Extraction of a Comprehensive Steroid Panel from Horse Hair Using ISOLUTE® SLE+ Prior to LC/MS-MS Analysis

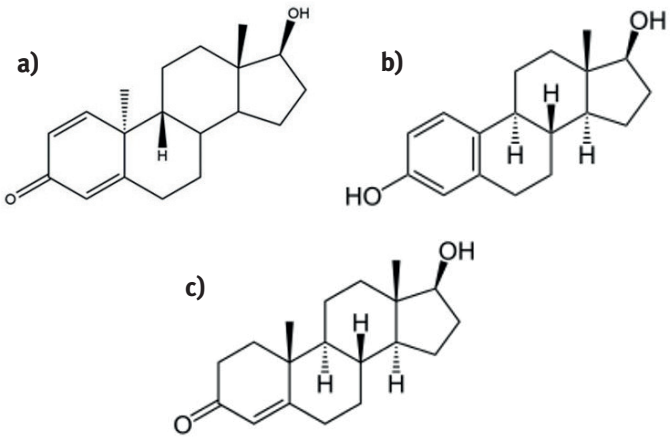

Figure 1. Structures of (a) Boldenone, (b) Estradiol and (c) Testosterone.

Introduction

This application note describes the extraction of a panel of 18 steroid hormones from horse hair using ISOLUTE® SLE+ Supported Liquid Extraction plates prior to LC/MS-MS analysis. The simple sample preparation procedure delivers clean extracts and analyte recoveries greater than 80% with RSDs lower than 5% for most analytes. Linearity of greater than 0.999 is achieved for all analytes in the range 0.5-500 pg/mg of hair.

ISOLUTE® SLE+ Supported Liquid Extraction plates offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

Analytes

21-Deoxycortisol, Cortisone, Estradiol, Aldosterone, 11-Deoxycortisol, Corticosterone, Estrone, Dehydroepiandrosterone (DHEA), 17-OH-Progesterone, Testosterone, Dihydrotestosterone (DHT), Androstenedione, 11-deoxycorticosterone, Progesterone, α-trenbolone, β-trenbolone, α-boldenone, β-boldenone.

Internal Standards

Dihydrotestosterone- D_3 (DHT-D_3) and Aldosterone-D_4.

Sample Preparation Procedure

Format

ISOLUTE® SLE+ 400 µL sample capacity plate, (p/n) 820-0400-P01

Matrix Preparation

Weigh 20 mg of hair into 2 mL Biotage® Lysera tubes containing 4 x 2.8 mm stainless steel beads.

Micropulverization Procedure 1:

Grind the hair sample using Biotage® Lysera (conditions: 5 x 6.45 m/s for 45 sec with a 15 sec dwell).

Add 1 mL of 0.1% (v/v) NH_4OH in propan-2-ol (IPA) to each ground hair sample. Spike with 100 µL of a 10 pg/µL methanolic ISTD to give a final concentration of 50 pg/mg of hair.

Micropulverization Procedure 2:

To ensure complete transfer of the analytes into the extraction solvent, process the sample again using Biotage® Lysera (conditions: 3 x 5.3 m/s for 60 sec with a 20 sec dwell).

Centrifuge tubes for 10 minutes at 13,300 rpm.

Post Micropulverization

Transfer 200 µL of extract and load directly onto the ISOLUTE® SLE+ 400 µL plate.

Sample Loading

Load up to 200 µL of horse hair extract into each ISOLUTE® SLE+ well. Using a Biotage® PRESSURE+96 Positive Pressure Manifold, apply a pulse of pressure to load samples onto the sorbent. Wait 5 minutes for the sample to equilibrate on the sorbent.

Analyte Extraction

Apply an aliquot of Dichloromethane (DCM) (500 µL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of DCM (500 µL) and allow to flow under gravity for 5 minutes. For complete removal apply a pulse of positive pressure at 10 psi (10–20 seconds). Collect extracts into a 2 mL, 96-well collection plate.

Post Elution and Reconstitution

Evaporate extracts at 40 °C, for 30 mins at a flow rate of 20–40 L/min using the Biotage® SPE Dry 96. Reconstitute extracts in a mix of mobile phase A/mobile phase B (50:50, v/v, 200 µL). Vortex mix. Cover plate with a sealing mat prior to injection.

© Biotage 2019

UHPLC Conditions

Instrument
Shimadzu Nexera X2 UHPLC

Column
ACE C18 (100 mm x 2.1 mm, 1.7 µm) (Hichrom (VWR), UK) with EXP Guard column holder fitted with a C-18 cartridge (Thames Restek, UK)

Mobile Phase
A: 0.2 mM Ammonium Fluoride (aq)

B: Methanol

Flow Rate
0.4 mL/min

Column Temperature
40 °C

Injection Volume
5 µL

Table 1. UHPLC Gradient.

Time (min)	%A	%B	Column Oven
0.1			Divert to waste
2	50	50	
3			To MS
5	40	60	
8	10	90	
9	5	95	
9.1	5	95	
9.2	50	50	

MS Conditions

Instrument
Shimadzu 8060 Triple Quadrupole MS using ES interface

Nebulizing Gas Flow
3 L/min

Drying Gas Flow
3 L/min

Heating Gas Flow
17 L/min

Interface Temperature
400 °C

DL Temperature
250 °C

Heat Block Temperature
400 °C

Interface Temperature
400 °C

CID Gas Flow
270 kPa

For optimum sensitivity, data was acquired in both positive and negative ion modes, as appropriate, shown in Table 2.

Table 2. MS conditions and retention times for target analytes in positive and negative mode.

Analytes	MRM Transition	Collision Energy	Ion Mode
β-trenbolone	271.0>253.0 (271.0>199)	-19	+
α-trenbolone	271.0>253.0 (271.0>199)	-21	+
β-boldenone	287>121 (287>135)	-23	+
Cortisone	361.3>163.15 (361.30>329.15)	-22	+
21-Deoxycortisol	347.1>311.2 (347.10>269.20)	-16	+
Estradiol	271.1>145.2 (271.10>183.25)	39	-
Aldosterone-D_4	363.1>190.3	19	-
Aldosterone	359.1>189.25 (359.00>297.15)	18	-
α-boldenone	287>121 (287>135)	-23	+
11-Deoxycortisol	347.3>109.25 (347.30>283.15)	-27	+
Corticosterone	347.3>329.25 (347.30>283.15)	-16	+
Estrone	269.2>145.2 (269.20>143.20)	37	-
11-Deoxycorticosterone	331.3 > 109.05 (331.30>97.25)	-25	+
DHEA	271.10>253.20 (271.10>213.20)	-13	+
Testosterone	289.3>97.05	-23	+
DHT-D_3	294.4>258.25	-16	+
DHT	291.3>255.25	-15	+
Androstenedione	287.3>97.2 (287.30>109.20)	-21	+
17-OH-Progesterone	331.3>97.1	-22	+
Progesterone	315.2>97.2 (331.30>109.15)	-22	+

© Biotage 2019

Biotage®

Results

This simple sample preparation method delivers clean extracts and analyte recoveries greater than 80% with RSDs lower than 5% for most analytes (see Figure 2), and LLOQs as low as 1 pg/mg for the steroids. Figure 2. below shows recoveries using 400 µL capacity ISOLUTE® SLE+ plates, loading 200 µL of non-aqueous extract directly onto the plate.

Figure 3. demonstrates representative chromatography obtained from horsehair spiked at 50 pg/mg. Satisfactory resolution of the various isobars was obtained using the ACE C18 UHPLC column. In order to achieve low level detection of analytes in positive and negative ion modes a combination of 0.2 mM NH_4F (aq) and MeOH was utilized in the mobile phase.

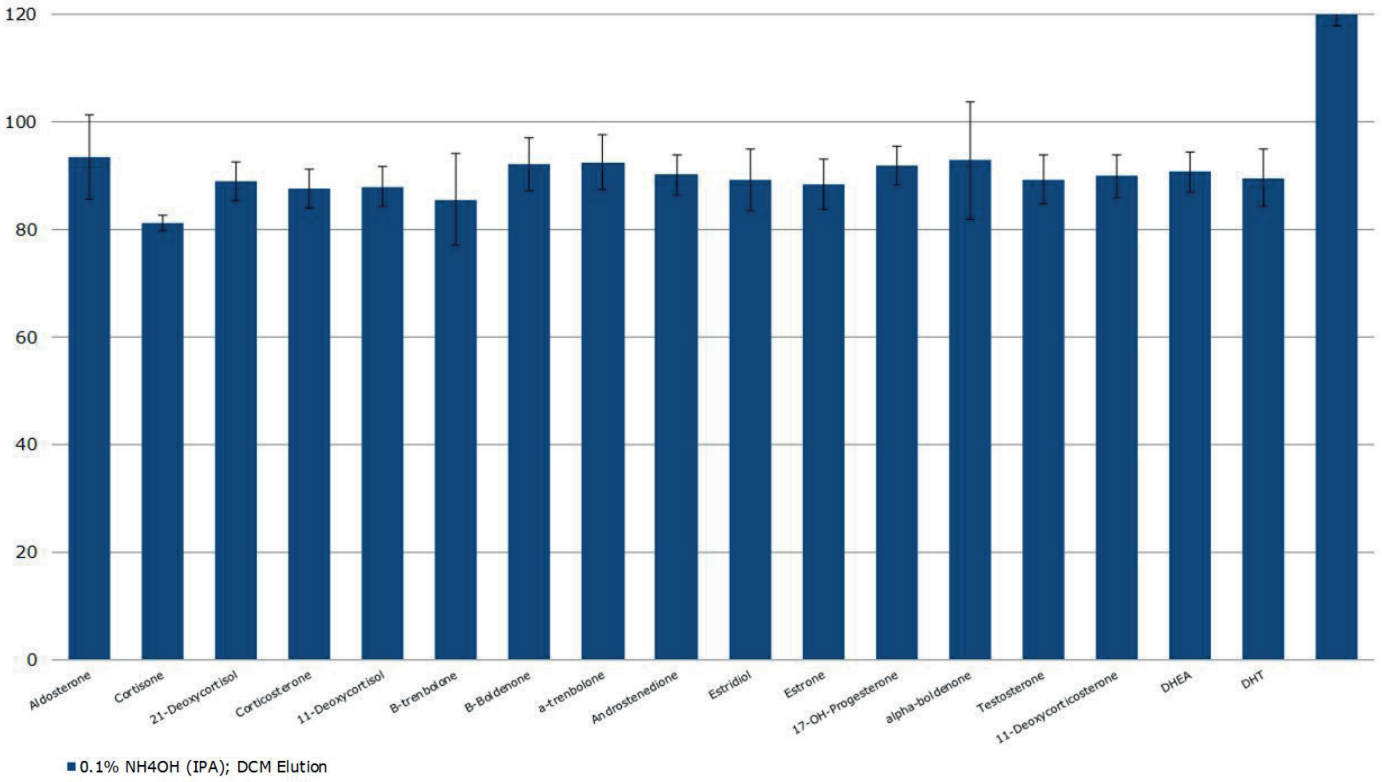

Figure 2. Typical analyte extraction recoveries (n=7) loading 200 µL of non-aqueous extract onto a 400 µL capacity ISOLUTE® SLE+ plate.

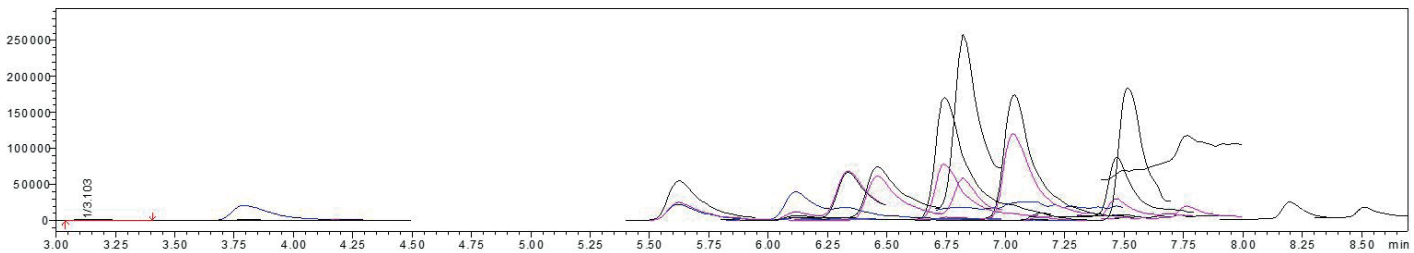

Figure 3. Representative chromatography for analytes spiked at 50 pg/mg in horse hair.

© Biotage 2019

Biotage®

Calibration curve performance was investigated using hair from a female horse spiked between 0.5–500 pg/mg. Good linearity was observed for all analytes typically delivering r^2 values greater than 0.999. Table 3. details linearity performance and associated LOQ for each analyte loading 200 µL of horse hair extract. Selected calibration curves loading 200 µL are shown in Figure 4.

Table 3. Analyte calibration curve r^2 and LOQ performance.

Analytes	r^2	LLOQ (pg/mg)
β-trenbolone	0.9993	<5
α-trenbolone	0.9997	0.5
β-boldenone	0.9995	<0.5
Cortisone	0.9997	1
21-Deoxycortisol	0.9996	<5
Estradiol	0.9995	<10
Aldosterone	0.9995	25
α-boldenone	0.9993	<5
11-Deoxycortisol	0.9995	0.5
Corticosterone	0.9995	<1
Estrone	0.9994	0.5
11-Deoxycorticosterone	0.9994	5
DHEA	0.9991	25
Testosterone	0.9991	1
DHT	0.9995	<5
Androstenedione	0.9994	1
17-OH-Progesterone	0.9995	10
Progesterone	0.9991	<25*

*Progesterone LOQ was estimated from endogenous levels of a female horse based on peak height and blank baseline. Endogenous levels were approximately 250 pg/mg.

Chemicals and Reagents

- Propan-2-ol (IPA) (LC-MS grade), Ultra-Pure methanol (Gradient MS) and dichloromethane were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards, deuterated internal standards and ammonium fluoride were purchased from Sigma-Aldrich Company Ltd. (Gillingham, UK).
- Water (18.2 MΩ-cm), was drawn fresh daily from a Direct-Q5 water purifier (Merck Millipore, Watford, UK).
- Mobile phase A (0.2 mM ammonium fluoride (aq)) was prepared by adding 7.4 mg of ammonium fluoride to 1 L of purified water.
- Internal standards (5 pg/mg) were prepared from a 1 ng/µL stock solution by adding 10 µL of each of to 950 µL of MeOH. 100 µL of this solution was then added to each calibration sample.
- Reconstitution solvent was made by measuring out 50 mL of mobile A and 50 mL of mobile phase B and adding them to the same bottle.

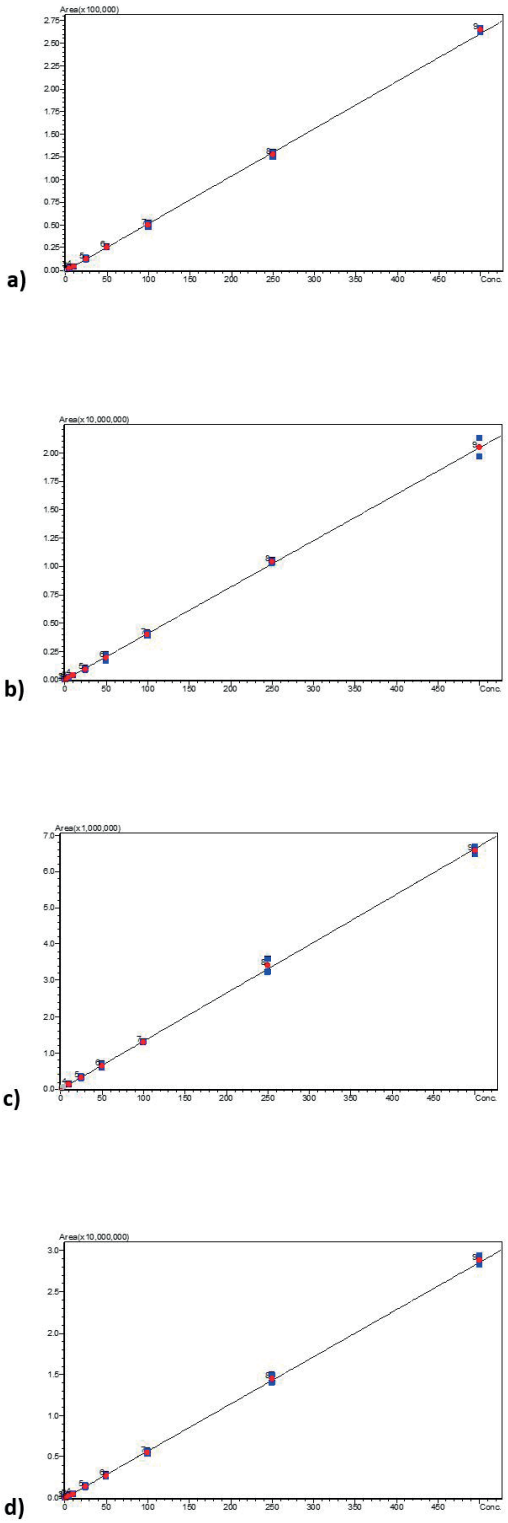

Figure 4. Calibration curves for Estradiol (a), α-trenbolone (b), 17-OH-Progesterone (c) and Androstenedione (d).

© Biotage 2019

Additional Information

- All data shown in this application note was generated using horsehair from the tail or mane provided by healthy horses.
- Ammonium fluoride increased sensitivity in both positive and negative ion modes.
- Steroids can exhibit non-specific binding to plastic collection plates. Different plastics exhibit different binding characteristics. Addition of 2 µL of ethylene glycol to the collection plate prior to evaporation can mitigate this issue. Note: No ethylene glycol was used in generation of the data shown in this application note, utilizing collection plate p/n 121-5203.
- Two Lysera methods were incorporated for this method. The grinding method was used to disrupt the analytes from the hair. The solvent extraction method was then used to ensure the analytes were sufficiently mixed into the solvent.

Ordering Information

Part Number	Description	Quantity
19-060	Biotage® Lysera	1
19-649	2 mL Reinforced Tubes with screw caps (Bulk pack)	1000
19-640	2.4 mm Metal Beads - 500 grams	1
820-0055-B	ISOLUTE® SLE+ 400 µL Sample Volume Columns	50
820-0400-P01	ISOLUTE® SLE+ 400 µL Capacity Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry 96 Sample Evaporator 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120 V	1
121-5203	Collection Plate, 2 mL Square	50
121-5204	Piercable Sealing Mat	50

EUROPE
Main Office: +46 18 565900
Toll Free: +800 18 565710
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
Order Fax: +1 434 296 8217
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
Outside US: +1 704 654 4900
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 68162810
Fax: +86 21 68162829
cn_order@biotage.com
cn-1-pointsupport@biotage.com

KOREA
Tel: + 82 31 706 8500
Fax:+ 82 31 706 8510
korea_info@biotage.com
kr-1-pointsupport@biotage.com

INDIA
Tel: +91 22 4005 3712
india@biotage.com

Distributors in other regions are listed on www.biotage.com

Part Number: AN921
© 2019 Biotage. All rights reserved. No material may be reproduced or published without the written permission of Biotage. Information in this document is subject to change without notice and does not represent any commitment from Biotage. E&OE. A list of all trademarks owned by Biotage AB is available at www.biotage.com/legal. Other product and company names mentioned herein may be trademarks or registered trademarks and/or service marks of their respective owners, and are used only for explanation and to the owners' benefit, without intent to infringe.

Biotage®