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Derivatization and Separation of Natural Estrogens (estrone, 17β-estradiol, and estriol) Using Gas Chromatography with Flame Ionization Detection (GC-FID)

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Derivatization and Separation of Natural Estrogens

(estrone, 17β-estradiol, and estriol) Using Gas Chromatography with Flame Ionization Detection (GC-FID)

by

Dena L. Guptill

THESIS

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Abstract

A major cause of concern for estrogens found in the environment is that even at very low concentrations (10–100 ng/L) they can have a negative effect on aquatic ecosystems by disrupting endocrine systems. Due to the increasing importance of monitoring the levels of estrogens in the environment, a detection method was developed to identify and quantify three natural estrogens (estrone, 17β-estradiol and estriol) using gas chromatography with flame ionization detection (GC-FID). In addition, the efficacy of two derivatizing agents, N,O-bis(trimethylsilyl) acetamide (BSTA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), was evaluated. The GC detector (FID) was most responsive with E3, followed by E1 and E2. The lowest concentration detected in this study was 0.1 ng/μL for E1 and 1.0 ng/μL for E2. E3 was detectable at the lowest concentration examined (0.01 ng/μL) and is likely detectable at a much lower concentration. The response of the GC-FID to all three estrogens was linear from 0.01-100 ng/μL. The data suggest also that both derivatizing agents are effective for the three natural estrogens. This analytical method will be used in future studies to detect and quantify natural estrogens from environmental samples, and will be particularly useful when exploring the relative concentration of various estrogen compounds spatially and/or temporally.

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I. Introduction

Exposure to estrogen has been linked to a reduction in fertility, a disruption in growth rates and sex reversal in aquatic wildlife (Hanselman et al. 2006). This is particularly concerning when considering the research findings from the U.S. Geological Survey that found estrogen in effluents from municipal treatment plants, presumably due to improperly disposal of pharmaceuticals and urine (Kolpin et al. 2001). The International Programme on Chemical Safety and the EPA's Endocrine Disruptor Screening and Testing Advisory Committee clearly defined natural estrogens as endocrine disruptors, and suggested that they be added to the screening program under the Safe Drinking Water Act (EPA: Federal Register, 1998). Currently, the EPA has no established acceptable levels of estrogen in potable water. Furthermore, traditional wastewater treatment processes are not effective at removing most estrogens from influent wastewater (Kolpin et al. 2001).

Natural Estrogens, Synthetic Estrogens and Estrogen Mimics

Natural estrogens are a group of endocrine hormones synthesized from cholesterol that are responsible for regulating metabolism, cellular growth and reproduction (Mandal, 2015), and are primarily produced in the ovaries of females that regulate estrous and menstrual reproductive cycles (Mandal, 2015). Estrogens are also produced in small amounts in male testes and responsible for the production of healthy sperm (Mandal, 2015). Small amounts are also manufactured in the adrenal glands, fat cells, and the liver, which is used to maintain bone, collagen, the blood supply and cholesterol levels (Mandal, 2015). There are three types of naturally occurring estrogens, which are turned on or off

depending on a woman's life cycle: estrone (E1), 17β-estradiol (E2), and estriol (E3) (Mandal, 2015).

Synthetic estrogens (e.g. 17α-ethylnylestradiol, EE2) are pharmaceuticals that are widely used in birth control pills and hormone replacement therapies (HRT) for women experiencing menopause (Magda et al. 2013). Estrogen mimickers are manufactured chemicals compounds not intended to be used as hormonal supplements, but intended for other purposes. For example, bisphenol A (BPA) that is commonly used in plastics and butylated hydroxyanisole (BHA) that is used as a food preservative are able to bind to estrogen receptors (Hanselman et al. 2006).

Endocrine Disruption

Endogenous estrogens can become endocrine disruptors when they are taken in excess, which disrupts the body's normal response to hormone levels (Cooper, 2000). Estrogens responsible for signaling when certain genes should be turned on or off may cause an over-response, block a response, or a cause a response at inappropriate times (EPA: EDSP, 2011). Because synthetic estrogens and their mimics have similar molecular structure to natural estrogens, they have the ability to be detected by estrogen receptors in a similar fashion (Brian et al. 2005). Endocrine disruptors can increase the risk of heart disease, stroke, blood clots, breast and uterine cancer (Mayo Clinic, 2015). Generally, estrogens taken in excess can increase cell proliferation and promote tumor development (Cooper, 2000).

Environmental Impact of Estrogens

From 1999-2000, the U.S. Geological Survey conducted the first nationwide study to identify the occurrence and concentration of organic wastewater contaminants (OWCs) throughout the United States. They targeted 95 OWCs that ranged from industrial by-products, household cleaning agents and pharmaceuticals, from 139 streams across 30 states (Kolpin et al. 2001). The sites of water sample collected were downstream of highly urbanized areas and livestock farms. Their results showed that OWCs are passing through the purification process of municipal wastewater treatment plants and residential septic tanks. In fact, 82 of the 95 OWCs that were targeted in the study were identified in 80% of 139 streams tested (Kolpin et al. 2001).

The presence of natural estrogens, synthetic estrogens, and other pharmaceutical chemicals that mimic estrogens, in waterways is particularly concerning. Current scientific studies show a link between natural and synthetic estrogen found downstream from municipal wastewater discharge and the disruption of normal endocrine function of aquatic organisms. Estrogen and their mimics are a major environmental concern because they can disrupt reproductive cycles and cause population decline in wild fish populations at concentration levels as low as 10-100 ng/L (Hanselman et al. 2006). For example, vitellogenin (VTG), a protein normally produced by female fish that promotes the production of egg yolk when stimulated by estrogen, has been found in male fish that were exposed to extremely low concentrations of estrogens in aquatic habitats (Brian et al. 2005).

The effects of estrogens, such as 17β-estradiol, on fish populations suggests that similar responses in terrestrial organisms, particularly humans, may be expected. For example, research has shown that E2 exposure may lead to growth abnormalities in children, as well as early and late onset puberty with females and males, respectively (ATSDR 2007). After puberty, exposure to environmental E2 has been linked to cancer of reproductive organs, heart disease, osteoporosis, and Alzheimer's disease (Heffron 2014, Wright-Walters and Volz 2007).

Several studies have investigated environmental exposure to synthetic estrogens as well, such 17α-ethylnylestradiol (EE2). For example, during a 7 year study at the Experimental Lakes Area in Ontario Canada, researchers tested the effects of chronic exposure of 17α-ethylnylestradiol (EE2), a synthetic estrogen used in birth control pills, on fathead minnow populations. The research was led by Dr. Karen Kidd, an NSERC-funded biology professor at the University of New Brunswick (Saint John) and the Canadian Rivers Institute (Kidd et al. 2007). Low concentrations (5-6 ng/L) of 17a-ethylnylestradiol were added to an experimental lake (Lake 260) over a period of 3 years, which was compared to two reference lakes (Lake 114 and Lake 442). Biomarkers used to measure endocrine disruption included fish abundance, concentrations of VTG in whole-body homogenates of both male and female, and histological changes in gonadal tissue. Females produced more VTG after exposure to EE2 when compared to reference samples, and males produced three times as much when compared to reference samples of whole–body homogenates. Cross-sections of

female ovarian tissue showed normal oocyte development from both reference lakes (114 and 442), but delayed development when compared to lake 260. Testicular tissue of males expressed normal development from reference lakes; however all males from both experimental lakes displayed spermatogenesis, widespread fibrosis and malformations of tubules. Moreover, 4 of 9 males captured after the third year of exposure to EE2 showed the presence of primary stage oocytes.

Gas Chromatography and Derivatization

Derivatization is an important procedure necessary for many compounds prior to injection into a gas chromatography system. Such compounds are typically not volatile at temperatures typical GC systems (e.g. <350°C). Organic compounds with polar functional groups that have active hydrogens attached, such as COOH, NH, and OH are not typically suitable for gas chromatography detection. A molecule with these functional groups attached causes the target compound to lack volatility and thermal stability, properties necessary for GC analysis (Shareef et al. 2005). When organic compounds with active hydrogens are exposed to hot injection temperatures, they tend to break down or thermally decompose before separation occurs (Jolanta et al. 2013). Derivatization allows hydrogens to be replaced with another functional group that is more non-polar, and therefore, more amendable for gas chromatography analysis.

Silylation is the preferred derivatization procedure for steroids, usually by replacing hydrogen with trimethylsilyl functional group. N,O-bis(trimethylsilyl) acetamide (BSTA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) are two

common derivatizing agents that have been shown to be effective in forming stable trimethylsilyl derivatives suitable for GC analysis. BSTFA is also commonly used in tandem with trimethylchlorosilane, which acts as a catalyst to enhance derivatization even further or prevent incomplete derivatization (Orata, 2012). Derivatization lowers the boiling point of estrogens (normally 445-469°C) so they are detectable in a GC system.

II. Research Objectives

The purpose of this study was to develop an analytical method for the detection and separation of estrone, 17β-estradiol, and estriol using gas chromatography with a flame ionization detector (GC-FID). Two different derivatizing agents (BSTA and BSTFA) were explored as a means to optimize the derivatization process. This analytical method will be used in future studies to detect and quantify these natural estrogens from environmental samples.

III. Materials and Methods

Chemicals and Reagents

Estrone (E1), 17β-estradiol (E2), estriol (E3), N,O-bis(trimethylsilyl) acetamide (BSTA), and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; contained 1% trimethylchlorosilane) (Figure 1) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific (Waltham, MA).

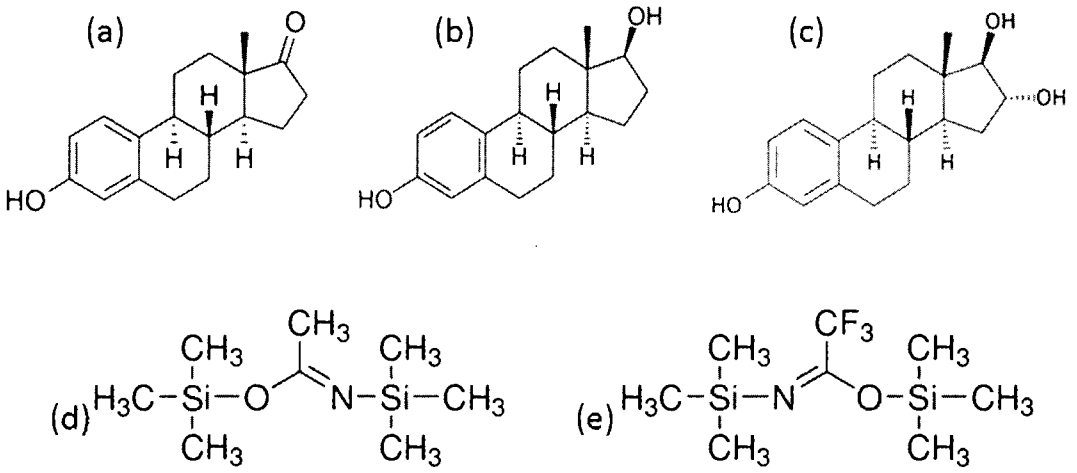

Figure 1. The chemical structures of estrone (a), 17β-estradiol (b), and estriol (c), N,O-bis(trimethylsilyl) acetamide (d), and N,O-bis(trimethylsilyl)-trifluoroacetamide (e).

Stock Preparation and Derivatization

Stock solutions of E1, E2, and E3 were prepared by dissolving them in compatible organic solvents. A polarity index was referenced to determine suitable solvents. Methylene Chloride was used as the solvent for both E1 and

E2. Pyridine was used as a solvent for E3, due to its incompatibility with methylene chloride as a result of its increased polarity compared to E1 and E2. Working samples of the estrogens were derivatized using 50 µL of estrogen sample, 250 µL of pyridine and 250 µL of derivatization agent (BSTA or BSTFA). Samples were then placed in a dry block heater for 30 min at 60°C before being used immediately or stored at 4°C.

Gas Chromatography Parameters

All experiments were performed using a Trace 1310 gas spectrometer equipped with a flame ionization detector (ThermoFisher Scientific, Waltham, MA). A TG-5MS (5% phenyl phase; ThermoFisher Scientific) capillary column (30 m x 0.25 mm x 0.25 µm) was used as the stationary phase with nitrogen as the carrier gas. The injection and detector temperatures were set at 250°C and 300°C, respectively. The oven program was: 150°C for 1.5 min, 50°C/min to 260°C, 260°C for 5 min, 10°C/min to 270°C, 270°C for 20 min. Samples were loaded as 1 µL injections using an autosampler. Data were collected and processed using Chromeleon 7.1 software (ThermoFisher Scientific).

IV. Results and Discussion

The United States Environmental Protection Agency (EPA) is responsible for upholding the 1972 Clean Water Act and the 1974 Safe Drinking Water Act. Although the EPA considers estrogen-based compounds endocrine disruptors, they currently have no established limits for acceptable concentrations of these chemicals in waterways. Acceptable limits are defined as the maximum contamination level in which there is no known or expected risk to health (Center for Disease Control, 2015). In addition, estrogen-based compounds are currently not on the EPA's list of primary or secondary contaminants list. However, E1, E2, and E3 are on EPA's contaminant candidate list of endocrine disruptors (EPA: CCL3, 2014). In order for a contaminant candidate to be placed in the primary contaminant list it must proceed through three phases of evaluation and review. The multi-step process includes: Phase 1 (data availability phase), Phase 2 (data evaluation phase), and Phase 3 (regulatory determination phase). E1, E2, and E3 are still in phase one because methods for identifying each type of estrogen are still being developed (Federal Registry: Daily Journal of the US Government, 2014).

A comprehensive method needs to be established before the EPA can allow estrogen-based compounds to be covered under the Safe Drinking Water Act as well as the Clean Water Act. In the following study, our goal was to develop a method that will detect natural estrogens using gas spectrometry with flame ionization detection (GC-FID). The estrogens were derivatized using two common derivatization agents (BSTA and BSTFA) to explore the effects these compounds may have on their detection using GC-FID.

Separation of Natural Estrogens by GC-FID

A detection method that will reliably separate natural estrogens (E1, E2, and E3) is necessary to detect and quantify these compounds in environmental samples. Our results show that we were able to resolve each estrogen at a unique retention time with both derivatizing reagents. For both derivatizing agents and at concentrations of 100 ppm, clear peaks were identified for E1 at approximately 14 min (Figures 2 and 3), for E2 at 14.5 min (Figures 4 and 5), and for E3 at 16.5 min (Figures 6 and 7). Figure 5 (E2 derivatized with BSTFA) shows a chromatogram with very low overall response, which is likely due to an anomaly with the GC-FID system for that particular sample. Nevertheless, an E2 peak was detected at 14.5 min. When these individual chromatograms were overlaid according to the derivatization agent used, all three estrogen peaks were clearly visible and distinct with 0.5 min being the shortest retention time difference, between E1 and E2 (Figures 8 and 9). A minimum separation of 0.5 min is typically sufficient for confidently identifying compounds from a sample.

Detection Range

Establishing the limit of detection, which is the lowest quantity a substance can be detected using an analytical instrument, is important for analysis of compounds that occur at low concentrations. In the present study, a dilution series (100 ng/μL, 10 ng/μL, 1.0 ng/μL, 0.1 ng/μL, and 0.01 ng/μL) was used to explore the lower detection range for each of the three natural estrogens explored in this study, where each sample contained E1, E2, and E3 at a given concentration. In all cases, peaks for all three estrogens at 100 and 10 ng/μL

were easily detected. Chromatograms for the three lowest concentrations were overlaid to explore the detection limits of the GC-FID (Figures 10 and 11). Although each estrogen from a given sample had identical concentrations (e.g. 1.0 ng/μL), the peak heights were unique due to fact that each of these compounds is detected differently by the detector. The data show that the lowest concentration that could be detected for E1 using either derivatization agent was 0.1 ng/μL, 1.0 ng/μL for E2, and 0.01 ng/μL for E3. It should be noted that the dilution series used in these experiments provides a general concentration range for the lower detection limit. Further analysis should be conducted with additional concentrations within a given lower range for each compound. For instance, E2 should be explored in increments of 0.1 ng/μL from 1.0 ng/μL to 0.1 ng/μL to get a more accurate estimate of the detection limit. It should also be noted that the lower detection range for E3 is likely much lower than 0.01 ng/μL. Further experiments need to be conducted with lower concentrations to provide a more accurate detection limit for this compound.

Linearity Test

A linearity test was performed to investigate whether the response of the detector was proportional to sample concentration. We plotted concentration against peak area for each estrogen for both BSTA and BSTFA from 100-0.01 ng/μL (Figures 12 and 13). The linear fit of the data (r^2 values between 0.996 and 1.000) suggest that the response of the FID to the estrogens was proportional to their concentrations over the given range. As observed with previous samples

from this study, the response of the detector was greater for E3 than E1 or E2, with the latter having the lowest response.

V. Conclusions

We have developed a GC-FID method to detect and separate three natural estrogens. Both derivatizing agents allowed effective derivatization with all three estrogens. The choice of derivatization agent for future experiments is likely to involve cost of the reagent and personal preference. The lowest detection range, regardless of derivatization agents, was 0.1 ng/μL for E1, 1.0 ng/μL for E2, and 0.01 ng/μL for E3. However, more accurate limits will need to be determined in the future, especially for E3 where detectable concentrations below the lowest tested in this experiment 0.01 ng/μL are expected. These concentration ranges are far above the expected concentrations for natural estrogens from environmental samples. For example, a study conducted by Kyle Heffron at Eastern Illinois University investigated E2 concentrations of the influent, mixed liquor and effluent from the Charleston Waste Water Treatment Plant, where the effluent had an average discharge level of 3.6 ng/L (Heffron, 2014). However, environmental samples could be concentrated using solid phase extraction to increase the concentration of the estrogens to be within the detection range of the GC-FID. This GC-based strategy will be particularly useful when exploring the relative concentration of various estrogen species spatially and/or temporally.

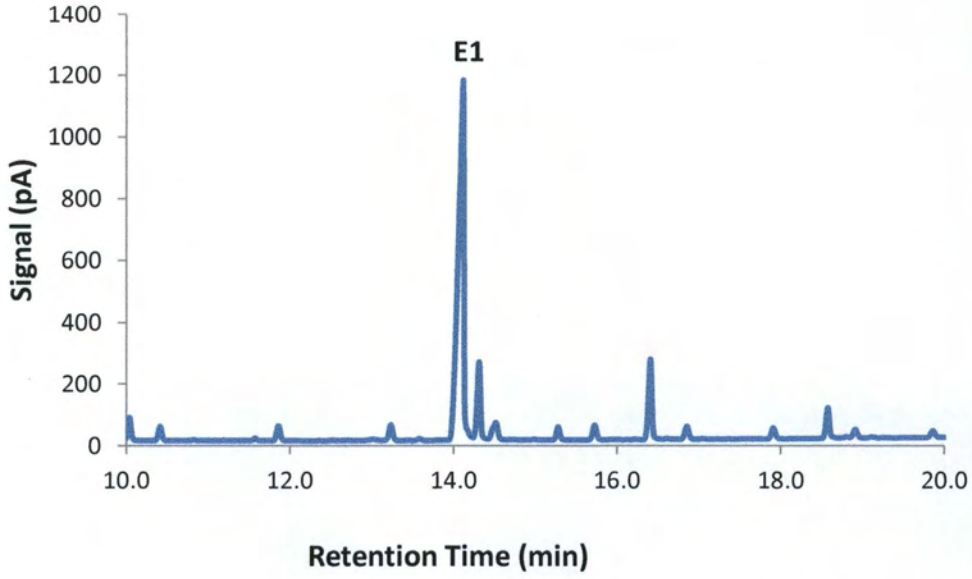

Figure 2. Chromatogram of the BSTA derivative of estrone (100 ng/µL).

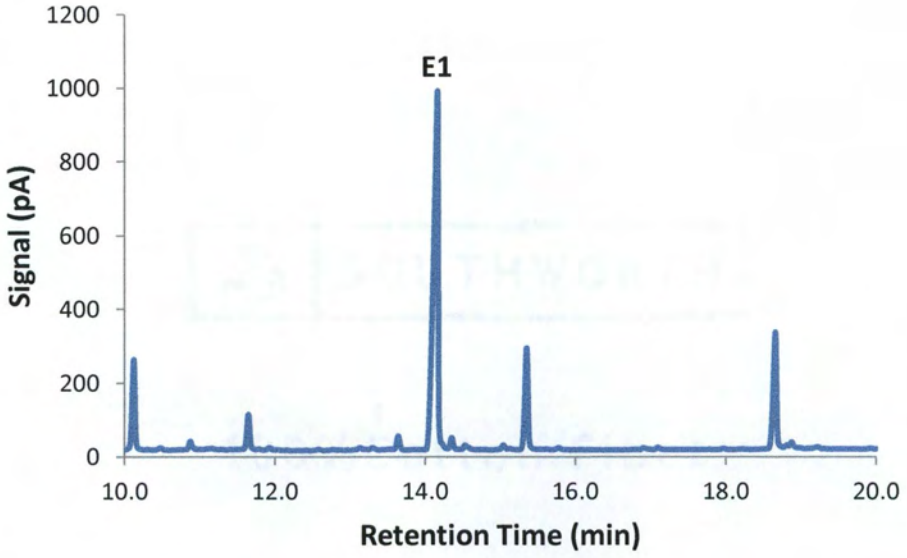

Figure 3. Chromatogram of the BSTFA derivative of estrone (100 ng/µL).

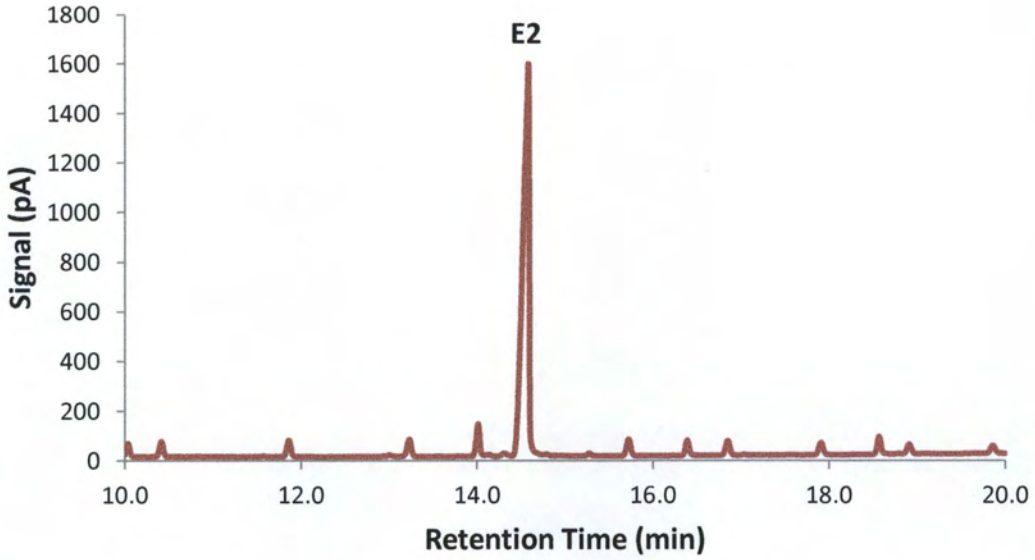

Figure 4. Chromatogram of the BSTA derivative of 17β-estradiol (100 ng/μL).

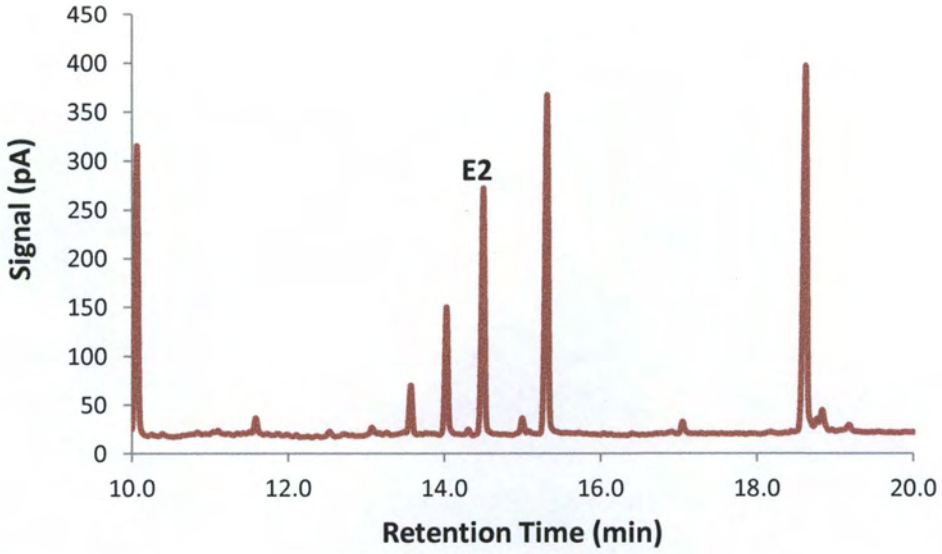

Figure 5. Chromatogram of the BSTFA derivative of 17β-estradiol (100 ng/μL).

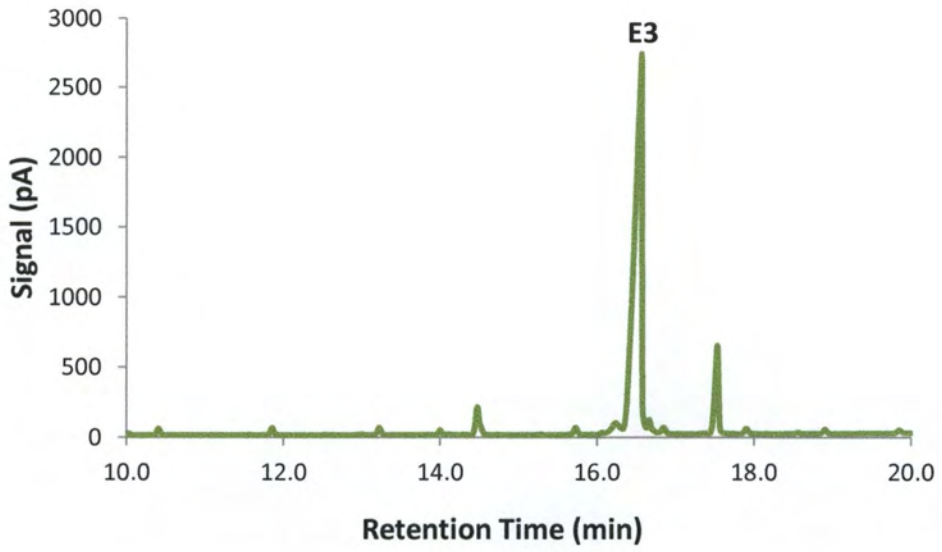

Figure 6. Chromatogram of the BSTA derivative of estriol (100 ng/µL).

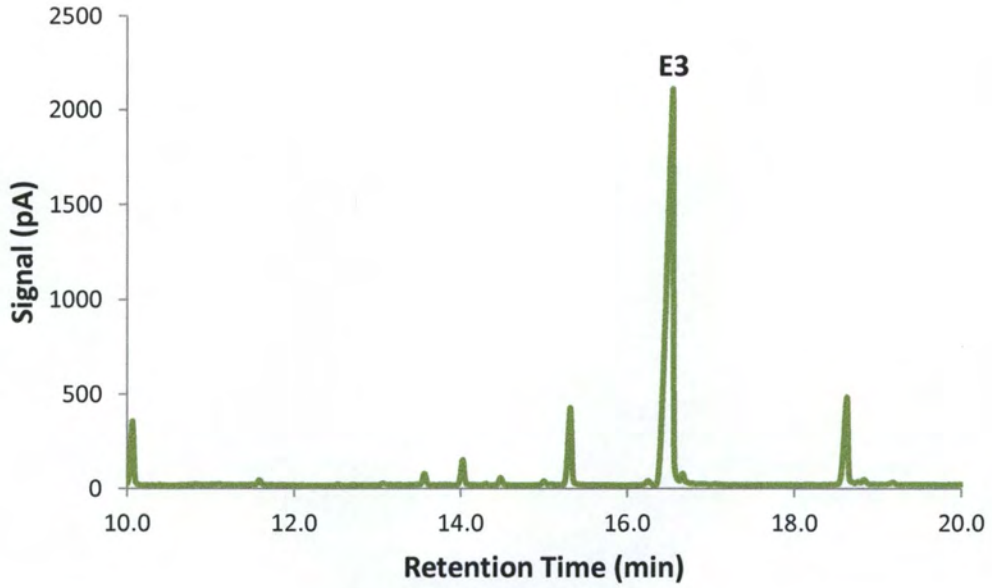

Figure 7. Chromatogram of the BSTFA derivative of estriol (100 ng/µL).

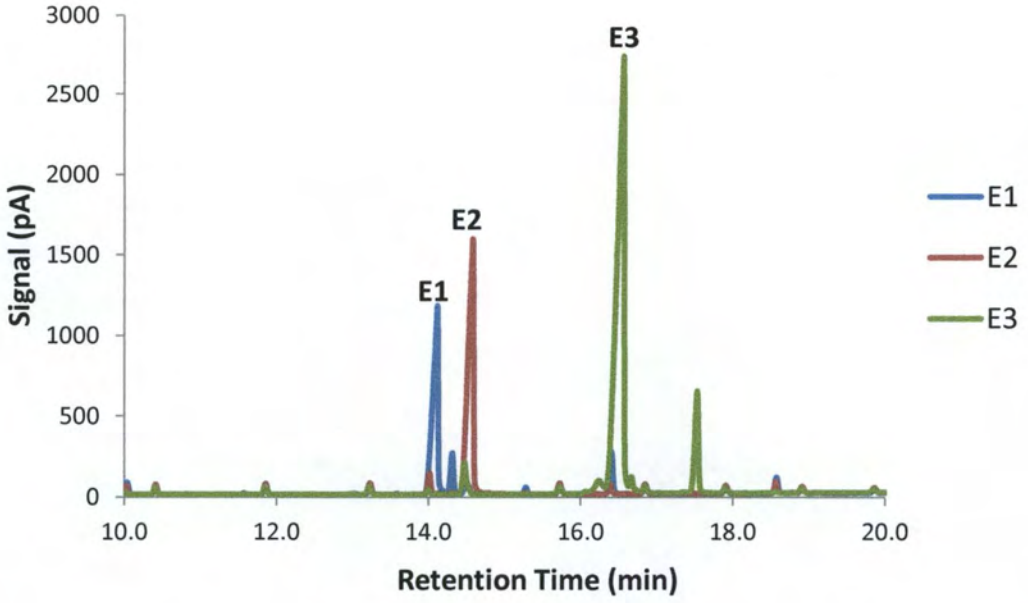

Figure 8. Chromatogram overlays of the BSTA derivatives of estrone, 17β-estradiol, and estriol (100 ng/μL).

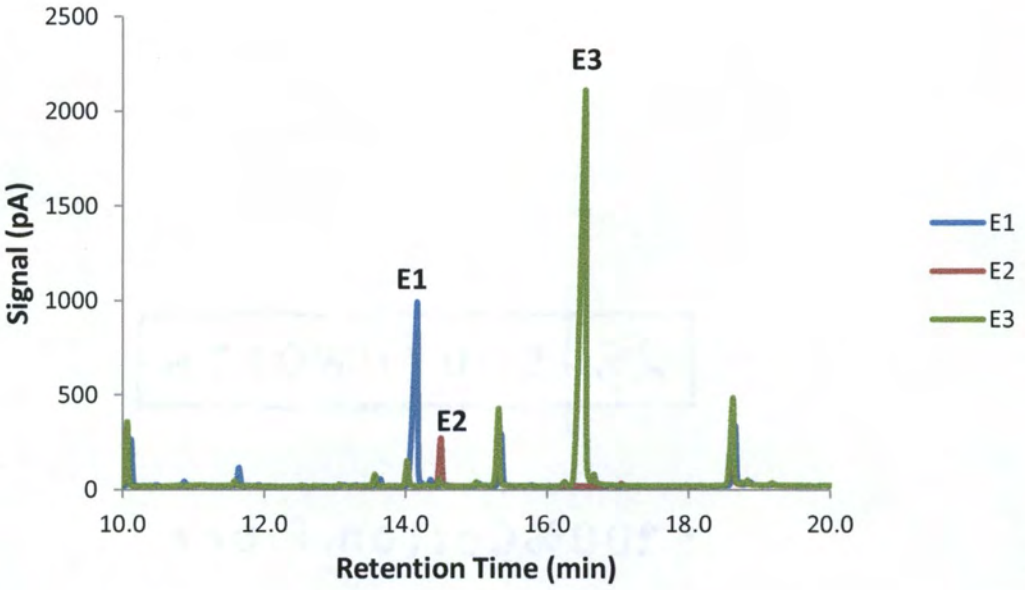

Figure 9. Chromatogram overlays of the BSTFA derivatives of estrone, 17β-estradiol, and estriol (100 ng/μL).

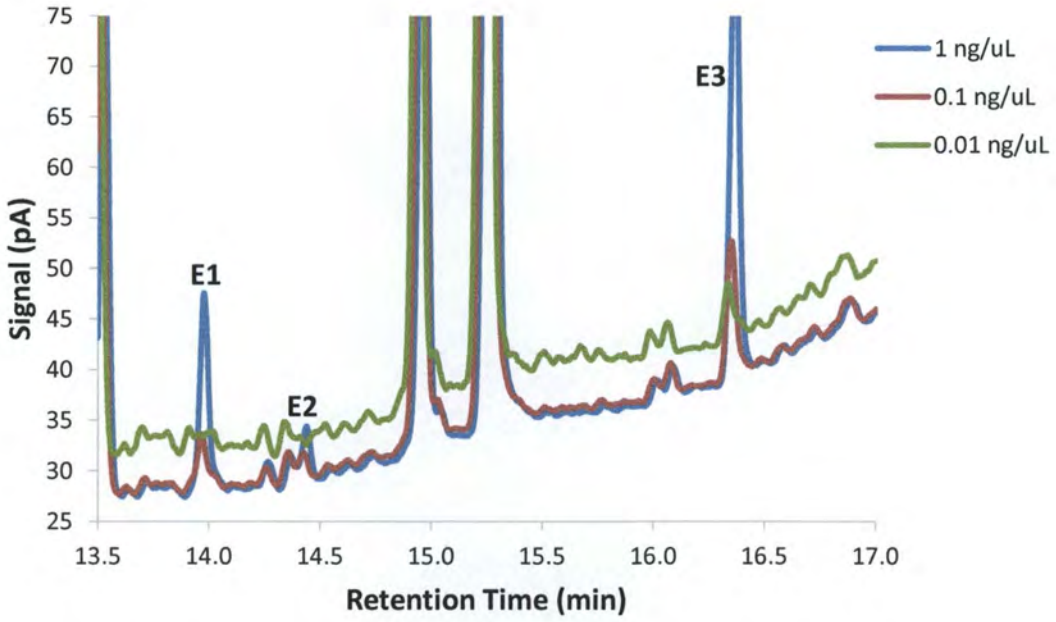

Figure 10. Chromatogram overlays of the BSTA derivatives of estrone, 17β-estradiol, and estriol at varying concentrations.

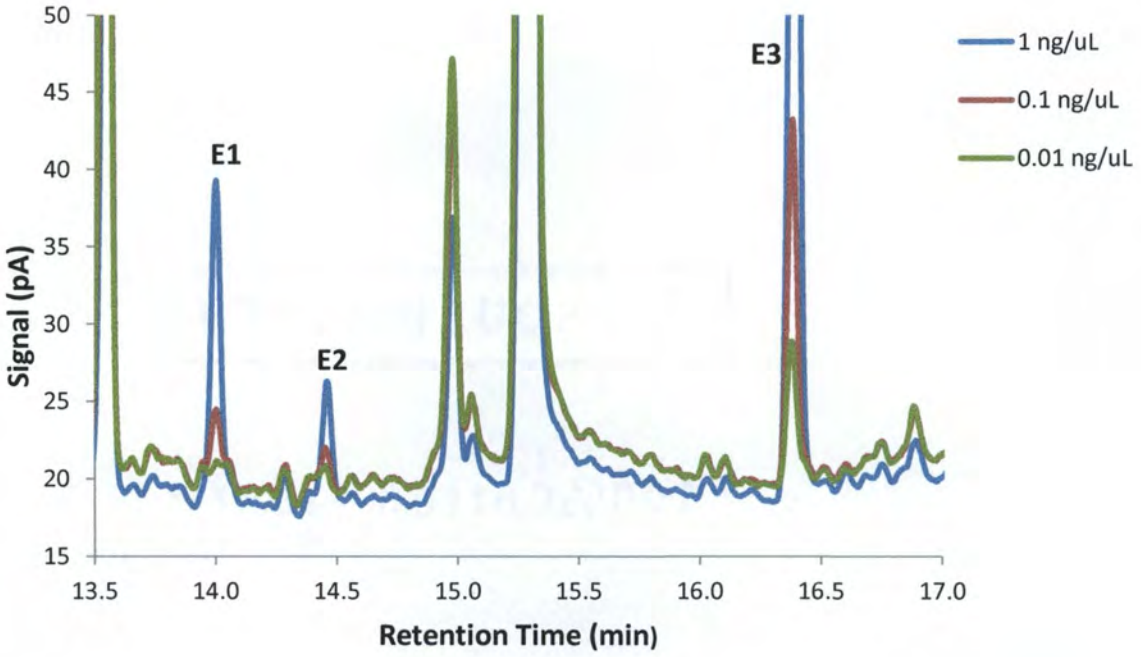

Figure 11. Chromatogram overlays of the BSTA derivatives of estrone, 17β-estradiol, and estriol at varying concentrations.

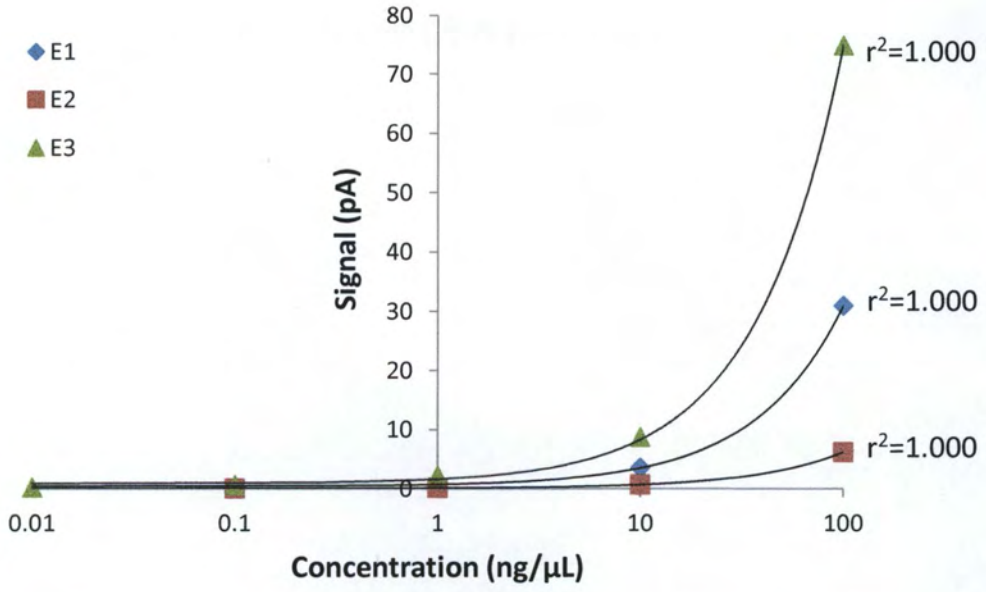

Figure 12. Dilution series (100, 10, 1.0, 0.1 and 0.01 ng/µL) of the BSTA derivatives of estrone, 17β-estradiol, and estriol. E1 and E2 at 0.01 ng/µL were not detectable.

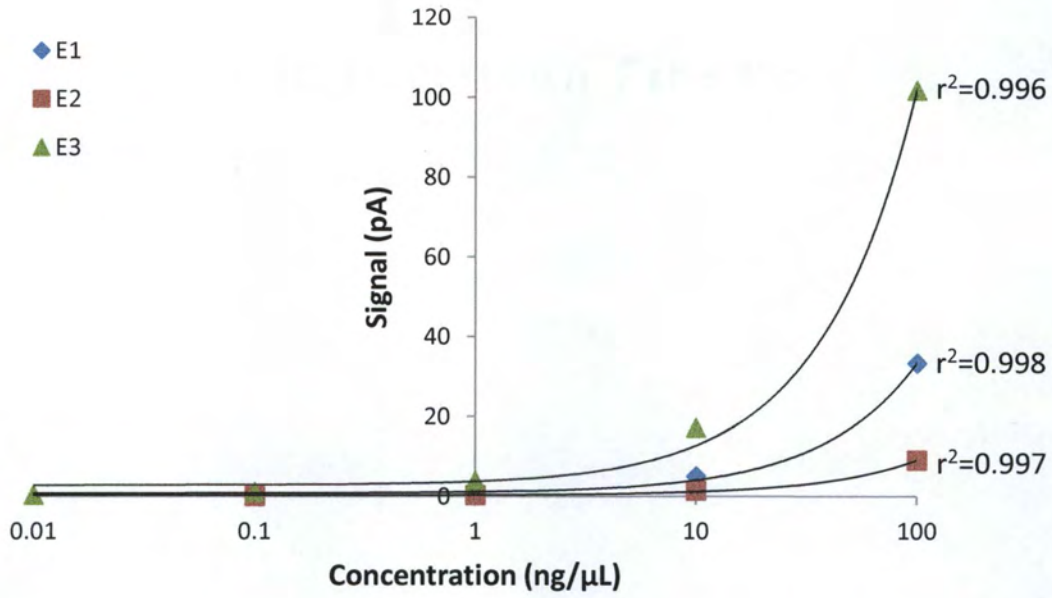

Figure 13. Dilution series (100, 10, 1.0, 0.1 and 0.01 ng/µL) of the BSTFA derivatives of estrone, 17β-estradiol, and estriol. E1 and E2 at 0.01 ng/µL were not detectable.

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