	0.03	0.51	0.03	0.19
0-2	0.06	0.48	0.03	0.10
0-3	0.03	0.14	0.06	0.10
25-1	0.31	26.90	0.09	16.80
25-2	0.35	28.58	0.04	14.10
25-3	0.17	29.02	0.09	15.85
50-1	0.85	41.30	0.33	38.28
50-2	0.20	49.67	0.21	36.35
50-3	0.40	49.32	0.17	41.79
75-1	0.07	62.72	0.41	70.17
75-2	0.22	64.59	0.21	59.53
75-3	0.06	54.47	0.20	62.36
100-1	0.93	99.59	0.44	64.72
100-2	0.77	67.70	0.35	64.71
100-3	0.81	93.58	0.29	69.64

0, 25, 50, 75, 100: equilibration spiking concentrations. -1, -2, -3: triplicates.

Table A-2. Equilibration and extraction concentrations for E2 using the forested A horizon soil.

	Pure Water		LF Effluent	
	E2 (µg L^{-1})	**E2 (µg kg^{-1})**	**E2 (µg L^{-1})**	**E2 (µg kg^{-1})**
0-1	1.20	0.36	0.12	1.03
0-2	0.13	0.36	0.13	0.22
0-3	0.14	0.30	0.14	0.39
25-1	0.28	9.04	0.13	4.39
25-2	0.31	7.83	0.09	7.53
25-3	0.18	7.93	0.14	4.86
50-1	0.73	14.34	0.13	12.20
50-2	0.19	14.30	0.13	10.59
50-3	0.09	13.24	0.13	14.80
75-1	0.13	16.47	0.13	27.55
75-2	0.13	19.11	0.13	36.23
75-3	0.13	14.12	0.13	18.86
100-1	0.30	25.43	0.21	17.51
100-2	0.32	16.58	0.13	16.69
100-3	0.15	23.13	0.13	18.48

0, 25, 50, 75, 100: equilibration spiking concentrations. -1, -2, -3: triplicates.

Table A-3. Equilibration and extraction concentrations for EE2 using the forested A horizon soil.

	Pure Water		LF Effluent	
	EE2 (µg L^{-1})	**EE2 (µg kg^{-1})**	**EE2 (µg L^{-1})**	**EE2 (µg kg^{-1})**
0-1	0.20	0.60	0.35	0.44
0-2	0.15	0.89	0.15	0.43
0-3	0.15	1.21	0.27	0.77
25-1	0.60	18.23	0.15	10.04
25-2	0.15	18.69	0.15	7.56
25-3	0.36	17.70	0.15	8.21
50-1	0.89	23.69	0.15	22.23
50-2	0.15	30.41	0.26	20.56
50-3	0.33	32.18	0.20	22.49
75-1	0.15	34.63	0.45	39.05
75-2	0.25	40.36	0.20	34.12
75-3	0.25	32.40	0.15	39.18
100-1	0.98	55.86	0.15	35.55
100-2	0.15	34.96	0.28	35.67
100-3	0.15	53.88	0.44	39.56

0, 25, 50, 75, 100: equilibration spiking concentrations. -1, -2, -3: triplicates.

Table A-4. Average estrogen extraction recoveries (% ± SD) from each soil and each pure water equilibration concentration (N=3).

	E1	E2	EE2
Pure Water			
Cropped A			
($\mu g\ L^{-1}$)			
25	96.8 ± 6.7	40.3 ± 1.9	70.2 ± 1.1
50	97.7 ± 11.0	39.3 ± 4.5	67.4 ± 9.9
75	97.7 ± 3.7	40.0 ± 1.9	70.3 ± 4.0
100	86.6 ± 9.1	32.1 ± 1.9	60.8 ± 4.4
Cropped B			
25	56.5 ± 0.2	47.8 ± 1.8	55.3 ± 1.8
50	47.2 ± 1.3	39.5 ± 1.8	45.3 ± 1.5
75	52.1 ±2.7	42.1 ± 1.5	49.1 ± 0.9
100	42.4 ± 0.9	33.3 ± 1.0	39.1 ± 0.5
Forested A			
25	57.0 ± 2.1	16.7 ± 1.4	37.0 ± 0.9
50	47.2 ± 4.5	14.1 ± 0.7	29.0 ± 4.3
75	40.5 ± 3.6	11.1 ± 1.7	24.0 ± 42.8
100	43.9 ± 11.4	10.9 ± 3.1	24.2 ± 7.6

Table A-5. Average estrogen extraction recoveries (% ± SD) from each soil and each effluent equilibration concentration (N=3).

	E1	E2	EE2
LF Effluent			
Cropped A			
($\mu g\ L^{-1}$)			
25	98.1 ± 3.5	26.6 ± 1.3	62.3 ± 2.6
50	88.6 ± 4.8	23.0 ± 0.8	54.0 ± 2.6
75	108.1 ± 6.1	30.1 ± 1.8	68.5 ± 4.2
100	96.3 ± 3.6	26.2 ± 0.4	61.2 ± 2.6
Cropped B			
25	55.4 ± 3.1	46.0 ± 12.6	51.1 ± 7.3
50	59.4 ± 4.1	40.8 ± 2.8	57.2 ± 4.4
75	52.2 ± 4.8	39.2 ± 1.8	49.2 ± 4.0
100	40.6 ± 1.6	30.2 ± 2.3	37.5 ± 2.1
Forested A			
25	31.2 ± 2.8	11.2 ± 3.4	17.3 ± 2.6
50	39.0 ± 2.7	12.6 ± 2.1	21.9 ± 1.0
75	42.8 ± 3.8	18.4 ± 8.2	25.1 ± 2.0
100	33.3 ± 1.4	8.8 ± 0.5	18.5 ± 1.2
UP Effluent			
Cropped A			
($\mu g\ L^{-1}$)			
100	75.6 ± 3.8	25.3 ± 1.2	48.9 ± 3.5

APPENDIX B

Living Filter and Sinnemahoning Creek Groundwater Data

The tables below indicate the raw estrogen concentration data that was used to calculate the averages presented in Table 5-5 and Table 5-6.

Table B-1. Raw estrogen concentration data (ng L^{-1}) from the Living Filter groundwater wells.

Well	E1	EE2	E2
W-2	4.32	2.28	3.54
W-2	40.80	10.19	25.71
W-2	3.85	2.36	4.19
W-7	9.15	12.65	5.28
W-7	3.33	2.83	3.72
W-7	8.22	5.40	8.70
G-12	3.08	3.19	5.18
G-12	3.95	3.58	3.74
G-12	4.78	4.88	8.88
G-10	3.48	1.84	2.61
G-10	37.43	8.42	22.70
G-10	2.43	3.04	2.21
P-2	3.31	1.56	3.58
P-2	6.96	3.30	4.19
P-2	6.52	4.51	7.79
F-3	49.95	10.65	31.24
F-3	3.29	1.69	3.85
F-3	3.14	1.53	2.68
P-3	2.81	1.53	2.50
P-3	2.29	1.56	2.69
P-3	3.53	1.61	3.70
W-6	1.84	1.53	2.01
W-6	24.67	4.92	14.04
W-6	7.84	5.18	6.24
W-5	4.34	2.87	3.04
W-5	1.75	1.54	2.32
W-5	1.79	1.53	2.26

Table B-1 continued. Raw estrogen concentration data (ng L^{-1}) from the Living Filter groundwater wells.

Well	E1	EE2	E2
P-1	3.10	1.53	3.55
P-1	1.76	1.56	1.26
P-1	17.42	4.81	11.64
UN-14	3.02	3.49	2.48
UN-14	5.68	2.72	7.31
UN-14	2.63	2.95	2.37
P-4	19.95	5.88	12.83
P-4	1.64	1.53	2.36
P-4	1.93	1.53	1.26
P-5	3.45	1.60	3.05
P-5	1.48	1.53	1.26
P-5	1.63	1.56	2.44
W-1	19.31	5.66	13.09
W-1	1.60	1.53	1.26
W-1	3.28	1.72	2.45

Averages were not calculated when two or more values were below LOD or LOQ.

Table B-2. Raw estrogen concentration data (ng L^{-1}) from the Sinnemahoning Creek Watershed Sampling.

Sample ID	Triplicate	E1	E2	EE2
W08606	1-1-CC1	18.05	4.20	9.51
	1-1-CC2	24.77	7.37	12.58
	1-1-CC3	18.47	4.18	10.48
W08608	2-1-CC1	48.74	41.47	7.34
	2-1-CC2	14.33	2.67	4.49
	2-1-CC3	11.95	1.43	3.24
W08607	2-2-CC1	39.27	55.24	14.35
	2-2-CC2	12.13	4.07	7.41
	2-2-CC3	12.87	9.70	8.64
W08609	2-3-CC1	11.66	1.63	3.29
	2-3-CC2	20.52	1.92	4.42
	2-3-CC3	20.72	1.63	3.32
W08610	3-1-CC1	9.45	1.13	2.45
	3-1-CC2	9.39	2.46	3.37
	3-1-CC3	39.49	47.95	15.24
W08611	4-1-CC1	13.91	3.51	6.61
	4-1-CC2	23.34	3.72	7.36
	4-1-CC3	9.91	1.86	3.31
W08612	5-1-CC1	45.75	42.93	6.50
	5-1-CC2	11.17	1.88	4.06
	5-1-CC3	13.57	2.10	4.40
W08613	6-1-CC1	14.80	1.83	4.09
	6-1-CC2	16.53	3.03	6.33
	6-1-CC3	7.97	1.58	3.29
W08614	6-2-CC1	12.70	1.65	3.41
	6-2-CC2	12.47	4.05	3.72
	6-2-CC3	9.72	1.57	3.26

Table B-2 continued. Raw estrogen concentration data (ng L^{-1}) from the Sinnemahoning Creek Watershed Sampling.

Sample ID	Triplicate	E1	E2	EE2
W08615	7-1-CC1	13.59	2.76	6.50
	7-1-CC2	12.68	2.26	4.63
	7-1-CC3	32.28	31.42	5.58
W08616	7-2-CC1	11.40	1.95	3.63
	7-2-CC2	12.84	2.62	4.61
	7-2-CC3	35.50	30.93	6.05
W08617	8-1-CC1	14.99	2.05	4.46
	8-1-CC2	11.05	1.80	3.72
	8-1-CC3	8.27	1.69	3.24
W08618	9-1-CC1	218.98	1.56	3.13
	9-1-CC2	10.56	1.61	3.53
	9-1-CC3	31.77	32.34	4.20
W08619	10-1-CC1	12.89	1.98	4.32
	10-1-CC2	11.22	1.92	3.85
	10-1-CC3	10.90	2.06	3.52
W08620	11-1-CC1	12.31	2.21	3.86
	11-1-CC2	8.81	1.88	4.18
	11-1-CC3	8.46	1.84	3.87
W08622	12-1-CC1	37.10	38.73	7.03
	12-1-CC2	9.02	2.03	3.40
	12-1-CC3	21.53	7.32	6.31
W08621	12-2-CC1	32.50	9.56	15.38
	12-2-CC2	44.67	18.19	34.93
	12-2-CC3	34.15	14.79	23.79
W08623	12-3-CC1	11.56	3.58	5.19
	12-3-CC2	34.92	35.69	7.56
	12-3-CC3	11.67	3.90	6.40
W08624	13-1-CC1	9.99	2.05	3.65
	13-1-CC2	11.18	2.48	4.70
	13-1-CC3	8.65	2.51	4.60

Table B-2 continued. Raw estrogen concentration data (ng L^{-1}) from the Sinnemahoning Creek Watershed Sampling.

Sample ID	Triplicate	E1	E2	EE2
W08625	13-2-CC1	-	-	-
	13-2-CC2	-	-	-
	13-2-CC3	-	-	-

Averages were not calculated when two or more values were below LOD or LOQ. The E1 concentration at W08618, 9-1-CC1 was considered an outlier and was not used to calculate the average seen in Table 5-6. For the W08625 samples, the labeled standard was not recovered; therefore, accurate field concentrations could not be quantified.

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