9-1-2012

Using High-Performance Liquid Chromatography with Fluorescence to Determine the Effectiveness of Wastewater Treatment Plants in the Removal of Natural and Synthetic Estrogens

Katie Seehusen
Winona State University

Jeanne Franz
Winona State University

Appendix C

RESEARCH / CREATIVE PROJECT ABSTRACT / EXECUTIVE SUMMARY
FINAL REPORT FORM

Title of Project
Using High-Performance Liquid Chromatography with Fluorescence to Determine the Effectiveness of Wastewater Treatment Plants in the Removal of Natural and Synthetic Hormones

Student Name Katherine Seehusen

Faculty Sponsor D. Jeanne Fray

Department Chemistry

Abstract
The presence of hormones in the environment is becoming an increasingly popular topic in environmental and analytical chemistry. This research aims to develop a method to quantify the amount of two types of hormones in wastewater treatment plant effluents. The two hormones analyzed are 17β-estradiol (E2), a naturally produced hormone, and 17α-ethinylestradiol (EE2), a synthetic hormone commonly found in oral contraceptives. It has been hypothesized that synthetic hormones are significantly harder for wastewater treatment plants to remove, and the final goal of this research is to be able to test samples to determine the effectiveness of the wastewater treatment plants. The research thus far has focused on method development using high-performance liquid chromatography with fluorescence and standard E2 and EE2 solutions. This report shows the work completed when trouble-shooting the fluorimeter and developing the method.

The end product of this project in electronic format has been submitted to the Provost/Vice President for Academic Affairs via the Office of Grants & Sponsored Projects Officer (Maxwell 161, rpeterson@winona.edu).

Student Signature Katie Seehusen Date 5-8-13

Faculty Sponsor Signature Jeanne Fray Date 5-8-13

Using High-Performance Liquid Chromatography with Fluorescence to Determine the Effectiveness of Wastewater Treatment Plants in the Removal of Natural and Synthetic Estrogens

Research Completed Fall 2012/Spring 2013

Katie Seehusen and Dr. Jeanne Franz

Winona State University

‘

Abstract

The presence of hormones in the environment is becoming an increasingly popular topic in environmental and analytical chemistry. This research aims to develop a method to quantify the amount of two types of hormones in wastewater treatment plant effluents. The two hormones analyzed are 17β-estradiol (E_2), a naturally produced hormone, and 17α-ethinylestradiol (EE_2), a synthetic hormone commonly found in oral contraceptives. It has been hypothesized that synthetic hormones are significantly harder for wastewater treatment plants to remove, and the final goal of this research is to be able to test samples to determine the effectiveness of the wastewater treatment plants. The research thus far has focused on method development using high-performance liquid chromatography with fluorescence and standard E_2 and EE_2 solutions. This report shows the work completed when trouble-shooting the fluorimeter and developing the method.

Introduction

The presence of hormones in the environment has become a growing concern over the past few decades. In the body, hormones play a main role in controlling growth and development, metabolism, tissue function, and reproduction. There are two main types of hormones: natural and synthetic. The two specific hormones studied in this research are 17β-estradiol (E2) and 17α-

ethinylestradiol (EE2). These compounds are very similar in structure; however one is natural and one is synthetic. E2 is naturally secreted by women, and EE2 is the main synthetic hormone found in oral contraceptive pills. EE2 is increasingly being found as a major pollutant in wastewater today.

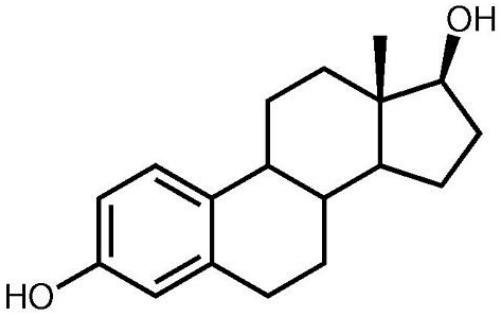

Figure 1.0. Chemical Structure of E2

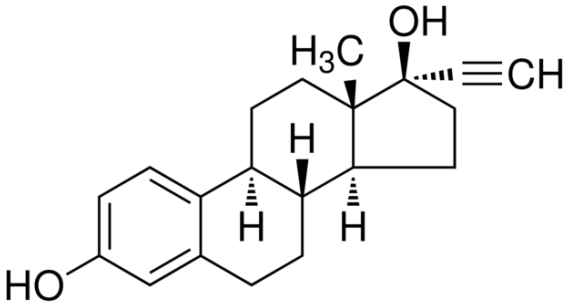

Figure 1.1 Chemical Structure of EE2

Much skepticism has risen about how well wastewater treatment plants (WTPs) remove these toxic compounds. The consequences of the inability of WTPs to remove estrogens are most commonly being observed in aquatic life. These effects were first verified in 1994. It was discovered that caged male rainbow trout contained elevated levels of vitellogenin, a yolk protein usually produced in female fish, when exposed to domestic effluents.[1] Since this study, the concern for this process, called feminization, has grown. Research has not verified a single pollutant as the origin of the estrogens in the environment. Removal of natural estrogens is generally greater than 90%; however, the aquatic life can be affected even at concentrations in units of parts per trillion.[2] The effects of the small concentration of estrogens are seen in reproductive abilities and gonadal development.

Overall, reports discussing the estrogenic activity were given little attention until toxicological effects were seen in fish. The observed increase of intersex fish has led to questioning about the risks posed to humans. Research today focuses on the possibility that the excess hormones excreted from the use of oral contraceptives (OCs) could be a cause of hormones in the groundwater. It is estimated that less than 1% of the total estrogens excreted by humans is EE2.[3] However, the average dose of EE2 is 30-35 μg/pill, and the body is able to absorb only 20-48% of the daily dose. This means that about 60% of the daily intake of EE2 is excreted by the body through urine or feces. Women naturally secrete E2 at concentrations of 2-20 μg/person/day.[4] In addition, often times the metabolized form of the hormones are excreted

and then conjugated back into the original form in the environment.

Wastewater Treatment Plants have trouble removing the synthetic form of estrogen in particular because the biodegradation of pharmaceuticals is slow. This is because the substances are designed to have long-term effects in humans. For example, the natural occurring hormone, 17β-estradiol (E2) biodegrades relatively quickly, while 17α-ethinylestradiol (EE2) biodegrades slowly due to its synthetic properties.[5] Reports on the presence of hormones in water were first published in 1965, showing that they were not completely eliminated by WTPs.

In an experiment conducted to potentially gain support for a new drug containing EE2, it was observed that in a 5-day aerobic sludge experiment, 100% of EE2 remained, concluding that EE2 is less biodegradable in the environment than the natural occurring hormones. [5] Wastewater treatment plants are having difficulty finding relatively inexpensive ways to remove estrogen from water. The most common method of removal is by the activated sludge process, and a number of studies have shown that nitrifying sludges can co-metabolically remove EE2.[6] WTPs are the main source of removal of these micropollutants. It has been found that tertiary treatments are needed to fully remove the compounds, however, tertiary treatments increase the overall cost by 10-15%.[7] In an experiment in which estrogen was added to the water, it was discovered that algae, bacteria, and invertebrates were unaffected by the hormones. Even the smallest fish, however, suffered a decline in population and a trend in feminization.[8]

The overall effectiveness of wastewater treatment plants has been examined; however, to our knowledge, no treatment has been designed to specifically remove estrogens in water. In addition, we have not seen research done using HPLC with fluorescence to analyze actual WTP influent and effluent samples. The long-term goal of this research project is to carry out experiments using water samples from treatment facilities to determine which treatment method is most efficient in removing natural and synthetic estrogens. To do this, standard samples of 17β-estradiol and 17α-ethinylestradiol were used this semester in attempt to create analytical methods that would aid in the analysis of samples from wastewater treatment plants in future research. The method developed was based on the procedure used in *HPLC- fluorescence detection and adsorption of bisphenol A, 17β-estradiol, and 17α- ethynyl estradiol on powdered activated carbon*.[9]

Experimental Details

Creation of Standard Solution

A stock solution of 1 mM E2 was created by adding 0.0137 g E2 to a 50 mL volumetric flask and diluting to volume with HPLC grade MeOH.

17β- estradiol

$$0.0137g\ E2 * \frac{1\ mol\ E2}{272.38\ g\ E2} \div 0.05\ L = 0.0010\ M$$

For use in the HPLC based on the procedure in *HPLC- fluorescence detection and adsorption of bisphenol A, 17β-estradiol, and 17α- ethynyl estradiol on powdered activated carbon*[9] the solutions were analyzed at a concentration of 15 mg/L; therefore the stock solutions were diluted as shown below.

$$15\frac{mg\ E2}{L} * \frac{1\ g}{1000\ mg} * \frac{1\ mol\ E2}{272.38\ g} = 5.5\ x\ 10^{-5} M\ E2$$

$$(0.001M)(V_1) = (5.5\ x\ 10^{-5}M)(0.05\ L)$$

$$V_2 = 0.00275\ L = 2.75\ mL$$

Therefore 2.75 mL of the 1 mM stock solution of E2 were added to a 50 mL volumetric flask and diluted to volume with HPLC grade water. The 15 mg/L solution of E2 was then analyzed using the method listed in Table 1.0 below.

Table 1.0 HPLC Method		
Method Type		Binary
Pump A	Solvent	HPLC grade MeOH
	Pump Conc.	55%
Pump B	Solvent	10 mM H_3PO_4

	Pump Conc.	45%			
SPD-10 Avp λ		277 nm			
RF-10 Axl	Excitation λ	280 nm			
	Emission λ	310 nm			
Time Program		Time	Module	Action	Value
		0.01	Pumps	Pump B Conc.	55
		30.00	Controller	Stop	-

Preparation of 10 mM H_3PO_4:

$$10\ mM\ Phosphoric\ Acid\ Stock$$

$$M_1V_1 = M_2V_2$$

$$(0.001\ M)(V_1) = (0.01\ M)(1\ L)$$

$$V_1 = 0.001\ L = 1\ mL$$

To a 1-L volumetric flask, 1 mL of 10 mM Phosphoric acid was added and diluted to volume with distilled water.

A sample of 5.5 x 10^{-5} M E2 was also analyzed by a RF-1501 Shimadzu Spectrofluorophotometer to test that the compound did in fact fluoresce. The excitation wavelength was set to 280 nm and the emission wavelength was set to 310 nm, similar to the HPLC method shown above.

Results and Discussion

Based on previous research, the 15 mg/L E2 was diluted to 1 x 10^{-7} M for use on the HPLC. This was completed by adding 0.18 mL of the 15 mg/L stock solution to a 100 mL volumetric flask and diluting to volume with distilled water. This concentration was run on the HPLC using the method above, and the spectrum can be seen in Figure 2.0.

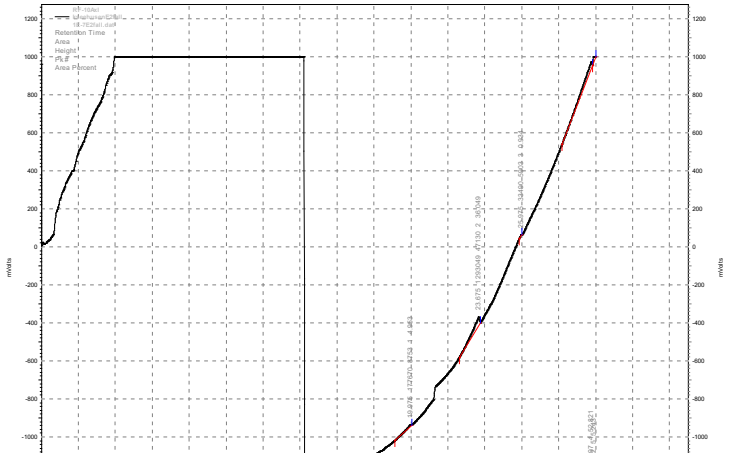

Figure 2.0. RF-10 Axl spectrum of 1 x 10^{-7} E2

Sample Dilution Calculation

$$M_1V_1 = M_2V_2$$

$$(0.055\ M)(V_1) = (1\ x\ 10^{-7}\ M)(0.1\ L)$$

$$V_1 = 0.18\ L = 0.18\ mL$$

This dilution process was repeated to obtain concentrations of 1 x 10^{-9} M, 1 x 10^{-11} M, and 1 x 10^{-13} M, and each were run on the HPLC in attempt to lower the reading of the RF-10 Axl. Because after each of the serial dilutions the results of the fluorescence detector still showed no signs of deterring from the rise and fall output, a sample was tested by a RF-1501 Spectrofluorophotometer to test for fluorescence. The value given by a 5.5 x 10^{-5} M sample of E2 was 130.278. This proved that the compound should fluoresce at the given wavelength, and it was concluded that an error was occurring within the HPLC device. It was suspected that the fault was coming from the RF-10 Axl because the SPD-10 Avp was presenting reasonable spectra. Shimadzu was then contacted and the situation was explained. To determine the cause of the error, the first suggestion was to use a new bottle of mobile phase solvent to see if an impurity in the methanol was causing the odd RF trace. The bottle was changed; however, this did not solve the issue.

The second suggestion was to increase the pump B concentration of 80% in attempt to flush the column, then re-equilibrate for 30 minutes using the original method. This did not improve the RF trace, and the rise and fall was still observed. It was then suggested that a sample of distilled water was run because no rise and fall or peaks should be observed with simply water. The results can be shown in Figures 2.1 and 2.2 below.

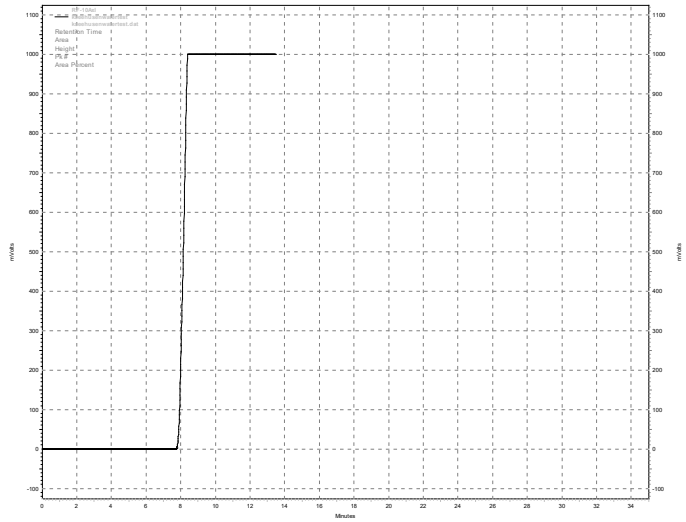

Figure 2.1. RF-10 Axl spectrum of distilled water

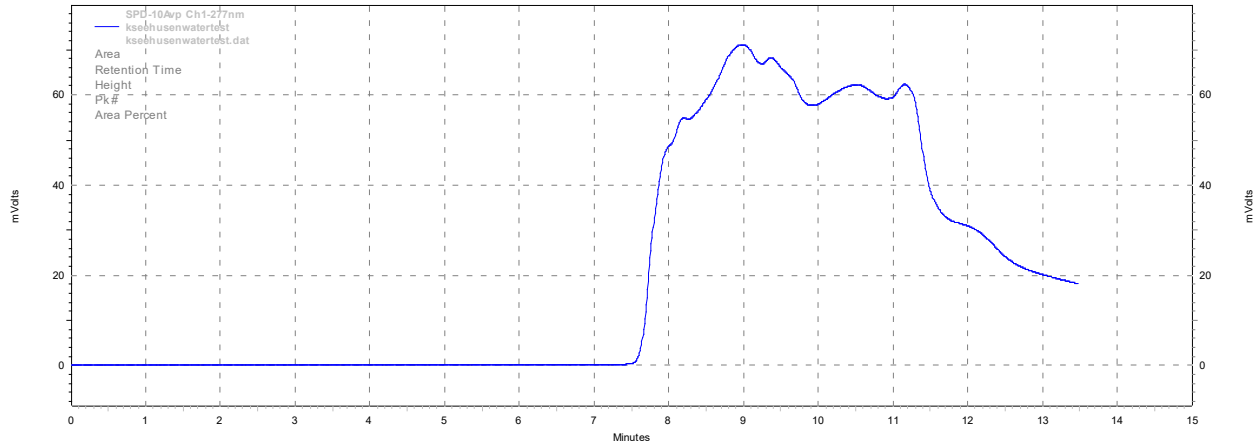

Figure 2.2. SPD- 10 Avp spectrum of distilled water

Because a peak in the SPD-10 Avp was seen with water, it was concluded that an unknown substance was coming off the column. To confirm this thought, the column was removed, and the results can be seen in Figure 2.3

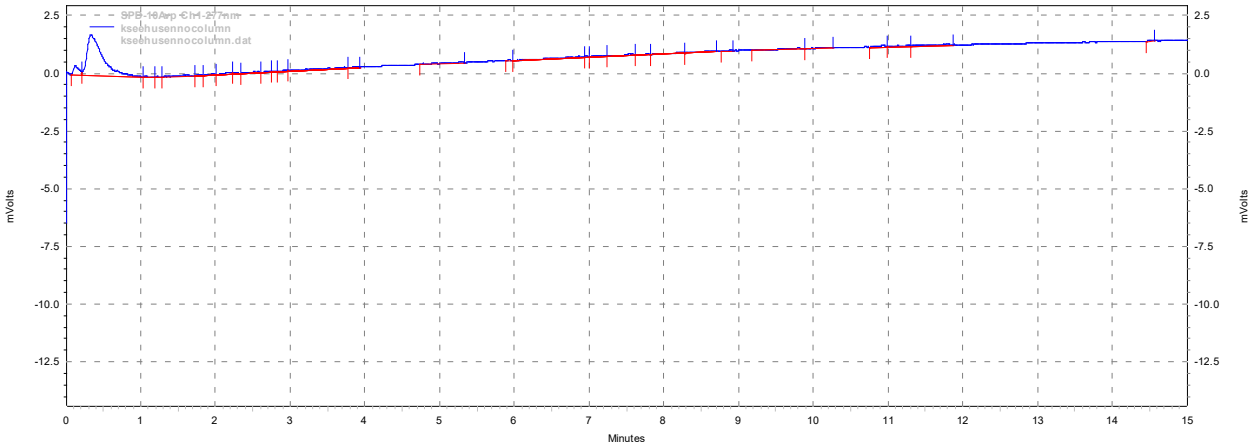

Figure 2.3. 10-SPD Avp spectrum- no column

The run showed no significant peaks or rise and fall on both the fluorimeter and the UV-vis spectrophotometer; therefore it was concluded that a new column was needed in order to obtain accurate results when running samples.

Both a new column and guard column were installed. This, however, did not solve the rise and fall of the signal in the spectra, even when only water was tested. To solve this issue, the column was removed, and the detector was flushed with HPLC grade MeOH in attempt to remove any trapped air bubbles in the flow cell. Subsequently, the flow cell was flushed with HPLC grade water. The next step was to apply the Raman test by checking the signal to noise ratio. The value found was 1090.50.

Based on the S/N value, it was determined that the detector was not functioning properly. The sample and reference energy was then checked with the excitation wavelength set to 350 nm and the emission wavelength set to 450 nm. The reference energy was 96 and the sample energy was at 28. The goal numbers for sample and reference energy were greater than 100 and less than 2.0, respectively. Since neither the sample nor reference energy were within the correct range, it was determined that because the lamp had more than 500 hours on it, it needed to be replaced.

A new 150 W xenon lamp, part number 228-34216, was then ordered, installed, and aligned properly. The maximum value when aligning the lamp was approximately 500. A new standard of 17β-estradiol was prepared and run using the previously developed method. The spectra is shown in Figure 1.0.

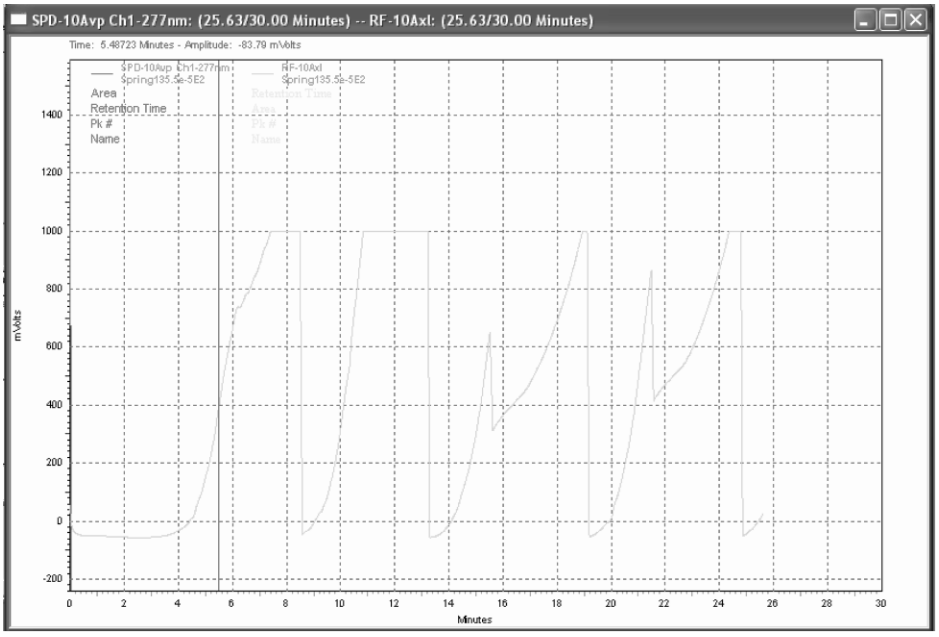

Figure 1.0. Spectrum of E_2 with new Xe lamp.

The sample and reference energy were tested again, reporting values of 160-190 and 102, respectively. Because the sample energy was so large, it was recommended to check again. After re-performing the test, it was found that the sample energy remained around 12, but would occasionally jump to 100, while the reference energy remained at 102. The jumping sample energy indicates air bubbles flowing through the flow cell, getting stuck in the flow cell, or a cracked flow cell. It was determined that the flow cell was not cracked, but may have some bubbles inside. It is possible that because the syringe used for flushing the flow cell is not specially made for the RF-Axl, it is not getting flushed properly. The next step would be to continue to flush the flow cell, and possibly purchase a new syringe.

Acknowledgements

I would like to thank Erika DeLoache from Shimadzu Technical Support for her help in determining the faults in the HPLC instrument. Her feedback was greatly appreciated.

References

(1) Kuch, H. M.; Ballschmiter, K. Environ. Sci. Technol. 2001, 35, 3201–3206.

(2) Shappell, N.; Vrabel, M.; Madsen, P.; Harrington, G.; Billey, L.; Hakk, H.; Larsen, G.; Beach, E.; Horwitz, C.; Ro, K.; Hunt, P.; Collins, T. Environ. Sci. Technol. 2008, 42, 1296– 1300.

(3) Wise, A.; O'Brien, K.; Woodruff, T. Environ. Sci. Technol. 2011, 45, 51–60.

(4) Belfroid, A. C.; Van de Horst, A.; Vathaak, A. D.; Schafer, A. J.; Rijs, G. B. J.; Wegener, J.; Confino, W. P. The Science of the Total Environment 1999, 225, 101–108.

(5) Christensen, F. M. Regulatory Toxicology and Pharmacology 1998, 28, 212–221.

(6) Yi, T.; Mackintosh, S.; Aga, D. S.; Harper Jr., W. F. Water Research 2011, 39, 1369–1377.

(7) Forrez, I.; Boon, N.; Verstraete, W. Comprehensive Biotechnology 2011, 6, 485-494.

(8) Dery, L. Troubled Waters: Removing Estrogen from Our Water Supply. 2011; http:// scientiareview.org/pdfs/169.pdf.

(9) Yoon, Y.; Westerhoff, P.; Snyder, S. A.; Esparza, M. Water Research 2003, 37, 3530-3537.

Detailed budget for Account # 213-776 Katie Seehusen

C-18 guard column for HPLC(to replace old, worn out one)

$157.25

Xenon lamp for detector (to replace burned out one)

$182.85

Flow cell for HPLC (to replace old, stained one)

$259.90

note: cost was split with other accounts

Total expenditures $600.00