Analytical Methods for the Detection and Quantification of Trenbolone Acetate Metabolites, Altrenogest and Related Photoproducts via Liquid Chromatography Tandem Mass Spectrometry

Philip T. Kenyon

A thesis submitted for the partial fulfillment

of the requirements for the degree of

Master of Science in

Civil Engineering

University of Washington

2015

Committee:

Edward Kolodziej

Joel Baker

Program Authorized to Offer Degree:

Civil & Environmental Engineering

University of Washington

Abstract

Analytical Methods for the Detection and Quantification of Trenbolone Acetate Metabolites, Altrenogest and Related Photoproducts via Liquid Chromatography Tandem Mass Spectrometry

Philip T. Kenyon

Chair of Supervisory Committee: Dr. Edward P. Kolodziej

Department of Civil and Environmental Engineering

Photoreaction coupled with liquid chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS) was used to develop an analytical method for the detection and quantification of trenbolone metabolites, altrenogest, and related photoproducts in water. Target parent analytes were 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), trendione (TBO), and altrenogest (ALT); target photoproducts were the metastable 5-hydroxy- and 12-hydroxy-photoproducts of 17α/17β-TBOH and TBO, and the cyclo-addition and hydroxy-cyclo-addition photoproducts of altrenogest. Photoproducts were generated with a solar simulator reacting trenbolone metabolite (or altrenogest) samples for 6 hrs (>10 half lives). Samples were extracted with C18 solid phase extraction (SPE) cartridges before liquid chromatographic separation with a reverse-phase C18 column with water and methanol mobile phases and ESI+ MS detection. Method detection limits (MDL's) and quantification limits (MQL's) for all compounds were near or below 1 ng L^{-1} except for 17β-TBOH photoproducts, which had MDL's <2 ng L^{-1} and MQL's <4 ng L^{-1}. Matrix suppression was <30% using lake water, and SPE recovery rates were near/above 100%, except for 17β-TBOH photoproducts, with recoveries >75%. Intra-day relative standard deviations (RSD's) were <30% for TBO and its products, <20% for all others.

Acknowledgements

I would firstly like to thank my adviser, Dr. Ed Kolodziej, for enabling and advising my research at the University of Nevada, Reno, and the University of Washington. I would also like to thank my second committee member, Dr. Joel Baker, for his support and guidance throughout my studies at the University of Washington.

I would like to thank my fellow researchers, Dr. Gerrad D. Jones,, Dr. Bowen Du, Phil Benedetti, Emily Ruskowitz, Tianlin Song, Rachel Weber, Jasmine Miller, Sam Randall, and Alex Gipe, for their advice and contributions to my research.

I also wish to thank my family and friends for their support throughout the duration of my studies, both in Reno and in Tacoma. I would especially like to thank Adrien Goucher, Bryan Kenyon, Linda Kenyon, Patrick Kenyon, and Mark Urlacher.

Lastly, I wish to thank the United States Department of Agriculture and the National Science Foundation for their research grant support, which fueled my research efforts and enabled my success.

Table of Contents

List of Figures

Manuscript Figures

List of Tables

Manuscript Tables

Chapter 1: Introduction

Endocrine Disrupting Compounds

In recent decades, contaminants which have the capacity to disrupt natural biological processes in animals have become a research priority for identification and control. Once such group of contaminants are labeled "endocrine disrupting compounds" (EDC's), which the National Institute of Environmental Health Services (NIEHS) defines as "chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife" [1]. EDC's are primarily synthetic, and are often described as "contaminants of emerging concern" (CEC's), which the EPA defines as "chemicals [that] are being discovered in water that previously had not been detected or are being detected at levels that may be significantly different than expected" [2]. EDC's are an increasing concern near agricultural outflows, where high concentrations of steroidal and pharmaceutical metabolites may be present in agricultural runoff, and could potentially affect exposed organisms.

Synthetic EDCs include dioxin-like compounds, polychlorinated biphenyls (PCBs), steroid hormones, pesticides, herbicides, and plasticizers[1]. Among steroid hormones, the growth promoter trenbolone (as trenbolone acetate) has been identified as an endocrine active aquatic contaminant[3-6]. The ability to detect and quantify these compounds using common analytical methods, such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) is imperative to understand their ultimate fate and transport within environmental systems.

Growth Promoter Uses

Growth promoters are used in animal agriculture to promote the growth of animals for slaughter. While antibiotics may sometimes be considered growth promoters (they promote growth by minimizing infection), steroidal growth promoters rely upon anabolic properties to improve the conversion of feed into muscle mass. Testosterone is the endogenous androgen, and mimicking its androgenic and anabolic effects is the synthetic androgen trenbolone (see Figure 1). Trenbolone is one of three synthetic hormone growth promoters used widely in the United States, along with the progestin melengestrol acetate and the estrogen zeranol[7]. Trenbolone acetate is applied to cattle as an ear implant, time-releasing trenbolone to the animals and increasing their muscle mass. This results in an important incremental economic effect, with estimates that its use is worth $1 billion USD per year[8]. Metabolized trenbolone is excreted as the metabolites 17α-trenbolone (17α-TBOH, the primary metabolite), 17β-trenbolone (17β-TBOH), and trendione (TBO) in urine and manure (see Figure 1)[9].

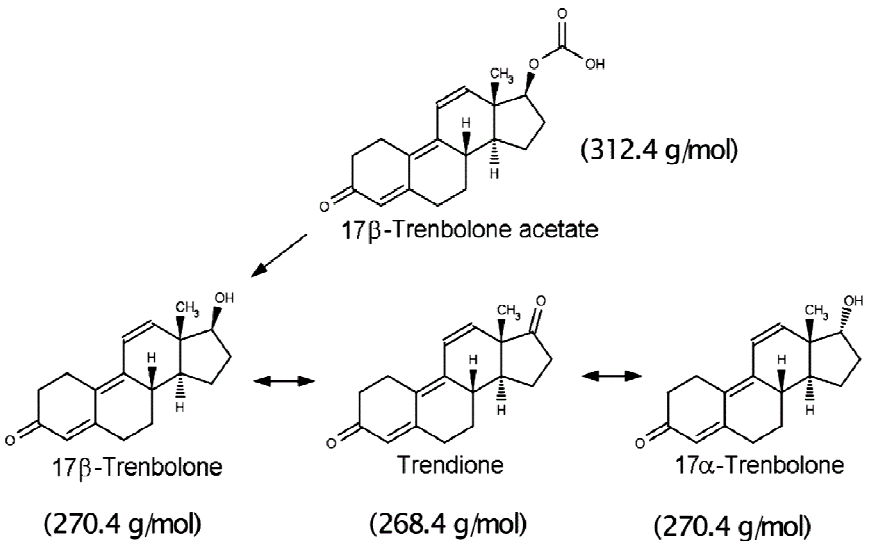


Figure1. Trenbolone acetate (TBA) metabolism. Figure adapted from Khan et al. 2008

Concerns over the environmental implications of metabolites in agricultural runoff reaching surface waters bearing sensitive fish populations have prompted all European countries to ban the use of trenbolone acetate in cattle, however trenbolone is still widely used in the U.S., Australia, and China, among other countries[10,11]. Table 1 shows commercially available formulations of trenbolone acetate (and other steroids) which are commonly used in the beef cattle industry.

Table 1. Commercially available implant formulations currently used by the beef cattle industry[12].

Hormone	Method of Administration	Dosage	Duration of Effect (days)	Growth Response
Trenbolone Acetate	Pellet implant	200 mg (heifers, cull cows)	60–90	5%–12%
Trenbolone Acetate + Estradiol Benzoate	Pellet implant	200 mg TBA + 28 mg EB (steers, heifers)	90–120	10%–20%
		100 mg TBA + 14 mg EB (steers)	90–120	10%–20%
Trenbolone Acetate + 17β-Estradiol	Pellet implant	200 mg TBA + 20 mg E (steers, heifers)	90–120	
		120 mg TBA + 24 mg E (steers)	90–120	
		140 mg TBA + 14 mg E	90–120	

		(heifers)		
		80 mg TBA + 16 mg E (steers)	90–120	
		80 mg TBA + 8 mg E (heifers)	90–120	
		40 mg TBA + 8 mg E (steers and heifers grazing pasture)	90–120	
Trenbolone Acetate + 17β-Estradiol	Pellet implant	200 mg TBA + 40 mg E (steers)	200	
Zeranol	Pellet implant	36 mg zeranol	90–120	10%–15%
		12 mg zeranol	90–120	10%–15%
Melengestrol Acetate	In feed	0.25–0.5 mg/day, PO	As long as it is given	3%–10%

TBA = trenbolone acetate; EB = estradiol benzoate; E = 17β-estradiol; MGA = melengestrol acetate

Progestin Uses

Progestins are synthetic progestogens, and are primarily used for hormonal contraception and for use in hormone therapy to treat endometrial hyperplasia. Progestins (like progestogen) suppress ovulation during pregnancy, which is useful both for human use and for veterinary uses. Altrenogest is a progestin, and is administered orally (animal feed) either as an estrus synchronizer, to maintain pregnancy, or to postpone estrus after weaning[13-16]. Swine typically receive 210-360 mg doses of altrenogest over 12-18 days (for horses, 230-400 mg doses over 15 days) for estrus synchronization. Pregnancy maintenance in horses typically involves the

additional dosage of 3000-6000 mg over a 120 day period, with unusual cases allowing for up to 15,000 mg over the entire gestation period of 300 or more days[17-18]. Estimating the annual usage of altrenogest is difficult (as is common with veterinary pharmaceuticals due to their industrial-scale use and minimal use regulations), but the most common form, Regu-Mate® has claimed to have sold more than 20 million doses over its 30 year life, at 2.2 mg for every 110 lbs of animal[17]. This is particularly important, given that concentrations at sub ng L^{-1} concentrations of other synthetic progestins, can reduce fecundity in fish[19-21].

Environmental Fate and Transport of Steroids

Scientific understanding of the fate and transport of many synthetic steroids used as human pharmaceuticals or in animal agriculture is incomplete, making difficult the accurate risk assessment to fish and other organisms from exposure to these compounds. In animal agriculture (specifically cattle) metabolites from implanted steroids (or endogenous steroids) can be detected in manure runoff at 26-370 ng/L[22]. High mass excretion of steroids via manure and urine (as conjugate steroids), despite dilution, results in these compounds being regularly detected downstream of outflows from animal agriculture. Trenbolone metabolites have been detected downstream at concentrations as high as 10-20 ng/L[23]. Natural biodegradation pathways show contrasting evidence between aerobic and anaerobic biodegradation of TBA. Aerobic half lives for TBA metabolites are on the order of 1-5 days[10,24] while half-lives of TBA metabolites under anaerobic biodegradation show that they are much more stable (half lives of 260 days)[25]. This suggests that under aerobic conditions (as in a river), TBA metabolites will transform readily, but under anaerobic conditions (as in a manure pile), TBA metabolites are far more likely to remain stable over time.

Additionally, sorption characteristics for trenbolone metabolites indicate that partitioning can be largely affected by soil type and the molecular properties, such as the octanol-water partitioning coefficient. Table 2 shows the organic carbon-water partitioning coefficients and octanol-water partitioning coefficients for the metabolites of trenbolone[26,27]. From their moderate log K_{ow} values it is clear that TBA metabolites will partition to soil over water, decreasing their aqueous transport rate, but increasing persistence in solids-rich environments. No altrenogest sorption data is available, but computer-modeling based on molecular structure suggests that its log K_{ow} is 3.94[28].

Table 2. Summary of log K_{oc} and log K_{ow} for TBA metabolites.

	Average ± SD[a]		
Metabolite	log K_{oc} [b, 26]	log K_{ow} [c, 26]	log K_{ow} [c, 27]
17α-trenbolone	2.77 ± 0.12	2.72 ± 0.02	2.72
17β-trenbolone	3.08 ± 0.10	3.08 ± 0.03	3.08
Trendione	3.38 ± 0.19	2.63 ± 0.05	2.63

[a] Standard deviation. [b] Organic-carbon normalized sorption coefficient. [c] Octanol-water partition coefficient.

Concern has grown over the transport of synthetic hormones downstream of animal agriculture due to their high bioactivity at low concentrations. Synthetic hormones are notably more resistant to microbial breakdown than natural hormones, limiting their transformation (and deactivation) pathways, and suggesting that they may be more persistent than natural hormones[11]. For example, studies demonstrate that trenbolone is stable in manure under

anaerobic conditions for over 260 days[11], suggesting that synthetic compounds are of greater concern to ecosystem stability than natural hormones. Data on the biodegradation of altrenogest is unavailable, and environmental degradation of progestins in general is also lacking.

In addition to concerns of the effects of trenbolone metabolites and altrenogest, transformation products can also act as EDCs. Trenbolone metabolites undergo phototransformation with product-to-parent reversion (described later), while altrenogest undergoes a cyclo-addition photoreaction followed with a similar reversible photohydration, a characteristic which could apply to other, structurally similar compounds[13,29,30]. Additional recent research suggests that trenbolone photoproducts may have their own separate bioactivity of the parent metabolites, meaning that photoreacted metabolites could also impact ecosystems[31]. In the case of the photoproducts of any steroid, methods for their detection and quantification have not yet been developed, leaving a gap in the measurement of steroids, due to the failure to detect photoproducts in addition to parent metabolites.

Research Objective

The critical research objective was to develop an analytical method to simultaneously detect trenbolone metabolites, altrenogest, and related photoproducts, which is sensitive enough to detect all target compounds at environmentally relevant concentrations.

Analytical Methods for the Detection and Quantification of Trenbolone Acetate Metabolites and Altrenogest plus Photoproducts via Liquid Chromatography Tandem Mass Spectrometry

Phil Kenyon

Edward Kolodziej

University of Washington

For submission to Journal of Chromatography A

Abstract

Photoreaction coupled with liquid chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS) was used to develop an analytical method for the detection and quantification of trenbolone metabolites, altrenogest, and related photoproducts in water. Target parent analytes were 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), trendione (TBO), and altrenogest (ALT); target photoproducts were the metastable 5-hydroxy- and 12-hydroxy- photoproducts of 17α/17β-TBOH and TBO, and the cyclo-addition and presumptive 5-hydroxy-cyclo-addition photoproducts of altrenogest. Because they are not commercially available, photoproducts were generated with a solar simulator reacting trenbolone metabolite (or altrenogest) samples for 6 hrs (>10 half lives). Samples were extracted with C18 solid phase extraction (SPE) cartridges before liquid chromatographic separation with a reverse-phase C18 separation column with water/methanol mobile phases and ESI+ MS detection. Method detection limits (MDL's) and quantification limits (MQL's) for all compounds were near or below 1 ng L^{-1} except for 17β-TBOH photoproducts, which had MDL's <2 ng L^{-1} and MQL's <4 ng L^{-1}. Matrix suppression was <30% using lake water, and SPE recovery rates were near/above 100%, except for 17β-TBOH photoproducts, with recoveries >75%. Intra-day relative standard deviations (RSD's) were <30% for TBO and its products, <20% for all others.

1.0 Introduction

In a resource constrained world with a growing population, increased use of concentrated animal feed operations (CAFO's), a form of intensive industrial agriculture, has resulted in concentrated discharges of pharmaceuticals to natural systems near animal agriculture facilities. Many of these pharmaceuticals are synthetic, and may pose ecological risks through mechanisms of carcinogenicity, teratogenicity, or endocrine disruption [1-7]. Of interest here are steroidal pharmaceuticals used as growth promoters and estrous synchronizers, specifically the metabolites of the synthetic androgen trenbolone acetate (TBA) and the synthetic progestin altrenogest (ALT) (Figure 1). Trenbolone acetate is employed as a growth promoter in beef production, and its use represents significant incremental economic value to the beef industry [8], while altrenogest is used widely as an estrus synchronizer in breeding mares and swine [9-11].

The trenbolone metabolites 17α-trenbolone (17α-TBOH) and 17β-trenbolone (17β-TBOH) have been identified as endocrine disrupting compounds [1-3, 6], and are linked to masculinization and fecundity reduction in fathead minnows and zebrafish [2, 6]. 17α-TBOH concentrations of 11 ng L^{-1} significantly reduce fecundity in fathead minnows [3], and concentrations of 9 ng L^{-1} resulted in male skewed sex ratios in zebrafish [12]. Altrenogest and its photoproducts exhibit potent androgenic effects in *in vitro* cell assays [13]. Endogenous & synthetic steroid hormones have been detected downstream of animal agriculture [8, 14, 15], suggesting that synthetic hormones, such as trenbolone and altrenogest, should be expected to occur in some receiving waters impacted by animal agriculture.

In the photic zone, metabolites of trenbolone react rapidly (k_{obs}=0.0254 - 0.213 min^{-1}), forming photohydration products [16, 17] (Figure 2). These photoproducts also exhibit thermal dehydration in the dark, reverting to parent structures [18]. Up to 80-90% of 17α-TBOH concentrations can revert to the parent structure from products; reversion is accelerated by higher temperatures and can be nearly instantaneous under strongly acidic or basic conditions (Figure 3) [18]. These mechanisms of reversible transformation can result in higher persistence and increased transport potential of trenbolone metabolites than would be expected based upon initial rates of phototransformation alone [19]. Additionally, evidence suggests that the primary photoproducts of trenbolone metabolites may have bioactivity distinct from that of the parent molecules [20].

Under irradiation, altrenogest reacts rapidly (~30 s) to form a cyclo-addition photoproduct (ALT-CAP) (k_{obs}=2.54 s^{-1}), which will then quickly react (3-5 hours) to form a hydrated cyclo-addition photoproduct (ALT-CAP-OH) (Figure 4) [13]. By analogy to trenbolone metabolites, it is very likely that photohydration occurs at carbon-5 in ALT-CAP, although no insights into stereochemistry are possible. The hydrated photoproduct can revert to the cyclo-addition product in the dark, but will not revert back to the altrenogest parent structure [13]. These complex dynamics highlight the need for the quantitative and comprehensive analysis of these compounds in aquatic systems, accounting for the dynamic behavior of trenbolone metabolites, altrenogest, and their primary and secondary transformation products.

To properly characterize and explore the transport pathways of trenbolone metabolites, altrenogest, and their photoproducts, a method for their detection and quantification is needed. Such methods already exist for trenbolone metabolites [8, 21-23], but not photoproducts; limited options exist for the detection of altrenogest [13], and no method is available for the detection of

altrenogest photoproducts. Thus the purpose of this study was to develop an analytical method for the detection and quantification of trenbolone metabolites, altrenogest, and related photoproducts via liquid chromatography tandem mass spectrometry (LC-MS/MS). Additionally, we needed to build a method with sufficient sensitivity and selectivity to detect photoproducts at realistic environmental concentrations, in this case, 1-10 ng L^{-1} in a complex environmental matrix, typical for systems affected by agricultural runoff [21-23].

2.0 Experimental

2.1 Reagents and Materials

17β-TBOH was purchased from Shenzhen Shijingu Technology Co. Ltd (Shanghai, China). 17α-TBOH and 17α-16,16,17-d3-TBOH (deuterated internal standard for 17α-TBOH) (17α-d3-TBOH) were purchased from BDG Synthesis (Wellington, New Zealand). Trendione (TBO) was purchased from Steraloids (Newport, Rhode Island). Altrenogest was purchased from Fluka (Sigma Aldrich, St. Louis, MO). LC-MS grade methanol and LC-MS grade water were purchased from Fisher Scientific (Pittsburgh, Pennsylvania).

To provide a realistic sample matrix, river water and lake water were used in these studies; river water was collected from the South Fork of the Snoqualmie River (Ollalie State Park, WA, 47.4372° N, 121.6533° W) and used as the primary matrix for method development. This high purity water, near circumneutral (pH=7.2), was used in lieu of LC-MS grade water, which was more acidic (pH=5.5) and would require pH correction (photoproduct reversion is acid catalyzed). Sensitivity decreased due to cationic adduct formation during spectrometry (see section 3.2), so we avoided buffer solutions or pH adjustment to the extent possible. Therefore, this river water source was used for most of our analytical efforts. River water was filtered

through a 0.45 μm polyethersulfone (PES) membrane (Fisher Scientific, Pittsburgh, Pennsylvania) to remove particulates. For an organic rich complex matrix representative of agricultural runoff, lake water was collected from Wapato Lake (Tacoma, WA, 47.1952° N, 122.4567° W), a highly eutrophic system, to validate the method.

2.2 Sample Preparation

For each metabolite, individual standards (100 ng L^{-1} to 100 μg L^{-1}) were serial dilutions from a stock solution of 10 mg L^{-1} (metabolite) in methanol, kept at 4° C. Samples for irradiation were either 2 mL HPLC vials (ambered borosilicate glass) with PTFE septa and plastic screw caps, used for injection of samples before/after reaction, or 40 mL samples (clear borosilicate glass), used when solid phase extraction (SPE) was employed. Unless otherwise noted, all data described here was acquired using 40 mL samples concentrated via SPE. All glassware except for transfer pipettes was silanized with 10% (v/v) dichlorodimethylsiloxane in toluene prior to each use. Coelution of metabolites and primary photoproducts in irradiated samples of mixed 17α/17β-TBOH prevented simultaneous quantification of all primary photoproducts, so parent compounds were irradiated individually to facilitate method development.

2.3 Photoreaction

During photoreaction, samples rested horizontally on a rack in a recirculating 7° C water bath to minimize photoproduct dehydration, such that the water bath elevation did not rise above 1/3 to 1/2 the diameter of each vial. The water bath was placed underneath a solar simulator, (EYE Lighting Int. Model # 93510, EYE Lighting, Mentor, OH) that used four high pressure, 150 W xenon bulbs (Solarlux 150R, EYE Lighting, Mentor, OH). Per manufacturer specification, the solar simulator was rated to class ABB, simulating sunlight at sea level with

89 low air pollution (rural setting). Trenbolone metabolite samples were irradiated for 6 hours,
90 representing >10 half lives of phototransformation, to maximize photoproduct production [16],
91 and altrenogest samples were irradiated for 1 hour, to maximize the production of the
92 photoproduct ALT-CAP-OH. After irradiation and prior to extraction, internal standard (17α-d3-
93 TBOH, 2.5 ng L^{-1}) was added to all samples as 40 µL of a 5 µg L^{-1} methanolic standard of 17α-
94 d3-TBOH to facilitate quantification via isotope dilution techniques.

95 As an alternative to direct photoproduct detection, we also evaluated indirect
96 photoproduct quantification by exploiting the temperature and pH sensitivities of the thermal
97 dehydration reaction (see section 3.6). After irradiation, samples were either placed in a heat bath
98 at 45^{o} C for 15 hours (overnight, to mimic maximum reversion possible naturally), or were
99 acidified with 200 µL of 1.2 M HCl prior to extraction (final pH ~2.0). Estimates of
100 photoproduct concentration were derived indirectly by comparing observed difference in
101 reversed-photoproduct peak area from unmanipulated trenbolone photoproducts.

102 *2.4 Solid Phase Extraction*

103 Resprep 6 mL C18 SPE cartridges (Restek, Bellefonte, Pennsylvania) (one cartridge per
104 sample) were pre-conditioned with three volumes of methanol added to each cartridge, then
105 allowed to drain and dry. Prior to sample extraction, one volume of water was added, then
106 samples were immediately extracted onto the SPE cartridges under vacuum (4 mL min^{-1}).
107 Samples were eluted with pure methanol using three 1.5 mL aliquots, then blown down to ~1 mL
108 with nitrogen gas from a dry down manifold (Biotage TurboVap LV, Biotage, Charlotte, North
109 Carolina). Samples were then transferred to a 2 mL ambered HPLC vial, and river water was
110 added (50:50, v:v) prior to injection to improve chromatography in LC-MS/MS analysis.

2.5 Instrumental analysis

An Agilent (Santa Clara, California) 1290 Infinity binary pump liquid chromatograph (LC) coupled with an Agilent 6430 triple quadrupole tandem mass spectrometer (MS/MS) was used for analysis. To minimize reversion and thus maximize photoproduct response, processing time and sample temperatures were minimized to the extent possible. Samples were injected via chilled autosampler tray (4^{o} C) and separated using an Agilent Poroshell 120Å EC C18 column (3.0mm i.d. X 50mm, 2.7µm) preceded by a SecurityGuard Gemini C18 guard column (2.0mm i.d. X 4mm) (Phenomenex, Torrance, California), both at 11^{o} C.

Mobile phase (0.2 mL min^{-1}) solutions were A: LC-MS grade water (pH=5.5) and B: LC-MS grade methanol, with the following separation gradient used: 55% B initially, increased to 75% over 5 min, increased to 100% B over 3 min, isocratic at 100% B for 3 min, decreased to 55% over 1 min, isocratic at 55% B for 5 min, for a total runtime of 17 min. Agilent Jetstream Electrospray Ionization Positive (AJS ESI+) mode was used with 2.5 kV capillary voltage, 1.0 kV nozzle voltage, 350^{o} C desolvation gas temp, 400^{o} C sheath gas temp, 12.0 L min^{-1} desolvation and sheath gas flows, 40 psi nebulizer pressure, and 400 V positive multiplier voltage (delta EMV). For all transitions, dwell time was 200 ms and collision cell acceleration voltage was 4 V.

3.0 Results and Discussion

3.1 Optimization of LC-MS/MS Conditions

LC conditions were optimized via serial injection of 100 µg L^{-1} samples of individual analytes. The LC gradient was adjusted to achieve peak separation of parent metabolites and photoproducts. The method was optimized for 17α-TBOH because it is the predominant

metabolite excreted [24]. Photoproduct transitions were determined by photo reacting samples and then scanning for product ions. MS/MS fragmentation conditions were optimized with Agilent Masshunter software (version B.06.00) by varying (in order): fragmenter voltage, collision energy, collision cell accelerator voltage, nebulizer pressure, capillary voltage, and sheath and desolvation gas flows and temperatures; the value corresponding to the best response (highest signal to noise ratio (S/N) and peak area) was selected. The software included an MS/MS auto optimizer, which was used to identify additional confirmatory MS/MS transitions. Auto-optimizer MRM transition and collision energy recommendations were poorly correlated to manually estimated optimization transitions and energies, so these manual optimization transitions and energies were selected to maximize sensitivity. Table 1 shows the optimized retention times, MRM transitions, fragmenter voltages, and collision energies for each of the target steroids, their photoproducts, and the deuterated standard used.

17α/17β-TBOH had the same precursor mass-to-charge ratio (m/z) of 271.2 Da for their $[M+H]^+$ ions, while their primary photoproducts (5-OH-17α-TBOH and 12-OH-17β-TBOH) had an expected precursor m/z of 289.2 Da for their $[M+H]^+$ ions. However, 289.2 Da was never observed via LC-MS/MS, only dehydrated photoproduct ($[M+H-H_2O]^+$) ions with m/z 271.2 Da were observed in LC-MS/MS analysis (Table 1, Figure 8, Figure 9), consistent with in-source dehydration as previously documented [17]. TBO had a precursor m/z of 269.2 Da, while TBO photoproducts observed (at m/z 269.2 Da) were also dehydrated. ALT and its primary photoproduct (ALT-CAP) both had precursor m/z of 311.2 Da, and the secondary photoproduct (ALT-CAP-OH) had a precursor m/z of 329.2 Da, but only the dehydrated ions ($[M+H-H_2O]^+$) with m/z 311.2 Da were observed for the secondary photoproduct.

156 *3.2 Cationic Adduct Formation*

157 When concentrations were high enough to allow for full scan detection, $[M+H]^+$ parent
158 ions could be resolved from identical mass $[M+H-H_2O]^+$ photoproduct ions by observation of
159 sodium adducts. Spectral analysis revealed that sodium present at the MS ionization source
160 would form an $[M+Na]^+$ adduct with all parent compounds (17α-TBOH, 17β-TBOH, TBO, and
161 ALT), resulting in additions of 22 Da (293.2 Da for 17α/17β-TBOH, 291.2 for TBO, 333.2 for
162 ALT) (Figure 8). In photoproducts, sodium replaced a proton on the hydrated molecule $[M+H]^+$
163 (289.2 Da for 17α/17β-TBOH, 287.2 for TBO, 329.2 for ALT-CAP-OH), resulting in $[M+Na]^+$.
164 However, no in-source dehydration of the photoproduct adducts occurred, resulting in 40 Da
165 additions (compared with protonated photoproduct ions) to detected photoproduct ions (311.2 Da
166 for 17α/17β-TBOH, 309.2 for TBO, 351.2 for ALT-CAP-OH) (Figure 9). This pattern was
167 consistently observed for both trenbolone metabolites and altrenogest.

168 Sodium adduct formation also limited method sensitivity, and maximizing method
169 performance required identification and mitigation of adduct sources. Sodium adduct formation
170 was found to be independent of solvent (lake water or river water) and independent of solid-
171 phase extraction. Sodium adduct formation occurred in all samples; the largest single source of
172 sodium contamination was from glassware, but could be minimized via silanization, and all LC
173 lines were purged prior to testing. Sodium adduct formation varied from day to day, and was
174 further aggravated by other instrument user sample matrices.

175 *3.3 Chromatography and Spectrometry*

176 During analysis of samples that contained similar concentrations of 17α-TBOH and 17β-
177 TBOH photoproducts, the similar polarity of TBA metabolites and photoproducts resulted in

peak coelution, rendering true simultaneous multi-residue standard analysis imprecise with these chromatographic conditions. Despite this limitation, which can be addressed by using slower LC gradients at the expense of analytical runtime, the simultaneous occurrence of multiple TBOH metabolite photoproduct groups is unlikely, as 17α-TBOH typically dominates environmental occurrence [24]. Figure 5 shows chromatograms and spectra for 17α-TBOH, 17β-TBOH and TBO metabolites, ALT, and the isotopic standard 17α-d3-TBOH. Figures 6 and 7 show the same for TBOH metabolite and ALT photoproducts [13, 25].

Given photoproduct instability, several analytical challenges were evident relative to these time scales (days). For example, two peaks were observed in SPE extracts (compounds (4) and (5) in Table 1) of photo reacted samples of 17α-TBOH that were not observed during direct injection of similar samples (see Figure 6b). Spectral analysis revealed that the peak at 6.34 min (compound (4)) has similar spectra to the peaks at 6.02 min (5-OH-17α-TBOH) and 6.53 min (12-OH-17α-TBOH) and the peak at 7.25 min (compound (5)) has similar spectra to the parent 17α-TBOH peak at 6.96 min. These peaks (6.34 min and 7.25 min) are possible analogs of the primary photoproduct (5-OH-17α-TBOH) and 17α-TBOH respectively, due to the near identical elution time differences from the "new" peaks to their respective parent compounds (0.32 minutes +/- 0.03 minutes each). This behavior is not exhibited in dark controls, suggesting that the photoproduct forms an analog in the SPE process, which then reverts to a metabolite analog. Time lapse data (see Figure 6c) shows that the 5-OH-17α-TBOH photoproduct, and its potential analog (compounds (2) and (4)), disappear after 1 day (likely due to reversion to parent structures) but 12-OH-17α-TBOH, 17α-TBOH, and its potential analog (compounds (1), (3), and (5)) all remain. Such observations clearly point to the dynamic nature of photoproduct analysis and highlight the challenges inherent to analysis of complex mixtures.

3.4 Limits of Detection and Quantification

The method detection limit (MDL) and quantification limit (MQL) were calculated by running matrix blanks (no analyte) 9 times and calculating the average and standard deviation of the background noise, where MDL is defined as the average plus 3 times the standard deviation, and MQL is the average plus 10 times the standard deviation [26-28]. For trenbolone metabolites, altrenogest, and their photoproducts, the MDL's and MQL's were sub ng L^{-1} regardless of sample matrix, with the exception of 12-OH-17β-TBOH in lake water and 5-OH-17β-TBOH in river water, both with MDL's <2 ng L^{-1} and MQL's <4 ng L^{-1} (see Table 2). The photoproducts were more difficult to quantify relative to the parent compounds, due to a.) their reactive nature, and b.) the formation of multiple products, thus reducing available analytical mass (relative to parents).

The following assumptions were made to quantify photoproducts: 1.) all metabolite mass is conserved to the photoproducts observed (i.e.; that the sum of photoproduct peak areas at a given concentration equates to the metabolite peak area measured at that concentration); 2.) quantitative transition response of photoproducts is equal to metabolites (i.e. 10 ng L^{-1} of 5-OH-17α-TBOH measured using m/z 271.2->211.2 is equal to 10 ng L^{-1} of 17α-TBOH measured using m/z 271.2->253.2); and 3.) all photoproducts have the same unit response to detection (i.e. 10 ng L^{-1} of 5-OH-17α-TBOH has the same peak area as 10 ng L^{-1} of 12-OH-17α-TBOH).

Comparing direct injections of parents and photoproducts showed that photoproduct raw peak area response is considerably less than raw parent response (with no change in internal standard response): for 17α-TBOH, photoproduct response was 57+/-7% of parent response; for 17β-TBOH, photoproduct response was 53+/-6%; for TBO, photoproduct response was 78+/-

223 8%; and for ALT, photoproduct response was 11%+/-3% of parent response. $[M+Na]^+$ adduct
224 formation is likely to explain some of this difference but regardless, photoproduct peak areas at
225 equal spiked concentrations were reduced from observed parent peak areas, reducing ultimate
226 method sensitivity of photoproducts relative to parents, observable in Table 2.

227 *3.5 Repeatability*

228 Intra-day relative standard deviations (RSD) were calculated (Table 2) for all compounds
229 in both river water and lake water (n=3 for all), at environmentally relevant concentrations (1
230 ng/L and 10 ng/L, respectively). 17α-TBOH and 17β-TBOH metabolites and photoproducts were
231 all below 20% intra-day RSD in river water (40% in lake water), but TBO photoproducts
232 exhibited slightly higher RSD's (RSD <30%) in river water due to lower sensitivity. Intra-day
233 RSD's for ALT and its photoproducts, in both river water and lake water, were all below 20%.
234 Inter-day RSD was based on internal standard response in control samples throughout all
235 experiments, and varied by 69.8% (n=22), largely because detector response was sensitive to use
236 by other researchers and by scheduled maintenance. For this reason, thorough cleaning and
237 purging procedures were employed prior to each instrument use to minimize influence from
238 other users.

239 *3.6 Reversibility*

240 Reversion of trenbolone metabolite photoproducts to parents during sample processing
241 was undesirable, but could be exploited for analysis of ALT-CAP due to its reversible
242 photohydration to ALT-CAP-OH, and because ALT-CAP formation from ALT is irreversible.
243 For trenbolone metabolites and altrenogest, direct injection post-irradiation revealed that
244 complete reaction to photoproducts (and ALT-CAP-OH) consistently occurred, with no

observable parent left. However, in samples SPE concentrated prior to analysis, <11% reversion (<30% reversion for ALT-CAP-OH) to parent over 3-5 hours occurred due to sample processing time (see Table 2), resulting in some loss of analytical sensitivity. Given the dynamic system, we consider the losses during sample processing and analysis acceptable.

Indirect quantification of photoproducts was assessed by comparing the difference in peak areas between photoreacted samples and photoreacted samples which were injected then intentionally reverted using heat or acid (quantification by difference) (see Figure 10). Direct measurement of TBA metabolite photoproducts (5-OH- and 12-OH- columns in Figure 10) resulted in lower total peak area responses than reverted parent measurements (heat and acid columns), but forcing reversion prevents the measurement of individual photoproducts in the case of 17α-TBOH and 17β-TBOH. In the case of TBO, reversion was inefficient (heat/acid compared with dark control), resulting in the best measurements coming from direct injection samples. Additionally, comparison of the heat and acid results shows that acid reversion in 17α-TBOH is the only instance where reversion is ~100%. This complicates indirect analysis for all the other metabolites, as incomplete reversion is an additional variable affecting accurate quantification. Thus accurate quantification of 17α-TBOH photoproducts via indirect analysis is viable, though limited due to the inability to distinguish individual photoproducts. Indirect photoproduct analysis is not recommended for either 17β-TBOH or TBO due to limited reversibility.

For ALT-CAP-OH, indirect analysis (by quantification of ALT-CAP) was viable when acidification was used, but heat resulted in incomplete reversion (as with trenbolone metabolites) (see Figure 10). Indirect analysis after acidification resulted in nearly identical response compared to direct analysis of ALT-CAP-OH, and in the acidified sample, no residual ALT-

CAP-OH was detected (suggesting ~100% reversion of ALT-CAP-OH to ALT-CAP). What's more, this technique was the only reliable method to directly detect ALT-CAP, as quantification after a short photoreaction period resulted in either residual ALT (unreacted parent) or significant subsequent photohydration to ALT-CAP-OH. It is highly recommended that acidification be used to directly measure ALT-CAP for calibration in the future.

3.7 Recoveries and Matrix Effects

Matrix effects were calculated by comparing internal standard peak areas from (a) direct injection samples with (b) samples of SPE concentrated matrix (no analyte), in which analyte was added immediately before injection [29]. Recovery rates were calculated using 10 ng L^{-1} spikes of analyte, added before extraction. Table 2 shows matrix effects and recoveries for this method for each compound in river water or lake water. River water was found to have an insignificant matrix effect (i.e. peak areas did not change as a result of concentrating river water during SPE), but lake water concentration resulted in a 10-30% decrease in response (matrix suppression). Recovery rates (n=3) were above 103-125% for 17α-TBOH and its photoproducts, 105-119% for 17β-TBOH and 75-93% for its photoproducts, 95-116% for TBO and its photoproducts, and 88-116% for ALT and its photoproducts.

3.8 Archived sample results

Archived samples were provided from the United States Geological Survey (USGS), dated 06/2009 to 04/2011, collected from a variety of sources: feces, lagoon solids, lagoon liquid, barn flush solids, barn flush liquid, and urine. Samples were previously concentrated via solid-phase extraction, and when received were dehydrated. Samples were reconstituted in 200 µL of methanol and 100 µL of nanopure water, then tested for trenbolone metabolites,

altrenogest, and related photoproducts, however, no target analytes were detected. Due to the dehydrated nature of the samples when received, it is unknown whether any target analytes were present when sampling occurred and subsequently transformed during storage. Attempts to analyze more recent samples which might contain target analytes are recommended for future analysis.

4.0 Conclusions

A multi-residue LC-MS/MS method coupled with photoreaction and SPE concentration was developed and optimized for the detection of trenbolone metabolites, altrenogest, and their related photoproducts. Initial efforts utilized filtered, circumneutral-pH river water, followed by method validation using the more complex matrix of Wapato Lake water to better mimic receiving waters. This method allowed for consistent detection of trenbolone metabolites, altrenogest, and related photoproducts, relying on a short sample preparation time (3 hours) to minimize product-to-parent reversion, and minimized variability (intra-day RSD <20% for 17α-TBOH photoproducts). Using 1-10 ng L^{-1} spike concentrations, most recoveries were 90-120%, and limited matrix suppression (<30%) was observed. Critically, method detection and quantification limits were at or below environmentally relevant concentrations (1-10 ng L^{-1} concentrations) for all metabolites and all photoproducts, and calibration curves were linear across ~3 orders of magnitude. Further sensitivity and selectivity could be achieved with the use of a dedicated instrument to minimize inter-day variability and sodium adduct formation, or via the use of a longer LC gradient to improve separation of metabolite photoproducts, (at the expense of longer runtime and increased reversion potential). This method could be applied to water samples affected by agricultural runoff in lieu of existing detection methods for trenbolone

312 metabolites and altrenogest, while also allowing for detection of their photoproducts, potentially
313 critical aspects of environmental risk assessment of these agricultural pharmaceuticals.

314

315

316

Works Cited

1. Howell, W. M.; Black, D. A.; Bortone, S. A., Abnormal Expression of Secondary Sex Characters in a Population of Mosquitofish, Gambusia affinis holbrooki: Evidence for Environmentally Induced Masculinization. *Copeia.* (1980), 676-681.

2. Sone, K.; M. H.; Itamoto, M.; Katsu, Y.; Watanabe, H.; Urushitani, H.; Tooi, O.; Guillette Jr., L. J.; Taisen, I. Effects of an androgenic growth promoter 17β trenbolone on masculinization of Mosquitofish (Gambusia affinis affinis). *Gen. Comp. Endocrinol.* (2005) ***143,*** 151–160.

3. Jensen, K. M.; E. A. M.; Kahl, M. D.; Ankley, G. T. Effects of the Feedlot Contaminant 17α Trenbolone on Reproductive Endocrinology of the Fathead Minnow. *Environ. Sci. Technol.* (2006) ***40,*** 3112-3117.

4. Duguet, J. P.; Bruchet, A.; Mallevialle, J. Pharmaceuticals and endocrine disruptors in the water cycle. *IWA Yearb*. (2004) 41-46.

5. Orlando, E. F.; Kolok, A. S.; Binzcik, G. A.; Gates, J. L.; Horton, M. K.; Lambright, C. S.; Gray, L. E.; Soto, A. M.; Guillette, L. J. Endocrine disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environ. Health Perspect.* (2004) *112,* 353-358.

6. Seki, M., S. Fujishima, T. Nozaka, M. Maeda & K. Kobayashi. Comparison of response to 17 beta estradiol and 17 beta trenbolone among three small fish species. *Environ. Toxicol. Chem.* (2006) *25,* 2742-2752.

7. Kumar, V.; Johnson, A.C.; Trubiroha, A.; Tumova, J.; Ihara, M.; Grabic, R.; Kloas, W.; Tanaka, H.; Kroupova, H.K. The challenge presented by progestins in ecotoxicological research: A critical review. *Envrion. Sci. Techol*. (2015), *49,* 2625-2638.

8. Khan, B.; Lee, L. S., Estrogens and synthetic androgens in manure slurry from trenbolone acetate/estradiol implanted cattle and in waste receiving lagoons used for irrigation. *Chemosphere* (2012), *89,* 1443-1449.

9. Squires, E.L.; Heesemann, C.P.; Webel, S.K.; Shideler, R.K.; Voss, J.L. Relationship of altrenogest to ovarian activity, hormone concentrations and fertility of mares. *J. Anim. Sci.* (1983), *56,* 901-910.

10. van Leeuwen, J.J.J.; Williams, S.I.; Martens, M.R.T.M.; Jourquin, J.; Driancourt, M.A.; Kemp, B.; Soede, N.M. The effect of post weaning altrenogest treatments of primiparous sows on follicular development, pregnancy rates, and litter sizes. *J. Anim. Sci*. (2011), *89,* 397-403.

11. Willmann, C.; Schuler, G.; Hoffmann, B.; Parvizi, N.; Aurich, C. Effects of age and altrenogest treatment on conceptus development and secretion of LH, prosgesterone, and eCG in early-pregnant mares. *Theriogenology. (*2011), *75,* 421-428.

12. Morthorst, J.E.; Holbech, H.; Bjerregaard, P. Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations. *Aquat. Toxicol*. (2010). *98(4)*, 336-343.

13. Wammer, K. H.; Anderson, K. C.; Erickson, P. R.; Kliegman, S.; Moffatt, M.; Berg, S. M.; Heitzman, J. A.; McNeill, K.; Martinovic-Weigelt, D.; Cwiertny, D. M.; Kolodziej, E. P. Environmental photochemistry of altrenogest: photoisomerization to a bioactive product with increased environmental persistence via reversible photohydration. In review.

14. Kolodziej, E. P. & D. L. Sedlak (2007) Rangeland grazing as a source of steroid hormones to surface waters. *Environ. Sci. Technol.* (2007)**,** *41***,** 3514-3520.

15. Gall, H. E.; Sassman, S. A.; Lee, L. S; Jafvert, C. T. Hormone discharges from a midewest tile-drained agroecosystem receiving animal wastes. *Environ. Sci. Technol*. (2011). *45(20)*, 8755-8764.

16. Qu, S.; Kolodziej, E. P.; Cwiertny, D. M., Phototransformation Rates and Mechanisms for Synthetic Hormone Growth Promoters Used in Animal Agriculture. *Environ. Sci. Technol.* (2012)**,** *46*, 13202-13211.

17. Kolodziej, E. P.; Qu, S.; Forsgren, K. L.; Long, S. A.; Gloer, J. B.; Jones, G. D.; Schlenk, D.; Baltrusaitis, J.; Cwiertny, D. M. Identification and Environmental Implications of Photo Transformation Products of Trenbolone Acetate Metabolites. *Environ. Sci. Technol.* (2013), *47*, 5031-5041.

18. Qu, S.; Kolodziej, E. P.; Long, S. A.; Gloer, J. B.; Patterson, E. V.; Baltruisaitis, J.; Jones, G. D.; Benchetler, P. V.; Cole, E. A.; Kimbrough, K. C.; Tarnoff, M. D.; Cwiertny, D. M. Product to Parent Reversion of Trenbolone: Unrecognized Risks for Endocrine Disruption. *Sci.* (2013), *342*, 347-351.

19. Ward, A. S.; Cwiertny, D. M.; Kolodziej, E. P.; Brehm, C. C. Coupled reversion and stream hyporheic exchange processes increase environmental persistence of trenbolone metabolites. *Nat. Commun*. (2015), 6:7067, 1-10.

20. Forsgren, K. L.; Qu, S.; Lavado, R.; Cwiertny, D.; Schlenk, D. Trenbolone acetate metabolites promote ovarian growth and development in adult Japanese medaka (*Oryzias Latipes*). *Gen. Comp. Endocrinol.* (2014), *202*, 1-7.

21. Khan, B.; Lee, L. S.; Sassman, S. A. Degradation of Synthetic Androgens 17α and 17β Trenbolone and Trendione in Agricultural Soils. *Environ. Sci. Technol.* (2008)**,** *42*, 3570-3574.

22. Webster, J. P.; Kover, S. C.; Bryson, R. J.; Harter, T.; Mansell, D. S.; Sedlak, D. L.; Kolodziej, E. P. Occurrence of Trenbolone Acetate Metabolites in Simulated Confined Animal Feeding Operation (CAFO) Runoff. *Environ. Sci. Technol.* (2012), *46*, 3803-3810.

23. Durhan, E. J.; Lambright, C. S.; Makynen, E. A.; Lazorchak, J.; Hartig, P. C.; Wilson, V. S.; Gray, L. E.; Ankley, G. T. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. *Environ. Health Perspect*, (2006), *114***,** 65-68.

24. Jones, G. D.; Benchetler, P. V.; Tate, K. W.; Kolodziej, E. P. Mass balance approaches to characterizing the leaching potential of trenbolone acetate metabolites in agro-ecosystems. *Environ. Sci. Technol*. (2014). *48(7)*, 3715-3723.

25. Baltrusaitis, J.; Patterson, E. V.; O'Connor, M.; Qu, S.; Kolodziej, E. P.; Cwiertny, D. Reversible photohydration of trenbolone acetate metabolites: mechanistic understanding of product to parent reversion through complementary experimental and theoretical approaches. *J. Am. Chem. Soc*. (2015), In review.

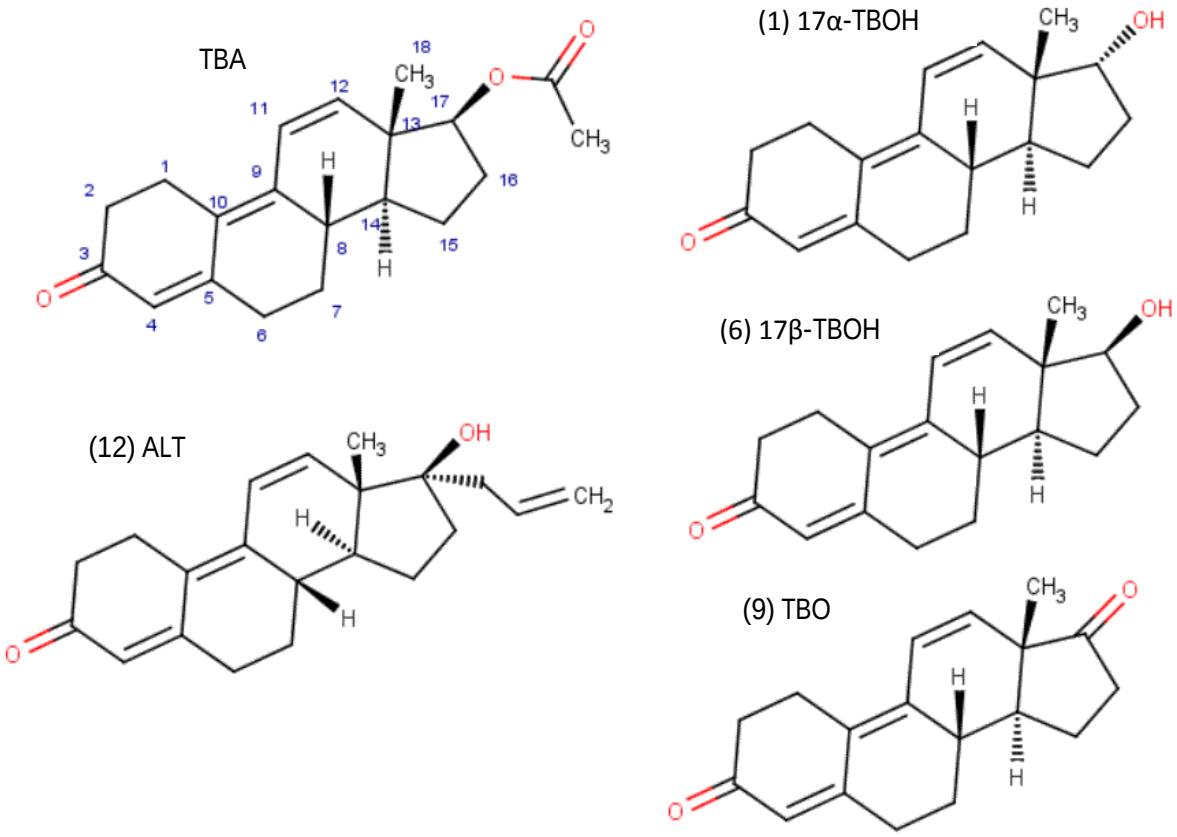
26. Farre, M.; Pico, Y.; Barcelo, D. Application of ultra-high pressure liquid chromatography linear ion-trap orbitrap to qualitative and quantitative assessment of pesticide residues. *J. Chrom. A.* (2014), *1328*, 66-79.

27. Mou, R.; Chen, M.; Cao, Z.; Zhu, Z. Simultaneous determination of triazine herbicides in rice by high-performance liquid chromatography coupled with high resolution and high mass accuracy hybrid linear ion trap-orbitrap mass spectrometry. *Analytica Chimica Acta.* (2011), *706(1)*, 149-156.

28. Fernandez-Arauzo, L.; Pimentel-Trapero, M.; Hernandez-Carrasquilla, M. Simultaneous determination of resorcylic acid lactones, β and α trenbolone and stilbenes in bovine urine by UHPLC/MS/MS. *J. Chrom. B.* (2014), *973*, 89-96.

29. Gosetti, F.; Mazzucco, E.; Zampieri, D.; Gennaro, M. C. Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *J. Chrom A*. (2010). *1217(25)*, 3929-3937.

410 **List of Figures**

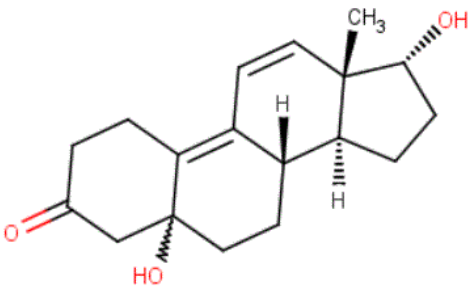


411

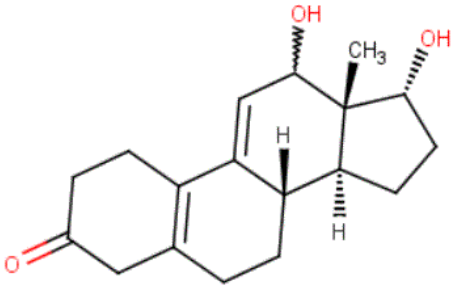
412 **Figure 1:** Structures of trenbolone acetate (with carbon numbering), 17α-trenbolone, 17β-

413 trenbolone, trendione, and altrenogest.

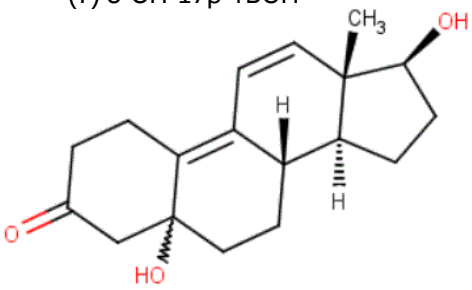
(2) 5-OH-17α-TBOH



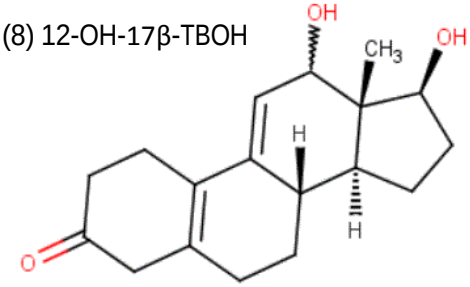
(3) 12-OH-17α-TBOH



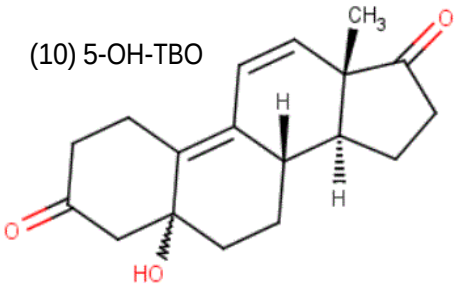
(7) 5-OH-17β-TBOH



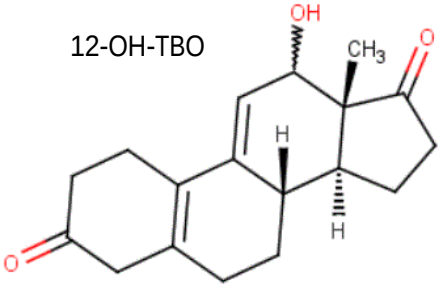
(8) 12-OH-17β-TBOH



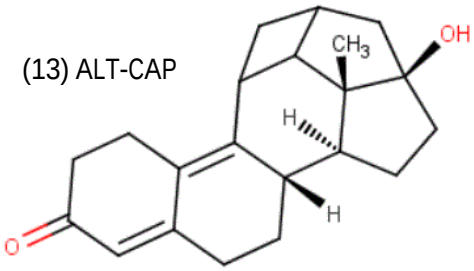
(10) 5-OH-TBO



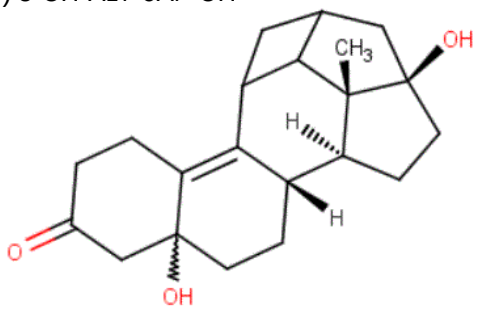
12-OH-TBO



(13) ALT-CAP



(14) 5-OH-ALT-CAP-OH

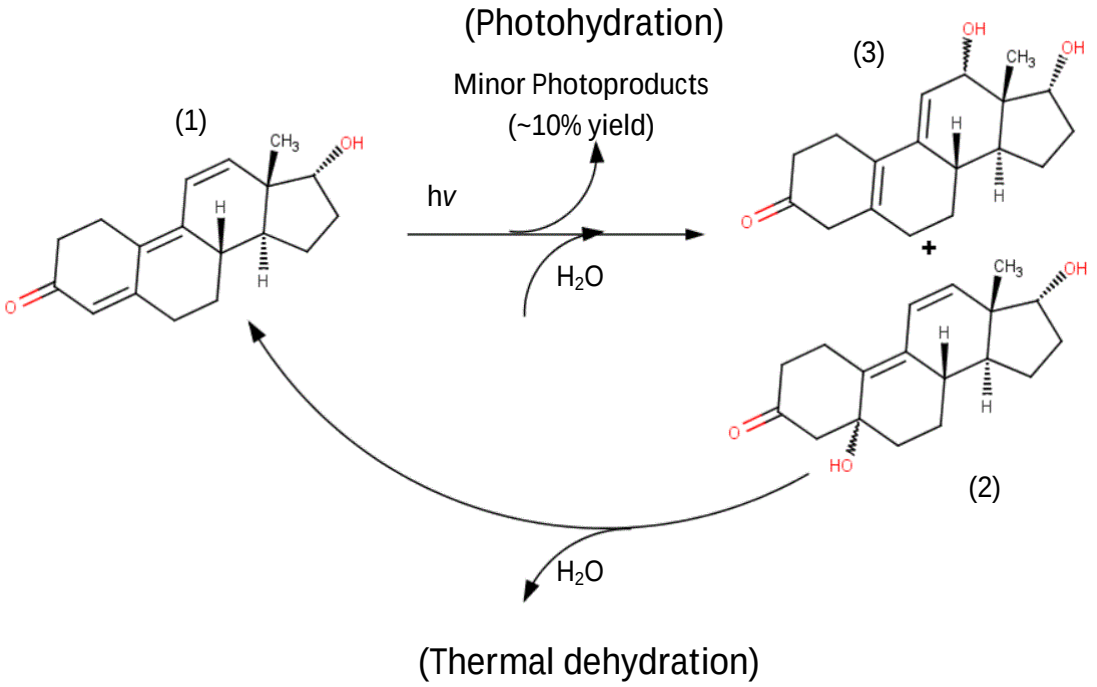


414

415 **Figure 2:** Chemical structures of the 5-hydroxy and 12-hydroxy photoproducts of 17α-

416 trenbolone, 17β-trenbolone, and trendione, and the cyclo-addition and hydroxy-cyclo-addition

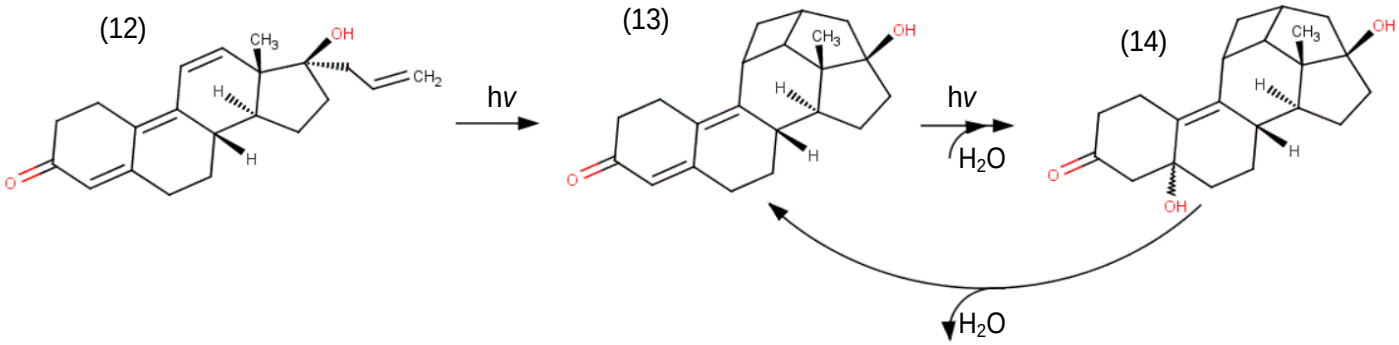
417 photoproducts of altrenogest.



418

419 **Figure 3:** Coupled photohydration-thermal dehydration reversion process of trenbolone acetate

420 metabolites. Reaction shown for 17α-TBOH, which also applies to 17β-TBOH, and TBO [17].

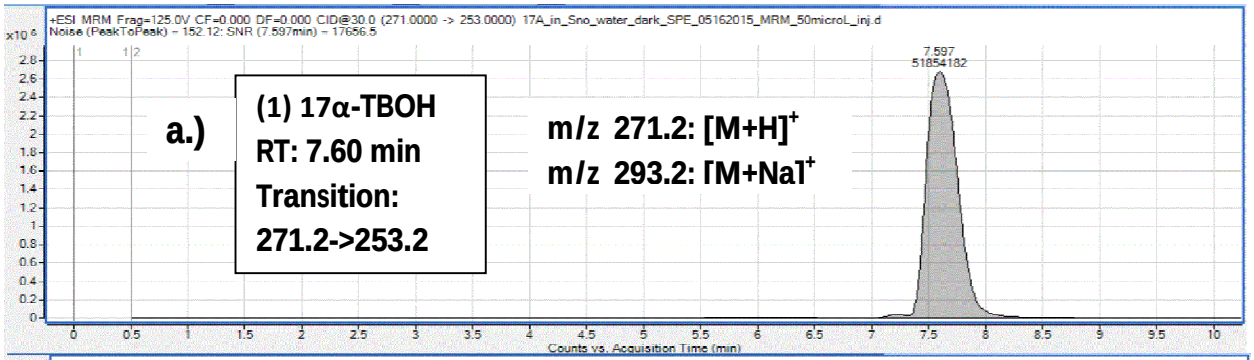


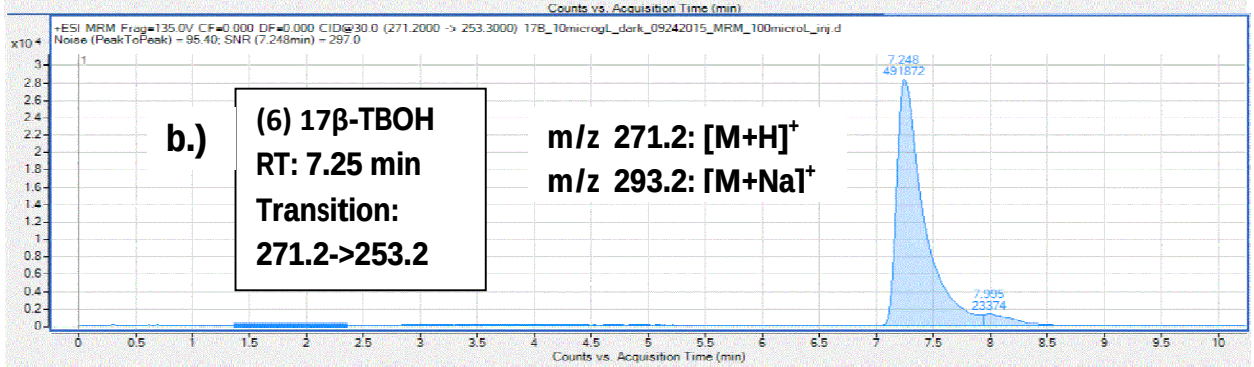
42

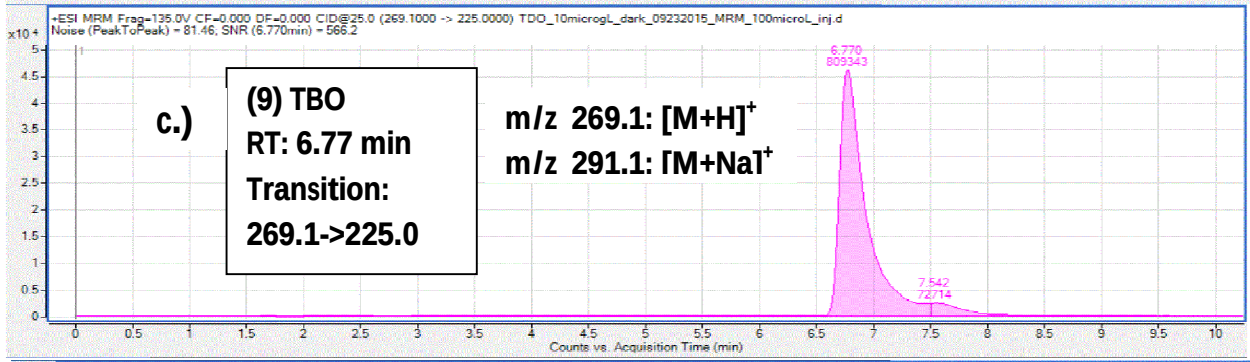
422 **Figure 4:** Photoisomerization of altrenogest to the cyclo-addition photoproduct, and subsequent

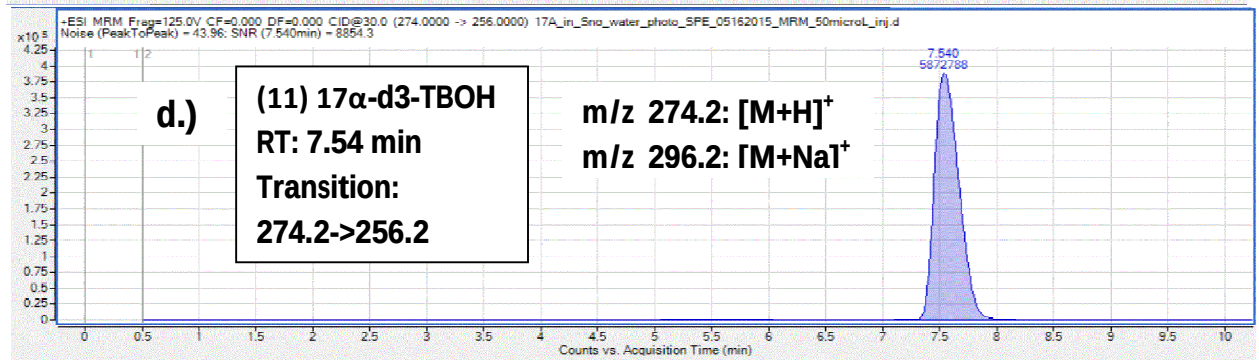
423 coupled photohydration-thermal dehydration reversion process of the cyclo-addition and

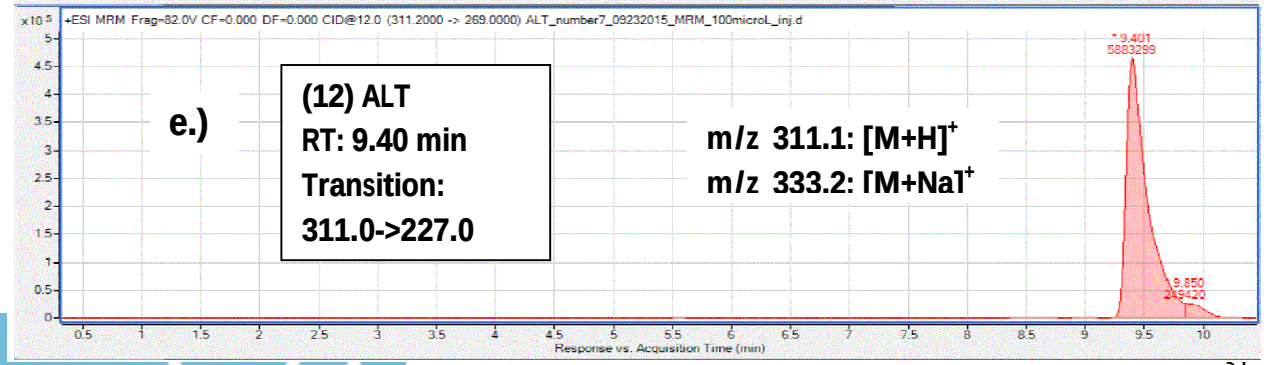
424 hydroxy-cyclo-addition photoproducts of altrenogest [13].







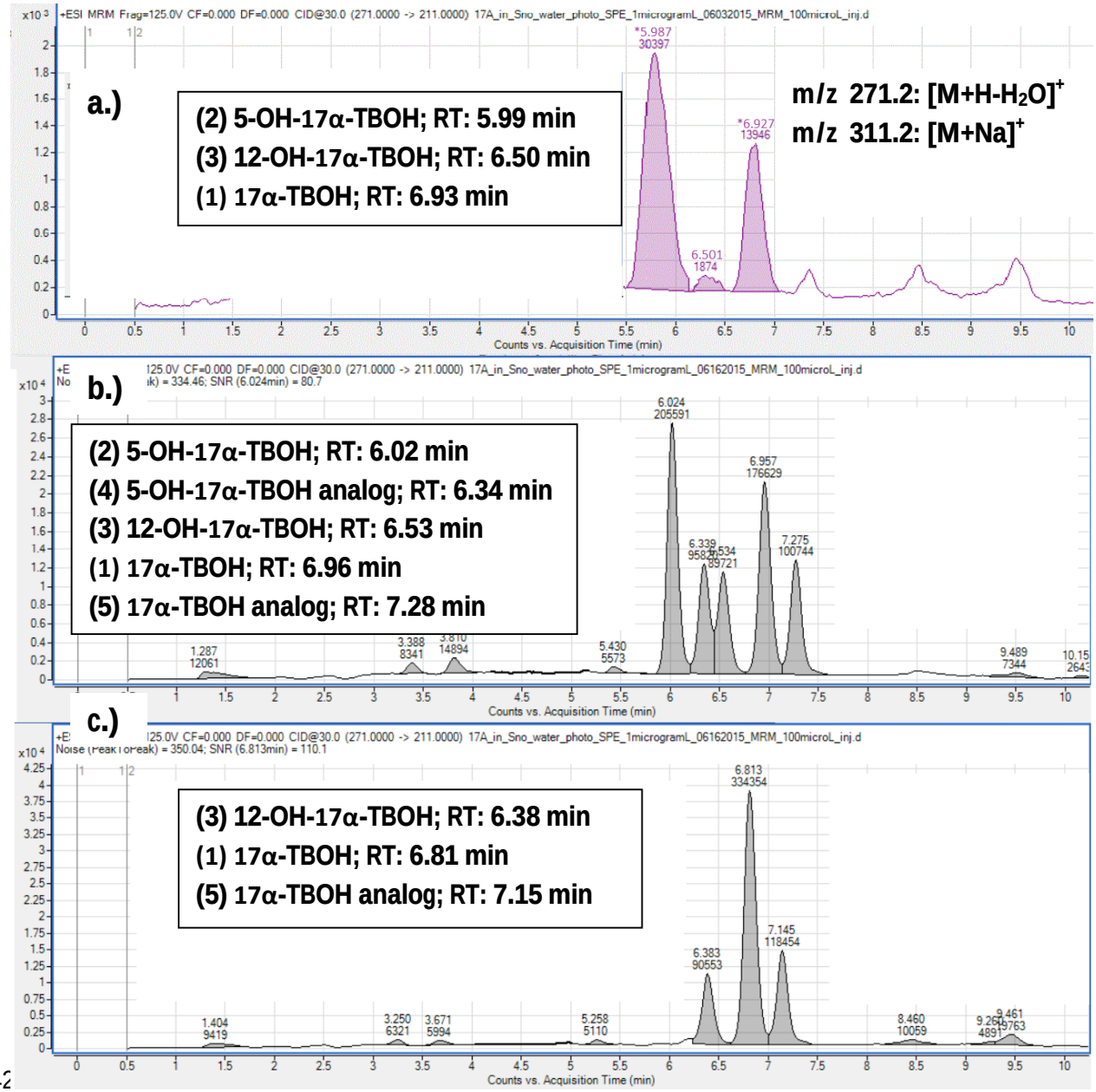




426 **Figure 5:** Chromatograms for a.), 17α-TBOH, b.) 17β-TBOH, c.) TBO, d.) 17α-d3-TBOH, and

427 e.) ALT, including retention time (RT), quantitative MRM transition, and most abundant mass-

428 to-charge ratios (m/z) for each. Concentrations for each are 10 ng/L.



42

430 **Figure 6:** Chromatograms and Spectra for a.) 17α-TBOH, photo-reacted, not solid phase

431 extracted, b.) 17α-TBOH, photo-reacted, solid phase extracted, c.) same sample from b.) tested

again at T=24hrs, Includes prevalent ions and their retention times (RT), most common mass to charge ratios (m/z), and transition of each chromatogram (note that 271.2->211.2 applies for Figures 6a-c). Figures 6b-c are of the same sample, tested one day apart, where 6b was run immediately, and 6c was run after overnight reversion at room temperature was allowed to take place.

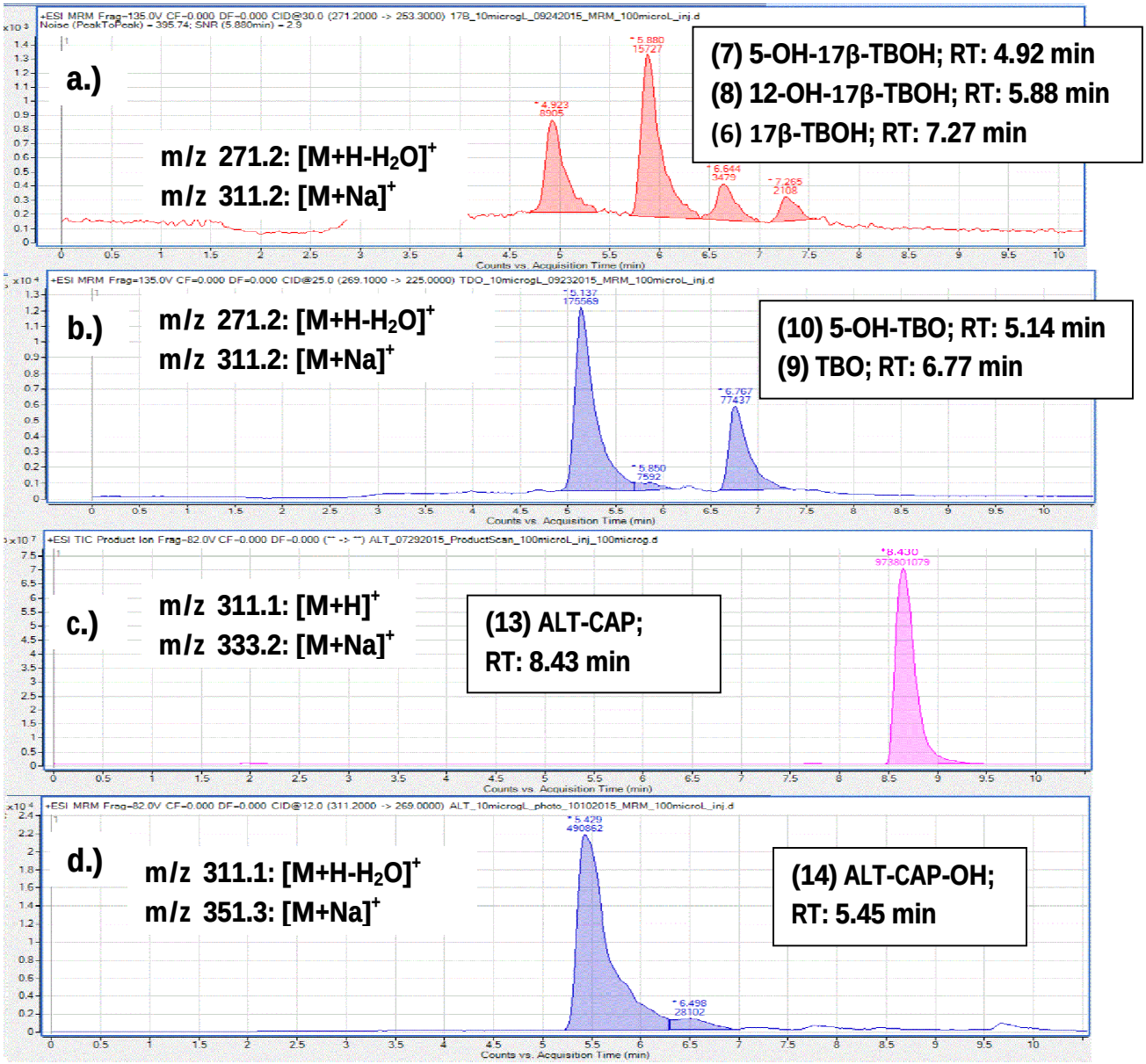


Figure 7: Chromatograms for a.) 17β-TBOH, photo-reacted, solid phase extracted, b.) TBO, photo-reacted, solid phase extracted, c.) ALT, photo-reacted, acidified, solid phase extracted, and d.) ALT, photo-reacted, solid phase extracted. Includes prevalent ions and their retention times (RT), most common mass to charge ratios (m/z), and transition of each chromatogram. Figure 7a shows m/z 271.2->211.2, Figure 7b shows m/z 269.1->169.0, Figure 7c shows m/z 311.2->227.0, and Figure 7d shows m/z 311.2->269.0.

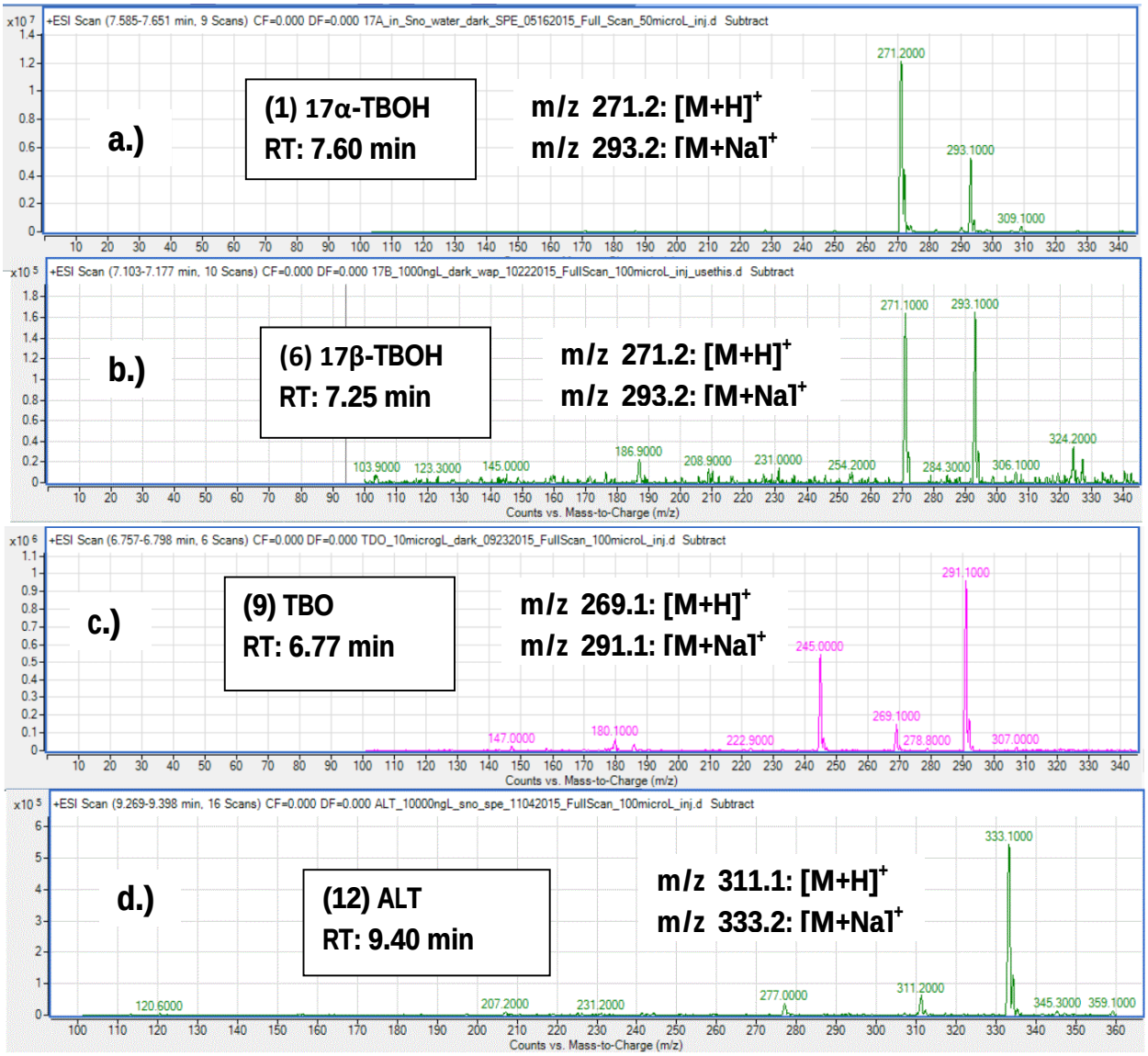


Figure 8: Spectra for a.), 17α-TBOH, b.) 17β-TBOH, c.) TBO, and d.) ALT, including retention time (RT) and most abundant mass-to-charge ratios (m/z) for each. Concentrations for each are 10 ng/L. Figure 9a shows spectra representative of compounds (2), (3), and (4), and Figure 9b shows spectra representative of compounds (7) and (8).

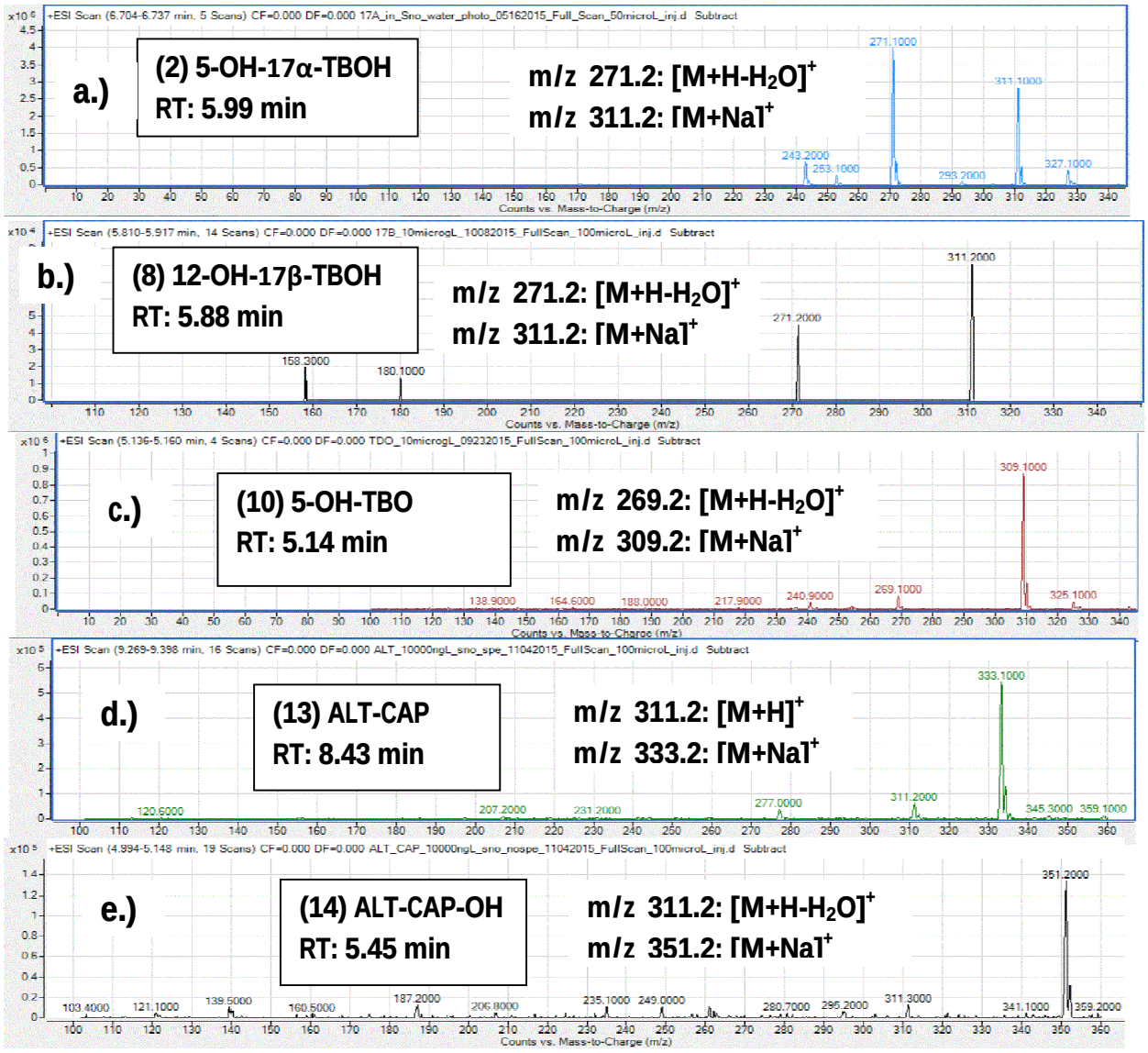
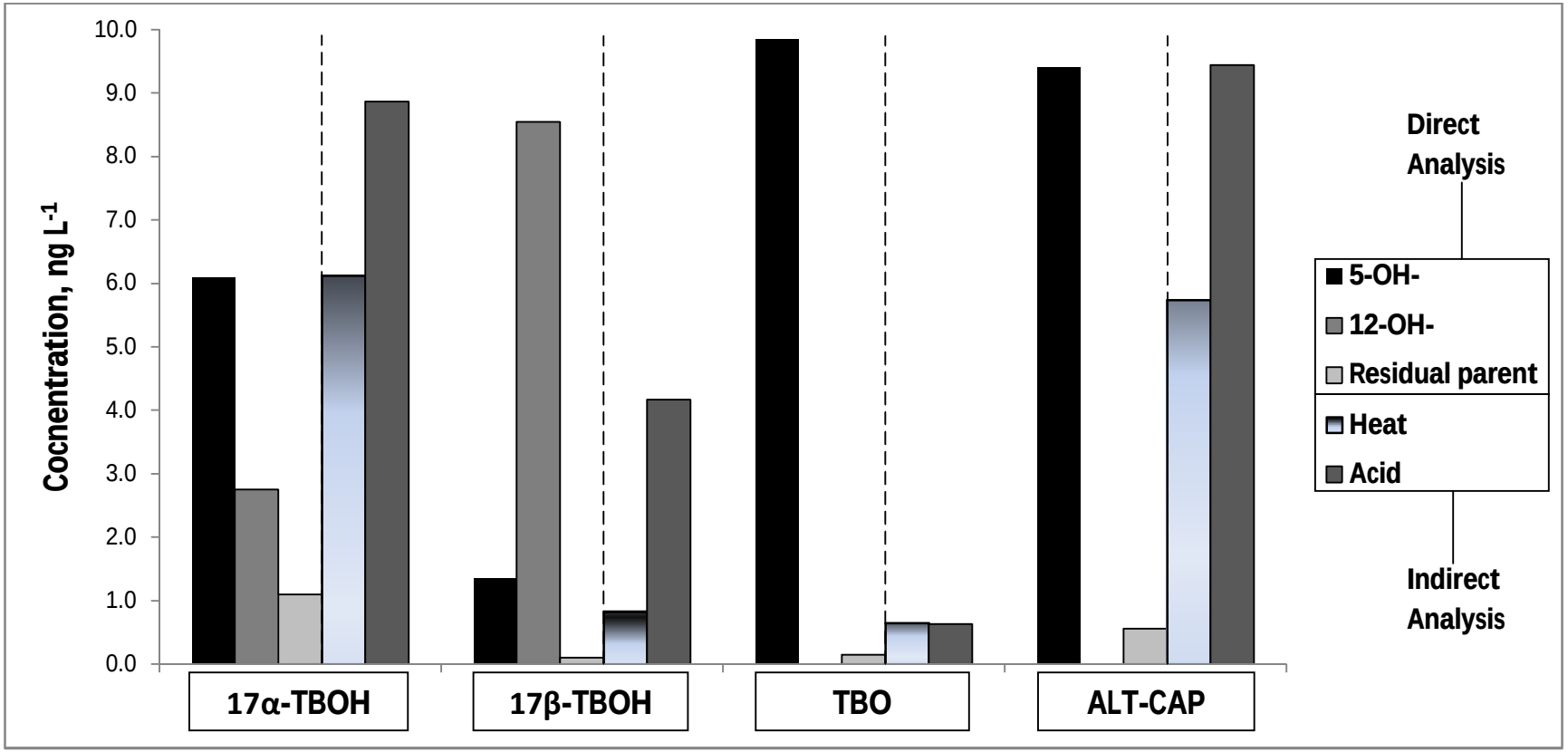


Figure 9: Spectra for a.), 5-OH-17α-TBOH, b.) 12-OH-17β-TBOH, c.) 5-OH-TBO, d.) ALT-CAP, and e.) ALT-CAP-OH, including retention time (RT) and most abundant mass-to-charge ratios (m/z) for each. Concentrations for each are 10 ng/L.



454

455 **Figure 10:** Indirect photoproduct analysis via acidification or heat, for the primary photoproducts of trenbolone metabolites and ALT-
456 CAP-OH. All concentrations are recovered from 10 ng L^{-1} spikes. Acidified samples were adjusted to pH~2 using HCl after
457 photoreaction. and heated samples were left in a 50^{o} C water bath for 15 hours. 5-OH-, 12-OH- photoproducts and residual parent
458 values are direct photoproduct measurements taken from samples photoreacted without purposeful reversion (left of dashed lines).
459 Heat and acid results were the change in parent concentration after reversion (right of dashed lines).

460 **List of Tables**

461 **Table 1:** Elution times, Multiple reaction monitoring (MRM) transitions, fragmenter voltages, and collision energies for trenbolone
462 metabolites, altrenogest, and photoproducts. MRM transitions, fragmenter voltages, and collision energies shown are quantitative
463 (confirmatory) for each compound.

	Compound	Retention time (min)	MRM Transitions (m/z)	Fragmenter (V)	Collision Energy (eV)
(1)	17α-TBOH	7.55	271>253 (271>211)	135 (135)	30 (30)
(2)	5-OH-17α-TBOH	6.62	271>211 (271>253)	135 (135)	30 (30)
(3)	12-OH-17α-TBOH	7.13	271>211 (271>253)	135 (135)	30 (30)
(4)	5-OH-17α-TBOH analog	6.93	271>211 (271>253)	135 (135)	30 (30)
(5)	17α-TBOH analog	7.87	271>211 (271>253)	135 (135)	30 (30)
(6)	17β-TBOH	7.23	271>253 (271>211)	135 (135)	30 (30)
(7)	5-OH-17β-TBOH	4.87	271>211 (271>253)	135 (135)	30 (30)
(8)	12-OH-17β-TBOH	5.85	271>211 (271>253)	135 (135)	30 (30)
(9)	TBO	6.65	269>225 (269>169)	135 (135)	28 (28)
(10)	5-OH-TBO	5.14	269>169 (269>225)	135 (135)	28 (28)
(11)	17α-d3-TBOH	7.58	274>256 (274>214)	135 (135)	30 (30)
(12)	ALT	9.37	311>227 (311>269)	82 (82)	24 (12)
(13)	ALT-CAP	8.41	311>269 (311>227)	82 (82)	12 (24)
(14)	ALT-CAP-OH	5.43	311>269 (311>227)	82 (82)	12 (24)

464

465 **Table 2:** Method detection limits (MDL) and quantification limits (MQL), linear regression slopes, intercepts, coefficients of
466 determination (R^2), matrix effects, recoveries from solid phase extraction, relative standard deviations (RSD) at high (10 ng L^{-1}) and
467 low (1 ng L^{-1}) spikes, reversion from photoproducts to parent metabolites, and number of points used in regression, for trenbolone
468 metabolites, altrenogest, and photoproducts. Responses of compounds (3), (4), (5), and (8) in lake water were below detection at tested
469 levels, and were not pursued at higher concentrations since they are minor photoproducts or analogs.

470

471

472

	Compound	Solvent	MDL (ng/L)	MQL (ng/L)	Slope	Intercept	R^2	Matrix Effect	Recovery	RSD (low spike)	RSD (high spike)	Reversion
(1)	17α-TBOH	river water	**0.03**	**0.04**	0.075	-0.053	0.999	100%	115%	10%	14 %	N/A
		lake water	**0.16**	**0.23**	0.18	-0.32	0.996	84%	103%	39%	19%	N/A
(2)	5-OH-17α-TBOH	river water	**0.01**	**0.03**	0.021	0.066	0.991	100%	104%	14%	12%	9%
		lake water	**0.45**	**0.78**	0.014	0.008	0.997	83%	107%	13%	11%	11%
(3)	12-OH-17α-TBOH	river water	**0.23**	**0.53**	0.002	0.01	0.991	100%	125%	6%	4%	9%
(4)	5-OH-17α-TBOH analog	river water	**0.17**	**0.36**	0.003	0.015	0.991	N/A	N/A	34%	22%	9%
(5)	17α-TBOH analog	river water	**0.61**	**1.19**	0.001	-0.003	0.989	N/A	N/A	51%	48%	N/A
(6)	17β-TBOH	river water	**0.07**	**0.10**	0.014	-0.011	0.999	100%	105%	13%	18%	N/A
		lake water	**0.57**	**0.80**	0.024	-0.159	0.998	85%	119%	26%	14%	N/A
(7)	12-OH-17β-TBOH	river water	**0.36**	**0.79**	0.026	0.144	0.998	100%	81%	8%	13%	1%
		lake water	**1.02**	**1.41**	0.028	-0.019	0.999	88%	93%	17%	1%	9%
(8)	5-OH-17β-TBOH	river water	**1.51**	**3.63**	0.004	-0.008	0.999	100%	75%	10%	26%	1%
(9)	TBO	river water	**0.05**	**0.07**	0.051	-0.029	0.997	100%	115%	7%	14%	N/A
		lake water	**0.26**	**0.30**	0.106	-0.072	0.999	117%	107%	17%	23%	N/A
(10)	5-OH-TBO	river water	**0.33**	**0.57**	0.019	-0.359	0.996	100%	116%	26%	8%	11%
		lake water	**0.59**	**0.94**	0.047	0.401	0.998	108%	95%	15%	7%	10%
(12)	ALT	river water	**0.03**	**0.03**	0.745	-1.71	0.997	100%	96%	4%	10%	N/A
		lake water	**0.05**	**0.06**	1.135	-2.132	0.996	74%	90%	15%	13%	N/A
(13)	ALT-CAP	river water	**0.03**	**0.03**	0.407	-0.929	0.997	100%	104%	9%	3%	100%
		lake water	**0.07**	**0.10**	0.369	-0.717	0.997	79%	88%	3%	12%	100%
(14)	ALT-CAP-OH	river water	**0.08**	**0.09**	0.06	0.027	0.991	100%	110%	10%	15%	30%
		lake water	**0.34**	**0.44**	0.105	-0.37	0.995	84%	99%	15%	21%	26%

473

Chapter 3: Thiol Adduct Formation

Introduction

Previous research at the University of Nevada, Reno discovered a potential adduct between 17β-TBOH and the amino acid L-cysteine during experiments to determine the rates of anaerobic biodegradation trenbolone metabolites under anaerobic and anoxic conditions. Observations of controls of 17β-TBOH in microcosms reduced with L-cysteine indicated that the 17β-TBOH would rapidly disappear. Subsequent tests via diode-array-detector HPLC strongly suggested that 17β-TBOH (peak absorbance 350 nm[10]) and L-cysteine (peak absorbance 250 nm[31]) formed a thiol adduct likely via Michael addition[32] (see Figure 2).

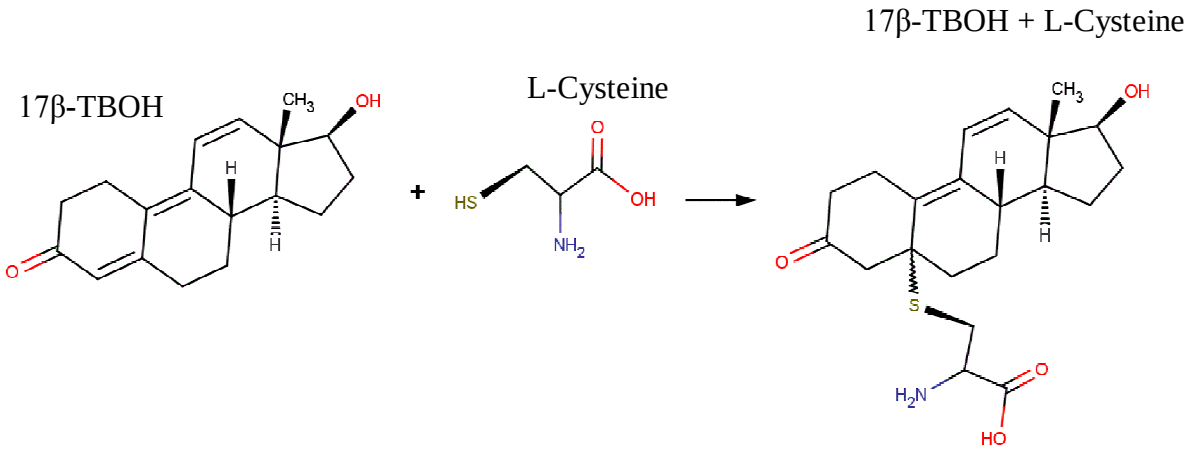


Figure 2: Possible pathway for thiol-adduct formation between 17β-TBOH and L-cysteine.

L-cysteine is a common amino acid, one of the building blocks of proteins, and many anoxic or anaerobic environments contain similar compounds. Therefore in cattle treated with trenbolone acetate, it is possible that trenbolone metabolites could form adducts with L-cysteine, along with other compounds with free thiols (such as glutathione) in the animal gut or manure. If

this were the case, then MS/MS analysis, a common method for the detection of trenbolone metabolites[10,23,33], would not easily detect an L-cysteine -- trenbolone adduct.

More importantly, there exist many examples of reversibility in thiol binding, suggesting that adducts might represent metastable products subject to reversible reactions when water quality conditions change. Like the reversible photochemical hydration-thermal dehydration reactions, the possibility may exist of reversibility in systems with steroids and reduced sulfur. If this were the case, then trenbolone metabolite conjugates could remain stable in anaerobic systems (manure piles) for extended periods of time, then reverse and release trenbolone metabolites into natural systems representing new transport pathways.

Research Objective

The critical research objective was to investigate whether thiol adducts between L-cysteine and trenbolone are reversible upon changes in solution conditions, and if so, whether such changes can occur at environmental conditions.

Experimental

Samples were processed in an anaerobic glove box (nitrogen filled) to minimize potential oxidation of L-cysteine to L-cystine (see Figure 3). Inside the glove box, a single sample was created comprised of an aqueous solution split into three 250 mL glass bottles, (each baked at 100^{o} C for an hour prior to use). The solution was prepared in nitrogen-sparged water (NSW) by adding approximately 246 mL of NSW to the bottle, adding 2.0 mL of a 2500 mg L^{-1} methanolic stock of 17β-TBOH to the solution, then adding L-cysteine-anhydride (89 mg) to the solution, and adding 2 mL of 1 M aqueous NaOH solution to the bottle (20 mg L^{-1} 17β-TBOH and 357 mg L^{-1} L-cysteine (40:1 L-cysteine:17β-TBOH)). Samples were reacted for 48 hours in the glove

box. Previous tests had shown that the half-life of the forward reaction was approximately 15 hours, and that the reaction reached equilibrium at 24-30 hours, with all available 17β-TBOH transforming to adducts.

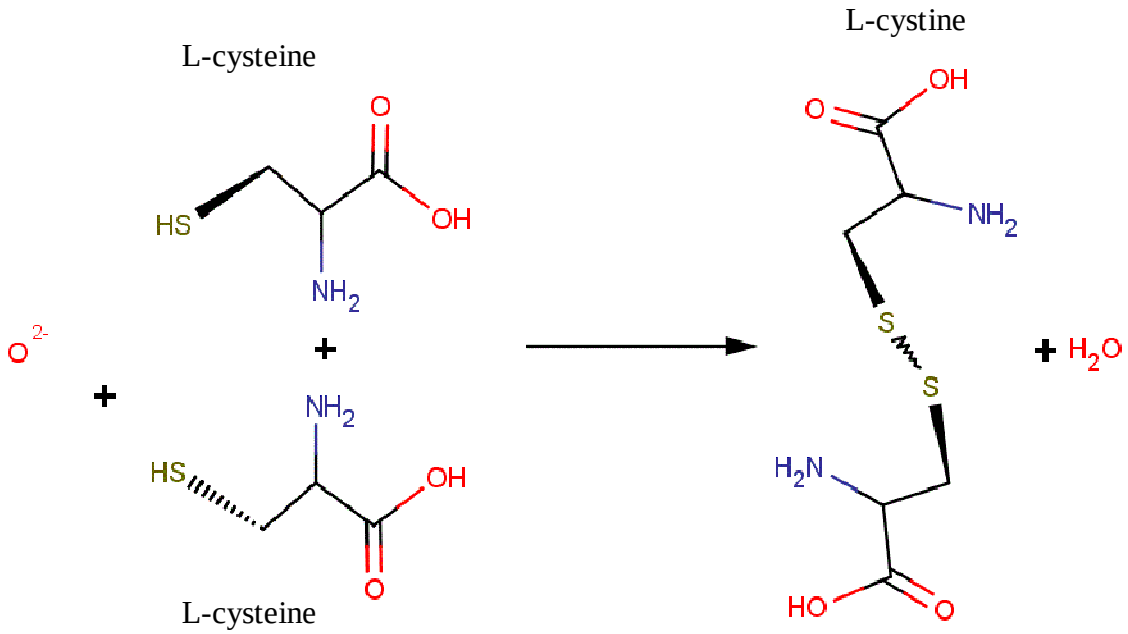


Figure 3: L-cysteine oxidation to L-cystine in the presence of oxygen.

After the samples (250 ml) were allowed to react, they all were solid-phase extracted onto a single C18 cartridge. Each cartridge was eluted with 9 mL of 95% methanol, 5% DDW, dried down (completely) using a rotating evaporator, and then resuspended in methanol (2 mL). The sample was then injected onto a preparation-scale HPLC to separate the parent compounds from the adduct(s) (the products of the forward reaction). Peaks (including parents and products) in the chromatogram were previously identified using HPLC-DAD (Agilent 1260, Agilent, Santa Clara, California) and smaller samples, thus enabling accurate isolation of larger quantities of

product on the prep-scale HPLC. Prep-scale HPLC conditions were 50 mL min^{-1} flow rate, C18 column, solvents were A: DDW with 0.1% acetic acid, and B: Acetonitrile (ACN) with 0.1% acetic acid. Separation was accomplished with the following gradient: 25% B initially, increased to 72% over 18 min, increased to 100% B over 0.01 min, isocratic at 100% B for 1 min, decreased to 25% over 0.01 min, isocratic at 25% B for 6 min, for a total runtime of 25 min[30].

Deaerating Water

To optimize the stoichiometry of L-cysteine - trenbolone adduct formation, steps were taken to deaerate water to reduce the oxidative-reductive potential of the system and the mass of L-cysteine required. Titanium (III) citrate was produced according to Jones and Pickard[35], and added to NSW prior to L-cysteine and trenbolone addition to reduce the amount of L-cysteine required to form adducts. However, the use of this deaerated water resulted in the formation of titanium oxides which clogged SPE cartridges, preventing sample analysis. It is recommended that another reducing agent be used for further efforts (keeping in mind that any reducing agent with an active thiol group on it (diothiothreitol, glutathione, etc.) will theoretically react with trenbolone just as L-cysteine does.

Adduct Formation

L-cysteine and 17β-TBOH were detected via HPLC-DAD (see figure 4). When a molar excess of L-cysteine was present, 17β-TBOH-L-cysteine adducts formed (see figure 5). After the adduct was formed, efforts were made to isolate it for NMR analysis, and adduct reversibility was investigated as a function of changing solution conditions. Samples were fraction collected as described above, and either lyophilized (freeze-dried via submersion in liquid nitrogen, then placed under vacuum) or dried down under vacuum at room temperature. Samples were then re-

suspended in deuterated solvents for NMR analysis (University of Nevada, Reno, Chemistry department). Due to interference from residual water, analysis could not determine conclusively any adduct structures.

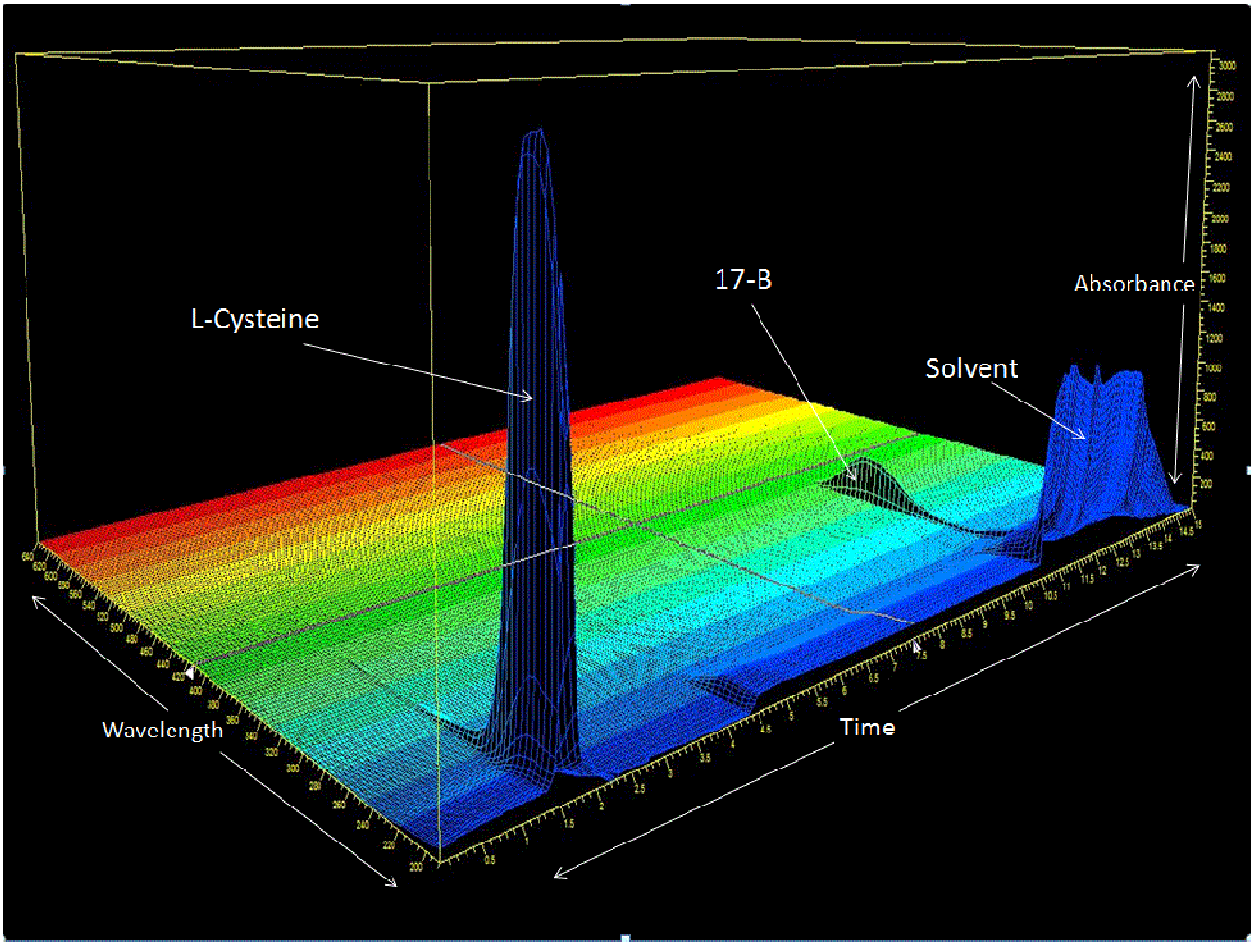


Figure 4: 3-dimensional chromatogram showing L-cysteine (retention time ~2 min, peak absorbance 220 nm) and 17β-TBOH (retention time ~11.5 min, peak absorbance 346 nm), measured via HPLC-DAD, prior to reaction.

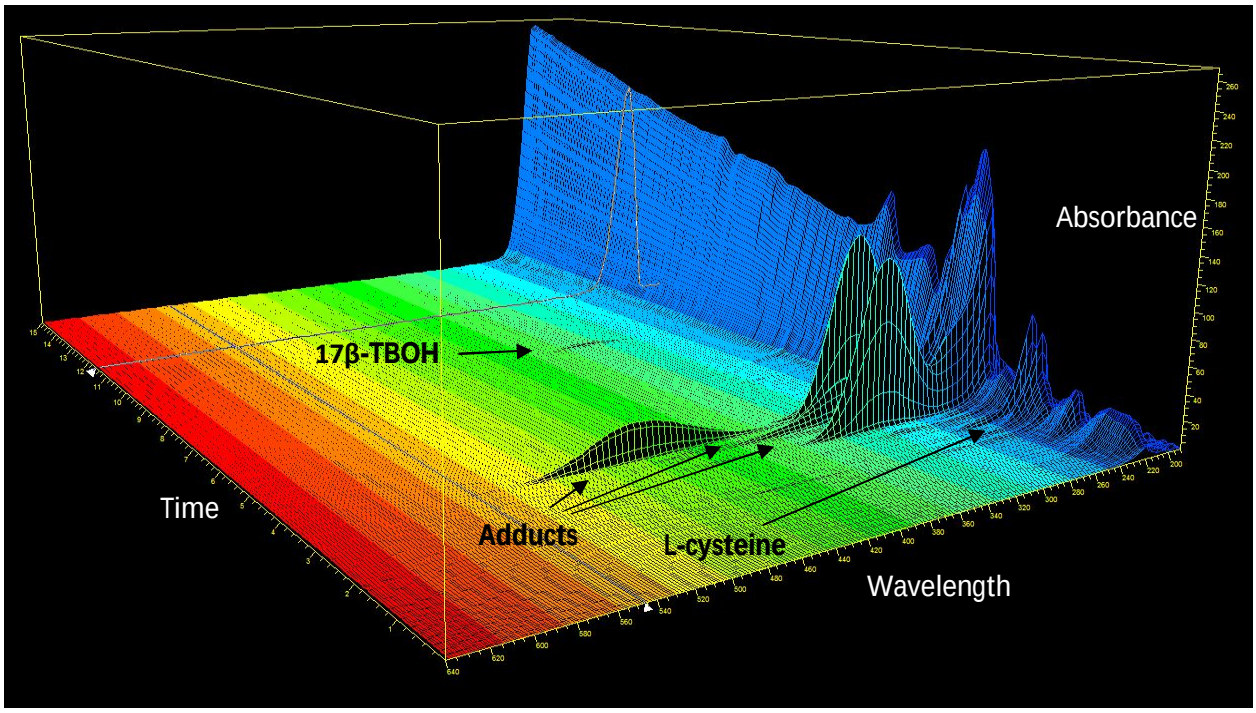


Figure 5: 3-dimensional chromatogram showing 17β-TBOH-L-cysteine adducts (at ~3-3.5 min), with some residual 17β-TBOH and L-cysteine after reaction. Note the "wall" of background signal at low wavelengths (~200 nm).

Adduct Stability

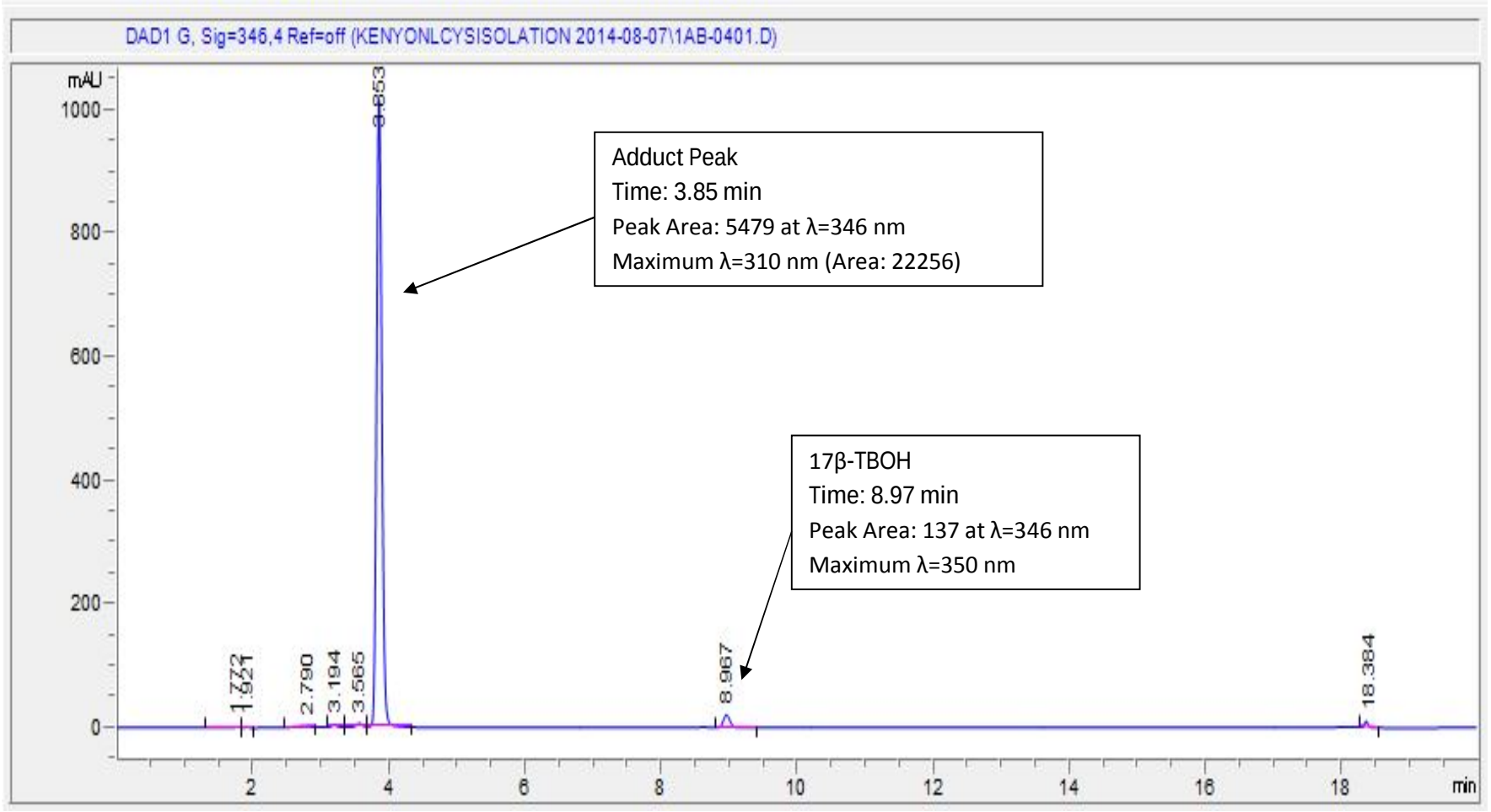
To explore whether adduct reversibility could regenerate trenbolone, solution conditions were changed in adduct samples post fractionation. After fraction collection, two vials (totaling approximately 30 mL of solution) of product were isolated from the prep-scale HPLC, and split into a total of eleven 2 mL samples for testing. Two of these samples were controls (pH measured was 4). Three samples were heated at 80^{o} C for 2 hours using a water bath and mercury thermometer. Three samples were pH-adjusted to pH 13 using 1 M NaOH, and three samples were adjusted to pH 13 and subsequently heated for 2 hours at 80^{o} C in a water bath.

Neither aerobic conditions alone, nor combined with heating or alkaline conditions altered adduct stability (see Figures 6-8), but aerobic, alkaline conditions with subsequent heating did regenerate 17β-TBOH (see Figure 9). However, analysis of peak areas showed that while a decrease in adduct peak did occur, it did not decrease proportionally to the amount of 17β-TBOH which reappeared, suggesting insufficient analytical sensitivity or an unidentified source of complexed 17β-TBOH. It is also possible that 17β-TBOH has a lesser peak area response factor in UV-DAD than its adduct with L-cysteine, such that the small difference in the adduct peak could correspond to the increase in 17β-TBOH.

Conclusions

Regarding the first research objective (a single method to detect all metabolites and photoproducts of trenbolone acetate, as well as altrenogest and its photoproducts), a method was successfully developed. With separation, simultaneous detection is possible, however due to coelution between metabolites and photoproducts of 17α-TBOH and 17β-TBOH in fast chromatography runs (<20 min), accurate quantification in mixed residue systems can be compromised. Fortunately, 17α-TBOH is typically the dominant metabolite present, and thus 17β-TBOH, TBO, and related photoproducts would usually be expected to occur at lower concentrations as compared with 17α-TBOH.

With regard to the second research objective, efforts to test the reversibility of 17β-TBOH-L-cysteine adducts yielded some insights into adduct stability. Future efforts maintaining cysteine stability in solution, include improving analytical methods to observe adducts and more comprehensive reversibility characteristics. Also, the fraction-collection and subsequent NMR analysis of adducts to determine their structure would help to understand this system.

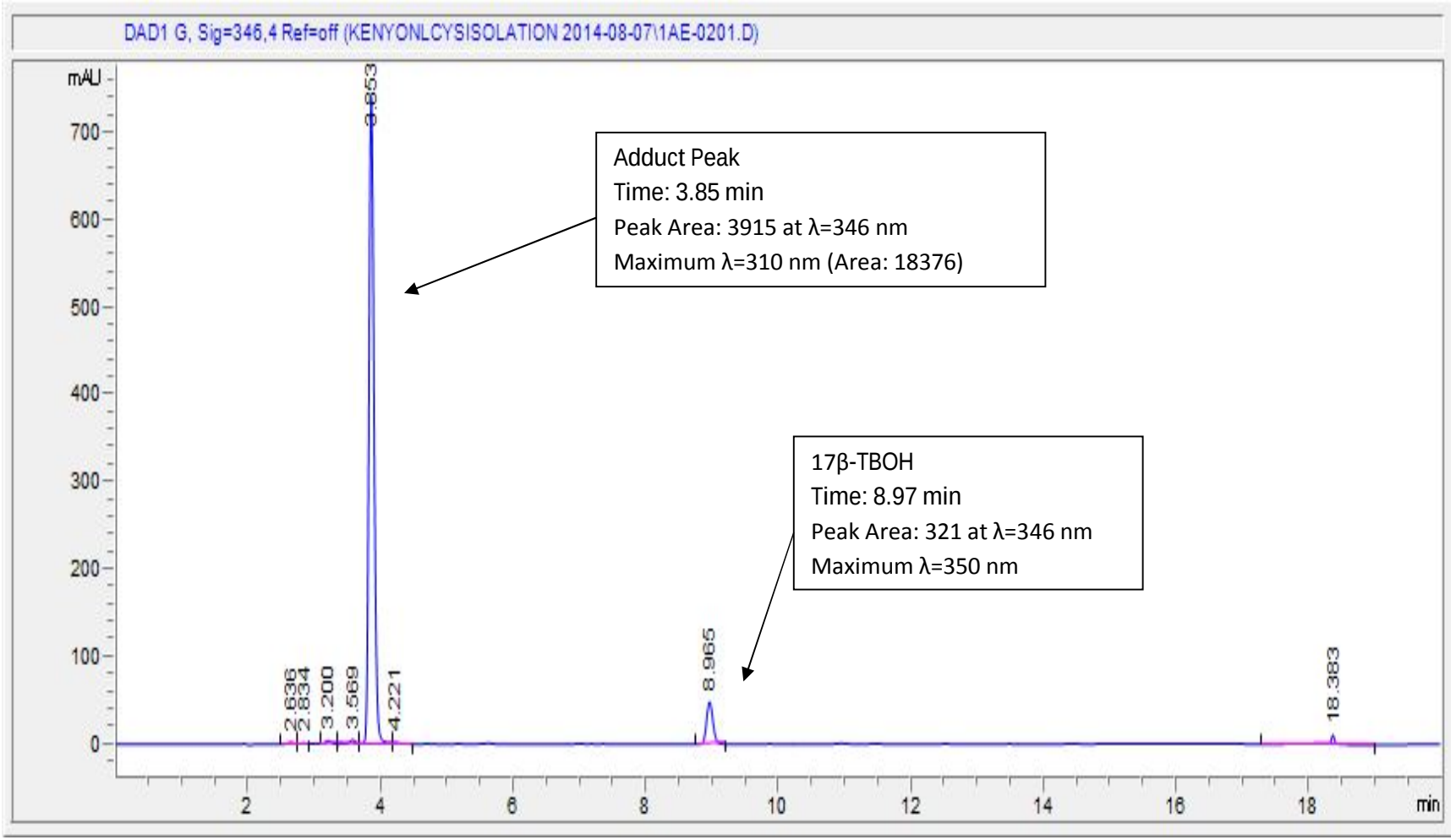


586

587 Figure 6: Fraction-collected L-cysteine-17β-TBOH adduct, control (pH=4).

588

589



590

591 Figure 7: Fraction-collected L-cysteine-17β-TBOH adduct, heated (80° C water bath for 2 hours) (pH=4).

592

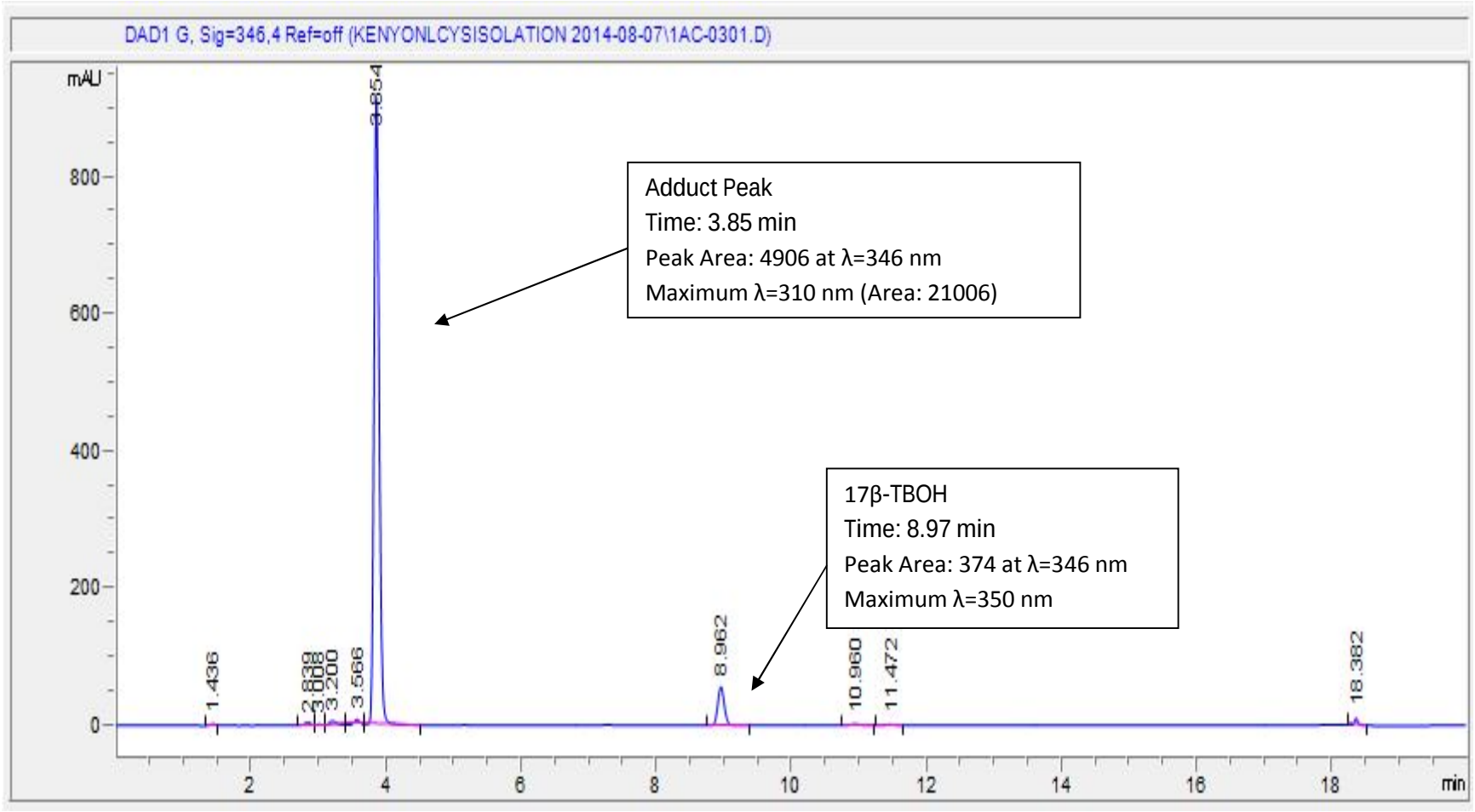


Figure 8: Fraction-collected L-cysteine-17β-TBOH adduct, under alkaline (pH=13) conditions.

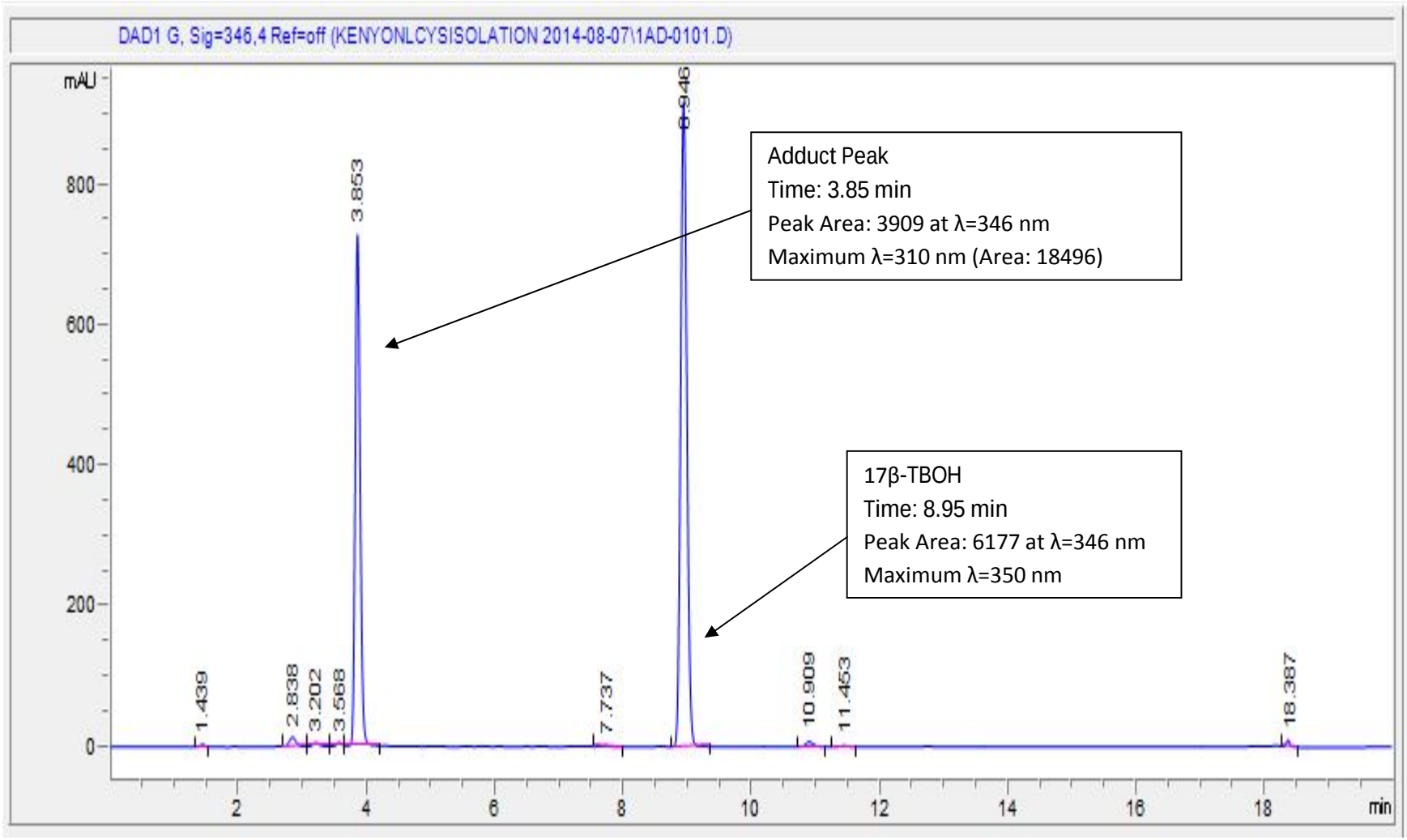


Figure 9: Fraction-collected L-cysteine-17β-TBOH adduct, under alkaline (pH=13) conditions, then heated (80° C water bath for 2 hours) .

Works Cited

(1) Endocrine Disruptors. National Institute of Environmental Health Services. August 11, 2015. http://www.niehs.nih.gov/health/topics/agents/endocrine/

(2) Contaminants of Emerging Concern. United States Environmental Protection Agency. May 8, 2014. http://water.epa.gov/scitech/cec/

(3) Sone, K.; M. H.; Itamoto, M.; Katsu, Y.; Watanabe, H.; Urushitani, H.; Tooi, O.; Guillette Jr., L. J.; Taisen, I. Effects of an androgenic growth promoter 17β trenbolone on masculinization of Mosquitofish (Gambusia affinis affinis). *Gen. Comp. Endocrinol.* (2005) ***143,*** 151–160.

(4) Jensen, K. M.; E. A. M.; Kahl, M. D.; Ankley, G. T. Effects of the Feedlot Contaminant 17α Trenbolone on Reproductive Endocrinology of the Fathead Minnow. *Environ. Sci. Technol.* (2006) ***40,*** 3112-3117.

(5) Seki, M., S. Fujishima, T. Nozaka, M. Maeda & K. Kobayashi. Comparison of response to 17 beta estradiol and 17 beta trenbolone among three small fish species. *Environ. Toxicol. Chem.* (2006) ***25,*** 2742-2752.

(6) Morthorst, J.E.; Holbech, H.; Bjerregaard, P. Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations. *Aquat. Toxicol*. (2010). *98(4),* 336-343.

(7) Orlando, E. F.; Kolok, A. S.; Binzcik, G. A.; Gates, J. L.; Horton, M. K.; Lambright, C. S.; Gray, L. E.; Soto, A. M.; Guillette, L. J. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinal species, the fathead minnow. *Environ. Health Perspect*. (2004). *112(3),* 353-258.

(8) Khan, B.; Lee, L. S., Estrogens and synthetic androgens in manure slurry from trenbolone acetate/estradiol implanted cattle and in waste receiving lagoons used for irrigation. *Chemosphere* (2012), *89,* 1443-1449.

(9) Pottier, J.; Cousty, C.; Heitzman, R. J.; Reynolds, I. P. Differences in the biotransformation of 17-beta-hydroxylated steroid, trenbolone acetate, in rat and cow. *Xenobiotica*. (1981), *11(7),* 489-500.

(10) Khan, B.; Lee, L. S.; Sassman, S. A. Degradation of Synthetic Androgens 17α and 17β Trenbolone and Trendione in Agricultural Soils. *Environ. Sci. Technol.* (2008)**,** *42,* 3570-3574.

(11) Lange, I. G.; Daxenberger, A.; Schiffer, B.; Witters, H.; Ibarreta, D.; Meyer, H. H. D. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. *Analytica Chimica Acta.* (2002). *473,* 27-37.

(12) Steroid Hormones. The Merck Veterinary Manual. December 2013. http://www.merckvetmanual.com/mvm/pharmacology/growth_promotants_and_production_enhancers/steroid_hormones.html

(13) Wammer, K. H.; Anderson, K. C.; Erickson, P. R.; Kliegman, S.; Moffatt, M.; Berg, S. M.; Heitzman, J. A.; McNeill, K.; Martinovic-Weigelt, D.; Cwiertny, D. M.; Kolodziej, E. P. Environmental photochemistry of altrenogest: photoisomerization to a bioactive product with increased environmental persistence via reversible photohydration. In review.

(14) Squires, E.L.; Heesemann, C.P.; Webel, S.K.; Shideler, R.K.; Voss, J.L. Relationship of altrenogest to ovarian activity, hormone concentrations and fertility of mares. *J. Anim. Sci.* (1983). *56*, 901-910.

(15) van Leeuwen, J.J.J.; Williams, S.I.; Martens, M.R.T.M.; Jourquin, J.; Driancourt, M.A.; Kemp, B.; Soede, N.M. The effect of post weaning altrenogest treatments of primiparous sows on follicular development, pregnancy rates, and litter sizes. *J. Anim. Sci*. (2011). *89*, 397-403.

(16) Willmann, C.; Schuler, G.; Hoffmann, B.; Parvizi, N.; Aurich, C. Effects of age and altrenogest treatment on conceptus development and secretion of LH, prosgesterone, and eCG in early-pregnant mares. *Theriogenology* (2011). *75*, 421-428.

(17) Regu-Mate® Product label. Regu-Mate®. 2010. http://www.regu-mate.com/label.asp

(18) Estienne, M.J.; Harper, A.F.; Horsley, B.R.; Estienne, C.E.; Knight, J.W. Effects of P.G. 600 on the onset of estrus and ovulation rate in gilts treated with Regu-mate. *J. Anim. Sci.* (2001). *79*, 2757-2761.

(19) Zeilinger, J.; Steger-Hartmann, T.; Maser, E.; Goller, S.; Vonk, R.; Lange, R. Effects of synthetic gestagens on fish reproduction. *Environ. Tox. Chem.* (2009). *28(12)*, 2663-2670.

(20) Säfholm, M.; Ribbenstedt, A.; Fick, J.; Berg, C. Risks of hormonally active pharmaceuticals to amphibians: a growing concern regarding progestagens. *Philos. T. Roy. Soc. A.* (2014). *369*, 20130577.

(21) Kumar, V.; Johnson, A.C.; Trubiroha, A.; Tumova, J.; Ihara, M.; Grabic, R.; Kloas, W.; Tanaka, H.; Kroupova, H.K. The challenge presented by progestins in ecotoxicological research: A critical review. *Envrion. Sci. Techol*. (2015). *49*, 2625-2638.

(22) Jones, G. D.; Benchetler, P. V.; Tate, K. W.; Kolodziej, E. P. Trenbolone acetate metabolite transport in rangelands and irrigated pasture: observations and conceptual approaches for agro-ecosystems. *Environ. Sci. Technol*. (2014). *48(21)*, 12569-12576.

(23) Durhan, E. J.; Lambright, C. S.; Makynen, E. A.; Lazorchak, J.; Hartig, P. C.; Wilson, V. S.; Gray, L. E.; Ankley, G. T. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. *Environ. Health Perspect*, (2006), *114*, 65-68.

(24) Cole, E. A.; McBride, S. A.; Kimbrough, K. C.; Lee, J.; Marchand, E. A.; Cwiertny, D. M.; Kolodziej, E. P. Rates and product identification for trenbolone acetate metabolite biotransformation under aerobic conditions. *Environ. Tox. Chem.* (2015). *34(7),* 1472-1484.

(25) Schiffer, B.; Daxenberger, A.; Meyer, K.; Meyer, H. H. D., The Fate of Trenbolone Acetate and Melengestrol Acetate after Applicationas Growth Promoters in Cattle: Environmental Studies. *Environ. Health Perspect.* (2001). *109*, 7.

(26) Khan, B.; Qiao, X. L.; Lee, L. S. Stereoselective sorption by agricultural soils and liquid-liquid partitioning of trenbolone (17 alpha nad 17 beta) and trendione. *Environ. Sci. Technol.* (2009). *43(23),* 8827-8833.

(27) Card, M. L.; Chin, Y.-P.; Lee, L. S.; Khan, B. Prediction and experimental evaluation of soil sorption by natural hormones and hormone mimics. *J. Agric. Food Chem.* (2012), *60,* 1480−1487.

(28) Altrenogest. Properties-Predicted-EpiSuite. Chemspider. 2015. http://www.chemspider.com/Chemical-Structure.8216634.html?rid=da4d7a65-097e-41b2-8b82-649f22b8295a

(29) Qu, S.; Kolodziej, E. P.; Cwiertny, D. M., Phototransformation Rates and Mechanisms for Synthetic Hormone Growth Promoters Used in Animal Agriculture. *Environ. Sci. Technol.* (2012)**,** *46*, 13202-13211.

(30) Kolodziej, E. P.; Qu, S.; Forsgren, K. L.; Long, S. A.; Gloer, J. B.; Jones, G. D.; Schlenk, D.; Baltrusaitis, J.; Cwiertny, D. M. Identification and Environmental Implications of Photo Transformation Products of Trenbolone Acetate Metabolites. *Environ. Sci. Technol.* (2013), *47,* 5031-5041.

(31) Forsgren, K. L.; Qu, S.; Lavado, R.; Cwiertny, D.; Schlenk, D. Trenbolone acetate metabolites promote ovarian growth and development in adult Japanese medaka (*Oryzias Latipes*). *Gen. Comp. Endocrinol.* (2014), *202*, 1-7.

(32) Avi-Dor, Y.; Mager, J. A spectrophotometric method for determination of cysteine and related compounds. *J. Biol. Chem.* (1956). *222*, 249-258.

(33) Nair, D. P.; Podgorski, M.; Chatani, S.; Gong, T.; Xi, W.; Fenoli, C. R.; Bowman, C. N. The thiol-Michael addition click reaction: a powerful and widely used tool in materials chemistry.

(34) Webster, J. P.; Kover, S. C.; Bryson, R. J.; Harter, T.; Mansell, D. S.; Sedlak, D. L.; Kolodziej, E. P. Occurrence of Trenbolone Acetate Metabolites in Simulated Confined Animal Feeding Operation (CAFO) Runoff. *Environ. Sci. Technol.* (2012), *46*, 3803-3810.

(35) Jones, G. A.; Pickard, M. D. Effect of titanium (III) citrate as reducing agent on growth of rumen bacteria. *Appl. Environ. Microbiol.* (1980). *39(6),* 1144-1147.