2009

Environmental Analysis of Selected Estrogens and androgens: Applying Ultra-Performance Liquid Chromatography and Combating Matrix Interference

Stacie L. Rice
College of William and Mary - Virginia Institute of Marine Science

Follow this and additional works at: https://scholarworks.wm.edu/etd

Part of the Analytical Chemistry Commons, and the Environmental Health and Protection Commons

Recommended Citation
https://dx.doi.org/doi:10.25773/v5-7ggw-qg35

This Thesis is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Dissertations, Theses, and Masters Projects by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
Environmental Analysis of Selected Estrogens and Androgens:
Applying Ultra-Performance Liquid Chromatography and
Combating Matrix Interference

A Thesis
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
of the Requirements for the Degree of
Master of Science

by
Stacie L. Rice
2009
APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Science

Stacie L. Rice

Approved, by the Committee, July 2009

Robert C. Hale, PhD
Committee Chairman/Advisor

Michael A. Unger, PhD

Peter A. Van Veld, PhD

Elizabeth A. Canuel, PhD

Daniel J. Fisher, PhD
University of Maryland
Queenstown, MD
This work is dedicated to the one constant in my life:

God

And in loving memory of Mary Peworchik; may we all find the fire she had at 90.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>2</td>
</tr>
<tr>
<td>Hormones as Contaminants</td>
<td>2</td>
</tr>
<tr>
<td>Toxicity</td>
<td>5</td>
</tr>
<tr>
<td>Molecular Structure</td>
<td>7</td>
</tr>
<tr>
<td>Transport</td>
<td>8</td>
</tr>
<tr>
<td>Transformation</td>
<td>9</td>
</tr>
<tr>
<td>Detection</td>
<td>11</td>
</tr>
<tr>
<td>Chromatographic Separation</td>
<td>13</td>
</tr>
<tr>
<td>Sample Preparation</td>
<td>15</td>
</tr>
<tr>
<td>Matrix Effects</td>
<td>17</td>
</tr>
<tr>
<td>Objective</td>
<td>19</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>Chemicals</td>
<td>20</td>
</tr>
<tr>
<td>(ESI)MS$^2$</td>
<td>21</td>
</tr>
<tr>
<td>UPLC</td>
<td>23</td>
</tr>
<tr>
<td>Instrumental Quantitative Boundaries</td>
<td>25</td>
</tr>
<tr>
<td>SPE</td>
<td>26</td>
</tr>
</tbody>
</table>

iv
ACKNOWLEDGEMENTS

The instrumentation, chemical standards, laboratory supplies, and federal permits utilized in this study were provided by Dr. Robert Hale, with technical support from Ellen Harvey and Dr. Drew Luellen. Samples were supplied by Serena Ciparis, Lance Yonkos, and Dr. Daniel Fisher. Throughout this degree program, I was fortunate to receive the financial support of the VIMS Dean’s Office and Dr. Hale. I am also grateful for the intellectual input of my committee members and their understanding in instances of methodological and instrumental set-backs.

The research herein greatly benefited from the instruction, moral support, and friendship provided by labmates Elizabeth Bush, Da Chen, Gregg Mears, and Mark LaGuardia.

I am most grateful for the constant support of my family and friends. The Rices and Popsos have cheered me on for 25 years, suffering every failure and celebrating every success. The Mitras encouraged me to pursue a graduate degree and introduced me to VIMS. The Nichols and Baers have been my Virginia friends and guardian angels. Special thanks to Paul for his relentless friendship. The Jenkins have filled my final year with fun, and Kenny has shown me beauty in the world and myself, giving me vision beyond my failure and doubt.
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (ESI)MS² compound-dependent parameters</td>
<td>36</td>
</tr>
<tr>
<td>2. (ESI)MS² source/gas-dependent parameters</td>
<td>36</td>
</tr>
<tr>
<td>3. Estrogen UPLC method</td>
<td>38</td>
</tr>
<tr>
<td>4. Androgen UPLC method</td>
<td>38</td>
</tr>
<tr>
<td>5. Repeatability of UPLC/(ESI)MS² hormone analyses</td>
<td>40</td>
</tr>
<tr>
<td>6. Modified androgen UPLC method</td>
<td>46</td>
</tr>
<tr>
<td>7. (APCI)MS² compound-dependent parameters</td>
<td>53</td>
</tr>
<tr>
<td>8. (APCI)MS² source/gas-dependent parameters</td>
<td>53</td>
</tr>
<tr>
<td>9. Estrogen plus testosterone UPLC method</td>
<td>55</td>
</tr>
<tr>
<td>10. Quantitative boundaries of UPLC/(APCI)MS² analyses</td>
<td>56</td>
</tr>
<tr>
<td>11. Repeatability of UPLC/(APCI)MS² hormone analyses</td>
<td>56</td>
</tr>
<tr>
<td>12. ESI vs. APCI repeatabilities for estrogens and testosterone</td>
<td>70</td>
</tr>
<tr>
<td>13. Inter-study comparison of quantitative boundaries</td>
<td>70</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Hormone structures</td>
</tr>
<tr>
<td>2.</td>
<td>Hormone metabolism</td>
</tr>
<tr>
<td>3.</td>
<td>(ESI)MS² schematic</td>
</tr>
<tr>
<td>4.</td>
<td>Steps in handling and analysis of samples for hormone contaminants</td>
</tr>
<tr>
<td>5.</td>
<td>(APCI)MS² schematic</td>
</tr>
<tr>
<td>6.</td>
<td>Modifier addition for androgens</td>
</tr>
<tr>
<td>7.</td>
<td>Total ion chromatograms and UPLC flow regimes for ESI analyses</td>
</tr>
<tr>
<td>8.</td>
<td>Quantitative boundaries for UPLC/(ESI)MS² analyses</td>
</tr>
<tr>
<td>9.</td>
<td>Hormone elution solvent distribution</td>
</tr>
<tr>
<td>10.</td>
<td>SPE method recoveries</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of storage on apparent recoveries</td>
</tr>
<tr>
<td>12.</td>
<td>Recoveries from surface water vs. DI</td>
</tr>
<tr>
<td>13.</td>
<td>Total ion chromatogram for androgens in pond water</td>
</tr>
<tr>
<td>14.</td>
<td>Total ion chromatogram and UPLC flow regime of modified androgen method</td>
</tr>
<tr>
<td>15.</td>
<td>Recoveries across matrices with UPLC/(ESI)MS² quantification</td>
</tr>
<tr>
<td>16.</td>
<td>Affect of purification on recoveries</td>
</tr>
<tr>
<td>17.</td>
<td>Matrix effect in standard-spiked effluent extract</td>
</tr>
<tr>
<td>18.</td>
<td>Matrix effect during infusion</td>
</tr>
<tr>
<td>19.</td>
<td>Modifier addition in APCI</td>
</tr>
</tbody>
</table>
20. Total ion chromatogram and UPLC flow regime for APCI analysis............................55
21. Recoveries of analytes from effluent using UPLC/(APCI)MS² quantification............57
22. SEC fractionation of mixed standard...........................................................................58
23. Recoveries using SPE/SEC/(APCI)MS².........................................................................59
24. Effect of SEC on hormone recoveries.............................................................................68
ABSTRACT

Investigations of environmental hormone contamination commonly utilize solid-phase extraction (SPE) followed by high-performance liquid chromatography / (electrospray ionization) tandem mass spectrometry (HPLC/(ESI)MS²) in the detection of estrogens. Matrix interference is widely reported. In this study, androgens were targeted alongside estrogens as environmentally co-introduced endocrine disrupting chemicals. Analytical methods were developed in parallel for detection of several hormones from each class, with comparison of protocols and instrumental parameters. Ultra-performance LC (UPLC®), an emerging technology advertised for reduced retention times, was used in place of HPLC for hormone separation. Applicability to diverse aqueous samples was tested. Matrix interference was combated with two rarely used techniques: atmospheric pressure chemical ionization (APCI) MS² and extract purification by size exclusion chromatography (SEC). Infusion and spiking tests were performed in (ESI)MS² and (APCI)MS², demonstrating relative matrix disruption of hormone signals. An SEC fractionation protocol was developed and applied to extracted wastewater samples prior to UPLC/MS² analysis. Hormone recoveries were compared to those obtained without SEC purification. In accordance with these experiments, it was found that estrogen and androgen contaminants can be simultaneously extracted using C₁₈ SPE. This approach reduces the amount of sample, supplies, and time required in preparation for instrumental analysis. In UPLC separation, a broader gradient, slower flow rate, and increased run time were used for the androgens to counteract structural similarity and reduced polarity. The androgens were most readily detected using positive (ESI)MS², versus negative for the estrogens, with modifier addition for signal enhancement. Electron delocalization in the estrogen and testosterone molecular structures facilitated ionization, permitting MS² detection at or below 23 pg on-column versus 500 pg for the androgens lacking bond conjugation. Recoveries of all analytes from deionized water were 67-112% using UPLC/(ESI)MS². Use of UPLC reduced retention times and solvent usage in comparison to HPLC, permitting adequate resolution of the hormones within 7 min in the presence of clean solvents. In environmental samples, the rapid analyses proved susceptible to matrix interference, with lack of signal resolution amidst unresolved complex mixtures. The application of (APCI)MS² to complex samples showed promise in combating matrix interference, permitting detection of hormones spiked into wastewater that were not observed using (ESI)MS². The ionization methods tended toward opposite matrix effects, with 140-410% recoveries (i.e. ion enhancement) from effluents using APCI and 5-120% (i.e. ion suppression) using ESI. Application of SEC prior to instrumental analysis removed some interfering compounds, allowing recoveries of 48-98% for several hormones using UPLC/(APCI)MS².
Environmental Analysis of Selected Estrogens and Androgens:
Applying Ultra-Performance Liquid Chromatography and
Combating Matrix Interference
INTRODUCTION

Hormones as Contaminants. Scientists worldwide have entered into the third decade of environmental estrogen research. Spurred by observations of hermaphroditic fish proximal to wastewater treatment plants (WWTPs), exposure studies were undertaken in the mid-1980s and continue today [1-4]. The natural female steroids estrone (E1), 17β-estradiol (E2), and estriol (E3) and the synthetic contraceptive 17α-ethynylestradiol (EE2), have been the primary foci of this research [5-11].

Following the early studies of Purdom et al. [1], confirming a connection between feminized male fish and WWTP effluent, further investigation into the suspect chemicals and their concentrations became widespread. In the early 1990s, Shore et al. [12] analyzed the aqueous waste entering a biological treatment facility, incubating in a digestion tank within the plant, and exiting as treated waste. During three sampling dates, removal rates of 20-88% of the total estrogens present were reported, with levels of 6.5-50 ng/L in the effluent. In a similar study, Koh et al. [9] reported comparable in-plant removal, with effluent concentrations of 3.0, 0.7, 1.0, and 1.0 ng/L for E1, E2, E3, and EE2, respectively. Although these levels vary daily within and between WWTPs, estrogens are commonly reported at low ng/L levels in effluents [13-15]. Direct
introduction of effluents into surface waters with varying dilution capabilities has resulted in commonplace detection of female hormones at similar levels in streams, rivers, and lakes throughout the world [5,7,10,12,16].

In communities which do not have access to municipal sewer systems, individual or communal septic tanks may be used. These likewise collect and emit aqueous solutions of hormones, potentially contaminating groundwater supplies. Swartz et al. [17] detected E1 and E2 in Cape Cod residential systems at 16-19 and 49-74 ng/L, respectively.

Non-point source pollution is also suspect in contemporary observations of wildlife exhibiting endocrine disruption. The presence of male fish containing egg cells, a condition known as intersex, in the Potomac and Shenandoah Rivers of Virginia has been under investigation since 2003 [18,19]. One suspect cause is the presence of estrogens in runoff contaminated with animal waste from agricultural facilities. Rockingham County in the Shenandoah Valley, for example, is the top turkey producing county in the U.S [19-20].

The male counterpart to the estrogens, the androgens, have been far less studied and documented, with testing of compounds other than testosterone (T) not appearing until the early 2000s [21-22]. Consequently, fewer analytical methods, monitoring data, and fate studies have been published for androgens relative to those concerning estrogens. Searching the electronic databases Web of Science and CSA Illumina, and that of the American Chemical Society, reveals 4.4-4.9 times the number of references associated with “estrogens AND environmental” versus “androgens AND environmental” published over the past 20 years.
Androgen research has in part been driven by endocrine disruption in aquatic wildlife proximal to paper mills. Jenkins et al. [22] studied a river which received paper mill effluent and contained masculinized female mosquitofish (*Gambusia hoboooki*). They identified and attributed androgenic activity to androstenedione and its precursor, progesterone. These were believed to derive from microbial transformation of phytosterols present in the tree pulp used in paper production and are precursors to T and additional androgenic hormones [22-23].

Like estrogens, androgens are also introduced into environmental waters via WWTP discharge [7,21]. In 1993, Shore et al. [12] reported 60-77% removal of T during biological treatment, with exiting concentrations of 6.8-123 ng/L. It was not until 2002 that Thomas et al. [21] tested for additional androgens, and detected dehydrotestosterone, androstenedione, androsterone (A), and epiandrosterone (EA) at estimated concentrations of 172, 100, 83, and 33 ng/L, respectively.

Androgens can also be deposited with animal waste and incorporated in runoff along with estrogens. Steroidal growth promotion in cattle housed in concentrated animal feeding operations (CAFOs) is one such source of concern [24-25]. The synthetic androgen trenbolone acetate is a common growth promoter that degrades within the body to produce 17α- and 17β-trenbolone, which can be found at concentrations of 14 and 1 ng/g, respectively, in fresh dung [25]. Contaminated feces have been found to contribute low ng/L levels of the stereoisomers to CAFO run-off, endangering receiving water bodies [24].
Toxicity. The original study by Purdom et al. [1] naming wastewater as a source of endocrine disruption was published in 1994. A radioimmunoassay was used to measure the blood plasma concentrations of vitellogenin (VTG) in rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) caged outside of a WWTP. Under natural conditions, VTG is inherent only to egg-producing females. Over a three week period, male trout experienced steadily rising VTG levels, reaching 33, 192, and 373 pg/ml blood plasma at the end of the first, second, and third weeks, respectively, while controls remained near the detection limit, at 0.1, 0.06, and 0.04 µg/ml. Similar testing was pursued outside of 30 facilities across England and Wales. At 50% of the sites, all test fish were dead by the end of three weeks. At the remaining fifteen, male trout VTG concentrations reached those typical of egg-producing females (mg/ml).

Purdom et al. [1] suspected that EE2 contributed to the observed estrogenic activity, but were unable to successfully apply methods for the detection of individual hormones in the surface water. They instead carried out laboratory injection and immersion studies using only EE2. Muscular injections of 1, 100, 500, and 1,000 µg/kg EE2 into rainbow trout were tested against equivalent doses of E2. The synthetic estrogen resulted in up to 720 times the VTG concentrations induced by the natural hormone, with 88-20,300 µg/ml blood plasma levels versus 10-4,295 µg/ml across the injection range for EE2 and E2, respectively. Immersion in 10, 25, and 50 ng/L EE2 for 10 d also resulted in elevated VTG in the trout, with average levels of 630, 4,970, and 11,200 µg/ml plasma, respectively. Although immersed carp also exhibited increased VTG levels at 25 and 50 ng/L EE2, increases were minor compared to those recorded in the trout, with final plasma levels of 0.84 and 216 µg/ml).
The estrogenic potency and popularity of EE2 as a contraceptive have led to a number of additional exposure studies incorporating diverse target species, estrogenic mixtures, and markers of endocrine disruption [2-4,26]. Coe et al. [4] recorded reduced male parentage, decreases in 11-ketotestosterone to below detection limits, and suppressed competition between males upon exposure of zebrafish (*Danio rerio*) to 10 ng/L EE2 for 25 d. Nash et al. [26] examined cross-generational effects of EE2 and E2 on zebrafish. In the first generation, neither chemical effected the growth or weight of the fish. Egg production and viability were likewise unaffected upon exposure to 0.5 or 5 ng/L EE2 or 5 ng/L E2, while 11-ketotestosterone in males was decreased to 30, 5, and 8%, respectively, that measured in controls. At 50 ng/L, offspring produced in the first few days of exposure exhibited spinal deformities and suppressed gonad growth. Egg production ceased by day 10. In the second generation, the number of nonviable eggs produced by zebrafish exposed to 0.5 ng/L EE2 or 5 ng/L E2 had ≥2 times as many nonviable eggs as the controls. Those in the 5 ng/L EE2 treatment were 100% phenotype females with no possibility for reproduction.

Though androgen research lags that of estrogens, a small number of studies have been published concerning the effects of trenbolone on mixed fathead minnow (*Pimephales promelas*) populations [27-28]. A number of the same endpoints are monitored in these studies as those discussed above, including plasma VTG and 11-ketotestosterone levels, weight, and egg production. Jensen et al. and Ankley et al. [27-28] also closely monitored females for thickening and darkening of the dorsal pad and the formation, number, and size of nuptial tubercles as indicators of masculinization, as these are naturally inherent to only reproducing males. Exposure tests were carried out for 21 d
in aqueous concentrations of 3-100 and 175-7,000 ng/L of 17α-trenbolone [28] and 5, 50, 500, 5,000, and 50,000 ng/L of 17β-trenbolone [27]. Reproduction halted at and above 175 ng/L 17α-trenbolone and 500 ng/L 17β-trenbolone. Concentrations of 17α-trenbolone as low as 30 ng/L reduced female VTG levels and initiated the development of nuptial tubercles. From 3-100 ng/L, egg production declined in a time- and concentration-dependent manner. Similarly, 17β-trenbolone caused physical masculinization of the female fatheads and reduced egg production at 50 ng/L.

Molecular Structure. The structures of several representative estrogens and androgens are provided in Figure 1. Both hormone classes are members of the same biological system, with the androgens derived from progesterone and the estrogens from the androgens [23]. The molecular structures of the estrogens, however, exhibit greater aromaticity and polarity due to the benzene ring in the steroid A ring position and 1-3 hydroxyl functional groups. The pictured androgens have a single hydroxyl group and lack electron delocalization, with the exception of T, which has a double bond conjugated with the carbonyl group.

These hormones act as ligands to estrogen and androgen receptors of the endocrine system [29]. Fang et al. [29] used computer modeling to characterize the fit of 202 chemical compounds into an androgen receptor with the same active site as the human androgen receptor. They demonstrated that diverse chemicals can interact with the binding site, given well-placed functional groups and coinciding polarity distribution and molecular size. This observation accounts for endocrine activation and/or disruption
by synthetic hormones and non-biological chemicals. Fang et al. [29] found trenbolone to have nearly six times the affinity for the androgen receptor as T.

![Hormone structures](image)

**Figure 1. Hormone structures.** The molecular structures of several representative estrogens and androgens. The steroid rings under the estrogen heading are lettered according to convention. The androgen bonds are simply labeled for reference here.

**Transport.** Hormones are mobilized throughout the environment via dissolution in runoff, surface waters, pore water, and groundwater [5,10,17,24,30-32]. Because of moderate logKow values (e.g. 2.45-4.15), these compounds also partition to colloidal organic carbon and sediment [33-34].

It is common practice in some regions to apply treated wastewater and solid waste from both municipal WWTPs and CAFOs to agricultural fields for irrigation and fertilization [25,30,35]. Runoff from treated fields can carry detectable levels of estrogens and/or androgens. A study of fields in southern California irrigated with WWTP effluent reported 52, 3, and 3 ng/L E1, E2, and T in runoff from onion and pepper fields [30].
Subsurface transport away from septic tanks and CAFO waste lagoons has also been observed. Swartz et al. [17] detected E1 and E2 in wells meters away from a septic tank leachate pit, with E1 concentrations as high as 120 ng/L, while groundwater testing of 2000 sites prone to agricultural input demonstrated maximum concentrations of 1.6, 0.79, 0.16, and 0.94 ng/L E1, E2, E3, and EE2, respectively. Arnon et al. [32] further detected estrogens and T in sediment 32 and >45 m beneath a dairy farm lagoon.

While the hydroxyl functional groups enhance the polarity and, consequently, the solubility of estrogens and androgens, the aliphatic and aromatic ring backbones encourage sorption to organic matter [34]. Holbrook et al. [34] suggested that removing greater quantities of colloidal organic carbon (COC) from WWTP effluent prior to discharge would reduce the hormone contamination of receiving waters. They quantified the partitioning of E2 and EE2 to colloidal organic carbon (K_{COC}), and found that exact values varied widely with season, colloidal size fraction, and treatment processes. The log K_{COC} values spanned 1.4-2.3 for E2 and 1.7-2.6 for EE2 across two WWTPs in May and August for colloidal size fractions <30 kD, 100 kD, 0.22 μm, and 1.5 μm. In a separate series of batch equilibrium experiments carried out by Lee et al. [33], the partitioning coefficients of E2, EE2, and T were consistent across several soils and sediments when normalized to organic carbon content (K_{OC}) of the solid matrix. Average log K_{OC} values were 3.34, 2.99, and 3.34 for E2, EE2, and T, respectively.

Transformation. Throughout their sorption study, Lee et al. [33] noted substantially greater recoveries of E2 when including the mass of E1 detected, and of T when including the masses of androstenedione and an additional unknown compound. This
was considered indicative of hormone degradation and is in agreement with biological pathways [23] (Figure 2). Degradation was not observed after 12 h of equilibration, but was significant at 72 h for E2 and 24-31 h for T. Measured E1 constituted as much as 72% of E2 recoveries, while the product androgens generally accounted for only a few percent of overall T.

\[
\begin{align*}
17\alpha\text{-Hydroxy-pregnenolone} & \rightarrow \text{Dehydroepiandrosterone} \rightarrow 3\beta, 17\beta\text{-Dihydroxyandrost-5-ene (DHEA)} \\
17\alpha\text{-Hydroxy-progesterone} & \rightarrow \text{Androst-4-ene-3,17-dione} \leftrightarrow \text{Testosterone (T)} \\
5\alpha\text{-Androstane-3,17-dione} & \leftrightarrow 19\text{-Hydroxyandrost-4-ene-3,17-dione} \\
& \downarrow 19\text{-Oxo-androst-4-ene-3,17-dione} \downarrow 19\text{-Oxotestosterone} \\
& \downarrow \text{ Estrone (E1)} \leftrightarrow 17\beta\text{-Estradiol (E2)} \\
& \downarrow \text{ Estriol (E3)}
\end{align*}
\]

Figure 2. Hormone metabolism. Biological pathways of hormone transformation [23].

Documented removal of hormones during treatment of wastewater [12] may be due to one or more physical, chemical, or biological purification processes, including filtration, flocculation and sedimentation, aerobic and/or anaerobic digestion, chlorination, nitrogen removal, and ozonation [12,36]. Microbial decomposition of EE2 by \textit{Sphingobacterium} sp. JRC5 collected from the activated sludge of a WWTP has been reported, with 87% losses in 10 d [37]. Haiyan et al. [37] proposed initial transformation to E1, followed by reactions producing detectable concentrations of 2-hydroxy-2,4-dienevaleric acid, 2-hydroxy-2,4-diene-1,6-dioic acid, and 3,4-dihydroxy-9,10-secoestrone-1,3,5(10)-diene-9,17-dione.
The use of UV radiation to destroy hormones in wastewater prior to environmental release has also come under recent investigation [38]. The TiO\textsubscript{2}-catalyzed half lives of E\textsubscript{1} and E\textsubscript{2} were less than an hour (48 min) at 150 W of 238-579 nm light. They were reduced to merely 18 min at 15 W of 253 nm light. The rates did not appear dependent on initial hormone concentrations. The rate constants increased with increasing pH over 2-7.6 and humic acid concentrations of 0.01-10 mg/L.

In many instances, the exact mechanism(s) responsible for measured hormone losses remains elusive. In a study of declining trenbolone metabolite concentrations in stored liquid manure and solid dung, as well as soil fertilized with animal waste, mechanisms of degradation were not investigated, but the authors listed microbial degradation, photodegradation, incorporation into runoff, and soil penetration as possibilities [25].

Detection. In environmental hormone research published in the early 1990s, such as that pursued by Purdom et al. [1] and Shore et al. [12], viable analytical methods for the identification and measurement of individual estrogens were not readily available. Rather, assays were used to estimate cumulative sample estrogenicity. Some such methods remain popular today. Assays utilize the binding of hormones to antibodies or endocrine receptors as a means of quantification and include radioimmunoassays (RIAs), the yeast estrogen screen (YES), and enzyme-linked immunosorbent assays (ELISAs) [31-32,39]. These types of assays have been used by Shore et al. [12], Labadie et al. [31], and Farré et al. [39], respectively, to report total estrogenicity, E\textsubscript{2} equivalents, and E\textsubscript{2} concentrations. An assay measuring androgenicity in units of dehydrotestosterone
equivalents has also been developed [21]. In some cases, assays have proven more sensitive than instrumental detection methods, but are prone to overestimation via cross-reactivity, in which non-target compounds contribute to the response [12,32,39].

Due to cross-reactivity and inadequate differentiation in bioassays, alternative detection methods have become popular. Although UV detection has been used for estrogens on occasion [33], mass spectrometry (MS) is the most commonly used method in current studies. Single MS recognizes compounds based on their unique fragmentation patterns [6,8,17,30,38]. Broad scans can be used to view the mass/charge ratios (m/z values) of all fragments during a run, while sensitivity and specificity can be increased by establishing a selected ion monitoring (SIM) program, in which only fragments associated with compounds of interest are monitored. Tandem MS (MS²) is an extended instrumental set-up, affording greater selectivity. It contains three quadrupoles (versus one in MS), allowing detection of the m/z values of the ionized molecules prior to collision (i.e. the precursor ions), in addition to those of the fragments [8-10,14-15,30,39-41]. In the same way that SIM programming enhances single MS detection, multiple reaction monitoring (MRM), in which only relevant precursor/fragment ion m/z pairs are investigated, improves the sensitivity of MS².

Upon MS² injection, molecules are ionized and vaporized through one of several possible mechanisms operated with positive or negative polarity. Negative electrospray ionization (ESI) is the most common of these in estrogen research [9,15,39-40]. Additional options remain largely unexplored. Yamamoto et al. [7] reported initial success with positive atmospheric pressure photoionization (APPI). Positive atmospheric pressure chemical ionization (APCI) has also shown promise, but remains overshadowed
by ESI [14,30,41]. Enhanced sensitivity may be achieved in some cases through the incorporation of a chemical modifier, such as formic acid, into the sample stream prior to injection [37].

Testosterone is occasionally included in estrogen studies [7, 12, 33], however, methods specific to several androgens are uncommon, as noted previously. Thomas et al. [21] published an MS procedure and Yamamoto et al. [7] a means of positive APPI detection, but affirmational and alternative studies remain scarce.

Diverse instrumental methods can be compared based on their sensitivity. Sensitivity is commonly expressed using two parameters: the instrumental detection limits (IDLs) and limits of quantitation (LOQs) for analytes of interest. These can be calculated in a number of ways, with relatively lower values indicating greater sensitivity. Typically, these parameters are determined through injection of increasingly lower standard concentrations with coincident monitoring of the signal to noise ratios (S/N values) of the resultant signals. The IDLs are those concentrations giving S/N values of 3 and LOQs, those of 10 [6,13,15,39-40]. There are also mathematical equations that can be applied to estimate these values [41].

_Chromatographic Separation._ Detection via MS is regularly preceded by chromatographic separation of sample components [5-10,14-15,17,21,30,37-41]. Chromatography may also be used prior to assay application [21,22].

Gas chromatography (GC) was used in early quantitative estrogen studies, and is still used today [5,8,21,30,38]. An additional sample preparation step, derivatization, must be incorporated when using GC for hormone separation, due to the polarity, low
volatility, and thermal lability of such compounds [8,42]. Derivatization is the masking of polar functional groups through reactions with reagents such as N-methyl-N trifluoroacetamide with trimethylchlorosilane, N-trimethylsilylimidazole, or N,O-bis[trimethylsilyl]trifluoroacetamide [42]. Typical separation columns for GC are 30 m long, with 0.25 mm internal diameters and 0.25 μm of internal thin film coating. To achieve separation, columns are often temperature programmed to hundreds of degrees Celsius. Common carrier gases include hydrogen and helium [42].

Liquid chromatography (LC) has supplanted GC in many estrogen studies due to the additional preparation time and cost of derivatization, as well as the higher detection limits observed using GC [30]. High-performance liquid chromatography (HPLC) is currently the most widely-used instrumentation [6-7,9-10,14-15,17,21,28,33,37,39,41]. Common columns for HPLC are 50-300 mm long with 2-10 mm internal diameters and solid-phase particle diameters of 3-5 μm. Column heating, typically only slightly above ambient temperatures, may be used, principally to increase reproducibility. Liquid phases vary widely in composition and flow rate. Elution with mixtures of water/methanol or water/acetonitrile at 0.2-5 ml/min is typical. These solvents may also be modified by chemicals such as formic acid and ammonium hydroxide to alter molecular interactions. Sample aliquots of 10-50 μl are injected and column elution programs often run for ~20-45 min. The vast majority of these elution programs utilize mobile phase gradients to optimize hormone separation.

Recently, ultra-performance liquid chromatographs (UPLCs®) have become available for compound separation [39-40]. Literature utilizing this technology in contaminant hormone research remains scarce. Although similar to HPLC in mobile
phase compositions and column lengths, the UPLC has smaller stationary phase particles (1.7 μm diameter), necessitating lower flow rates (0.05-0.4 ml/min) and producing higher backpressures. The UPLC methods may incorporate smaller injection volumes as well (e.g. <10-20 μl). This technology can reduce run times to <10 min, with narrower peak widths compared to HPLC [39].

Analysis of androgens in the environment remains rare, and few detection methods exist for multiple male hormones. Yamamoto et al. [7] and Thomas et al. [21], however, have published LC/(APPI)MS² and GC/MS methods, respectively, for collections of natural hormones, such as T, A, EA, DHEA, dehydrotestosterone, androstenedione, and androstanedione.

**Sample Preparation.** Treatment of environmental samples collected for contaminant analyses varies widely. Aqueous samples (50-5000 ml) are often pulled through glass fiber filters, with pore sizes ranging from 0.55 to 3.0 μm [6,14-15,17,30,39-40]. Upon collection, preservatives may also be added to deter microbial degradation of analytes during transport and storage. Addition of hydrochloric or sulfuric acid to a pH of 2-3 is one such practice [15,40-41]. Formaldehyde treatment is an alternative [45]. In lieu of chemical preservation, samples may be maintained at 4° C [13-14,30,39].

Prior to separation and detection of contaminants via chromatography, assays, and/or other instrumental techniques, samples are commonly subjected to an extraction procedure, meant to concentrate the analytes of interest [6-9,12-15,17,21,26,28,30-33,38-41,45]. Extraction methods vary across laboratories and sample matrices.
For aqueous samples, the most common method of concentration is solid-phase extraction (SPE), which involves drawing the sample through a solid material, selected to bind the compounds of interest, packaged in a disk, cartridge, or column. The compounds are then eluted off of the material with organic solvent(s). The vast majority of published methods use C$_{18}$ as the solid phase [6,9,12-14,21,32,39,45]. Hydrophilic-lipophilic balance material is an alternative solid matrix [15,38,41]. Though C$_{18}$ is common in many studies, the exact protocols for its usage vary widely. In general, the solid phase is first conditioned with 5-20 ml methanol, followed by 5-20 ml purified water. A sample of 0.5-5 L surface water, 0.5-1 L ground water, 0.05-1 L WWTP effluent, or 0.1 L WWTP influent is then pulled through the C$_{18}$ cartridge at 4-15 ml/min. The solid matrix may then be washed with additional solvent and/or water before drying for 2-60 min. Final elution of the solid phase is typically achieved through 2-20 ml of acetonitrile, dichloromethane, ethyl acetate, methanol, or a mixture of those organic solvents. In nearly all methods, extracts are taken to complete dryness and recovered in a solvent suitable for further purification or instrumental injection.

Purification is aimed at reducing non-target compounds coincidently eluted from the SPE fixture with those of interest. An additional solid phase column containing a different sorbent (e.g. florisil, silica, or amine) is often incorporated, with conditioning and elution steps as in SPE [7-8,13-14,45]. Limited study has demonstrated that automated gel permeation size exclusion chromatography (SEC) shows promise, as well [9].

Different sample preparation procedures are applied to solid matrices such as soil, sediment, suspended particles, and biological tissue. Shake-flask (i.e. solid-liquid)
extractions for instance, involve the infiltration of a sample with solvent via shaking or sonication. Centrifugation is then used to separate the phases, and the supernatant (containing the compounds of interest) is removed and concentrated for further preparation or analysis [8,21,28,32-33]. Automated methods are also available. Most utilize the general principle of mixing the solid sample with a fluid and removing the fluid for analysis. This may be performed under non-ambient temperatures, pressures, and radiation, however. Examples include microwave-assisted solvent extraction and supercritical carbon dioxide extraction [30-31,42].

Matrix Effects. In addition to DLs and LOQs, the quality of analytical methods is further gauged by the percentage recoveries of the target analytes (i.e. the masses detected in the final extract normalized to those in the raw sample). The latter are derived through spike recoveries, in which known amounts of target compounds are added to a matrix, which is then processed according to the established protocol. Comparing published methods based on this criteria is difficult, however, as the analytes are spiked at a variety of concentrations into diverse matrices [6-7,9-10,13-15,28,30,40-41]. Published spikes encompass 2-500 ng/L hormones into purified water, drinking water, surface water, and WWTP effluent and influent.

In general, those analytical methods that attempt to encompass large groups of diverse chemicals typically result in lower and more variable overall recoveries [30,40]. For instance, Kasprzyk-Hordern et al. [40] investigated 25 pharmaceuticals and personal care products, including antibiotics, analgesics, lipid regulators, sunscreen agents, and preservatives, and achieved recoveries of 8-134% from purified water and 6-102% from
surface water. Similarly, Pedersen et al. [30] developed a method for various medicines, plasticizers, and caffeine, in addition to hormones in aqueous solution. Recoveries of E1, E2, E3, EE2, and T ranged from 33-87%. In contrast, the method by Koh et al. [9], targeting only estrogens, achieved 83-100% recoveries for E1, E2, E3, and EE2.

Gomes et al. [6] demonstrated steadily declining recoveries of E1, E2, and E3 with spike recoveries into drinking water, lake water, WWTP effluent, and WWTP influent. Such observations suggest matrix interference, the skewing of analytical procedures or signals by extraneous sample components. Such is common in the analysis of complex environmental samples [6,8-10,13-15,40-41,46]. Areas particularly vulnerable include the retentive capacity of the chosen SPE material and the efficiency of MS(/MS) ionization [6,40].

Matrix interference of MS(/MS) signals can take the form of ion suppression or ion enhancement, resulting in signal reduction or augmentation, respectively [47]. A number of equations have been proposed for quantification of these effects. For example, Kaspryzk-Hordern et al. [40] reported percentage signal suppression (SS) with respect to signal intensity of an analyte in surface water (I_{sw}) versus deionized water (I_{di}).

\[
SS = (1 - \frac{I_{sw}}{I_{di}}) \times 100\%
\]

Despite such efforts to pinpoint matrix effects, they remain elusive and are currently the focus of numerous scientific discussions, including those between the Federal Drug Administration and pharmaceutical industry [47].

There are several proposed solutions for matrix interference. The preparation of calibration standards in the sample matrix or use of standard addition sample processing have been suggested by Hao et al. [10]. Others suggest the use of precise surrogate
standards or dilution of final extracts [40]. When SPE appears insufficient in isolating analytes from major interferents, use of the additional purification columns described above is recommended by Koh et al. [9]. Some who observe interference using ESI MS\(^2\) suggest that APCI may be less susceptible [41,46]. These measures, however, are prone to increased sample processing time or decreased sensitivity.

**Objective.** The objective of this study was to expand current analytical techniques for hormones in environmental matrices beyond the HPLC/(ESI)MS\(^2\) analysis of estrogens, as a response to the matrix interference observed in such analyses, the emerging availability of UPLC technology, and the demonstrated toxicity of the androgenic hormones. Specific objectives included:

1. Developing LC/(ESI)MS\(^2\) analytical methods for both estrogenic and androgenic steroid hormones
2. Comparing the analytical parameters and considerations for the two hormone classes
3. Utilizing emerging UPLC technology in the analysis of environmental samples for trace hormone contaminants
4. Applying overlooked MS\(^2\) ionization techniques and/or extract purification methods in the event of matrix interference
MATERIALS AND METHODS

Chemicals. Three natural (E1, E2, E3) and one synthetic (EE2) estrogen, plus four natural androgens (T, A, EA, DHEA), were selected for analysis and purchased from Sigma-Aldrich (St. Louis, MO) (Figure 1). Surrogate standards estrone-2,4,16,16-Cl (E1-d4, Isotec; St. Louis, MO) and testosterone-d3 (T-d3, Toronto Research Chemicals; Ontario, Canada) were incorporated for calculation of compound recoveries during sample analysis. Internal calibration via 17β-estradiol-16,16,17-d3 (E2-d3, Isotec) and 6β-hydroxy-testosterone-d3 (OH-T-d3, Toronto Research Chemicals) was used when possible.

Acetonitrile, methanol, and ultrapure water (LC-MS grade) used in the preparation of standard solutions and UPLC analyses were purchased from Burdick and Jackson (Muskegon, MI), Fisher Scientific (Fair Lawn, NJ), and J.T. Baker (Phillipsburg, NJ). Additional solvents used in the development of an extraction procedure (i.e. dichloromethane and acetone) were purchased from Honeywell (Muskegon, MI) and ammonium acetate and formic acid modifiers from Fisher Scientific.
A 3200 Q TRAP® MS² (Applied Biosystems/MDS Sciex; Toronto, Canada) was equipped with a TurboIonSpray® ESI probe for hormone detection. Upon insertion into the source housing, the ESI probe emits a high ionspray voltage (-4500 - +5500 V) to ionize molecules, thereby producing precursor ions, in the incoming solution, which has been nebulized by ion source gas 1 (0-90 psi, zero air). A proximal turbo heater (0-750 °C), aided by the pressure of ion source gas 2 (0-90 psi, zero air), vaporizes these ions, which are subsequently guided from quadrupole 0 to quadrupole 1 by the curtain gas (10-50 psi, ultra high purity nitrogen). Declustering (0-400 V) and entrance (2-15 V) potentials aid in this movement by minimizing clusters and focusing the ions, respectively. The m/z values of the precursor ions are monitored in quadrupole 1. Additional pressure and voltage from the CAD gas (low-high, ultra high purity nitrogen) and collision energy (5-130 V) facilitate fragmentation of the precursor ions in quadrupole 2, and the collision cell exit potential (0-55 V) forces the fragment ions into quadrupole 3, where their m/z values are monitored [48]. Together, the above parameters constitute the compound- and source/gas-dependent parameters and are set via vendor-supplied instrument control software (Analyst 1.4.2). Figure 3 depicts the MS² components and parameters.
Individual standard solutions of the target hormones were prepared at 70 μg/ml by adding methanol in 5 ml aliquots to 25 ml volumetric flasks containing massed amounts of powdered individual analytes until complete dissolution was achieved. Acetonitrile was then used to constitute the remaining volume. Use of 100% acetonitrile was inadequate to achieve complete dissolution of all compounds.

Aliquots (0.5 ml) of individual solutions were later diluted 1:1 v/v with purified water, or ammonium acetate (0.5 mM) or formic acid (0.1%) modifiers. To establish the compound-dependent parameters, each solution was infused into the MS² at 10 μl/min via a 1 ml syringe pump (Hamilton; Reno, NV). Positive and negative scans of quadrupole 1 were monitored for the m/z values and intensities of the most prominent precursor ions. Respective declustering and entrance potentials were then optimized. Scans of quadrupole 3 revealing fragmentation patterns were studied to select the most intense fragments, and their m/z values were noted. The collision energies and collision cell exit potentials were optimized for each precursor/fragment ion pair. The most
intense ion transition for each compound was selected for quantitative detection, and the second most intense for qualitative confirmation.

As a result of key differences in MS$^2$ requirements for the analysis of estrogens versus androgens (explained below), a single method could not be developed to encompass all analytes. The compound-dependent parameters for the analytes in each hormone class were thus merged separately to form two MRM programs.

One representative estrogen and androgen were separately infused into the MS$^2$ to optimize the source/gas-dependent parameters for each class. The ionspray voltage, heater temperature, and pressures of the curtain and CAD gases and ion source gases 1 and 2 were adjusted individually to maximize MRM signal intensities. In the event that the temperature and gas pressures in the source housing were insufficient to vaporize the solution stream (evidenced by condensation on the curtain plate), the parameters were increased until the curtain plate appeared dry. The remaining analytes were later tested under the set conditions to ensure general viability.

For those compounds whose MRM signals were intensified with the addition of a chemical modifier, seven aqueous solutions of 0.29-1.7 mM ammonium acetate were prepared and infused via a 10 ml syringe pump at 10 µl/min during hormone analysis. The intensities (i.e. heights) and shapes of the MRM peaks were monitored to select the most effective modifier concentration.

UPLC. An Acuity UPLC® (Waters, Ireland), equipped with a bridged ethyl hybrid (BEH) C$_{18}$ column (100 mm, 2.1 mm i.d., 1.7 µm particle diameter) and photo-diode array (PDA) detector, was used for chromatographic separation. The individual hormone
solutions were combined and diluted to achieve final concentrations of 8 \( \mu \text{g/ml} \) each. Initial method development was pursued using the PDA, as it is more easily programmed than the MS\(^2\), albeit, less sensitive in detection. When several analytes proved unresponsive to UV/Vis excitation, methods were finalized using the MS\(^2\). The UPLC injection volume was set to 5 µl, and the column was held at 40°C for ESI or 45°C for APCI, above the widely fluctuating room temperature (climate control issues were periodically experienced in the instrumental laboratory due to faulty building HVAC equipment).

Acetonitrile and water were tested as the strong and weak mobile phase solvents, respectively [8,13,39]. A preliminary trial attempted separation of the compounds using a gradient of 10-90% acetonitrile over 15 min. The gradient range and rate of change were systematically adjusted to achieve adequate resolution of the analytes. Columns of both 100 mm and 150 mm lengths were tested, with flow rates between 0.2 and 0.4 ml/min. After each individual change in elution program, the resolution, intensities, widths, symmetry, and retention times of the MRM peaks were noted. When the elution programs were optimized, two minutes of reequilibration to initial run parameters were added to the end of each flow method.

The solvent composition of the injected sample was later tested for its effect on chromatography. Standards dissolved in acetonitrile were analyzed against those in an acetonitrile/water cocktail similar to that of the initial mobile phase composition.

Finalized UPLC programs were merged with MS\(^2\) MRM programs to produce complete UPLC/MS\(^2\) acquisition methods. Optimum parameters were compared for the analysis of estrogens versus androgens.
Instrumental Quantitative Boundaries. Mixed standard solutions of the unlabelled analytes were initially prepared in 50:50 v/v acetonitrile/water at seven concentrations, ranging from 0.5-420 ng/ml to test for detection and quantification ranges. Solutions were later prepared at five concentrations spanning 10-400 ng/ml or 450-4000 ng/ml depending on hormone class detectability. The solutions in each series (a 1 ml vial per concentration) were spiked with 100 µl of internal standard (10 µg/ml) and analyzed according to the respective UPLC/MS² program. The Quantitation Wizard component of the Analyst software was used to integrate resultant MRM peaks and plot the analyte/internal standard peak area ratios versus mass ratios for each target hormone. A linear regression was performed to determine the coefficients of regression (r² values).

To assess the repeatability of instrumental analyses, each calibration standard was injected and analyzed in triplicate. The percentage relative standard deviation (%RSD) of the analyte/internal standard area ratio was calculated for each hormone at each calibration level, with a lower RSD indicating greater repeatability.

To establish IDLs and LOQs for the UPLC/MS² analytical methods, the least concentrated calibration standard for each hormone class was progressively diluted, with each new standard analyzed in triplicate. This was repeated until concentrations were found for each compound which resulted in MRM peaks having average S/N ratios of 3(±1) and 10(±1). These were deemed the IDL and LOQ, respectively. These concentrations were also converted to on-column masses.

Detection and quantitation ranges, as well as repeatability, were compared between the estrogens and androgens. Any exceptions to class generalizations were noted.
Though C\textsubscript{18} is the most commonly recommended material for SPE, the extreme diversity in published elution methods necessitated experimentation to establish a viable procedure which could be used to extract both estrogens and androgens on the available laboratory apparatus. All spike recoveries and samples were extracted in duplicate.

A six-sample extraction manifold (J.T. Baker; Phillipsburg, NJ) with glass reservoirs and vacuum filtration was used for SPE via Bakerbond C\textsubscript{18} Speedisks\textsuperscript{TM} (J.T. Baker; Phillipsburg, NJ). Aliquots (1000 ml each) of deionized water were spiked with 0.5 ml of concentrated estrogen (420 ng/ml) and androgen (4000 ng/ml) mixed standards and 100 µl of a surrogate standard solution (10 µg/ml each E1-d\textsubscript{4} and T-d\textsubscript{3}) and mixed well. In all cases, the SPE cartridges were conditioned with 10 ml of the first elution solvent, 10 ml methanol, and 2x10 ml deionized water, as recommended by the manufacturer. Each aliquot was pulled through the cartridge by vacuum, with a small amount of the final aliquot of water held to maintain cartridge moisture until sample application. The spiked samples were added to the reservoirs and drawn through at \~15 ml/min. The cartridges were then dried under vacuum for 15 min during initial tests, but for as long as 45 min in the finalized method. Longer drying times were found to facilitate subsequent extract concentration steps via removal of water from the solid phase.

The solvent composition and volume for elution were the first variables addressed. Initial spike recoveries were eluted with 2x5 ml acetonitrile, followed by 2x5 ml methanol, and finally 2x5 ml dichloromethane, with each 5 ml fraction collected in an individual 15 ml conical tube. All fractions were blown to dryness under nitrogen in a water bath at 41°C. Residues were reconstituted by rinsing tubes with small amounts of
acetonitrile/water 1:1 v/v and transferring the rinses to 1.5 ml LC vials with split-septa screw caps for extract volumes of 1 ml. Just prior to UPLC/MS$^2$ analysis, 100 μl of the internal standard solution (10 μg/ml each E2-d₃ and OH-T-d₃) were added to each vial. The internal standard was also spiked into calibration standards run in the same batch.

Four additional spike recoveries into deionized water were carried out as above, with variation in the elution regime. Solutions of 0, 10, 50, and 100% methanol in acetonitrile were used in 3x5 ml aliquots, with quantification of recovery in each 5 ml aliquot. The average and %RSD in recovery was calculated for each hormone across the four acetonitrile/methanol solvent ratios. If the %RSD was greater than that resulting from imperfect instrument repeatability (see above), the effect of the elution solvent ratio on hormone recovery was deemed significant.

Extract Stability. To test stability of hormones during storage, the final extracts of 5 ml acetonitrile SPE elution fractions from duplicate spike recoveries were held at 4°C for 25 and 27 d after initial estrogen and androgen UPLC/MS$^2$ analyses, respectively. Vials were reanalyzed and recoveries compared with those calculated from the run performed immediately upon completion of sample preparation. As in the previous experiment, the %RSDs were compared to those measuring instrument repeatability to determine significance.

UPLC/(ESI)MS$^2$ Application. Pond water was collected in 4 L amber glass bottles from a small, freshwater system in the watershed of the York River in Virginia, U.S.A. and immediately transported to the laboratory. Within 2 h of collection, duplicate 650 ml
aliquots were pulled via vacuum through GF/F filters (i.e. 0.7 μm), spiked with 100 μl surrogate standards (10 μg/ml), and processed according to the SPE/UPLC/(ESI)MS² method developed above. Duplicate deionized water samples were processed in parallel as laboratory blanks. Spike recovery studies (200 ng estrogens, 2000 ng androgens) were also conducted using pond water samples containing no detectable levels of the target analytes.

Effluent samples from three WWTPs were collected in the watershed of the Shenandoah River in Virginia. The first sampling site, WWTP 1, processes 0.975 MGD using aeration/chlorination; WWTPs 2 and 3 incorporate biological/chlorination treatment for 1 and 16 MGD. Samples were collected in 4 L amber glass bottles, preserved with hydrochloric acid (to pH 3), and stored at 4°C prior to analysis. They were later equilibrated to room temperature, and 1000 ml aliquots were GF/F filtered, spiked with surrogates, and processed in duplicate. Deionized water was again used for laboratory blanks. A flow chart of the sample processing steps is provided in Figure 4.

---

**Figure 4. Steps in handling and analysis of samples for hormone contaminants.**

The pond water and effluent represent matrices expected to be more complex than deionized water. Percentage recoveries and MRM chromatographs were consequently
examined for signs of matrix interference. Substantial changes in general recovery levels, baseline noise, the appearance of unresolved complex mixtures, and/or distortion of internal standard peaks were deemed suspect.

Where matrix effects were observed, attempts were made to attenuate the influence on hormone recoveries. For surface water interference, the chromatography was extended. For wastewaters, more aggressive changes, including extended sample preparation and an alternative MS$^2$ ionization technique were evaluated.

**Silica and LLE Purification.** When significant matrix interference was encountered, several attempts were made to remove interferents from the extracts before reanalyzing them for the target hormones. Silica gel-based columns (1 g, 3 ml, 60 Å pore diameter, Isolute) and liquid-liquid extraction (LLE) were included in this process.

In the first silica gel test, a column was washed with 2.5 column-volumes of acetonitrile. An effluent extract (1.1 ml) was then transferred from the LC vial to the column, along with 3x0.5 ml acetonitrile rinses. The column was eluted with 3 ml of acetonitrile followed by 2x3 ml methanol. Each fraction was dried under nitrogen and recovered in the mobile phase. In a second trial, the extracts of spike recoveries into effluent were dried under nitrogen and recovered in 0.5 ml hexane. Silica columns were washed with hexane. The sample was transferred to the column with 3x0.2 ml hexane rinses of the container. The column was sequentially eluted with 4 ml each of hexane, dichloromethane, acetone, methanol, and acetonitrile, with each fraction collected in a separate 15 ml conical test tube, dried under nitrogen, and recovered in the mobile phase. In each trial, all fractions were analyzed separately for hormone recoveries.
Another of the effluent extracts was liquid-liquid extracted with hexane. The original extract (1.1 ml) was transferred from the LC vial to a 15 ml conical tube with 3 acetonitrile rinses and brought to 10 ml with acetonitrile. This volume was transferred to a separatory funnel with 3x0.2 ml acetonitrile rinses and shaken with 10 ml hexane. The hexane fraction was discarded and the acetonitrile dried under nitrogen, and recovered in the mobile phase.

All extracts subjected to these purification trials were analyzed via the developed UPLC/(ESI)MS² methods. The appearances of the MRM chromatograms and the quantified recoveries were compared before and after application of the additional preparation steps.

**ESI vs. APCI.** As noted in the introduction, the peer-reviewed literature suggests that alternative MS² ionization techniques may be less susceptible to matrix interference than ESI, but few evidential studies are available. The ESI probe used previously in this study was thus removed from the MS² and replaced with an APCI probe. This set-up differs from the original in the mechanics in the source housing. In contrast to ESI, vaporization is facilitated by a ceramic heater (0-750°C) prior to ionization via the nebulizing current (-5 – +5 μA) from a Corona discharge needle (vaporization and ionization take place in reverse order in ESI) (Figure 5).
One representative estrogen (E2) and androgen (A) were selected, and a concentrated standard (70 μg/ml) of each was individually infused into the MS\(^2\) to establish respective compound- and source/gas-dependent parameters for APCI. These standards were then spiked (43 ng E2, 445 ng A) into effluent extracts which had no ESI-detectable hormone levels. The extracts were then analyzed in turn using ESI and APCI for the two chosen hormones.

At this point in method development, the androgens A, EA, and DHEA were removed from the list of analytes due to substantially different levels of detection and time restrictions of the study period. Remaining analytes included T, E1, E2, EE2, and E3, in addition to the deuterated standards E2-d\(_3\) and E1-d\(_4\).

Matrix interference of ESI and APCI were further explored for the representative estrogen through infusion of the concentrated E2 standard into the MS\(^2\) during UPLC analysis of an effluent extract with no ESI-detectable hormone levels. Infusion was carried out at 0.2, 0.3, 0.5, 1.0, and 10 ml/min during ESI and then APCI analysis. The
continuity of the E2 signal, or lack thereof, was monitored for each. Modifications were considered indicative of matrix disturbances in ionization.

(APCI)MS2 Application. Compound- and source/gas-dependent parameters were developed for the APCI analysis of the truncated list of hormone analytes. Once again, each analyte was infused as a concentrated standard diluted 1:1 v/v with water, or the ammonium acetate or formic acid modifier. In APCI, the ionspray voltage of ESI is replaced with a nebulizing current, and ion source gas 2 is unnecessary (e.g. set to zero), as vaporization occurs in the probe, rather than upon exiting. Parameters were combined to create new MRM programs, each encompassing both T and the estrogens, but specific to the ions produced in the presence of either modifier.

Solutions of ammonium acetate or formic acid of five concentrations spanning 0.5-20 mM were infused into the solvent stream between the UPLC and MS2. The MRM signal intensities were compared between modifiers, across modifier concentrations, and against modifier absence.

A new UPLC method was also developed to incorporate T into the estrogen chromatography program, with optimization aimed at maximizing peak intensity, symmetry, retention times, resolution, and overall run time. Flow rates of 325-425 μl/min, column temperatures of 30-45°C, and diverse initial acetonitrile/water mobile phase ratios were tested to determine optimum values.

The instrumental quantitative boundaries for the UPLC/(APCI)MS2 method were established as for the ESI method. Calibration standards of 10-500 ng/ml were tested for linearity and repeatability. The least-concentrated standard was diluted to obtain the
IDLs and LOQs. These values were compared to the sensitivity measurements of ESI analysis.

Duplicate effluent samples were processed in parallel with replicate spike recoveries into effluent according to the $C_{18}$ SPE method. Extracts were concentrated, recovered in the mobile phase specific to the UPLC/(APCI)MS$^2$ method, and analyzed without additional purification. Percentage recoveries were compared to those obtained through ESI application.

**SEC Purification.** Chen et al. [49] successfully incorporated SEC via HPLC for routine clean-up of environmental samples prior to analysis of organic chemicals. Their method involved sample injection followed by fractional collection of the eluent, with only the retention window containing compounds of interest reserved for further analysis. An attempt was made here to use such technology in hormone analyses. A Waters 600E System Controller pump module, Waters 717 Plus Autosampler, and Waters Fraction Collector III (Waters, Ireland) were used with a Phenomenex (Torrance, CA) Envirosep ABC column (350 x 21.20 mm, predominantly styrene divinylbenzene).

To determine which portion of the run should be collected, 5 ml of a concentrated mixed standard solution were injected and eluted with dichloromethane at 5 ml/min. Retention times from 0.0-15.0 min were discarded. One minute fractions were collected from 15.0-26.9 min, resulting in 12x5 ml fractions. Each fraction was dried under nitrogen, recovered in the mobile phase, and analyzed for the target compounds according to the UPLC/(APCI)MS$^2$ method. In response to the results from this test, the procedure was repeated with collection of 10.0-19.9 min.
To evaluate the cleanup procedure for a complex environmental matrix, effluent samples were collected in amber glass bottles from WWTP 4, which utilizes aeration/chlorination treatment for approximately 0.4 MGD. Samples were immediately acidified to pH 3 with HCl and stored at 4°C, as above, until analysis. They were then equilibrated to room temperature and filtered (GF/F). Duplicate effluent samples (500 ml) and spike recoveries (75 ng each hormone) into the effluent were subjected to the SPE/SEC/UPLC/(APCI)MS² protocols. The SEC was set for a 30 min elution, with cumulative collection across those minutes during which the target hormones were eluted. Recoveries were compared to those achieved without the purification step.
RESULTS

(ESI)MS². In first quadrupole scans for precursor ions, negative ionization was found to produce constant, high-intensity signals for the estrogens, while androgen signals were faint and sporadic. Conversely, positive ionization readily revealed the androgens and failed to permit estrogen detection. This fundamental difference in instrumental parameters dictated the separation of hormone classes during instrumental analysis.

The compound-dependent parameters for (ESI)MS² analysis of the target analytes are provided in Table 1 and the source/gas-dependent parameters in Table 2. It was difficult to deduce unique ion pairs for T and DHEA, and for A and EA, and so their MRM traces contained peaks representative of one another. Consequently, carefully defined chromatography was required.
Table 1. (ESI)MS\textsuperscript{2} compound-dependent parameters.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Polarity</th>
<th>DP (V)</th>
<th>EP (V)</th>
<th>Quantifier (m/z:m/z)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
<th>Qualifier (m/z:m/z)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
<th>RT\textsubscript{1} (min)</th>
<th>RT\textsubscript{2} (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>-</td>
<td>-110</td>
<td>-4</td>
<td>269/145</td>
<td>-60</td>
<td>-3</td>
<td>269/143</td>
<td>-75</td>
<td>-2</td>
<td>3.13</td>
<td>N/A</td>
</tr>
<tr>
<td>E1-d\textsubscript{4}</td>
<td>-</td>
<td>-91</td>
<td>-10</td>
<td>273/147</td>
<td>-50</td>
<td>-1</td>
<td>273/145</td>
<td>-71</td>
<td>-1</td>
<td>3.11</td>
<td>N/A</td>
</tr>
<tr>
<td>E2</td>
<td>-</td>
<td>-93</td>
<td>-5</td>
<td>271/145</td>
<td>-50</td>
<td>-3</td>
<td>271/183</td>
<td>-51</td>
<td>-1</td>
<td>2.63</td>
<td>N/A</td>
</tr>
<tr>
<td>E2-d\textsubscript{3}</td>
<td>-104</td>
<td>-4</td>
<td>274/145</td>
<td>-58</td>
<td>-1</td>
<td>274/185</td>
<td>-54</td>
<td>-1</td>
<td>2.60</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>EE2</td>
<td>-</td>
<td>-80</td>
<td>-8</td>
<td>295/145</td>
<td>-55</td>
<td>-2</td>
<td>295/143</td>
<td>-75</td>
<td>0</td>
<td>2.94</td>
<td>N/A</td>
</tr>
<tr>
<td>E3</td>
<td>-</td>
<td>-95</td>
<td>-10</td>
<td>287/171</td>
<td>-51</td>
<td>-1</td>
<td>287/145</td>
<td>-58</td>
<td>0</td>
<td>1.35</td>
<td>N/A</td>
</tr>
<tr>
<td>T</td>
<td>+</td>
<td>78</td>
<td>9</td>
<td>289/97</td>
<td>38</td>
<td>4</td>
<td>289/81</td>
<td>60</td>
<td>3</td>
<td>2.01</td>
<td>4.35</td>
</tr>
<tr>
<td>T-d\textsubscript{3}</td>
<td>+</td>
<td>78</td>
<td>9</td>
<td>292/97</td>
<td>35</td>
<td>3</td>
<td>292/109</td>
<td>37</td>
<td>3</td>
<td>1.97</td>
<td>4.31</td>
</tr>
<tr>
<td>OH-T-d\textsubscript{3}</td>
<td>+</td>
<td>59</td>
<td>5</td>
<td>308/290</td>
<td>16</td>
<td>5</td>
<td>308/272</td>
<td>28</td>
<td>6</td>
<td>1.27</td>
<td>1.80</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>100</td>
<td>10</td>
<td>291/255</td>
<td>20</td>
<td>5</td>
<td>291/273</td>
<td>5</td>
<td>12</td>
<td>3.32</td>
<td>6.00</td>
</tr>
<tr>
<td>EA</td>
<td>+</td>
<td>70</td>
<td>10</td>
<td>291/273</td>
<td>15</td>
<td>4</td>
<td>291/135</td>
<td>25</td>
<td>2</td>
<td>2.50</td>
<td>5.17</td>
</tr>
<tr>
<td>DHEA</td>
<td>+</td>
<td>69</td>
<td>10</td>
<td>289/271</td>
<td>10</td>
<td>4</td>
<td>289/253</td>
<td>15</td>
<td>4</td>
<td>2.30</td>
<td>4.84</td>
</tr>
</tbody>
</table>

(DP) declustering potential, (EP) entrance potential, (m/z:m/z) mass/charge ratios of precursor/fragment ions, (CE) collision energy, (CXP) collision cell exit potential, (RT\textsubscript{1}) retention time in original UPLC method, (RT\textsubscript{2}) retention time in modified UPLC program, (N/A) original UPLC program was not modified.

Table 2. (ESI)MS\textsuperscript{2} source/gas-dependent parameters.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>IS (V)</th>
<th>TEM (C)</th>
<th>GS1 (psi)</th>
<th>GS2 (psi)</th>
<th>CUR (psi)</th>
<th>CAD (psi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogens</td>
<td>-4500</td>
<td>450</td>
<td>20</td>
<td>7</td>
<td>35</td>
<td>medium</td>
</tr>
<tr>
<td>Androgens</td>
<td>5000</td>
<td>450</td>
<td>20</td>
<td>14</td>
<td>35</td>
<td>medium</td>
</tr>
</tbody>
</table>

(IS) ionspray voltage, (TEM) temperature, (GS1) ion source gas 1, (GS2) ion source gas 2, (CUR) curtain gas, (CAD) CAD gas.

Whereas estrogen MRM signals decreased with increasing modifier concentrations, androgen peak heights were increased and shapes improved through incorporation of the additional chemicals into the UPLC effluent stream prior to MS\textsuperscript{2} injection. Although both modifiers enhanced androgen signal intensity, peaks heights were greatest in the presence of ammonium acetate. The modifiers likely encouraged the formation of positive precursor ions through donation of protons.
Ammonium acetate solutions were prepared at 0.3, 0.6, 0.7, 0.9, 1.1, 1.7, and 2.3 mM and infused during androgen analyses (Figure 6). Peak intensities of EA and DHEA steadily increased between 0.29 and 1.7 mM ammonium acetate with no additional benefit from 2.3 mM. Heights of the A and T signals generally increased over the tested concentrations. The appearance of splitting in T peaks at higher modifier levels, however, resulted in the selection of 0.92 mM as the optimal concentration of ammonium acetate infused at 10 µl/min into the MS² to aid in ESI of the androgens.

Figure 6. Modifier addition for androgens. The effect of increasing concentrations of infused ammonium acetate on the MRM signal intensity of target androgens. Like symbols at the same concentration indicate results from analysis of duplicate samples.

UPLC. Development of a method for the chromatographic separation of the estrogens was readily achieved using the PDA detector at 280 nm. With the exception of T, which responded at 244 nm, the androgens were not conducive to UV analysis, and MS² was necessary. As MS² detection of all hormones could not be achieved simultaneously, two chromatographic methods were also developed, one for each hormone class.
The estrogens proved less difficult to separate in retention time, while T and DHEA tended to elute in close proximity. The latter proved problematic, as unique (ESI)MS\(^2\) transitions could not be established for the androgens. Hence, greater effort had to be invested in androgen chromatography. For both hormone classes, the 100 mm column was replaced with a 150 mm column of the same stationary phase to improve resolution. A compromise was drawn for mobile phase flow rates, as faster flow resulted in narrower peaks, but decreased resolution. The estrogen flow rate was set to 375 \(\mu\)l/min and that of the androgens to 325 \(\mu\)l/min. The initial mobile phase composition for elution of the two hormone classes also differed. A weaker cocktail of 40:60 v/v acetonitrile/water was used for the estrogens, while the androgens required the stronger 60:40 v/v acetonitrile/water. The final programs were each 7 min long, with the last two minutes devoted to re-equilibration to initial UPLC conditions. The flow regimes are given in Tables 3 and 4, and the retention times in Table 1 (as RT\(_1\)). The column temperature was set to 40°C for both analyses. Standards constituted in a solvent cocktail similar to the initial mobile phase produced narrower MRM peaks of greater intensity and symmetry than those prepared in 100% of a single mobile phase component.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow ((\mu)l/min)</th>
<th>ACN (%)</th>
<th>H(_2)O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>375</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>1.88</td>
<td>375</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>4.00</td>
<td>375</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>5.00</td>
<td>375</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>7.00</td>
<td>375</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 4. Androgen UPLC method.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow ((\mu)l/min)</th>
<th>ACN (%)</th>
<th>H(_2)O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>325</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>4.00</td>
<td>325</td>
<td>62.5</td>
<td>37.5</td>
</tr>
<tr>
<td>4.50</td>
<td>325</td>
<td>62.5</td>
<td>37.5</td>
</tr>
<tr>
<td>5.00</td>
<td>325</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>7.00</td>
<td>325</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

(ACN) acetonitrile.
The total ion chromatograms of the mixed estrogen and androgen standard solutions are provided in Figures 7A and 7B, respectively, with cumulative intensity along the left-hand y-axis. The UPLC flow regimes are superimposed over the peaks, with the percentage acetonitrile (i.e. 100% minus percentage water) constituting the mobile phase along the right-hand y-axis.

Figure 7. Total ion chromatograms and UPLC flow regimes for ESI analyses. The chromatograms of cumulative ion signal, with intensity along the left-hand y-axis, for the estrogens (A) and androgens (B). The UPLC flow regimes are superimposed as the percentage of acetonitrile in the mobile phase, given along the right-hand y-axis.

Some of the more dilute of the original seven calibration standards (0.5-10 ng/ml) failed to produce detectable signals for the estrogens and T, while A, EA, and DHEA were generally undetectable across the entire range (0.5-420 ng/ml). Consequently, future calibration standards were prepared at five concentrations of 12-420 ng/ml for the
estrogen analytes and 450-4000 ng/ml for the androgens. The relationships between all analyte/internal standard area versus mass ratios proved linear ($r^2 \geq 0.99$).

Hormone analyte/internal standard MRM peak area ratios ($n=3$) had moderate repeatability (Table 5), with unlabelled estrogens exhibiting an average %RSD of 11, with a median of 10 and range of 2.8-30, and the androgens, an average of 9.8, median of 9.4, and range of 3.3-19. These values were unchanged by the incorporation of surrogate standard area ratios into the calculations.

Table 5. Repeatability of UPLC/(ESI)MS	extsuperscript{2} hormone analyses.

<table>
<thead>
<tr>
<th>Concentrations (ng/ml)</th>
<th>Repeatability (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen</td>
<td>Androgen</td>
</tr>
<tr>
<td>420</td>
<td>4000</td>
</tr>
<tr>
<td>220</td>
<td>2500</td>
</tr>
<tr>
<td>120</td>
<td>1250</td>
</tr>
<tr>
<td>60</td>
<td>750</td>
</tr>
<tr>
<td>12</td>
<td>450</td>
</tr>
</tbody>
</table>

(N/A) not available.

As indicated by the differences in calibration ranges, the androgens A, EA, and DHEA exhibited IDLs and LOQs 1-2 orders of magnitude greater than those of T and the estrogens (Figure 8). The estrogens and T could all be detected at or below 23 pg on-column, equivalent to an extract concentration of 4.6 ng/ml, given a 5 µl injection volume, while A, EA, and DHEA were detectable down to 500 pg on-column, or 100 ng/ml. For an approximate translation to levels in environmental samples, these ng/ml values would be those of the final extracts concentrated three orders of magnitude from a starting sample volume of 1 L. The detection limits would thus be ~4.6 ng/L for the
estrogens and testosterone and ~100 ng/L for A, EA, and DHEA, if 100% recovery was achieved.

\[ \text{SPE. In the initial spike recovery into deionized water, acetonitrile proved an apt elution solvent, with a majority of the recovered mass of each analyze in the first 2x5 ml aliquots. For the estrogens, the first 5 ml fraction contained nearly three times the mass in the second, while the androgens were more equally divided between the two (Figure 9). Subsequent elution with methanol and dichloromethane recovered small masses of E1 (8 and 5%), E2, (1 and 0%), EE2 (7 and 3%), T (3 and 0%), DHEA (4 and 1%), A (9 and 0%), and T-d3 (21 and 3%). In an additional test in which elution was carried out using 2x5 ml acetonitrile plus two additional 5 ml aliquots, recoveries were not detected beyond the first 10 ml.} \]
Figure 9. Hormone elution solvent distribution. Recoveries of estrogen (A) and androgen (B) analytes across sequential 2x5 ml aliquots of acetonitrile (ACN), methanol (MeOH), and dichloromethane (DCM). Like-colored double bars indicate results from analysis of duplicate spike recoveries.

Elution of the SPE cartridges with methanol (0, 10, 50, or 100%) in acetonitrile further revealed a lack of recovery beyond the first 10 ml. Estrogen recoveries did not differ significantly across the four elution cocktails, as compared to routine instrumental repeatability. The androgens were also unaffected, with the exception of T and T-d3, which were recovered to the greatest extent without methanol addition (i.e. 100% acetonitrile).

Total recoveries in 2x5 ml acetonitrile ranged from 67-94% for the estrogens and 81-112% for the androgens. They are depicted in Figure 10. Double bars for each hormone indicate recoveries from duplicate spiking experiments.
Extract Stability. Percentage RSDs between recoveries at 1 and 25 or 27 d were considered significant if they exceeded the bounds of instrument repeatability, as above. Following storage, E1, E1-d4, and EE2 were significantly depleted, while the apparent recoveries of T-d3 and EA were enhanced (Figure 11). Average losses in estrogen recoveries ranged from 11-54% during the study period. Androgen recoveries were decreased by as much as 8% and increased up to 39% from day 1 to day 27. Note that the analyzed extracts were concentrated from only one of the two 5 ml acetonitrile aliquots used to elute the SPE cartridges and thus never did contain the total recovered masses.
Figure 11. Effect of storage on apparent recoveries. Recoveries of hormones immediately following sample preparation and after storage in the dark at 4°C for 25 d before follow-up analysis for the estrogens and 27 d for that of the androgens. Note that recoveries are for one 5 ml aliquot out of two such aliquots used for each SPE elution. Like-colored double bars indicate results from analysis of duplicate samples.

UPLC/(ESI)MS² Application. The target analytes were not found at detectable levels in pond water samples or in laboratory blanks. Recoveries did not differ significantly across duplicate deionized water and pond water spike recoveries run in parallel, except for E3, T, and EA, for which average recoveries were reduced by 57, 32, and 50% in pond water (Figure 12). Chromatograms for estrogen analyses appeared largely unaffected by natural matrix components, with only slight elevation of the E2 and E3 baselines. Interference was more visible and problematic for the androgens, with gross distortion of the internal standard baseline rendering the signal irresolvable and necessitating the use of external calibration. The baselines of DHEA, A, and EA were also elevated (Figure 13 versus Figure 7B).
Interference with the determination of the androgens during UPLC/MS due to the pond water matrix was attenuated via modification of the androgen chromatographic method. The initial method was nearly isocratic, while the new program contained a definitive gradient in the elution regime (Table 6 versus Table 4). The initial mobile phase was also weakened to the same composition as that of the estrogen method, and the flow rate alternated between 325 and 375 μl/min. The program was 2 min longer, with a return to the initial mobile phase composition by 6 min and the flow rate by 7 min, with
re-equilibration until 9 min. The altered retention times can be found in Table 1 (RT).

A total ion chromatogram of the modified androgen method as applied to a standard solution is provided in Figure 14 with the UPLC flow regime overlaid, as above.

Table 6. Modified androgen UPLC method.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (μl/min)</th>
<th>ACN (%)</th>
<th>H₂O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>325</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>2.00</td>
<td>325</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>5.00</td>
<td>375</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>6.00</td>
<td>375</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>7.00</td>
<td>325</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>9.00</td>
<td>325</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure 14. Total ion chromatogram and UPLC flow regime of modified androgen method. The chromatogram of cumulative ion signal, with intensity along the left-hand y-axis, with the UPLC flow regime superimposed as the percentage of the acetonitrile mobile phase, given along the right-hand y-axis.

Wastewater proved an even greater analytical challenge than pond water, with interference in the analysis of both hormone classes. Internal standard MRM signals were reduced in intensity, distorted in shape, and submerged within expanses of unresolved complex mixtures. The E2-d₃ peak areas were reduced sporadically by 1-2 orders of magnitude in comparison to those in calibration standards. Those of OH-T-d₃
were randomly reduced by ~1 order of magnitude. The internal standard inconsistencies and failures to resolve dictated the use of external calibration.

Laboratory blanks were free of contamination, while the effluent extracts contained peaks consistent with some analytes. These signals, however, were below the lowest calibration standard. Peaks believed to correspond to EE2 were found in all effluent extracts (i.e. both duplicates of WWTP1-3), those for E2 in both WWTP2 duplicates, for DHEA in all samples except one duplicate of WWTP1, and for A in both WWTP3 extracts and one duplicate from each of the other two sites.

Figure 15 provides a comparison of surrogate recoveries from extracted deionized water, pond water, and WWTP effluent. Recoveries of E1-d₄ were visibly reduced in the effluents versus simpler matrices, but reproducibility across duplicates was high. Recoveries of the androgen T-d₃, conversely, were generally enhanced with greater disparity between duplicates.

![Figure 15. Recoveries across matrices with UPLC/(ESI)MS² quantification. Recoveries of estrogen and androgen surrogate compounds from deionized water (DI), pond water, and effluent from sites 1-3 (WWTP1-3) using the SPE/UPLC/(ESI)MS² procedure. Like-colored double bars indicate results from analysis of duplicate samples.](image-url)
Purification. The application of LLE and silica gel clean-up to the effluent extracts failed to sufficiently remove matrix interferences, and external calibration was still necessary for quantification. The LLE procedure decreased recoveries of E1-d$_4$ and T-d$_3$ to 1.3 and 22%, respectively. In the silica column trials, the hormones were eluted from the silica in acetonitrile and methanol, whether the column was conditioned in acetonitrile or hexane. Conditioning in and initial elution with acetonitrile resulted in higher surrogate recoveries, 53 and 110% for E1-d$_4$ and T-d$_3$, respectively. Of the three purification tests, only the silica clean-up with hexane conditioning was tested during a spike recovery, thereby providing a look at recoveries of all individual analytes (the other methods were tested on extracts spiked with only the surrogate and internal standards). It revealed wildly inconsistent recoveries for duplicates. Percentages for E3 and EE2, for instance, were 7.8 for both compounds in the first spike recovery and 0 and 27%, respectively, in the second. Furthermore, A, EA, and DHEA were not recovered at detectable levels in either duplicate. Figure 16 depicts the results of these purification tests. For comparison, averages of the duplicate recoveries achieved using the original C$_{18}$ SPE method without purification, lifted from Figure 15, are included as WWTP1-3.
Figure 16. Affect of purification on recoveries. Recovery of surrogate estrogen and androgen using the SPE/UPLC/(ESI)MS² procedure on wastewater without further purification (WWTP1-3), with clean-up using a silica column conditioned with acetonitrile (Si/acn) or hexane (Si/hex) or liquid-liquid extracted with hexane (LLE).

ESI vs. APCI. An effluent extract spiked with E2 and A demonstrated the efficacy of APCI versus ESI for analyzing hormones in the presence of extraneous matrix components. The APCI analysis allowed detection of both compounds, neither of which were observed using ESI (Figure 17).
Figure 17. Matrix effect in standard-spiked effluent extract. MRM analysis of extract spiked with E2 and A and analyzed using UPLC/(ESI)MS² analysis (A,C) and UPLC/(APCI)MS² analysis (B,D).
The extended utility of APCI was further demonstrated via infusion of E2 during UPLC/MS\(^2\). In the absence of matrix effects, the signal would be expected to remain constant across the entire analysis time. The time at which matrix interference would have been of greatest concern, however, was that proximal to the E2 retention time, 2.6 min. At 10 \(\mu\)l/min infusion, the intensity of the signal in ESI dropped two orders of magnitude at 0.8 min, remained so until 3 min (beyond the elution time of E2) (Figure 18A). In APCI, the signal intensity dropped significantly at 0.6 min, but was largely restored after 1 min. Signal suppression was less than an order of magnitude by 1.05 min and 58% of the starting intensity by 2.6 min (Figure 18B). Infusion of the 76 \(\mu\)g/ml standard at 10 \(\mu\)l/min corresponds to an injection of a 2000 ng/ml extract at the UPLC flow rate of 375 \(\mu\)l/min.
Progressively slower infusion rates were tested to determine the level at which the suppression effectively eliminated the E2 signal. The ESI signal at 2.6 min disappeared by 0.5 µl/min infusion (100 ng/ml UPLC flow). The APCI pattern observed at 10 µl/min, continued across 1, 0.5, 0.3, and 0.2 µl/min, and it was not until 0.2 µl/min (40 ng/ml UPLC flow) that the signal was negligible (data not shown).

**UPLC/(APCI)MS² Method.** In contrast to ESI analyses, the APCI precursor ion (Q1) signals were more intense for all estrogens and T in the positive ionization mode. The ion transitions for each analyte and the associated compound-dependent parameters are provided in Table 7, with source/gas-dependent parameters in Table 8.
Table 7. (APCI)MS² compound-dependent parameters.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Polarity</th>
<th>DP (V)</th>
<th>EP (V)</th>
<th>Quantifier (m/z:m/z)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
<th>Qualifier (m/z:m/z)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>+</td>
<td>50</td>
<td>5</td>
<td>271/133</td>
<td>35</td>
<td>4</td>
<td>271/253</td>
<td>20</td>
<td>6</td>
<td>6.31</td>
</tr>
<tr>
<td>E1-d₄</td>
<td>+</td>
<td>40</td>
<td>3</td>
<td>275/257</td>
<td>20</td>
<td>4</td>
<td>275/135</td>
<td>30</td>
<td>2</td>
<td>6.26</td>
</tr>
<tr>
<td>E2</td>
<td>+</td>
<td>30</td>
<td>2</td>
<td>255/159</td>
<td>30</td>
<td>3</td>
<td>255/133</td>
<td>35</td>
<td>4</td>
<td>5.42</td>
</tr>
<tr>
<td>E2-d₃</td>
<td>+</td>
<td>30</td>
<td>9</td>
<td>258/159</td>
<td>25</td>
<td>5</td>
<td>258/133</td>
<td>30</td>
<td>3</td>
<td>5.39</td>
</tr>
<tr>
<td>EE2</td>
<td>+</td>
<td>40</td>
<td>3</td>
<td>279/133</td>
<td>20</td>
<td>2</td>
<td>279/159</td>
<td>35</td>
<td>2</td>
<td>6.04</td>
</tr>
<tr>
<td>E3</td>
<td>+</td>
<td>30</td>
<td>4</td>
<td>271/253</td>
<td>15</td>
<td>4</td>
<td>271/133</td>
<td>35</td>
<td>3</td>
<td>5.39</td>
</tr>
<tr>
<td>T</td>
<td>+</td>
<td>50</td>
<td>3</td>
<td>289/109</td>
<td>35</td>
<td>4</td>
<td>289/97</td>
<td>45</td>
<td>2</td>
<td>5.8</td>
</tr>
</tbody>
</table>

(DP) declustering potential, (EP) entrance potential, (m/z:m/z) mass/charge ratios of precursor/fragment ions, (CE) collision energy, (CXP) collision cell exit potential, (RT) retention time.

Table 8. (APCI)MS² source/gas-dependent parameters.

<table>
<thead>
<tr>
<th>NC (V)</th>
<th>TEM (C)</th>
<th>GS1 (psi)</th>
<th>GS2 (psi)</th>
<th>CUR (psi)</th>
<th>CAD (psi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>400</td>
<td>45</td>
<td>0</td>
<td>10</td>
<td>medium</td>
</tr>
</tbody>
</table>

(NC) nebulizing current, (TEM) temperature, (GS1) ion source gas 1, (GS2) ion source gas 2, (CUR) curtain gas, (CAD) CAD gas.

Initial infusion of the analytes with and without chemical modifiers resulted in similar MRM transition intensities. Further testing was thus done to determine if intensities were affected by modifier concentration. With increasing concentrations of ammonium acetate, signal intensities generally decreased (Figure 19A). Intensities were greater in the presence of formic acid than ammonium acetate, but not greater than those recorded in the absence of a modifier (Figure 19B). It was therefore decided to continue APCI analyses without a chemical modifier.
Figure 19. Modifier addition in APCI. Effect of ammonium acetate (A) and formic acid (B) modifiers on the intensity of the MRM signals of the estrogens and T in (APCI)MS^2. Like symbols at the same concentration indicate results from analysis of duplicate samples.

As in the ESI-related UPLC program development, it was observed that peak intensities, shapes, and resolution were not optimal for all compounds at the same parameter values. The flow regime optimizing the greatest number of these factors for the greatest number of analytes was selected, with 1.50 minutes of re-equilibration time added after elution (Table 9). The optimum column temperature was 45°C. Retention times are shown above in Table 7. A total ion chromatogram and the UPLC flow regime are depicted in Figure 20.
Table 9. Estrogen plus testosterone UPLC method.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (μl/min)</th>
<th>ACN (%)</th>
<th>H₂O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>375</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>2.00</td>
<td>375</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>2.50</td>
<td>375</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>6.50</td>
<td>375</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>7.50</td>
<td>375</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>9.00</td>
<td>375</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

(ACN), acetonitrile.

The initial mobile phase composition selected for combination with the APCI method was weaker than those associated with the ESI analyses. The run was longer and the gradient breadth larger than the ESI-related estrogen program. This was done in anticipation of matrix interference. Testosterone eluted amidst the estrogens.

The IDLs and LOQs for the UPLC/(APCI)MS² method are listed in Table 10. The method was most sensitive for T. All analytes could be detected at or below 64 pg on-column, equivalent to an extract concentration of 12.8 ng/ml, given a 5 μl injection volume.

Figure 20. Total ion chromatogram and UPLC flow regime for APCI analysis. The chromatogram of cumulative ion signal, with intensity along the left-hand y-axis, with the UPLC flow regime superimposed as the percentage of the acetonitrile mobile phase, given along the right-hand y-axis.
Table 10. Quantitative boundaries of UPLC/(APCI)MS² analyses.

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E1-d4</th>
<th>E2</th>
<th>EE2</th>
<th>E3</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDL (pg on-column)</td>
<td>18</td>
<td>64</td>
<td>18</td>
<td>7.0</td>
<td>48</td>
<td>3.0</td>
</tr>
<tr>
<td>LOQ (pg on-column)</td>
<td>127</td>
<td>143</td>
<td>126</td>
<td>30</td>
<td>252</td>
<td>11</td>
</tr>
</tbody>
</table>

All calibration curves were linear ($r^2>0.99$). Repeatabilities across the calibration standards are given below (Table 11). They were moderate, falling between 1.1 and 18%, and increased irregularly from high to low concentrations. The average RSD was 5.6% and the median, 4.7%.

Table 11. Repeatability of UPLC/(APCI)MS² hormone analyses.

<table>
<thead>
<tr>
<th>Concentrations (ng/ml)</th>
<th>Analytes</th>
<th>Surrogate</th>
<th>E1</th>
<th>E1-d4</th>
<th>E2</th>
<th>EE2</th>
<th>E3</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500</td>
<td>4000</td>
<td>2.6</td>
<td>5.3</td>
<td>2.5</td>
<td>1.1</td>
<td>3.3</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>2400</td>
<td>1.3</td>
<td>6.6</td>
<td>4.2</td>
<td>5.3</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>1200</td>
<td>7.2</td>
<td>7.4</td>
<td>3.6</td>
<td>5.5</td>
<td>3.7</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>360</td>
<td>1.2</td>
<td>7.8</td>
<td>4.2</td>
<td>6.9</td>
<td>14</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>80</td>
<td>13</td>
<td>5.3</td>
<td>18</td>
<td>8.5</td>
<td>11</td>
<td>5.6</td>
</tr>
</tbody>
</table>

(APCI)MS² Application. Effluent samples and spike recoveries into effluent clearly revealed ion enhancement for most analytes when using APCI. The internal standard MRM signal was exaggerated and erratic, with peak areas an order of magnitude greater than in the calibration standards, with an RSD of 53% across samples (the RSD across calibration standards was 7%). External calibration was thus used. Analyte recoveries ranged from 95-415%, with poor reproducibility across replicate spike recoveries for E1, E1-d₄, and EE2 (Figure 21).
Figure 21. Recoveries of analytes from effluent using UPLC/(APCI)MS\(^2\) quantification. Like-colored double bars indicate results from analysis of duplicate spike recoveries.

Surrogate recoveries in the unspiked effluent samples were 218 and 223%. Peaks corresponding to unlabelled E1, E3, and T were found in the unspiked effluents within the calibration range. Without correction for surrogate recovery, duplicate effluent samples were found to contain 58 and 64 ng/L E1, 0 and 56 ng/L E3, and 4 and 3.2 ng/L T.

SEC Purification. Collection of the eluent (5 ml/min) from 15.0-26.9 min (the 15\(^{th}\)-26\(^{th}\) minutes) in HPLC elution achieved cumulative recoveries ≥93% for the estrogens, but only 37% for T. All analytes were detected in the 15\(^{th}\)-17\(^{th}\) minutes, with additional recovery of most in the 18\(^{th}\) and 19\(^{th}\), and small percentages (<2%) of E2, EE2, and E3 in the 20\(^{th}\)-22\(^{nd}\) minutes.

Collection from 10.0-19.9 (the 10\(^{th}\)-19\(^{th}\) minutes) provided cumulative recoveries of ≥93% for all estrogens, except E3 (82%), and 100% for T. Estrogen detection spanned the 14\(^{th}\)-19\(^{th}\) minutes, and T the 12\(^{th}\)-19\(^{th}\).
Graphs depicting elution distribution are provided below (Figure 22). The recoveries are given as percentages of the total spiked mass, not of the total recovered mass. The finalized method consisted of discarding 0.0-11.9 min, collecting 12.0-21.9 min, and discarding 22.0-30.0.

![Graphs depicting elution distribution](image)

**Figure 22. SEC fractionation of mixed standard.** Percentages of the total spiked mass in individual collection fractions using an elution rate of 5 ml/min. **Trial 1 (A)** incorporated collection from 15.0-26.9 min in a 30 min run, and **trial 2 (B),** the central 10 min of a 30 min run.

In application of the SPE/SEC/UPLC/(APCI)MS² procedure to unspiked effluent, none of the analytes were found at detectable levels, and E1-d₄ surrogate recoveries were
of 22 and 32% in duplicate samples. In the duplicate spiking experiments, the surrogate was recovered to 36 and 21%, but recoveries of E1, E2, EE2, and T were greater, ranging from 48-91%. These results are depicted in Figure 23. E3 was not detected in either duplicate.

Figure 23. Recoveries using SPE/SEC/(APCI)MS². Recovery of analytes from a spike recovery into effluent processed using the SPE/SEC/UPLC/(APCI)MS² protocols. Like-colored double bars indicate results from analysis of duplicate spike recoveries.
DISCUSSION

Estrogens vs. Androgens. Estrogenic and androgenic steroid hormones are co-introduced into surface waters through dispersal of biological waste [12,30]. Both classes can act as endocrine disruptors in exposed wildlife [1-4,22,26-28]. In light of such facts, the present study investigated estrogens and androgens, a rare practice in current environmental hormone research. It was herein demonstrated that the classes can be successfully co-extracted from aqueous samples, which reduces the volume of sample that must be collected, as well as the materials and time devoted to processing each. Divergences in instrumental detection were investigated and explained in reference to differences in molecular structure and polarity, with complete methods for UPLC/(ESI)MS² analysis developed for six estrogens and six androgens.

Ionization of the analytes during MS² occurred according to the polarity leading to the most stable precursor ions. The estrogens analyzed here contain an aromatic A ring adjacent to a hydroxyl functional group. Loss of the hydroxyl proton through negative ESI resulted in a charge that was stabilized by electron delocalization throughout the aromatic ring. In contrast, the loss of the hydroxyl proton from the androgens during ESI would have resulted in a negative charge localized on an oxygen
atom bonded to an aliphatic ring, producing an unstable ion. Hence, it was more effective to add protons onto the carbonyl oxygen atoms through positive ionization. It is common for the estrogens to be subjected to negative ionization during ESI analyses [6,9,11,13,15,17,37,39,45], while there is little published literature describing androgen MS2 methods.

Chemical modifiers are used for enhancement of MS2 ionization. The stability of the estrogen precursor ions eliminated the need for such additives. Data from Zuelke et al. [13] corroborate this. The formation of positive androgen ions, however, was facilitated by the protons donated by ammonium ions in the ammonium acetate solution. This was of particular importance for A, EA, and DHEA. Although the intensity of the T signal increased with modifier concentration, unmodified signals were already an order of magnitude more intense than those of the remaining androgens.

Differences in precursor ion stability can also be used to explain the dichotomy in the IDLs and LOQs of the estrogens and T versus A, EA, and DHEA. Greater stability ensures that a larger portion of the ions that have been formed in the ESI probe reach the detector. This decreases the number of injected molecules required to amass a distinguishable signal. Electron delocalization across the aromatic rings in the estrogens and conjugation between the carbonyl group and A ring in T stabilized the negative and positive charges, respectively. For A, EA, and DHEA no such delocalization was possible, rendering such precursor ions less stable than those of either the estrogens or T. In the publication by Yamamoto et al. [7], containing a similar list of analytes, the IDLs of A, EA, and DHEA were slightly greater than those of the estrogens (1.0-4.0 ng/ml
versus 0.41-1.2) and more than an order of magnitude greater than that of T (0.08 ng/ml). This reduced dichotomy may be due to the use of APPI.

Although MS² was applicable for all analytes, PDA detection was not. The PDA is less sensitive, but useful for preliminary UPLC method development when concentrated standards are used. The estrogens and T produced reliable signals due to the conjugation inherent to their molecular structures. Such delocalization is necessary for excitation by and emission of UV radiation. Without such chromophores, A, EA, and DHEA failed to respond. This may also explain why the androgens were less prone to degradation during storage than the estrogens. Although degradation mechanisms were not investigated, photodegradation was one possible mechanism [38]. The extracts were stored in the dark, but held in clear glass vials and exposed during instrumental preparation and incidentally during analysis.

The reasons for increased recoveries of the surrogate T-d₃ and those of additional androgens during storage remain unknown. It is possible that the internal standard or another of the analytes degraded; however, there was no striking decrease in the area of the internal standard after storage, and the net decrease in androgen recovery did not balance the gain. Alternatively, the formation of ion-enhancing, modifier-like compounds during storage could have influenced recoveries. Determination of the exact mechanisms at work during storage was beyond the scope of this study.

The similarity of the androgens to one another, in terms of molecular weight and structure, further contrasted their analysis with that of the estrogens. The estrogen precursor/fragment ion MRM transitions were unique from one another, while the androgens displayed commonalities. The estrogens differ in the number and types of
functional groups bound to the steroid D ring and span a difference of 26 amu in mass. The androgen stereoisomers A and EA are of equal mass and differ only in orientation of the hydroxyl group, while DHEA and T differ in structure but share a common molecular mass. Such phenomena necessitated well-defined chromatographic separation prior to MS\(^2\). The original androgen UPLC method used a lower flow rate than that of the estrogen method. The modified method also included a broader gradient and longer run time.

Finally, the lesser polarity of the androgens, due to a smaller number of hydroxyl groups compared to the estrogens, resulted in greater affinity for the BEH C\(_{18}\) UPLC column and C\(_{18}\) SPE cartridge. This was exemplified by the greater percentage of the strong mobile phase solvent (i.e. acetonitrile) used in segments of both the original and modified androgen UPLC methods. In addition, it explains the ~1:1 elution of the androgens across the 2x5 ml SPE elution aliquots, versus the ~3:1 observed for the estrogens.

In the analyses of effluent samples, the estrogen and androgen surrogates suffered opposite matrix effects using ESI analyses. As T-d\(_3\) was the androgen surrogate, however, and T differed from the remaining androgens in several respects, its behavior in the complex sample may not be indicative of that of A, EA, and DHEA. According to the preliminary ESI versus APCI tests with A, ion suppression may occur for those target compounds. Regardless, the accuracy of hormone quantification was compromised by elution with extraneous matrix components. Those extraneous compounds entering the MS\(^2\) with the estrogens tended to reduce ionization efficiency of the target analytes. Such ion suppression may be caused by the scavenging of ionization voltage or inhibition
of ionization by coeluters, thereby reducing the proportion of target analytes reaching the
detector. Alternatively, extraneous compounds that elute with the androgens may
enhance ionization, behaving like modifiers. Municipal waste includes chemicals from
household use, hospitals, runoff, and industries. Thus, analytical interferents in WWTP
effluent may include steroidal pharmaceuticals, microbial sterols, and/or vegetative
phytosterols.

In retrospect, alternative surrogate standards could have been selected, especially
for androgen analyses. The behavior and results for T-d₃ may not closely track those of
A, EA, and DHEA due to the differences discussed above. Gabet et al. [50] further
claimed that E1-d₄ is a poor surrogate due to storage instability. In this study, E1-d₄
degraded during the 25 d storage trial period, along with only E1 and EE2. The %RSDs
in area ratio used to assess repeatability of the ESI and APCI MRM signals were also on
the high end for E1-d₄, as well as APCI quantitation boundaries (ESI boundaries were not
determined for the surrogates).

**UPLC.** UPLC is an emerging technology with a limited history of environmental
application [39,40]. This study demonstrates the ability of UPLC to separate six
compounds, either estrogenic or androgenic, in a 7 min program, including re-
equilibration to initial conditions. Brief retention times and slow flow rates (<400
μl/min) minimized mobile phase solvent usage, reducing the costs of purchase and waste
disposal. The research herein further tested UPLC technology through application to
surface waters and WWTP effluents.
The brevity of the UPLC programs was challenged by the complexity of extracts derived from the effluents. The presence of unresolved complex mixtures in the chromatograms of both hormone classes interfered with quantification. This demonstrated an important consideration in the application of this emerging technology:

In a UPLC system having a column of equal length (L) to that in an HPLC, the smaller packing particle diameter (d) of the former results in a greater number of theoretical plates (N), according to the following equation [43].

\[ N \approx \frac{3500 \, L}{d} \]

This, in turn, implicates a reduced plate height (H) in UPLC [43].

\[ N = \frac{L}{H} \]

A lower height is mathematically associated with narrower peak widths (W). Longer retention times (t_R) would also reduce the height according to the following equation, but UPLC tends to shorten retention, as observed here and by Farré et al. [39,43].

\[ H = \frac{(L \, W^2)}{(16 \, t_R^2)} \]

Reduced peak widths for analytes 1 and 2 improve resolution (R) if the spread in retention times is unchanged [43].

\[ R = \frac{2 \, (t_{R2} - t_{R1})}{(W_1 + W_2)} \]

If retention time span is reduced, however, then resolution may not be enhanced. Farré et al. [39] have indeed demonstrated that peak spacing may be reduced upon transition from HPLC to UPLC [39,44]. Method development for UPLC separation should, therefore, focus on reduced retention times or increased resolution, depending on the application. Results of this study suggested that resolution should not be sacrificed for increased sample throughput in environmental application. The development of elution regimes
encouraging longer hormone retention times, similar to those of HPLC methods, would result in greater resolving power due to narrower UPLC peaks, according to the equation above.

Although enhanced chromatographic resolution may be useful in counteracting matrix interference, it alone may be insufficient in highly complex samples. Kaspryzk-Hordern et al. [40] used the same UPLC system and stationary phase employed here for detection of 25 pharmaceuticals in surface water. The elution conditions were drastically weaker than those used in this study. Their run time was 20 min, with a flow rate of 50 µL/min and column temperature of 22°C, with retention times spanning 5-13 min. Even so, matrix interference in the form of ion suppression was encountered as a result of coelution of extraneous compounds with the analytes of interest. In such cases, the incorporation of supplemental extract purification methods may be unavoidable [9,39-40].

**Purification.** Many protocols for the removal of interfering matrix components involve the manual processing of extracts through disposable columns packed with various solid phases [7,13-14,45]. In contrast, this study examined a method utilizing SEC as a means of sample purification. The incorporation of SEC reduces laboratory waste through repeated usage of a single analytical column. It also reduces the number of man-hours devoted to sample processing, as the entire procedure is automated.

The goals of purification trials carried out here were to (1) reduce the background interference of the internal standard MRM signals so that internal calibration could be used, (2) reduce the background interference with remaining MRM signals so that all
analytes could be reliably integrated, and (3) achieve acceptable hormone recoveries (i.e. 70-110%).

The silica column and LLE tests failed to eliminate the need for external calibration. They also failed to increase the recovery of E1-d₄ above 53%. For T-d₃, only the silica column conditioned in acetonitrile elevated the recovery to ~100%, while the remaining methods using hexane resulted in less than 25% recovery.

Although rarely applied in hormone analyses, SEC showed promise as a purification technique. Koh et al. [9] published a method incorporating its usage late in 2007, and followed it with anion exchange chromatography using an amine column. The application of SEC in this study achieved recoveries from effluents that exceeded the (ESI)MS² values that were as low as 4.8% and reduced the exaggerated (APCI)MS² values reaching 420%. These data, provided individually in the Results section, have been plotted together for comparison in Figure 24. Note that effluent spike recoveries were not completed during ESI testing and, hence, only data for the surrogates are provided.
When testing the viability of SEC through application to a standard solution, E3 was recovered to $\geq 82\%$, but was removed below detection upon application to WWTP effluent extracts. The presence of additional compounds in the effluent may have affected the elution or there may have been complications during APCI that were not observed when injecting simple solutions. Ingrand et al. [14] also lost E3 during clean-up with florisil columns. They suggested that E3 remained sorbed to the florisil, while the other estrogens were effectively eluted using dichloromethane/acetonitrile (95:5 v/v). The E3 molecule contains the greatest number of hydroxyl groups, which may result in stronger associations with the sorbents. In the SEC method used here, 100% dichloromethane was used for elution. A stronger mobile phase may therefore be needed for elution. Koh et al. [9], for example, eluted a PLgel SEC column with dichloromethane/methanol (90:10 v/v) for the purification of extracts prior to analysis for estrogens.
ESI vs. APCI. Environmental hormone research to date has largely relied on ESI for MS², with mere speculation about additional ionization mechanisms. This study delved into APCI usage, providing an MRM method for several hormones and testing its application to complex matrices. The ESI- and APCI-based methods developed here required different polarization modes and exhibited disparate repeatabilities, quantitative boundaries, and responses to matrix interferences. APCI was found promising in the detection of estrogens and androgens that were not revealed via ESI when analyzing wastewater extracts.

During optimization of the compound-dependent parameters at the start of APCI method development, it was immediately observed that the estrogens were more readily detected in positive ionization, in contrast to negative ionization in ESI. Upon inspection of the precursor ions, it was also noted that all m/z values in APCI were not indicative of a proton transfer (i.e. molecular weight ± 1 amu), as they were in ESI. Those of E₁, E₁-d₄, and T did exhibit precursor ions of molecular weight + 1 amu, but those of E₂, E₂-d₃, EE₂, and E₃ all had values of molecular weight minus 17 amu. The only functional group common to the latter four analytes, but not found in E₁, is a hydroxyl group on the steroidal D ring. The hydroxyl has a mass of 17 amu and would leave a positive charge on the remaining molecule, although loss of the A ring hydroxyl group would be more stable due to adjacent aromaticity. The E₁ and T molecules each have a hydroxyl and carbonyl functional group. According to stabilization arguments, the proton would associate with the E₁ hydroxyl group and the T carbonyl oxygen, although such possibilities cannot be directly proven or refuted from this study.
The calibration ranges and linearity were the same for the two techniques. The 
APCI analyses had improved repeatability, however (Table 12). The ESI methods had 
lower quantitative boundaries, although differences were generally less than an order of 
magnitude (Table 13).

<table>
<thead>
<tr>
<th>Table 12. ESI vs. APCI repeatabilities for estrogens and testosterone.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability (%) RSD</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Average</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 13. Inter-study comparison of quantitative boundaries.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrumental Detection Limits</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>UPLC/ (ESI)MS2*</td>
</tr>
<tr>
<td>UPLC/ (APCI)MS2*</td>
</tr>
<tr>
<td>HPLC/ (ESI)MS2[39]</td>
</tr>
<tr>
<td>HPLC/ (APPI)MS2[7]</td>
</tr>
<tr>
<td>Estradiol (E1)</td>
</tr>
<tr>
<td>Estradiol (E2)</td>
</tr>
<tr>
<td>Estriol (EE2)</td>
</tr>
<tr>
<td>Estriol (E3)</td>
</tr>
<tr>
<td>Estriol (T)</td>
</tr>
<tr>
<td>Androgen (A)</td>
</tr>
<tr>
<td>Androgen (EA)</td>
</tr>
<tr>
<td>DHEA</td>
</tr>
</tbody>
</table>

* Methods developed in this study.

Table 13 provides the IDLs characteristic of the HPLC/(ESI)MS2 and 
HPLC/(APPI)MS2 methods developed by Farré et al. [39] and Yamamoto et al. [7], 
respectively, alongside those developed here. The values are presented as both on-
column masses and extract concentrations. The former eliminates the influence of 
injection volume. The methods developed here utilized 5 µl injections, while Farré et al. 
[39] and Yamamoto et al. [7] incorporated larger volumes of 25 and 10 µl, respectively.
This parameter was limited by the instrumental set-up, with a 10 µl UPLC sample loop set to partial loop with needle overfill having a maximum injection volume of 7.5 µl.

The estrogen IDLs of the UPLC/(APCI)MS\textsuperscript{2} method developed in this study generally exceeded those of the other methods. When considering concentration values, the UPLC/(ESI)MS\textsuperscript{2} method given here is slightly less sensitive than the referenced methods, while the mass values are on par with them. A larger sample loop could be installed in the UPLC to increase the injection volume and thereby decrease the IDL concentrations. Farré et al. [39], for instance, used the same UPLC system, but with 20 µl injections (the IDLs are not provided for that method, as time-of-flight MS was used for detection). Such would decrease the UPLC/(ESI)MS\textsuperscript{2} and UPLC/(APCI)MS\textsuperscript{2} IDLs given above by a factor of 4.

Few published methods are available for comparison of androgen detection methods. The quantitative boundaries of Yamamoto et al. [7] for HPLC/(APPI)MS\textsuperscript{2} given above are consistently lower than those observed here using UPLC/(ESI)MS\textsuperscript{2}. The same pattern exists, however, in which the IDL of T is 1-2 orders of magnitude less than those of the other androgens.

Returning to the comparison of the ESI and APCI techniques presented in this study, the response to matrix components differed between the two ionization approaches, as demonstrated by the spiking of effluent with concentrated E2 and A solutions. The APCI technique permitted detection despite the presence of matrix interference that inhibited ESI detection. In the subsequent infusion experiments, ESI was shown to succumb to ion suppression resulting in a signal loss of two orders of
magnitude at an extract concentration of 2000 ng/ml. In contrast, the APCI signal persisted and was not diminished to background levels until 40 ng/ml.

While the infusion results seemed to suggest that APCI was subject to ion suppression, the effect was less pronounced than in ESI. In contrast, the spike recovery work demonstrated ion enhancement for the analytes, and the results of spiking E2 and A into effluent could have resulted from either matrix effect. Of the three, only the spike recovery had an established baseline, that of 100% recovery. The observed values of >400% clearly demonstrated ion enhancement. In retrospect, the spiking and fusion should have been performed on a clean solvent solution in addition to the effluent extract, in order to provide a baseline from which to gauge matrix effect.
CONCLUSIONS

Many current studies involving environmental hormone research incorporate HPLC/(ESI)MS or HPLC/(ESI)MS\(^2\) for the detection of estrogens, with occasional inclusion of supplemental silica or florisil purification. This study extended that research to recognize additional steroidal hormone contaminants, the availability of alternative instrumental setups, and the potential for more efficient clean-up methods.

It was demonstrated that estrogens and androgens could be extracted in the same analytical step. Extracts were then analyzed twice using LC/MS\(^2\), once for each hormone class. The androgens required more robust chromatography than the estrogens and benefited from the addition of a chemical modifier for MS\(^2\). Ionization in (ESI)MS\(^2\) produced stable ions in the negative and positive modes for the estrogens and androgens, respectively. The estrogens E1, E2, EE2, and E3, as well as T, could be detected at low pg on-column masses, while A, EA, and DHEA exhibited higher IDLs and LOQs, due to a lack of bond conjugation in their molecular structures.

The use of UPLC allowed analytical separation of the various hormones in simple solvent solutions within 7 min, reducing mobile phase usage and processing time. Coelution of analytes with interferents present in WWTP effluent samples indicated that
resolution must be emphasized over brevity of analysis when testing more complex matrices.

Although the use of APCI did not eliminate matrix effects in MS² analysis, it did generate hormone MRM signals that were not produced via ESI. The use of an SEC purification step, in combination with APCI showed promise for quantitative hormone analysis in the face of matrix interference.
FUTURE RESEARCH

Hormone research should continue to expand in the directions taken in this study. The inclusion of multiple androgens, emerging instrumental technology, and less frequently used MS$^2$ ionization techniques should be pursued farther.

The target compound list pursued at the onset of this study should be maintained and augmented with progesterone, the estrogen and androgen precursor, androstenedione, the natural androgen associated with paper mill effluent, and synthetic androgens, such as trenbolone. Methods for (APCI)MS$^2$ analysis should be developed for androgens in addition to T in order to determine if multiple estrogens plus androgens could be detected using a shared MS$^2$ method. This would further streamline analysis.

Future UPLC usage should focus on prolonged hormone retention times. If later elution effectively bypasses early-eluting unresolved complex mixtures, the advertised resolution capability of UPLC might be demonstrated. In order to delay elution of the hormones incorporated in this study, the initial mobile phase should be weak (i.e. mostly aqueous) and held for several minutes. Alternatively, different stationary phases could be evaluated.
It was shown here that SEC is a promising technique for sample purification and that the estrogen and T analytes could be recovered at $\geq 82\%$ from clean standard solutions. SEC application to effluent extracts did result in the loss of E3. A different column packing could be used, but first, minor adjustments of the current SPE elution method should be tested, such as alteration of the mobile phase solvent. The molecular sizes of the hormones were similar enough to result in elution across a common fractionation volume. Minor separation was observed within that window, however, based on polarity, with T recovery peaking first, followed by E1, EE2, E2, and E3. The 100% dichloromethane mobile phase could be strengthened with a small percentage of a more polar solvent to encourage elution of E3, the most polar of the analytes. In addition, SEC should be tested as a purification method preceding (ESI)MS$^2$ analysis, as it was only tested in tandem with (APCI)MS$^2$ here.

As interest in hormone contamination continues to spread, inter-laboratory comparisons of analytical techniques should be pursued. This can be done round-robin fashion, with multiple groups analyzing the same surface or wastewater samples. A reference standard with established concentrations would be especially useful in this field. Challenges in establishing a reference standard include the observed analyte instability and the compositional variations in animal waste and composites reaching WWTPs.
REFERENCES


VITA

STACIE L. RICE

Raised in Vestal, NY. Graduated from Vestal Senior High School in 2002. Earned a B.S. in Chemistry from Binghamton University, a State University of New York, in 2006. Entered the Masters program at the Virginia Institute of Marine Science, the School of Marine Science of the College of William and Mary, in 2006.