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

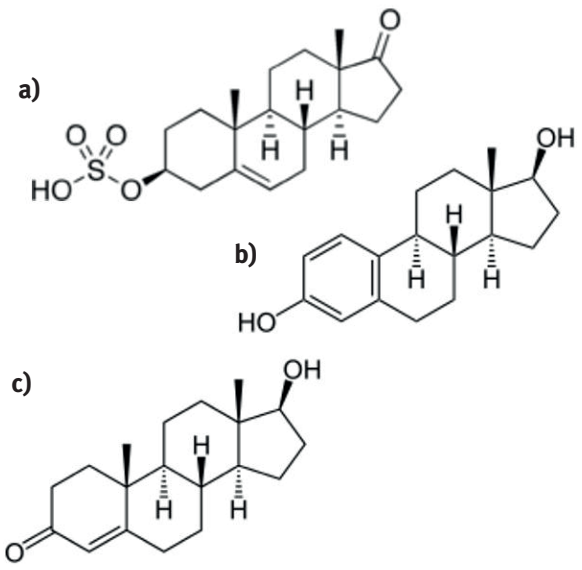

Figure 1. Structures of (a) DHEAS, (b) Estradiol and (c) Testosterone.

Introduction

This application note describes the extraction of a panel of 19 steroid hormones from human urine 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 90% with RSDs lower than 10% for all analytes. Linearity of greater than 0.999 is achieved for all analytes in the range 1-1000 pg/mL.

Manual sample preparation was performed using the Biotage® Pressure+ 96 Positive Pressure Manifold. The sample preparation method is automatable using the Biotage® Extrahera™. See Appendix for automation parameters and comparative data generated using the automated method.

ISOLUTE® SLE+ Supported Liquid Extraction products 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

Cortisol, 18-OH-Corticosterone, 21-Deoxycortisol, Cortisone, Estradiol, 17-OH-Pregnenolone, Aldosterone, 11-Deoxycortisol, Corticosterone, Estrone, Dehydroepiandrosterone (DHEA), 17-OH-Progesterone, Dehydroepiandrosterone sulfate (DHEAS), Testosterone, Dihydrotestosterone (DHT), Pregnenolone, Androstenedione, 11-deoxycorticosterone, Progesterone

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

Sample Pre-treatment

Add 10 µL of a 100 pg/µL methanolic ISTD solution to 1 mL of human urine and mix to give a final concentration of 1 ng/mL.

Sample loading

Apply up to 400 µL of pre-treated sample into each well of the ISOLUTE SLE+ plate. Using a Biotage® PRESSURE+96 Positive Pressure Manifold*, apply a pulse of pressure (2–5 psi) to load samples onto the sorbent. Wait 5 minutes for the sample to equilibrate on the sorbent.

*Conditions for automated processing using Biotage® Extrahera are shown in Appendix.

Analyte Extraction

Apply an aliquot of ethyl acetate (600 µL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of ethyl acetate (600 µL) and allow to flow under gravity for 5 minutes. Apply a pulse of positive pressure at 10 psi (10–20 seconds) to remove any remaining extraction solvent. Collect extracts in a 2 mL collection plate (p/n 121-5203).

Post Elution and Reconstitution

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

UHPLC Conditions

Instrument
Shimadzu Nexera X2 UHPLC

Column
ACE C18 (100 mm x 2.1 mm, 1.7 µm) (Advanced Chromatography Technologies Ltd, Aberdeen, UK) with EXP Guard column holder fitted with a C-18 cartridge (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
0	50	50
2	50	50
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 for target analytes in positive and negative mode.

Analytes	MRM Transition	Collision Energy	Ion Mode
DHEAS	367.1>97.05 (367.1>191.05)	33	-
Cortisol	363.4>121.25 (363.40>327.15)	-24	+
18-OH-Corticosterone	363.3>269.2 (363.30>121.10)	-16	+
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	-
17-OH-Pregnenolone	315.3>297.2 (315.30>251.00)	-13	+
11-Deoxycortisol	347.3>109.25 (347.30>283.15)	-27	+
Corticosterone	347.3>329.25 (347.30>283.15)	-16	+
Esterone	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	+
Pregnenolone	299.3>159.25 (299.30>281.20)	-20	+
17-OH-Progesterone	331.3>97.1	-22	+
Progesterone	315.2>97.2 (331.30>109.15)	-22	+

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Results

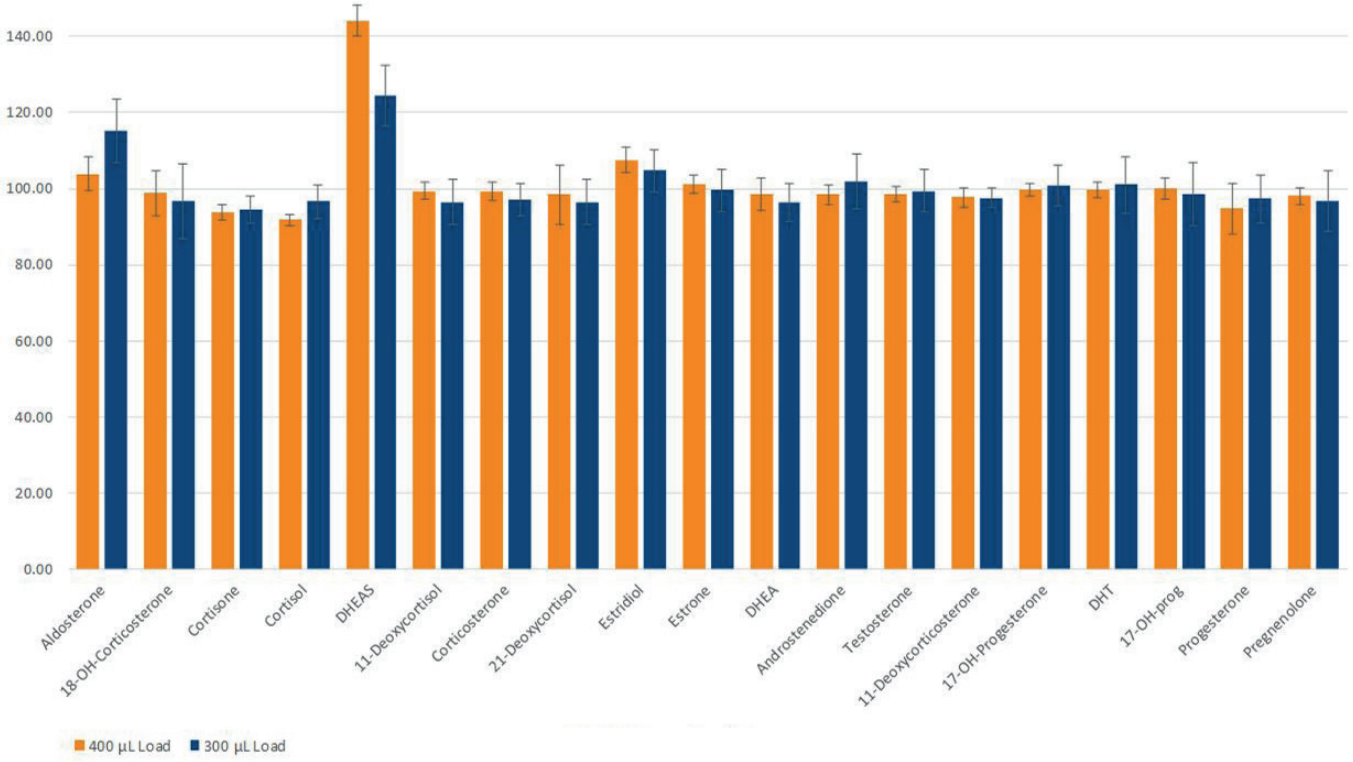

Figure 2. Typical analyte % extraction recoveries (n=7) loading 400 µL or 300 µL urine.

This simple sample preparation method delivers clean extracts and analyte recoveries mostly greater than 90% with RSDs lower than 10% for all analytes (see fig 2), and LLOQs below 10pg/mL for most of the steroids. Figure 2. below shows recoveries using 400 µL capacity ISOLUTE SLE+ plates loading either 400 µL or 300 µL sample volumes.

Figure 3. illustrates representative chromatography obtained from stripped urine spiked at 5 ng/mL. 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.

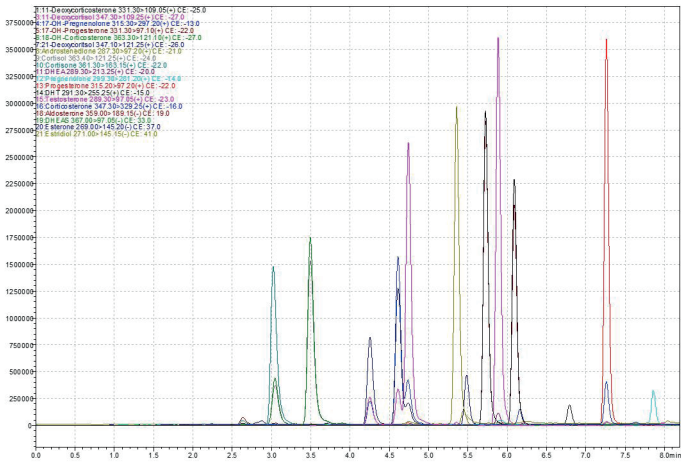

Figure 3. Representative chromatography for analytes spiked at a concentration of 5 ng/mL in stripped urine.

Calibration curve performance was investigated from stripped urine spiked in the range 1–1000 pg/mL. 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 400 and 300 µL of urine. Selected calibration curves loading 400 µL are demonstrated in Figure 4.

Table 3. Analyte calibration curve r^2 and LOQ performance (manual processing).

Analytes	r^2 400 µL Load	LLOQ (pg/mL) 400 µL Load	r^2 300 µL Load	LLOQ (pg/mL) 300 µL Load
DHEAS	0.9991	50	0.9991	50
Cortisol	0.9994	< 5	0.9993	< 5
18-OH-Corticosterone	0.9991	< 5	0.9992	< 5
Cortisone	0.9993	< 10	0.9991	< 10
21-Deoxycortisol	0.9998	5	0.9997	5
Estradiol	0.9995	10	0.9996	10
Aldosterone	0.999	100	0.9993	100
17-OH-Pregnenolone	0.9993	< 250	0.999	100
11-Deoxycortisol	0.9993	1	0.9995	< 1
Corticosterone	0.9998	< 1	0.9997	< 1
Estrone	0.9993	< 5	0.9991	1
11-Deoxycorticosterone	0.999	< 5	0.9994	< 5
DHEA	0.9994	50	0.9996	50
Testosterone	0.9994	5	0.9993	< 5
DHT	0.9991	< 10	0.9995	< 5
Androstenedione	0.9993	5	0.999	5
Pregnenolone	0.9993	< 100	0.999	< 100
17-OH-Progesterone	0.9993	5	0.9993	< 10
Progesterone	0.9991	< 50	0.9992	< 50

Chemicals and Reagents

- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS) and ethyl acetate 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 purified water.
- Internal standards (100 pg/µL) were prepared from a 10 ng/µL stock solution by adding 10 µL of each to 950 µL of MeOH. 10 µL of this solution was then added to each calibration sample.
- Reconstitution solvent was made by mixing 50 mL of mobile A and 50 mL of mobile phase B

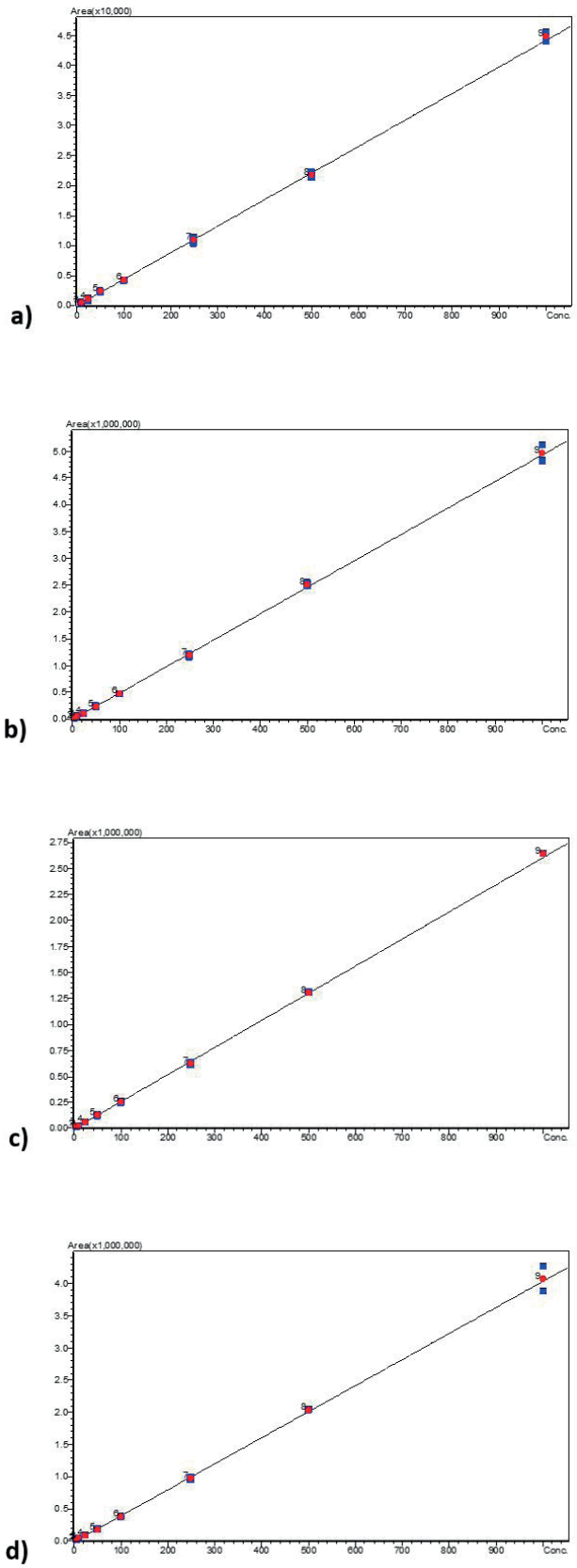

Figure 4. Calibration curves for Estradiol (a), Testosterone (b), 17-OH-Progesterone (c) and Androstenedione (d).

Additional Information

- All data shown in this application note was generated using fresh urine provided by healthy human volunteers.
- Ammonium fluoride increased sensitivity in both positive and negative ion modes.
- Other strategies for increasing sensitivity:
  - Decrease reconstitution solvent volume below 200 µL
  - Increase injection volumes above 10 µL.
- 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.

Ordering Information

Part Number	Description	Quantity
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	Pierceable Sealing Mat	50

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Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated using Biotage® Extrahera™ and ISOLUTE® SLE+ plates. This appendix contains the software settings required to configure Extrahera to run this method.

Comparable results were obtained using both manual and automated processing methods.

Sample Name:	Urinary Steroids - SLE+
Sample Plate/Rack:	Plate
Extraction Media:	400 µL ISOLUTE® SLE+

Settings

"Sample" Tab

Sample Type:	Aqueous Sample
Starting Sample Volume (µL):	410
Method Comment:	

Pre-treatment	
No. of steps	1
Pause after last step	No
Dispose tips after last step	No

Solvent
1
2
3
4

	1	2	3	4
Volume (µL)				
Wait Time (min)				

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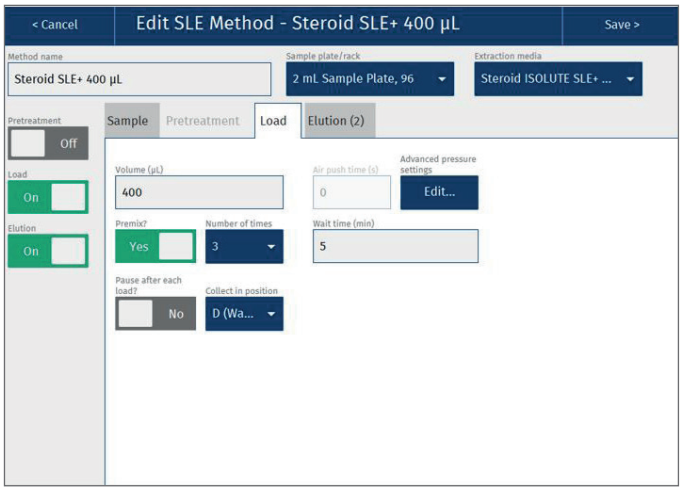
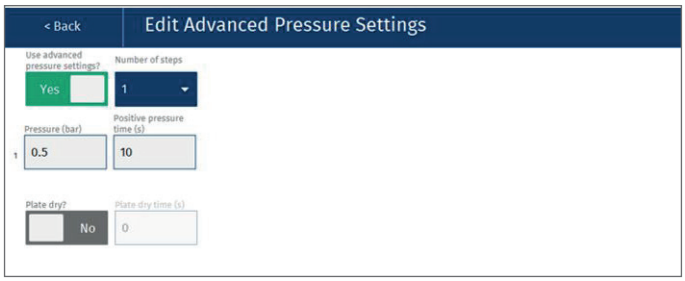
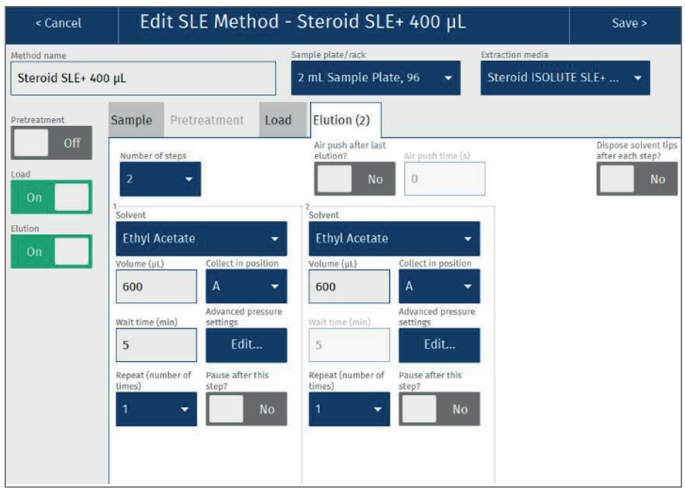


Load	
Pressure	0
Pause after each load	No
Volume	400
Collect in position	D
Positive pressure time	0
Premix	Yes
Number of times	3
Wait time (min)	5



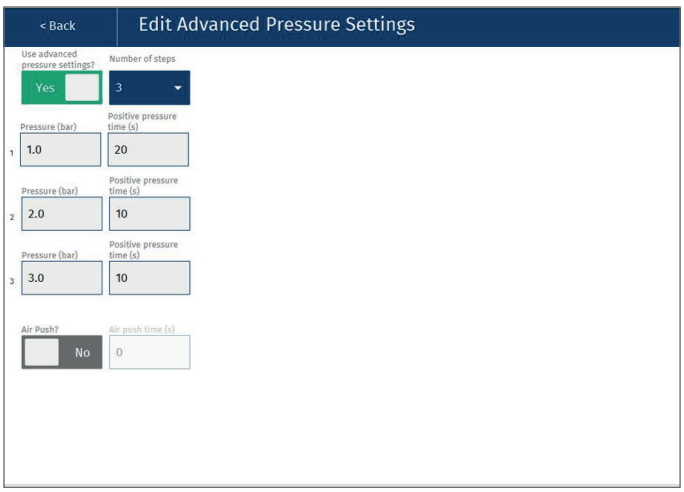
Advanced Settings
Advanced Pressure: 1 Step; 0.5 Bar for 10 seconds



Elution	
No. of steps	2
Pressure	
Plate Dry	No
Dry time	
Wait time (min)	5

Solvent	
1	Ethyl Acetate
2	Ethyl Acetate
3	
4	

	1	2	3	4
Volume	600	600		
Position	A	A		
Pressure time	0	Advanced		
Repeat	1	1		
Pause	No	No		

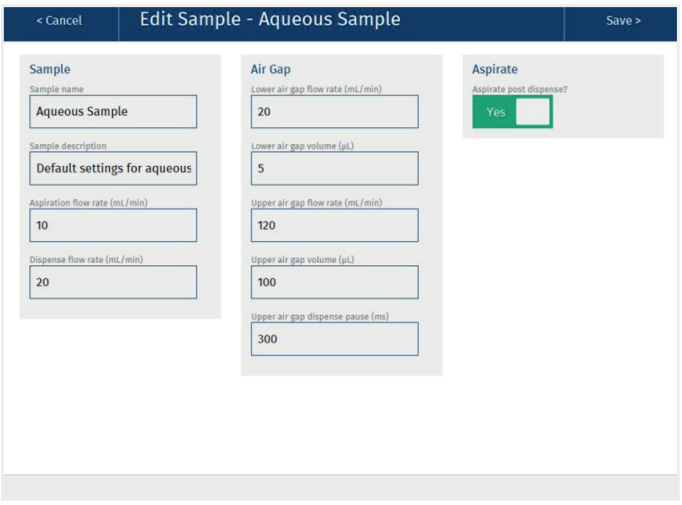


Advanced settings
Advanced Pressure: 3 Steps; 1.0 Bar for 20 seconds; 2.0 bar for 10 seconds; 3.0 bar for 10 seconds

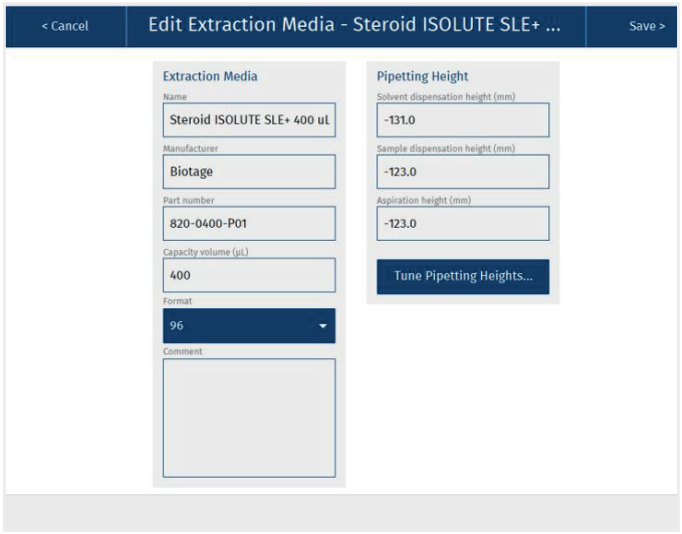
Solvent Properties

	Solvent Description
1	Ethyl Acetate

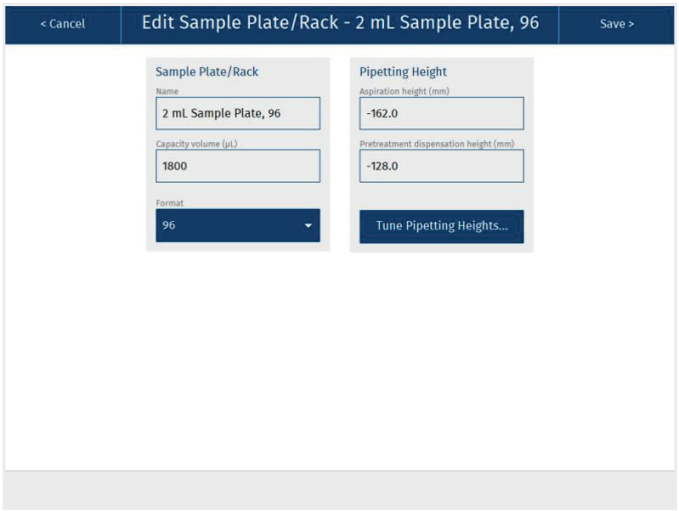
Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type		**Refillable**						**Non Refillable**		
Capacity	10									
Aspiration flow rate (mL/min)	20									
Dispense flow rate (mL/min)	20									
Lower air gap flow rate (mL/min)	5									
Lower air gap volume (µL)	120									
Upper air gap flow rate (mL/min)	100									
Upper air gap volume (µL)	300									
Upper air gap dispense pause	Yes									
Conditioning?	2									
Conditioning number of times	10									
Conditioning flow rate (mL/min)	No									
Chlorinated	No									
Serial dispense										



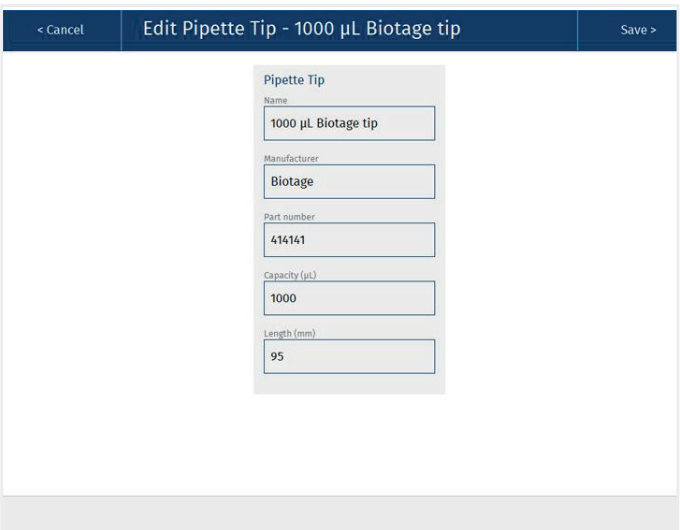
"Sample" Screen	
Sample name	Aqueous sample
Sample description	Default settings for Aqueous
Aspiration flow rate	10
Dispense flow rate	20
Lower air gap flow rate	20
Lower air gap volume	5
Upper air gap flow rate	120
Upper air gap volume	100
Upper air gap dispense pause	300



"Extraction Media" Screen	
Name	ISOLUTE® SLE+ 400 uL Plate
Manufacturer	Biotage
Part number	820-0400-P01
Capacity volume	400
Format	96
Comment	
Solvent dispensation height	-131
Sample dispensation height	-123
Aspiration height	-123



"Sample Plate/Rack" Screen	
Name	2 mL Sample plate, 96
Capacity volume	1800
Format	96
Aspiration height	-162
Pretreatment dispensation height	-128



"Pipette tip" Screen	
Name	1000 µL Biotage Tip
Manufacturer	Biotage
Part number	414141
Capacity (µL)	1000
Length (mm)	95

Results

Using the method parameters described in this appendix, analyte recoveries, %RSDs, linearities and LOQs were comparable for both manually processed and automated methods.

A comparison of analyte recoveries with RSDs obtained through manual and automated processing are shown in figure 5 (400 µL sample load) and 7 (300 µL sample load).

Table 4 shows linearity and LOQ data for the automated processing method.

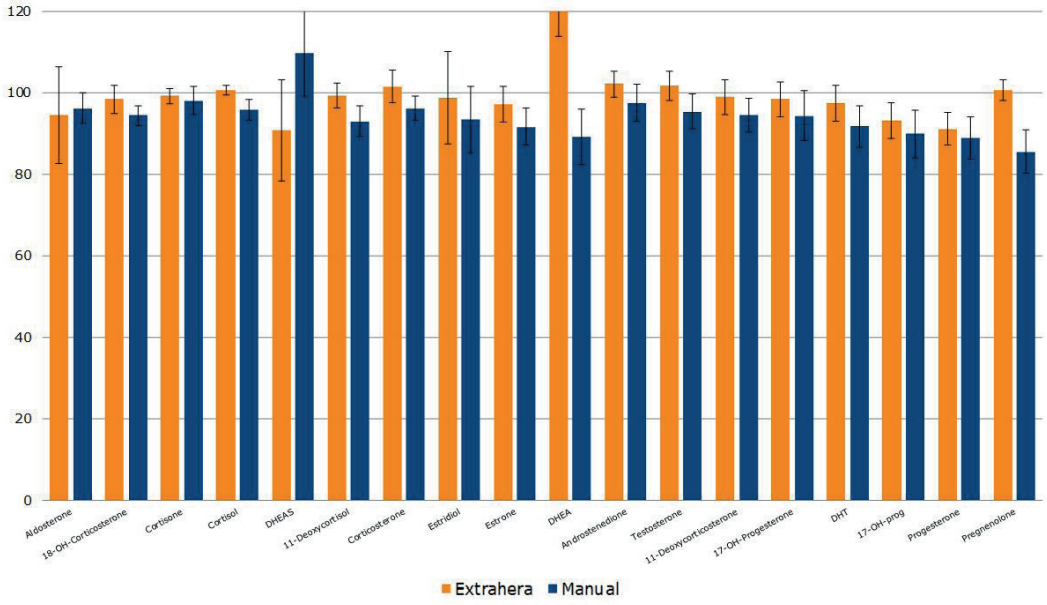


Figure 5. Comparison of steroid recovery and RSD for 400 µL sample load.

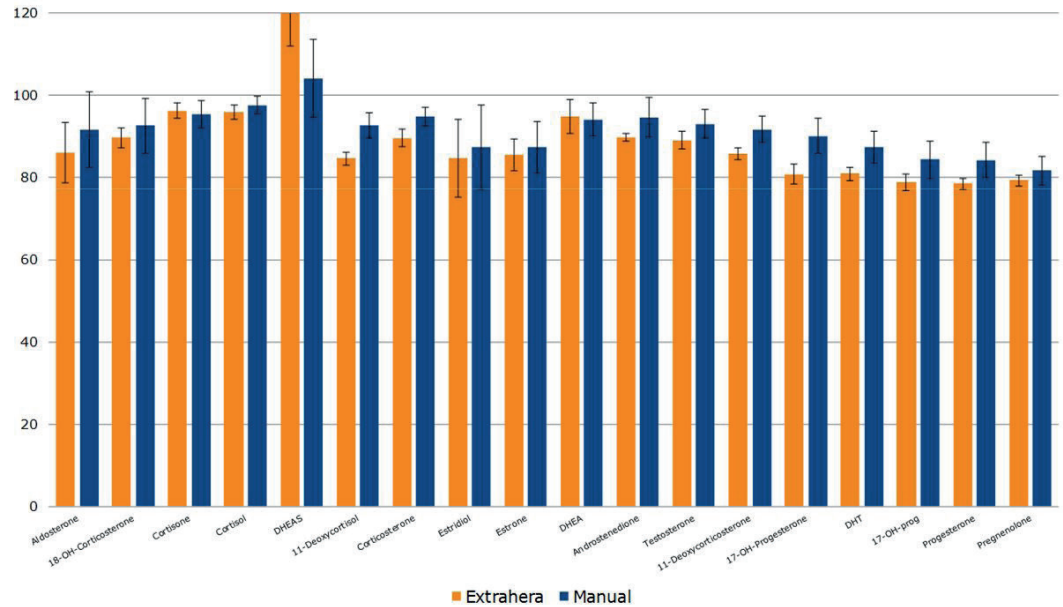


Figure 6. Comparison of steroid recovery and RSD for 300 µL sample load.

Table 4. Analyte calibration curve r^2 and LOQ performance (automated processing).

Analytes	r^2 400 µL Load	LLOQ (pg/mL) 400 µL Load	r^2 300 µL Load	LLOQ (pg/mL) 300 µL Load
DHEAS	0.9991	100	0.9991	<100
Cortisol	0.9992	<5	0.9992	5
18-OH-Corticosterone	0.9995	<10	0.9990	<25
Cortisone	0.9995	<10	0.9995	<10
21-Deoxycortisol	0.9998	<5	0.9997	<5
Estradiol	0.9990	10	0.9990	25
Aldosterone	0.9994	100	0.9994	100
17-OH-Pregnenolone	0.9991	<250	0.999	100
11-Deoxycortisol	0.9994	<5	0.9991	<5
Corticosterone	0.9993	<5	0.9993	10
Estrone	0.9996	<5	0.9991	1
11-Deoxycorticosterone	0.9992	<5	0.9994	10
DHEA	0.9996	100	0.9996	50
Testosterone	0.9992	<10	0.9993	10
DHT	0.9995	<25	0.9995	25
Androstenedione	0.9995	<10	0.9990	10
Pregnenolone	0.9996	100	0.999	100
17-OH-Progesterone	0.9991	5	0.9993	<10
Progesterone	0.9990	25	0.9992	<50

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