

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

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**Abstract**

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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 17 $\alpha$ -trenbolone (17 $\alpha$ -TBOH), 17 $\beta$ -trenbolone (17 $\beta$ -TBOH), trendione (TBO), and altrenogest (ALT); target photoproducts were the metastable 5-hydroxy- and 12-hydroxy-photoproducts of 17 $\alpha$ /17 $\beta$ -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<sup>-1</sup> except for 17 $\beta$ -TBOH photoproducts, which had MDL's <2 ng L<sup>-1</sup> and MQL's <4 ng L<sup>-1</sup>. Matrix suppression was <30% using lake water, and SPE recovery rates were near/above 100%, except for 17 $\beta$ -TBOH photoproducts, with recoveries >75%. Intra-day relative standard deviations (RSD's) were <30% for TBO and its products, <20% for all others.

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## **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" <sup>1</sup>. 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" <sup>2</sup>. 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<sup>1</sup>. Among steroid hormones, the growth promoter trenbolone (as trenbolone acetate) has been identified as an endocrine active aquatic contaminant<sup>3-6</sup>. 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<sup>7</sup>. 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<sup>8</sup>. Metabolized trenbolone is excreted as the metabolites 17 $\alpha$ -trenbolone (17 $\alpha$ -TBOH, the primary metabolite), 17 $\beta$ -trenbolone (17 $\beta$ -TBOH), and trendione (TBO) in urine and manure (see Figure 1)<sup>9</sup>.

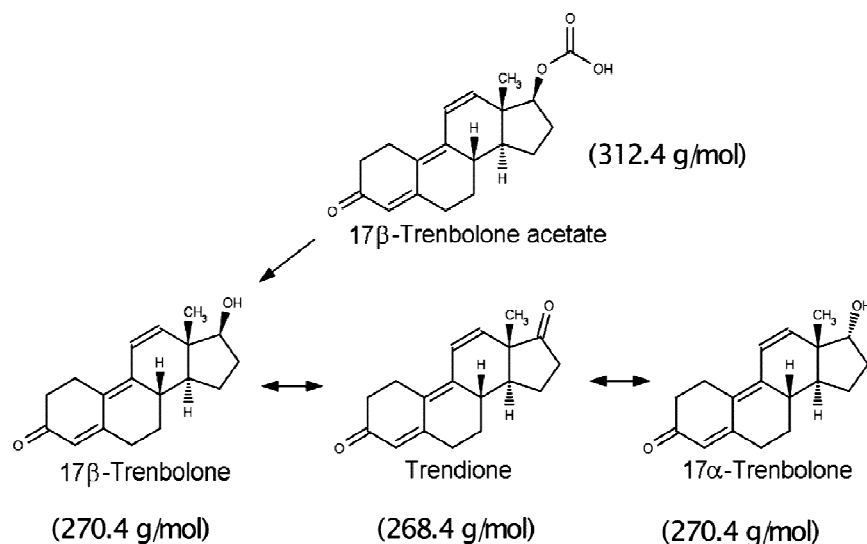


Figure1. Trenbolone acetate (TBA) metabolism. Figure adapted from Khan et al. 2008

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<sup>10,11</sup>. 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<sup>12</sup>.

<b>Hormone</b>	<b>Method of Administration</b>	<b>Dosage</b>	<b>Duration of Effect (days)</b>	<b>Growth Response</b>
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 + 17 $\beta$ -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 + 17 $\beta$ - 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 = 17 $\beta$ -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<sup>13-16</sup>. 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<sup>17-18</sup>. 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<sup>17</sup>. This is particularly important, given that concentrations at sub ng L<sup>-1</sup> concentrations of other synthetic progestins, can reduce fecundity in fish<sup>19-21</sup>.

### **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<sup>22</sup>. 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<sup>23</sup>. 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<sup>10,24</sup> while half-lives of TBA metabolites under anaerobic biodegradation show that they are much more stable (half lives of 260 days)<sup>25</sup>. 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<sup>26,27</sup>. 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<sup>28</sup>.

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

Metabolite	Average $\pm$ SD <sup>a</sup>		
	log $K_{oc}$ <sup>b, 26</sup>	log $K_{ow}$ <sup>c, 26</sup>	log $K_{ow}$ <sup>c, 27</sup>
17 $\alpha$ -trenbolone	2.77 $\pm$ 0.12	2.72 $\pm$ 0.02	2.72
17 $\beta$ -trenbolone	3.08 $\pm$ 0.10	3.08 $\pm$ 0.03	3.08
Trendione	3.38 $\pm$ 0.19	2.63 $\pm$ 0.05	2.63

<sup>a</sup> Standard deviation. <sup>b</sup> Organic-carbon normalized sorption coefficient. <sup>c</sup> 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<sup>11</sup>. For example, studies demonstrate that trenbolone is stable in manure under

anaerobic conditions for over 260 days<sup>11</sup>, 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<sup>13,29,30</sup>. 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<sup>31</sup>. 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**

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**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 17 $\alpha$ -trenbolone (17 $\alpha$ -TBOH), 17 $\beta$ -trenbolone (17 $\beta$ -TBOH), trendione (TBO), and altrenogest (ALT); target photoproducts were the metastable 5-hydroxy- and 12-hydroxy- photoproducts of 17 $\alpha$ /17 $\beta$ -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<sup>-1</sup> except for 17 $\beta$ -TBOH photoproducts, which had MDL's <2 ng L<sup>-1</sup> and MQL's <4 ng L<sup>-1</sup>. Matrix suppression was <30% using lake water, and SPE recovery rates were near/above 100%, except for 17 $\beta$ -TBOH photoproducts, with recoveries >75%. Intra-day relative standard deviations (RSD's) were <30% for TBO and its products, <20% for all others.

## 1 **1.0 Introduction**

2           In a resource constrained world with a growing population, increased use of concentrated  
3 animal feed operations (CAFO's), a form of intensive industrial agriculture, has resulted in  
4 concentrated discharges of pharmaceuticals to natural systems near animal agriculture facilities.  
5 Many of these pharmaceuticals are synthetic, and may pose ecological risks through mechanisms  
6 of carcinogenicity, teratogenicity, or endocrine disruption [1-7]. Of interest here are steroidal  
7 pharmaceuticals used as growth promoters and estrous synchronizers, specifically the  
8 metabolites of the synthetic androgen trenbolone acetate (TBA) and the synthetic progestin  
9 altrenogest (ALT) (Figure 1). Trenbolone acetate is employed as a growth promoter in beef  
10 production, and its use represents significant incremental economic value to the beef industry  
11 [8], while altrenogest is used widely as an estrus synchronizer in breeding mares and swine [9-  
12 11].

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

22 In the photic zone, metabolites of trenbolone react rapidly ( $k_{\text{obs}}=0.0254 - 0.213 \text{ min}^{-1}$ ),  
23 forming photohydration products [16, 17] (Figure 2). These photoproducts also exhibit thermal  
24 dehydration in the dark, reverting to parent structures [18]. Up to 80-90% of  $17\alpha$ -TBOH  
25 concentrations can revert to the parent structure from products; reversion is accelerated by higher  
26 temperatures and can be nearly instantaneous under strongly acidic or basic conditions (Figure 3)  
27 [18]. These mechanisms of reversible transformation can result in higher persistence and  
28 increased transport potential of trenbolone metabolites than would be expected based upon initial  
29 rates of phototransformation alone [19]. Additionally, evidence suggests that the primary  
30 photoproducts of trenbolone metabolites may have bioactivity distinct from that of the parent  
31 molecules [20].

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

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

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

## 51 **2.0 Experimental**

### 52 *2.1 Reagents and Materials*

53 17 $\beta$ -TBOH was purchased from Shenzhen Shijingu Technology Co. Ltd (Shanghai,  
54 China). 17 $\alpha$ -TBOH and 17 $\alpha$ -16,16,17-d3-TBOH (deuterated internal standard for 17 $\alpha$ -TBOH)  
55 (17 $\alpha$ -d3-TBOH) were purchased from BDG Synthesis (Wellington, New Zealand). Trendione  
56 (TBO) was purchased from Steraloids (Newport, Rhode Island). Altrenogest was purchased from  
57 Fluka (Sigma Aldrich, St. Louis, MO). LC-MS grade methanol and LC-MS grade water were  
58 purchased from Fisher Scientific (Pittsburgh, Pennsylvania).

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

67 through a 0.45  $\mu\text{m}$  polyethersulfone (PES) membrane (Fisher Scientific, Pittsburgh,  
68 Pennsylvania) to remove particulates. For an organic rich complex matrix representative of  
69 agricultural runoff, lake water was collected from Wapato Lake (Tacoma, WA, 47.1952° N,  
70 122.4567° W), a highly eutrophic system, to validate the method.

## 71 *2.2 Sample Preparation*

72 For each metabolite, individual standards (100 ng L<sup>-1</sup> to 100  $\mu\text{g L}^{-1}$ ) were serial dilutions  
73 from a stock solution of 10 mg L<sup>-1</sup> (metabolite) in methanol, kept at 4° C. Samples for irradiation  
74 were either 2 mL HPLC vials (ambered borosilicate glass) with PTFE septa and plastic screw  
75 caps, used for injection of samples before/after reaction, or 40 mL samples (clear borosilicate  
76 glass), used when solid phase extraction (SPE) was employed. Unless otherwise noted, all data  
77 described here was acquired using 40 mL samples concentrated via SPE. All glassware except  
78 for transfer pipettes was silanized with 10% (v/v) dichlorodimethylsiloxane in toluene prior to  
79 each use. Coelution of metabolites and primary photoproducts in irradiated samples of mixed  
80 17 $\alpha$ /17 $\beta$ -TBOH prevented simultaneous quantification of all primary photoproducts, so parent  
81 compounds were irradiated individually to facilitate method development.

## 82 *2.3 Photoreaction*

83 During photoreaction, samples rested horizontally on a rack in a recirculating 7° C water  
84 bath to minimize photoproduct dehydration, such that the water bath elevation did not rise above  
85 1/3 to 1/2 the diameter of each vial. The water bath was placed underneath a solar simulator,  
86 (EYE Lighting Int. Model # 93510, EYE Lighting, Mentor, OH) that used four high pressure,  
87 150 W xenon bulbs (Solarlux 150R, EYE Lighting, Mentor, OH). Per manufacturer  
88 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 (17 $\alpha$ -d3-  
93 TBOH, 2.5 ng L<sup>-1</sup>) was added to all samples as 40  $\mu$ L of a 5  $\mu$ g L<sup>-1</sup> methanolic standard of 17 $\alpha$ -  
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 $^{\circ}$  C for 15 hours (overnight, to mimic maximum reversion possible naturally), or were  
99 acidified with 200  $\mu$ L 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<sup>-1</sup>).  
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.

## 111 2.5 Instrumental analysis

112 An Agilent (Santa Clara, California) 1290 Infinity binary pump liquid chromatograph  
113 (LC) coupled with an Agilent 6430 triple quadrupole tandem mass spectrometer (MS/MS) was  
114 used for analysis. To minimize reversion and thus maximize photoproduct response, processing  
115 time and sample temperatures were minimized to the extent possible. Samples were injected via  
116 chilled autosampler tray (4°C) and separated using an Agilent Poroshell 120Å EC C18 column  
117 (3.0mm i.d. X 50mm, 2.7µm) preceded by a SecurityGuard Gemini C18 guard column (2.0mm  
118 i.d. X 4mm) (Phenomenex, Torrance, California), both at 11°C.

119 Mobile phase (0.2 mL min<sup>-1</sup>) solutions were A: LC-MS grade water (pH=5.5) and B: LC-  
120 MS grade methanol, with the following separation gradient used: 55% B initially, increased to  
121 75% over 5 min, increased to 100% B over 3 min, isocratic at 100% B for 3 min, decreased to  
122 55% over 1 min, isocratic at 55% B for 5 min, for a total runtime of 17 min. Agilent Jetstream  
123 Electrospray Ionization Positive (AJS ESI+) mode was used with 2.5 kV capillary voltage, 1.0  
124 kV nozzle voltage, 350°C desolvation gas temp, 400°C sheath gas temp, 12.0 L min<sup>-1</sup>  
125 desolvation and sheath gas flows, 40 psi nebulizer pressure, and 400 V positive multiplier  
126 voltage (delta EMV). For all transitions, dwell time was 200 ms and collision cell acceleration  
127 voltage was 4 V.

## 128 3.0 Results and Discussion

### 129 3.1 Optimization of LC-MS/MS Conditions

130 LC conditions were optimized via serial injection of 100 µg L<sup>-1</sup> samples of individual  
131 analytes. The LC gradient was adjusted to achieve peak separation of parent metabolites and  
132 photoproducts. The method was optimized for 17α-TBOH because it is the predominant



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

145  $17\alpha/17\beta$ -TBOH had the same precursor mass-to-charge ratio (m/z) of 271.2 Da for their  
146  $[M+H]^+$  ions, while their primary photoproducts (5-OH- $17\alpha$ -TBOH and 12-OH- $17\beta$ -TBOH) had  
147 an expected precursor m/z of 289.2 Da for their  $[M+H]^+$  ions. However, 289.2 Da was never  
148 observed via LC-MS/MS, only dehydrated photoproduct ( $[M+H-H_2O]^+$ ) ions with m/z 271.2 Da  
149 were observed in LC-MS/MS analysis ([Table 1](#), [Figure 8](#), [Figure 9](#)), consistent with in-source  
150 dehydration as previously documented [17]. TBO had a precursor m/z of 269.2 Da, while TBO  
151 photoproducts observed (at m/z 269.2 Da) were also dehydrated. ALT and its primary  
152 photoproduct (ALT-CAP) both had precursor m/z of 311.2 Da, and the secondary photoproduct  
153 (ALT-CAP-OH) had a precursor m/z of 329.2 Da, but only the dehydrated ions ( $[M+H-H_2O]^+$ )  
154 with m/z 311.2 Da were observed for the secondary photoproduct.

155

### 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 ( $17\alpha$ -TBOH,  $17\beta$ -TBOH, TBO, and  
161 ALT), resulting in additions of 22 Da (293.2 Da for  $17\alpha/17\beta$ -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  $17\alpha/17\beta$ -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  $17\alpha/17\beta$ -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  $17\alpha$ -TBOH and  $17\beta$ -  
177 TBOH photoproducts, the similar polarity of TBA metabolites and photoproducts resulted in

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

185           Given photoproduct instability, several analytical challenges were evident relative to  
186 these time scales (days). For example, two peaks were observed in SPE extracts (compounds (4)  
187 and (5) in [Table 1](#)) of photo reacted samples of  $17\alpha$ -TBOH that were not observed during direct  
188 injection of similar samples (see [Figure 6b](#)). Spectral analysis revealed that the peak at 6.34 min  
189 (compound (4)) has similar spectra to the peaks at 6.02 min (5-OH- $17\alpha$ -TBOH) and 6.53 min  
190 (12-OH- $17\alpha$ -TBOH) and the peak at 7.25 min (compound (5)) has similar spectra to the parent  
191  $17\alpha$ -TBOH peak at 6.96 min. These peaks (6.34 min and 7.25 min) are possible analogs of the  
192 primary photoproduct (5-OH- $17\alpha$ -TBOH) and  $17\alpha$ -TBOH respectively, due to the near identical  
193 elution time differences from the "new" peaks to their respective parent compounds (0.32  
194 minutes +/- 0.03 minutes each). This behavior is not exhibited in dark controls, suggesting that  
195 the photoproduct forms an analog in the SPE process, which then reverts to a metabolite analog.  
196 Time lapse data (see [Figure 6c](#)) shows that the 5-OH- $17\alpha$ -TBOH photoproduct, and its potential  
197 analog (compounds (2) and (4)), disappear after 1 day (likely due to reversion to parent  
198 structures) but 12-OH- $17\alpha$ -TBOH,  $17\alpha$ -TBOH, and its potential analog (compounds (1), (3), and  
199 (5)) all remain. Such observations clearly point to the dynamic nature of photoproduct analysis  
200 and highlight the challenges inherent to analysis of complex mixtures.

### 201 3.4 Limits of Detection and Quantification

202 The method detection limit (MDL) and quantification limit (MQL) were calculated by  
203 running matrix blanks (no analyte) 9 times and calculating the average and standard deviation of  
204 the background noise, where MDL is defined as the average plus 3 times the standard deviation,  
205 and MQL is the average plus 10 times the standard deviation [26-28]. For trenbolone  
206 metabolites, altrenogest, and their photoproducts, the MDL's and MQL's were  $\text{sub ng L}^{-1}$   
207 regardless of sample matrix, with the exception of 12-OH-17 $\beta$ -TBOH in lake water and 5-OH-  
208 17 $\beta$ -TBOH in river water, both with MDL's  $<2 \text{ ng L}^{-1}$  and MQL's  $<4 \text{ ng L}^{-1}$  (see [Table 2](#)). The  
209 photoproducts were more difficult to quantify relative to the parent compounds, due to a.) their  
210 reactive nature, and b.) the formation of multiple products, thus reducing available analytical  
211 mass (relative to parents).

212 The following assumptions were made to quantify photoproducts: 1.) all metabolite mass  
213 is conserved to the photoproducts observed (i.e.; that the sum of photoproduct peak areas at a  
214 given concentration equates to the metabolite peak area measured at that concentration); 2.)  
215 quantitative transition response of photoproducts is equal to metabolites (i.e.  $10 \text{ ng L}^{-1}$  of 5-OH-  
216 17 $\alpha$ -TBOH measured using  $m/z$  271.2- $\rightarrow$ 211.2 is equal to  $10 \text{ ng L}^{-1}$  of 17 $\alpha$ -TBOH measured  
217 using  $m/z$  271.2- $\rightarrow$ 253.2); and 3.) all photoproducts have the same unit response to detection (i.e.  
218  $10 \text{ ng L}^{-1}$  of 5-OH-17 $\alpha$ -TBOH has the same peak area as  $10 \text{ ng L}^{-1}$  of 12-OH-17 $\alpha$ -TBOH).

219 Comparing direct injections of parents and photoproducts showed that photoproduct raw  
220 peak area response is considerably less than raw parent response (with no change in internal  
221 standard response): for 17 $\alpha$ -TBOH, photoproduct response was  $57 \pm 7\%$  of parent response; for  
222 17 $\beta$ -TBOH, photoproduct response was  $53 \pm 6\%$ ; for TBO, photoproduct response was  $78 \pm$

223 8%; and for ALT, photoproduct response was 11%+/-3% of parent response. [M+Na]<sup>+</sup> 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). 17 $\alpha$ -TBOH and 17 $\beta$ -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

245 observable parent left. However, in samples SPE concentrated prior to analysis, <11% reversion  
246 (<30% reversion for ALT-CAP-OH) to parent over 3-5 hours occurred due to sample processing  
247 time (see [Table 2](#)), resulting in some loss of analytical sensitivity. Given the dynamic system, we  
248 consider the losses during sample processing and analysis acceptable.

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

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

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

### 273 *3.7 Recoveries and Matrix Effects*

274 Matrix effects were calculated by comparing internal standard peak areas from (a) direct  
275 injection samples with (b) samples of SPE concentrated matrix (no analyte), in which analyte  
276 was added immediately before injection [29]. Recovery rates were calculated using 10 ng L<sup>-1</sup>  
277 spikes of analyte, added before extraction. [Table 2](#) shows matrix effects and recoveries for this  
278 method for each compound in river water or lake water. River water was found to have an  
279 insignificant matrix effect (i.e. peak areas did not change as a result of concentrating river water  
280 during SPE), but lake water concentration resulted in a 10-30% decrease in response (matrix  
281 suppression). Recovery rates (n=3) were above 103-125% for 17 $\alpha$ -TBOH and its photoproducts,  
282 105-119% for 17 $\beta$ -TBOH and 75-93% for its photoproducts, 95-116% for TBO and its  
283 photoproducts, and 88-116% for ALT and its photoproducts.

### 284 *3.8 Archived sample results*

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

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

#### 295 **4.0 Conclusions**

296 A multi-residue LC-MS/MS method coupled with photoreaction and SPE concentration  
297 was developed and optimized for the detection of trenbolone metabolites, altrenogest, and their  
298 related photoproducts. Initial efforts utilized filtered, circumneutral-pH river water, followed by  
299 method validation using the more complex matrix of Wapato Lake water to better mimic  
300 receiving waters. This method allowed for consistent detection of trenbolone metabolites,  
301 altrenogest, and related photoproducts, relying on a short sample preparation time (3 hours) to  
302 minimize product-to-parent reversion, and minimized variability (intra-day RSD <20% for 17 $\alpha$ -  
303 TBOH photoproducts). Using 1-10 ng L<sup>-1</sup> spike concentrations, most recoveries were 90-120%,  
304 and limited matrix suppression (<30%) was observed. Critically, method detection and  
305 quantification limits were at or below environmentally relevant concentrations (1-10 ng L<sup>-1</sup>  
306 concentrations) for all metabolites and all photoproducts, and calibration curves were linear  
307 across ~3 orders of magnitude. Further sensitivity and selectivity could be achieved with the use  
308 of a dedicated instrument to minimize inter-day variability and sodium adduct formation, or via  
309 the use of a longer LC gradient to improve separation of metabolite photoproducts, (at the  
310 expense of longer runtime and increased reversion potential). This method could be applied to  
311 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

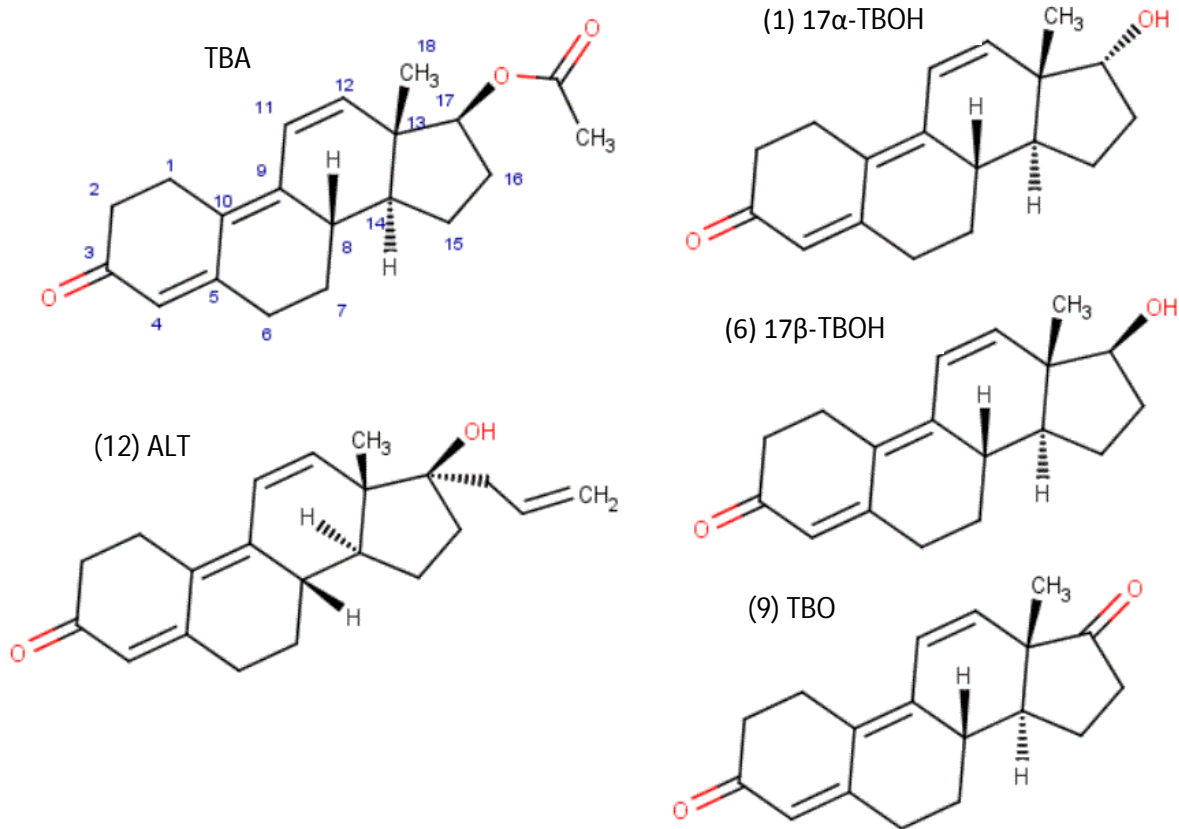
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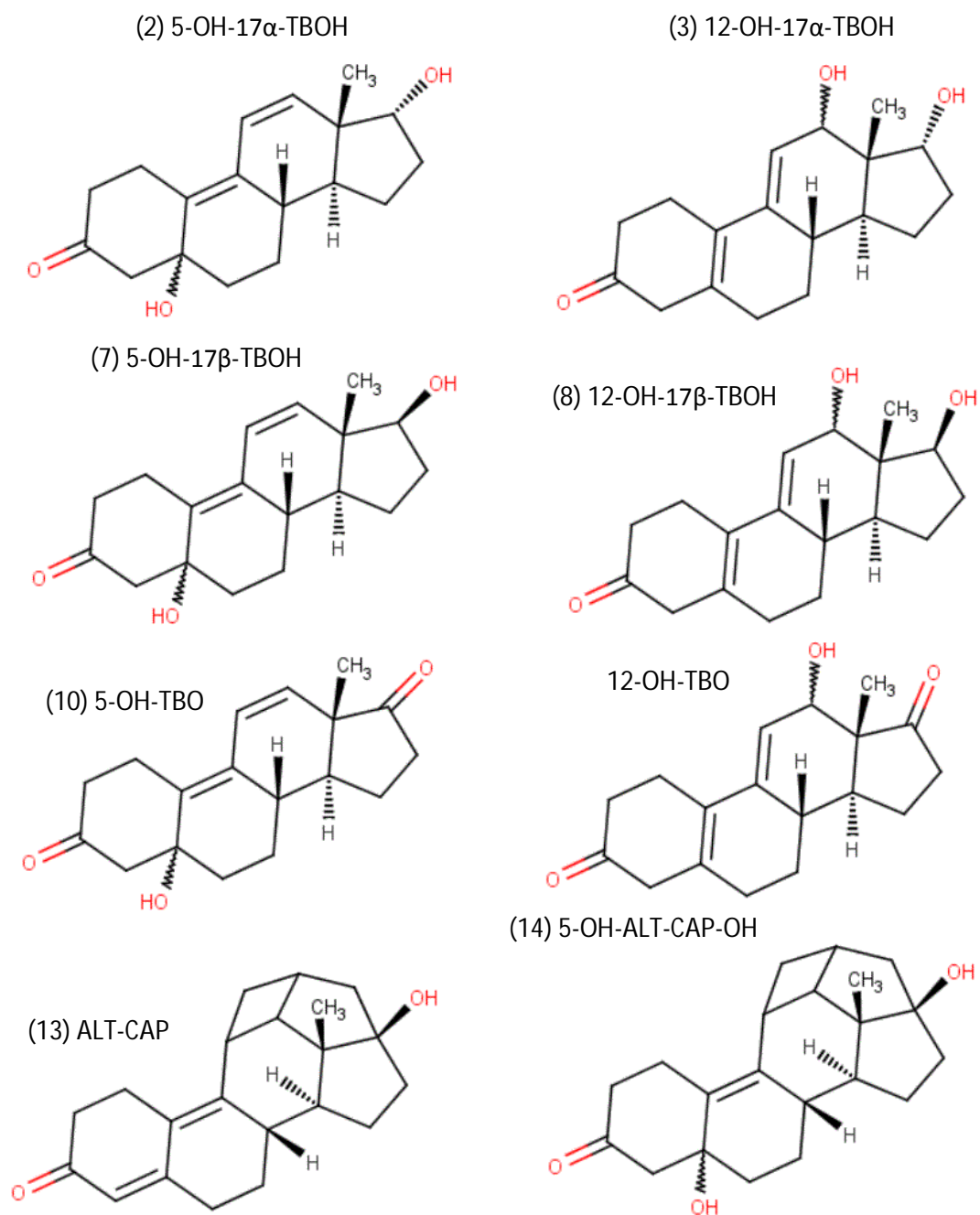
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410 **List of Figures**



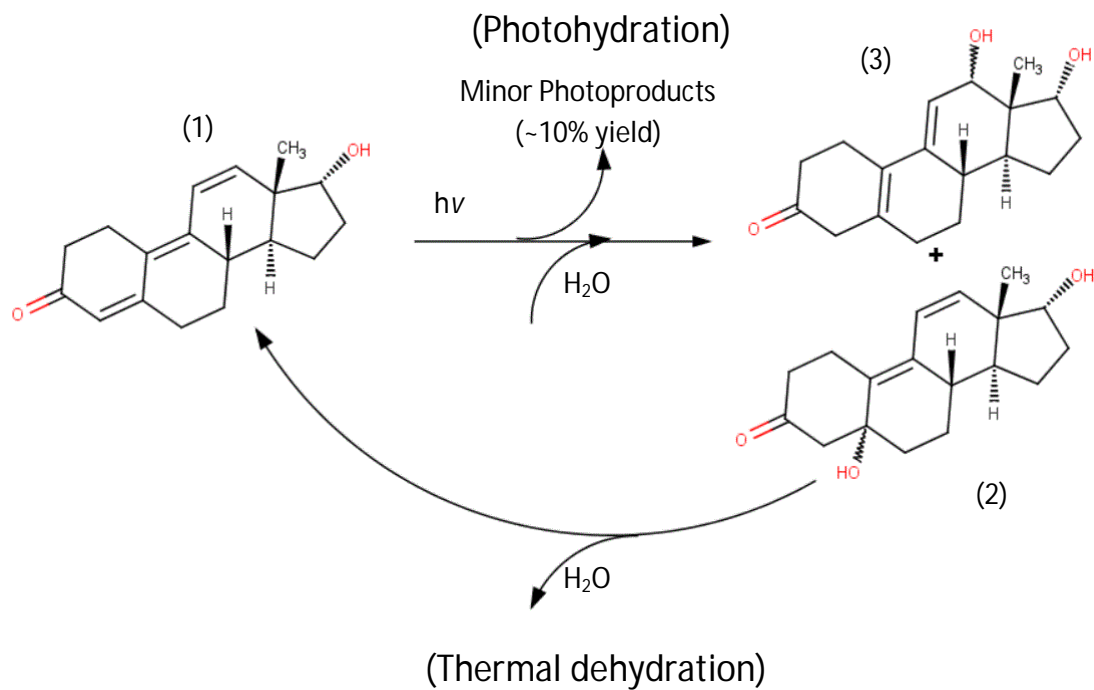
411

412 **Figure 1:** Structures of trenbolone acetate (with carbon numbering), 17 $\alpha$ -trenbolone, 17 $\beta$ -  
413 trenbolone, trenbolone, and altrenogest.



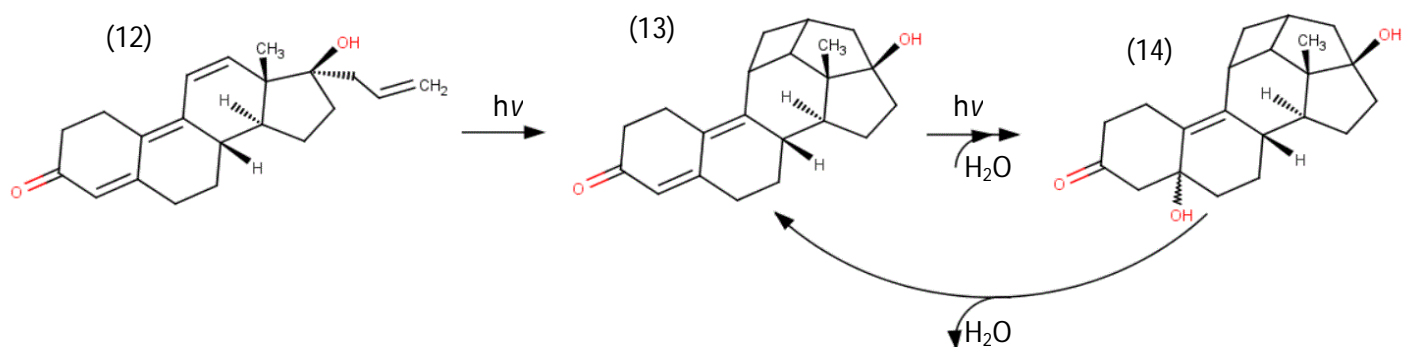
414

415 **Figure 2:** Chemical structures of the 5-hydroxy and 12-hydroxy photoproducts of 17 $\alpha$ -  
 416 trenbolone, 17 $\beta$ -trenbolone, and trendione, and the cyclo-addition and hydroxy-cyclo-addition  
 417 photoproducts of altrenogest.



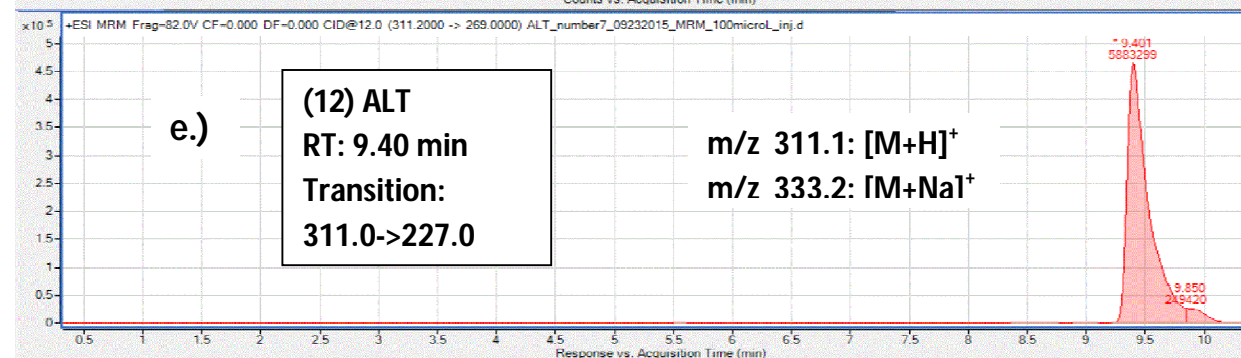
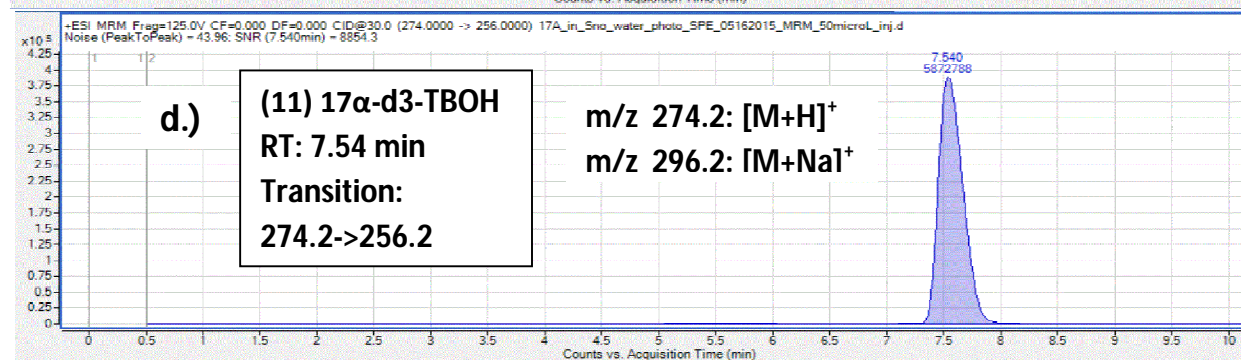
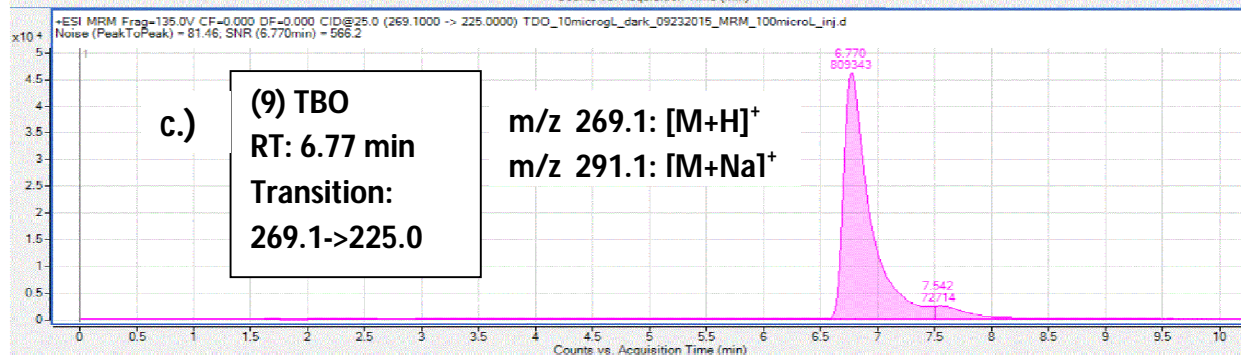
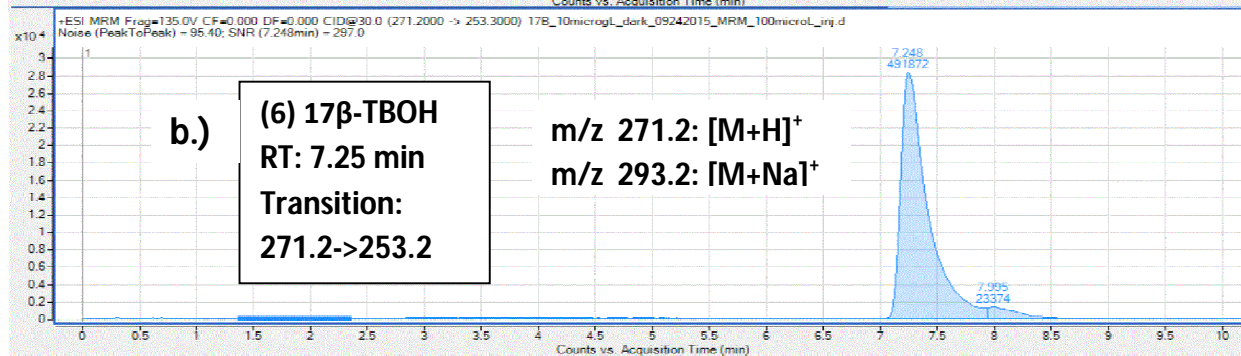
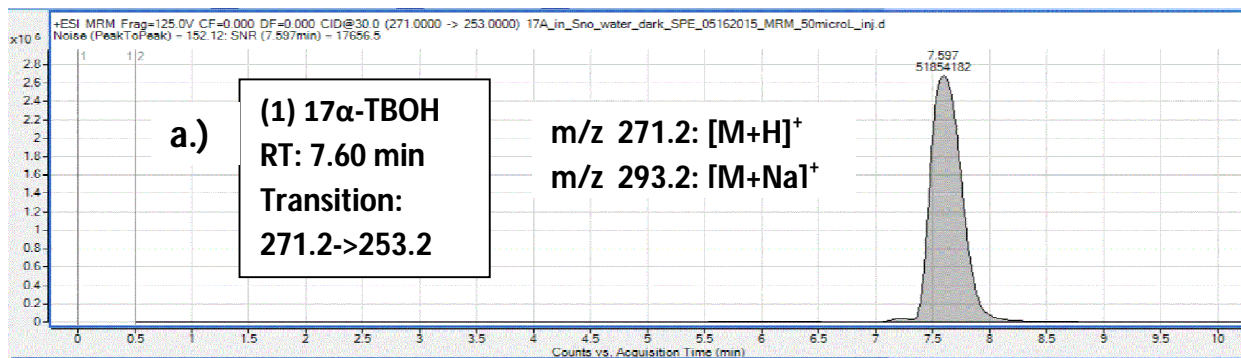
418

419 **Figure 3:** Coupled photohydration-thermal dehydration reversion process of trenbolone acetate  
 420 metabolites. Reaction shown for 17 $\alpha$ -TBOH, which also applies to 17 $\beta$ -TBOH, and TBO [17].



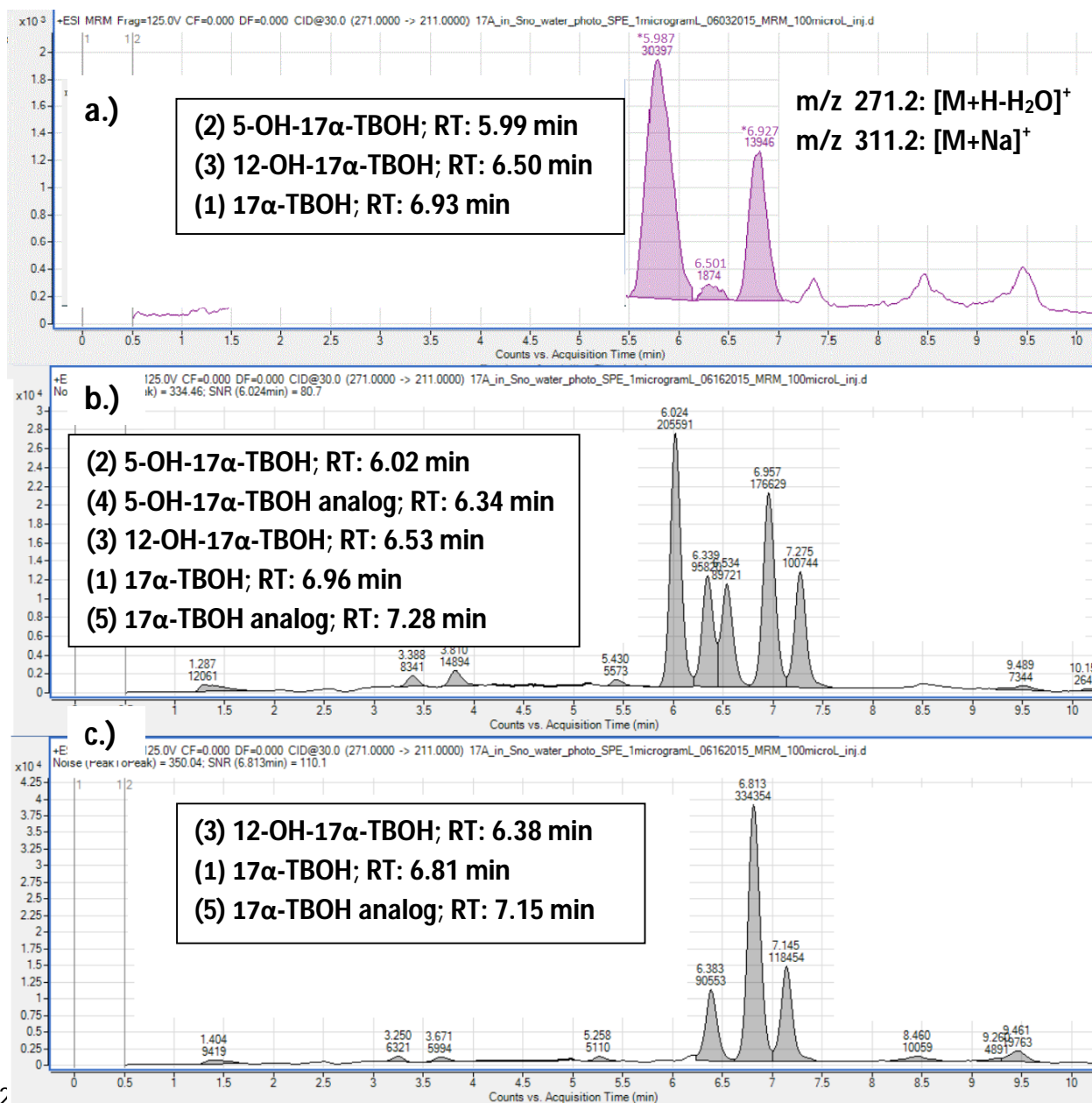
42

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].



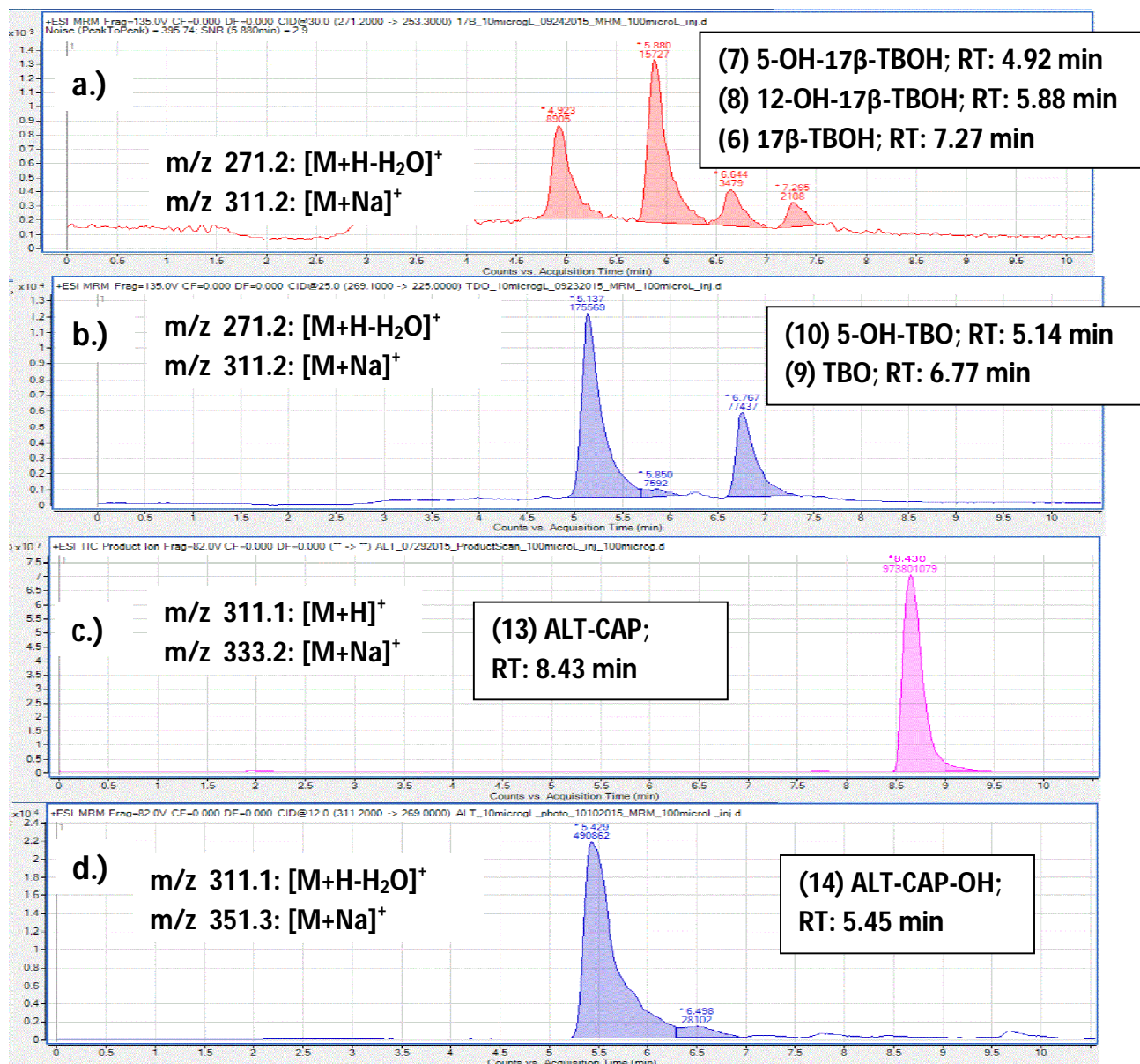


426 **Figure 5:** Chromatograms for a.), 17 $\alpha$ -TBOH, b.) 17 $\beta$ -TBOH, c.) TBO, d.) 17 $\alpha$ -d3-TBOH, and  
 427 e.) ALT, including retention time (RT), quantitative MRM transition, and most abundant mass-  
 428 to-charge ratios (m/z) for each. Concentrations for each are 10 ng/L.



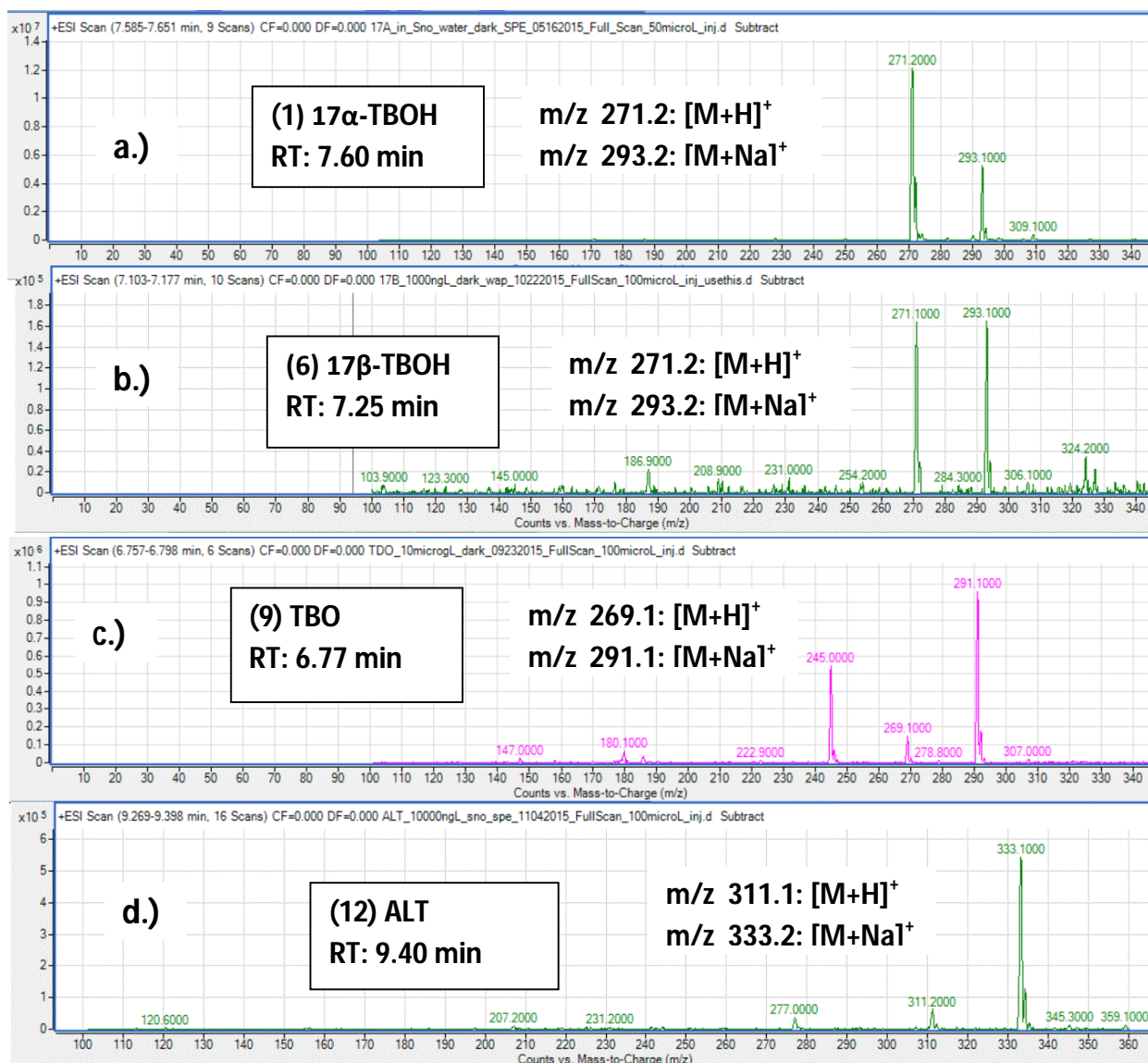
429 **Figure 6:** Chromatograms and Spectra for a.) 17 $\alpha$ -TBOH, photo-reacted, not solid phase  
 430 extracted, b.) 17 $\alpha$ -TBOH, photo-reacted, solid phase extracted, c.) same sample from b.) tested  
 431

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



437

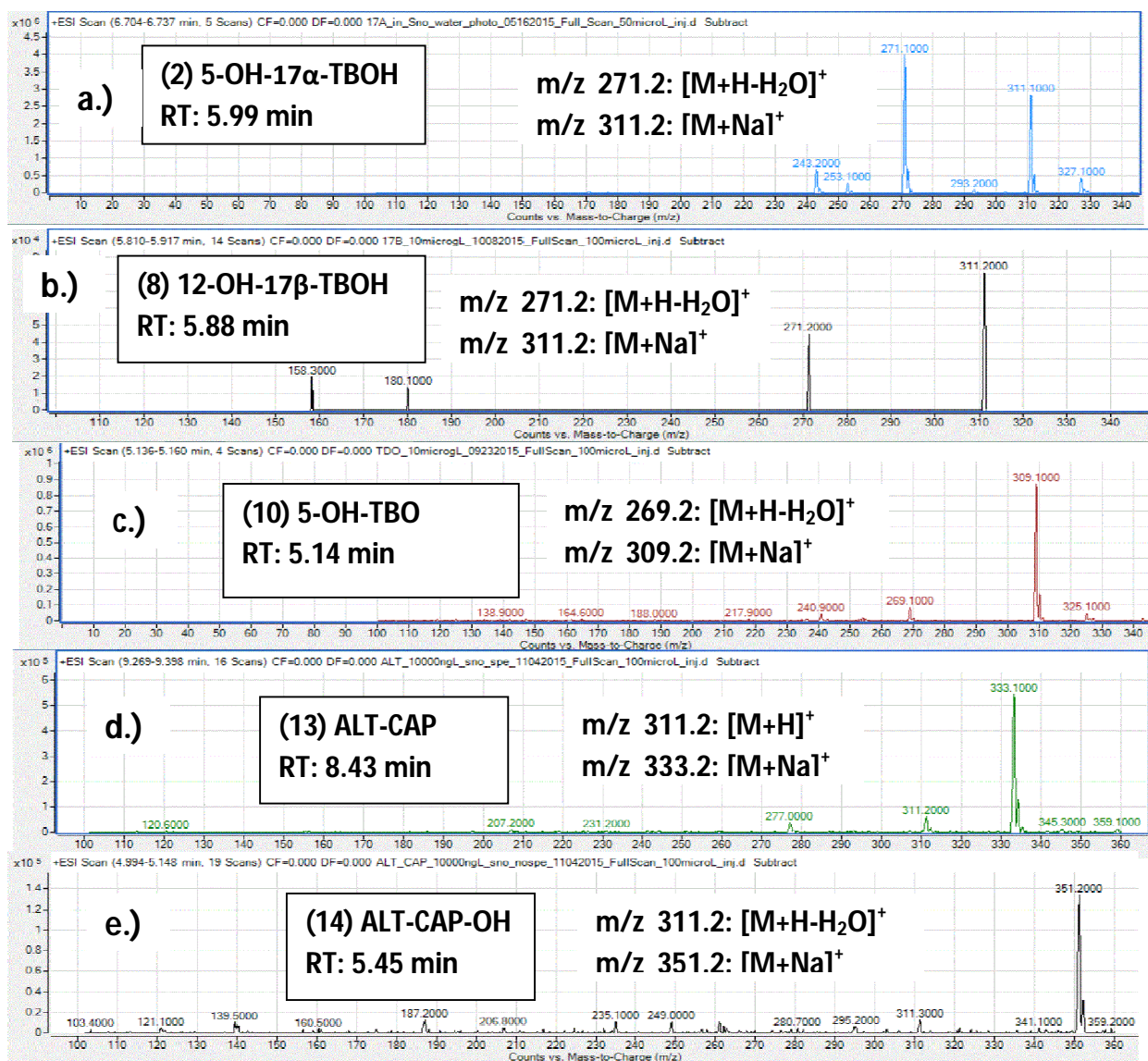
438 **Figure 7:** Chromatograms for a.) 17 $\beta$ -TBOH, photo-reacted, solid phase extracted, b.) TBO,  
 439 photo-reacted, solid phase extracted, c.) ALT, photo-reacted, acidified, solid phase extracted, and  
 440 d.) ALT, photo-reacted, solid phase extracted. Includes prevalent ions and their retention times  
 441 (RT), most common mass to charge ratios (m/z), and transition of each chromatogram. Figure 7a  
 442 shows m/z 271.2->211.2, Figure 7b shows m/z 269.1->169.0, Figure 7c shows m/z 311.2-  
 443 >227.0, and Figure 7d shows m/z 311.2->269.0.



444

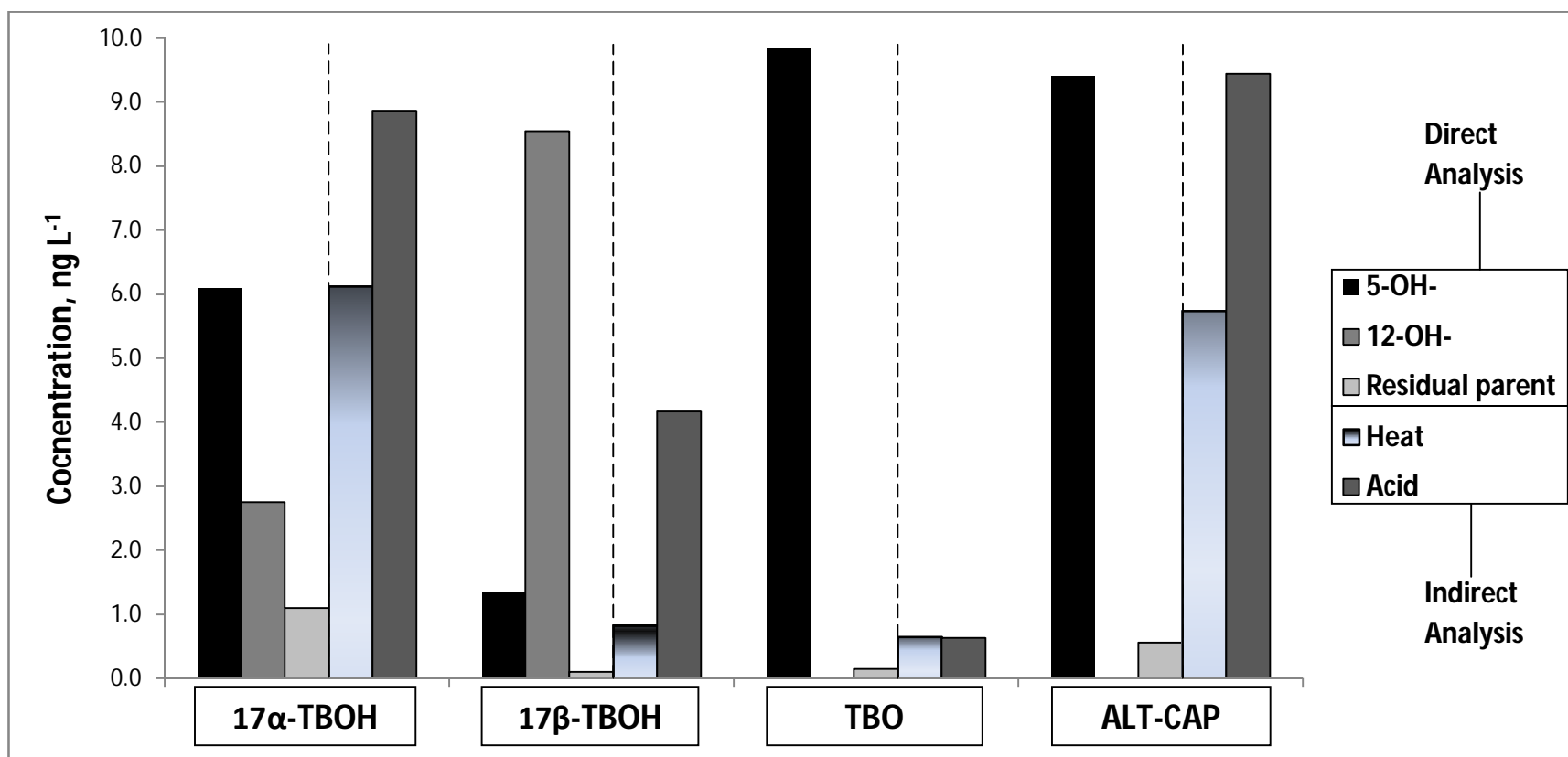
445

446 **Figure 8:** Spectra for a.)  $17\alpha$ -TBOH, b.)  $17\beta$ -TBOH, c.) TBO, and d.) ALT, including retention  
 447 time (RT) and most abundant mass-to-charge ratios (m/z) for each. Concentrations for each are  
 448 10 ng/L. Figure 9a shows spectra representative of compounds (2), (3), and (4), and Figure 9b  
 449 shows spectra representative of compounds (7) and (8).



450

451 **Figure 9:** Spectra for a.) 5-OH- $17\alpha$ -TBOH, b.) 12-OH- $17\beta$ -TBOH, c.) 5-OH-TBO, d.) ALT-  
 452 CAP, and e.) ALT-CAP-OH, including retention time (RT) and most abundant mass-to-charge  
 453 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<sup>-1</sup> spikes. Acidified samples were adjusted to pH~2 using HCl after  
 457 photoreaction. and heated samples were left in a 50° 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) 17 $\alpha$ -TBOH	7.55	271>253 (271>211)	135 (135)	30 (30)
(2) 5-OH-17 $\alpha$ -TBOH	6.62	271>211 (271>253)	135 (135)	30 (30)
(3) 12-OH-17 $\alpha$ -TBOH	7.13	271>211 (271>253)	135 (135)	30 (30)
(4) 5-OH-17 $\alpha$ -TBOH analog	6.93	271>211 (271>253)	135 (135)	30 (30)
(5) 17 $\alpha$ -TBOH analog	7.87	271>211 (271>253)	135 (135)	30 (30)
(6) 17 $\beta$ -TBOH	7.23	271>253 (271>211)	135 (135)	30 (30)
(7) 5-OH-17 $\beta$ -TBOH	4.87	271>211 (271>253)	135 (135)	30 (30)
(8) 12-OH-17 $\beta$ -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) 17 $\alpha$ -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<sup>-1</sup>) and  
 467 low (1 ng L<sup>-1</sup>) 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

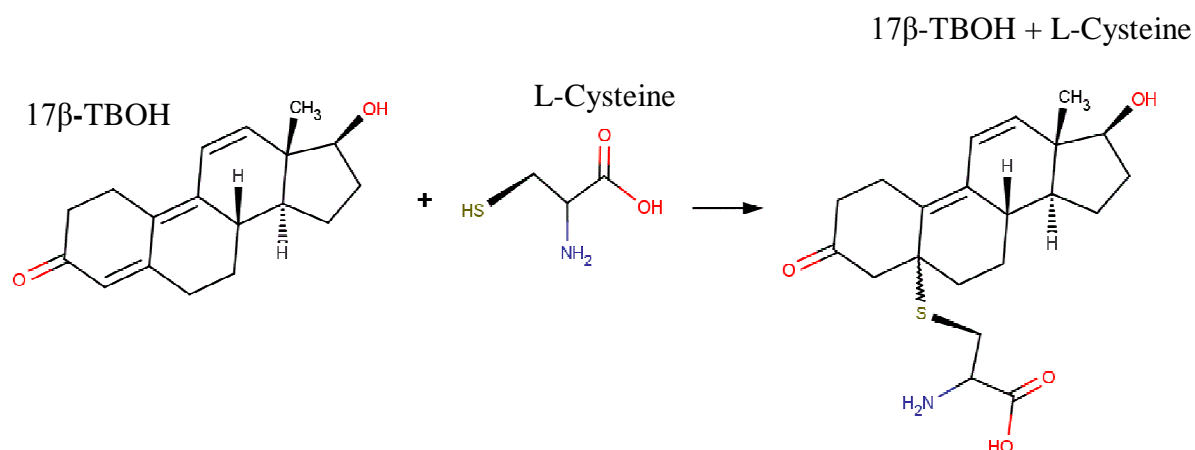
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	Compound	Solvent	MDL (ng/L)	MOQ (ng/L)	Slope	Intercept	R <sup>2</sup>	Matrix Effect	Recovery	RSD (low spike)	RSD (high spike)	Reversion
(1)	17 $\alpha$ -TBOH	river water	<b>0.03</b>	<b>0.04</b>	0.075	-0.053	0.999	100%	115%	10%	14 %	N/A
		lake water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

## 474 Chapter 3: Thiol Adduct Formation

### 475 Introduction

476 Previous research at the University of Nevada, Reno discovered a potential adduct  
477 between 17 $\beta$ -TBOH and the amino acid L-cysteine during experiments to determine the rates of  
478 anaerobic biodegradation trenbolone metabolites under anaerobic and anoxic conditions.  
479 Observations of controls of 17 $\beta$ -TBOH in microcosms reduced with L-cysteine indicated that the  
480 17 $\beta$ -TBOH would rapidly disappear. Subsequent tests via diode-array-detector HPLC strongly  
481 suggested that 17 $\beta$ -TBOH (peak absorbance 350 nm<sup>10</sup>) and L-cysteine (peak absorbance 250  
482 nm<sup>31</sup>) formed a thiol adduct likely via Michael addition<sup>32</sup> (see Figure 2).



484 **Figure 2:** Possible pathway for thiol-adduct formation between 17 $\beta$ -TBOH and L-cysteine.

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



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

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

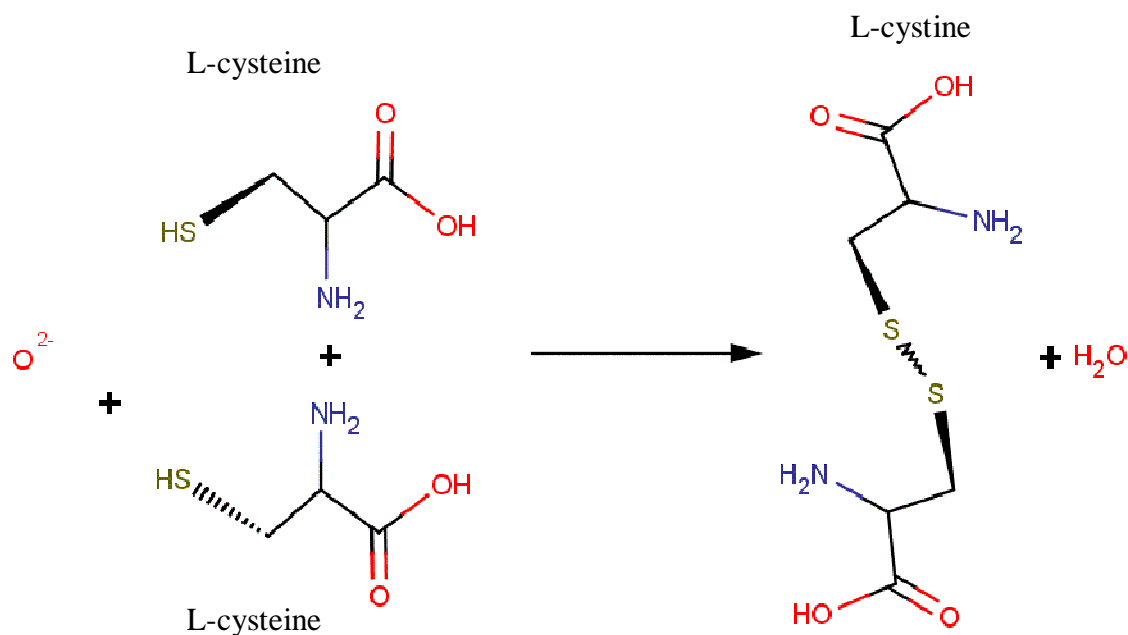
#### 498 **Research Objective**

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

#### 502 **Experimental**

503 Samples were processed in an anaerobic glove box (nitrogen filled) to minimize potential  
504 oxidation of L-cysteine to L-cystine (see Figure 3). Inside the glove box, a single sample was  
505 created comprised of an aqueous solution split into three 250 mL glass bottles, (each baked at  
506 100° C for an hour prior to use). The solution was prepared in nitrogen-sparged water (NSW) by  
507 adding approximately 246 mL of NSW to the bottle, adding 2.0 mL of a 2500 mg L<sup>-1</sup> methanolic  
508 stock of 17β-TBOH to the solution, then adding L-cysteine-anhydride (89 mg) to the solution,  
509 and adding 2 mL of 1 M aqueous NaOH solution to the bottle (20 mg L<sup>-1</sup> 17β-TBOH and 357 mg  
510 L<sup>-1</sup> L-cysteine (40:1 L-cysteine:17β-TBOH)). Samples were reacted for 48 hours in the glove

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



514

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

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

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

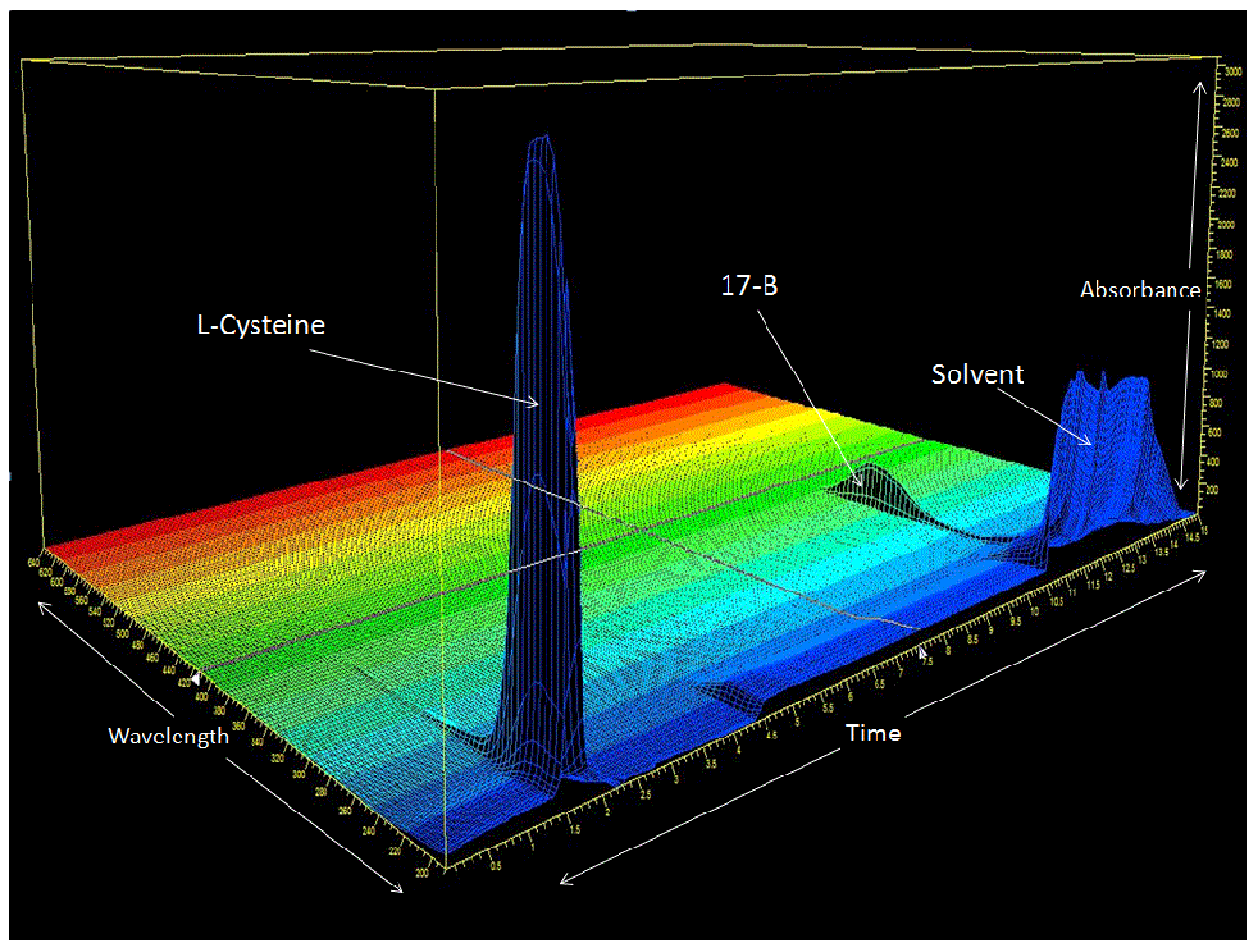
## 528 **Deaerating Water**

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

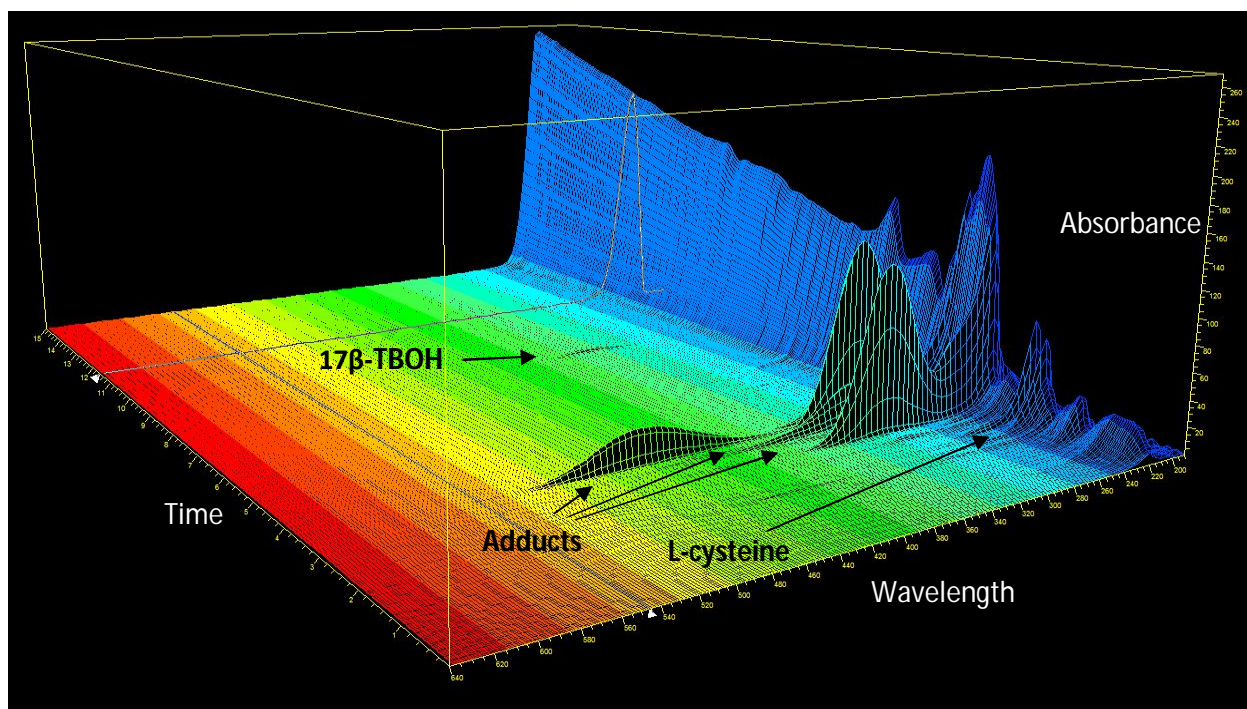
## 538 **Adduct Formation**

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

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



548  
549 Figure 4: 3-dimensional chromatogram showing L-cysteine (retention time ~2 min, peak  
550 absorbance 220 nm) and 17 $\beta$ -TBOH (retention time ~11.5 min, peak absorbance 346 nm),  
551 measured via HPLC-DAD, prior to reaction.



552

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

### 556 **Adduct Stability**

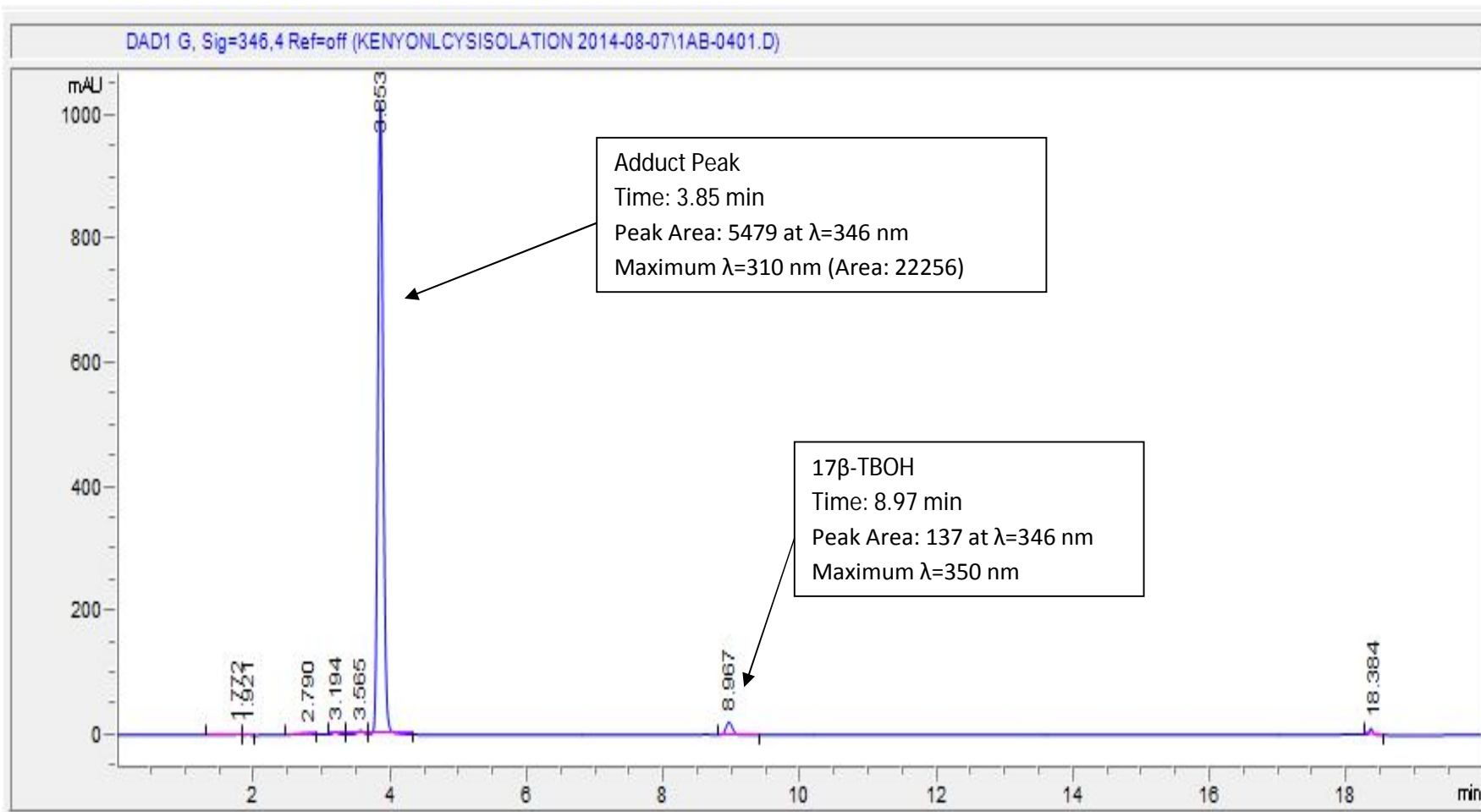
557 To explore whether adduct reversibility could regenerate trenbolone, solution conditions  
 558 were changed in adduct samples post fractionation. After fraction collection, two vials (totaling  
 559 approximately 30 mL of solution) of product were isolated from the prep-scale HPLC, and split  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

564           Neither aerobic conditions alone, nor combined with heating or alkaline conditions  
565 altered adduct stability (see Figures 6-8), but aerobic, alkaline conditions with subsequent  
566 heating did regenerate 17 $\beta$ -TBOH (see Figure 9). However, analysis of peak areas showed that  
567 while a decrease in adduct peak did occur, it did not decrease proportionally to the amount of  
568 17 $\beta$ -TBOH which reappeared, suggesting insufficient analytical sensitivity or an unidentified  
569 source of complexed 17 $\beta$ -TBOH. It is also possible that 17 $\beta$ -TBOH has a lesser peak area  
570 response factor in UV-DAD than its adduct with L-cysteine, such that the small difference in the  
571 adduct peak could correspond to the increase in 17 $\beta$ -TBOH.

## 572 **Conclusions**

573           Regarding the first research objective (a single method to detect all metabolites and  
574 photoproducts of trenbolone acetate, as well as altrenogest and its photoproducts), a method was  
575 successfully developed. With separation, simultaneous detection is possible, however due to  
576 coelution between metabolites and photoproducts of 17 $\alpha$ -TBOH and 17 $\beta$ -TBOH in fast  
577 chromatography runs (<20 min), accurate quantification in mixed residue systems can be  
578 compromised. Fortunately, 17 $\alpha$ -TBOH is typically the dominant metabolite present, and thus  
579 17 $\beta$ -TBOH, TBO, and related photoproducts would usually be expected to occur at lower  
580 concentrations as compared with 17 $\alpha$ -TBOH.

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

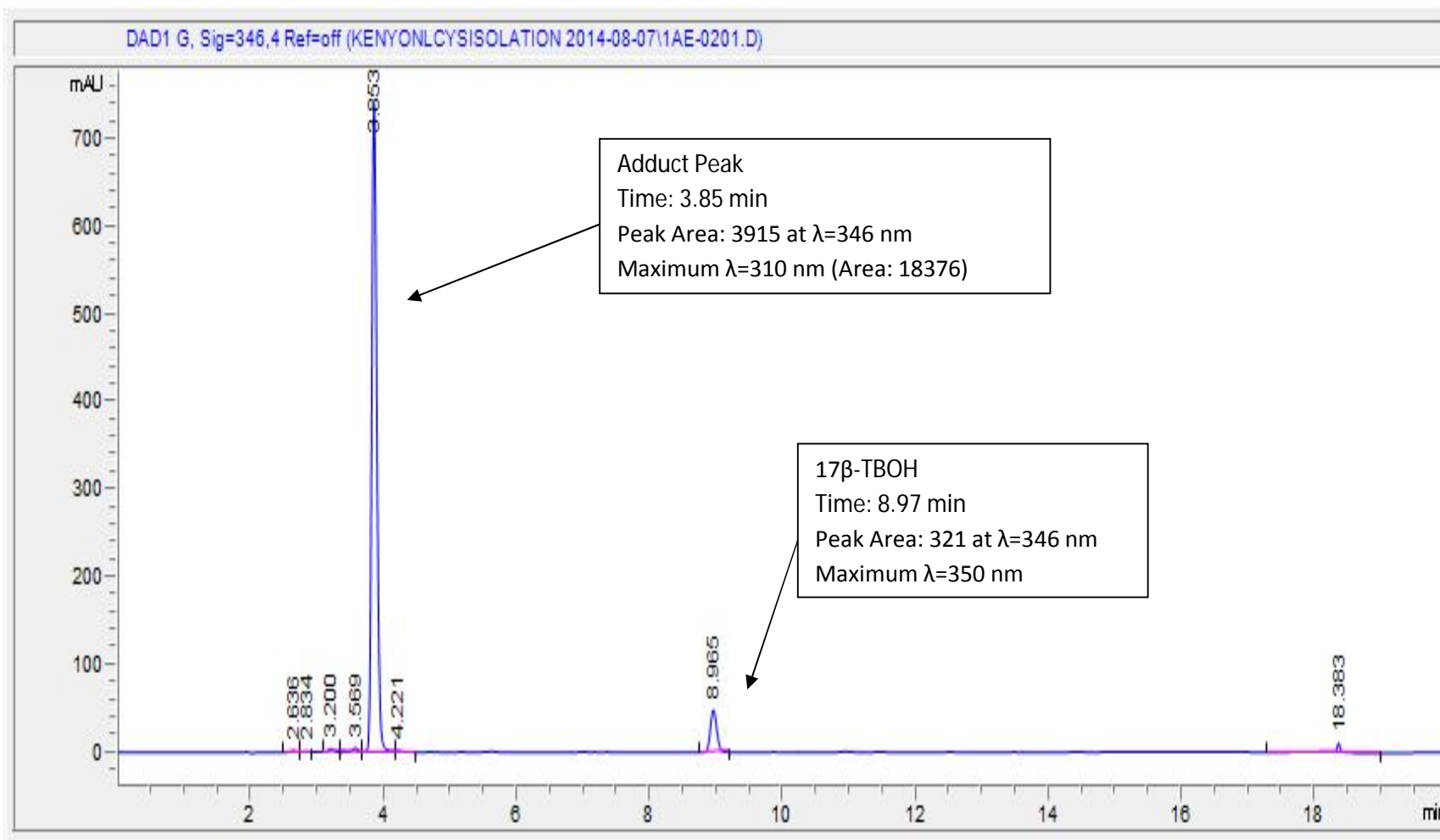


586

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

588

589

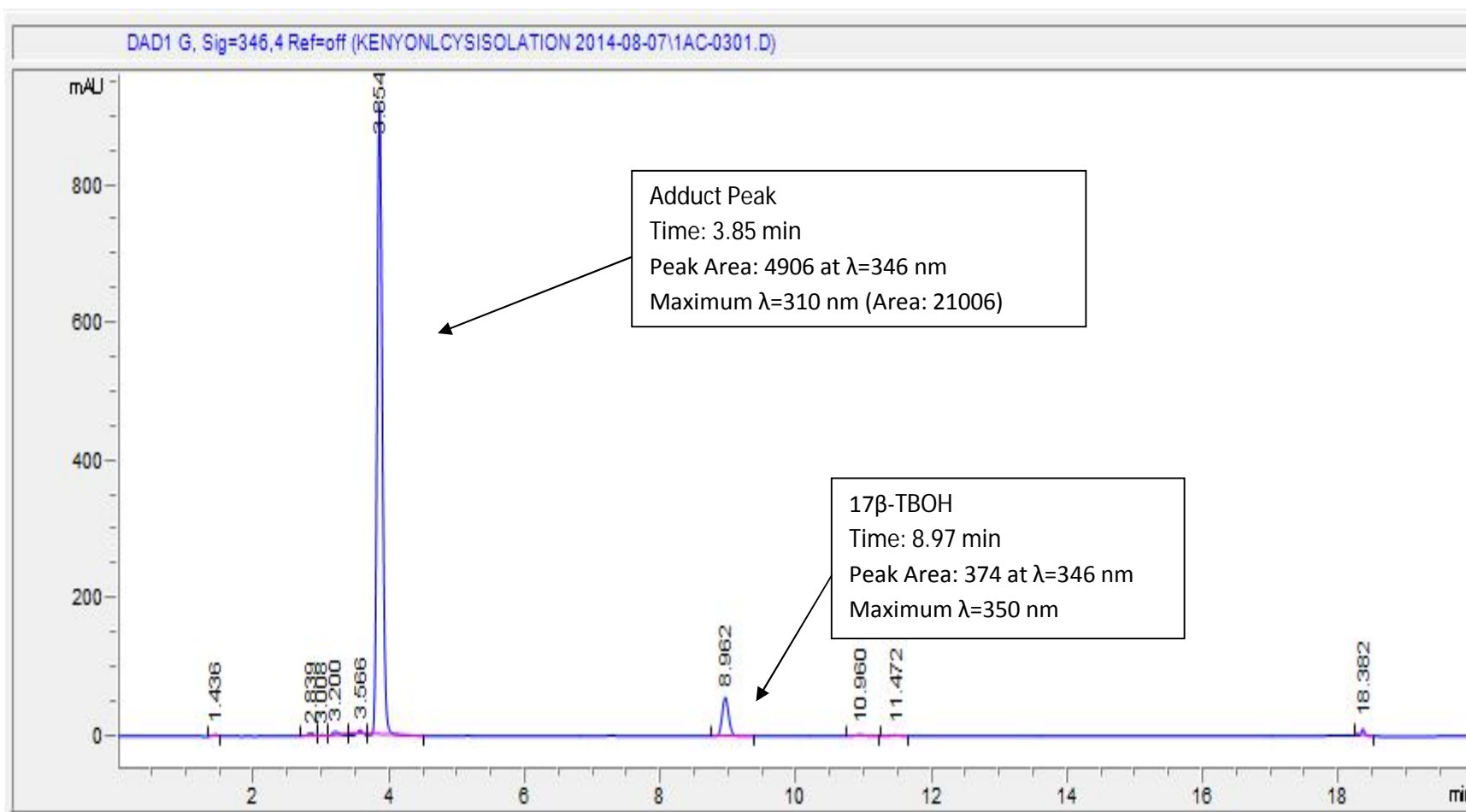


590

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

592

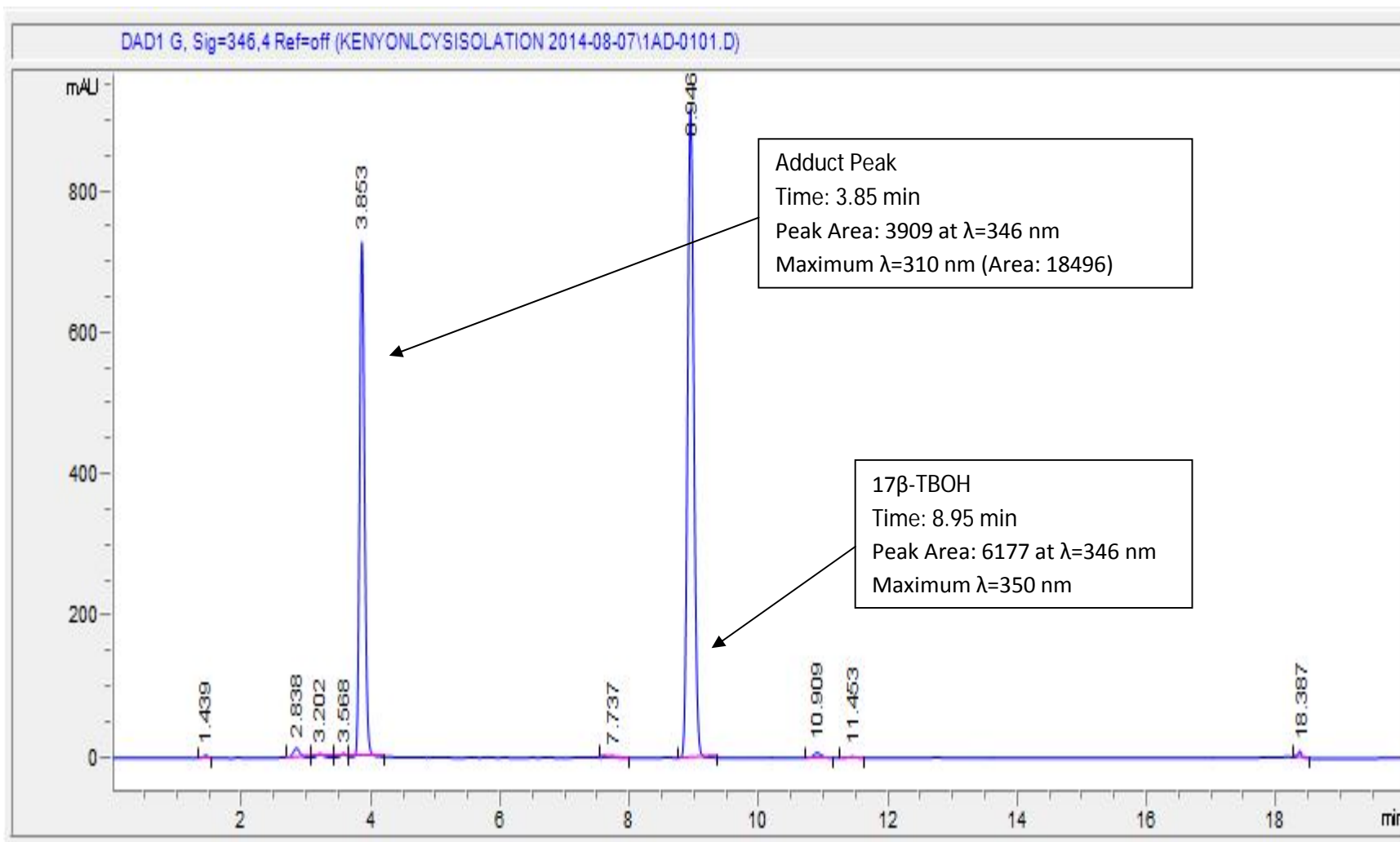




593

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

595



596

597 Figure 9: Fraction-collected L-cysteine-17 $\beta$ -TBOH adduct, under alkaline (pH=13) conditions, then heated (80 $^{\circ}$  C water bath for 2

598 hours) .

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