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Extraction of a Comprehensive Steroid Panel from Human Urine Using EVOLUTE® EXPRESS ABN Prior to LC/MS-MS Analysis

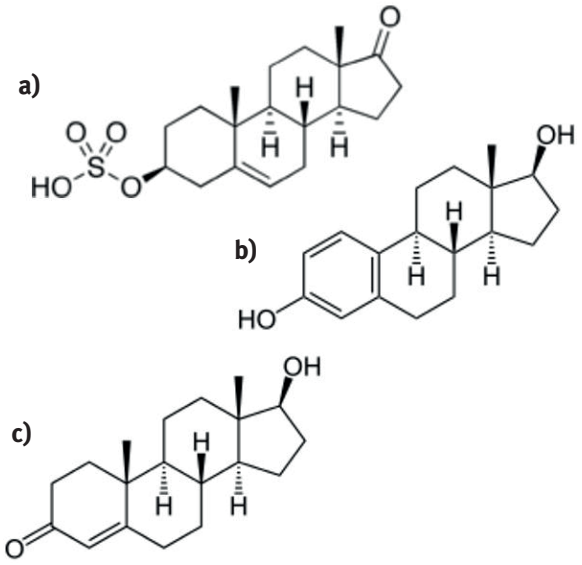

Figure 1. Structures of (a) DHEAS, (b) Estradiol and (c) Testosterone.

Introduction

This application note describes the extraction of a panel of 19 steroid hormones from human urine using EVOLUTE® EXPRESS ABN solid phase extraction plates prior to LC-MS/MS analysis. The simple sample preparation procedure delivers clean extracts and analyte recoveries greater than 75% with RSDs lower than 5% for most 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.

EVOLUTE EXPRESS products dramatically improve flow characteristics and enhance sample preparation productivity.

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_3

Sample Preparation Procedure

Format

EVOLUTE® EXPRESS ABN 10 mg plate, (p/n) 600-0010-PX01

Sample Pre-treatment

Add 20 µL of a 10 pg/µL methanolic ISTD solution to 200 µL of human urine and mix to give a final concentration of 1 ng/mL. Dilute sample 1:1 (v/v) with water and mix.

Condition (optional)

Condition wells with methanol (500 µL)

Equilibration (optional)

Equilibrate wells with 0.1% Formic Acid (aq) (500 µL)

Sample loading

Load 400 µL of pre-treated urine into each well

Wash 1

Elute interferences with H_2O (500 µL)

Wash 2

Elute interferences with H_2O:MeOH (60:40, v/v, 500 µL)

Elution

Elute analytes with methanol (200 µL)

Post Elution and Reconstitution

Dry the extract in a stream of air or nitrogen using a Biotage® SPE Dry at 40 °C, at a flow rate of 20 to 40 L/min, for approximately 20 minutes.

Reconstitute evaporated samples with mobile phase A: mobile phase B (50:50, v/v, 200 µL) and vortex mix. Cover plate with a sealing mat prior to injection.

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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

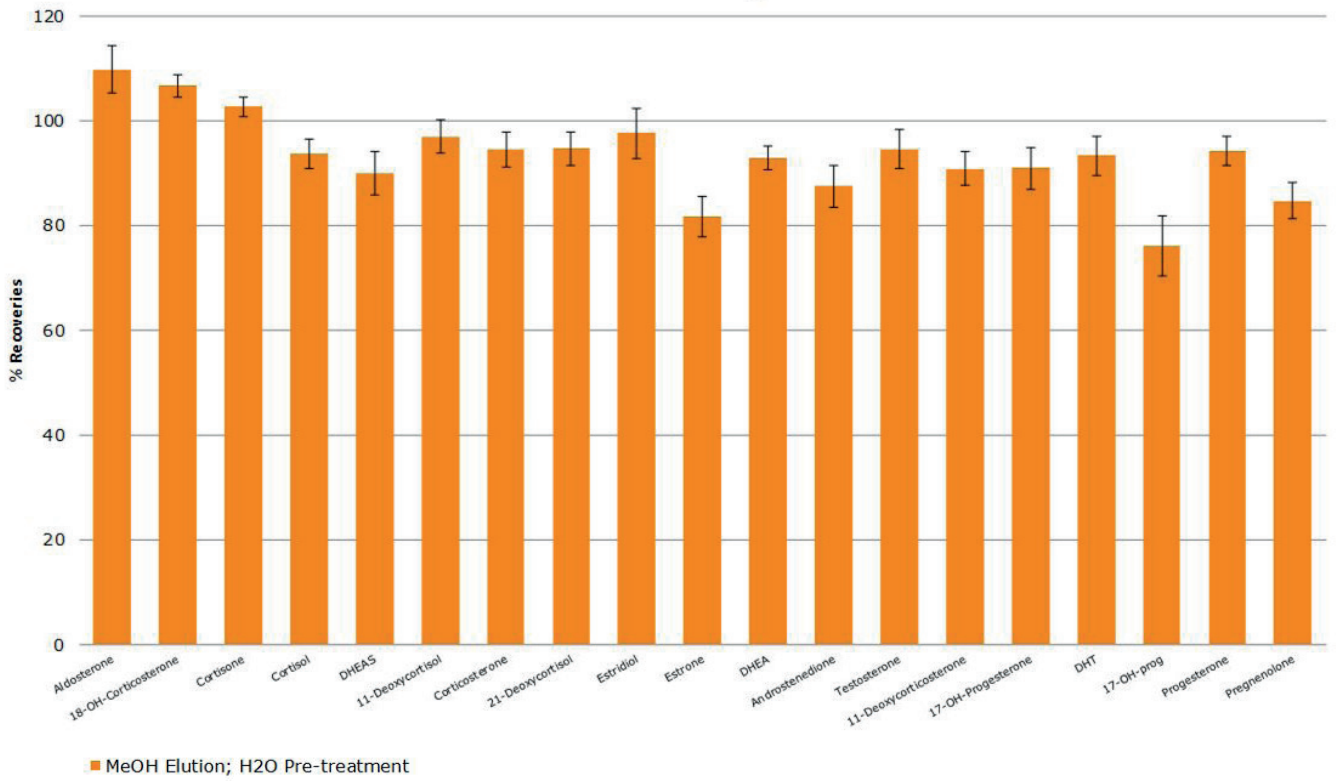

Figure 2. Typical analyte % extraction recoveries (n=7) using methanol as the elution solvent.

This optimized SPE protocol delivers typical analyte recoveries above 75%, with corresponding RSDs below 5%, with a few exceptions. Inclusion of the polar DHEAS metabolite requires methanol as the elution solvent. If DHEAS is not required, alternative elution solvents such as ethyl acetate may be used. Recovery data for methanol elution is shown below in Figure 2.

Figure 3. demonstrates 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.

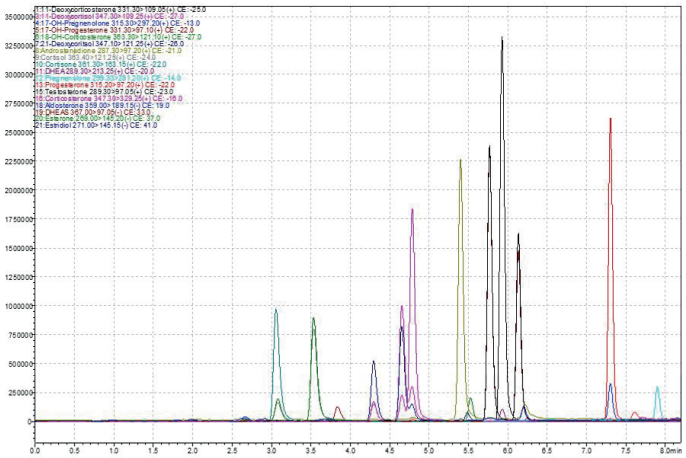

Figure 3. Representative chromatography for stripped urine spiked at 5 ng/mL.

Calibration curve performance was investigated from female 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. Selected calibration curves are demonstrated in Figure 4.

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

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

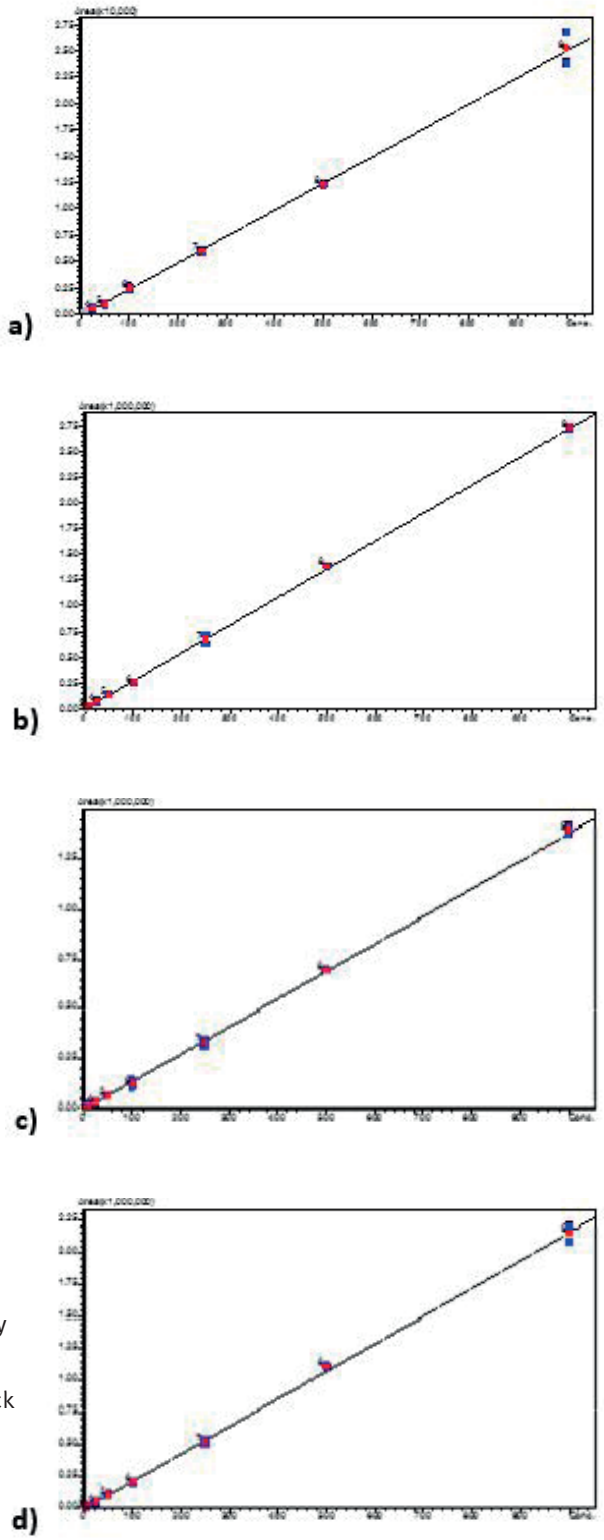

Figure 4. Calibration curves for Estradiol (a), Testosterone (b), 17-OH-Progesterone (c) and Androstenedione (d).

Chemicals and Reagents

- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS) and formic acid (98%) were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards and deuterated internal standards 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 with purified water.
- Internal standards (100 pg/µL) were prepared from a 10 ng/µL stock solution by adding 10 µL of each of to 950 µL of MeOH. 10 µL of this solution was then added to each calibration sample.
- Equilibration solvent (0.1% formic acid) was made by adding 100 µL of formic acid to 99. 9mL of water (18.2 MΩ-cm)
- Wash 2 solvent (H_2O:MeOH, 60:40, v/v) was made up by adding 60 mL of water to 40 mL of methanol and mixing.
- Reconstitution solvent was made by mixing 50 mL of mobile A and 50 mL of mobile phase B.

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:
  - Increase matrix volumes above 200 µL
  - 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
600-0010-PX01	EVOLUTE® EXPRESS 10 mg ABN 96 fixed well 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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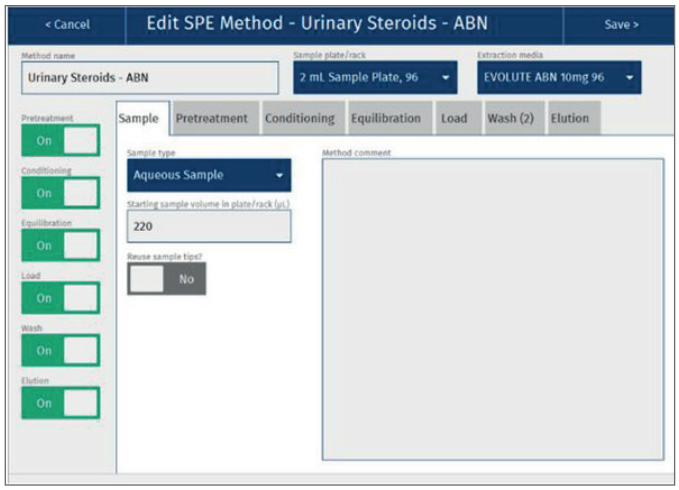
Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated using Biotage® Extrahera™ and EVOLUTE® EXPRESS ABN 10 mg 96-well 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.

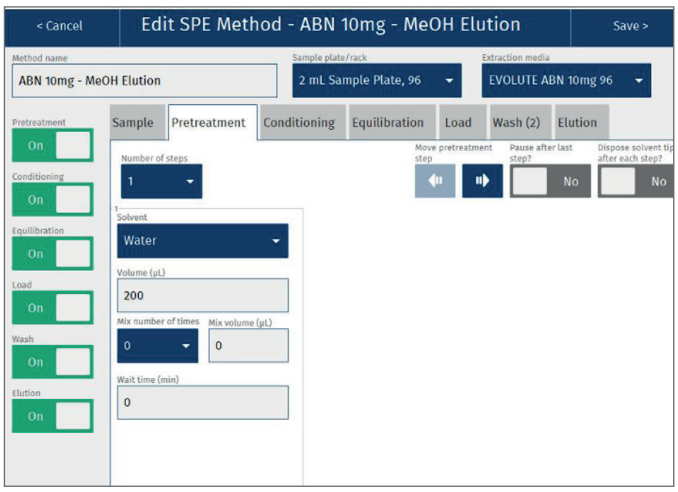
Sample Name:	Urinary Steroids - ABN
Sample Plate/Rack:	2 mL Sample Plate, 96
Extraction Media:	EVOLUTE® EXPRESS ABN 10 mg 96



Settings

"Sample" Tab

Sample Type:	Aqueous Sample
Starting Sample Volume (µL):	220
Method Comment:	



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

	Solvent
1	Water
2	
3	
4	

	1	2	3	4
Volume (µL)	200			
Wait Time (min)				

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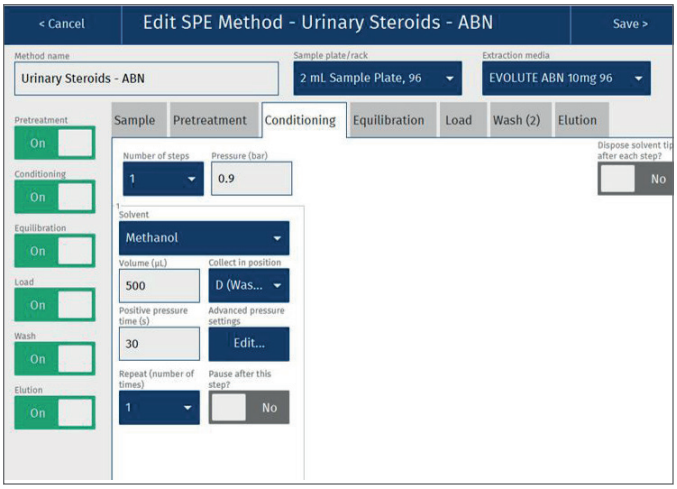
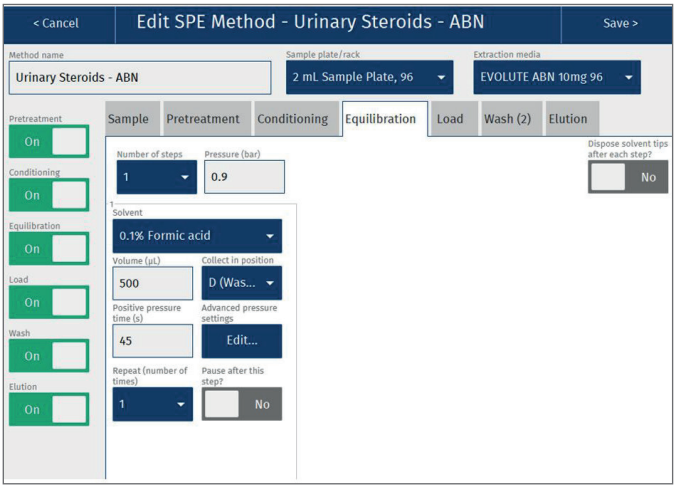


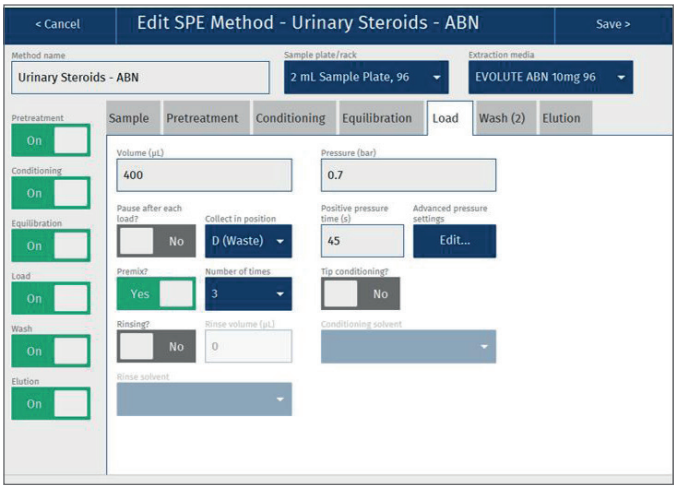
Conditioning	
Pressure	0.9
Pause after each load	No
Volume	500
Collect in position	D
Positive pressure time	30
Advanced Pressure	No
Number of times	1

	Solvent
1	Methanol
2	
3	
4	

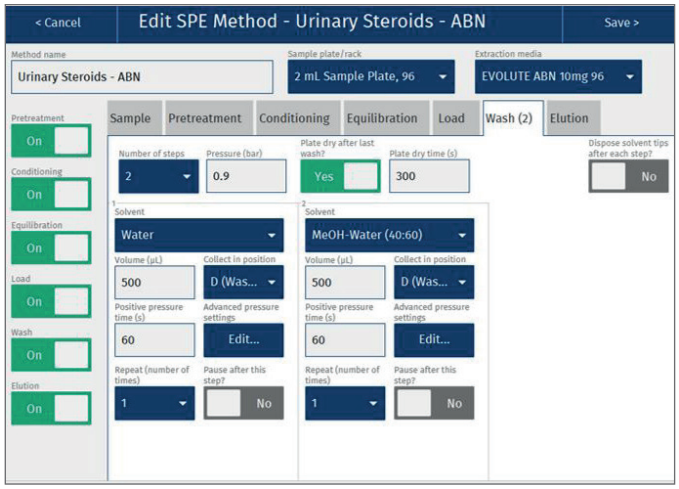


Equilibration	
Pressure	0.9
Volume	500
Collect in position	D
Positive pressure time	45
Advanced Pressure	No
Number of times	1
Number of times	1

	Solvent
1	0.1% Formic Acid



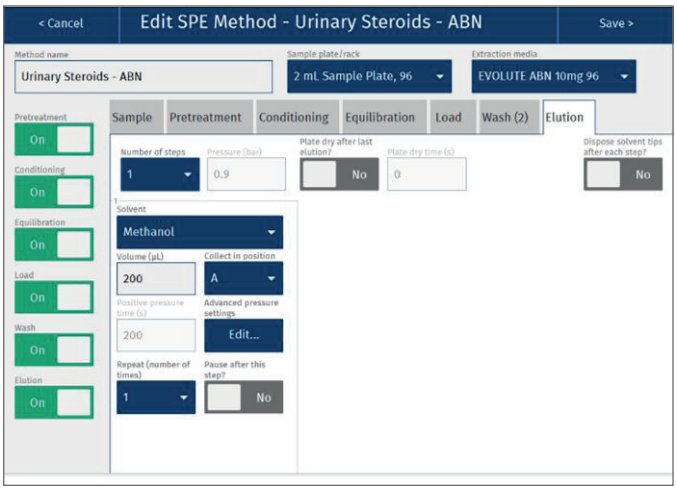
Load	
Pressure	0.7
Pause after each load	No
Volume	400
Collect in position	D
Positive pressure time	45
Premix	Yes
Number of times	3



Wash 1	
Pressure	0.9
Volume	500
Collect in position	D
Positive pressure time	60
Advanced Pressure	No
Number of times	1

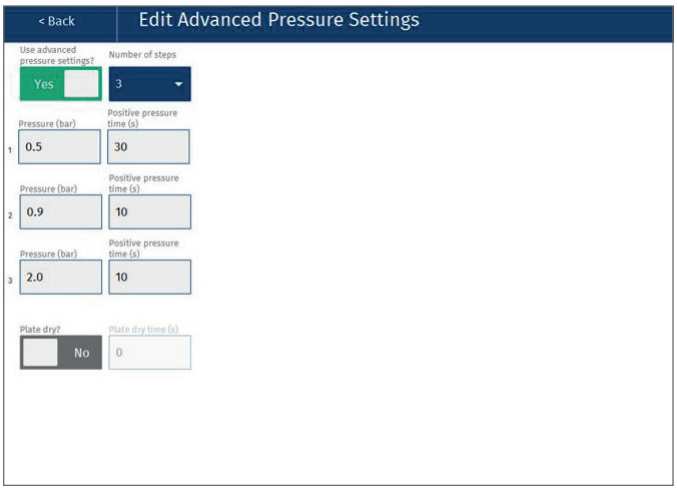
Wash 2	
Pressure	0.9
Volume	500
Collect in position	D
Positive pressure time	60
Advanced Pressure	No
Number of times	1
Plate Dry	YES

	Solvent
1	Water
2	60:40 Water:MeOH



Elution	
No. of steps	1
Pressure	
Plate Dry	No
Dry time	
Wait time (min)	

	Solvent
1	Methanol



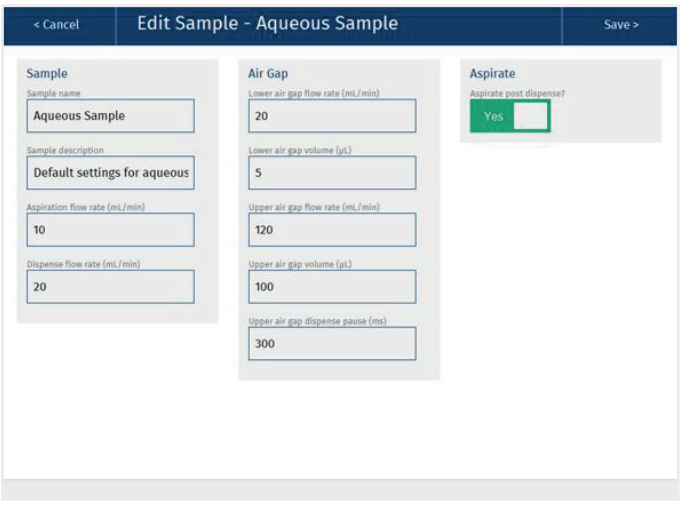
	1
Volume	200
Position	A
Pressure time	0
Repeat	1
Pause	No

Advance Settings
Advanced Pressure:
2 Steps; 0.5 bar for 30 seconds; 0.9 Bar for 10 seconds; 2.0 bar for 10 seconds

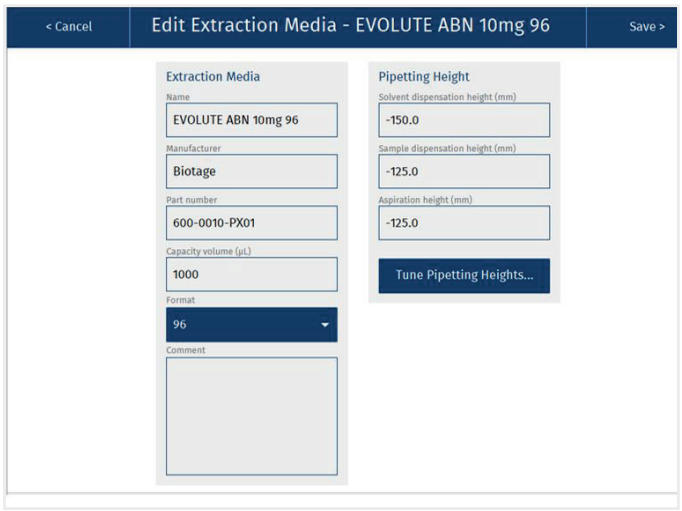
Solvent Properties

	Solvent Description
1	Methanol
2	Water
3	0.1% Formic Acid
4	Water
5	60:40 Water:Methanol

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

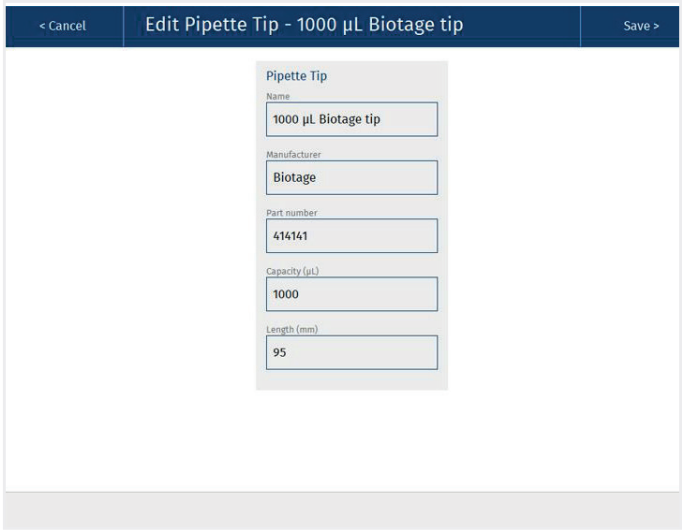


"Sample" Screen	
Sample name	Aqueous sample
Sample description	Default settings for Aqueous
Aspiration flow rate	10
Dispense flow rate	20
Lower air gap flow rate	20
Lower air gap volume	5
Upper air gap flow rate	120
Upper air gap volume	100
Upper air gap dispense pause	300



"Extraction Media" Screen	
Name	EVOLUTE® EXPRESS ABN 10 mg 96 well plate
Manufacturer	Biotage
Part number	600-0010-PX01
Capacity volume	1000
Format	96
Comment	
Solvent dispensation height	-150
Sample dispensation height	-125
Aspiration height	-125

"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 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 obtained through manual and automated processing is shown in figure 5.

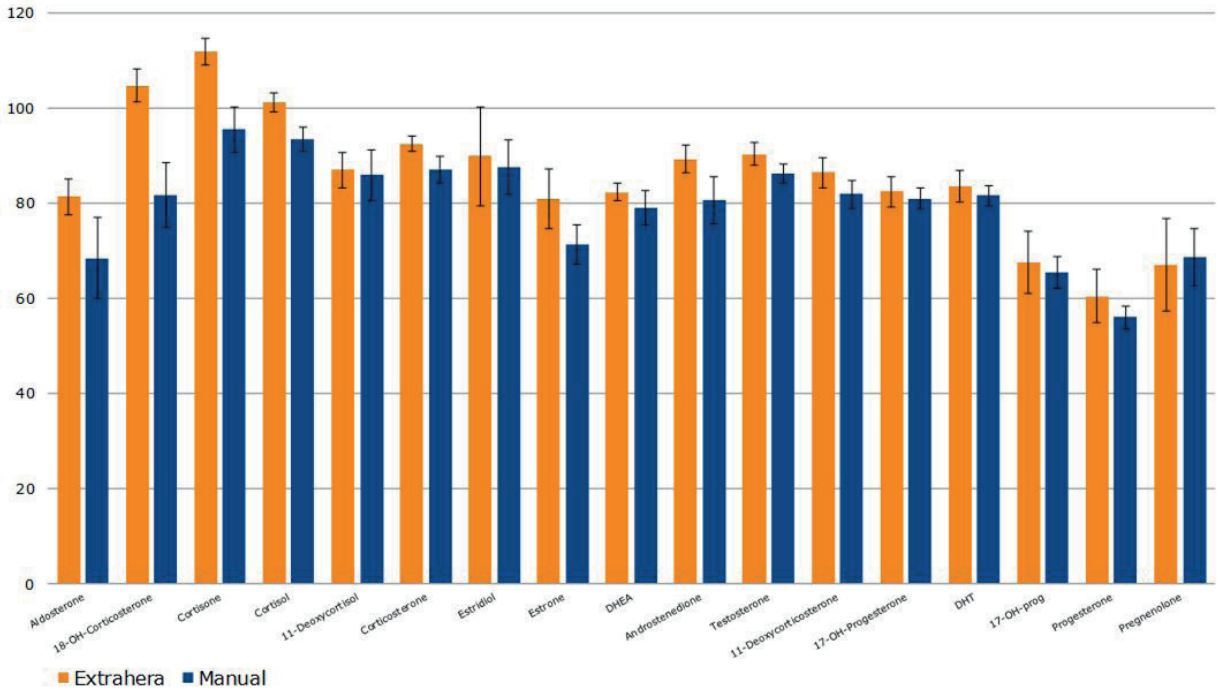


Figure 5. Comparison of steroid recovery and RSD (manual vs automated processing)

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