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Analysis of trenbolone acetate metabolites and melengestrol in environmental matrices using gas chromatography–tandem mass spectrometry

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ABSTRACT

Studies demonstrate that exposure to steroid hormones in receiving waters can adversely impact reproduction of aquatic organisms. In particular, exogenous steroid hormones widely used as growth promoters in animal agriculture are of high concern, yet no gas chromatography–tandem mass spectrometry (GC/MS/MS) analytical methods for the detection of these compounds in complex environmental matrices is described in the literature. This study utilizes analytical methods based upon *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide-iodine (MSTFA-I_2) derivatization for the analysis of metabolites of trenbolone acetate (TBA), including 17α-trenbolone, 17β-trenbolone, and trendione, and melengestrol acetate in receiving waters and surface soils associated with animal agriculture. Results suggest method detection levels of 0.5–1 ng/L for the trenbolone metabolites, while detection of melengestrol is qualitative only. Isotope dilution methods employing d3-17β-trenbolone were used to improve steroid quantification. Method recoveries in spiked samples collected from a variety of representative receiving waters generally ranged from 80–120% with consistent and low standard deviation (generally < 10%) for replicate analysis. Analysis of a storm water runoff sample from a commercial confined animal feeding operation (CAFO) that used TBA implants detected 17β-trenbolone and trendione at concentrations of 31 and 52 ng/L, respectively. Analysis of surface soils at a commercial CAFO using TBA implants detected 17α-trenbolone at concentrations between 4–6 ng/g dry weight. Method development efforts suggested that the concentration of I_2 in MSTFA, the removal of I_2 from sample extracts after derivatization, and the use of Florisil clean-up to reduce organic matter matrix were vital aspects of steroid hormone quantification at low (< 30 ng/L) concentrations in complex environmental matrices.

1. Introduction

The occurrence of steroid hormones that can act as endocrine disrupting compounds in surface waters has been linked to feminization, altered morphology, and reproductive disruption in fish upon exposure to steroid hormones [1–3]. Of particular interest are potent exogenous steroid hormones that have the potential to cause catastrophic, population-wide effects due to disruption of reproductive function in sensitive aquatic organisms. For example, a population of fathead minnows (*Pimephales promelas*) continuously exposed to 5 ng/L ethynyl estradiol over 3 years neared local extinction due to disrupted spermatogenesis in male fish, suggesting that exogenous steroids can pose a substantial ecological risk in affected receiving waters [4].

Androgens also have demonstrated endocrine disrupting potential at similar low concentrations, although these steroids are not as well studied as the estrogens [5–7]. Sources of endogenous and exogenous androgens into the natural environment include wastewater treatment plant effluent [8], pulp and paper mill effluent [9], and animal agriculture sources such as confined animal feeding operations (CAFOs) [10,11]. In particular, the synthetic androgen 17β-trenbolone, a potent anabolic steroid, is of considerable interest as an environmental pollutant due to its widespread use and potential for endocrine disruption in sensitive species of fish.

For example, over 90% of beef cattle raised in the United States receive at least one growth implant, and the vast majority of these implants contain trenbolone acetate (TBA) [12]. TBA use is widespread in cattle production because of demonstrated increases in weight gain and carcass quality of TBA-implanted cattle [13]. After implantation, TBA is metabolized to 17β-trenbolone (the most biologically potent compound), 17α-trenbolone and

Abbreviations: CAFOs, confined animal feeding operations; GC/MS, gas chromatography–mass spectrometry; GC/MS/MS, gas chromatography–tandem mass spectrometry; LC, liquid chromatography; MGA, melengestrol acetate; MDLs, method detection limits; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide; MRM, multiple reaction monitoring mode; S/N, signal-to-noise; SPE, solid phase extraction; TBA, trenbolone acetate; TMS, trimethylsilyl

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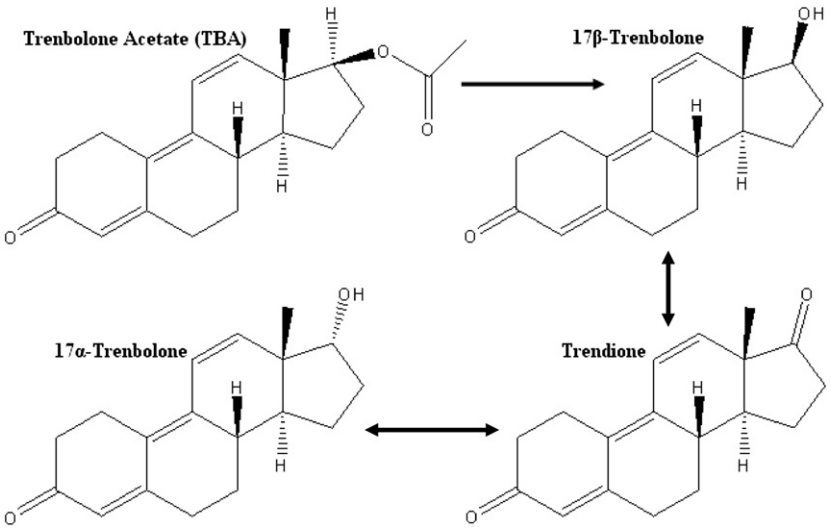

Fig. 1. Primary pathway of trenbolone acetate (TBA) metabolism in cattle. Figure adapted from Khan et al. [14].

trendione (Fig. 1) [14]. Of these metabolites, 17α-trenbolone accounts for ~95% of the total trenbolone mass excreted by the implanted animal; thus this compound is expected to dominate occurrence in the aquatic environment, although transformations between the different metabolites likely occur [14]. Exposure to low concentrations (10–30 ng/L) of either 17α-trenbolone and 17β-trenbolone can result in significant reductions in fecundity in some species of fish [10,11]. Similarly, other growth promoters such as melengestrol acetate (MGA) are used in animal agriculture, though not as widely as TBA, and metabolites of MGA such as melengestrol also are likely contaminants in receiving waters near animal agriculture operations utilizing growth promoters [14].

While the discharge of untreated CAFO runoff directly into surface waters is prohibited, the widespread use of growth promoters and the potential for incidental CAFO runoff discharges suggest that TBA metabolites such as trendione, 17α-trenbolone and 17β-trenbolone along with other steroidal contaminants linked to animal agriculture can occur in receiving waters. TBA metabolites have been detected in receiving waters at ecologically relevant concentrations via overland flow, CAFO effluent discharges, and runoff from fields receiving animal manure [14,15]. The ubiquitous use of TBA and MGA growth promoters suggests that assessing the environmental fate of these exogenous steroids and their metabolites is a critical aspect of evaluating the risk these compounds pose to sensitive aquatic organisms.

To date, gas chromatography–mass spectrometry (GC/MS) analytical methods have been used to detect trenbolone metabolites in biological matrices (*e.g.* urine and tissue) [16,17]. Analytical methods for the detection of these compounds in environmental matrices (*e.g.* water and solids) often utilize liquid chromatography (LC) methods due to the simpler sample processing steps required for LC along with difficulties associated with thermal instability and problematic derivatization of trenbolone for GC analysis [10,14,18–20]. One study used a simple GC/MS method (no derivatization) for the confirmatory analysis of 17α-trenbolone and 17β-trenbolone in an agricultural receiving water, though with very poor sensitivity and variable performance [10]. However, no gas chromatography–mass tandem spectrometry (GC/MS/MS) analytical methods for detecting low concentrations of the metabolites of TBA and MGA in complex environmental matrices such as receiving waters and solids samples (*e.g.* soil, manure) have been published to date. GC/MS/MS can be advantageous due to its reduced costs in comparison to other analytical instruments and sometimes offers better chromatographic resolution of analytes in difficult sample matrices. Tandem mass spectrometry also offers improved sensitivity, especially in the very heterogeneous, high suspended solids, high organic carbon matrices typical of field sampling efforts. In comparison, liquid chromatography–tandem mass spectrometry methods are often preferred choices for steroid analysis due to their ability to analyze compounds without using a preparatory derivatization step, but can experience substantial matrix effects.

The objective of this research was to apply GC/MS/MS analytical methods to quantitatively detect trace levels of the metabolites of TBA and MGA in environmental samples such as receiving waters and solids. While GC/MS analytical methods for analysis of 17β-trenbolone and 17α-trenbolone in biological matrices exist [16,17], the unique aspects of this method include optimization for aqueous and soil samples and the incorporation of trendione and melengestrol as analytes. No published GC/MS/MS analytical method exists for these compounds. The basic derivatization procedure for trenbolone was first presented by Maume et al. and Marchand et al. [16,17]. Optimization of this derivatization method to quantify exogenous steroid metabolites at low concentrations in complex environmental matrices employed C18 solid phase extraction followed by Florisil cleanup, *N*-methyl-*N*-(trimethylsilyl)trifluoro-acetamide (MSTFA)-I_2 derivatization, and subsequent GC/MS/MS analysis.

2. Materials and methods

2.1. Materials

All steroids and standards used in these studies were obtained at the highest possible commercially available purity. 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). Deuterated 17β-trenbolone (d3-17β-hydroxy-estra-4,9,11-trien-3-one) was obtained from the Bank of Reference Standards (RIVM, Netherlands). 17α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one) was purchased from NMI (Pymble, NSW, AU). Trendione (estra-4,9,11-trien-3,17-dione) was synthesized from 17β-trenbolone using the protocol outlined in Khan et al. [14], although this compound is recently

commercially available. Derivatization grade *N*-methyl-*N*-(trimethylsilyl) trifluoro-acetamide (MSTFA) and iodine (I_2, 99.999% pure) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Florisil cartridges were purchased from Restek (Bellefonte, PA, USA), while Sep-Pak C18 solid phase extraction cartridges were obtained from Waters (Milford, MA, USA).

Stock solutions (2–10 mg/L) for each of the steroid analytes were prepared in silanized volumetric glassware using HPLC grade methanol (Sigma-Aldrich, Milwaukee, WI, USA), then serially diluted to create working standard standards over the range of 1–500 μg/L. The derivatization reagent MSTFA-I_2 was prepared by dissolving 1.4 mg of I_2 per mL of MSTFA (0.14% m/v). Standard solutions and derivatization reagents were stored at −20 °C to reduce the potential degradation of trenbolone steroids as suggested by manufacturer protocols. Standard and reagent solution storage periods did not exceed three months in any cases prior to remaking solutions, though in many cases reagents were prepared as needed without storage.

2.2. Field sample collection and processing

To validate method performance, receiving water samples were collected from a variety of water sources representative of agriculturally impacted systems. The first receiving water sample type "Surface Water" was collected from a small creek in a mixed use agriculture-urban watershed near Reno, NV. The second receiving water sample type "Constructed Wetland" was collected from a ~1 ha constructed wetland designed to collect and treat storm water runoff. The third receiving water sample type "Irrigation Canal" was collected in an irrigation canal located near Marysville, CA whose flow largely consisted of agricultural return water from irrigated pastureland used for cattle grazing. Prior to use, no analytes were detected in any of these sample types (data not shown). To validate method performance and demonstrate detection of these analytes in receiving waters and soils impacted by animal agriculture, a sample of storm water runoff from a commercial CAFO located in Iowa that used TBA implants was collected. Also, spatially composited surface soils (top 10 cm) collected from a commercial CAFO in Nevada that used TBA implants were used as a representative solids matrix to assess environmental occurrence and extraction efficiency. Along with the CAFO soil, washed and sieved sand (Stead, NV) also was used for extraction efficiency studies.

Aqueous samples for steroid analysis and method development were collected in 1 L amber glass bottles and immediately refrigerated or stored on ice for transport. Sample filtration and extraction were usually performed within 24 h of sample collection. To reduce suspended particulates, samples were vacuum filtered through 0.7 μm glass fiber prefilters (AP40, Millipore, Billerica, MA, USA). No further processing of particle-associated analytes captured on the filters was performed. To determine method recoveries and validate method limits of detection, collected samples were spiked (5–100 ng/L) with analyte stock solutions in methanol after filtration using no more than 1 mL of analyte stock to spike steroids into samples. After filtration, 1 mL of 100 μg/L d3-17β-trenbolone stock in methanol was added as an isotopic surrogate standard per each 1 L of sample, yielding 100 ng/L concentrations of the deuterated standard in all solutions. Deuterated versions of 17α-trenbolone and trendione were not available; therefore, d3-17β-trenbolone was used for isotopic correction for all of the trenbolone metabolites. Processing of solids samples consisted of solvent extracting 5 g (wet weight) soil samples with methanol via sonication (10 min) using three successive 25 mL methanol aliquots. The methanolic aliquots were then combined, centrifuged at 3500 rpm for 6 min for solids separation, and the supernatant collected. The supernatant was diluted with deionized water (Millipore, Billerica, MA, USA) to 1 L, spiked with 1 mL 100 ppb d3-17β-TbOH surrogate standard, and processed as for aqueous samples. To quantify the extraction efficiency of the method, recovery experiments consisted of s piking 5 g of oven dried (103 °C) CAFO soil and sand samples that were spiked to a concentration of 40 ng/g-dw using 2 mL of a concentrated methanolic stock solution (which included the deuterated standard) of the analytes. The CAFO soils used were samples where the analytes were not detected in unspiked samples, and spiking concentrations were environmentally realistic [15]. After spiking, the samples were homogenized, and allowed to air dry at room temperature for 24 h prior to solvent extraction as described above.

2.3. Solid phase extraction and cleanup

Prior to sample extraction, C18 solid phase extraction (SPE) cartridges were conditioned with four 5 mL aliquots of methanol and water (95:5 v/v), passed through the cartridges under vacuum at a flow rate of 5–10 mL/min. SPE cartridges were then conditioned with four 5 mL aliquots of deionized water. Samples were then extracted under vacuum at flow rates of 1–5 mL/min, taking care to not exceed 5 mL/min. Cartridges were not allowed to vacuum dry between conditioning and sample addition. If necessary, vacuum dried extraction cartridges were stored indefinitely at −20 °C prior to sample elution. C18 cartridges were eluted by sequentially percolating three aliquots (2 mL) of methanol and water (95:5 v/v) through the cartridges under vacuum. The eluate (~6 mL) was dried to completeness under a gentle stream of nitrogen at room temperature. Dried residues were 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 varied between 1 and 24 h, with the longer times used for samples with complex sample matrices and higher concentrations of residual organic matter. During these solvent transfer steps, samples were held at room temperature in a capped vial in the dark. For all samples with little visible organic matter residue, one hour of time for mass transfer is sufficient. For complex samples with significant color and residual organic matter, 3–12+ h may be necessary to dissolve residues and achieve quantitative mass transfer in the extracts.

Sample clean-up of the extracts was performed with normal phase extraction using Florisil cartridges. Florisil cartridges were first conditioned by passing four aliquots (5 mL) of acetone through each cartridge with a vacuum drying period between each pass. After vacuum drying, the cartridges were conditioned with four aliquots (5 mL) of dichloromethane-methanol (95:5 v/v). Sample extracts were then passed through the conditioned cartridges at 1–2 mL/min, applying a vacuum if necessary. Next, each sample volume (~6 mL) was vacuum dried to ~1.5 mL and transferred into 2 mL SilCote deactivated vials (Restek, Bellefonte, PA, USA). Samples were then completely dried down under nitrogen gas to prepare for derivatization.

2.4. Derivatization procedure

As derivatization reaction yields are often sensitive to the presence of moisture, care was taken to fully dry sample extracts. In this case, azeotropic removal of residual water was performed by adding 1 mL of dichloromethane to each vial, vortexing, and evaporating with nitrogen gas. Fifty μL of the derivatization reagent MSTFA-I_2 (1.4:1000 m/v) was added to the dried extract and vortexed at room temperature for 15 s to insure mixing. The derivatization reagent MSTFA-I_2 can produce distinct and characteristic mass spectral differences between the 17α- and 17β-trenbolone stereoisomers [16], as these particular anabolic

steroids, based upon a 4,9,11 triene conjugated bond system structure, are analytically difficult by gas chromatography because they do not easily yield stable products with other derivatization reagents. Preliminary derivatization studies with trendione and melengestrol demonstrated the appearance of two product peaks in standards, suggesting the formation of partially derivatized products for these analytes. Partial silylation of trendione likely results from the difficult enolization of the C-17 keto group, limiting the addition of trimethylsilyl (TMS) to C-17, while data suggests partial α-nucleophilic substitution of N-methyltrifluoroacetamide at the C-4 position on melengestrol. To further investigate the derivatization conditions and to optimize method performance, the concentration of I_2 in the MSTFA derivatization reagent also was varied between 0–6.9 mg I_2 per mL of MSTFA.

As the derivatization reaction is nearly instantaneous for trenbolone [17], samples were immediately dried down under a gentle nitrogen stream. Small reddish–purple colored crystals (presumably containing iodine) were often observed to form during this dry down. For these samples, a continued flow of N_2 into the vial over 30–60 min sublimated these crystals. Preliminary studies suggested that it is critical to remove residual iodine from the extracts prior to further processing, especially for those samples with high concentrations of organic matrix, which seemed to trap iodine and hinder sublimation. Residual iodine was found to be detrimental to method performance, as it can form non-volatile steroid adducts [21]. Observationally, in any samples with reddish–purple color visible after drying, subsequent method performance (*e.g.* steroid peak area) decreased. For these same reasons, utilizing the lowest effective I_2 concentration in MSTFA during the derivatization step was desirable, as higher iodine concentrations were more likely to result in residual iodine color in sample extracts despite drying. After drying, extracts were resuspended in 100 μL of MSTFA and incubated for 40 min at 60 °C to ensure complete silylation of both C-3 and C-17 functional groups [22]. Proposed structures for the fully derivatized analytes (excepting d3-17β-trenbolone) can be found in Supporting Information (Fig. SI1).

2.5. 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). The GC column used was a RXI-5Sil MS, 30 m, 0.25 mm inner diameter (Restek, Bellefonte, PA, USA). Splitless injections (1.0 μL) into a 250 °C injection port were used. Ultra-high purity helium was used as the carrier gas at 1.0 mL/min. The temperature program was: 120 °C (held for 2 min), increased at 45 °C/min to 260 °C (held for 1 min), increased at 5 °C to 270 °C (held for 8 min), increased at 45 °C/min to 285 °C (held for 6 min), increased at 45 °C to 300 °C (held for 2 min). Total GC run time was approximately 22 min.

A 70 eV electron ionization energy was used in the mass spectrometer with a 180 °C ion source temperature and a 290 °C gas chromatograph transfer line temperature. Hexachlorobenzene was used as an injection standard to verify injection and monitor MS performance. To both quantify and confirm analyte identity, two multiple reaction monitoring mode (MRM) transitions were monitored for each analyte, excepting d3-17β-trenbolone where a confirmatory transition was not monitored. Identification and quantification criteria for analytes required: (1) consistent relative chromatographic retention times for analytes and surrogates as compared to calibration standards; (2) detection of both quantification and confirmatory MRM peaks; and (3) consistent quantification-confirmatory peak area ratio within 25% of calibration standards ratio. Full scan mass spectra for 17α- and 17β-trenbolone are found in Supporting Information (Fig. SI2). A chromatogram of a 100 ppb standard containing all of the analytes, including partially derivatized products (discussed later), is presented in Fig. 2. Chromatographic retention times, quantification and confirmatory MRM transitions, ion ratios, and MS collision energies for the analytes are presented in Table 1.

3. Results and discussion

3.1. Solid phase extraction and Florisil clean-up

To account for undesirable matrix effects and to improve steroid quantitation at trace levels, sample clean-up using Florisil extraction was critical to applying this method to complex environmental matrices. Qualitatively, the Florisil extraction step greatly reduces the color of sample extracts, and generally decreases the number and size of baseline impurity peaks in chromatograms. Extracts prior to Florisil extraction often are colored green–brown, while those after the extraction are typically colorless. These colorless extracts dried more rapidly and were more easily dissolved in derivatization solvents as compared to extracts without clean-up. Quantitatively, in a direct comparison of CAFO runoff samples processed with and without Florisil clean-up, the clean-up step increased the signal-to-noise ratios of <10 ng/L analyte peaks four-fold to twelve-fold, suggesting improved sensitivity at trace concentrations. Additionally, the Florisil clean-up increased the number of sample injections possible before the GC/MS/MS instrument needed routine maintenance such as replacing the GC injection port liner and cleaning the inner source of the mass spectrometer.

Concentration enrichment factors arising from the use of solid phase extraction were generally 10-fold for standards (1 mL of standard solution dried and resuspended to 100 μL for injection) and up to 10,000-fold for samples (*e.g.* 1 L of sample resuspended to 100 μL). A calibration curve for the TBA metabolites is presented in Supporting Information (Fig. SI3). Note that melengestrol is not included in the calibration curve, as the peak area response of melengestrol was found to be insufficient for trace (defined as low ng/L detection) analysis using MSTFA-I_2 derivatization. Therefore, despite efforts to improve performance, melengestrol analysis is considered as qualitative only using the MSTFA-I_2 derivatization.

3.2. Importance of I_2 concentration in MSTFA

The use of MSTFA-I_2 as a derivatization reagent for 4,9,11 conjugated triene steroids was first described by Maume et al. [16]. According to these researchers, the MSTFA-I_2 yielded "unexpected" EI mass spectra along with good analyte specificity, although the general application of MSTFA-I_2 derivatization to other steroids related to 17α and 17β-trenbolone is complicated by its "very particular" reactivity [16]. Therefore, one of the primary objectives of this study was to evaluate whether trendione could be derivatized with MSTFA-I_2 while still maintaining method performance for 17α- and 17β-trenbolone, yielding a quantitative method suitable for all three of the primary metabolites of TBA expected to occur in the aquatic environment.

Preliminary studies suggested that the concentration of I_2 in MSTFA was a key variable in both overall derivatization yield for all steroids studied and also the formation of partially silylated trendione product peaks. The iodine in the MSTFA reagent acts as a catalyst by facilitating the α-nucleophilic substitution of N-methyltrifluoroacetamide at the C-4 position with simultaneous silylation of the C-3 hydroxyl group, and is critical to

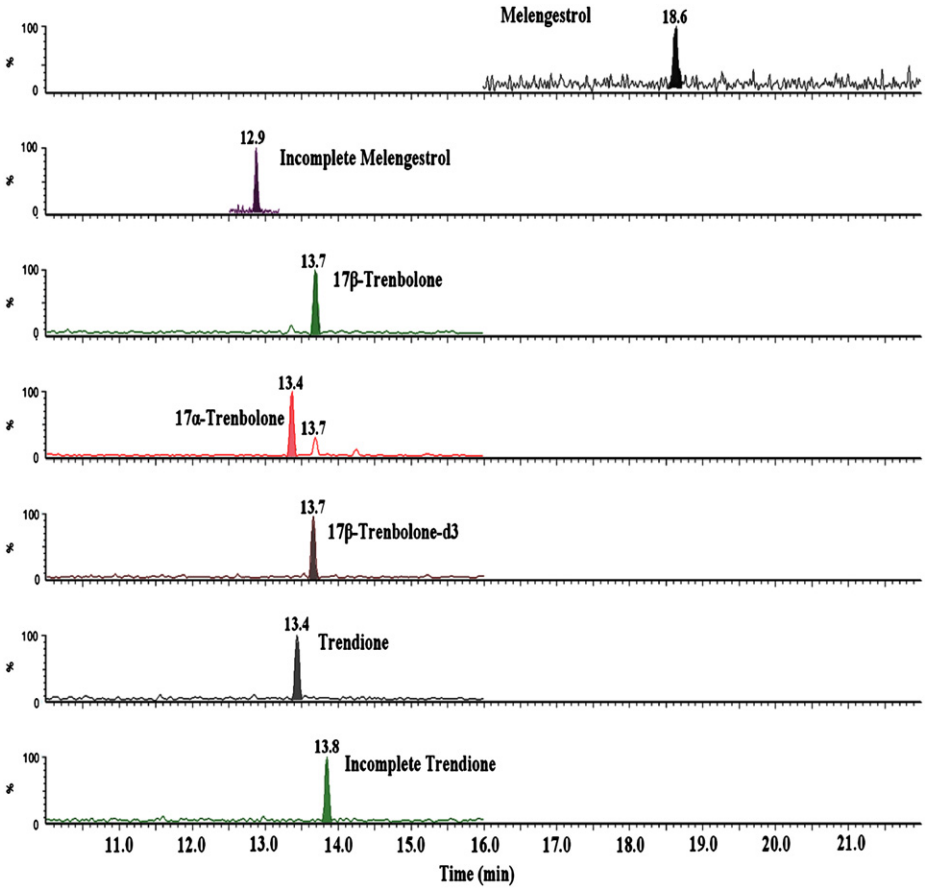

Fig. 2. Chromatogram of the steroid analytes in a 100 ppb standard, including observed partially and completely derivatized peaks for trendione and melengestrol.

Table 1
Retention times (RT), quantification and confirmatory MRM transitions, corresponding optimized collision energies and observed quantification/confirmatory ion ratios for synthetic steroid analytes.

Analyte	RT [min]	Quantification transition	Collision energy [V]	Confirmatory transition	Collision energy [V]	Quant./Confirm. ion ratio [%] (± std.dev.)
Androgen						
17α-trenbolone	13.4	449.3 > 323.2	8	380.2 > 323.2	24	74 (± 6)
d3-17β-trenbolone	13.7	445.3 > 383.3	30			NA[a]
17β-trenbolone	13.7	442.2 > 382.2	30	442.2 > 309.3	32	87 (± 6)
Trendione	13.8	368.2 > 338.2	22	368.2 > 352.2	22	76 (± 11)
(Partially Silylated) *Progestin* Melengestrol						
Partially derivatized	12.9	498.3 > 259.1	22	498.3 > 455.3	10	32 (± 19)
Completely derivatized	18.6	624.2 > 491.3	12	624.2 > 581.2	10	NA

[a] NA=Not applicable.

obtaining stoichiometric yield of derivatized products from parent analytes [16]. For example, 2 ppm standards of 17α- and 17β-trenbolone were derivatized with a range of I_2 concentrations in MSTFA from 0–2.2 mg I_2/mL MSTFA (Supporting Information, Fig. SI4). This data indicates that 17α-trenbolone has a higher area response factor than 17β-trenbolone when using MSTFA-I_2. More importantly, peak area response for both 17α- and 17β-trenbolone plateaus near 1 mg I_2/mL MSTFA, with no significant ($p < 0.05$) improvement in peak area response at higher I_2 concentrations. Based on this data, and the desire to optimize performance for 17α- and 17β-trenbolone in particular because of their higher ecological toxicity while still minimizing the amount of iodine used in the reaction, 1.4 mg I_2/mL MSTFA was selected as the most appropriate reagent composition for these analytes.

By contrast, partially and completely silylated product peaks are observed in chromatograms for trendione (Fig. 3), with the retention time 15.28 min peak exhibiting ~12–15% of the peak area response of the retention time 15.62 min peak using 1.4 mg I_2/mL MSTFA. The full scan spectra of these products suggests that the completely silylated 15.28 min product exhibits similar characteristics to the product spectra published for 17β-trenbolone [16], with a corresponding $[M]^+$ product at m/z 537, $[M\text{-}CH_3]^+$ at m/z 522, $[M\text{-}COCF_3]^+$ at m/z 440, and $[M\text{-}TMS\text{-}COCF_3]^+$ at m/z 368. In contrast with the completely silylated m/z 537 parent at 15.28 min, the spectra for the partially silylated 15.62 min peak

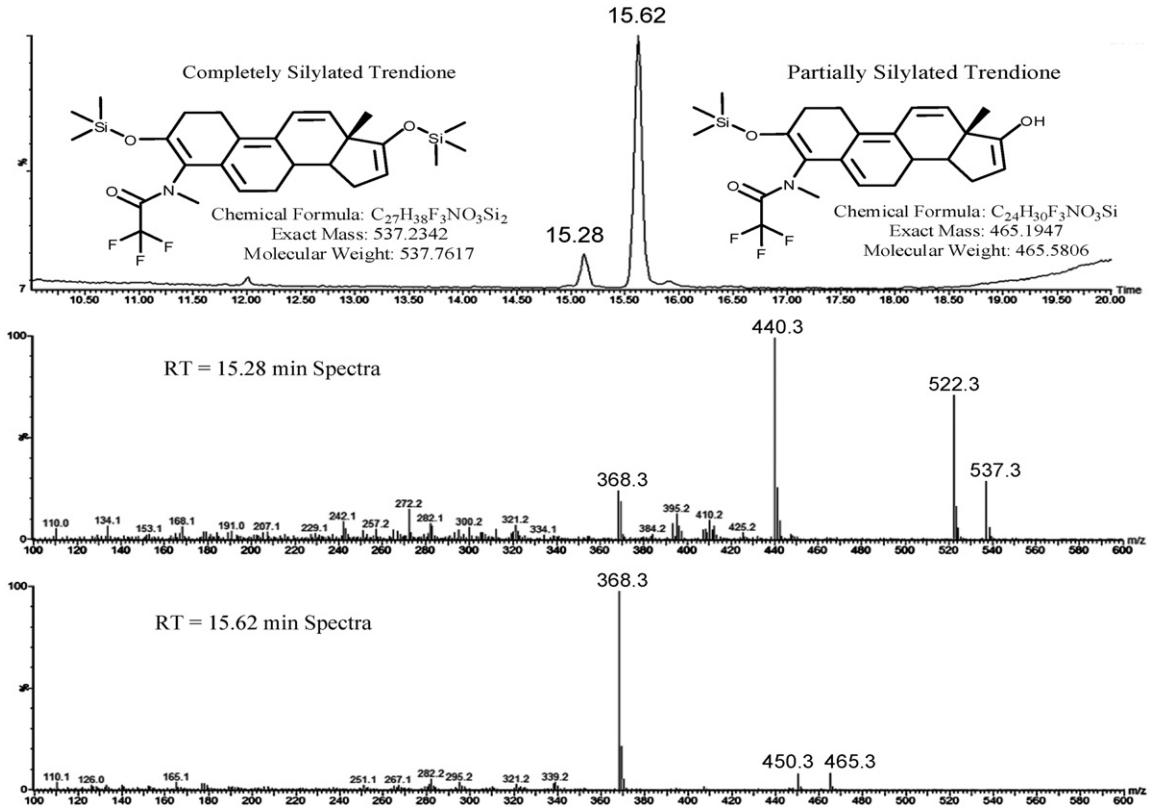

Fig. 3. Chromatogram, including proposed structures for derivatization products, and observed mass spectra for completely (RT=15.28 min) and partially (RT=15.62 min) silylated product peaks observed for 500 ppb trendione standard at 1.4 mg I_2/mL MSTFA.

suggests a $[M\text{-}TMS]^+$ parent product of *m/z* 465, with further products $[M\text{-}CH_3]^+$ at *m/z* 450 and $[M\text{-}COCF_3]^+$ at *m/z* 368. More interestingly, the observed ratio of partial to complete silylation products of trendione also is dependent upon the I_2 concentration in MSTFA (Supporting Information, Fig. SI5). Though the range of I_2 concentrations tested was not exhaustive, at 2.2 mg I_2/mL MSTFA, the partially silylated peak dominated peak response, while increasing the I_2 concentration up to 6.9 mg/mL resulted in improved silylation, with most of the peak area occurring in the completely silylated 15.28 min peak. This data implies that iodine also is involved in silylation of the C-17 carbonyl group of trendione as well as C-3 silylation. For example, the simultaneous C-3, C-4 α-nucleophilic substitution and silylation reaction structure is common to 17α- and 17β-trenbolone as well as trendione, and neither 17α- nor 17β-trenbolone demonstrates any propensity to form partially silylated products under derivatization conditions. If we then assume that the trendione C-17 enolization-silylation is the more challenging silylation reaction position of the two (based upon 17α- and 17β-trenbolone results), and also observe that increasing iodine concentration shifts product formation from partially to completely silylated products, then it also may be logical to conclude that iodine is directly involved with both C-3 and C-17 silylation of trendione. Further structural studies would be needed to evaluate this possibility, although they are beyond the scope of this study.

Due to its much higher peak area response, the partially silylated product was used to quantify trendione with good results at trace levels. Also, the partially silylated product formed at lower iodine concentrations (~1.4 mg I_2/mL), which is preferable for iodine removal during extract processing. An additional factor suggesting that the partially silylated peak was most suited for quantification is that the partially silylated product was stable in solution over at least 48 h (Supporting Information, Fig. SI6). However, the completely silylated product demonstrated a significant increase in observed peak area ($p < 0.01$) over the same 48 h storage period, suggesting potential error if the completely silylated product was used for quantification. Other analyte derivatives were stable for up to 3 months if stored at −20 °C.

Similar to trendione, melengestrol also exhibited partially and completely derivatized product peaks, though unlike trendione, each of these peaks was fully silylated. Unlike trendione, these peaks were widely separated in retention time, with the partially derivatized peak at 12.9 min. retention time and the completely derivatized peak at 18.6 min. retention time, suggesting a substantial structural difference between products. The retention time 12.9 min peak exhibited ~25% of the peak area response of the retention time 18.6 min peak at 1.4 mg I_2/mL MSTFA. The full scan spectra of these products (Fig. 4) suggest that the retention time 12.6 min product is silylated at both the C-3 and C-17 positions. However, α-nucleophilic substitution of N-methyltrifluoroacetamide at the C-4 position was apparently unsuccessful in the partially derivatized product, as no trace of N-methyltrifluoroacetamide fragments can be detected in the mass spectra. The partially derivatized parent $[M]^+$ product at *m/z* 498 in the spectra represents an addition of two TMS groups to melengestrol with likely enolization at the C-2, C-3 bond position. Other fragments observed in the partially derivatized mass spectra include $[M\text{-}C_2H_3O]^+$ at *m/z* 455, $[M\text{-}TMSOH\text{-}CH_3]^+$ at *m/z* 393, and $[M\text{-}TMSOH\text{-}C_2H_3O]^+$ at *m/z* 365. The mass spectra for the completely derivatized product suggests a $[M]^+$ parent product of *m/z* 624, with further products $[M\text{-}C_2H_3O]^+$ at *m/z* 581, $[M\text{-}TMS\text{-}C_2H_3O]^+$ at *m/z* 508 and $[M\text{-}TMSOH\text{-}C_2H_3O]^+$ at *m/z* 491. Due to its higher peak area response, the completely derivatized product was used for qualitative identification of melengestrol.

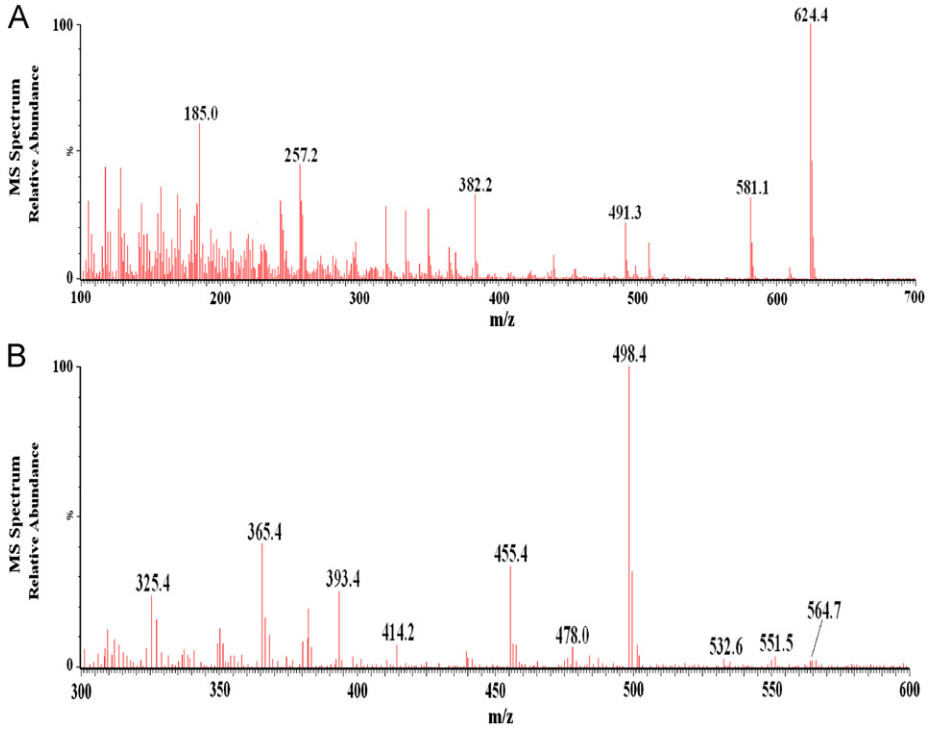

Fig. 4. Mass spectra for completely (A) and incompletely (B) derivatized melengestrol products.

Table 2
Method recoveries [%] of trenbolone metabolites in a variety of representative receiving waters. Standard deviations of triplicate analysis are reported in parentheses (± std.dev.).

Analyte	Spike concentration [ng/L]	Surface water ($n=3$)	Constructed wetland ($n=3$)	Irrigation canal ($n=3$)
17α-Trenbolone	5	105 (± 3.4)	108 (± 4.2)	89 (± 3.0)
	50	87 (± 6.9)	92 (± 18)	89 (± 10)
17β-Trenbolone	5	91 (± 0.6)	110 (± 1.4)	84 (± 2.1)
	50	119 (± 5.8)	126 (± 13)	112 (± 4.5)
Trendione	5	82 (± 1.1)	105 (± 10)	102 (± 9.3)
	100	106[a]	100[a]	95[a]

[a] $n=2$, so standard deviation not reported.

3.3. Method performance

Toxicological effects of steroid hormones on aquatic organisms are often observed to occur in the low ng/L range, and sometimes even as low as 1 ng/L [4–6,11]. For this reason, many analytical methods for steroid hormones seek method limits of detection near 1 ng/L. Given typical 1000-fold extract concentration though solid phase extraction, method sensitivity is often assessed by determining the signal-to-noise (*S*/*N*) ratio of the lowest point of calibration curves, often using a 1 ppb concentration standard and 1000X concentration enrichment factors as elements of the analytical method. This "lower level of detection" approach relies upon setting method detection limits (MDLs) by determining the lowest standard concentration in the calibration curve that consistently yields a *S*/*N* ratio of 3 or greater [23], with levels of quantification typically set as 3X higher than the level of detection. In this study, the lowest concentration standard in the calibration curve was 1 ppb, which yields 1 pg analyte on column for a 1 μL injection volume. Observed S/N ratios for this 1 ppb standard for 17α-trenbolone, 17β-trenbolone and trendione ranged from ~10 to near 200, and were a function of instrument maintenance cycle. These strong responses suggest that the lower level of detection for the method was below 1 ng/L for the analytes, and that low ng/L concentrations could be accurately quantified for all the trenbolone metabolites.

A second, more rigorous approach to assess method sensitivity specifically calculates MDLs using the observed standard deviations of seven replicate analyses of samples at concentrations near the expected method detection limit to determine a MDL [23,24]. Using this replicate analysis approach, MDLs of the analytical method were estimated to be 0.5 ng/L for 17α-trenbolone and trendione, and 1 ng/L for 17β-trenbolone. This MDL approach describes an analyte concentration that can be expected to produce a result with 99% probability that the analyte signal is different from a blank [23]. Under either approach, the MDLs suggest that analytical method sensitivity is capable of quantification of low ng/L concentrations of trenbolone metabolites in receiving waters.

Method recoveries and precision in a variety of receiving water types were assessed by spiking high (50–100 ng/L) and low (5 ng/L) concentrations of the trenbolone metabolites into surface water, constructed wetland, and irrigation canal samples. Recoveries of analytes in these representative matrices ranged from 82–126%, depending on the sample, with standard deviations generally at 10% or less for triplicate analysis (Table 2). No consistent variation in method performance with matrix type is evident in this data. No analytes were observed in concurrent analysis of deionized water blanks or receiving water samples not impacted by animal agriculture operations. To account for potential sample

processing loss, matrix effects, and instrument performance, recoveries were corrected by the isotopic standard d3-17β-trenbolone. Recovery of the isotopic standard exceeded 80% in all replicate samples. During our studies on the environmental occurrence of these compounds, we have conducted a number of recovery experiments using identical spiked samples on different days. Analytical results were generally consistent for the different sample runs, as these interday comparisons of method performance suggest 10% or less variation for identical samples is typical (data not shown). These results suggest good, reproducible method performance in a variety of different sample matrices and that d3-17β-trenbolone is a reasonable isotopic standard for quantification of the trenbolone metabolites.

To assess analyte extraction efficiency in solid samples, CAFO surface soil and sand samples were spiked to 40 ng/g (dry weight) with the three analytes, homogenized, and the solvent removed by air drying (24 h). Observed recoveries in the sand ($n=5$) were 106 (± 5.3)%, 105 (± 3.4)%, and 72 (± 30)% for 17α-trenbolone, 17β-trenbolone, and trendione, respectively. For the CAFO soil ($n=5$), observed recoveries were 86 (± 20)%, 106 (± 4.2)%, and 53 (± 30)% for 17α-trenbolone, 17β-trenbolone, and trendione, respectively. Observed trendione recoveries were somewhat lower than expected, which may be due to higher sorption affinities for trendione to soils relative to 17α-trenbolone and 17β-trenbolone [25]. It is likely that the higher sorption affinity of trendione is not well approximated by the use of the deuterated d3-17β-trenbolone internal standard for recovery correction, and the use of a deuterated trendione ISTD (if available) likely would further improve quantification and reduce the variation in recovery observed for trendione.

3.4. Analysis of field samples

To confirm method performance in unspiked field samples, a storm water runoff sample from a commercial CAFO in Iowa and surface soils from a commercial CAFO in Nevada were collected and analyzed. Both of these CAFOs used growth promoting implants that contained TBA in all of their cattle. The CAFO runoff sample demonstrates the concurrent detection of 17β-trenbolone and trendione at concentrations of 31 and 52 ng/L, respectively (Fig. 5). Analysis of three CAFO surface soils in triplicate observed the detection of 17α-trenbolone only, at concentrations between 4 and 6 ng/g dry weight in these CAFO surface soils (Supporting Information, Fig. SI7). Fig. 6 shows a representative chromatogram for the detection of 4.6 ng/g dry weight 17α-trenbolone in a CAFO surface soil sample. These results suggest that the described GC/MS/MS analytical method is capable of detecting low concentrations of trenbolone metabolites in complex environmental matrices such as CAFO runoff and soils. The results also suggest that TBA metabolites, including 17α-trenbolone, 17β-trenbolone, and trendione, can be present in environmental samples such as soils and runoff from CAFOs utilizing growth implants, suggesting that a potential for release of these endocrine disrupting compounds to the aquatic environment does exist.

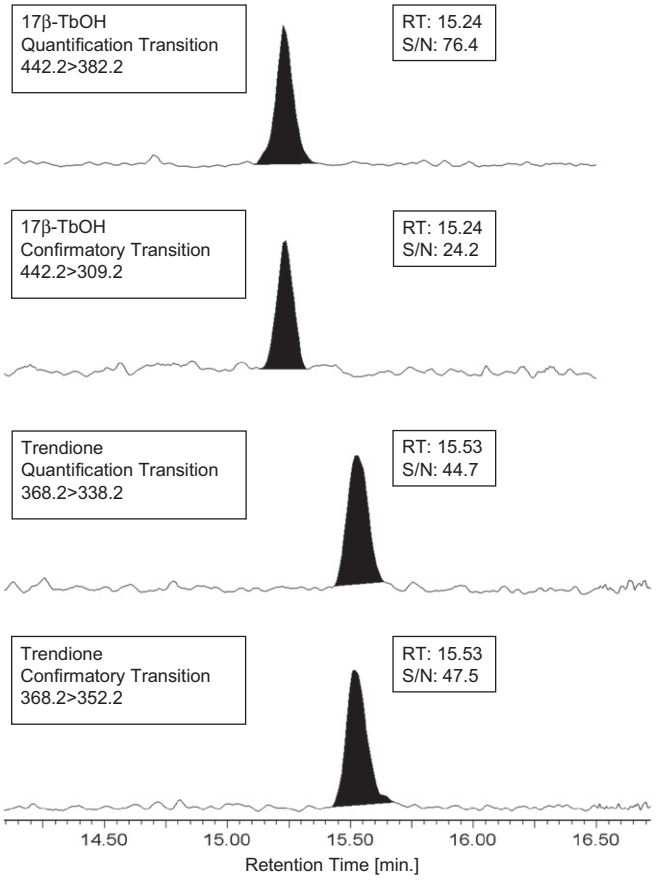

Fig. 5. Representative chromatogram for the environmental detection of 31 ng/L of 17β-trenbolone and 52 ng/L trendione in storm water runoff from a commercial CAFO using TBA implants.

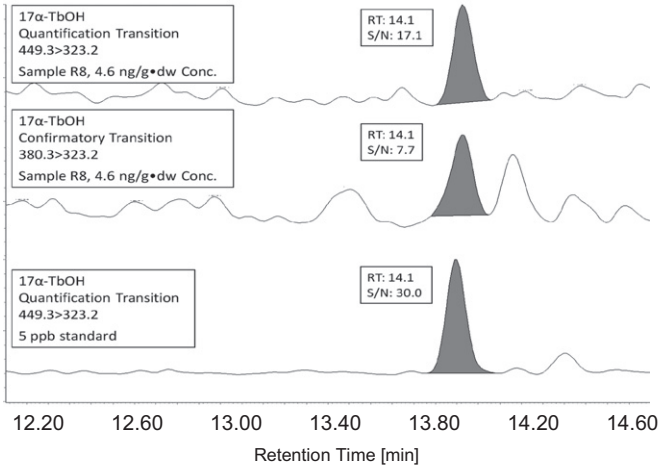

Fig. 6. Representative chromatogram for the environmental detection of 4.6 ng/g dry weight 17α-trenbolone in CAFO surface soils (Pen=*R*8).

4. Conclusion

The scientific literature suggests that the occurrence of potent growth promoting steroid hormones derived from animal agriculture is a concern for aquatic organism health in impacted receiving waters. Although analytical methods utilizing liquid chromatography–tandem mass spectrometry for the detection of these steroidal growth promoters in environmental matrices are described in the literature, no similar methods utilizing gas chromatography–tandem mass spectrometry are published. Using a previously described MSTFA-I_2 derivatization procedure, an analytical method was validated for metabolites of trenbolone acetate, extended to trendione and qualitative detection of melengestrol, and applied to analysis of complex environmental matrices.

Method performance was excellent for 17α-trenbolone, 17β-trenbolone, and trendione, with observed MDLs near or below 1 ng/L, while melengestrol exhibited weak response factors using this derivatization approach, resulting in variable performance,

especially at lower concentrations. For this reason, method performance for melengestrol is considered qualitative only. To insure accurate quantification, isotope dilution methods were applied for the trenbolone metabolites using d3-17β-trenbolone. Observed analyte recoveries in several representative sample types generally ranged from 80–120%, with consistently low ($<10\%$) standard deviation. Each of the trenbolone metabolites (17α-trenbolone, 17β-trenbolone, and trendione) was detected in at least one storm water runoff or surface soil sample collected from commercial CAFOs that used TBA implants. Method development efforts suggested that elements critical to consistent method performance included the use of Florisil cleanup to reduce extract organic matter, the concentration of I_2 in MSTFA, and its subsequent removal from extracts.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2012.05.046.

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