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Extraction of Veterinary Growth Promoters from Animal Urine Using ISOLUTE® HYDRO DME+ Prior to UPLC-MS/MS Analysis

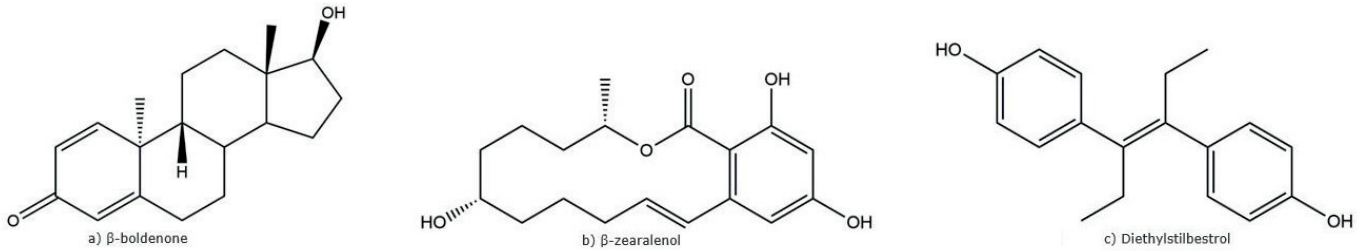

Figure 1. Representative veterinary growth promoter chemical structures.

This application note describes the extraction of 18 growth promoters (steroids, resorcylic acid lactones and stilbenes) using ISOLUTE® HYDRO DME+ prior to LC/MS analysis.

ISOLUTE HYDRO DME+ dual mode extraction columns and plates provide extremely efficient removal of matrix components and hydrolysis enzymes from urine samples, using a simple flow-through workflow. This application note provides an effective and efficient ISOLUTE HYDRO DME+ procedure using column and 96-well plate formats. This simple sample preparation delivers clean extracts and recoveries greater than 80% for most analytes. RSDs are typically less than 5% and lower than 10% for all analytes. LLOQ are typically less than the estimated linear range minima of 0.4 ng mL^{-1}, using a total load volume of 75 µL of urine.

Analytes

ß-trenbolone, α-trenbolone, ß-boldenone, α-boldenone, ß-nortestosterone, α-nortestosterone, methylboldenone, methyltestosterone, 16-ß-hydroxystanozolol, taleranol, zeranol, ß-zearalenol, α zearalenol, zearalenone, zearalanone, diethyl-stilbestrol, dienestrol, hexestrol

Internal Standards

N/A

Sample Preparation Procedure

Format

ISOLUTE® HYDRO DME+
400 mg columns, part number 970-0040-BZ

ISOLUTE® HYDRO DME+
400 mg plate, part number 970-0400-PZ01

Matrix Preparation

Hydrolyze 75 µL animal urine as follows: dilute 1:1 (v./v) with 200 mM ammonium acetate pH 5.2 containing 10 µL per mL of glucuronidase H-2 ex. *Helix pomatia*. Incubate at 60 °C for 2 hours. Cool to room temperature.

Sample Loading

Load 150 µL of hydrolyzed sample directly to ISOLUTE® HYDRO DME+ column/well. Add 900 µL of acetonitrile and mix with 5x aspirate/dispense steps.

Analyte Extraction

Using a Biotage® Pressure+ 48 or 96 Positive Pressure Manifold, apply approximately 5 psi pressure. Collect the extract.

Post Elution and Reconstitution

Evaporate the extract at 40 °C and reconstitute in 0.1% formic acid in 50% aq MeOH (200 µL).

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UHPLC Conditions

Instrument
Shimadzu Nexera UHPLC

Column
ACE UltraCore Super C18 2.5 µm 100 x 2.1 mm (CORE-25A-1002U), VWR International (Lutterworth, UK)

Mobile Phase
A: 0.5 mM ammonium acetate, 0.01% acetic acid, 30% MeOH (aq)

B: 0.5 mM ammonium acetate, 0.01% acetic acid, 95% MeOH (aq)

Flow Rate
0.5 mL min

Injection Volume
10 µL

Column Temperature
45 °C

MS Conditions

Instrument
Sciex Triplequad 5500 operating in dual polarity ESI mode

Source Temperature (TEM)
600 °C

Curtain Gas (CUR)
35

Source Gas 1 (GS1)
60

Source Gas 1 (GS2)
50

Table 1. Gradient and divert valve settings

Time (min)	%A	%B	Divert Valve
0.00	90.0	10.0	waste
7.00	NA	NA	MS
8.00	75.0	25.0	
12.00	62.5	37.5	
14.00	NA	NA	waste
14.50	50.0	50.0	
16.50	10.0	90.0	
18.00	10.0	90.0	
20.00	90.0	10.0	
24.50	90.0	10.0	

Table 2. Typical retention times.

Analyte	Retention Time (min)
taleranol	7.5
β-trenbolone	8.1
β-zearalenol	8.4
β-boldenone	9.1
α-trenbolone	9.4
β-nortestosterone	9.6
zeranol	10.5
methylboldenone	10.6
α-zearalenol	11.2
zearalanone	11.5
α-boldenone	11.7
diethylstilbestrol	11.9
zearalenone	12.0
α-nortestosterone	12.0
16-β-hydroxystanozolol	12.1
dienestrol	12.6
methyltestosterone	12.9
hexestrol	13.1

Table 3. MS parameters for target analytes.

Analyte	Transition (Da)	IS (V)	DP (V)	EP (V)	CE (V)	CXP (V)
taleranol	321.2 > 277.1	-3000	-112	-4	-30	-43
β-trenbolone	271.0 > 199.0	+4500	48	5	30	22
β-zearalenol	319.3 > 159.9	-3000	-85	-4	-38	-18
β-boldenone	287.2 > 135.0	+4500	73	9	21	18
α-trenbolone	271.0 > 199.0	+4500	30	4.5	32	22
β-nortestosterone	275.2 > 108.8	+4500	49	5.5	32	14
zeranol	321.2 > 277.0	-3000	-100	-4	-30	-38
methylboldenone	301.1 > 120.8	+4500	85	9.5	30	16.5
α-zearalenol	319.2 > 275.0	-3000	-180	-4	-28	-44
zearalanone	319.3 > 275.0	-3000	-90	-7	-28	-48
α-boldenone	287.2 > 120.9	+4500	62	7.5	31	18
diethylstilbestrol	267.1 > 237.0	-3000	-210	-8	-37	-48
zearalenone	317.2 > 131.0	-3000	-110	-4	-32	-17
α-nortestosterone	275.0 > 108.8	+4500	20	4.5	33	14
16-β-hydroxystanozolol	345.4 > 94.90	+4500	70	5	51	13
dienestrol	265.0 > 92.80	-3000	-130	-4	-31	-12
methyltestosterone	303.2 > 97.00	+4500	82	8	31	17
hexestrol	269.1 > 134.0	-3000	-74	-4	-20	-17

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Results

Recovery

Extraction recoveries were determined at 1ng mL^{-1}, equivalent to 75 pg when extracting 150 µL of pre treated hydrolyzed urine from four different species: bovine, equine, ovine and porcine.

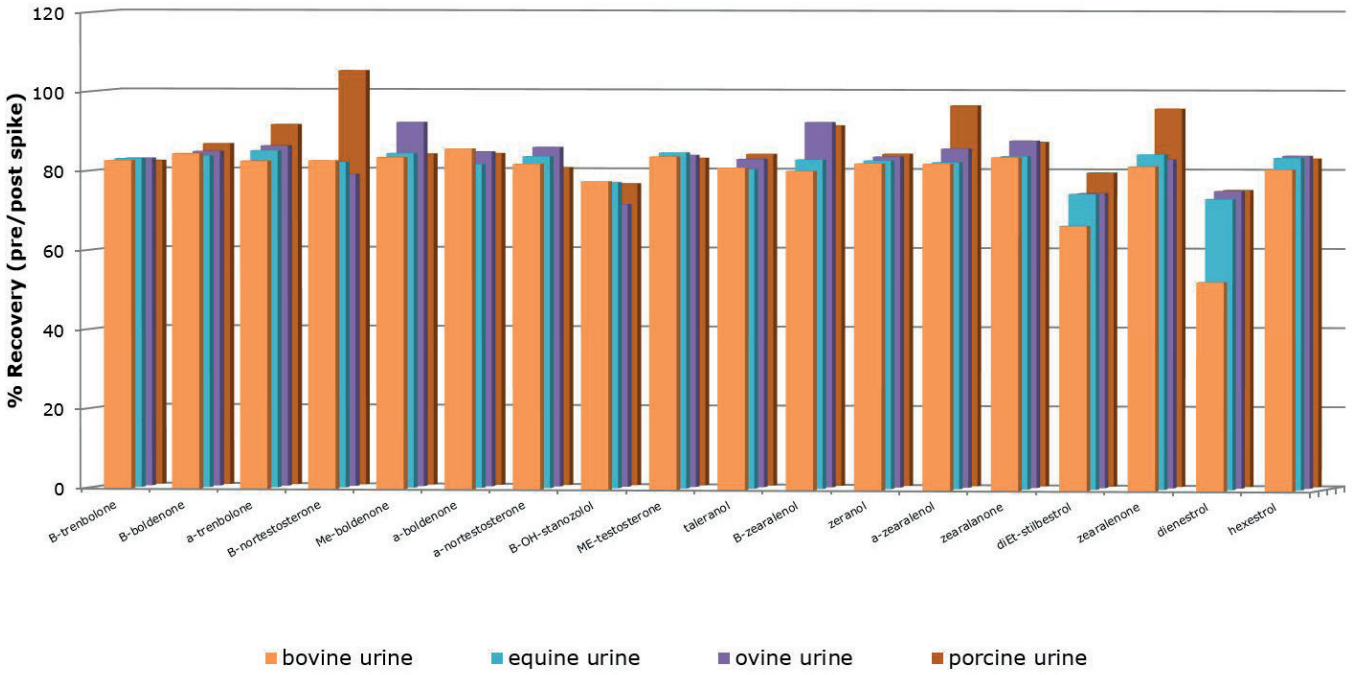

Figure 2. Representative analyte recoveries using an optimized ISOLUTE® HYDRO DME+ protocol (75 µL of urine extracted).

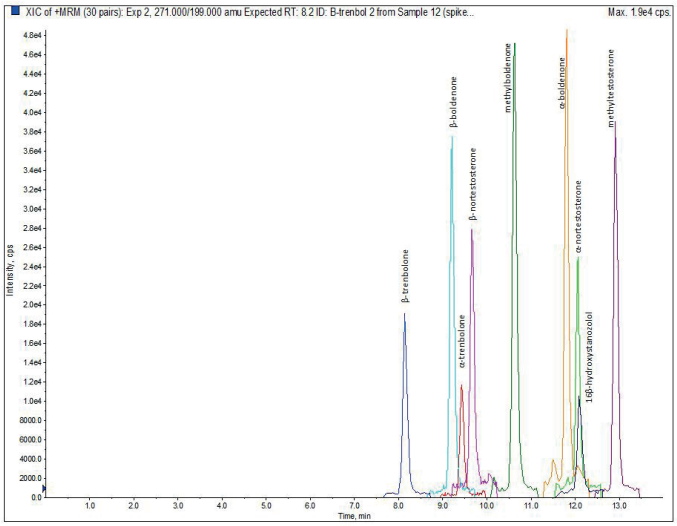

Figure 3. Representative +ESI target analyte chromatography (1 ng mL^{-1} spike).

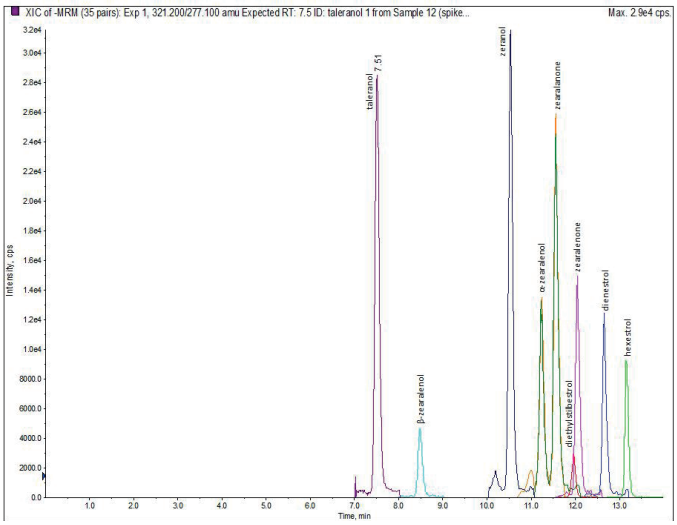

Figure 4. Representative -ESI target analyte chromatography (1 ng mL^{-1} spike).

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Linearity

Extraction linearity was determined between 0.4 and 10 ng mL^{-1} from a mixed stock spiked into bovine urine and serially diluting in bovine urine. Each calibration level was extracted in four replicates. Figure 4 demonstrates representative calibration curves. Table 4 details linearity performance and associated LOQ for each analyte. Good linearity was observed for all analytes typically delivering r^2 values greater than 0.999.

Table 4. Analyte calibration curve r^2 and LOQ performance.

Analyte	Coefficient (r^2)	Linear range, ng mL^{-1}	LOQ, ng mL^{-1}	% accuracy (RSD) at LOQ
taleranol	0.9975	0.4 to 10	0.4	95.8 (10.3)
β-trenbolone	0.9995	0.4 to 10	0.4	99.1 (3.7)
β-zearalenol	0.9970	0.4 to 10	0.4	97.1 (10.1)
β-boldenone	0.9994	0.4 to 10	0.4	98.1 (6.7)
α-trenbolone	0.9981	0.4 to 10	0.4	101 (5.8)
β-nortestosterone	0.9996	0.4 to 10	0.4	98.3 (3.5)
zeranol	0.9990	0.4 to 10	0.4	94.9 (2.7)
methylboldenone	0.9997	0.4 to 10	0.4	98.4 (4.9)
α-zearalenol	0.9982	0.4 to 10	0.4	99.8 (12.5)
zearalanone	0.9967	0.4 to 2.0	0.4	103 (6.5)
α-boldenone	0.9995	0.4 to 10	0.4	97.7 (1.1)
diethylstilbestrol	0.9969	0.4 to 10	0.4	102 (8.1)
zearalenone	0.9994	0.4 to 10	0.4	96.3 (7.4)
α-nortestosterone	0.9970	0.4 to 10	0.4	99.0 (6.0)
16-β-hydroxystanozolol	0.9984	0.4 to 10	0.4	95.9 (3.4)
dienestrol	0.9996	0.4 to 10	0.4	94.8 (5.9)
methyltestosterone	0.9996	0.4 to 10	0.4	99.8 (2.9)
hexestrol	0.9993	0.4 to 10	0.4	94.2 (4.0)

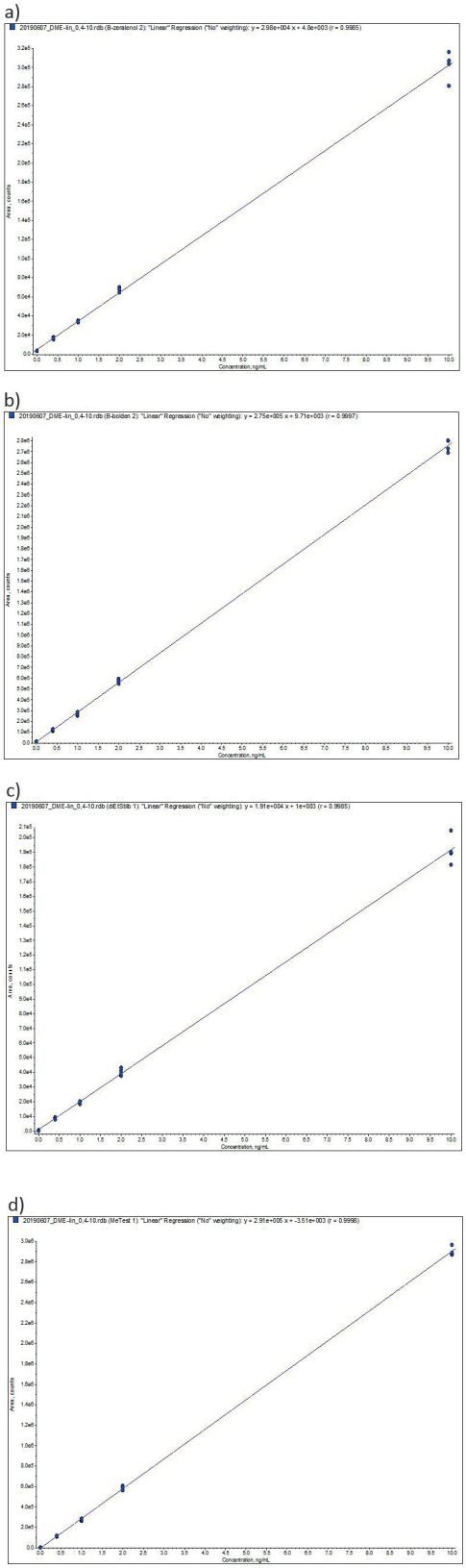

Figure 5. Representative Calibration curves (0.4 to 10 ng mL^{-1} extracted from bovine urine) a) β-zearalenol, b) β-boldenone, c) diethylstilbestrol, d) methyltestosterone.

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Chemicals and Reagents

Water (18.2 MΩ.cm) was drawn fresh daily from a Millipore Direct-Q5 water purifier (Watford, UK).

Organic solvents (methanol and acetonitrile) were HPLC or LC-MS grade and purchased from Honeywell Research Chemicals (Bucharest, Romania).

Acetic acid (LC-MS) was purchased from Fisher Scientific UK (Loughborough, UK).

Ammonium acetate (reagent and LC-MS grade), formic acid (LC-MS grade) and ß-glucuronidase (Type HP-2, aqueous solution ≥100,000 units/mL) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK).

Analytical standards were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK) or Toronto Research Chemicals (Toronto, Canada)

200 mM ammonium acetate pH 5.2 (aq) was prepared by dissolving 15.42 g of reagent grade ammonium acetate in 1 L deionized water, the pH was adjusted using concentrated formic acid.

Buffered ß-glucuronidase hydrolysis solution was prepared fresh daily by diluting 150 µL ß-glucuronidase solution in 14.85 mL 200 mM ammonium acetate pH 5.2 (aq) and vortex-mixing thoroughly.

50% methanol (aq), 0.1% formic acid (reconstitution solution) was prepared by adding 50 mL methanol to 50 mL deionised water, followed by 100 µL concentrated LC-MS grade formic acid.

Mobile phase A (0.5 mM ammonium acetate (aq), 0.01 % acetic acid) was prepared by dissolving 38.5 mg LC-MS grade ammonium acetate in 500 mL deionized water, adding 100 µL of concentrated acetic acid and making up to 1 L with deionized water.

Mobile phase B (0.5 mM ammonium acetate (methanol), 0.01 % acetic acid) was prepared by dissolving 38.5 mg LC-MS grade ammonium acetate in 500 mL LC-MS grade methanol, adding 100 µL of concentrated acetic acid and making up to 1 L with LC-MS grade methanol.

Additional information

Animal urine matrix samples were kindly donated by the State Laboratory, Celbridge, Ireland.

Ordering Information

Part Number	Description	Quantity
970-0400-PZ01	ISOLUTE® HYDRO DME+ 400 mg Plate	1
970-0040-BZ	ISOLUTE® HYDRO DME+ 400 mg/3 mL Columns	50
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
PPM-48	Biotage PRESSURE+ 48 Positive Pressure Manifold	1
45000	TurboVap® LV	1
SD-9600-DHS-NA	SPE Dry 96 Sample Concentrator System, 100/120V	1
SD-9600-DHS-EU	SPE Dry 96 Sample Concentrator System, 220/240V	1

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