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RESEARCH ARTICLE

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Bioformation of boldenone and related precursors/metabolites in equine feces and urine, with relevance to doping control

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Abstract

Boldenone (1-dehydrotestosterone) is an exogenous anabolic-androgenic steroid (AAS) but is also known to be endogenous in the entire male horse and potentially formed by microbes in voided urine, the gastrointestinal tract, or feed resulting in its detection in urine samples. In this study, equine fecal and urine samples were incubated in the presence of selected stable isotope labeled AAS precursors to investigate whether microbial activity could result in 1-dehydrogenation, in particular the formation of boldenone. Fecal matter was initially selected for investigation because of its high microbial activity, which could help to identify potential 1-dehydrogenated biomarkers that might also be present in low quantities in urine. Fecal incubations displayed Δ1-dehydrogenase activity, as evidenced by the use of isotope labeled precursors to show the formation of boldenone and boldione from testosterone and androstenedione, as well as the formation of Δ1-progesterone and boldione from progesterone. Unlabeled forms were also produced in unspiked fecal samples with Δ1-progesterone being identified for the first time. Subsequent incubation of urine samples with the labeled AAS precursors demonstrated that Δ1-dehydrogenase activity can also occur in this matrix. In all urine samples where labeled boldenone or boldione were detected, labeled Δ1-progesterone was also detected. Δ1-progesterone was not detected any non-incubated urine samples or following an administration of boldenone undecylenate to one mare/filly. Δ1-progesterone appears to be a candidate for further investigation as a suitable biomarker to help evaluate whether boldenone present in a urine sample may have arisen due to microbial activity rather than by its exogenous administration.

KEYWORDS

boldenone, equine, feces, urine, Δ1-progesterone

1 | INTRODUCTION

Boldenone (1-dehydrotestosterone, 17β-hydroxyandrosta-1,4-dien-3-one) is an anabolic-androgenic steroid (AAS) that is prohibited as a performance enhancer in equine sports in most racing and equestrian jurisdictions, similarly to human sports.

Boldenone is known to be endogenously produced in the entire male horse, necessitating an international urinary threshold in Thoroughbred racing.[1] Boldenone and its 17-oxo analog boldione (androsta-1,4-diene-3,17-dione) are naturally present in the equine testis, with the biosynthesis of these steroids arising from the conversion of testosterone (17β-hydroxyandrost-4-en-3-one) and

androstenedione (androsta-4-diene-3,17-dione).[2] No urinary threshold values are in place for mares/fillies and castrated males (geldings) because historically there was no scientific evidence that boldenone was produced endogenously in either, with the important exception of mares/fillies when in estrous. However, a recent publication demonstrated that boldenone was present in a urine sample of an untreated gelding at a concentration of 1 ng/mL when urine samples collected from a population of 47 geldings were analyzed.[3]

Boldenone may be formed by microbial activity in feed, the gastrointestinal tract, or voided urine. Therefore the origin of boldenone in an equine sporting context has to be considered. One hypothesis is that boldenone could originate from conversion of phytosterols present in animal feed by micro-organisms, such as *Mycobacterium* sp.[4] In addition, fungal species in corn have been reported to convert androstenedione to boldione and then further to boldenone.[5] However, in vitro experiments investigating fermentation reactions in the horse hindgut, using fecal inocula, showed that although boldione was converted to boldenone and sitosterol to androstenedione, no direct conversion of phytosterols to boldione or boldenone was observed.[6] It has also been reported that black oats, contaminated with fungus and insects, contained boldenone and boldione, and its ingestion resulted in the excretion of boldenone sulfate in equine urine.[7]

Boldenone has also been recently detected as a minor metabolite of testosterone in gelding urine following an intramuscular administration of a high dose (500 mg) of testosterone suspension.[8] Interestingly, it was not detected following in vitro incubations with liver homogenates, and thus it could have been produced by enterohepatic cycling when testosterone enters the intestine via bile and is converted to boldione by intestinal microbes, as theorized separately for humans by Schänzer et al.[9]

Urine is sterile in the bladder but it is exposed to microbes in the urinary tract, on skin, and in the environment which might lead to microbial growth in urine samples. In doping control, urine samples are not generally stabilized by the addition of chemical additives, but by adequate temperature control measures. It is theorized that boldenone could be formed in equine urine in particular where post-collection storage conditions are unfavorable (temperature/extended period). For example, Fidani et al[10] showed that boldione was detected in 10% of inappropriately stored equine urine samples spiked with testosterone glucuronide. Additionally, inadvertent exposure of a urine sample to feces may result in the formation of boldenone, as demonstrated in both human urine[11] and in bovine feces.[12] Boldenone has previously been detected in the equine feces of 2 mares/fillies in estrous but not in a reference population of 8 geldings and 12 mares/fillies.[13] Furthermore, it was shown that boldenone and boldione are excreted in feces following an administration of boldione to horses.[13]

Ideally, distinguishing exogenous administration of boldenone from putative microbial action would be shown by differences in the carbon isotope content of the steroid. Boldenone has been detected in urine samples of people not treated with anabolic steroids,[9] and its exogenous origin in human doping control samples is required to be confirmed by the use of carbon isotope ratio mass spectrometry.[14,15] The carbon isotope ratio approach is less suitable for doping control in equine sports. This is due to the diet of the horse containing high amounts of plant material deriving from similar carbon sources as synthetic steroids, as is also the case for the bovine, or due to interspecies differences in enzymatic digestion.[16] An alternative approach is therefore required to help ascertain whether microbial activity may have resulted in the formation of boldenone in an equine urine sample, with the focus described herein being on the formation of Δ1-steroids that may act as markers of such microbial activity.

If microbial conversion is one cause of the formation of boldenone, in theory this could be catalyzed by the activity of a 3-ketosteroid Δ1-dehydrogenase isoenzyme (hereafter referred to as a Δ1-dehydrogenase). This enzyme has been identified in *Arthrobacter*, *Comamonas*, *Mycobacterium*, *Pseudomonas testosteroni*, and *Rhodococcus* (formerly *Nocardia*).[17-19] The enzyme was found to catalyze the formation of a double bond between C1 and C2 in 3-ketosteroids with or without saturation at C4 by eliminating two hydrogen atoms, as demonstrated for the formation of boldione from androstenedione.[18] In addition to converting androgens, Δ1-dehydrogenase can also convert 19-norandrogens and progestogens, and 5-reduced 3-ketosteroids, although structural changes such as the loss of C19 reduce its reactivity.[18,19] However, the microbial isoforms of Δ1-dehydrogenase studied to date are apparently unable to convert 3-hydroxysteroids to Δ1-steroids.[18] To the best of the authors' knowledge, Δ1-dehydrogenase has not been reported in any mammalian species.

The aim of this study was to investigate whether Δ1-steroids (1-dehydrogenated AAS or progestogens only) could be formed in equine feces and urine, and to identify any precursors that could be used to aid the differentiation between microbial production and the exogenous administration of boldenone. Fecal samples were initially used for experimentation since previous work by Pompa et al[12] with bovine feces suggested high conversion rates to Δ1-steroids, which could be advantageous in the identification of potential boldenone precursors and potential urinary biomarkers of Δ1-steroid dehydrogenase activity. Subsequent to using horse feces, urine samples were incubated at room temperature for 3 days to encourage microbial activity, and to assess the applicability of the presence of Δ1-biomarker(s) to identify microbial 'reactive' urine. Urine samples were spiked at higher than expected endogenous concentrations of testosterone in geldings (1.0 ± 3.5 ng/mL)[20] and mares/fillies (5.1 ± 5.0 ng/mL),[21] androstenedione concentrations in geldings (0.7 ± 0.6 ng/mL) and mares/fillies (1.4 ± 1.3 ng/mL), and progesterone in mares/fillies (1.0 ± 1.6 ng/mL)[3] to facilitate the demonstration of these steroid conversions.

2 | MATERIAL AND METHODS

2.1 | Reagents and standards

Methoxyamine hydrochloride, *β-glucuronidase* from *Eschericia coli*, sulfuric acid, and ammonium acetate were obtained from Sigma-Aldrich

(Gillingham, UK). Methanol, hexane, diethyl ether, ethyl acetate, acetic acid, disodium hydrogen orthophosphate 2-hydrate, sodium dihydrogen orthophosphate 2-hydrate, sodium chloride, and sodium hydroxide were purchased from Fisher Scientific Ltd (Loughborough, UK). Laboratory water was purified using a Triple Red Duo Water system (Triple Red Laboratory Technology, Long Crendon, UK).

16,16,17α-D_3-testosterone, progesterone, testosterone, epitestosterone, boldenone, boldione, and androstenedione were purchased from Sigma-Aldrich (Gillingham, UK) whilst epiboldenone, 17α-OH-progesterone and Δ1-progesterone (pregn-1,4-diene-3,20-dione) were from Steraloids (Newport, RI, USA), 2,2,4,6,6,17α-21,21,21-D_9-progesterone from CDN Isotopes (Pointe-Claire, Quebec, Canada) and 2,3,4-$^{13}C_3$-androstenedione from Cerilliant (Sigma-Aldrich, Gillingham, UK).

2.2 | Administration of boldenone undecylenate

A selection of pre- and post-administration urine samples was analyzed following the intramuscular administration of boldenone undecylenate (Sybolin®, 25 mg/mL by Ranvet) at a dose of 125 mg to a Thoroughbred mare/filly (5-year-old, 434 kg). The administration study was conducted at the British Horseracing Authority (BHA) Centre for Racehorse Studies (CRS) under license from the Home Office according to the framework of the Animal Scientific Procedures Act (1986). Urine samples presented in this study were collected pre-administration and at 24 and 168 hours post-administration. The samples were stored at −20°C prior to analysis.

2.3 | Incubation and extraction of feces

Fresh fecal samples were collected from four geldings and two mares/fillies residing at the CRS and immediately prepared for analysis.

D_3-testosterone, D_9-progesterone. and $^{13}C_3$-androstenedione were spiked into feces (1 g), at a concentration of 2 μg/g (or methanol for control samples) and vortex mixed. Samples were incubated at room temperature alongside control samples from each horse for either 0 (non-incubated), 3, or 10 days. At each time point, the whole sample was extracted and analyzed. Additionally, aliquots of pooled fecal samples were spiked immediately before the extraction, with boldione, boldenone, epiboldenone, androstenedione, testosterone, epitestosterone, progesterone, and 17α-hydroxyprogesterone at concentrations between 1 and 100 ng/g to determine the detection capability of the method and to assist with the identification of the detected peaks. Recoveries of each steroid were assessed to determine the method suitability for equine feces. Recoveries were determined by spiking pooled fecal samples, in duplicate, at a concentration of 20 ng/g, pre and post extraction, and comparing the obtained peak areas.

Samples were extracted and analyzed for steroids in the free (unconjugated) fraction since previous studies have shown that the majority of steroids excreted in equine feces are not conjugated.[13,22] The extraction method in the current study was based on the Pompa et al[12] method. Briefly, 1 M acetate buffer at pH 4.7 (3 mL) was added and samples were sonicated in a water bath for 15 minutes. Then, pH was adjusted with 2 M sodium hydroxide (100 μL) and a double liquid–liquid extraction (LLE) was performed with diethyl ether. Samples were evaporated to dryness and reconstituted with methanol (250 μL) and 0.1 M phosphate buffer at pH 6.3 (2.5 mL). Solid-phase extraction (SPE) was performed using SepPak C18 (3 mL, 500 mg) cartridges to retain steroids. The cartridges were conditioned with methanol and water prior to sample loading. They were washed with methanol in water (5:95, v:v) followed by hexane prior to eluting with diethyl ether (4 mL). Samples were evaporated to dryness at 80°C and reconstituted in ethyl acetate (200 μL) before splitting samples in two portions. A portion was used for the analysis of underivatized steroids (reconstituted in 10% methanol in water) and another portion for methyloxime (MO)-derivatized steroids (reconstituted in methoxyamine HCl in 80% methanol and incubated at 80°C for an hour).

2.4 | Incubation and extraction of urine samples

Urine samples were collected from 23 geldings and 7 mares/fillies post-race as a part of the BHA's routine surveillance program. These samples were reported negative for all substances covered in the routine screening methods in place in the authors' laboratory, and subsequently anonymized and assigned as research samples.

D_3-testosterone, D_9-progesterone, and $^{13}C_3$-androstenedione were spiked into urine (1.5 mL) at a concentration of 250 ng/mL (at 125 ng/mL for $^{13}C_3$-androstenedione) and vortex mixed. Samples were incubated at room temperature for 3 days. Incubated samples were extracted and analyzed alongside a set of non-incubated control samples and post-administration samples. Additionally, pooled urine samples were spiked immediately before the extraction with boldione, boldenone, epiboldenone, androstenedione, testosterone, epitestosterone, progesterone, and Δ1-progesterone at concentrations between 0.05 and 50 ng/mL to determine the detection capability of the method and to enable to identification of the detected peaks. Recoveries of each steroid were assessed to determine the method suitability for these steroids in equine urine. Recoveries were determined by spiking pooled urine samples, in duplicate, at a concentration of 1 and 10 ng/mL prior to extraction and post-extraction, and comparing the obtained peak areas.

Samples were extracted to produce separate fractions for steroids in the free, sulfate, and glucuronide conjugated forms. Urine samples were diluted with 2 mL of 1 M phosphate buffer at pH 6.3 (including D_3-testosterone as an internal marker). The first SPE was performed using Bond Elut Nexus (3 mL, 60 mg) nonpolar polymeric cartridges to retain steroids. The cartridges were conditioned with methanol and water prior to sample loading. They were washed with water followed by hexane prior to eluting free steroids with diethyl ether (3 mL). Conjugated steroids were subsequently eluted with methanol (3 mL), evaporated to dryness and then enzyme hydrolysis was

TABLE 1 SRM transitions with collision energies (CE) and cone voltages for each non- and MO-derivatized steroid

Steroid and Retention Time	Isotope Labels	SRM Transition	CE (eV)	Cone (V)
Underivatized				
Boldione (5.0 minutes)	n/a	285.1 > 121.1	14	2
		285.1 > 151.1	12	
	D_2*	287.0 > 121.0	14	2
		287.0 > 153.0		
	D_4*	289.0 > 125.0	14	2
		289.0 > 151.0		
	$^{13}C_3$*	288.0 > 124.0	24	22
		288.0 > 151.0		
Boldenone/epiboldenone (6.0/7.5 minutes)	n/a	287.1 > 121.1	24	22
		287.1 > 135.1	14	
	D_2*	289.0 > 121.0	24	22
		289.0 > 137.0	14	
	D_3*	290.0 > 121.0	24	22
		290.0 > 138.0	14	
	$^{13}C_3$*	290.0 > 124.0	24	22
		290.0 > 135.0	14	
Androstenedione (6.9 minutes)	n/a	287.2 > 97.2	20	16
		287.2 > 109.1		
	D_2*	289.0 > 97.0	20	16
		289.0 > 109.0		
	D_5*	292.0 > 100.0	20	16
		292.0 > 113.0		
	$^{13}C_3$	290.2 > 100.2	22	80
		290.2 > 112.4		
Testosterone/epitestosterone (8.0/10.5 minutes)	n/a	289.2 > 97.2	20	44
		289.2 > 109.1		
	D_3	292.2 > 97.2	20	44
		292.2 > 109.1		
	D_5*	294.0 > 100.0	20	44
		294.0 > 113.0		
	$^{13}C_3$*	292.0 > 100.0	20	44
		292.0 > 112.0		
Progesterone (11.4 minutes)	n/a	315.3 > 97.1	44	38
		315.3 > 109.1		
	D_9	324.2 > 100.1	44	38
		324.0 > 113.0	42	
17α-OH-progesterone (9.3 minutes)	n/a	331.3 > 97.1	42	32
		331.3 > 109.1	48	
	D_8*	339.0 > 100.0	42	32
		339.0 > 113.0	48	
Δ1-progesterone** **(8.3 minutes)**	n/a	313.3 > 121.1	44	38
		313.3 > 147.0	42	
	D_8*	321.3 > 125.0	44	38
		321.3 > 151.0	42	
MO-derivatized				
Boldione bis-MO (6.3 + 6.4 minutes)	n/a	343.3 > 120.0	34	16
		343.3 > 150.1	26	
	D_2*	345.0 > 120.0	34	16
		345.0 > 150.0	26	
	D_4*	347.0 > 124.0	34	16
		347.0 > 154.0	26	
	$^{13}C_3$*	346.0 > 123.0	34	16
		346.0 > 153.0	26	
Boldenone/epiboldenone MO (4.8 + 5.1/5.2 + 5.4 minutes)	n/a	316.3 > 120.3	46	6
		316.3 > 150.2	42	

(Continues)

TABLE 1 (Continued)

Steroid and Retention Time	Isotope Labels	SRM Transition	CE (eV)	Cone (V)
	D_2*	318.0 > 120.0	46	6
		318.0 > 150.0	42	
	D_3*	319.0 > 120.0	46	6
		319.0 > 150.0	42	
	$^{13}C_3$*	319.0 > 123.0	46	6
		319.0 > 153.0	42	
Androstenedione bis-MO (6.8 + 7.0 minutes)	n/a	345.2 > 126.1	20	16
		345.2 > 260.3	58	
	D_2*	347.0 > 126.0	20	16
		347.0 > 260.0	58	
	D_5*	348.0 > 129.0	20	16
		348.0 > 265.0	58	
	$^{13}C_3$	350.0 > 129.0	20	16
		350.0 > 263.0	58	
Testosterone/epitestosterone MO (5.3 + 5.7 minutes)	n/a	318.2 > 126.1	25	50
		318.2 > 138.1		
	D_3	321.0 > 126.0	25	50
		321.0 > 138.0		
	D_5*	323.0 > 129.0	25	50
		323.0 > 142.0		
	$^{13}C_3$*	321.0 > 129.0	25	50
		321.0 > 141.0		
Progesterone bis-MO (8.7 + 8.9 + 9.2 + 9.4 minutes)	n/a	373.4 > 126.1	62	4
		373.4 > 286.3	64	
	D_9	382.5 > 104.0	62	4
		382.5 > 292.3	64	
17α-OH-progesterone bis-MO (7.1 + 7.3 minutes)	n/a	389.5 > 126.1	64	2
		389.5 > 286.2		
	D_8*	397.5 > 129.1	64	2
		397.5 > 291.3		
Δ1-progesterone bis-MO (8.0 + 8.1 minutes)**	n/a	371.4 > 120.2	40	16
		371.4 > 150.1		
	D_8*	379.0 > 124.0	40	16
		379.0 > 154.0		

*predicted SRM transitions where no reference standard was available.

**monitored only in urine analysis.

performed using *Escherichia coli* (100 μL) and 0.1 M phosphate buffer at pH 6.8 (2 mL) and incubating for 2 hours at 50°C. A second SPE was performed using the same cartridges. Diethyl ether (3 mL) was used to elute the deconjugated glucuronide fraction and solvolysis solvent [ethyl acetate:methanol:sulfuric acid 100:20:0.2 (v:v:v), (3 mL)] was used to elute sulfate conjugates prior to incubating them for 1 hour at 50°C. All the fractions were further cleaned by LLE with pentane and 1 M sodium chloride and a second LLE with pentane and 2 M sodium hydroxide. Samples (underivatized and MO-derivatized) were then prepared as described for the fecal samples.

2.5 | Liquid chromatography–tandem mass spectrometry

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was used for the detection of unlabeled, deuterium and carbon isotope labeled steroids, which were compared to corresponding unlabeled reference standards. Identification criteria for the detected peaks were based on the AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry.[23] It was performed using a Waters Acquity I-Class UPLC coupled with a Waters Xevo TQ-S triple quadrupole mass spectrometer in positive electrospray ionization mode at a capillary voltage of 3.2 kV, a source temperature of 150°C and a desolvation gas temperature of 500°C. Analysis was carried out in selected reaction mode (SRM) as detailed in Table 1. For labeled steroids, where no reference standard was available, the SRM transitions were predicted on the basis of likely considered fragmentation.

Chromatographic separation of steroids was achieved on an Acquity HSS T3 (100 mm x 2.1 mm, 1.8 μm) reversed phase UPLC column using 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B) as mobile phases; column temperature at 60°C and flow at 0.4 mL/min. For underivatized steroids the gradient ran at 25% organic for 1 minute, increased to 55% organic at 2 minutes, and held for 7 minutes before increasing to 99.9% organic at 12 minutes, held

TABLE 2 Steroids formed from endogenous steroids and in the presence of D_3-testosterone, $^{13}C_3$-androstenedione and D_9-progesterone in feces following incubation periods of 3 and 10 days at room temperature. Numbers in parentheses represent number out of six samples where the relevant steroids were detected

Steroid precursor	Day 3 – Steroids Produced	Day 10 – Steroids Produced
Unspiked	Progesterone (n = 6) Boldione (n = 5) Androstenedione (n = 6) 17α-OH-progesterone (n = 4) Δ1-progesterone (n = 3)	Progesterone (n = 6) Boldione (n = 6) Boldenone (n = 2) Epiboldenone (n = 2) Androstenedione (n = 6) 17α-OH-progesterone (n = 5) Δ1-progesterone (n = 6)
D_3-testosterone	D_3-boldenone (n = 6) D_2-epiboldenone (n = 6) D_2-androstenedione (n = 6) D_2-boldione (n = 6)	D_3-boldenone (n = 6) D_2-epiboldenone (n = 6) D_2-androstenedione (n = 6) D_2-boldione (n = 6)
$^{13}C_3$-androstenedione	$^{13}C_3$-boldenone (n = 4) $^{13}C_3$-epiboldenone (n = 6) $^{13}C_3$-boldione (n = 6) $^{13}C_3$-testosterone (n = 6)	$^{13}C_3$-boldenone (n = 6) $^{13}C_3$-epiboldenone (n = 6) $^{13}C_3$-boldione (n = 6) $^{13}C_3$-testosterone (n = 6)
D_9-progesterone	D_5-androstenedione (n = 5) D_4-boldione (n = 1) D_8-Δ1-progesterone (n = 2)	D_5-androstenedione (n = 6) D_4-boldione (n = 5) D_8-Δ1-progesterone (n = 6)

for 1 minute before resuming the initial conditions and re-equilibrating for 1 minute. For MO-derivatized steroids the gradient started at 20% organic, increased to 60% at 0.5 minutes, to 80% at 4.5 minutes, to 90% at 8 minutes and to 99.9% at 9.5 minutes, held for 1 minute before resuming the initial conditions and re-equilibrating.

2.6 | Liquid chromatography high-resolution mass spectrometry analysis

LC high resolution mass spectrometry (LC–HRAM) analysis was used to provide further evidence of the identity of the detected steroids and gain information on fragmentation pathways. LC–HRAM was performed using a Dionex Ultimate 3000 UPLC system coupled to a Thermo Scientific QExactive Focus Hybrid Quadrupole-Orbitrap. The MS was operated using a heated electrospray source in positive mode. The MS parameters included an electrospray voltage of 3,750 V, a capillary temperature of 320°C, sheath gas of 40, auxiliary gas of 10, a probe heater temperature of 425°C and S-lens RF level of 40. Data was acquired in full scan mode (m/z 50–500) with a resolution of 35,000. The steroids detected in full scan mode where further analyzed using parallel reaction monitoring (PRM) mode with stepped collision energy (15, 35, and 50 eV).

Chromatographic separation was achieved using an Atlantis T3 (100 mm x 2.1 mm, 3 μm) reversed phase high performance liquid chromatograph (HPLC) column using 0.1% acetic acid in acetonitrile (A) and 0.1% acetic acid in water (B) as mobile phases. The gradients were run with a temperature of 40°C, using the mobile phase compositions described for LC–MS/MS analysis of underivatized and MO-derivatized steroids.

3 | RESULTS

3.1 | Analytical methods

The fragmentation pathways of underivatized and MO-derivatized boldenone, boldione, testosterone, and androstenedione were postulated with the help of LC–HRAM and labeled steroids. Since no reference standards were available for the converted isotope labeled steroids, identification of fragmentation pathways in addition to the locations of potential deuterium exchange was crucial to help determining the SRM transitions that could be used by LC–MS/MS.

Boldenenone and boldione produced a common selective fragment ion at m/z 121 (underivatized) and m/z 120 and 150 (MO-derivatized) that were considered diagnostic for the 1-dehydrosteroid structure. Previously published studies have rationalized the pathway for the formation of the characteristic and abundant A-ring fragment ion at m/z 121 for underivatized 1-dehydrosteroids.[24-26] Additionally, it has been demonstrated that m/z 135 for 255 underivatised boldenone could originate from the A- and B-rings and the C- and D-rings.[25,26] In the current study, the use of deuterium and carbon isotope labeled reference standards indicated that the latter was the more prominent pathway. The fragment ion at m/z 151 for boldione was consistent with the undehydrated C- and D-ring fragment. The fragmentation pathways for MO-derivatized 1-dehydrosteroids have not been previously described in the literature, but the accurate mass data indicated that the characteristic fragment ions at m/z 120 and 150 were formed by the B-ring cleavage with and without the loss of a methoxy group.

The fragmentation pathways of underivatized 3-keto-4-ene steroids, such as testosterone, have been described comprehensively in the literature,[24-27] and the characteristic fragment ions produced at m/z 97 and 109 were consistent with the observation in the current

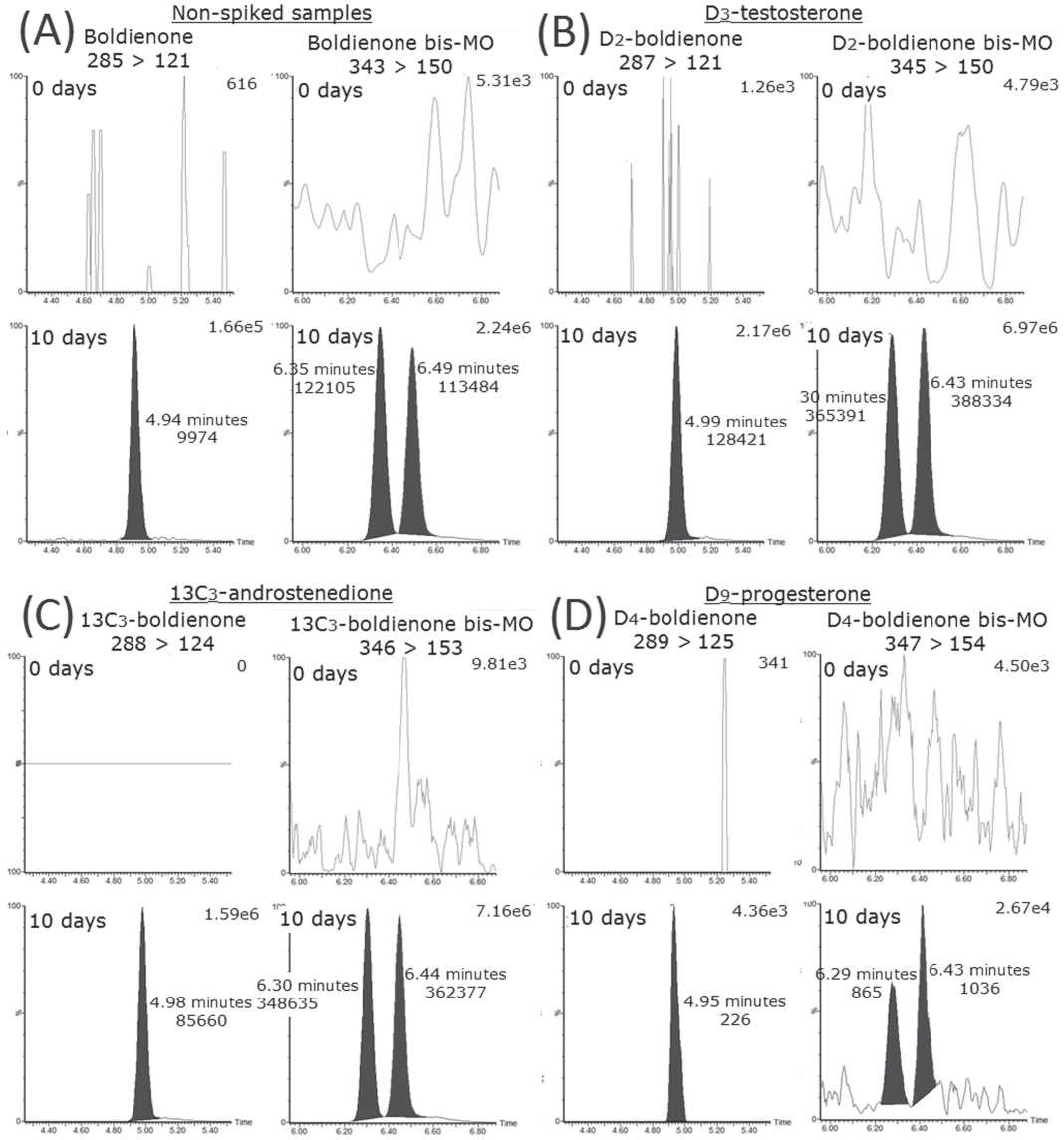

FIGURE 1 LC–MS/MS chromatograms of A, boldione;B, D2-boldione; C, 13C3-boldione; and D, D4-boldione, both underivatized and MO-derivatized in feces (unspiked and spiked with D3-testosterone, 13C3-androstenedione and D9-progesterone) following incubation periods of 0 and 10 days at room temperature

study. Additionally, fragmentation pathways of oxime derivatives of 3-keto-4-ene steroids have been described,[28] and they were consistent with the fragment ions at m/z 126 and 128 for MO-derivatives. The cleavage of the D-ring for androstenedione resulting in the formation of m/z 260 has also been reported previously.[28]

Samples were analyzed with and without derivatization to enable unambiguous identification of detected steroids. Additionally, MO-derivatization was utilized to increase analytical sensitivity by enhancing ionization of steroids containing keto functional groups. Although the derivatization results in formation of two geometric isomers (*syn* and *anti*) for the majority of steroids, considerable increases in overall responses were observed.

Complete chromatographic separation of all the steroids was not achieved; however, the steroids that co-eluted, ie, the first peak of MO-derivatized epiboldenone (5.2 minutes) and MO-derivatized testosterone (5.3 minutes), produced different product ions monitored permitting analytical specificity.

Suitability of the extraction methods for equine feces and urine was assessed in terms of analyte recovery and detection capability. Recovery of all analytes varied between 62 and 85% in fecal samples and between 65 and 85% in urine samples. MO-derivatization enhanced the detection of the steroids in both fecal and urine samples. In underivatized fecal samples, all the steroids were detected in the samples spiked at a concentration of 1 ng/g apart from epitestosterone (2 ng/g) and epiboldenone (5 ng/g). MO-derivatization increased the responses at least 2-fold even if two isomers were observed, resulting in the detection of all the steroids in samples spiked at 1 ng/g. In underivatized urine samples, Δ1-progesterone, progesterone and androstenedione were detected in the samples spiked at a concentration of 0.05 ng/mL, boldione at 0.1 ng/mL,

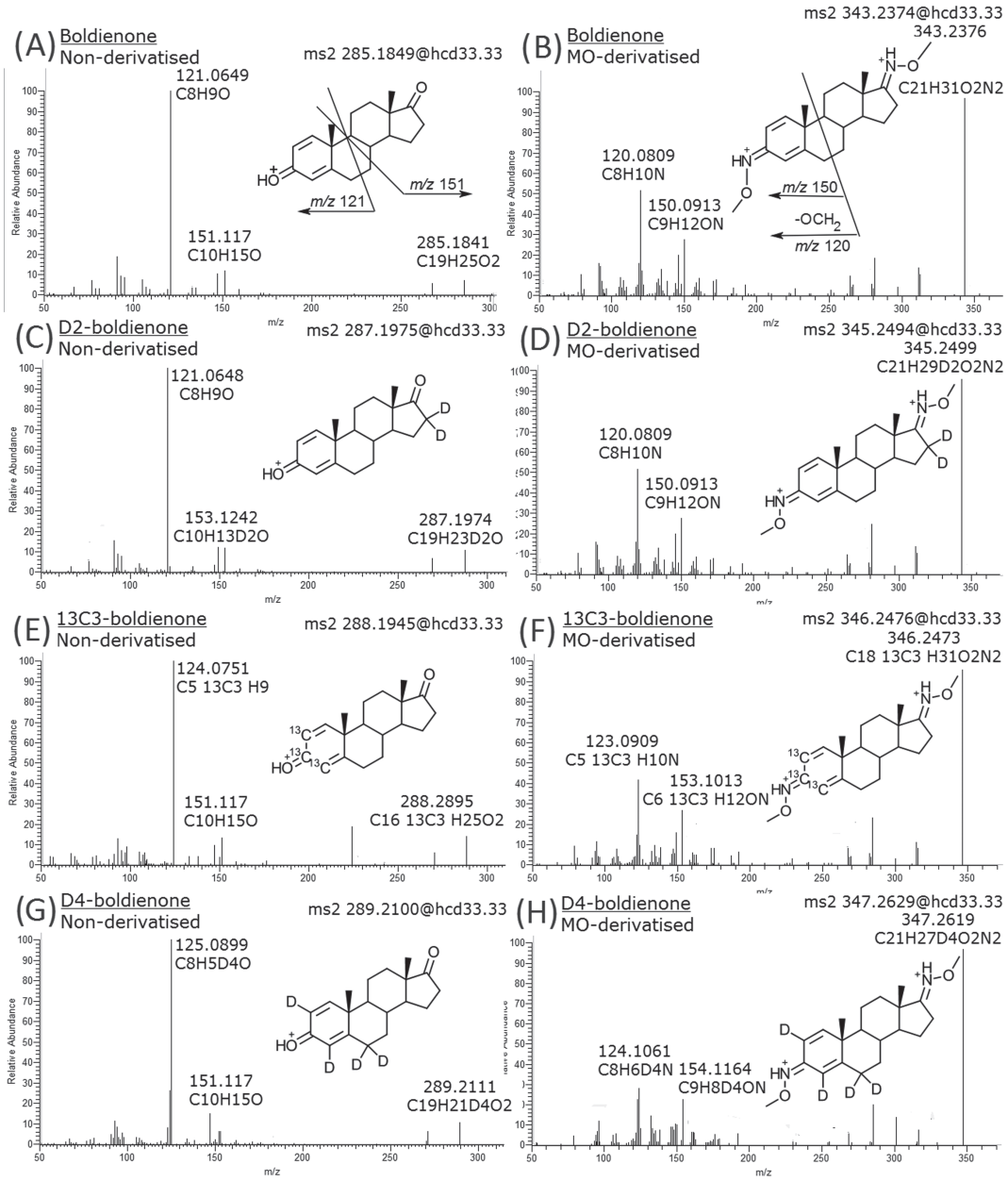

FIGURE 2 LC–HRAM spectra of underivatized and MO-derivatized boldione reference standard (a and B, respectively) and formed deuterium and carbon isotope labeled boldione in feces (C and D spiked with D_3-testosterone, E and F with $^{13}C_3$-androstenedione, and G and H with D_9-progesterone) following a 10-day incubation at room temperature.

boldenone, testosterone, and epitestosterone at 0.5 ng/mL, and epiboldenone at 5 ng/mL, whilst MO-derivatization resulted in the detection of all the steroids at 0.05 ng/mL, apart from epiboldenone which was detected at 0.1 ng/mL.

3.2 | Fecal analysis

The results for the steroids formed in six fecal samples, both unspiked and spiked with deuterium and carbon isotope labeled steroids are summarized in Table 2. The retention times and LC–HRAM spectra of the detected unlabeled and isotope labeled steroid matched those of unlabeled reference standards for boldione, boldenone, and epiboldenone supporting their identity. Analysis of unspiked fecal samples prior to incubation showed the presence of progesterone in MO-derivatized samples, but not the other monitored steroids or underivatized progesterone.

Boldione was detected in incubated unspiked samples, samples incubated in the presence of D_3-testosterone, $^{13}C_3$-androstenedione and D_9-progesterone (Figures 1 and 2). Unlabeled boldione (Figure 2 A and 2B for underivatized and MO-derivatized samples, respectively), produced characteristic fragment ions at m/z 121 and 151 for underivatized samples (Figure 2A), whilst m/z 120 and 150 were detected for MO-derivatized samples (Figure 2B).

Incubation in the presence of D_3-testosterone resulted in formation of D_2-boldione (Figure 2C and 2D for underivatized and MO-

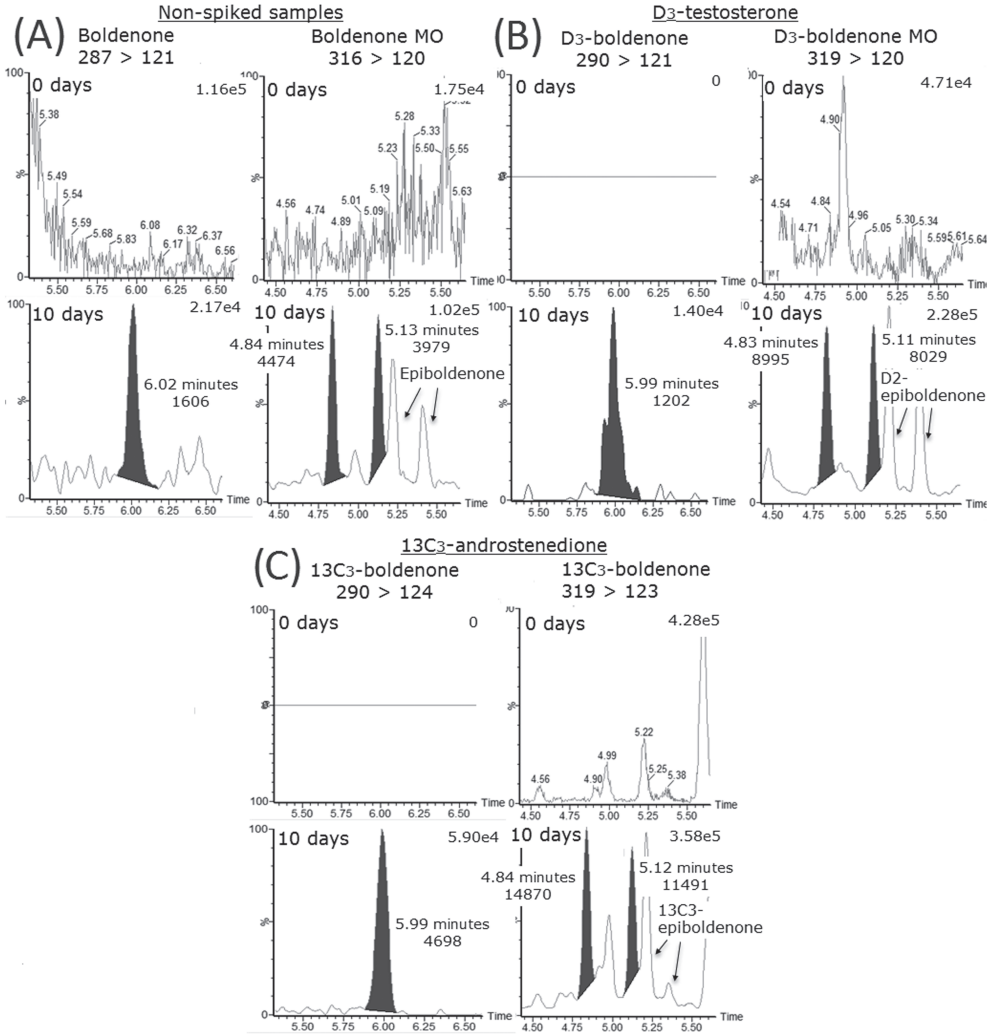

FIGURE 3 LC–MS/MS chromatograms of A, boldenone; B, D3-boldenone; and C, 13C3-boldenone, both underivatized and MO-derivatized in feces (unspiked and spiked with D3-testosterone and 13C3-androstenedione) following incubation periods of 0 and 10 days at room temperature.

derivatized samples, respectively) following 17-oxidation of the 17β-hydroxy group, which also led to an exchange of a deuterium atom at C17, and 1-dehydrogenation. Detection of fragment ions at m/z 121 and 153 (151 + 2) for underivatized samples (Figure 2C) and m/z 120 and 150 for MO-derivatized samples (Figure 2D) further supported this observation.

Meanwhile, incubation in the presence of $^{13}C_3$-androstenedione resulted in the formation of $^{13}C_3$-boldione (Figure 2E and 2F for underivatized and MO-derivatized samples, respectively) following 1-dehydrogenation alone. Again, this was supported by detection of fragment ions at m/z 124 (121 + 3) and 151 for underivatized samples (Figure 2E) and 123 (120 + 3) and 153 (150 + 3) for MO-derivatized samples (Figure 2F).

Incubation in the presence of D_9-progesterone resulted in formation of D_4-boldione (Figure 2G and 2H for underivatized and MO-derivatized samples, respectively) following cleavage of the C20 side chain, most likely following 17α-hydroxylase, 17,20-lyase, and Δ1-dehydrogenase activity. The first two enzymatic reactions also caused a cleavage of three deuterium atoms at C21 and an exchange of a deuterium atom at C17, and 1-dehydrogenation caused an exchange of a deuterium atom at C2. The fragment ions at m/z 125 (121 + 4) and 151 were detected for underivatized samples (Figure 2G) and m/z 124 (120 + 4) and 154 (150 + 4) were detected for MO-derivatized samples (Figure 2H).

Boldenone and epiboldenone were detected in incubated unspiked samples incubated in the presence of D_3-testosterone and $^{13}C_3$-androstenedione (Figures 3 and 4 for boldenone). Unlabeled boldenone and epiboldenone (Figure 4A and 4B for underivatized and MO-derivatized samples, respectively) detected in unspiked samples produced the fragment ions at m/z 121 and 135 for underivatized samples (Figure 4A) and m/z 120 and 150 for MO-derivatized samples (Figure 4B).

Incubation in the presence of D_3-testosterone resulted in formation of D_3-boldenone (Figure 4C and 4D for underivatized and MO-derivatized samples, respectively) and D_2-epiboldenone following 1-dehydrogenation alone or in conjunction with 17-oxidation and 17α-reduction leading to an exchange of a deuterium atom at C17. The fragment ions at m/z 121 and 138 (135 + 3) were observed for underivatized D_3-boldenone (Figure 4C), whilst fragment ions at m/z 121 and 137 (135 + 2) were observed for underivatized D_2-

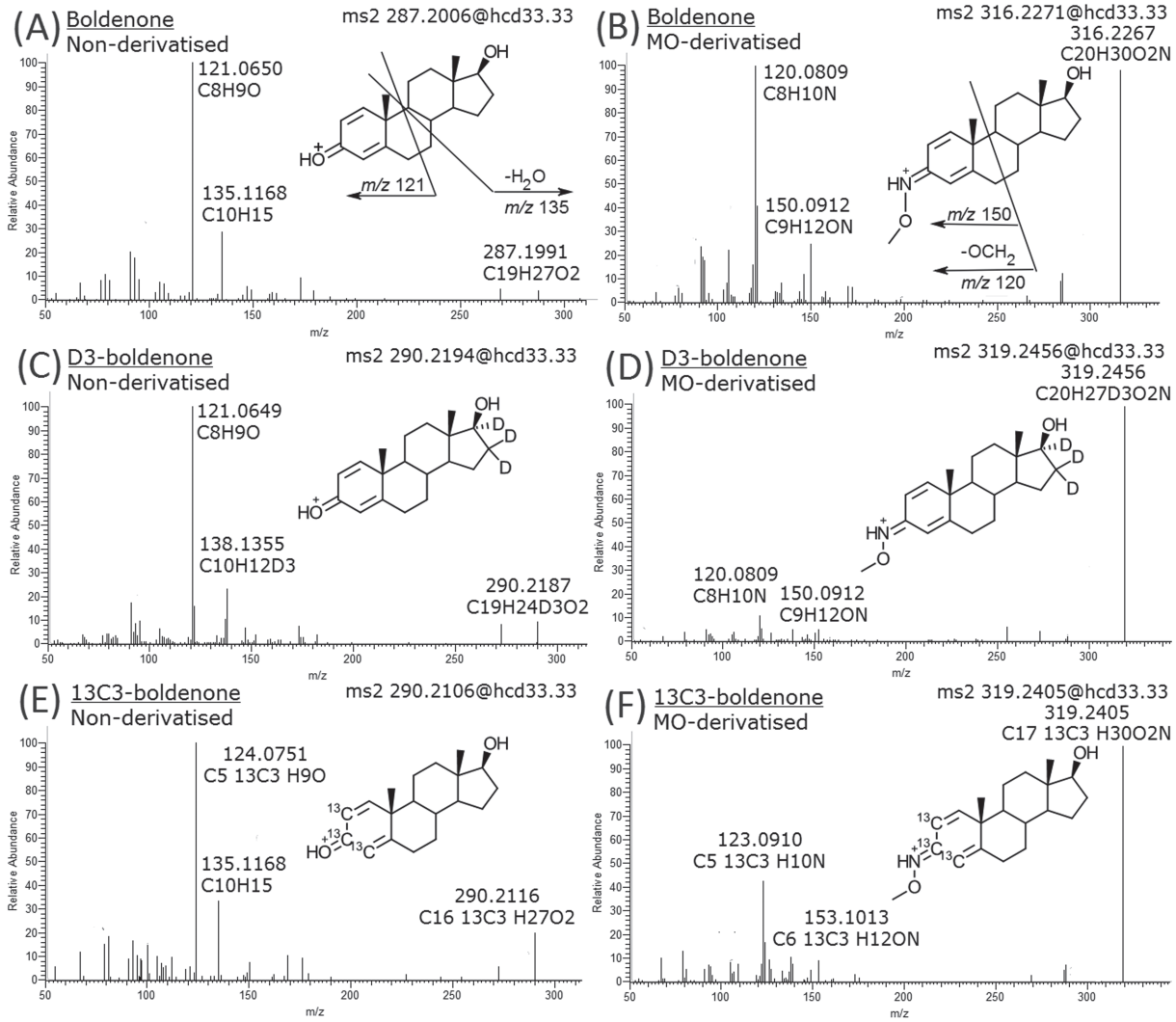

FIGURE 4 LC–HRAM spectra of underivatized and MO-derivatized boldenone reference standard (a and B, respectively) and formed deuterium and carbon isotope labeled boldenone in feces (C and D spiked with D_3-testosterone, and E and F with $^{13}C_3$-androstenedione) following a 10-day incubation at room temperature.

epiboldenone. MO-derivatized samples produced fragment ions at m/z 120 and 150 for both deuterium labeled steroids (Figure 4D).

Incubation in the presence of $^{13}C_3$-androstenedione resulted in formation of $^{13}C_3$-boldenone and $^{13}C_3$-epiboldenone (Figure 4E and 4F for underivatized and MO-derivatized samples, respectively) following 1-dehydrogenation in conjunction with either 17α- or 17β-reduction. Again, this was supported by detection of fragment ions at m/z 124 (121 + 3) and 135 and for underivatized samples (Figure 4E) and 123 (120 + 3) and 153 (150 + 3) for MO-derivatized samples (Figure 4F). Interestingly, 17α-reduction seemed to be a more prominent pathway than 17β-reduction as $^{13}C_3$-epiboldenone was detected in all samples following an incubation period of three days, whilst $^{13}C_3$-boldenone was detected in four samples. The peak intensities observed for boldenone and epiboldenone were similar in incubated samples. In spiked samples, the peak intensity observed for the epiboldenone was 10-fold lower than that of the boldenone spiked at the same concentration, indicating that epiboldenone concentrations detected in samples were higher than those of boldenone.

Additionally, a previously unreported equine 1-dehydrosteroid was identified and its structure subsequently supported by comparison against a reference standard as pregna-1,4-diene-3,20-dione (Δ1-progesterone) (Figures 5 and 6). This was detected in all incubated unspiked samples and samples incubated with D_9-progesterone. Unlabeled Δ1-progesterone (Figure 6A and B for underivatized and MO-derivatized samples, respectively) produced fragment ions at m/z 121 and 147 for underivatized steroids (Figure 6A) and m/z 120 and 150 for MO-derivatized steroids (Figure 6B), consistent with those observed for the reference standard. The detection of the fragment ion at m/z 100 for MO-derivatized samples was indicative of 20-keto structure, and is also observed with progesterone.[28]

Incubation in the presence of D_9-progesterone resulted in formation of D_8-Δ1-progesterone (Figure 6C and 6D for underivatized and MO-derivatized samples, respectively) following 1-dehydrogenation resulting in an exchange of a deuterium atom at C2. The fragment ions at m/z 125 (121 + 4) and 151 (147 + 4) were observed for underivatized D_8-Δ1-progesterone (Figure 6C), whilst fragment ions

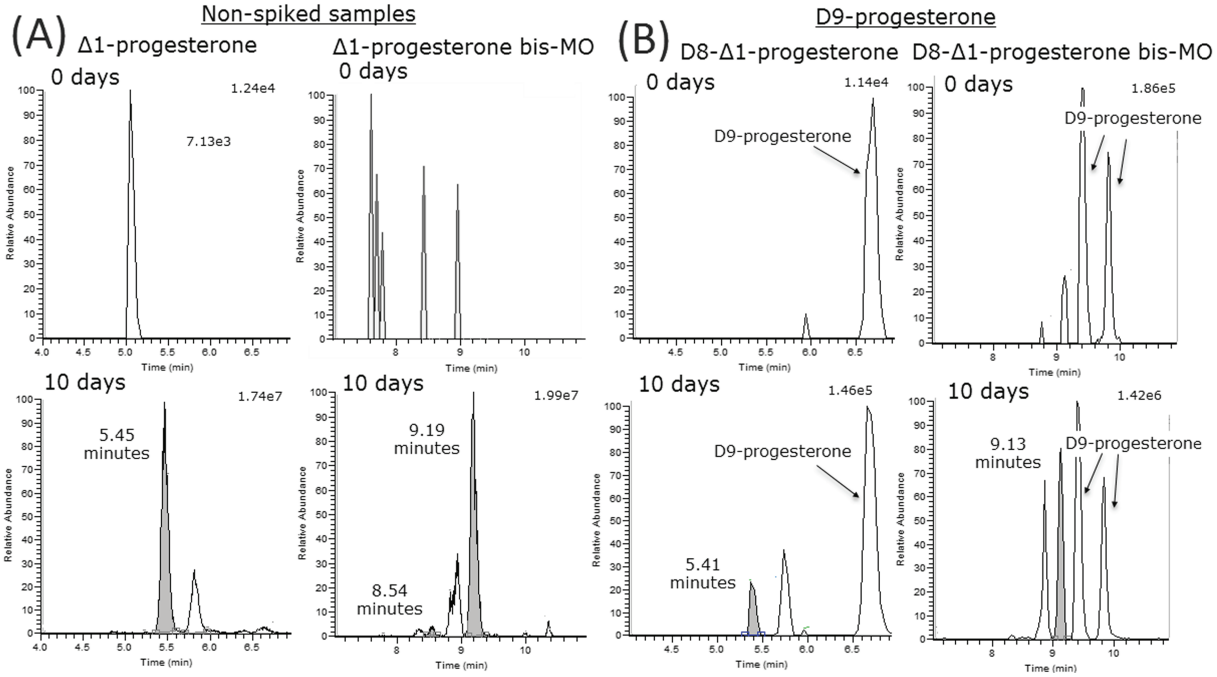

FIGURE 5 LC–HRAM chromatograms of A, Δ1-progesterone and B, D_8-Δ1-progesterone, both underivatized (m/z 313.2159 and m/z 321.2662, respectively, mass tolerance of 5 ppm) and MO-derivatized (m/z 371.2690 and m/z 379.3193, respectively, mass tolerance of 5 ppm) in feces (unspiked and spiked with D_9-progesterone) following incubation periods of 0 and 10 days at room temperature. [Colour figure can be viewed at wileyonlinelibrary.com]

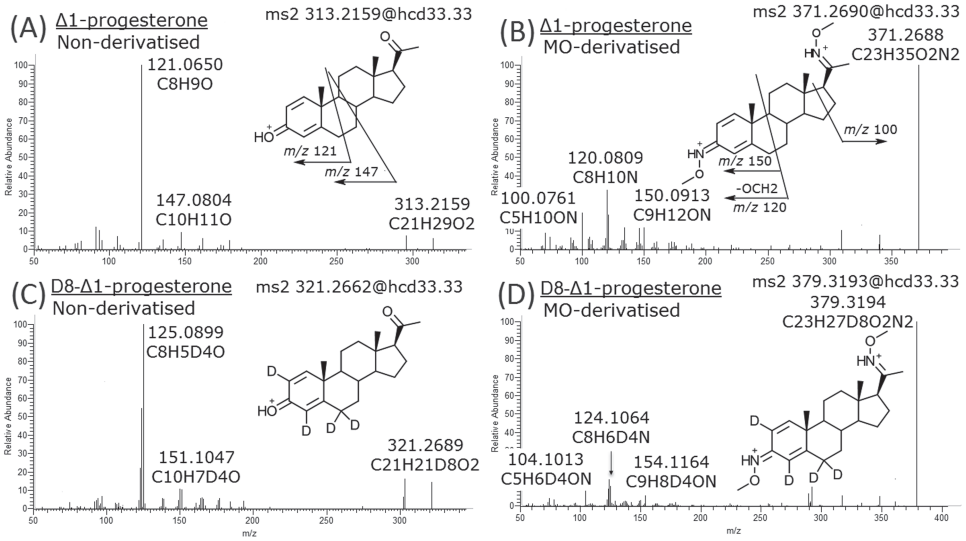

FIGURE 6 LC–HRAM spectra of underivatized and MO-derivatized Δ1-progesterone reference standard (a and B, respectively) and formed deuterium labeled Δ1-progesterone in feces (C and D spiked with D_9-progesterone) following a 10-day incubation at room temperature.

at m/z 124 (120 + 4) and 154 (150 + 4) were observed for MO-derivatized D_8-Δ1-progesterone (Figure 6D).

Additional peaks eluting close to Δ1-progesterone in fecal samples (one in underivatized samples and two in MO-derivatized samples) were likely caused by a related isomer since they produced the similar spectra to that of Δ1-progesterone and the same phenomenon was observed for progesterone following incubation. Although all these 1-dehydrosteroids were detected in underivatized fecal samples, MO-derivatization increased analytical sensitivity further.

Other steroids formed included progesterone and 17α-OH-progesterone in unspiked samples, androstenedione in all unspiked samples, and samples incubated in the presence of D_3-testosterone and D_9-progesterone and testosterone in all samples incubated in the presence of $^{13}C_3$-androstenedione.

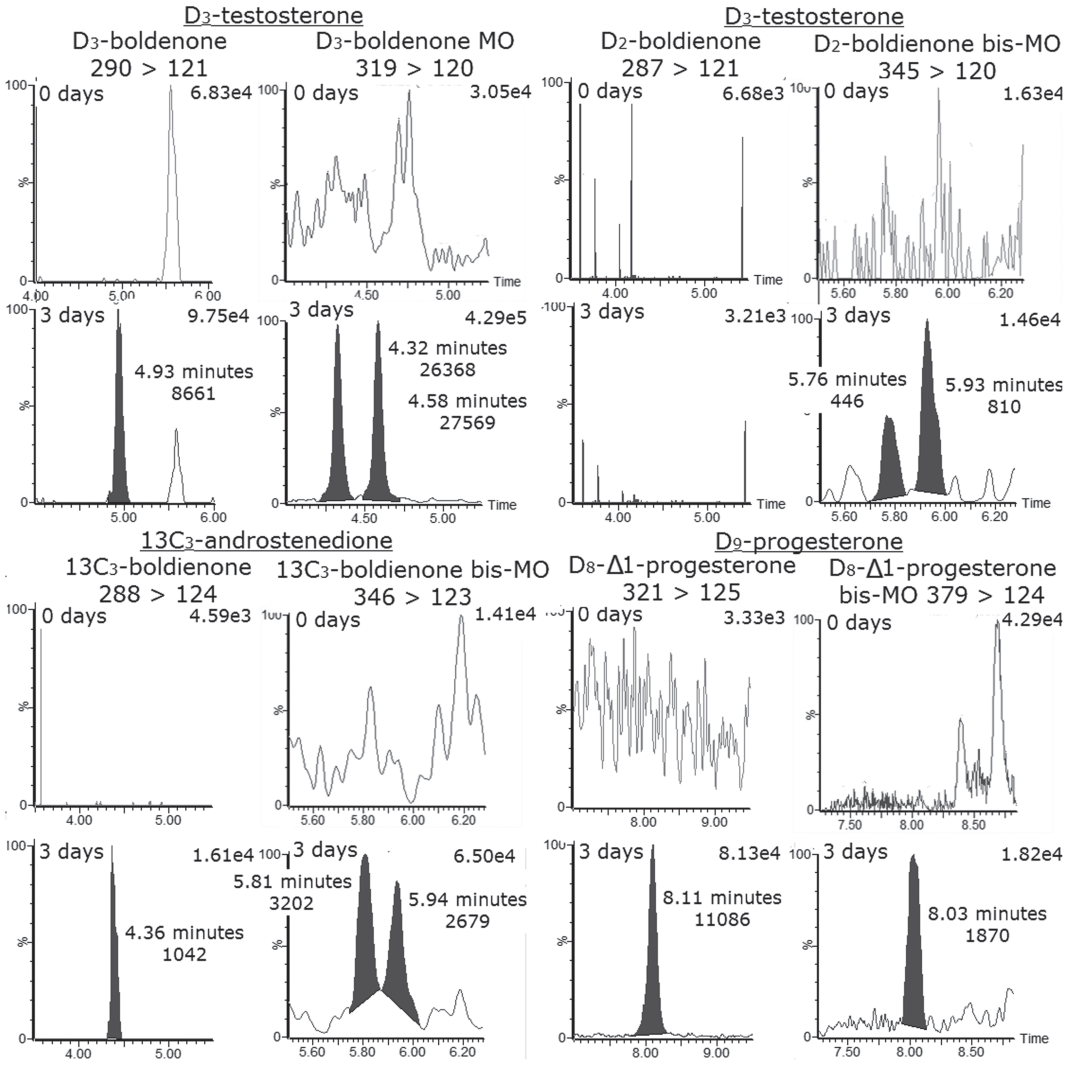

FIGURE 7 LC–MS/MS chromatograms of D_3-boldenone, D_2-boldione, $^{13}C_3$-boldione, and D_8-Δ1-progesterone, both underivatized and MO-derivatized in equine urine (spiked with D_3-testosterone, $^{13}C_3$-androstenedione, and D_9-progesterone) following incubation periods of 0 and 3 days at room temperature.

3.3 | Urine analysis

Following the identification of Δ1-progesterone as a potential biomarker of microbiological activity in equine feces, an incubation experiment was carried out to assess whether it could be used to discern the administration of boldenone and related compounds from microbial conversion in urine.

Analysis of 30 equine urine samples (underivatized and MO-derivatized) spiked with deuterium and carbon isotope labeled steroids (D_3-testosterone, D_9-progesterone, and $^{13}C_3$–4-androstenedione) following incubation periods of 0 and 3 days at room temperature demonstrated Δ1-dehydrogenase activity. $^{13}C_3$-boldione was detected in five samples, D_2-boldione in two samples (only MO-derivatized), D_3-boldenone in four samples, and D_8-Δ1-progesterone in seven samples (Figure 7). All the samples that produced peaks for either $^{13}C_3$-boldione or D_3-boldenone also produced peaks for D_8-Δ1-progesterone. These peaks were not detected in the samples extracted and analyzed prior to incubation. No unlabeled boldenone, boldione, or Δ1-progesterone was detected in the incubated samples analyzed, and thus these conversions could not be proven in samples without any added steroids.

The absence of Δ1-progesterone in non-incubated urine samples supported its use as a putative biomarker, although the sample size was small. Furthermore, the administration of boldenone undecylenate resulted in increases in boldenone and boldione concentrations, but no Δ1-progesterone was detected in these samples. Boldenone concentrations were estimated at 40 and 109 ng/mL in post-administration samples collected at 24- and 168-hours post-administration, respectively, whereas the boldione concentrations were estimated at 0.8 and 0.3 ng/mL, respectively.

4 | DISCUSSION

A high level of Δ1-dehydrogenase activity was observed with equine feces leading to formation of boldenone, boldione, and Δ1-

progestrone. Detection of Δ1-progesterone in fecal samples was very promising, since this steroid could potentially be used as a biomarker to distinguish the exogenous administration of boldenone and related compounds from microbial conversion to the same.

Conversion of D_9-progesterone to D_5-androstenedione and D_4-boldione showed that the majority of the enzymes required for steroidogenesis (17α-hydroxylase, 17,20-lyase and 17β-hydroxysteroid dehydrogenase) were present in fecal samples. However, the reduction of the 17-keto group was not as prominent a pathway as the oxidation of the 17β-OH group or Δ1-dehydrogenase activity. This is in line with a previously published study reporting that testosterone is mainly converted to androstenedione and boldione by bacteria, whilst androstenedione is mainly converted to boldione and to a smaller extent to testosterone and boldenone.[19]

17α-reduction appeared to be a more prominent pathway than 17β-reduction in this study, which was an unexpected finding since the published literature indicated that while 17β-reduction is widely observed in bacteria, 17α-reduction is not.[29] However, the formation of epiboldenone is known to occur in bovine feces[12] and it is possible that a 17α-precursor present in the urine may have been the substrate as opposed to 17α-reduction being catalyzed by the microbes themselves.

One theory is that the formation of progesterone and Δ1-progesterone in unspiked fecal samples could be due to conversion of excreted sterols. Equine feces has been demonstrated to contain high concentrations of sitosterol (218 ± 48 μg/g in 6 horses), cholesterol (39 ± 9 μg/g in 6 horses), and related metabolites.[30] Since actinobacteria present in feces are shown to express 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase, 3-ketosteroid Δ1-dehydrogenase, and cholesterol side-chain cleavage enzyme activity, this could result in the formation of progesterone and Δ1-progesterone in feces.[17] Subsequently, boldenone could be formed from progesterone via conversions to androstenedione and testosterone or from Δ1-progesterone via conversions to boldione (Figure 8).

The equine hindgut model utilizing fecal inocula has successfully showed the conversion of sitosterol to androstenedione, but conversions to Δ1-steroids were not demonstrated.[6] It is possible that 3-ketosteroid Δ1-dehydrogenase is active in aerobic conditions (in feces) but not in the normal anaerobic conditions in the stomach as proposed by the study using a human large intestinal model.[31] It is unclear what conditions are required to induce the production of Δ1-steroids in the gut. However, if boldenone or boldione are produced in the equine or human gut, it is also possible that Δ1-progesterone would be produced simultaneously.

Δ1-dehydrogenase activity was also observed in incubated urine samples. Since boldenone is most likely formed in mammalian tissues as a by-product of aromatase action[2] and progesterone is not a likely substrate for this enzyme, the detection of Δ1-progesterone with boldenone in a urine samples is an indicator that both have been formed by microbial Δ1-dehydrogenase activity. Indeed, detection of D_8-Δ1-progesterone in all incubated samples where either $^{13}C_3$-boldione or D_3-boldenone was detected indicated that it could be used as a biomarker to distinguish an exogenous administration of boldenone from microbial conversion to boldenone. However, these

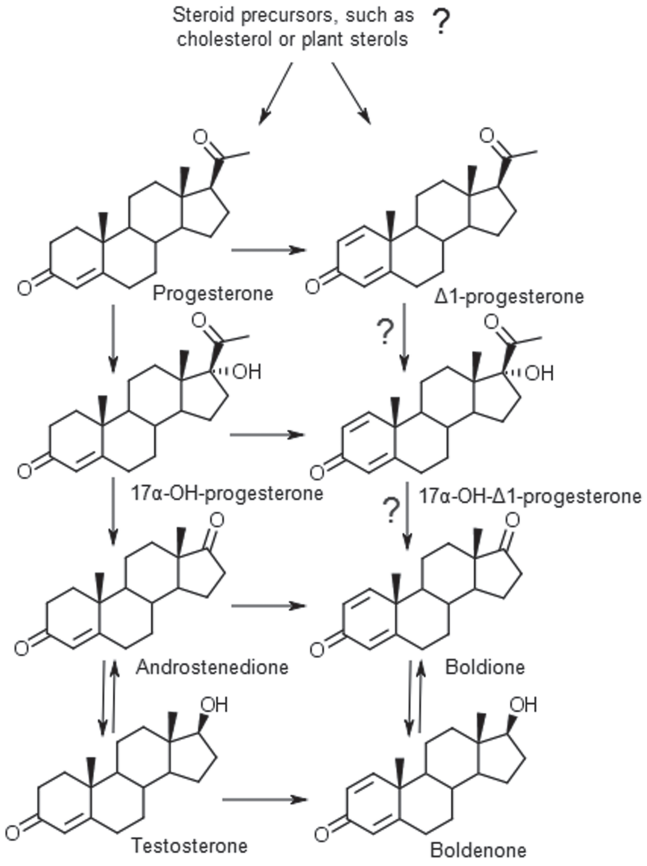

FIGURE 8 Postulated formation of boldenone by microbes as demonstrated in equine feces.

conversions could not be proven in samples without any added steroids. This is likely a result of not enough unlabeled precursor material being present in these urine samples and/or the levels of microbes in the samples not being high enough to demonstrate conversion. However, it is theorized that if boldenone or its precursor boldione had been produced by microbes, the detection of Δ1-progesterone would have been expected based on these results. Further studies including blank tests would be required to support the use of this putative biomarker. However, it was observed that the responses for boldenone and boldione in the urine samples were not as high as some of those observed in previous studies, so it is possible that Δ1-progesterone may have been detected if higher concentrations were present or if more sensitive methods were used.

Progesterone has been reported in unspiked equine urine samples at low concentrations. A study by Decloedt et al[3] reported urinary concentrations of 1.0 ± 1.6 ng/mL in 57 mares/fillies and stated that it could also be detected in gelding urine. Meanwhile, the metabolites of progesterone have been reported at higher concentrations in both genders.[32] For example, 5α-pregane-3β,20β-diol was detected at concentrations of 5–1,500 ng/mL in 113 mares/fillies and 1–100 ng/mL in 103 geldings and pregn-5-ene-3β,20β-diol at concentration of 3–160 ng/mL in mares/fillies, and 2–150 ng/mL in geldings. Although a number of these metabolites are more abundant in horse urine, 3-ketosteroid Δ1-dehydrogenase is unable to convert 3-hydroxysteroids,[18] and thus these metabolites could not be used to

identify microbial activity leading to the detection of Δ1-steroids. Additionally, 5-reduced 3-ketosteroids could be converted to Δ1-steroids via the Δ4-steroid structure, as demonstrated for 17β-hydroxyandrost-5-en-3-one, which was converted to boldione via androstenedione,[19] and thus Δ1-progesterone could be a suitable biomarker to detect Δ1-dehydrogenase activity in voided urine samples. However, further investigation will be carried out for other suitable putative biomarkers.

5 | CONCLUSION

The aim of this study was to investigate whether Δ1-steroids, in particular boldenone, could be formed in equine urine and feces from selected precursors, and to identify whether any biomarkers that could be used to distinguish the microbial production of boldenone from its exogenous administration. Δ1-dehydrogenase activity was demonstrated in equine feces resulting in formation of boldenone and boldione. Furthermore, the steroid Δ1-progesterone was identified in equine feces for the first time. Labeled testosterone and androstenedione were shown to be precursors for boldenone and boldione and labeled progesterone was shown to be a precursor for boldione and Δ1-progesterone. The fecal experiments indicated that Δ1-progesterone and/or related metabolites could be potential biomarkers to distinguish the exogenous administration of boldenone and related compounds from microbial production of the same. Following the urine analysis of samples stored at room temperature, Δ1-dehydrogenase activity was demonstrated in a total of seven urine samples by the formation of labeled boldenone, boldione, and/or Δ1-progesterone from labeled precursors. Since labeled Δ1-progesterone was detected in all samples where labeled boldenone and boldione were observed, this could act as a potential biomarker to identify microbial activity in urine samples.

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