University of Nevada, Reno

# Analytical Method Development for the Detection of the Metabolites of the Hormonal Steroids Trenbolone Acetate and Melengestrol Acetate in Complex Environmental Matrices using Gas Chromatography / Tandem Mass Spectrometry

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by

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# Abstract

The occurrence of hormonal steroids originating from cattle implanted with anabolic growth promoters in surface waters is of great consequence to sensitive aquatic species. Exposure to excreted metabolites of trenbolone acetate, the most prevalently used growth promoter in the U.S., has been shown to have deleterious, androgenic effects on sensitive aquatic species, such as decreased fecundity and altered secondary sexual characteristics. The potential risk of this exposure is population collapse in isolated, sensitive populations. These effects have been observed when exposure concentrations to trenbolone metabolites are as low as 0.3 ng/L.

In light of observed effects on aquatic organisms, a number of analytical techniques have been developed to detect trenbolone metabolites in environmental matrices. Such techniques include bio-assays and liquid or gas chromatography paired with mass and tandem mass spectrometry. While there are existing liquid chromatography / tandem mass spectrometry methods, to date, there are no gas chromatography / tandem mass spectrometry methods which detect the trenbolone acetate metabolites, 17α-trenbolone, 17β-trenbolone and trendione in environmental matrices. The research presented describes such an analytical method for environmental matrices.

The analytical method, presented here, draws on gas chromatography paired to mass spectrometry and tandem mass spectrometry methods developed for 17α- and 17β-trenbolone detection in biological matrices, and extends them to more complex environmental matrices. In addition, this method attempts to incorporate the third trenbolone acetate metabolite, trendione and the melengestrol acetate metabolite, melengestrol. Trendione was added to complete the family of trenbolone acetate metabolites, allowing for the possibility of conducting fate and

transport studies. Melengestrol was added to the method to examine the possibility of utilizing the established MSTFA/$I_2$ derivatization procedure for other classes of steroids such as progestagens.

Results indicate that 17α- and 17β-trenbolone can be recovered from environmental matrices with excellent chromatographic response at or below a concentration of 2.5 ng/L. Trendione, owing to the possible degradation of the derivatized product, has shown more difficulty with recoveries in environmental matrices. It is theorized that stabilizing trendione after derivatization or analyzing samples within 48 hours will allow for recoveries similar to that of 17α- and 17β-trenbolone. Attempts at matrix spike recoveries with melengestrol limited its detection in environmental matrices to 100 ng/L. An observed standard concentration of 20 µg/L was successfully analyzed for melengestrol which leads to the possibility that it can be analyzed at much lower concentrations within the GC/MS/MS. As with trendione, if a stable derivatization product can be formed for melengestrol, it is expected that it can be recovered in environmental matrices at relevant concentrations of 2.5 ng/L and below.

*The following is dedicated to my brother Wells... No longer with us, his presence is, and always will be.*

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# 1 Introduction

## 1.1 Endocrine Disrupting Compounds (EDCs)

The United States Environmental Protection Agency (EPA) defines an endocrine disruptor as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action and elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development and behavior" (United States Environmental Protection Agency, 2001). More broadly defined, an endocrine disruptor can be thought of as any chemical compound which, after exposure, produces a physiological effect in an organism which is contrary to normal endocrine operation.

One example of an endocrine disruptor is the pesticide DDT (Dichloro-Diphenyl-Trichloroethane) which was heavily used in the United States until it was banned by the EPA entirely in 1988 for all uses (United States Environmental Protection Agency, 2001). Research has shown that EDCs comprise a wide range of anthropogenic substances including surfactants, plasticizers, organohalogens and pesticides (United States Environmental Protection Agency, 2001). These compounds are found in common consumer products such as plastic water bottles and cosmetics. The use of compounds with endocrine disrupting potential in society is ubiquitous, and as such, they can be expected to appear in the aquatic environment as well.

An example of endocrine disruption in the an aquatic environment was documented when common free ranging male northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) in agricultural drains were physiologically compared to similar frogs living in habitats with little or no agricultural water input. McDaniel *et al.*, (2008) observed that male frogs living in areas with agricultural input exhibited ovarian follicles, a physiological trait reserved strictly for female frogs. Those male frogs living outside of agricultural exposure showed no signs of

ovarian follicles, leading to the conclusion that the agricultural water, likely pesticide contaminated, had caused endocrine disruption in the exposed male frogs.

While McDaniel *et al.*, (2008) illustrated that only a small population of male frogs exhibited feminization after exposure to commonly used pesticides, other studies have reported drastic population effects under exposure to environmentally relevant concentrations of hormonal steroids. Kidd *et al*., (2007) demonstrated that an entire population of isolated fathead minnows collapsed when exposed to a constant 5 ng/L concentration of the synthetic estrogen 17α-ethynylestradiol. Over a period of three years, male fathead minnows were feminized to the point of an inability to produce sperm, nearly causing extinction of the isolated population.

### 1.1.1 Initial Reports of Estrogenic Contaminants

One class of naturally and synthetically produced compounds which are inherent in endocrine processes is hormonal steroids. These compounds are responsible for normal endocrine operation in all vertebrate organisms. Some hormonal steroids are responsible for maintaining male sexual characteristics (androgens) while others are responsible for maintaining female sexual characteristics (estrogens). Endogenous hormones are those which are naturally produced and utilized by organisms. Estradiol and testosterone are well known examples of an endogenous estrogen and androgen.

This study focuses particularly on exogenous hormones, which are synthetically produced. Exposure of an organism to an exogenous hormone has the potential for endocrine disruption because the hormone mimics the structure of an endogenous steroid and binds to receptors which then produce a physiological result in the organism. Because the exposure was not endogenous, or intended by the organism, the physiological result has the potential to negatively impact endocrine operation within the organism.

One notable and prominent estrogen is 17α-ethynylestradiol, whose chemical structure is given in Figure 1-1. 17α-ethynylestradiol is a synthetic oral contraceptive used by over 80 million women worldwide (Wild, 1993).

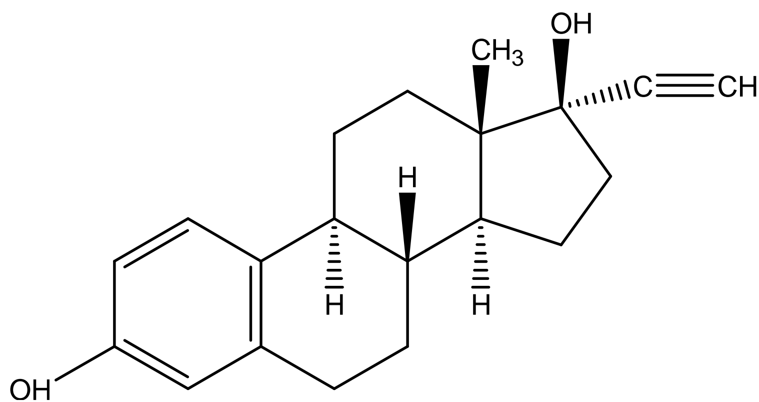


**Figure 1-1: Chemical Structure of 17α-ethynylestradiol (Fine *et al.*, 2003)**

The relatively strong chemical stability of 17α-ethynylestradiol and other exogenous steroidal hormones results in their appearance in wastewater treatment effluents in trace, yet ecologically significant levels (Lorenzen *et al.*, 2006). Because of the widespread use of 17α-ethynylestradiol, general scientific concern has been raised about its effects in the natural environment (Purdom *et al.,* 1994). It was proposed by Purdom *et al*., (1994) in the early 1990s that the occurrence of hermaphrodite fish living downstream of wastewater treatment effluents were the result of an estrogenic compound exposure.

To evaluate this hypothesis, rainbow trout (*Oncorhynchus mykiss*) of both sexes were kept in cages and exposed to wastewater treatment effluent over a 30 to 90 day exposure period. A bio-assay was used to evaluate feminization of male rainbow trout exposed to 17α-ethynylestradiol by measuring how vitellogenin concentrations in male fish correlated with increasing hormone exposure. Vitellogenin is a yolk precursor found in all egg laying animals

(Clemens, 1978), and its presence in male fish would indicate that the fish were exposed to estrogenic contaminants.

Purdom *et al*., (1994) demonstrated that male fish exhibited vitellogenin increases ranging from 500 to 100,000 fold, depending on sample site and exposure conditions. It was hypothesized that 17α-ethynylestradiol, or other estrogenic steroids, were the compounds most likely to have caused vitellogenin induction. To evaluate whether 17α-ethynylestradiol could be responsible, additional experiments were conducted using male rainbow trout that were injected with increasing doses of 17α-ethynylestradiol. It was found 17α-ethynylestradiol exposure doses as low as 1 ng/L could generate similar responses to those seen in the fish caged in sewage treatment effluent (Purdom *et al.*, 1994).

17α-ethynylestradiol is not the only steroidal compound to have shown endocrine disrupting potential, other estrogens such as 17β-estradiol, estrone and estriol can also act as EDCs. Irwin *et al.*, (2001) demonstrated that 17β-estradiol; an endogenous estrogen, has the potential to increase vitellogenin in male painted turtles (*Chrysemys picta*), as well as fish. Subsequent studies have examined estrone and estriol for their estrogenic effects on sensitive aquatic species (Johnson *et al*., 2000 and Jobling *et al*., 2003), both concluding that trace concentrations of these compounds induce sexual feminization of males. It is important to note that while significant progress has been made in researching potential estrogenic hormonal steroids and their effect on aquatic species, much less is known regarding the endocrine disrupting potential of non-estrogenic contaminants.

### 1.1.2 Androgenic Contaminants

As there are both endogenous and exogenous hormonal steroids that are estrogenic in the natural environment, there also exist androgenic hormonal steroids which can induce

masculinization or other androgenic effects in sensitive aquatic species. For instance, Howell *et al*., (1980) observed in 1980 that a population of mosquitofish (*Gambusia affinis holbrooki*) living downstream of a paper mill effluent exhibited abnormal sexual characteristics. Female mosquitofish were found to be strongly masculinzed, having physical secondary sex characteristics and showing some reproductive behavior of males. Conversely, juvenile male mosquitofish were shown to exhibit secondary sex characteristics of adult males, an indication of abnormally rapid maturation, and possible exposure to androgens. To illustrate that androgenic compounds in the effluent were responsible, female mosquitofish in the stream above the paper mill effluent and those in other control streams were shown to have normal sexual expression and behavior, with no sign of masculinization.

Denton *et al*., (1985) continuing on the research of Howell *et al*., (1980) demonstrated in laboratory experiments that microbially degraded plant sterols (beta-sitosterol and stigmastanol) induced masculinization in exposed mosquitofish. Microbially degraded plant sterols, which are present in pulp and paper mill effluents, have been implicated in other studies which have looked at the masculinization of sensitive species (Cody *et al.*, 1997 and Larsson *et al.*, 2000). Recent research has introduced the possibility that microbially derived androstenedione and androstadienedione could also be implicated in observed masculinzation of fish living downstream of pulp and paper mill effluents (Bandelj *et al.,* 2006).

Studying the masculinization of spawning channel catfish (*Ictalurus punctatus*) living in the Red River of the North, Hegrenes, (1999) observed that female catfish caught below sugar beet processing effluent and sewage treatment effluent exhibited secondary sex characteristics of male fish such as enlarged muscles on the surface of the head. Research by Hegrenes (1999), Denton *et al*., (1985) and Howell *et al*., (1980) and others demonstrated that androgenic compounds could occur in point sources such as waste water treatment effluent and pulp mill

effluent, and have potential significant ecological effects. While pulp and paper mill effluents have been shown to be have androgenic effects when introduced into the environment, recent research has been conducted on androgenic activity originating from agricultural operations. These studies have focused on the anabolic compound 17β- trenbolone, often used as a growth promoter in animals.

### 1.1.3 Trenbolone as a Potent Androgenic EDC

In a study examining androgenic, endocrine-disrupting effects in agricultural applications, Orlando *et al*., (2004) observed that wild fathead minnows exhibited altered reproductive biology when exposed to cattle feedlot effluent. For instance, male fish exhibited lower testicular testosterone synthesis, altered head morphometrics and smaller testis size after exposure. Female fish exhibited a decreased estrogen to androgen ratio of *in vivo* steroid hormone synthesis, an indication of exogenous androgenic exposure. The observations of altered reproductive biology in both sexes led to the possible conclusion that within feedlot effluent, there is likely a mix of androgenic and estrogenic substances. Orlando *et al.,* (2004) became the landmark study that would spawn research into what hormonal compounds are present in confined animal feeding operation (CAFO) lagoon effluents and how they enter into and persist in the natural environment. Orlando *et al.,* (2004) hypothesized that 17α- and 17β-trenbolone were likely the potent compounds causing the androgenic endocrine disruption that was observed.

One of the first studies to document the effects of trenbolone exposure on sensitive aquatic species, Ankley *et al*., (2003) demonstrated that fecundity of the fathead minnow was significantly reduced with a 21-day exposure to 17β-trenbolone at a concentration of 0.027 μg/L. A 0.027 μg/L concentration of 17β-trenbolone caused the appearance of male secondary sexual

characteristics in female fathead minnows (Ankley *et al*., 2003). In another study, Jensen *et al*., (2006) examined 17α- trenbolone under similar exposure conditions using the fathead minnow. Similar results were demonstrated, with an exposed concentration of 0.011 μg/L to 17α-trenbolone causing a significant reduction of fecundity in female fathead minnows. Other studies have observed similar results in other aquatic species such as the female mosquitofish (Sone *et al., 2005).*

The effects of 17β-trenbolone exposure on mosquitofish were investigated by Sone *et al*., (2005). Male and female mosquitofish, both adults and newborn fry, were exposed to 17β-trenbolone concentrations ranging from 0.1-10 μg/L for 28 days. In the mosquitofish, significant sexual changes were observed when the fish were exposed to a 17β-trenbolone concentration of 0.3 μg/L. All female fry exposed to a 17β-trenbolone concentration of 1 μg/L showed the growth of ovotestes, growth normally reserved only for male fry, and also observed an acceleration of testicular development in the majority of male fry (Sone *et al*., 2005).

17α-trenbolone, 17β-trenbolone and trendione occur in the aquatic environment due to the use of 17β-trenbolone acetate (TBA), a synthetic anabolic steroid which is used as a growth promoter throughout the United States and Canada. It has been estimated by the United States Department of Agriculture that approximately 90% of the beef cattle raised in the U.S. receive at least one TBA implant during their lives (USDA, 2000). While TBA is the compound given to cattle via implant, its metabolites 17β-trenbolone (the active form), 17α-trenbolone and trendione are subsequently found in animal wastes and introduced to the environment. The hypothesized path of TBA metabolism is given in Figure 1-2 (Schiffer, *et al., 2001).*

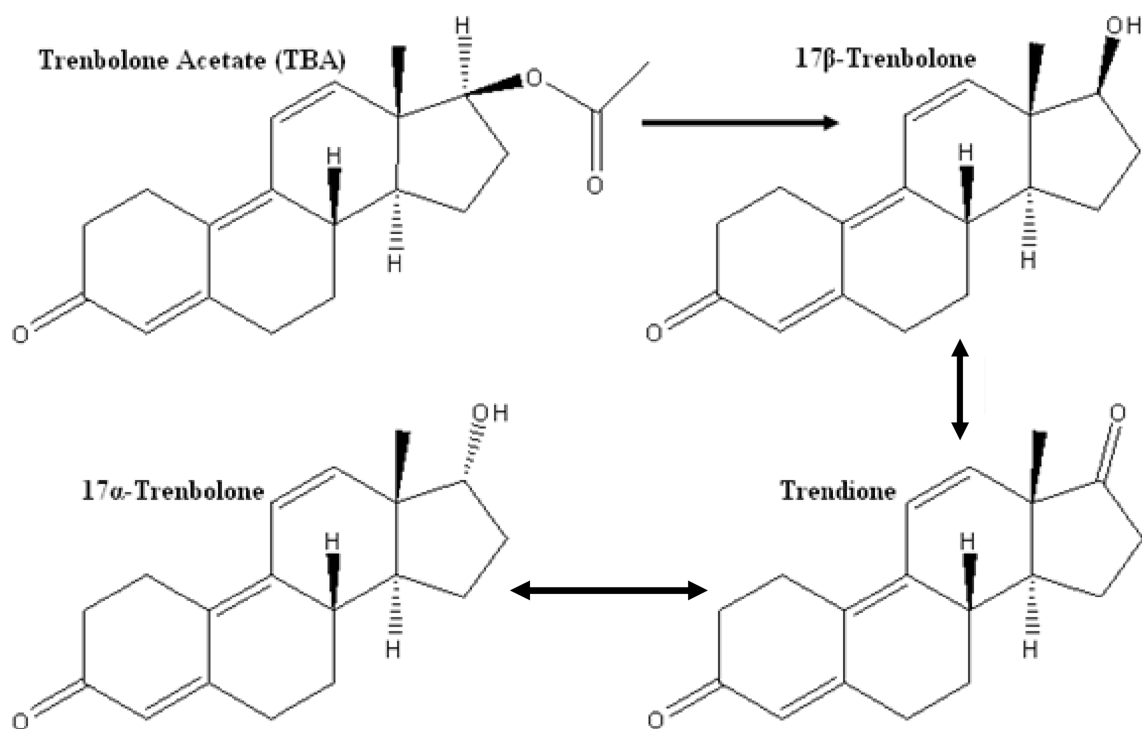


**Figure 1-2: Major TBA metabolism pathway as proposed by Schiffer *et al*., (2001).**

### 1.1.4 Persistence and Routes of Entry of Trenbolone and Melengestrol in the Natural Environment

The EPA reported in 1998 that the market forces of beef production in the U.S. favor high efficiency, large-scale confined feeding operations such as CAFOs (EPA, 1998) over smaller, family run operations. Theses CAFOs typically generate larger amounts of waste than small farming operations. When there is a lack of available land to apply this waste to as fertilizer, manure ends up stock piling and can serve as a potential point source of environmentally significant amounts of trenbolone and other contaminants. Two studies highlighted here examined the fate and transport and the degradation rates of trenbolone compounds.

Schiffer *et al*., (2001) was the first study to assess the degradation of trenbolone metabolites along with the progestagen melengestrol acetate (MGA). Examining the concentrations of the trenbolone metabolites and MGA in solid dung, liquid manure, and soil, Schiffer *et al*., (2001) observed that 17α- and 17β-trenbolone exhibited half-lives of nearly 260 days in liquid manure. These results would indicate that 17α- and 17β-trenbolone are very persistent in anaerobic liquid manure, similar to CAFO lagoon effluent. With respect to MGA, Schiffer *et al*., (2001) found greater stability in solid dung relative to the metabolites of trenbolone.

In a similar study, Khan *et al*., (2008) evaluated the persistence of trenbolone metabolites after land application on two contrasting agricultural soil types. Of the three metabolites, it was reported that trendione was the most persistent in the environment, due in part to its higher sorptive behavior and the low rate of conversion of 17α-trenbolone to trendione. While the 17α and 17β metabolites generally exhibited half lives between 4 and 40 hours, trendione in clay loam had a half life of nearly 100 hours.

Based on results from Khan *et al*., (2008) and Shiffer *et al.,* (2001), it is unlikely that trenbolone metabolites and MGA are able to travel any substantial distance within aerobic aquifers into surface waters. However, it is possible that they could reach shallow aquifers via anaerobic seepage from animal waste lagoons as the metabolites were shown to be relatively stable under such conditions (McNab, *et al*., 2007). Under these conditions the possibility of trenbolone compounds appearing in shallow aquifers with reasonable persistence is possible. While it is possible that trenbolone could reach surface waters via shallow aquifers, where sensitive aquatic species reside, it is more likely to do so through other routes.

Two proposed routes for trenbolone reaching surface waters include cattle having direct access to surface waters, and overland flow transport. In a study examining the potential release

of steroid hormones into surface waters, Kolodziej *et al*., (2007) found that 86% of rangeland creek samples had steroid hormones present when cattle were allowed direct access to the water. Similarly, Nichols *et al*., (1997) demonstrated that significant amounts of 17β-estradiol in poultry litter applied over a pasture could reach receiving waters via overland flow. In general, where CAFOs have lagoon treatment effluent entering surface waters, and where implanted cattle have direct access to surface waters, it can be expected that trenbolone will be present in those waters.

Given the amount of research conducted on the possibility of steroidal hormones reaching receiving waters, the potential exposure of trenbolone metabolites and MGA to sensitive aquatic species is likely. Further research is needed, specifically on the fate and transport of trenbolone metabolites and MGA to definitively identify where these compounds are most likely to come from and what steps can be taken to prevent their exposure to aquatic species.

Toxicology studies demonstrate the obvious danger of trenbolone metabolites to exposed fish species such as the fathead minnow. However, it is important to understand that the biological mechanisms (i.e., androgen receptors) which allow trenbolone to masculinize female fathead minnows are similar in all vertebrate species, including humans. Being a potent androgenic agonist, trenbolone has the potential to negatively affect whole populations of species. Due to a lack of published studies, the effects of MGA in aquatic environments are not known. However being a potent progestagen, it is likely to have endocrine disruption potential. With the increase in trenbolone metabolites and melengestrol entering aquatic environments and the documented danger they pose to sensitive species (in the case of trenbolone), further environmental assessments of these synthetic steroid hormones are merited.

### 1.1.5 Current Methods Available for Trenbolone Detection

To date, a limited number of analytical methods have been developed for trenbolone. Analytical method development began when the potential use of trenbolone as a growth promoter in cattle resulted in an outright ban on its use in the European Union in 1985. To enforce this ban, analytical methods had to be developed which could detect trace concentrations (ng/L) of trenbolone and other anabolic steroids in complex biological matrices such as bovine tissue. Methods have expanded to detect trenbolone compounds in human hair as a means of detecting illicit use of trenbolone in competitive sports.

Potential techniques for analysis must include the metabolites at trace concentrations. Such techniques include the use of enzyme-linked immunosorbent assays (ELISA) and both gas (GC) and liquid chromatography (LC) paired with mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Assays are analytical techniques used to detect trace compounds in a variety of matrices. Assay reagents attach to target analytes by exploiting their specific spatial shape. The reagent-analyte complex is then optically measured and the concentration of analyte is calculated. While there are a number of different assays that can be employed, radioimmunoassay (RIA), time-resolved fluroimmunoassay (TR-FIA), enzyme-linked immusorbent assays have been used in trace quantification of steroids (Wang, *et al.*, 2008).

For instance one of the first studies to examine the fate of trenbolone in agricultural soils and liquid manure, Schiffer *et al*. (2001) developed an enzyme immunoassay analytical method to detect 17α-trenbolone as low as 0.4 pg/g in soil and 4 pg/g in liquid manure. While offering limits of detection (LOD) for hormonal steroids in the low ng/L levels, immunoassays suffer from poor reproducibility and false positives due to cross-reactions and complex matrix effects (Wang *et al.*, 2008). As a result immunoassays have proven to be largely ineffective for detecting trace concentrations of hormonal steroids when complex environmental matrices are involved. The

result with employing assays in complex environmental matrices is a lack of confirmed specificity and the presence of false positives.

LC/MS/MS methods can positively identify a variety of endocrine disrupting compounds including trenbolone in environmental matrices (see Table 1-1). LC/MS/MS methods are often preferred choices for the analysis of steroids due to their ability to analyze compounds without using a preparatory derivatization step (Marques *et al.*, 2007). However, Vanderford, *et al.* (2003) reported that a signal suppression of up to 38% for progesterone was present in their LC/MS/MS method due to matrix interferences. In essence, for complex environmental matrices, the analysis of hormonal steroids like trenbolone could be complicated by the introduction of matrix interferences. To reduce signal suppression or enhancement, deuterated surrogate standards and isotope dilution techniques are often employed in LC/MS/MS analysis of environmental samples.

Because of its superior chromatographic separation capabilities and sensitivity, GC/MS/MS is theorized to be an ideal technique for trenbolone analysis in complex matrices, but requires more sample preparation (Le Bizec *et al.*, 2004). This theory has been validated by numerous methods developed for a variety of complex matrices (see Table 1-1). GC/MS/MS methods suffer from their need for labor-intensive derivatization procedures because many steroidal hormones like those in this study are relatively polar, have low volatility and are thermally labile (Vanderford *et al.*, 2003).

Much of the work done for this analytical method comes from studies published by a research group at the National Veternary School in Nantes, France (Maume, *et al*., 1998; Marchand *et al*., 2000; Le Bizec *et al*., 2004; and Rambaud *et al*., 2007). These methods employ the use of an MSTFA/$I_2$ reagent used for derivatization of 17α- and 17β-trenbolone as presented by Maume *et al.,* (1998) for use in detecting 17α- and 17β-trenbolone in bovine tissue, urine and

hair. These methods do not attempt to quantify trendione, perhaps the most environmentally persistent metabolite of all three TBA metabolites, and also they do not attempt to quantify any other exogenous steroids used in animal agriculture. Because these methods were intended for biological matrices they have not yet been tested for use in detecting trenbolone metabolites and MGA in environmental matrices.

**Table 1-1: Summary of published analytical methods for the detection of trenbolone compounds.**

| Study | Target Trenbolone Compounds | Matrix Considered | Method Employed |
|---|---|---|---|
| Maume *et al.*, 1998 | 17α & 17β | Bovine Urine | SPE-GC/MS |
| Le Bizec *et al.*, 2004 | 17α & 17β | Bovine Tissue | SPE-GC/MS |
| Marchand *et al.*, 2000 | 17α & 17β | Bovine Tissue | SPE-GC/MS |
| Fedeniuk *et al.*, 2004 | 17β | Bovine Serum | SPE-GC/MS |
| Schanzer *et al.*, 2005 | 17β | Human Urine | XAD-GC/MS & LC/MS/MS |
| Khan *et al.*, 2008 | 17α, 17β & Trendione | Agricultural Soils | HPLC/MS |
| Durhan *et al.*, 2006 | 17α & 17β | CAFO Runoff | SPE-GC/MS & HPLC |
| Wilson *et al.*, 2002 | 17β | In Vitro & In Vivo Rat | Binding Assays |
| Schiffer *et al.*, 2001 | 17α & 17β | Dung, Manure and Soil | SPE-HPLC-Immunoassay |
| Chang *et al.*, 2008 | 17β | Environmental Waters | UPLC/MS/MS |
| Rambaud *et al.*, 2007 | 17β, 17α & 17α-d3 | Animal Hair | SPE-SiOH-GC/MS/MS |
| Marques *et al.*, 2006 | 17α | Human Urine | SPE-GC/MS |
| Impens *et al.*, 2002 | 17β | Kidney Fat and Meat | SPE-GC/MS/MS |

(SPE = Solid Phase Extraction, GC/MS = Gas Chromatography/Mass Spectrometry, XAD = proprietary extraction cartridge, HPLC = High performance liquid chromatography, UPLC = ultra performance liquid chromatography, SiOH = silicon hydroxide)

### 1.1.6 Research Objectives

The primary objective of this research was to develop an analytical method which could quantitatively detect, using gas chromatography/tandem mass spectrometry, trace levels of the anabolic steroid trenbolone acetate's metabolites 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one), 17α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one), trendione (17β-hydroxyestra-4,9,11-trien-3,17-one), and melengestrol (6-methyl-16-methylene-prena-4,6,-diene-3,20-dione), the predominant metabolite of melengestrol acetate in environmentally complex matrices. It is important to note that while this method presents an analytical method for 17β-trenbolone and 17α-trenbolone, which have existing GC/MS/MS methods established, though not for complex environmental matrices, a objective of this method was to incorporate trendione, the internal standard 17β-trenbolone-d3 and melengestrol. No GC/MS/MS analytical method exists for these compounds especially when considering complex environmental matrices (See Table 1-1).

An analytical method capable of detecting the trenbolone family in environmental matrices at or below 1 ng/L is necessary because toxicity studies have demonstrated the deleterious effects of 17α- and 17β-trenbolone in this range on various fish species (Ankley *et al.,* 2001, Jensen *et al.,* 2006). The challenge in establishing such a method comes from the ability to separate and positively identify target analytes from organic matter. Overall, analytical methods suffer from time consuming and labor intensive aspects but attempting methods in organic-rich environmental matrices adds the necessary complication of solid phase extraction (SPE) and normal phase extraction (Florasil) clean up, both of which are potential processes where target analyte mass can be lost. This method incorporates both SPE and Florasil clean up processes along with a derivatization procedure and GC/MS/MS quantification parameters.

# 2 Experimental Section

### 2.1.1 Chemicals, Reagents and Cartridges

The steroids melengestrol (4,6-pregnadien-6-methyl-16-methylene-17-OL-3,20-dione) and 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) were purchased from Steraloids. Inc. (Newport, R.I., USA). 17β-trenbolone-d3 (17β-hydroxy-estra-4,9,11-trien-3-one-$d_3$) was obtained from the Bank of Reference Standards (RIVM, the Netherlands). 17α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one) was purchased from NMI (Pymble, NSW, AU). Trendione (17β-hydroxyestra-4,9,11-trien-3,17-one) was synthesized from 17β-trenbolone using the protocol outlined in Khan *et al*. (2008). Trendione synthesis was performed by Soma Maitra, a graduate student in the Department of Chemistry under the supervision of her advisor Dr. Liming Zhang at the University of Nevada, Reno. Derivatization grade *N*-methyl-*N*-(trimethylsiyl)trifluoro-acetamide (MSTFA) and iodine (99.999% pure) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Resprep 6 mL C18 and Florasil cartridges were purchased from Restek (Bellefonte, PA, USA). Samples of Oasis HLB, Sep-Pak SPE cartridges were obtained from Waters (Milford, MA, USA) and samples of Strata-X, C18-U and C18-E SPE cartridges were obtained from Phenomenex (Torrance, California, USA).

Stock solutions for each of steroid were prepared in HPLC grade methanol from Sigma-Aldrich (Milwaukee, WI, USA). The derivatization reagent was prepared by dissolving 0.2 mg of iodine per mL of MSTFA (0.02% m/v). Both stock solutions and the derivatization reagent were stored in a freezer at -18°C to reduce the potential degradation of the steroids.

### 2.1.2 Filtration of Samples

Samples were vacuum filtered (Fisherbrand 90 mm diameter glass microanalysis filter holder assembly, Fisher Scientific, Pittsburgh, PA, USA) through 0.7 µm pore Glass Fiber Prefilters (AP40) purchased from Millipore (Billerica, MA, USA), and then poured back into their original glass containers. After filtration, 100 µL of a 100 µg/L stock of 17β-trenbolone-d3 was added as an internal surrogate standard to each sample including laboratory samples spiked with predetermined amounts of 17β-trenbolone, 17α-trenbolone and trendione; this deuterated surrogate was used as means of quality control and assurance.

### 2.1.3 Solid Phase Extraction Procedure

Prior to further processing of sample filtrate, C18 solid phase extraction cartridges were conditioned per the manufacturers SPE specifications. Conditioning began with four 5-mL aliquots of a mixture of methanol and water (95:5 v/v), passed through the cartridges under vacuum to create a high flow rate in an extraction manifold (Waters, Milford, MA, USA) (See Figure 2-1). The cartridges were then conditioned with four 5-mL aliquots of deionized water. Cartridges were not allowed to vacuum dry in between conditioning and sample addition as per manufacturer's SPE specifications.

The samples, typically 1.0 L in volume, were extracted under vacuum in the extraction manifold. The flow rate through the extraction cartridge was 1 to 5 mL/min. Extra care was taken not to exceed a 5 mL/min flow rate, as per manufacturer's specifications. At this point the C-18 cartridges were eluted immediately or stored in a 4ºC refrigerator to be eluted at a later date.

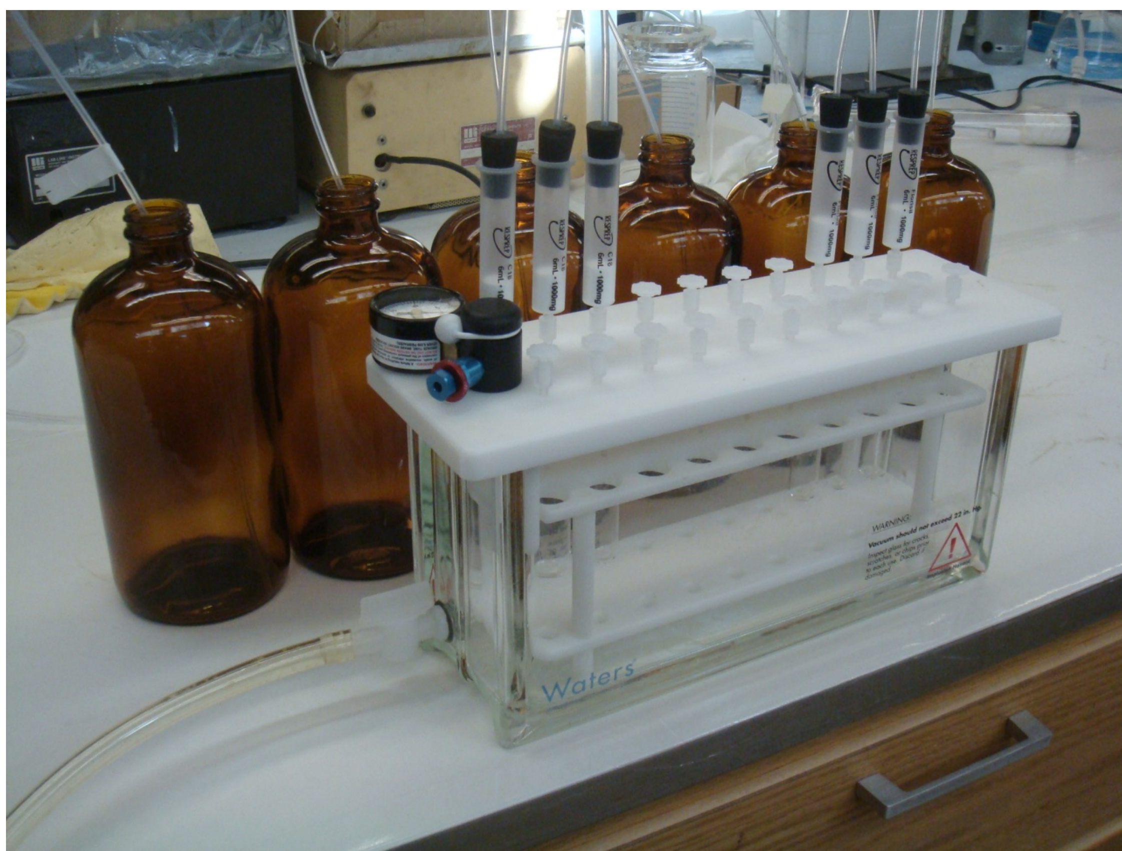


**Figure 2-1: Sample set up with collection bottles, cartridges and vacuum extraction manifold**

Elution of the steroids from the C18 cartridges was accomplished by percolating three 2-mL aliquots of methanol and water (95:5 v/v) through the cartridges under vacuum. The final sample volume (~ 6 mL) was then dried down under a gentle stream of $N_2$, leaving a dried residue which was then resuspended in 6 mL of dichloromethane and methanol (95:5 v/v). To allow for quantitative mass transfer the contact time between the residue and the solution was as long as 24 hours.

Sample clean-up was performed with normal phase extraction using Florasil cartridges. The cartridges are first conditioned by passing four, 5-mL volumes of acetone through each cartridge with a vacuum drying period in between each pass. After vacuum drying, the cartridges

are conditioned with four 5-mL final solvent passes of a dichloromethane and methanol mixture (95:5 v/v).

Samples are then passed through the conditioned Florasil cartridges, applying a vacuum if necessary. Each sample volume (~ 6 mL) is vacuum dried to ~1.5 mL and then poured into 2 mL SilCote deactivated vials (Restek, Bellefonte, PA, USA). Samples are then dried down under $N_2$ prior to derivatization.

### 2.1.4 Derivatization Procedure

A 1000 µL volume of dichloromethane is added to the vial, vortexed and then evaporated under $N_2$ for azeotropic removal of residual $H_2O$. Fifty µL of the derivatization reagent MSTFA-$I_2$ (1000:2 v/m) is added to the dried residue and vortexed at room temperature for 5 seconds. Because the derivatization reaction is instantaneous (Marchand *et al*., 2000) the sample is immediately dried down under a gentle $N_2$ stream. As small iodine colored crystals often formed during the dry down process, a continued flow of $N_2$ into the vial is used to evaporate the crystals, often taking 30 minutes depending on the flow rate of $N_2$. The dried residue is resuspended with 100 µL of MSTFA and incubated for 40 minutes at 60°C to ensure complete derivatization of the alcohol functional groups (Rambaud *et al.,* 2007).

The proposed derivatized structures for each steroid in this study (neglecting the internal standard, 17β-trenbolone-d3) are presented in Figure 2-2.

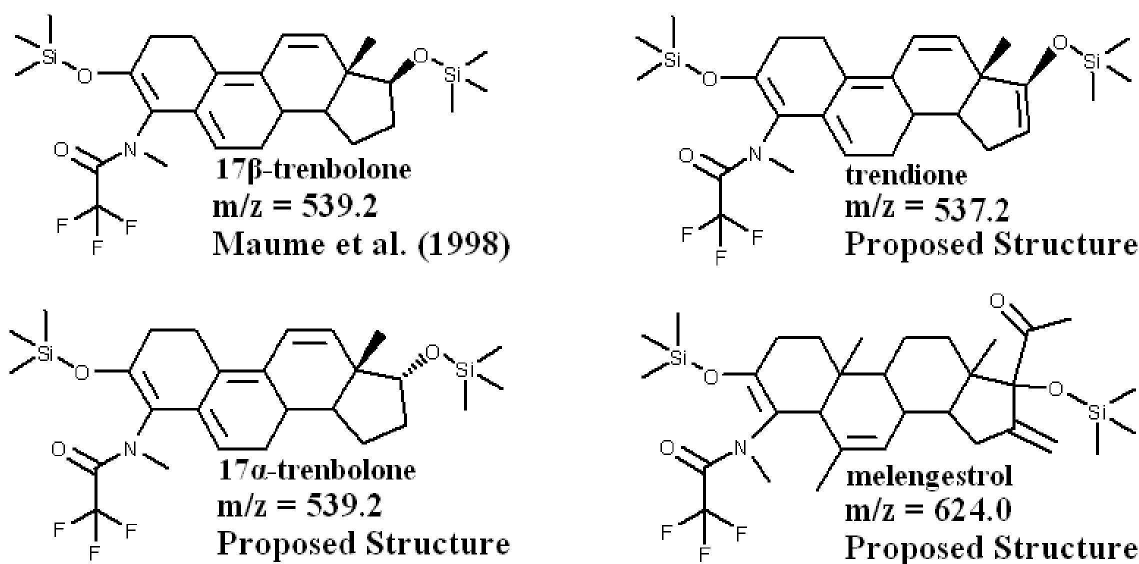


**Figure 2-2: The parent ion, molecular weight, and derivatized structure of studied steroids.**

### 2.1.5 Steroid Detection

Steroid derivatives were analyzed by GC/MS/MS using an Agilent 6890N Gas Chromatograph (Santa Clara, CA, USA) paired to a Waters Quattro Micro Mass Spectrometer (Milford, MA, USA) (See Figure 2-3). The GC column used for separation was a Restek Rxi®-5Sil MS, 30-meter long and 0.25- mm inner diameter (Restek, Bellefonte, PA, USA). Splitless injections of 1.0 µL into a 250°C injection port were used. UHP helium was used as the carrier gas at 1.0 mL/min. The temperature program was as follows: 120°C (held for 2 min), increased at 45°C /min to 260°C (held for 1 min), increased at 5°C/min to 270°C (held for 8 min), increased at 45°C/min to 285°C (held for 6 min), increased at 45°C/min to 300ºC (held for 2 minutes). The electron-impact ionization energy in the mass spectrometer was 70 eV. An ion source temperature of 180°C and a 290°C gas chromatograph transfer line temperature were maintained.

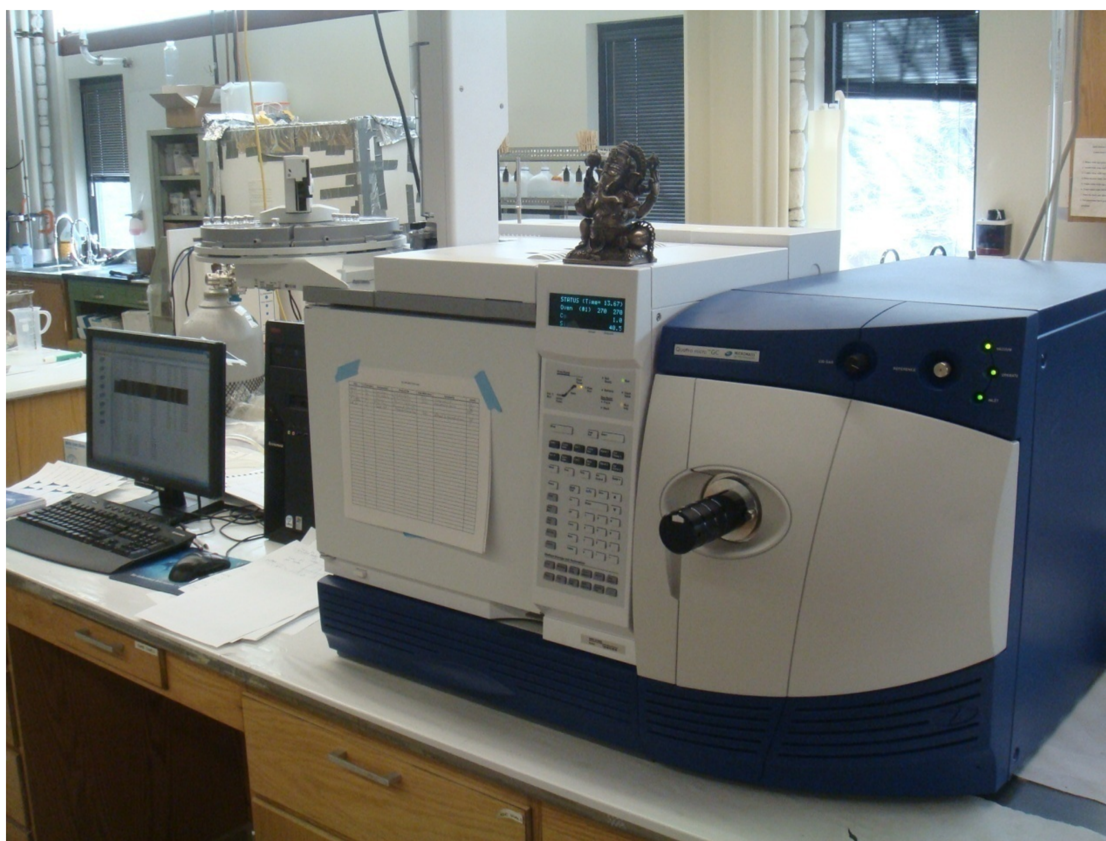

**Figure 2-3: Agilent 6890N Gas Chromatograph with a Waters Quattro Micro Mass Spectrometer.**

### 2.1.6 Multiple Reaction Monitoring (MRM) Development

MRM functions in the Quattro micro GC/MS/MS allow for simultaneous monitoring of multiple daughter ions and transitions. To positively identify a target compound like 17β-trenbolone in a complex matrix, the use of parent/daughter collisions is employed. In essence, a target compound with a known molecular weight (for MSTFA/$I_2$ derivatized 17β-trenbolone this is equal to 539.2) is isolated in the first quadrupole. The analyte is then passed from the first quadrupole into the collision cell where it collides with energized, characteristic argon gas. This collision fragments the "parent" compound into smaller fragments or "daughters." As these fragments pass from the collision cell into the second quadrupole they are again scanned for

molecular weight. Those transitions which meet the molecular weight requirements from predetermined fragment analysis are assigned a peak area based on their relative abundance. This process is referred to as multiple reaction monitoring.

Under carefully monitored conditions the parent structure of a target compound breaks apart into stable and repeatable fragments. When these transitions are observed in real-world samples, the target analyte is unambiguously identified.

### 2.1.7 MRM Development Example Using 17β-Trenbolone

To illustrate the process of how transitions are determined for each of the compounds, the MRM development procedure for 17β-trenbolone is given here. The process begins with a first quadrupole or "MS" scan of a sample containing 17β-trenbolone, also referred to as a full scan. The full scan spectrum for 17β-trenbolone, which contains all of the masses that were observed and arbitrarily quantified in the sample at a specified retention time, is given in Figure 2-4.

A spectrum of the MS scan is compiled which contains the parent ion (for 17β-trenbolone = 539.2). As an electron-impact ionization energy of 70 eV is used to ionize the sample during the full scan the compound of interest is often fragmented as opposed to remaining in the initial parent mass, which would be the preferred situation for tandem mass spectrometry. As this happens to be the case with 17β-trenbolone, the most abundant, unique and repeatable fragment is selected for further fragmentation and tandem mass spectrometry quantification.

Further MRM development includes a daughter scan with the highest intensity fragment (m/z = 442.2 for 17β-trenbolone). In a daughter scan the highest intensity fragment has a variety of collision energies applied to it. An example of a daughter scan, at a collision energy of 30V for the 17β-trenbolone mass 442.2 is given in Figure 2-5. Although the m/z = 539.2 is the actual parent mass for 17β-trenbolone the highest intensity fragment in the spectrum is m/z = 442.2, and

was therefore chosen as the parent fragment for further fragmentation in the collision cell. The spectrum for the collision of mass 442.2 is given in Figure 2-5. From the collision of mass 442.2 a number of fragment masses could be chosen as quantification and confirmatory transitions. After examining each for overall transition (i.e., 442.2>294.0) peak area, the transition with the largest, repeatable peak area, free of interferences is chosen. For 17β-trenbolone the 442.2>382.0 transition at a collision energy of 30 V gave the largest, repeatable peak area and was thus chosen as the quantification transition.

A similar procedure is performed to find a confirmatory transition that gives the next highest transition area. For 17β-trenbolone the confirmatory transition was 442.2>309.2 at collision energy of 32 V. The combination of the quantification and confirmatory transition unquestionably confirms the presence of 17β-trenbolone when found in actual test samples. A similar procedure as described here was performed for each of the compounds in the development of this analytical method (listed in Table 3-2).

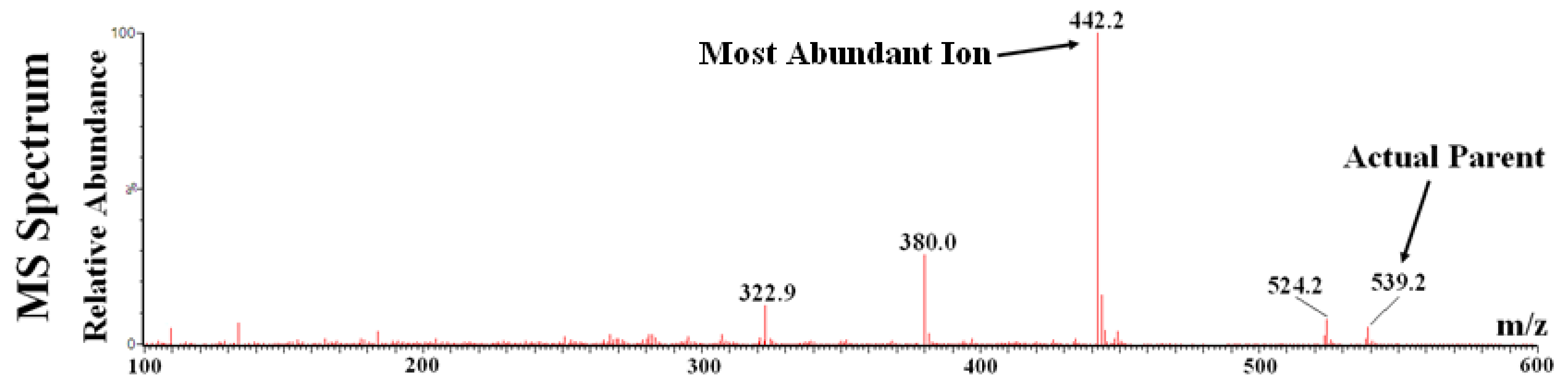

Figure 2-4: Full scan spectrum of a sample of 17β-trenbolone with the actual parent and most abundant ion highlighted.

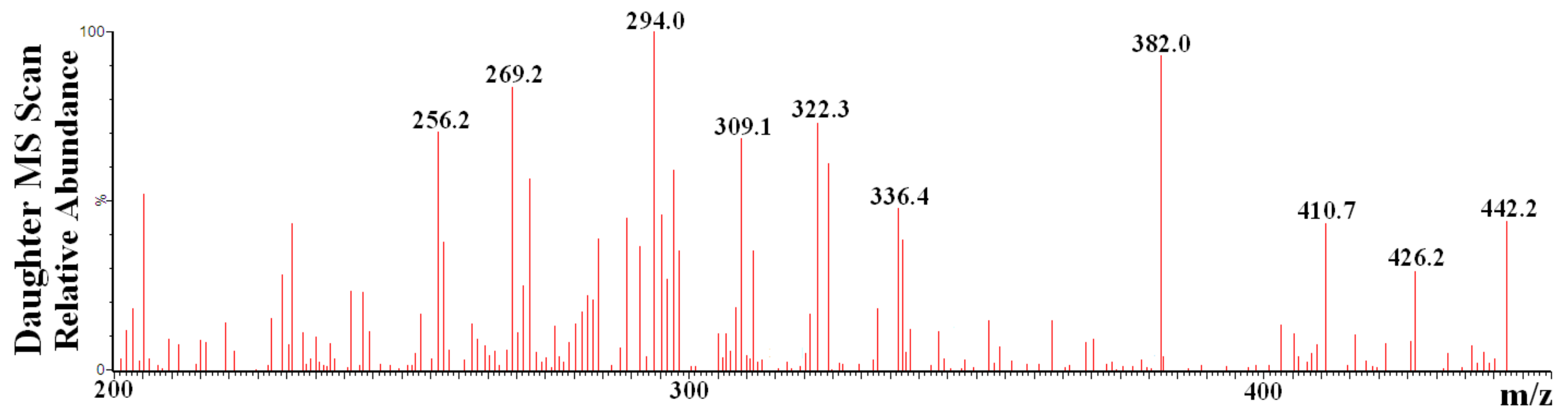

Figure 2-5: 17β-trenbolone daughter scan (30 V collision energy) of mass 442.2 with potential daughter masses.

# 3 Results

## 3.1 Literature Verification

For the steroids 17α-trenbolone and 17β-trenbolone, a number of studies have presented protocols for their derivatization and analysis using GC/MS/MS as a quantitative instrument (Refer to Table 1-1). Data and figures are provided here to ensure that similar results for these steroids were observed using the protocol outlined in this study. In addition, MRM development data are included for each compound.

In general, similar results were observed when verifying existing GC/MS/MS methods for 17α- and 17β-trenbolone. Because of differences in machinery, techniques, analyte matrices, and other unknown variables some different quantification and confirmation transitions at different collision energies were chosen for this method. A comparison of transitions and collision energies of known GC/MS/MS methods for 17α- and 17β-trenbolone and those of this study are given in Table 3-1. Similarities in the transitions were observed between this study and those of existing literature.

**Table 3-1: Comparison of method to other published method using the MSTFA/$I_2$ derivatization.**

| Study | Target Steroid | Parent Ion | Quantification Transition | Collision Energy (V) | Confirmatory Transition | Collision Energy (V) |
|---|---|---|---|---|---|---|
| Rambaud *et al.*, 2007 | 17α | 539.2 | 380.3>323.3 | 20 | 449>307.3 | 30 |
| Rambaud *et al.*, 2008 | 17β | 539.2 | 380.3>323.4 | 20 | 442.4>295.2 | 40 |
| Current Study | 17α | 539.2 | 449.3>323.2 | 8 | 380.2>323.2 | 24 |
| Current Study | 17β | 539.2 | 442.2>382.2 | 30 | 442.2>309.3 | 32 |

### 3.1.1 17α-Trenbolone Chromatogram/Spectrum

An 8 ppm standard of 17α-trenbolone was prepared using the method outlined in this study. The resulting chromatogram shows a strong chromatographic peak along with a similar spectrum as presented in Maume *et al.,* (1998) (See Figure 3-1). The quantification and confirmatory transitions (449.3>322.1 at 8 V and 380.2>323.2 at 24 V) for 17α-trenbolone are given in Figure 3-2. These were the transitions that gave the best response based on the procedure outlined in the methods section of this study.

In Figure 3-1 three total peaks are visible; the first represents the kinetic product, which for 17α-trenbolone and 17β-trenbolone is by far the most prevalent product. The second and third peaks represent the thermodynamic product and a di-substituted product, respectively. This analytical method does not account for these products as they are often only quantifiable when the steroid is present in high concentrations. For environmental matrices, where expected concentrations are in the ng/L as opposed to the mg/L range, thermodynamic and di-substituted products are virtually undetectable. Minimizing the thermodynamic and di-substituted products is accomplished by following the derivatization procedure without variation.

### 3.1.2 17β-Trenbolone Chromatogram/Spectrum

A 500 ppb standard of 17β-trenbolone was prepared using the method outlined in this study. The resulting chromatogram shows a strong chromatographic peak as expected, and features a very similar spectrum as presented in Maume *et al.*, (1998) (See Figure 3-3). The transitions (442.2>382.2 at 30 V and 442.2>309.3 at 32 V) for 17β-trenbolone are given in Figure 3-4.

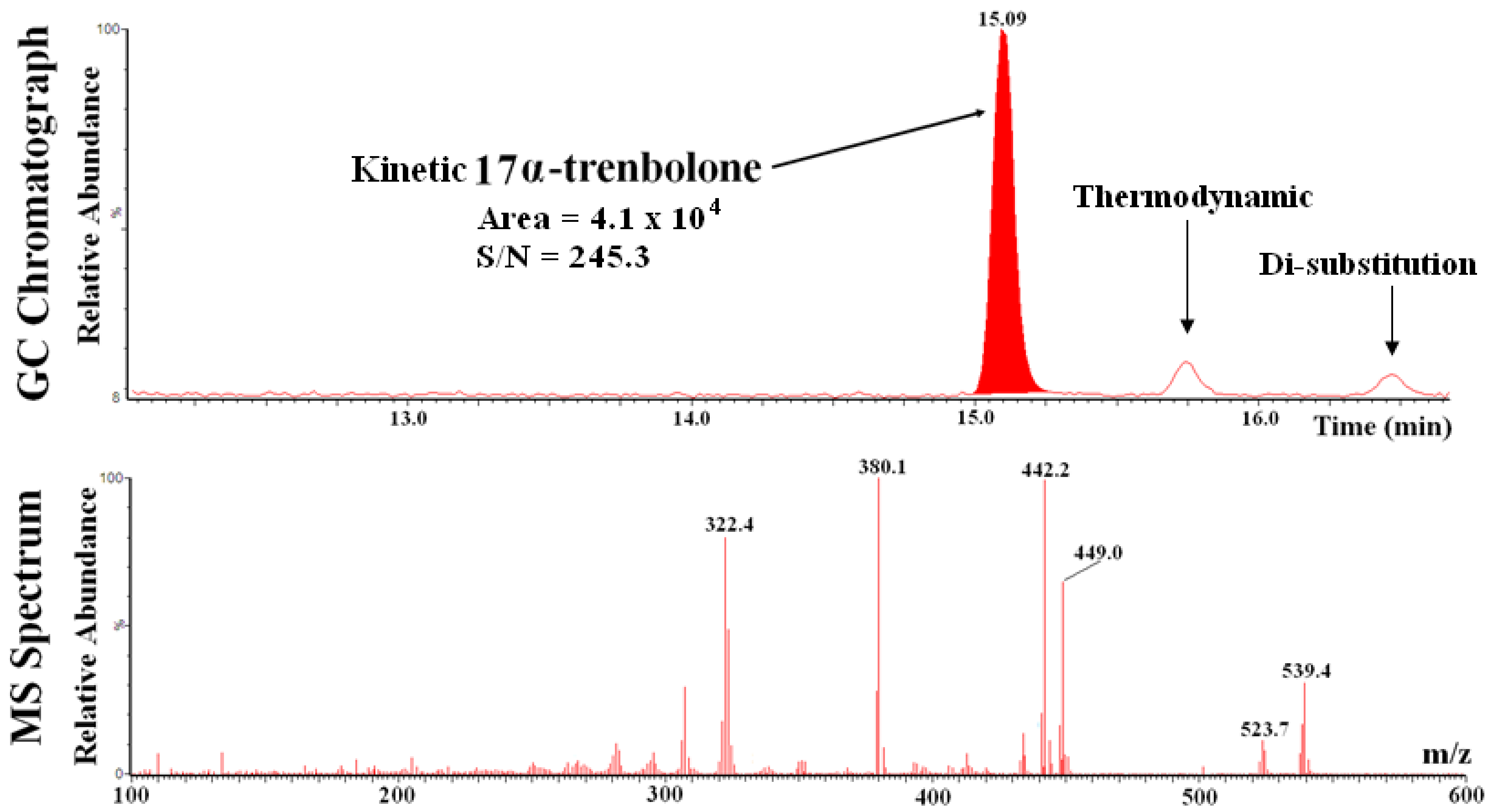


**Figure 3-1: An 8 ppm standard of derivatized 17α-trenbolone with chromatogram (top) and spectrum (bottom).**

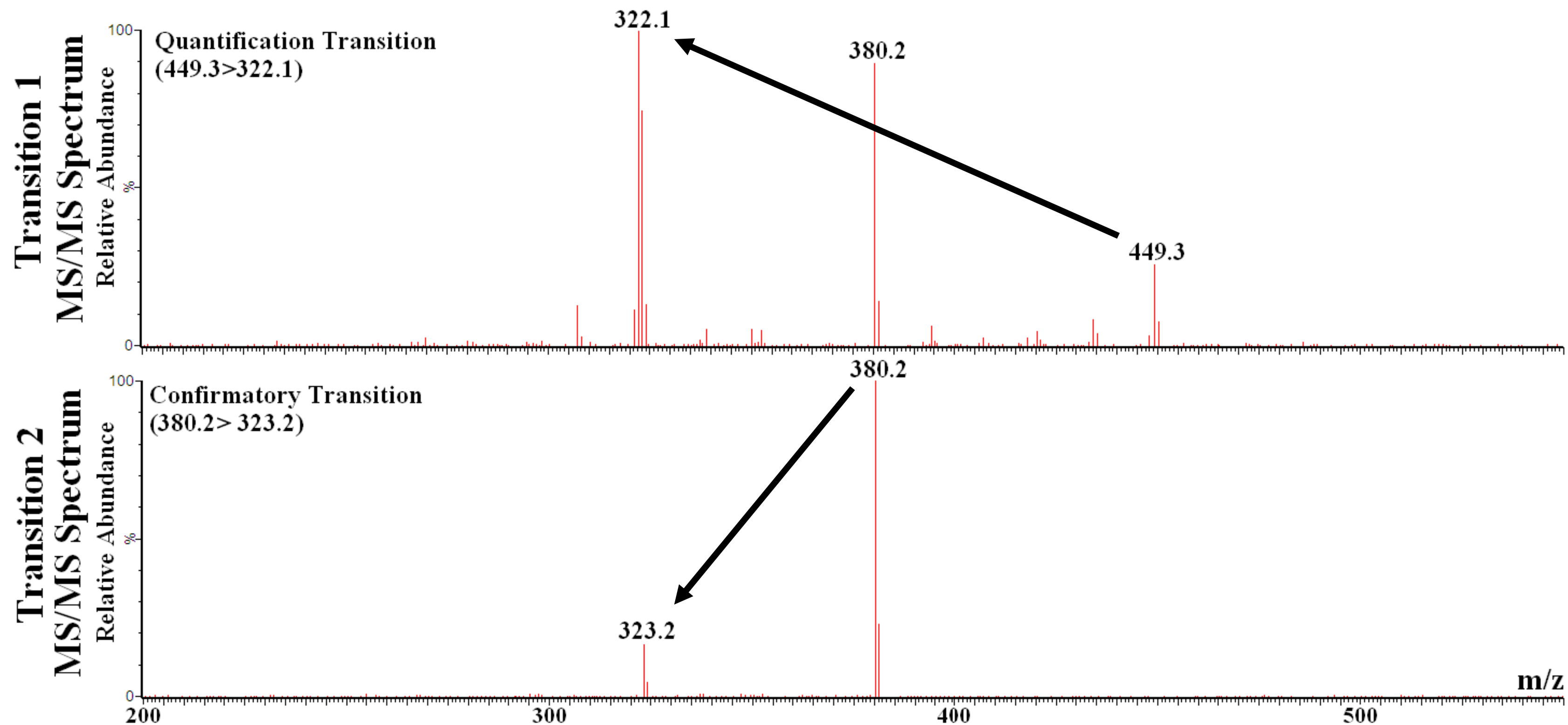


**Figure 3-2: MRM transitions one (top, 449.3>322.1 at 8 V) and two (bottom, 380.2>323.2 at 24 V) for 17α-trenbolone. As with other steroids, transition 1 is the quantification transition and transition 2 is the qualification or confirmatory transition.**

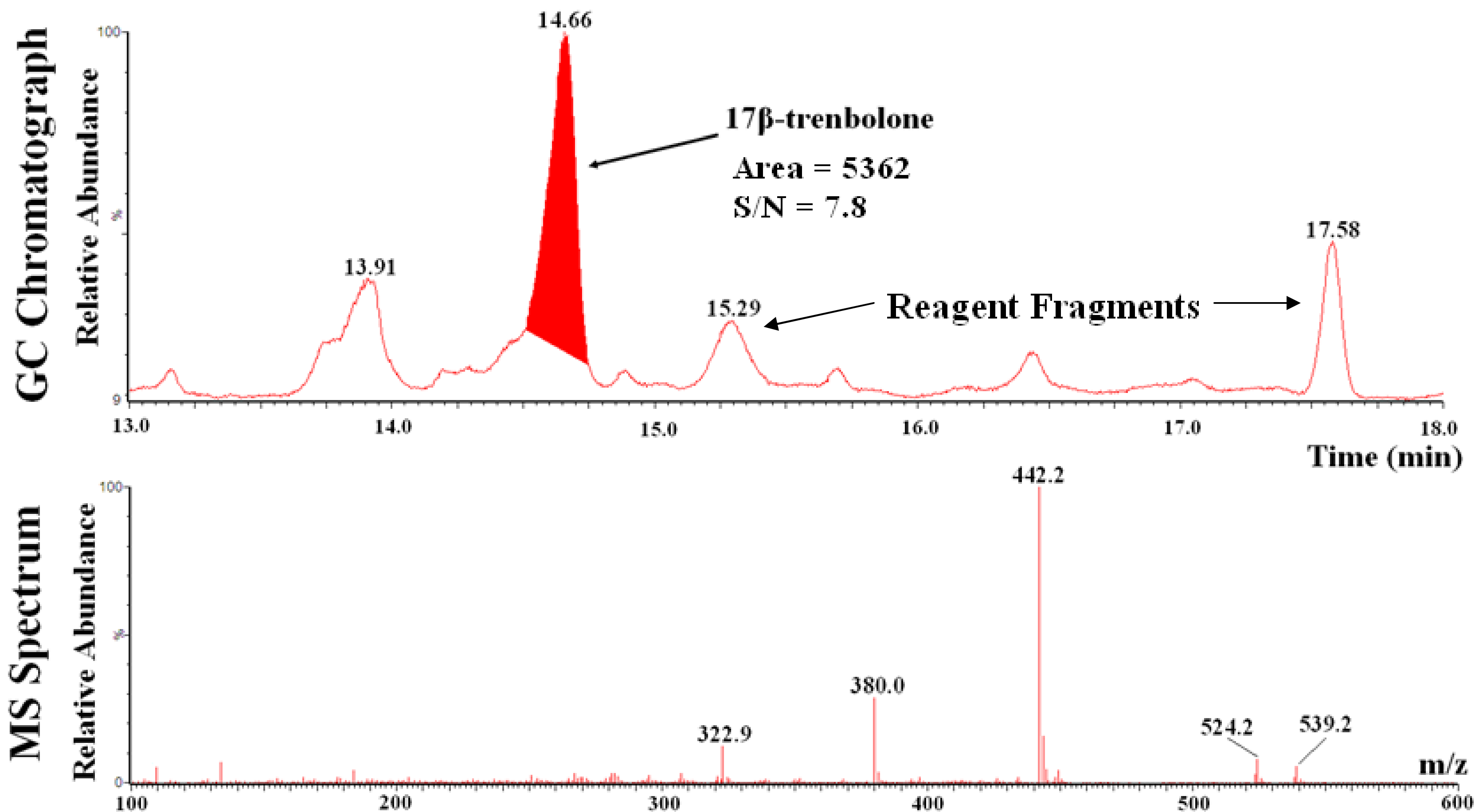


Figure 3-3: A 500 ppb standard of derivatized 17β-trenbolone with chromatogram (top) and spectrum (bottom).

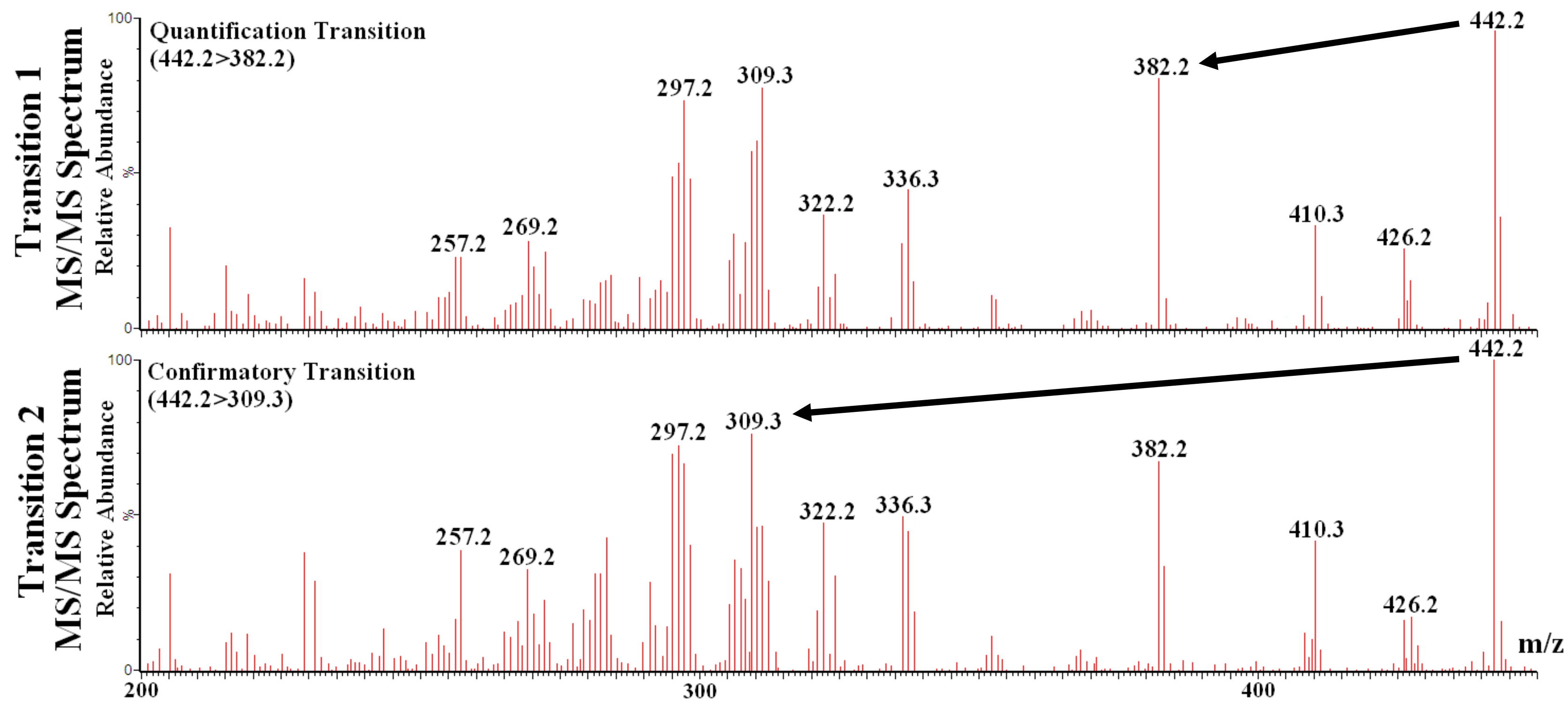


**Figure 3-4: MRM transitions one (top, 442.2>382.2 at 30 V) and two (bottom, 442.2>309.3 at 32 V) for 17β-trenbolone.**

## 3.2 Extension of Literature to Trendione, 17β-Trenbolone-d3 and Melengestrol

As described in the research objectives section of this study, there are no known GC/MS/MS methods using the MSTFA/$I_2$ derivatization approach for trendione and melengestrol especially using environmental matrices. Existing knowledge from Maume *et al.*, (1998), Marchand *et al.*, (2000), and Rambaud *et al.*, (2007) was used to develop the derivatization procedure presented in the experimental section of this study. The derivatization method was then applied to trendione, 17β-trenbolone-d3 (as a surrogate standard) and melengestrol. MRM transitions were established for each of the compounds, the results of which are presented in Figure 3-10, Figure 3-12 and Figure 3-14, respectively.

### 3.2.1 Trendione Chromatogram and Spectrum

As mentioned in the experimental section of this study, trendione was synthesized from 17β-trenbolone with the assistance of Soma Maitra, a graduate student in the Department of Chemistry and her advisor Dr. Liming Zhang at the University of Nevada, Reno. When the derivatization procedure outlined in this study was applied to a sample of the synthesized trendione, two chromatographic peaks were observed which are highlighted in Figure 3-7.

A variety of derivatization parameters (derivatization temperature, duration, volume used, etc.) were explored to maximize trendione yield and minimize what is hypothesized as the thermodynamic second peak. Experimental results indicate that only a fraction of the trendione mass within stock solutions could be derivatized to yield the “first peak” trendione product with the remainder going to the second product. The spectrum for the second product is given in Figure 3-8. It is theorized that the two peaks represent two products of derivatized trendione. Both theorized structures are represented in Figure 3-5.

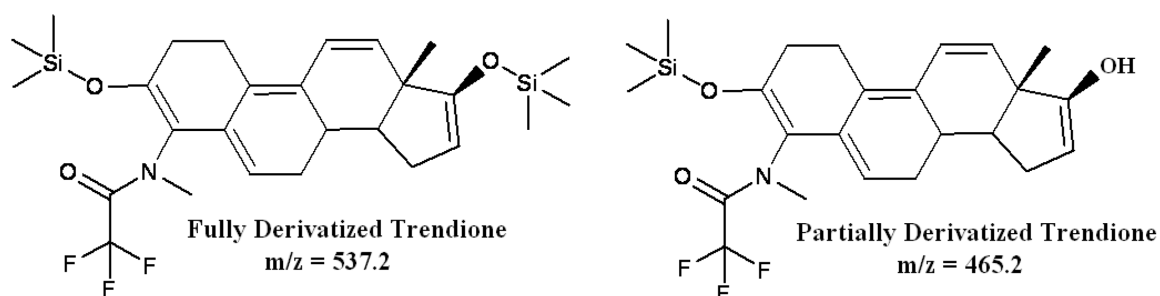


**Figure 3-5: Proposed structures of the first (fully derivatized) and second peaks (partially derivatized). Parent masses 537.2 and 465.2 closely resemble those of the first and second peaks represented in Figure 3-7 and Figure 3-8, respectively.**

As a response to incomplete trendione derivatization, an approximation was made as to what percentage of any given standard mass of trendione was actually going to the first product using the established derivatization method. Five concentrations of stock trendione, ranging from 1 mg/L to 26.8 mg/L, run in triplicate were analyzed for the percentage of peak area going into the first and second peaks. Established trendione stocks were then adjusted to reflect the approximate concentration that would be yielded using the established method. It was found with fair consistency (based on standard error) that with the established derivatization method, 15% of a known standard mass would show up in the first product while 85% would remain in the second product (See Figure 3-6). Based off of this assumption, stock solutions were numerically adjusted to account for the first peak yield. For example, a 26.8 mg/L stock solution would only yield 15% first peak trendione and thus became a 4.02 mg/L stock used to quantify the completely derivatized trendione product.

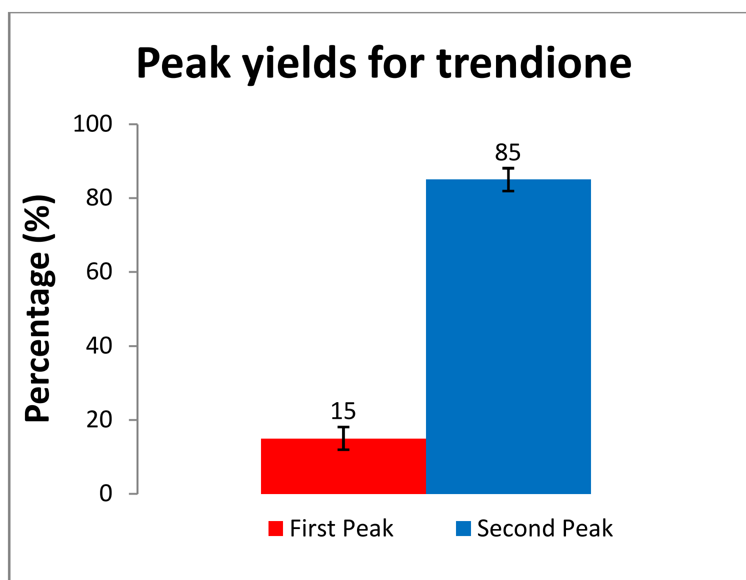


**Figure 3-6: A representation of peak area break-down into the first and second peaks. Percentages assume all trendione stock mass is derivatized into one peak or the other. Error bars represent standard error (%).**

A 2 ppm standard of corrected trendione was prepared using the procedure outlined in this method. The chromatogram and spectrum are presented in Figure 3-7. Both the first and second trendione peaks are represented. The quantification transition for the second trendione peak is given in Figure 3-9. The transitions for trendione (537.2>522.3 at 22 V and 440.3>410.3 at 10 V) are given in Figure 3-10.

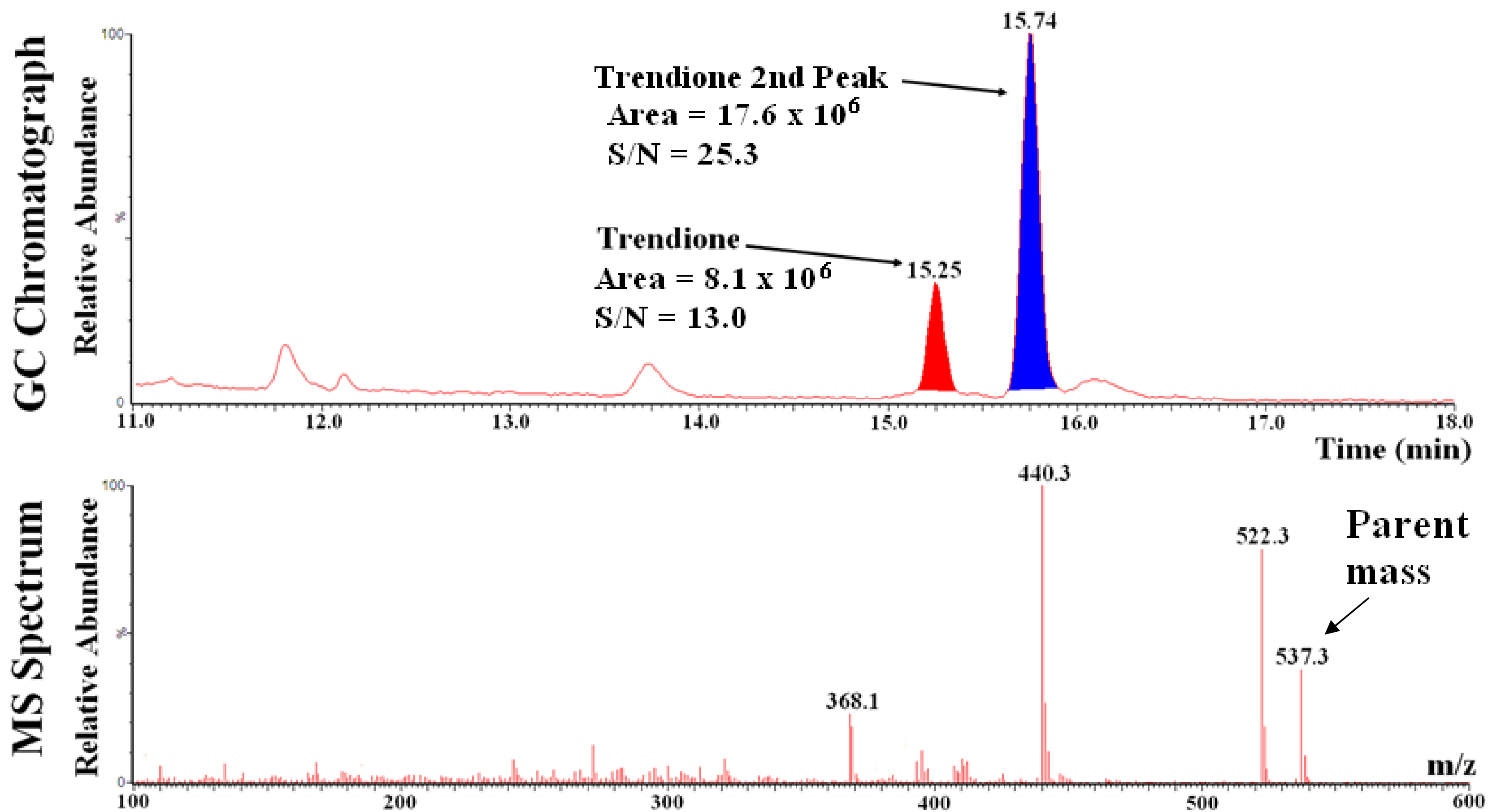


**Figure 3-7: A 2 ppm standard of derivatized trendione with both first and second peaks represented in the chromatograph (top) and spectrum for the first trendione peak (bottom).**

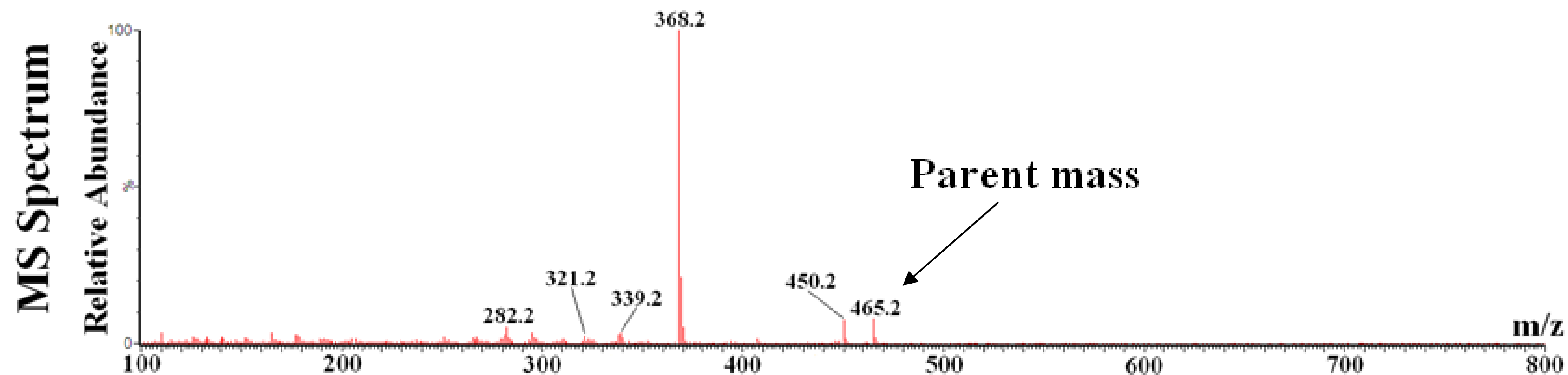


**Figure 3-8: Full scan of derivatized trendione second peak (R.T. = 15.74 min). The parent appears to be m/z = 465.2 and the most abundant ion appears to be 368.2.**

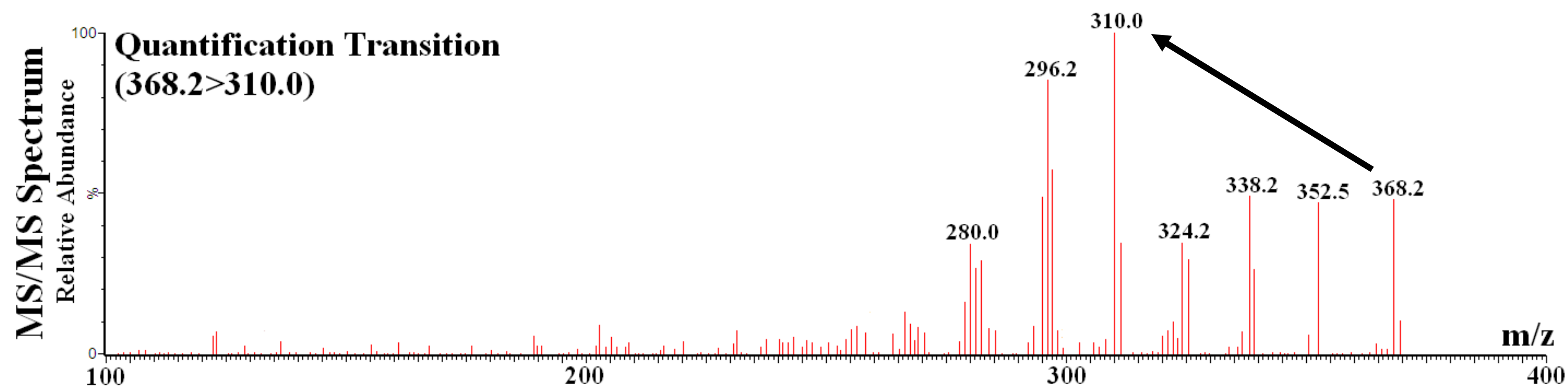


**Figure 3-9: Quantification transition for the second trendione peak (368.2>310.0), used to calculate matrix spike recoveries in place of trendione transitions.**

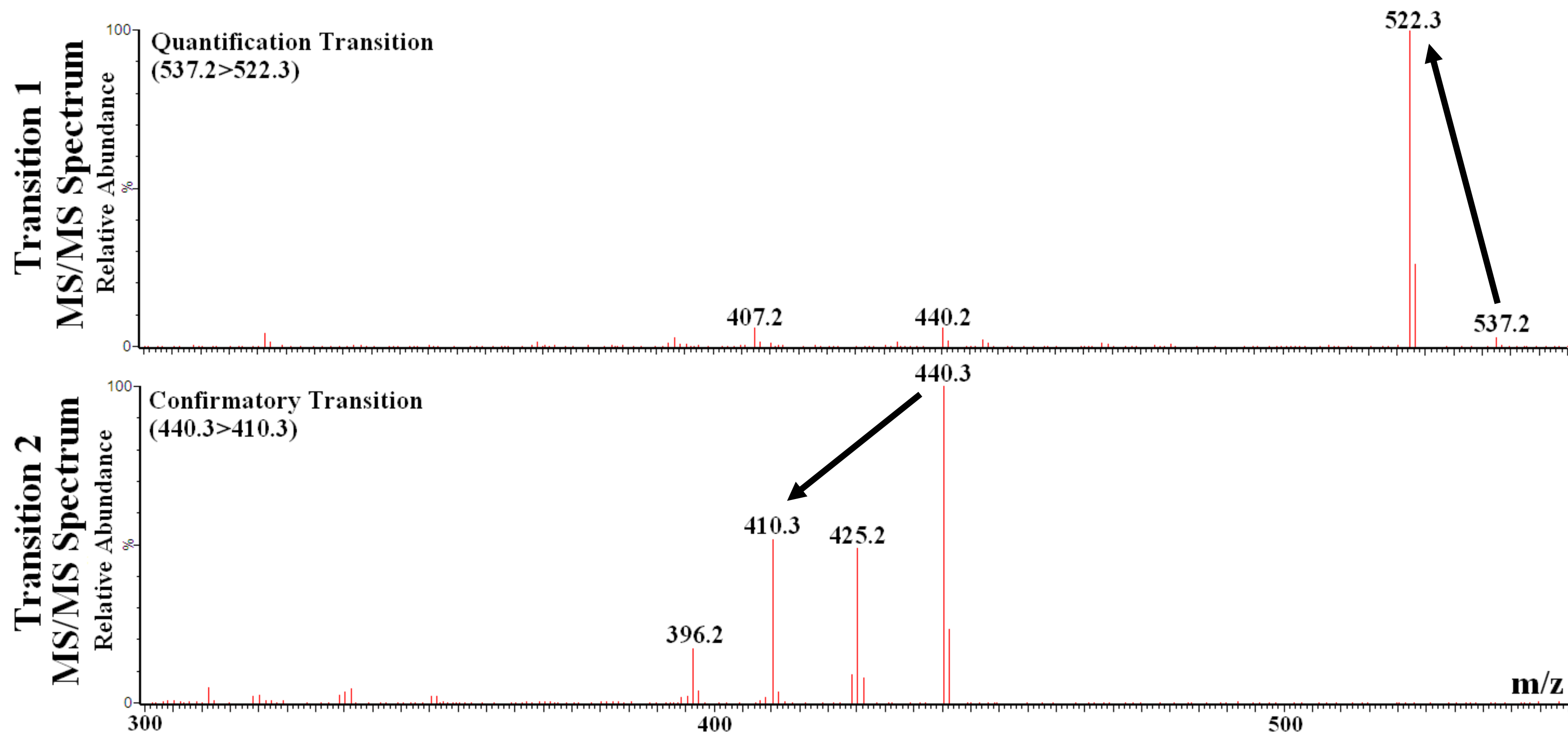


**Figure 3-10: MRM transitions one (top, 537.2>522.3 at 22 V) and two (bottom, 440.3>410.3 at 10 V) for the first trendione peak.**

### 3.2.2 17β-Trenbolone-d3 Chromatogram and Spectrum

A 500 ppb standard of 17β-trenbolone-d3 was prepared and analyzed using the method outlined in this study. The chromatogram and spectrum are given in Figure 3-11. Used as an internal standard, only one transition (445.3>383.3 at 30V) was established for 17β-trenbolone-d3 which is given in Figure 3-12.

### 3.2.3 Melengestrol Chromatogram and Spectrum

An 8 ppm standard of melengestrol was derivatized and analyzed using the protocol provided in this study. The chromatogram and spectrum are presented in Figure 3-13. The transitions for melengestrol (624.0>581.2 at 12V and 624>491.2 at 10V) are presented in Figure 3-14.

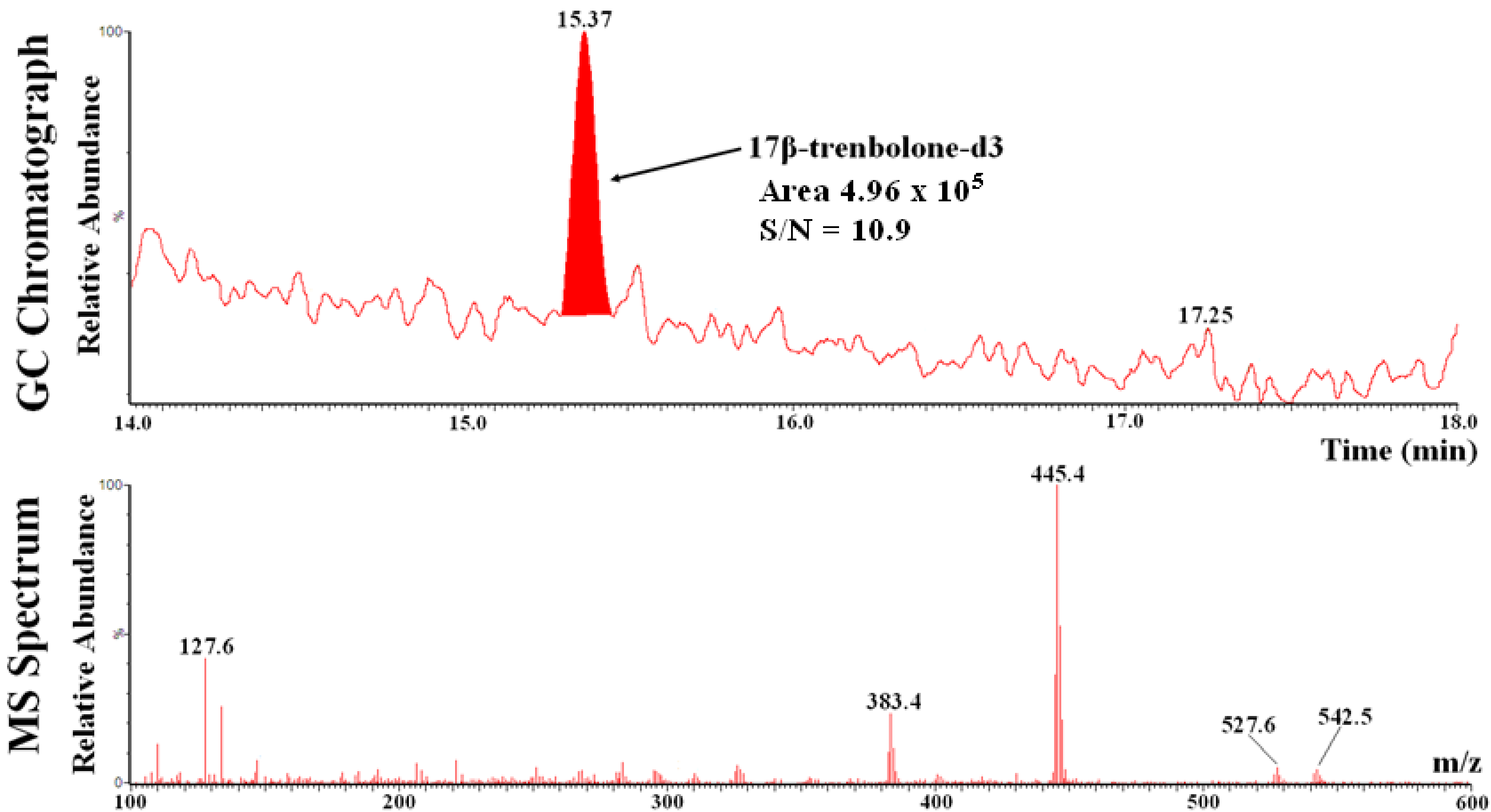


Figure 3-11: A 500 ppb sample of derivatized 17β-trenbolone-d3 with chromatogram (top) and spectrum (bottom).

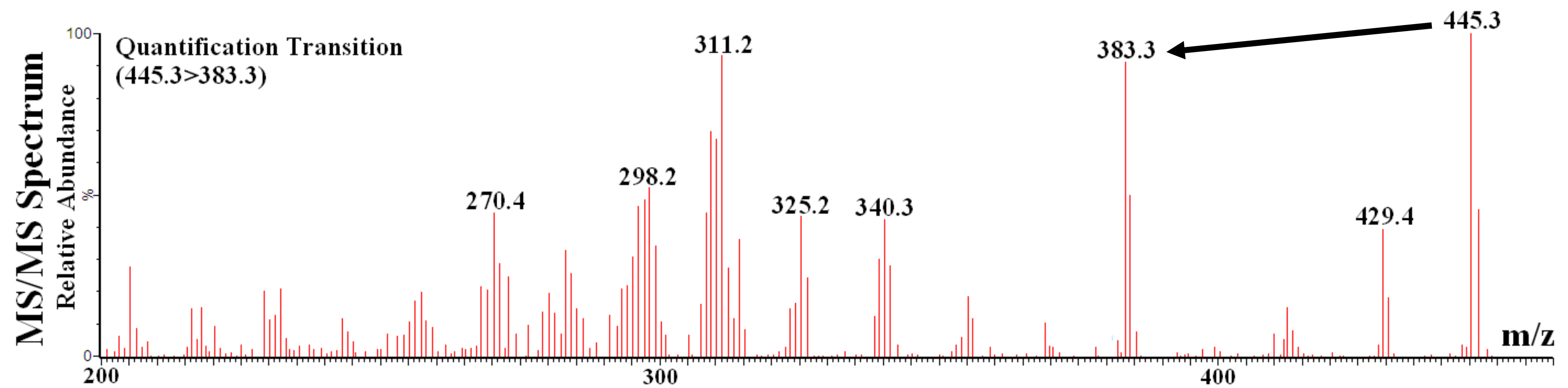


**Figure 3-12: MRM quantification transition for 17β-trenbolone-d3 (445.3>383.3 at 30 V). There is only one transition for 17β-trenbolone d3 because it is used as a surrogate standard and is always present in samples at significant concentrations, not requiring a confirmatory transition.**

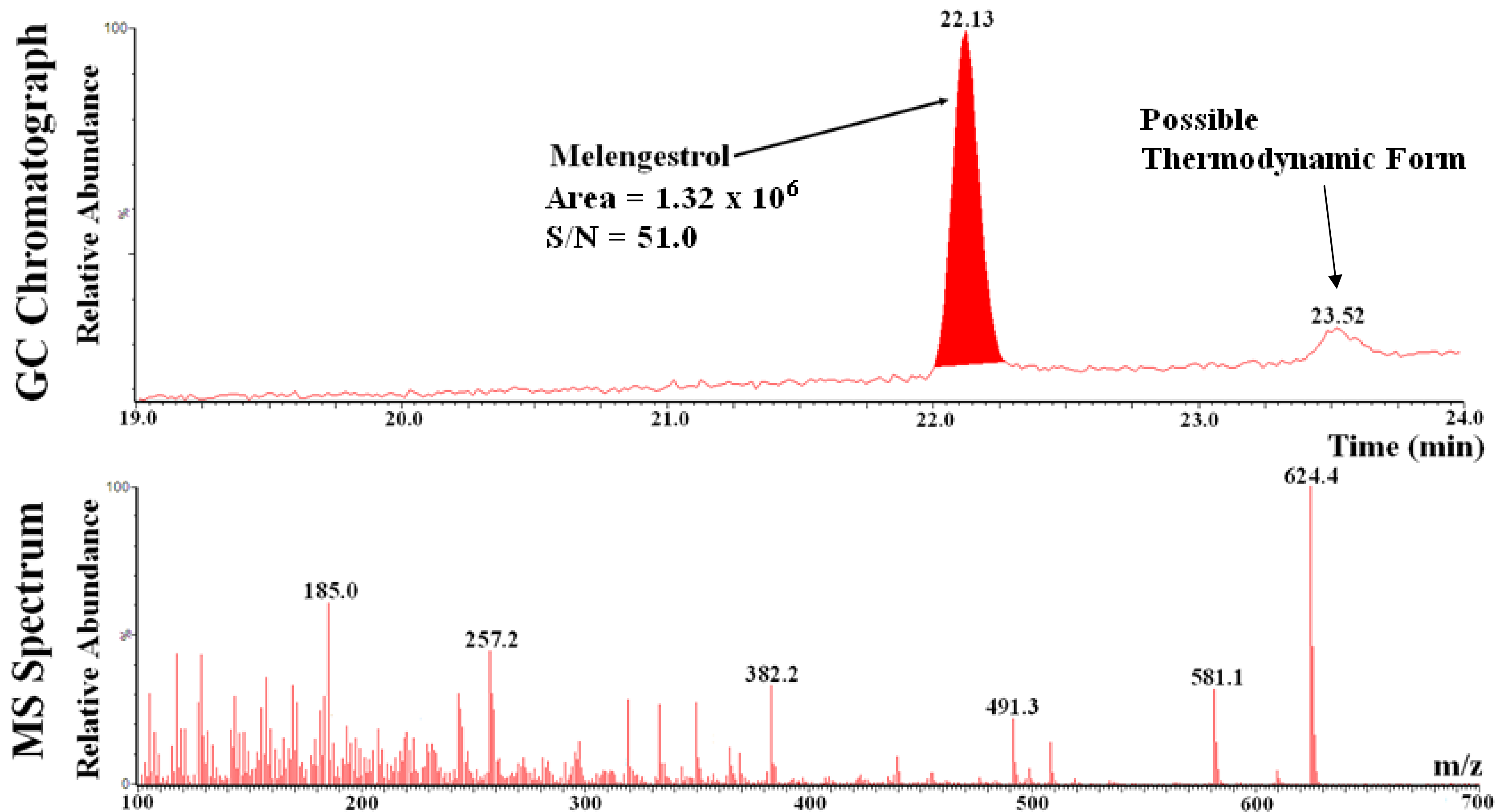


**Figure 3-13: An 8 ppm sample of derivatized melengestrol with chromatograph (top) and spectrum (bottom).**

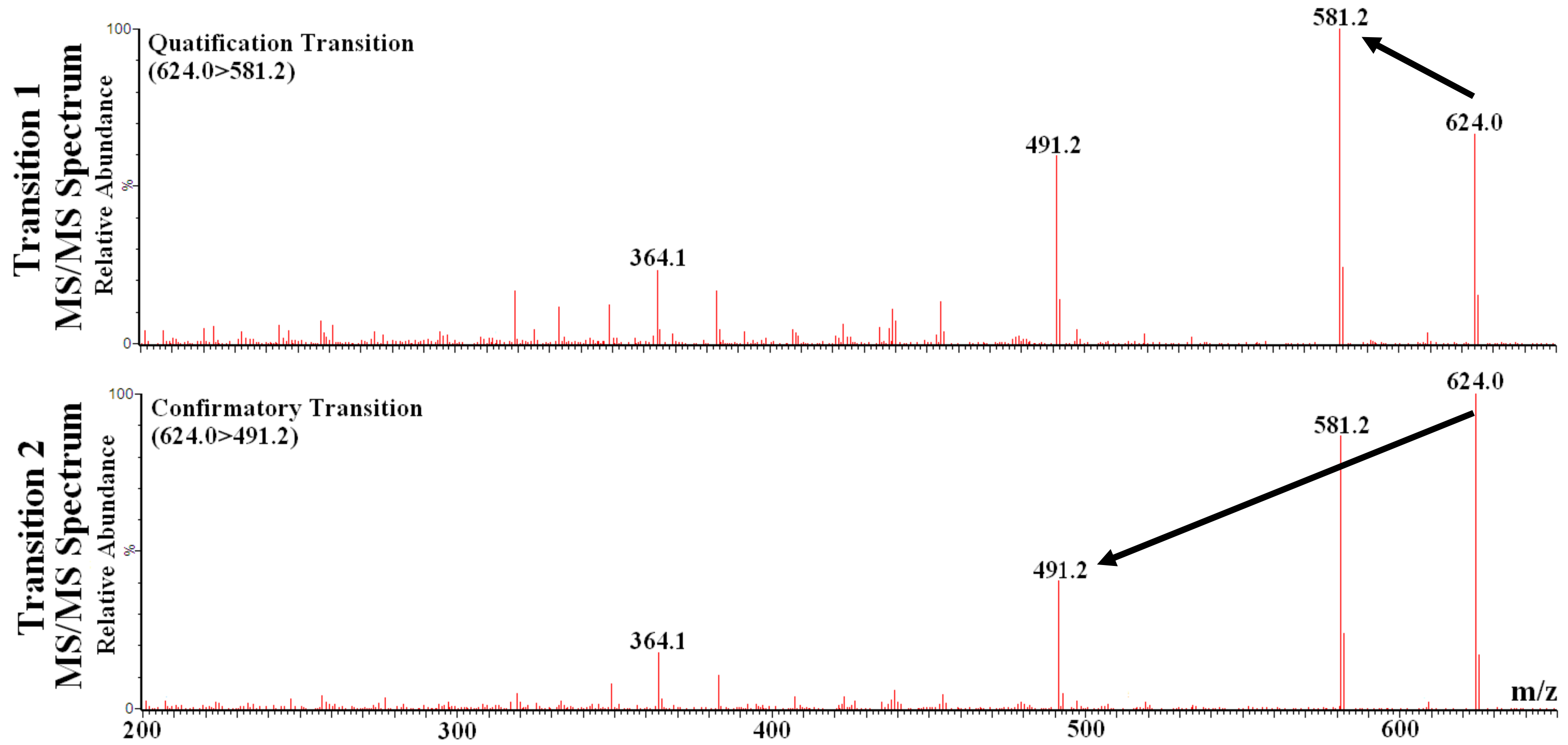


**Figure 3-14: MRM transitions one (top, 624.0>581.2 at 10V) and two (bottom, 624.0>491.2 at 12 V) for melengestrol.**

**Table 3-2: Retention times ($R_t$), parent ion (MW), Transions 1 and 2, and corresponding collision energies.**

| Steroid | $R_t$ (min) | MW | Quantification Transition | Collision Energy (V) | Confirmatory Transition | Collision Energy (V) |
|---|---|---|---|---|---|---|
| *Androgen* | | | | | | |
| 17α-Trenbolone | 14.22 | 539.2 | 449.3>323.2 | 8 | 380.2>323.2 | 24 |
| 17β-Trenbolone-d3 | 14.69 | 542.2 | 445.3>383.3 | 30 | | |
| 17β-Trenbolone | 14.71 | 539.2 | 442.2>382.2 | 30 | 442.2>309.3 | 32 |
| Trendione | 14.34 | 539.2 | 537.2>522.3 | 22 | 440.3>410.3 | 10 |
| | | | | | | |
| *Progestagen* | | | | | | |
| Melengestrol | 20.39 | 624.0 | 624.0>581.2 | 10 | 624.0>491.2 | 12 |

**(Retention times may differ from chromatographs as changes in machinery and processes during method development often affect retention times of compounds.)**

### 3.3 $I_2$ Concentration in MSTFA

The concentration of $I_2$ in MSTFA was found to be the primary variable in both overall derivatization yield for all steroids studied and the appearance and elimination of second or thermodynamic peaks in 17α- and 17β-trenbolone. The presence of $I_2$ in MSTFA acts as a catalyst by facilitating the nucleophilic substitution of a $N(CH_3)COCF_3$ at the 4-position and the silylation of the 3- and 17-oxygentated positions (Maume, *et al.,* 1998). The amount of $I_2$ added to MSTFA for use in derivatization was found to strongly impact the overall yield of steroids. For example, 2 ppm standards, run in triplicate, of 17α- and 17β-trenbolone were derivatized with increasing concentrations of $I_2$ in MSTFA beginning at zero and ending at 2.15 mg/mL. The yield results for these experiments, as measured each compounds fragment (m/z = 380.1), present in both 17α- & 17β-trenbolone are given in Figure 3-15 (17α-trenbolone) and Figure 3-16 (17β-trenbolone).

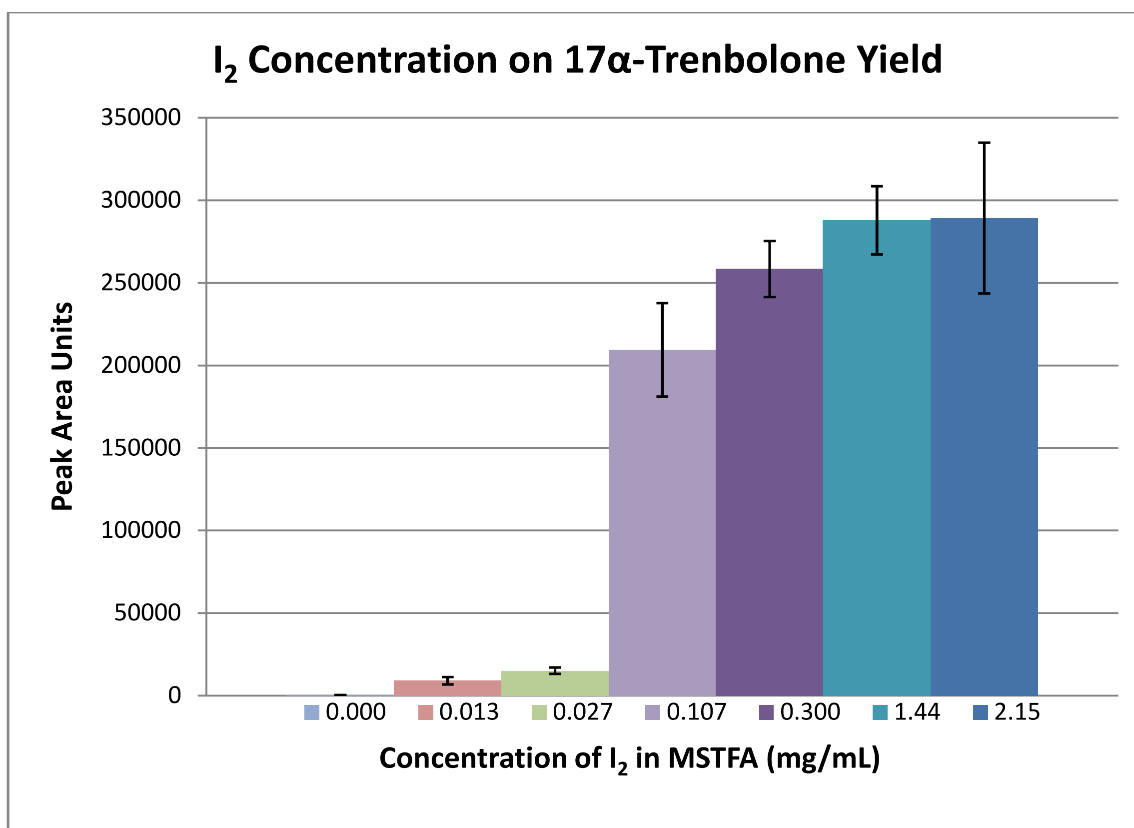


**Figure 3-15: Peak area responses from 2 ppm samples of 17α-trenbolone derivatized with different concentrations of $I_2$ in MSTFA. Error bars represent standard error of triplicate samples.**

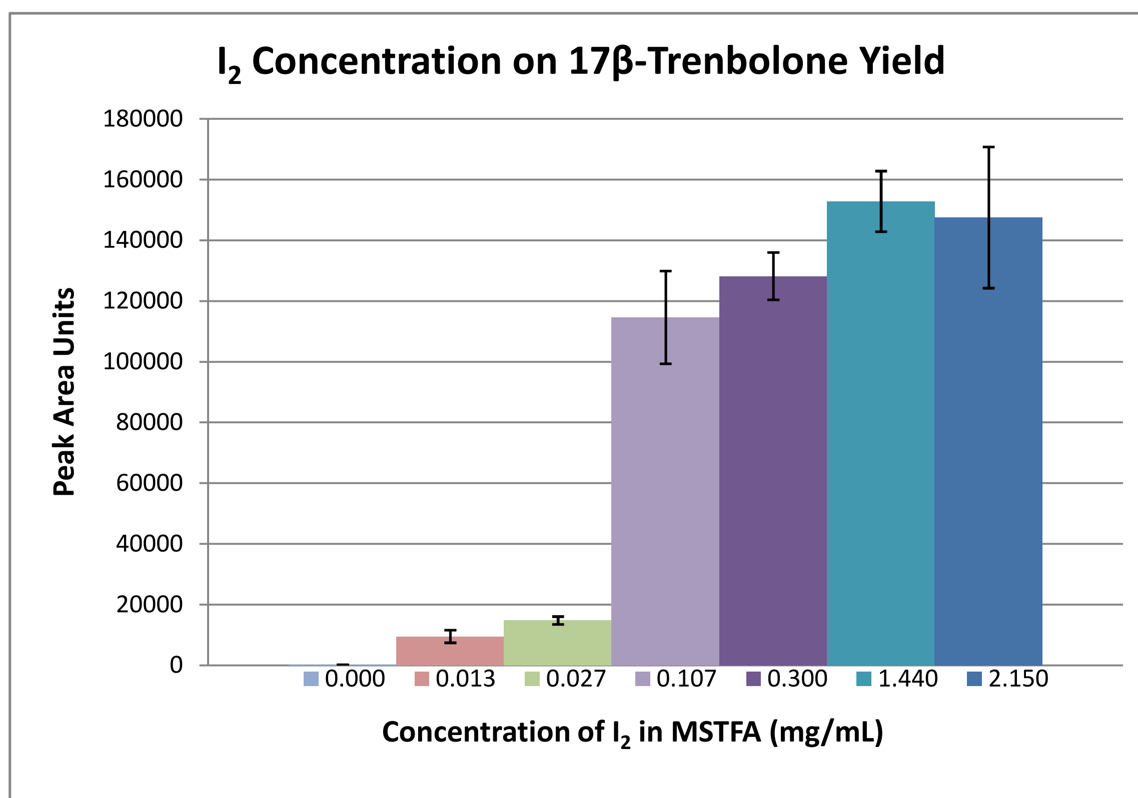


**Figure 3-16: Peak area responses from 2 ppm samples of 17β-trenbolone derivatized with different concentrations of $I_2$ in MSTFA. Error bars represent standard error of triplicate runs.**

Results from Figure 3-15 and Figure 3-16 would indicate, based on standard error, that beyond a concentration 0.107 mg/mL ($I_2$/MSTFA) the amount of $I_2$ present in MSTFA during derivatization does not affect yield.

### 3.3.1 Second Chromatographic Peak Appearance and Elimination

It was observed that at significantly lower concentrations of $I_2$ in MSTFA than literature values (4:1000 m/v) derivatization would yield the appearance of a second chromatographic peak in addition to the usual 17β-trenbolone peak. This second peak could result in an apparent false positive for 17α-trenbolone because both 17α- and 17β-trenbolone share this MRM transition. To investigate this relationship an experiment was devised where increasing

concentrations of $I_2$ in MSTFA were used to derivatize only 17β-trenbolone. Analogous results also appeared for 17α-trenbolone.

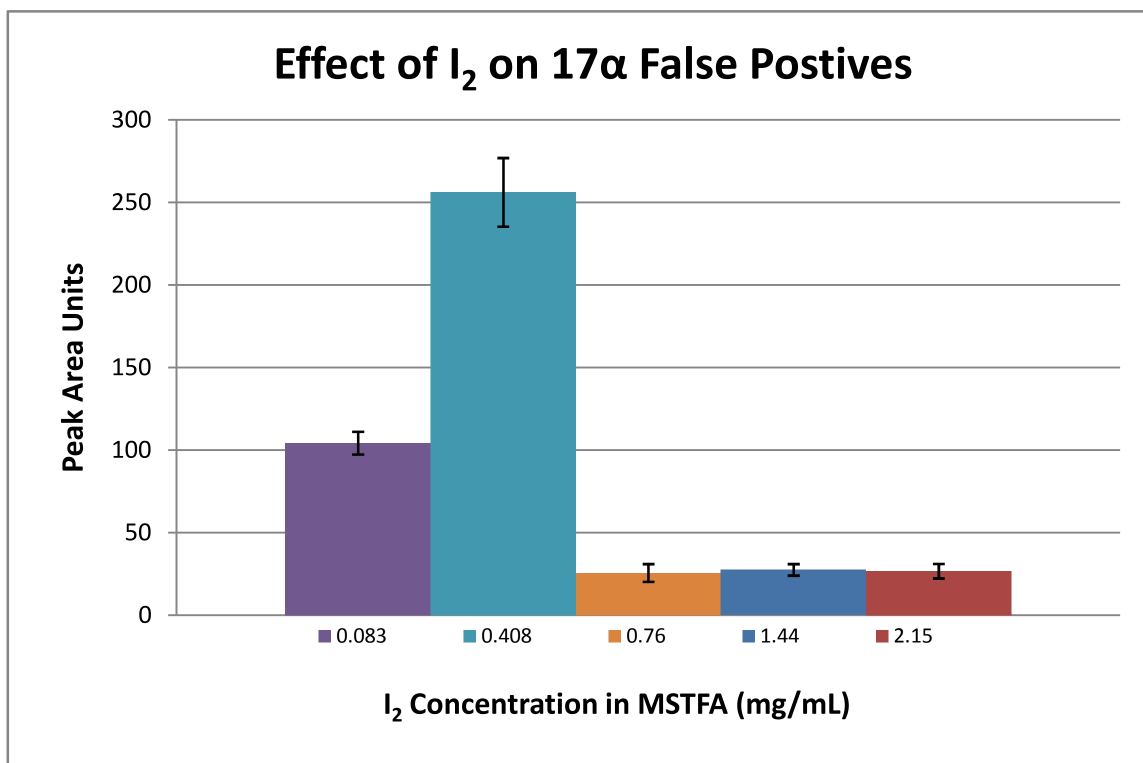


**Figure 3-17: The appearance of a 17α-trenbolone peak in a sample of 17β-trenbolone, a function of the $I_2$ concentration in MSTFA during derivatization.**

While it was not fully explored why false positives emerge at lower (< 0.76 mg/mL ($I_2$/MSTFA )), it is evident from the results in Figure 3-17 that concentrations exceeding 0.76 mg/mL ($I_2$/MSTFA) minimizes the second peak formation to < 5% of highest observed false positive values.

To further illustrate the importance of $I_2$ to MSTFA concentration in the derivatization reagent, two 1 mg/L standards of only 17β-trenbolone were derivatized, each with a different $I_2$/MSTFAconcentration (2.15 and 0.083 mg/mL) and were analyzed using the quantification transitions for both 17α- and 17β-trenbolone. The results of this experiment are illustrated in Figure 3-18. As the data from Figure 3-17 would suggest, a significant amount of 17α-trenbolone

is observed in the 0.083 mg/mL ($I_2$/MSTFA) derivatized 17β-trenbolone standard. 17α-trenbolone virtually disappears with the 2.15 mg/mL ($I_2$/MSTFA) derivatized 17β-trenbolone standard.

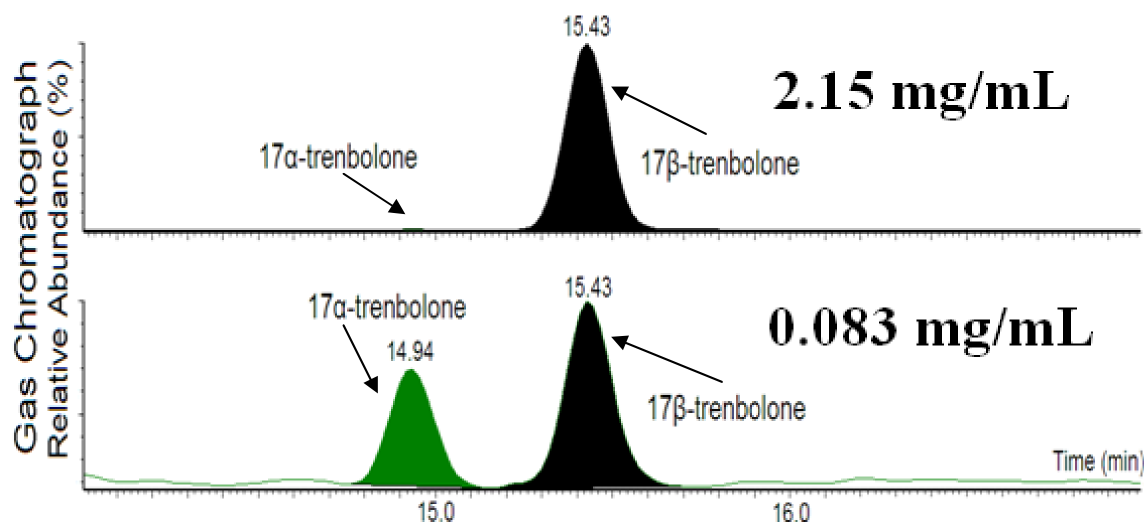


**Figure 3-18: Comparison of a 1 mg/L standard of derivatized 17β-trenbolone with 2.15 mg/mL (top) versus 0.083 mg/mL (bottom) (MSTFA/$I_2$).**

## 3.4 Limits of Detection and Quantification

The limits of detection (LOD) and limits of quantification (LOQ) were determined by comparing the signal (target peak area) to noise (baseline) within chromatograms. The limit of detection for the compounds studied was established at a signal to noise ratio of three or higher. The limit of quantification was established at a signal to noise ratio of six or higher.

17α-trenbolone is used as an example to illustrate how machine LODs and LOQs were established for each of the compounds studied. Two standard concentrations, 0.75 µg/L and 1.0 µg/L of 17α-trenbolone were derivatized using the method presented in this study. Their gas chromatograms along with their signal to noise ratios are given in Figure 3-19 and Figure 3-20, respectively. With a signal to noise ratio (S/N) of 8, 1.0 µg/L was found to be the limit of quantification of 17α-trenbolone for our GC/MS/MS instrument. A S/N of 4.29 established the

GC/MS/MS limit of detection at 0.75 µg/L for 17α-trenbolone. A summary of machine LODs and LOQs for all steroids in this method are given in Table 3-3.

**Table 3-3: Summary of machine and method LODs and LOQs for each of the steroids studied in this method.**

| | Machine LOD (µg/L) | Machine LOQ (µg/L) | Method LOD (ng/L) | Method LOQ (ng/L) |
|---|---|---|---|---|
| 17α-Trenbolone | 0.75 | 1.0 | <2.5 | <2.5 |
| 17β-Trenbolone | 0.75 | 1.0 | <2.5 | <2.5 |
| Trendione | 1.0 | 2.0 | <2.5 | <2.5 |
| Melengestrol | 20 | 20 | 100 | 100 |

The machine LOD and LOQ refer to the lowest possible concentrations that the GC/MS/MS can detect and quantify. The method LOD and LOQ refer to the lowest concentrations that can be detected and quantified in real samples which have gone through the SPE, Florasil clean up and derivatization processing. Method LODs and LOQs exhibit much lower concentrations because the volume analyzed decreases from its sample volume to the volume that is actually analyzed in the GC/MS/MS while the analyte mass is conserved. For example, a 1 L sample which contains 1 ng of 17β-trenbolone would have a concentration of 1 ng/L. If that 1 ng of 17β-trenbolone is completely conserved through processing, it is resuspended into 100 µL of MSTFA, resulting in a concentration of 10 µg/L for GC/MS/MS analysis.

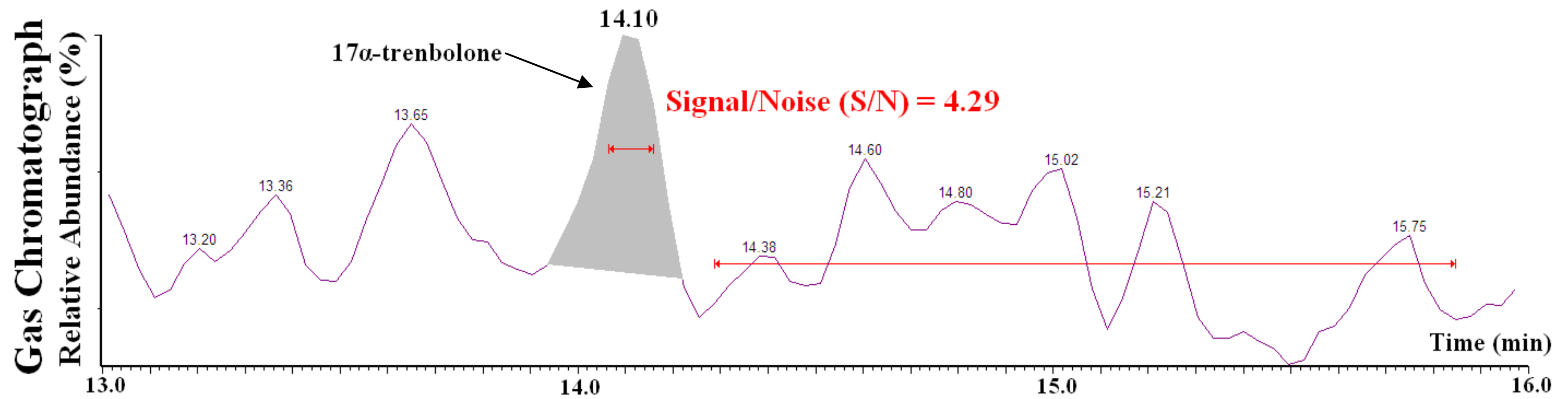


**Figure 3-19: Signal to noise ratio example establishing the machine LOD for 17α-trenbolone at 0.75 µg/L.**

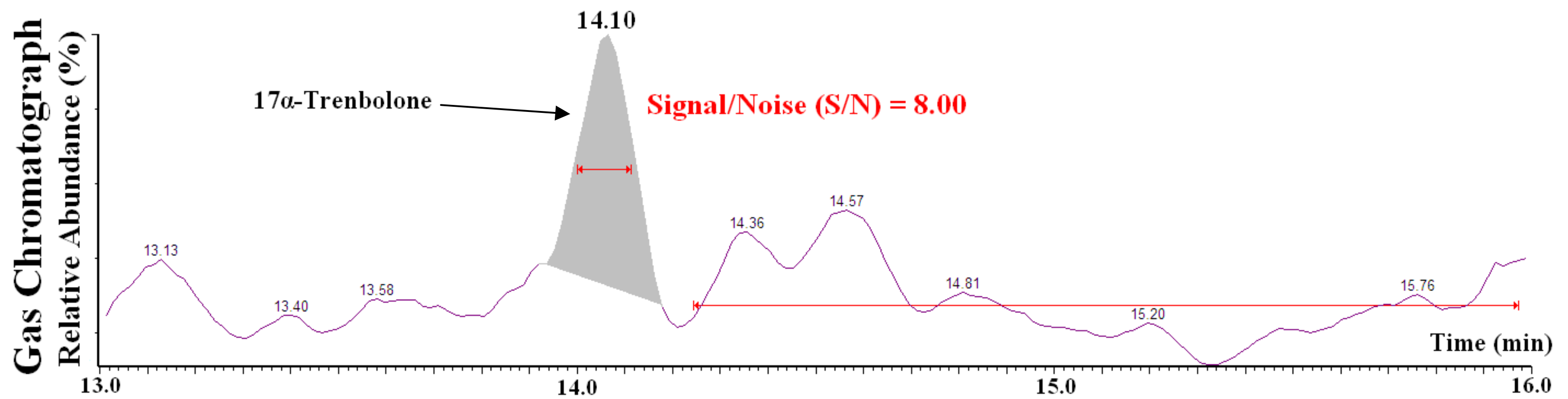


**Figure 3-20: Signal to noise ratio example establishing the machine LOQ for 17α-trenbolone at 1.0 µg/L.**

## 3.5 Calibration Curves

A representative, seven point calibration curve for each of the trenbolone family compounds, normalized to an internal standard is given in Figure 3-21. With a machine LOQ of 1 µg/L for 17α-trenbolone and 17β-trenbolone, the concentrations represented in Figure 3-21 are 1, 2, 5, 10, 20, 50 and 100 µg/L. With a machine LOQ of 2 µg/L for trendione, the concentrations represented in Figure 3-21 are 2, 5, 8, 10, 20, 50 and 100 µg/L. Melengestrol is not represented in this figure due to an inability to repeatedly detect it below 50 ppb.

Response, as represented in Figure 3-21 refers to the result of each concentrations peak area number in relation to the internal standard, 17β-trenbolone-d3, and is calculated using Equation 1. The internal standard did not vary outside of ± 7.0% in the making of the curves in Figure 3-21.

$$Response = Sample\ Concentration\left(\frac{Peak\ Area}{Internal\ Standard\ Peak\ Area}\right) \quad \text{Equation 1}$$

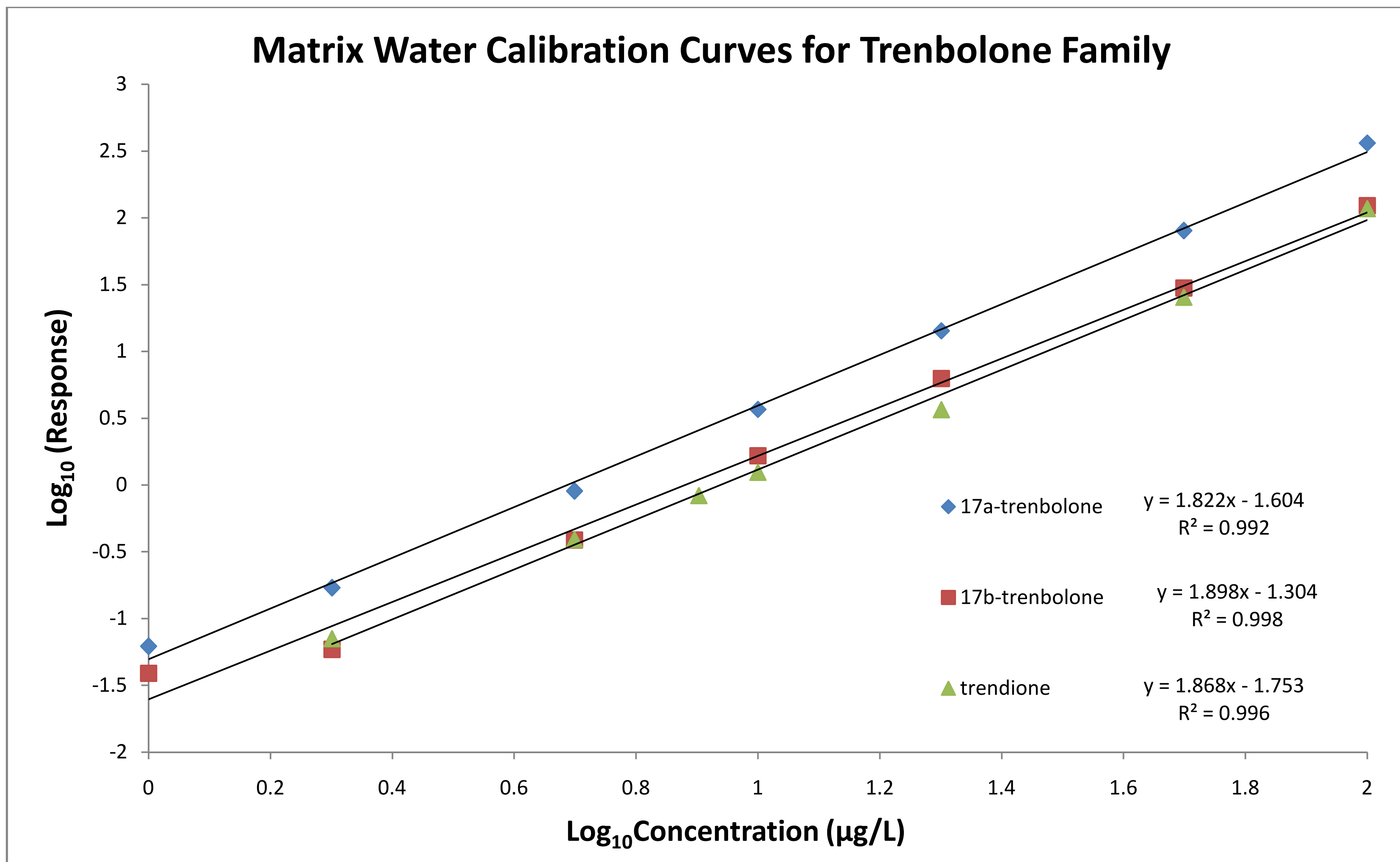


**Figure 3-21: Representative calibration curves for 17α-trenbolone, 17β-trenbolone and trendione.**

## 3.6 Comparison of SPE Cartridges

Selections of commercially available SPE cartridges were tested for recovery of the studied compounds in environmentally representative matrix water which featured organic content consistent with many agricultural runoff waters. The matrix water was obtained from the Sierra Foothills Research and Extension Center near Grass Valley, CA, USA. Samples were extracted through respective SPEs but were not followed by a Florasil clean up stage. The results are given in Figure 3-22.

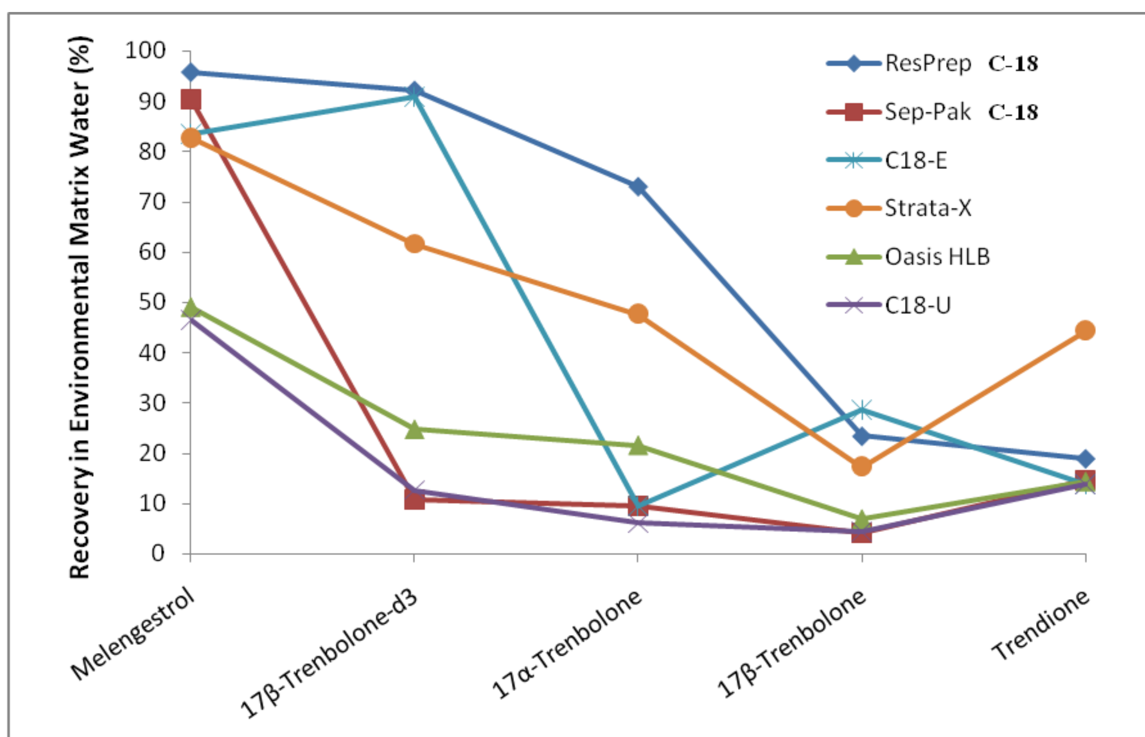


**Figure 3-22: Comparison of SPE cartridges on absolute recovery for 200 mL environmentally representative water samples spiked with 50 ng/L of each compound.**

It was expected prior to the SPE comparison test that the ResPrep C-18 cartridges would perform with the highest recovery efficiency, and in general the results confirm that assumption. The data however, appears noisy, and recovery efficiencies for all cartridges, including the ResPrep C-18 appear to be low for key analytes such as 17β-trenbolone and trendione. It is

hypothesized that the efficiency recoveries are low for these compounds because a Florasil clean up stage was not utilized, and thus matrix interferences were high. The data in Figure 3-22 support the necessity of a Florasil clean-up stage after SPE extraction to eliminate matrix interferences when complex environmental matrix samples are analyzed.

### 3.6.1 Method Validation

For method validation, matrix water samples were collected from three sites: The Sierra Foothills Research and Extension Center near Grass Valley, CA, USA (Grass Valley matrix water), Evans Creek which runs through the Rancho San Rafael park in Reno, NV, USA (Rancho San Rafael matrix water), and Steamboat Creek in Reno, NV, USA (Steamboat Creek matrix water). These three sites represent an array of environmental surface waters. Steamboat creek is a altered natural waterway which receives a large agricultural and urban storm water runoff. Evans creek is relatively pristine natural surface water which, at the time of collection was composed mainly of snowpack and rainwater with minimal inputs from other sources. The Grass Valley matrix water represents water which has been passed through agricultural fields, though it does not necessarily represent extremely complex environmental matrices like that of CAFO treatment lagoon effluent. The collection procedure was a grab-sample using a 5 L bottle. 1 L Aliquots were then taken from the 5 L bottle.

## 3.7 Laboratory Spike Results

Separate matrix spike recoveries were conducted on Grass Valley, Rancho San Rafael and Steamboat Creek matrix waters using two concentrations for all steroids in this method. Results are not provided for melengestrol, due to hypothesized derivatization complications which are discussed later in this report. The first concentration was 50 ng/L and the second was 2.5 ng/L.

The 50 ng/L spike recovery was attempted as an "easy" recovery that would likely ensure results that could be used to determine how much lower of a concentration the next spike recovery could achieve. It was determined that 2.5 ng/L would be attempted, as this is considered "environmentally relevant" and is a concentration that could be expected in many receiving waters in the natural environment where CAFO lagoon effluents are present.

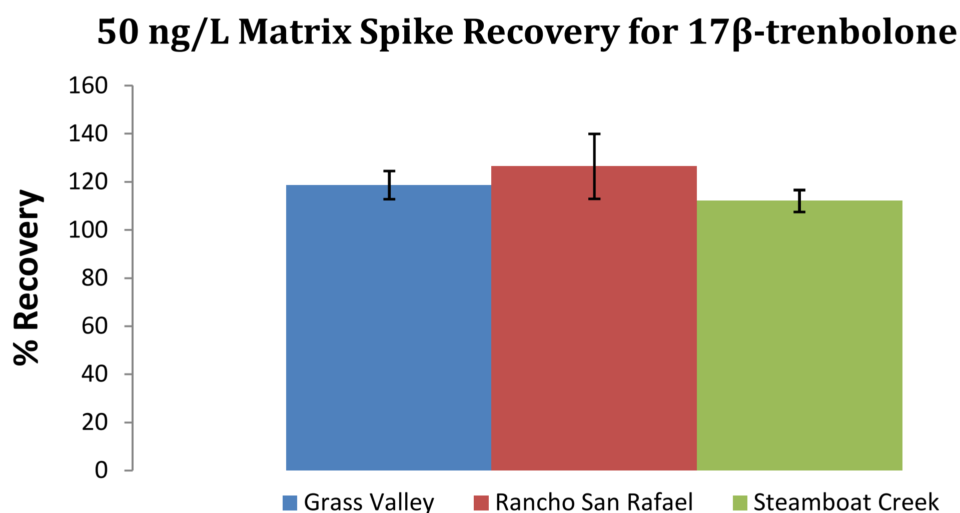


**Figure 3-23: Absolute recovery for three matrix water recoveries for the steroid 17β-trenbolone. Greater than 100 percent recoveries were observed. Error bars represent standard error based on triplicate runs.**

According to Figure 3-23 recoveries for 17β-trenbolone were above 100 percent in each matrix. It should be noted that for this and the other recoveries presented in this section that the calibration curve used to calculate recoveries was made from fully processed (SPE and Florasil clean up) Steamboat Creek matrix water. The intended reason for using a matrix water to make calibration curves was to possibly eliminate the effect of active sites on laboratory glassware. The presence of active sites on glassware can have the effect of physically adsorbing steroid analytes, decreasing overall yields.

Moreover, the recovery data presented here are absolute recoveries, which means that they are not corrected using the surrogate standard, 17β-trenbolone-d3. It was observed, in general, that when absolute recoveries are near or above 100 percent, as was the case with the data in Figure 3-23, correcting the data with a surrogate standard only increased recovery values, the exception being 17β-trenbolone (See Figure 3-24). As a result, recoveries using Steamboat matrix water increased beyond absolute values, and thus a surrogate standard correction is neglected.

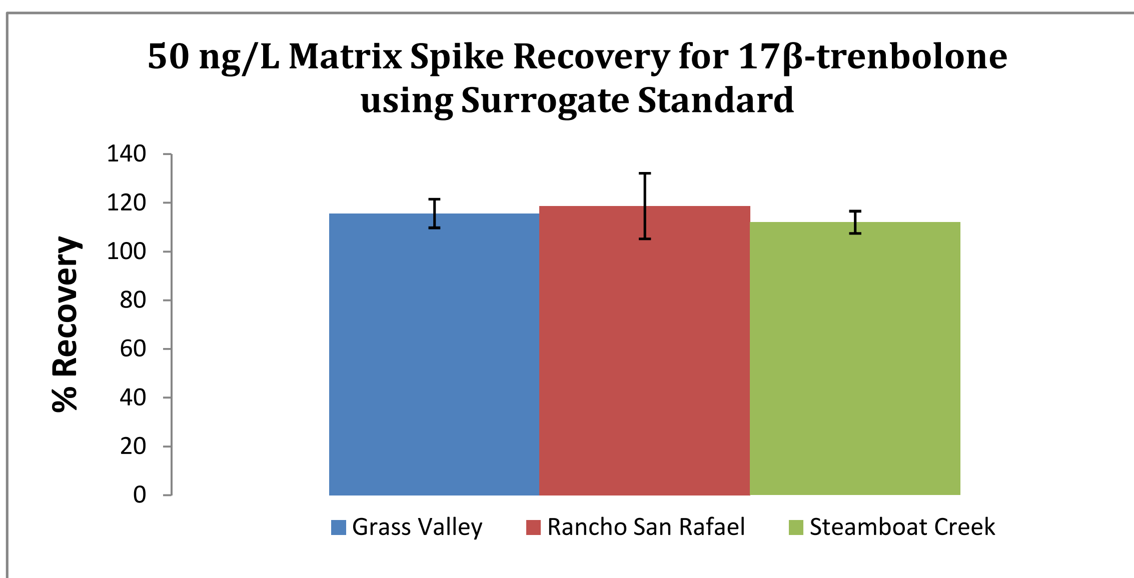


**Figure 3-24: Data from Figure 3-23 corrected with the surrogate standard 17β-trenbolone-d3. Error bars represent standard error from triplicate runs.**

An example of why absolute recoveries were used in spike recoveries as opposed to normalized recoveries using the surrogate standard is illustrated in Figure 3-25. Instead of correcting recoveries to reasonable levels (80% to 120%), the surrogate standard resulted in much larger recovery values than would be expected (~200-220%). This is evidence that for trendione, a deuterated form would be necessary for use as a surrogate standard to improve recovery estimates, though no such form may exist commercially.

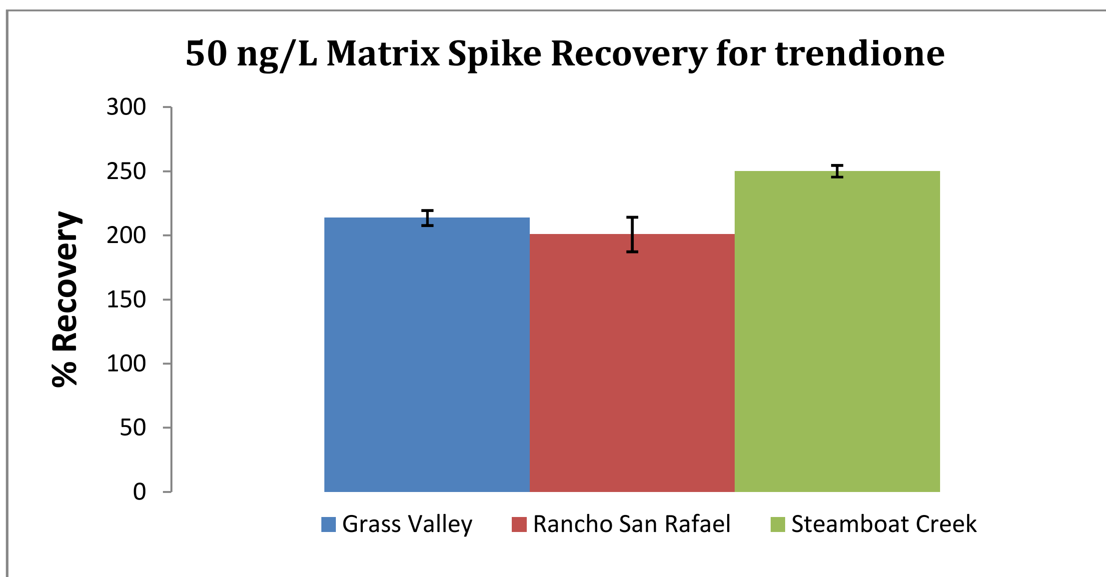


**Figure 3-25: 50 ng/L Matrix Spike Recovery for trendione using corrected data from the surrogate standard 17β-trenbolone-d3. In the case of trendione the use of the surrogate standard appeared to exaggerate recoveries. Error bars represent standard error for triplicate runs.**

A 2.5 ng/L matrix recovery was attempted in a similar fashion to the 50 ng/L matrix recovery illustrated in Figure 3-23. The results of this recovery are presented in Figure 3-26. As opposed to the 50 ng/L matrix spike recovery for 17β-trenbolone, Figure 3-26 illustrates results closer to those that were expected. Both 50 and 2.5 ng/L matrix spike recoveries were conducted for 17α-trenbolone, the results of which are represented in Figure 3-27 and Figure 3-28, respectively. 17α-trenbolone appeared to represent more realistic recoveries (80%-100%) in both the 50 and 2.5 ng/L cases.

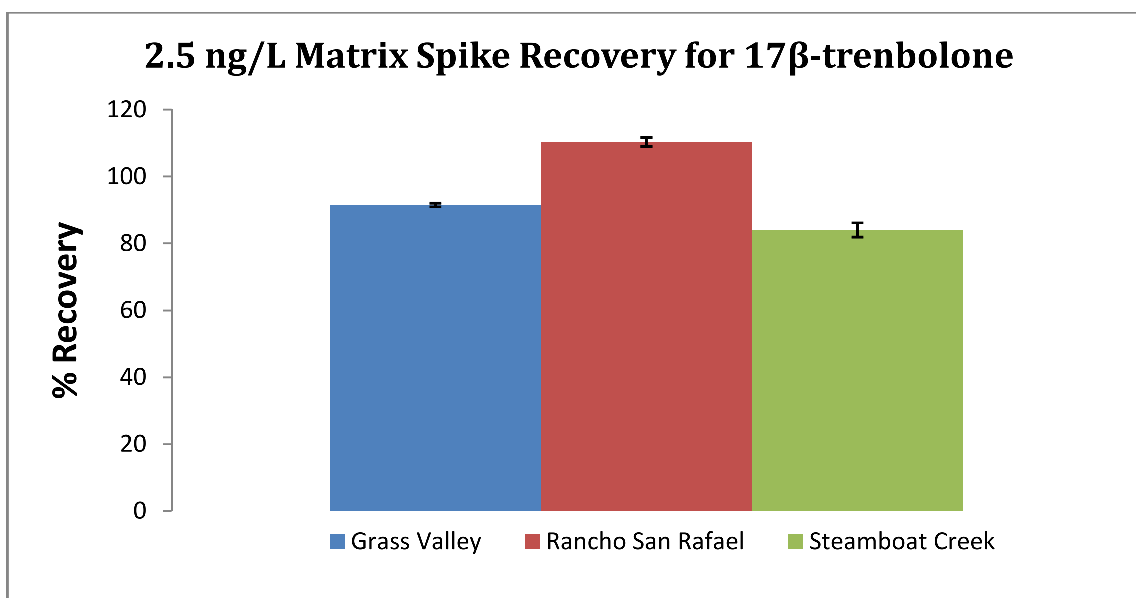


**Figure 3-26: Absolute matrix spike recoveries for 2.5 ng/L of 17β-trenbolone. Error bars are based on standard error for triplicate runs.**

Matrix spike recoveries were attempted at 50 and 2.5 ng/L standard concentrations of trendione. However, using the transitions from Figure 3-10 yielded sporadic results. It was observed that through processing of matrix water and derivatization dry down, a residual of natural organic matter was attached to the vial. It was theorized that this natural organic matter physically adsorbs $I_2$ that is not removed during dry down. When pure MSTFA is put back into the vial to resuspend the target analyte, $I_2$ is resuspended with it. As a consequence it is theorized that trendione is allowed to continue reacting to its eventual end-products. Conversely, monitoring the second trendione peak transition yielded less sporadic results and therefore, the matrix spike recoveries for trendione are based off of a second trendione peak calibration. Spike concentrations are adjusted to reflect this fact.

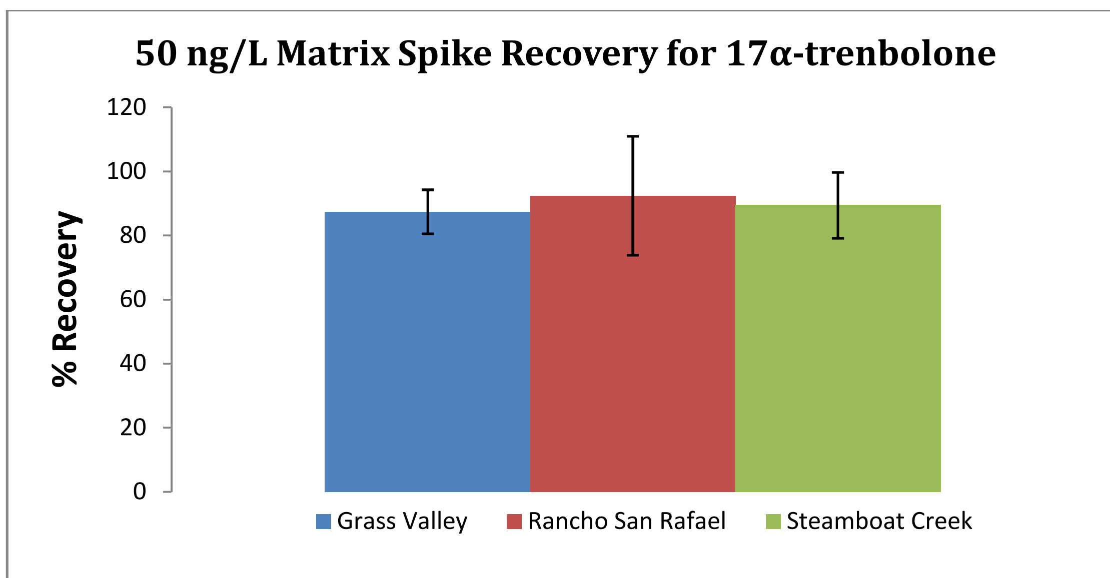


**Figure 3-27: 50 ng/L matrix spike recovery for 17α-trenbolone. Recoveries were above 80 percent for each matrix water. Error bars represent standard error for triplicate runs.**

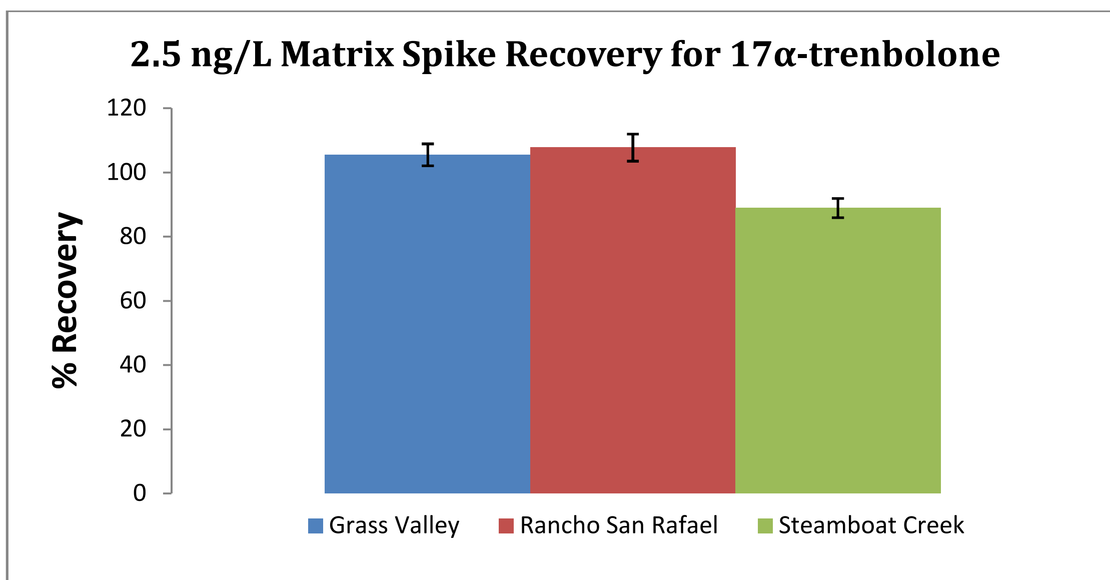


**Figure 3-28: 2.5 ng/L matrix spike recoveries for 17α-trenbolone. Grass Valley and Rancho San Rafael matrices recovered at or above 100 percent. Error bars represent standard error for triplicate runs.**

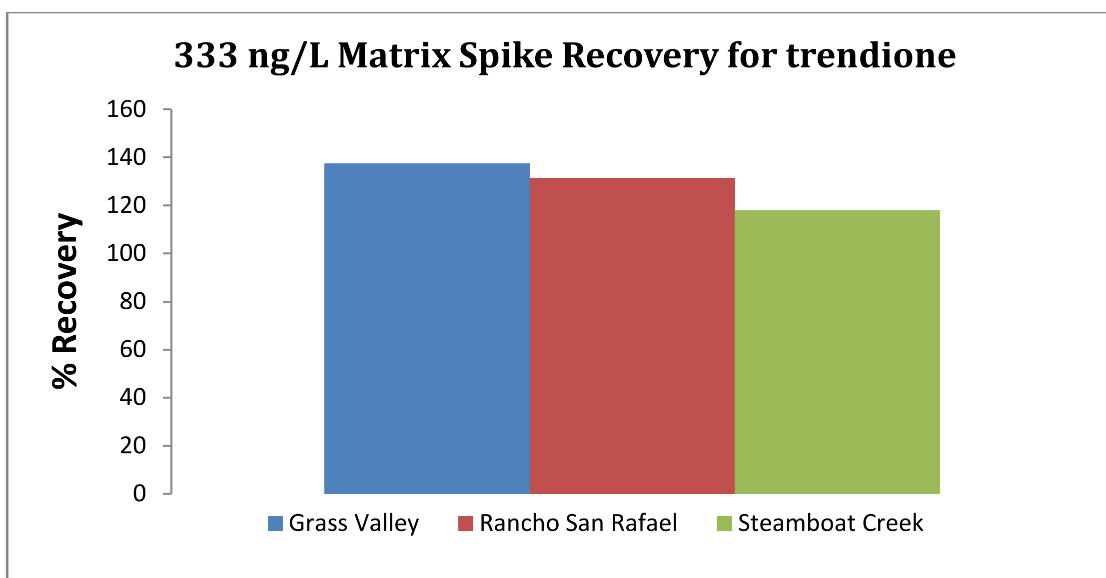


**Figure 3-29: 333 ng/L matrix spike recovery for trendione based off of a processed matrix water calibration curve using the second trendione quantification transition. No error bars are present because only two data points for each matrix water were detectable.**

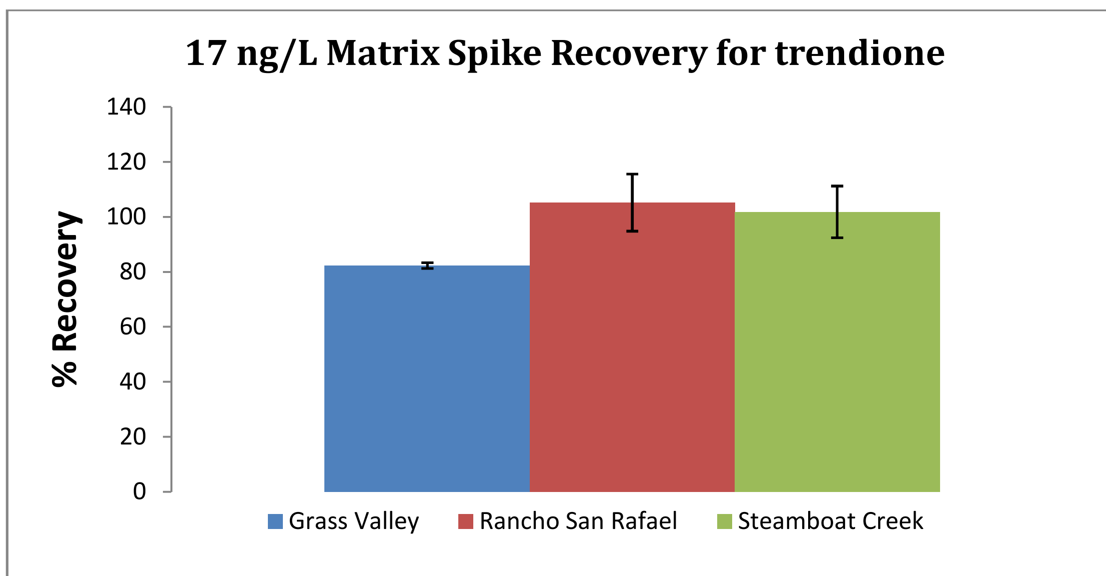


**Figure 3-30: 17 ng/L matrix spike recovery for trendione based off of similar conditions to the 333 ng/L matrix spike recovery. Error bars represent standard error in triplicate runs.**

## 3.8 Full Method Gas Chromatogram

To demonstrate that all steroids studied in this method can be analyzed using the MSTFA/$I_2$ derivatization method, a relatively high concentration (500 ppb) of each steroid was derivatized and analyzed. Using the experimental parameters given in the experimental section of this method a chromatogram was obtained (See Figure 3-31). Each steroid's quantification and confirmatory transition (excluding the internal standard, 17β-trenbolone-d3) was examined with strong peaks for each highlighted.

## 3.9 Melengestrol Complications

A linear, repeatable calibration curve for melengestrol was not obtained using the given method. It is unknown why melengestrol has sporadic responses and a number of variables were explored to correct the issue but no solution has yet been found. For example, a 20 ppb standard of melengestrol was derivatized and analyzed using the established method and gave an excellent response and signal to noise ratio as seen in Figure 3-32. Based on this data, the method should be comparably effective for melengestrol as for the trenbolone compounds. However, the protocols for melengestrol are still not robust and repeatable.

## 3.10 Trendione Complications

Trendione proved to have poor reproducibility when samples were repeatedly analyzed over time. A spiked standard of 200 ng/L in 250 mL of matrix water was processed through the SPE and Florasil clean up stages prior to derivatization and compared to a 500 ppb standard derivatized without processing. Both quantification and confirmatory transitions were monitored along with the trendione second peak quantification transition. The results are given in Figure 3-33, Figure 3-34 and Figure 3-35, respectively. Results indicate that the trendione concentration

in solution continually decreases with time, while the second trendione product peak results indicate more stable behavior. One possible explanation for the observed degradation of derivatized trendione could be due to reactions with light, which possibly photodegrades the derivatized product.

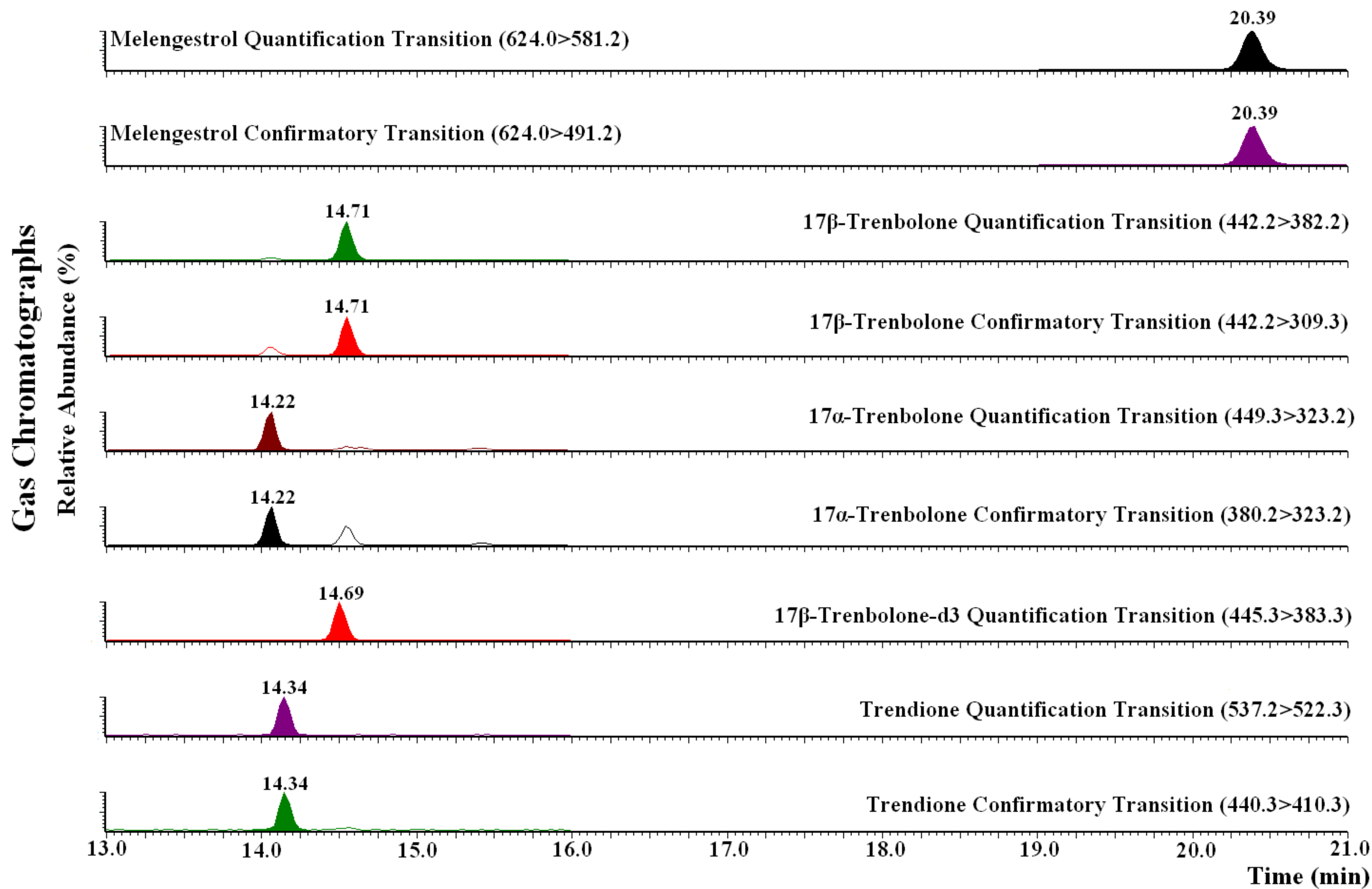


**Figure 3-31: Gas Chromatograph of each compound with a concentration of 500 µg/L.**

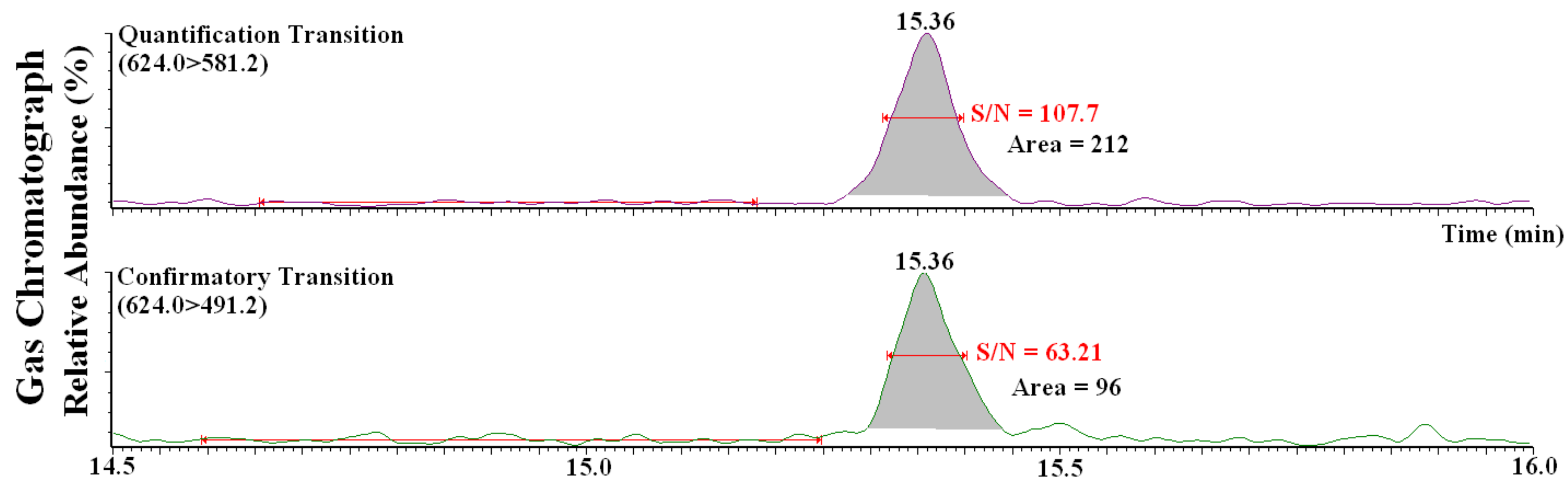


**Figure 3-32: A 20 ppb standard of melengestrol with excellent response and signal to noise. The retention time of melengestrol differs from reported values in this study because a different temperature program was utilized.**

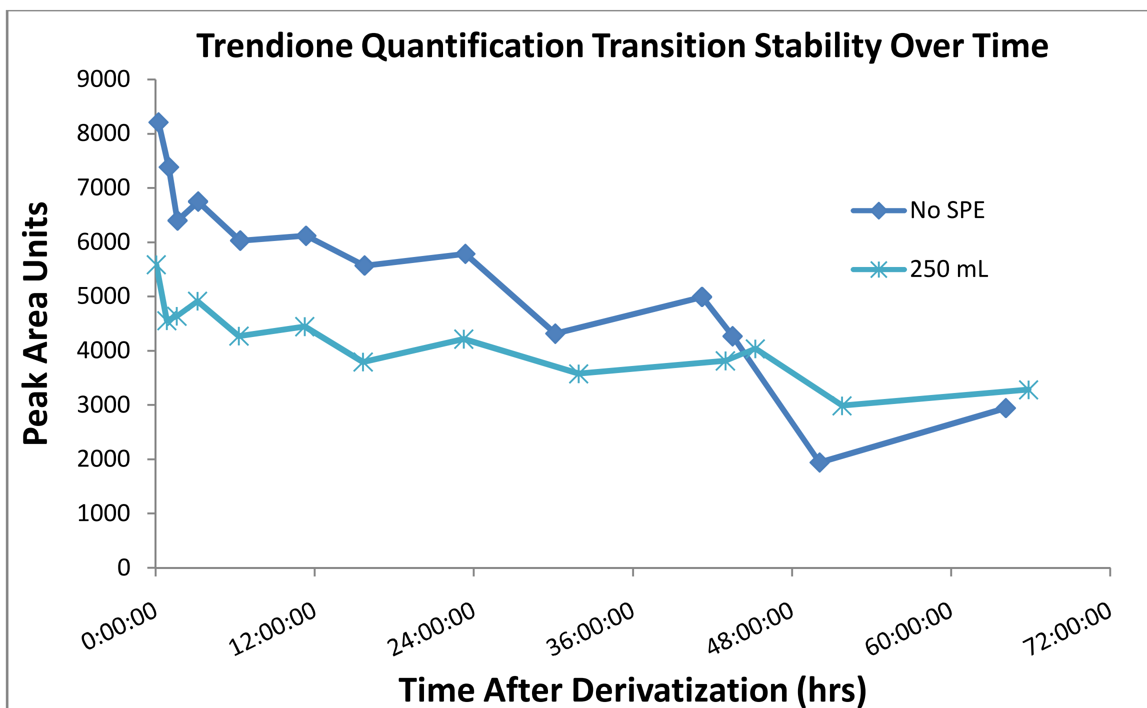


**Figure 3-33: Trendione quantification transition (537.2>522.3) stability over time showing continual decrease in both processed (250 mL of matrix water) and non-processed sample (No SPE). It should be noted that the 250 mL data represents a recovered concentration, which is less than the non processed data represented by No SPE data points.**

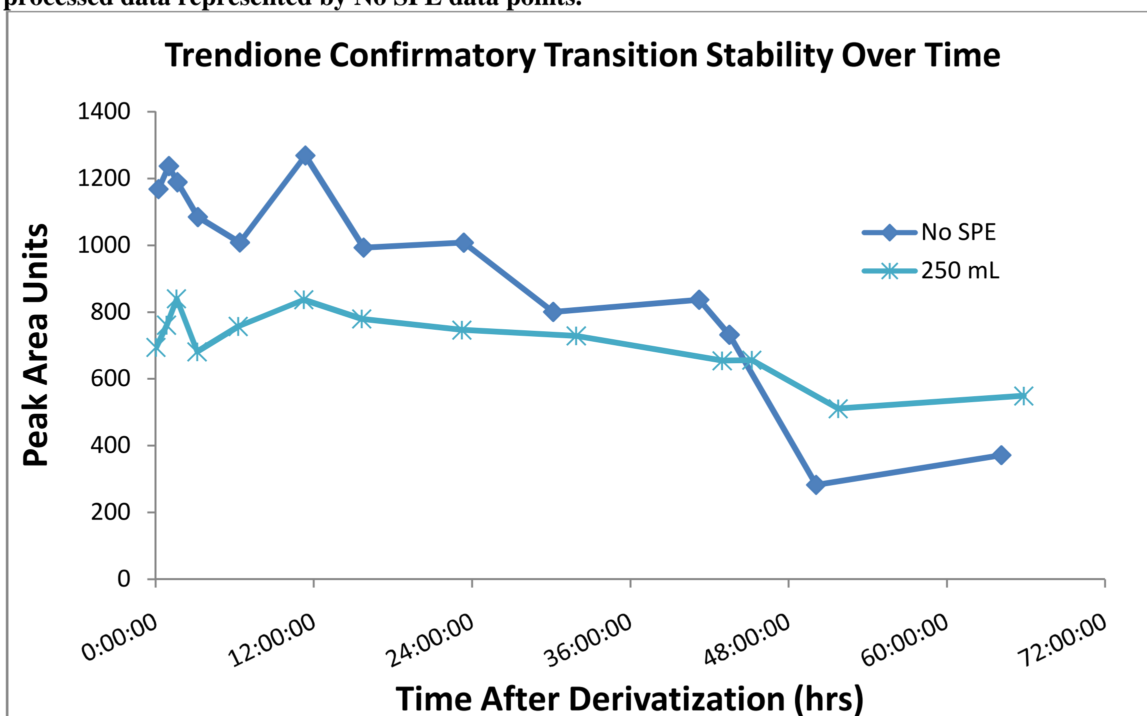


**Figure 3-34: Trendione confirmatory transition (440.3>410.3) stability over time. Similar results are observed with quantification stability.**

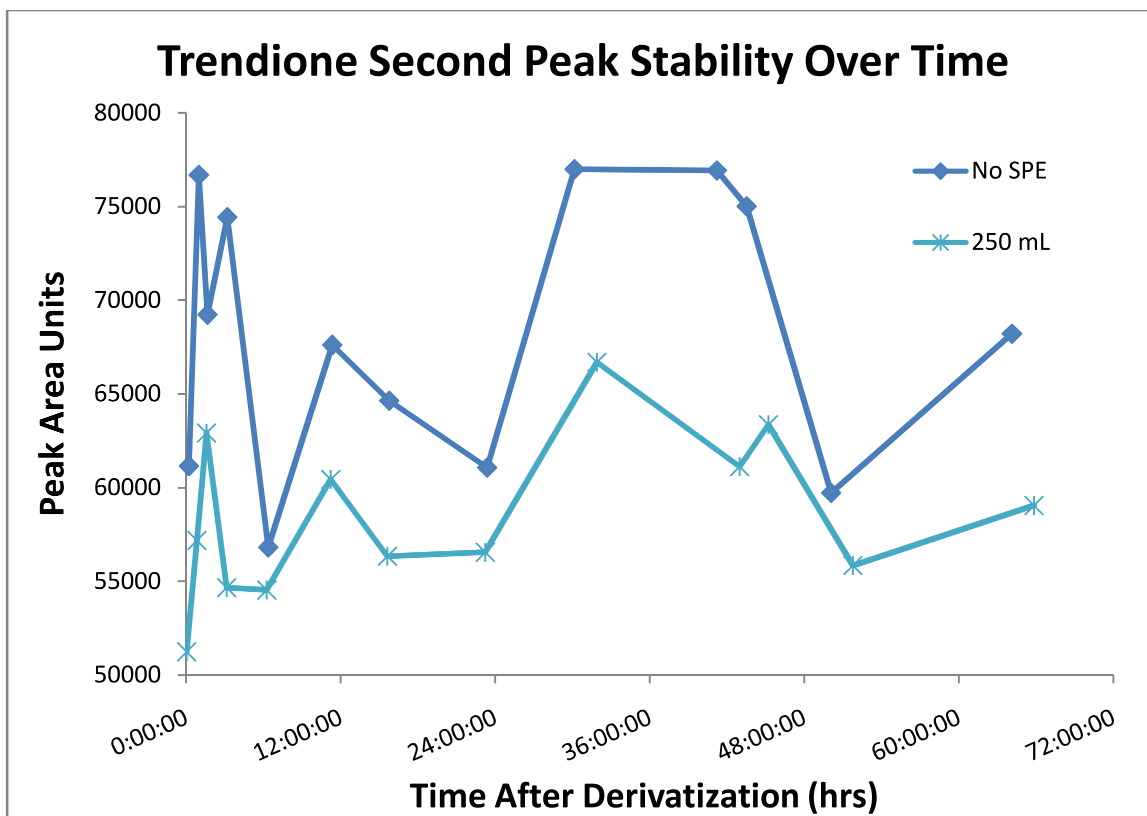


**Figure 3-35: Trendione second peak (monitored by quantification transition) stability over time.**

Results are erratic from Figure 3-35 but do not seem to have a continual decrease over time as was the case with the trendione transitions. A repeat of this experiment is necessary to conclude that the second product concentration does not decrease over time.

# 4 Discussion and Conclusions

The objective of this study was to develop an analytical method for the detection and quantification of the metabolites of trenbolone acetate and melengestrol acetate in complex environmental matrices. To date, no such method exists which utilizes gas chromatography / tandem mass spectrometry. The incorporation of trendione into a gas chromatography / tandem mass spectrometry method completes the family of trenbolone acetate metabolites and allows for the ability to analyze for trenbolone acetate metabolites in complex environmental matrices. This is critical considering that trenbolone metabolites have shown strong androgenic potential in sensitive aquatic species with exposures as low as 0.3 ng/L.

This method began with an existing derivatization method using a MSTFA/$I_2$ reagent developed by Maume, *et al.,* (1998) and Rambaud, *et al.,* (2007), intended for analyzing 17α- and 17β-trenbolone in biological matrices (bovine serum, tissue and urine). The derivatization procedure outlined by Maume, *et al.,* (1998) had to be repeated and reevaluated for its applicability to the particular GC/MS/MS used in our laboratory. Quantification and confirmatory transitions, along with their corresponding collision energies, were optimized for 17α- and 17β-trenbolone which were similar to those given by Rambaud, *et al.,* (2007). Transitions and corresponding collision energies were then established for trendione, melengestrol and the surrogate standard, 17β-trenbolone-d3.

As mentioned, the methods established by Maume, *et al.,* (1998) and Rambaud, *et al.,* (2007) were intended for use in biological matrices. Environmental matrices by comparison are more complex due to the presence of unknown organic matter, some of which may be similar in size and structure to target analytes. In effect, environmental matrices require the use of not only solid phase extraction, as used by methods intended for biological matrices, but also a matrix clean up stage. With a multitude of commercially available solid phase extraction cartridges

available, the ResPrep C-18 cartridge, manufactured by Restek was selected for the method. For a clean up stage after solid phase extraction, Florasil cartridges, also manufactured by Restek were employed.

Complex environmental matrices, because of the potential for matrix interferences, require an analytical instrument capable of unambiguously detecting target analytes to prevent false positives. Gas chromatography / tandem mass spectrometry with the inclusion of a Florasil clean up stage prior to analysis proved to be sufficient in filtering out matrix interferences from target analytes. Proof of the elimination of matrix interferences and excellent analyte recovery are given in this study by way of spiking known concentrations of steroids into environmental matrix waters. Results indicate that greater than 80% absolute recovery of target analytes is assured using the established method. The chosen surrogate standard 17β-trenbolone-d3 proved to be useful in correcting 17α- and 17β- trenbolone recoveries to nearly 100%. In addition, results indicate that a different surrogate standard may be necessary for melengestrol and trendione as matrix spike recoveries for these steroids appeared to be exaggerated when corrected using 17β-trenbolone-d3.

Because environmental waters can encompass a variety of suspended solids concentrations, different filtration methods were employed based on the water samples being analyzed. For instance, water samples with low to medium suspended solids, like those used in the matrix spike recoveries (presented in the results section of this study) required only vacuum filtration thru commercially available 0.7 µm pore size filters. However, certain samples were analyzed (data not presented) in this study that required much more extensive pressure filtration. Employing the use of a centrifuge may be necessary for water samples where high concentrations of suspended solids are present.

Extension of this method to confidently analyze for trendione in environmental matrices was complicated by two issues which require further research. The formation of two products from derivatized trendione was the first concern. It was observed that the established method yielded only 15% of derivatized trendione. The remaining 85% of trendione mass appeared to be incompletely derivatized into a second trendione product. Recoveries could not be improved without unduly impacting performance for 17α- and 17β-trenbolone. As a result, trendione calibrations were adjusted to reflect this phenomenon. The second issue encountered was that derivatized trendione appeared to degrade over time, perhaps into the second or thermodynamic product. Results indicate that as much as 20% degradation of derivatized trendione can occur within 48 hours. Therefore, the rapid (<48 hours) analysis of samples is merited. Further proposed experiments to alleviate these issues would include running full scans of derivatized trendione, monitoring the formation and degradation of products over time and reassessing transition parameters.

The extension of this method to the predominant metabolite of the progestagen melengestrol acetate, showed some success in terms of derivatization capability and recovery from environmental matrices. A 20 µg/L standard was analyzed using the derivatization procedure which gave a strong chromatographic peak, with a high signal to noise ratio. In addition, recoveries of melengestrol from environmental matrices were greater than 80% without a surrogate standard correction. These results would indicate that melengestrol can be recovered from environmental matrices at low ng/L concentrations. However, derivatized melengestrol displayed significant reproducibility issues. It is possible that like the trendione metabolites, derivatized melengestrol has a thermodynamic product which causes unstable derivatized products. Further research is needed to confidently detect and quantify melengestrol in

environmental matrices, and perhaps an adjustment to the derivatization procedure will provide a solution.

There are a few critical points in the method that are worth highlighting which maximized analyte recovery and derivatization yields. With respect to sample processing and maximum analyte recovery, it was found that maintaining a flow rate of roughly 2 mL/min, and not exceeding 5 mL/min through the solid phase extraction and Florasil cartridges was critical. In addition, achieving a complete dry down of the SPE elution and allowing adequate time for resuspension of analytes into the dichloromethane and methanol mixture was also critical for maximum analyte recovery. To achieve maximum derivatization yields, a number of points were found to be critical. Most importantly, to not only maximize yields but also to minimize false positives, a $I_2$/MSTFA concentration of 2.0 (mg/mL) and higher is necessary. Derivatization yields can be strongly affected if any solvent is remaining prior to the MSTFA/$I_2$ or pure MSTFA resuspension stages. Therefore, it is highly recommended to allow for extra $N_2$ dry down time prior to these steps.

Another critical parameter to be considered for maximum recovery that was not mentioned in this method is machine performance and sensitivity. Evaluating GC/MS/MS performance is critical when analyzing results. To ensure that machine sensitivity was at acceptable levels at all times, a constant concentration of hexachlorobenzene, a stable, relatively non-reactive compound was analyzed with every 10-20 samples. The assurance of a leak tight GC/MS/MS system was also found to be critical, not only for sensitivity issues but to keep the mass spectrometer’s inner source filament from fracturing, resulting in significant down-time. To ensure that the GC/MS/MS was leak tight at all times, the air/water spectra, which monitors the amount of water versus the amount of nitrogen in column was evaluated on a daily basis.

In conclusion, the analytical method presented in this study allows for the detection and quantification of trace concentrations of trenbolone acetate and melengestrol acetate metabolites in complex environmental matrices. As stated, significant issues with the derivatization of melengestrol and trendione require further research to provide confident results when analyzing actual environmental samples. Future directions to improve melengestrol and trendione derivatization and recovery will include full scan monitoring of derivatized standards over time and reassessing product transitions. In addition, the possibility of replacing MSTFA with another final solvent may remediate some of the issues highlighted in this study.

The extension of this method to melengestrol opens up the possibility for the incorporation of all exogenous metabolites that originate from confined animal feeding operations. This is a critical step when attempting to perform fate and transport studies on exogenous steroids that appear in surface waters. With existing studies demonstrating that hormonal steroids have deleterious effects on sensitive aquatic species, a comprehensive analytical method capable of detecting and quantifying all exogenous steroids originating from agricultural uses is highly desirable. The method developed in this study shows the potential for such an analytical method. Fate and transport studies along with a robust analytical method capable of detecting exogenous steroids in complex environmental matrices provides a platform to pursue better agricultural management practices which will limit or possibly eliminate the appearance of exogenous steroids in surface waters where sensitive aquatic species may reside.

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