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Automated Extraction of 11-Nor-9-Carboxy-THC from Human Urine with ISOLUTE® HAX SPE Columns Using Biotage® Extrahera™ HV-5000, Prior to GC-MS Analysis

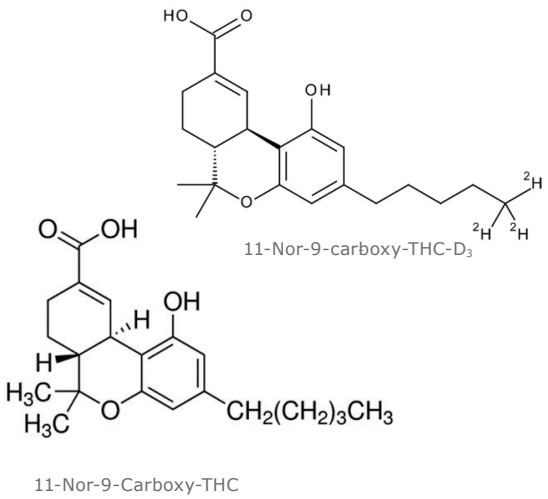

Figure 1. Structures of analyte and internal standard.

Introduction

This application note describes the extraction of 11-Nor-9-carboxy-THC from human urine using ISOLUTE® HAX SPE columns and Biotage® Extrahera™ HV-5000 prior to GC/MS analysis.

The simple sample preparation procedure, based on a mixed-mode/strong anion exchange extraction mechanism, delivers clean extracts and analyte recoveries greater than 90% with RSDs lower than 10%. Linearity of greater than 0.999 is achieved with a urinary concentration range of 1–100 ng/mL.

This application note includes optimized conditions for automated processing of the HAX columns (using Biotage® Extrahera™ HV-5000) and manual processing (using the Biotage® PRESSURE+ 48 positive pressure manifold). Data generated using both processing systems is shown.

Analytes

11-Nor-9-carboxy-Δ-9-tetrahydrocannabinol

Internal Standards

11-Nor-9-carboxy-Δ-9-tetrahydrocannabinol-D_3

Sample Preparation Procedure

Format:

ISOLUTE® HAX 200 mg/3 mL column, p/n 903-0020-B

Processing Conditions

Samples were processed manually using a Biotage® PRESSURE+ 48 positive pressure manifold. Each step below was processed at 2 to 4 psi using the adjustable flow setting. Drying steps were processed at 40 psi using the maximum flow setting.

Automated sample processing was performed using the Biotage® Extrahera™ HV-5000 system. Detailed processing conditions are included in the appendix.

Sample Pre-treatment:

Spike urine sample, QC or calibrator (2 mL) with internal standard solution, add 100 µL 10M NaOH and mix thoroughly in a suitable vial. Seal. Hydrolyze at 60 °C for 25 minutes, allow to cool and adjust to pH3 by adding 1 mL glacial acetic acid.

Transfer into 16 x 100 mm test tubes for extraction.

Note: Internal standard solution consists of a 10 ng/µL methanolic solution. 3 µL of this was added to 2 mL of urine to give a 15 ng/mL spike concentration.

Conditioning:

Condition each column with methanol (3 mL).

Equilibration:

Equilibrate each column with deionised (DI) water (3 mL) followed by sodium acetate buffer pH3 (aq) (1 mL).

Sample Loading:

Load the full volume of the pre-treated (hydrolyzed) urine sample to each column.

Wash 1:

Elute interferences with DI water (2 mL).

Wash 2:

Elute interferences with 100 mM HCl (aq):ACN (70:30, v/v, 2 mL).

Dry:

Dry columns for 10 mins under positive pressure (40 psi)

Wash 3:

Elute interferences with hexane (100 µL)

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Elution:
Elute analytes with hexane:ethyl acetate (50:50, v/v,) with 2% formic acid (3 mL). Elute into a 12 x 75mm test tube.

As THC-COOH contains an acid group, derivatisation of the extracts is required in order to obtain a stabilized analyte that can be analysed via GC/MS.

Post Elution & Reconstitution:
Transfer elution solvent tubes to the evaporation system. Dry the extract in a stream of air using a TurboVap® LV at 40 °C, 1.0 L/min, for approximately 14 minutes.

Reconstitute evaporated samples with ethyl acetate (20 µL) and BSTFA:TMCS (99:1, 20 µL). Vortex mix, transfer to high recovery GC/MS vial and derivatise on a heat block at 70° C for 25 minutes. Transfer to GC-MS for analysis.

Note: This extraction method can also be used for THC, Δ8-THC and THC-OH if required.

GC Conditions

Instrument
Agilent 7890A GC with purged Ultimate Union

Column
Agilent J&W DB-5ms 30 m, 0.25 mm, 0.25 µm

Mobile Phase
GC/MS grade helium

Inlet Temperature
260 °C Splitless

Flow Rate
1.5 mL/min

Injection Volume
1.5 µL

Table 1. GC Oven Gradient.

Ramp rate (°C/min)	Temp (°C)	Hold Time
	60	0
25	325	2

MS Conditions

Instrument:
Agilent 5975C

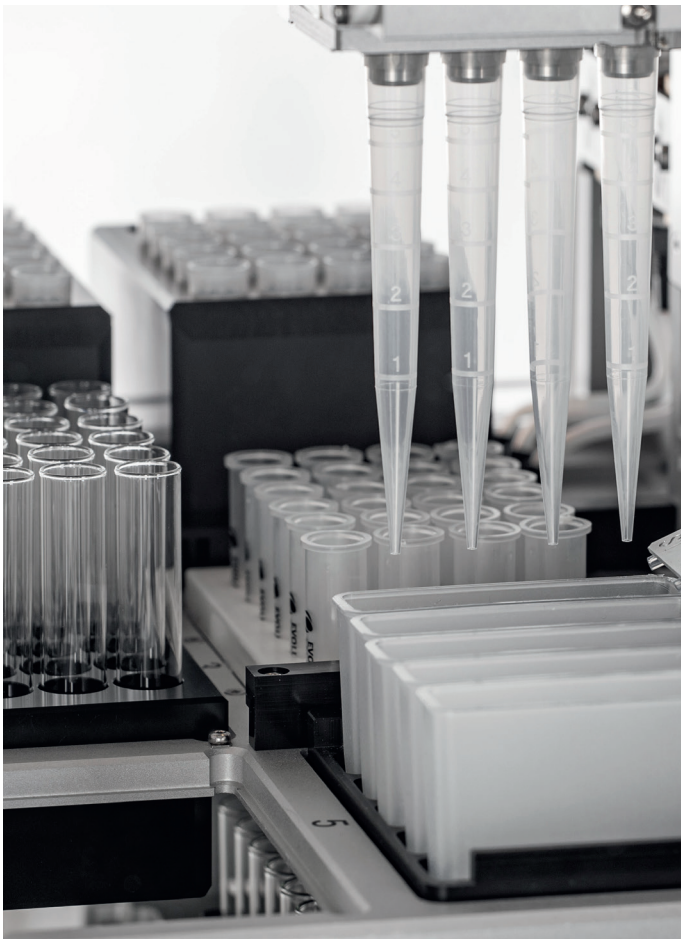
Transfer Line Temp:
280 °C

Source Temp:
230 °C

Quad Temp:
150 °C

Table 2. MS conditions for target analytes in positive mode.

Analytes	Quant ions	Qual ions
THC-COOH	371	488, 473
THC-COOH-D_3	374	476, 491



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Results

Recovery and Reproducibility

High (› 90%) and reproducible (RSD ‹ 10%) recoveries were achieved for THC-COOH using the method described in this application note using the ISOLUTE® HAX column format processed using the Biotage® Extrahera™ HV-5000 or Biotage® PRESSURE+ 48.

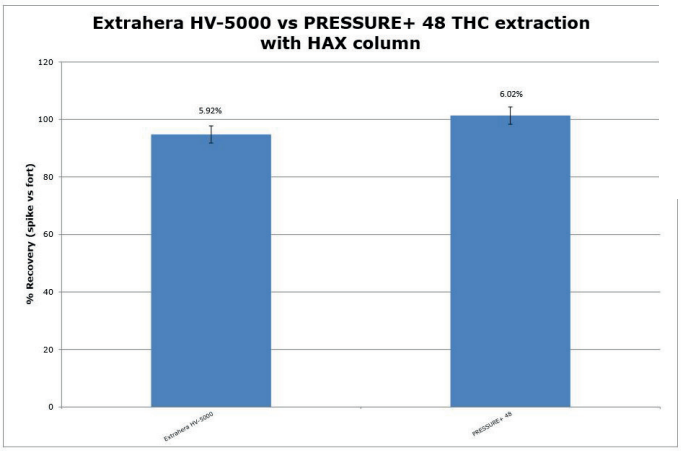

Figure 2. shows analyte % recoveries with RSDs (n=7) using the optimized ISOLUTE® HAX protocol described in this application note.

Linearity

Calibration curve performance was investigated from urine spiked between 1–100 ng/mL. Good linearity was observed for THC-COOH delivering r² values greater than 0.999. Table 3. details linearity performance for each method of extraction.

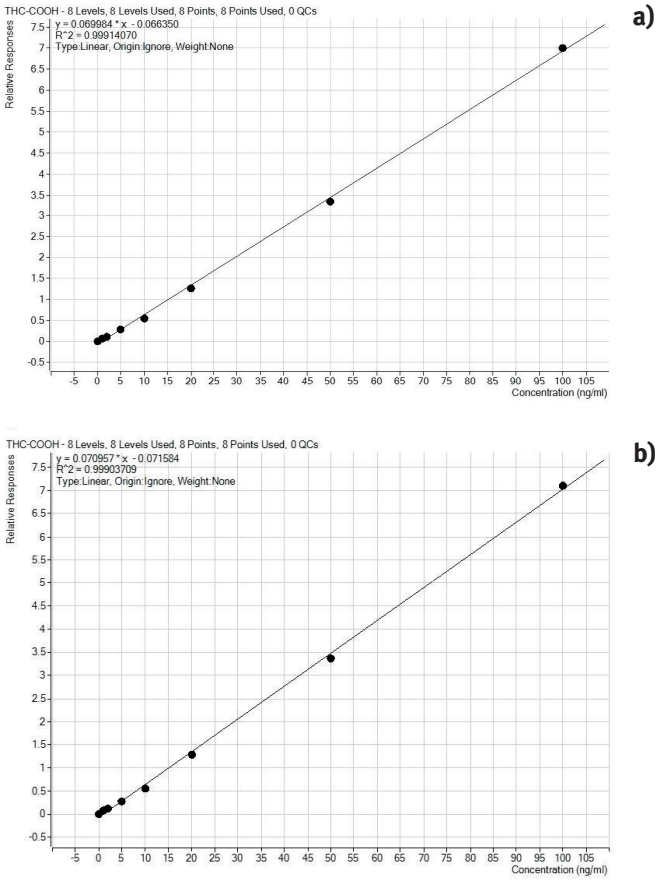

Figure 3. Calibration curves for THC-COOH extraction on the Biotage® Extrahera™ HV-5000 (a) and Biotage® PRESSURE+ 48 (b) using the ISOLUTE® HAX columns to extract hydrolyzed human urine.

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

Analyte	r^2
THC-COOH Automated	0.999
THC-COOH Manual	0.999

Calibrator Preparation

Calibration standards were prepared by spiking human urine with a 10 ng/µL THC-COOH stock solution to create a 100 ng/mL standard. This was then serially diluted in order to obtain further standards at concentrations of 50, 20, 10, 5, 2, 1 ng/mL.

Internal standard was added to 2 mL of each prepared urine standard and the extraction was performed as decribed.

Sample Preparation Time

For a batch size of 24 samples, processing times were:

Processing System	Processing Time
Biotage® PRESSURE+ 48	56 minutes
Biotage® Extrahera™ HV-5000	66 minutes

Conclusion

ISOLUTE® HAX solid phase extraction columns provided robust extraction of THC-COOH from hydrolyzed urine samples. Good, reproducible recoveries were achieved, with an overall processing time of 56 and 66 minutes using the Biotage® PRESSURE+ 48 or the Biotage® Extrahera™ HV-5000 respectively (excluding the common evaporation and derivatisation steps).

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Chemicals and Reagents

- Methanol (LC-MS grade), hexane, ethyl acetate and acetonitrile were purchased from Rathburn Chemicals Ltd (Walkerburn, UK).
- All analyte standards, deuterated internal standards, sodium hydroxide, hydrochloric acid, formic acid and acetic acid were purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- DI Water used was 18.2 MOhm-cm, drawn daily from a Direct-Q5 water purifier.
- Internal standard (10 ng/mL) was prepared from a 1 mg/mL stock solution by adding 10 µL to 990 µL of MeOH. 3 µL of this solution was then added to each calibration solution.
- Wash 2 solvent (100mM HCl (aq):ACN (70:30, v/v)) was made up by measuring out 70 mL of water (18.2 MOhm-cm) and 30 mL of acetonitrile and adding both to a bottle, then adding 833 µL of concentrated hydrochloric acid.
- Elution solvent (hexane:ethyl acetate (50:50, v/v with 2% formic acid)) was made up by measuring out 50 mL of hexane and 50 mL of ethyl acetate and adding both to a bottle with 2 mL formic acid.

Additional Information

All data shown in this application note was generated using human urine donated by healthy human volunteers.

Ordering Information

Part Number	Description	Quantity
903-0020-B	ISOLUTE® HAX columns, 200 mg/3mL	50
PPM-48	Biotage® PRESSURE+ 48 Positive Pressure Manifold	1
417002	Biotage® Extrahera™ HV-5000	1
417610	Configuration Kit 24 Positions Dual Flow - HV	1
414174SP	Column Rack 24 x 3 mL	1
415491	Sample/Collection Rack 12 x 75 mm, 24 Positions	1
414254SP	Sample Rack 16 x 100 mm, 24 Positions	1
415000	TurboVap® LV	1

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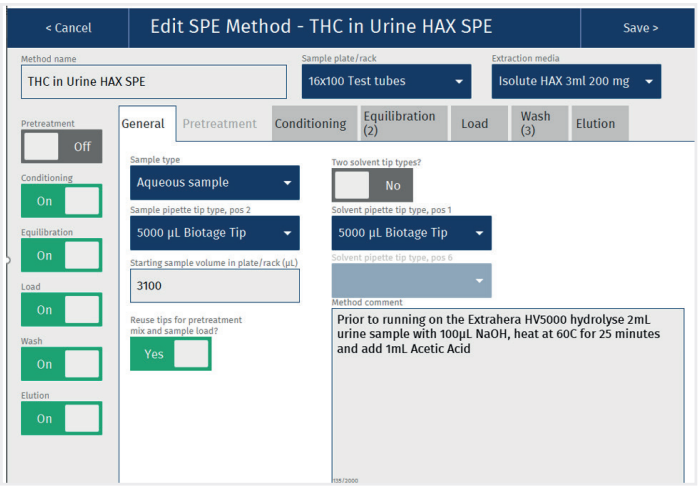
Appendix

Biotage® Extrahera™ HV-5000 Settings

The method described in this application note was automated on the Biotage® Extrahera™ High Volume 5000 using ISOLUTE® HAX 200 mg/3 mL columns. This appendix contains the software settings required to configure Extrahera™ to run this method. Screenshots may or may not match those here depending upon the instrument software version.

Sample Name:	11-Nor-9-carboxy-THC in urine
Sample Plate/Rack:	16 x 100 test tubes
Extraction Media:	ISOLUTE® HAX 200 mg/3 mL

Screenshot



Settings

"Sample" Tab

Sample Type:	Aqueous Sample
Starting Sample Volume (µL):	3100
Method Comment:	

Prior to running on the Extrahera™ HV-5000 hydrolyze 2 mL urine sample with 100 µL NaOH, heat at 60 °C for 25 minutes and add 1 mL Acetic Acid

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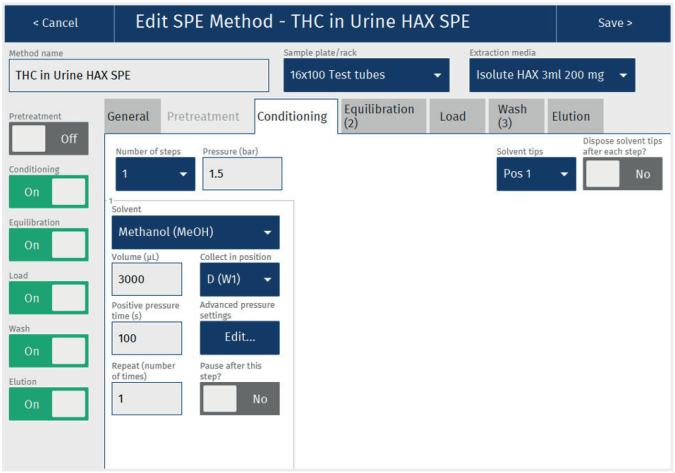
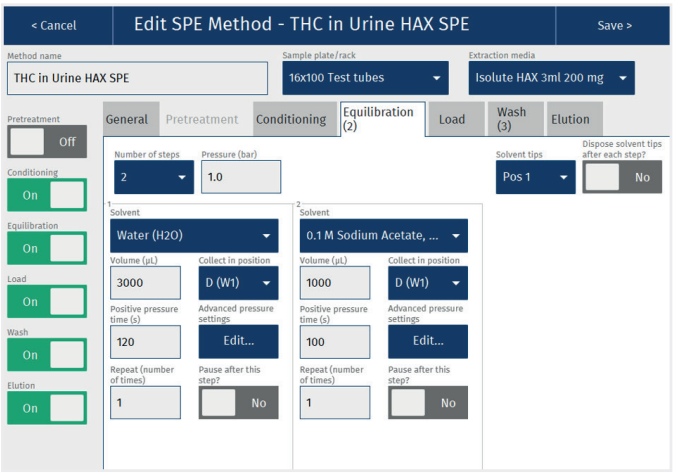
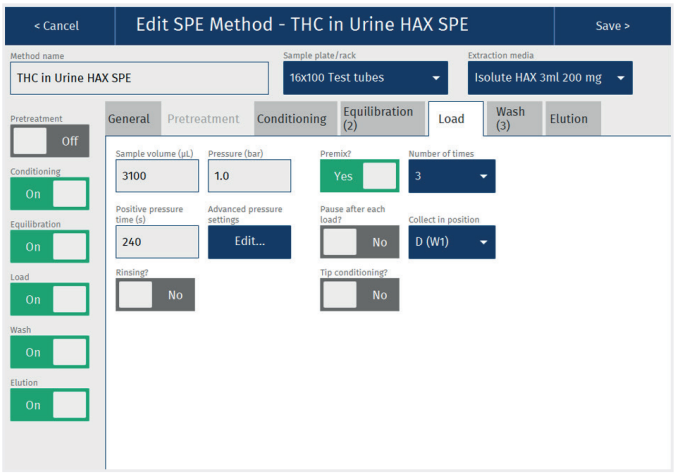
Screenshot







Settings

Conditioning	
Steps	1
Pressure	1.5
Solvent	Methanol (MeOH)
Volume (µL)	3000
Positive Pressure time (s)	100
Repeat (number of times)	1
Solvent tips	Pos 1
Dispose tips	No

Equilibration	
No. of steps	2
Pressure (Bar)	1.0
Solvent 1	Water (H2O)
Volume (µL)	3000
Collect position	D (W1)
Positive pressure time (s)	120
Repeat	1
Pause	No
Solvent 2	0.1M Sodium Acetate, pH3 (aq)
Volume (µL)	1000
Collect position	D (W1)
Positive pressure time (s)	100
Repeat	1
Pause	No
Solvent Tips	Pos 1
Dispose tips	No

Load	
Sample volume (µL)	3100
Pressure (bar)	1.0
Positive pressure time(s)	240
Rinsing	No
Premix?	Yes
Number of times	3
Pause after each load	No
Collect in position	D (W1)
Tip conditioning	No

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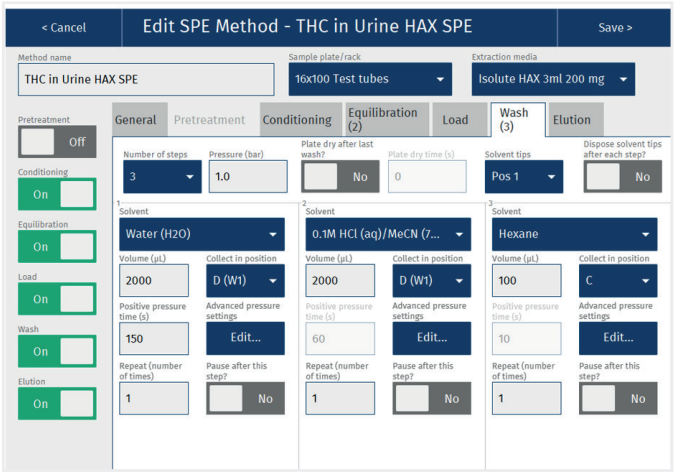
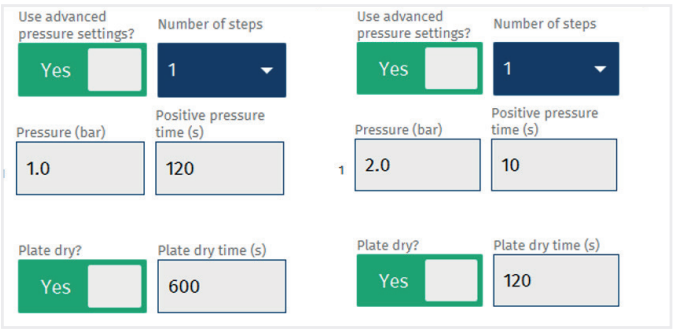
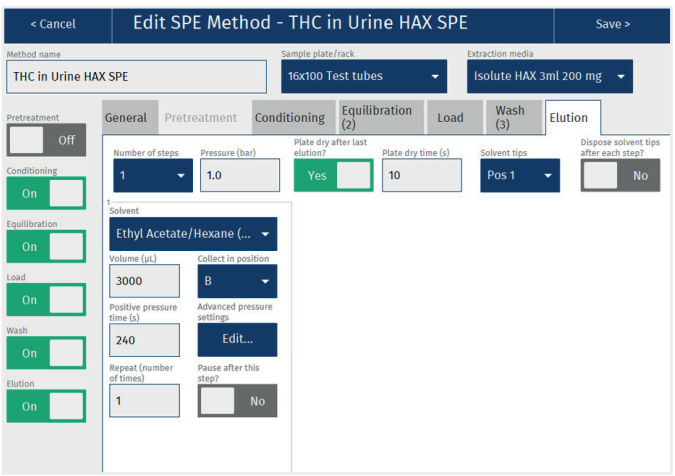


Wash	
Number of steps	3
Pressure (bar)	1.0
Plate dry after wash?	No
Solvent tips	Pos 1
Dispose tips	No
Solvent 1	Water (H2O)
Volume (µL)	2000
Collect in position	D (W1)
Positive pressure time (s)	150
Repeat (number of times)	1
Pause	No
Solvent 2	0.1M HCl (aq)/MeCN (70:30)
Volume (µL)	2000
Collect in position	D (W1)



Advanced pressure settings?	Yes
Number of steps	1
Pressure (bar)	1.0
Positive Pressure time (s)	120
Plate dry?	Yes
Plate dry time (s)	600
Repeat (number of times)	1
Pause	No
Solvent 3	Hexane
Volume (µL)	100
Collect in position	C
Advanced pressure settings?	Yes
Number of steps	1
Pressure (bar)	2.0
Positive pressure time (s)	10
Plate dry?	Yes
Plate dry time (s)	120
Repeat (number of times)	1
Pause	No



Elution	
Number of steps	1
Pressure (bar)	1.0
Plate dry after last elution?	Yes
Plate dry time (s)	10
Solvent tips	Pos 1
Dispose solvent tips?	No
Solvent	Ethyl Acetate/Hexane (50:50) 2% Formic Acid
Volume (µL)	3000
Collect in position	B
Positive pressure time (s)	240
Repeat	No
Pause?	No

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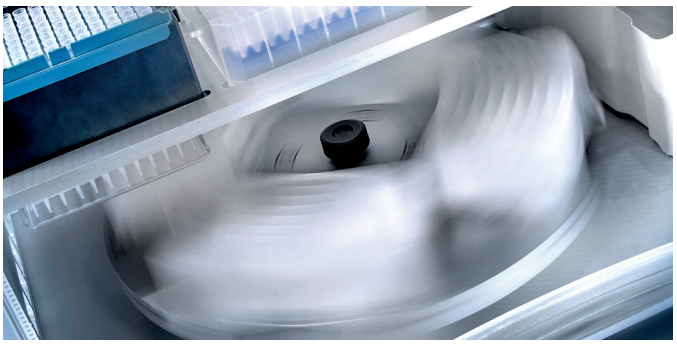
Solvent Properties

	Solvent Description
1	Methanol
2	Water (H2O)
3	0.1M Sodium Acetate, pH3 (aq)
4	0.1M HCl (aq)/MeCN (70:30)
5	Ethyl Acetate/Hexane (50:50) 2% Formic Acid
6	Hexane
7	
8	
9	
10	



Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type	**Refillable**					**Non Refillable**				
Capacity	N/A	N/A	N/A	N/A	N/A					
Aspiration flow rate	50	50	50	50	50	50				
Dispense flow rate	70	75	75	75	40	40				
Aspiration post dispense?	Yes	Yes	Yes	Yes	Yes	Yes				
Aspirate post dispense flow rate	7.5	7.5	7.5	7.5	7.5	7.5				
Aspirate post dispense volume	50	50	50	50	200	200				
Lower air gap flow rate	1.0	1.0	1.0	1.0	1.0	1.0				
Lower air gap volume	15	15	40	15	30	30				
Upper air gap flow rate	10	24	24	10	5	5				
Upper air gap volume	250	250	250	250	250	250				
Upper air gap dispense pause	1000	1000	1000	1000	2000	2000				
Conditioning?	Yes	Yes	Yes	Yes	Yes	Yes				
Frequency			1st Asp. only							
Cond. Times	3	2	2	3	4	4				
Cond. Flow rate	50	50	50	100	100	100				
Volume dependent conditioning	Yes	Yes	Yes	Yes	Yes	Yes				

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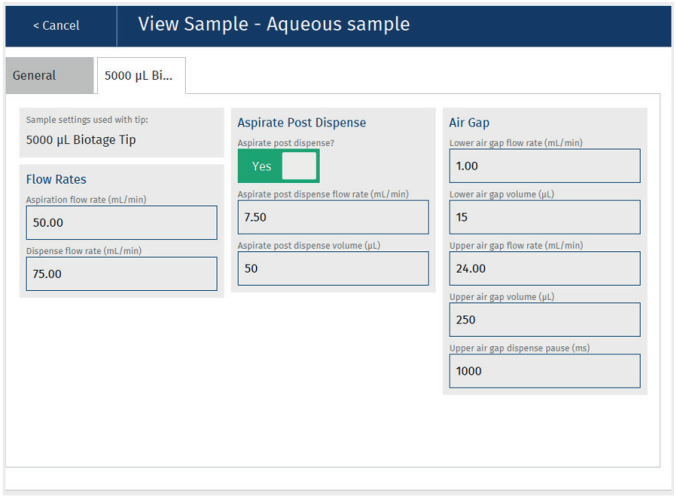
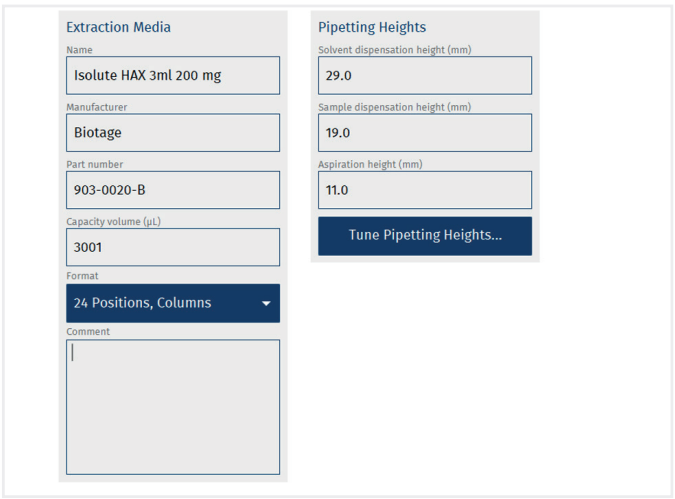


"Sample" Screen	
Sample name	Aqueous sample
Sample description	Default settings
Aspiration flow rate	50
Dispense flow rate	75
Aspirate post dispense?	Yes
Aspirate post dispense flow rate	7.5
Aspirate post dispense volume	50
Lower air gap flow rate	1.0
Lower air gap volume	15
Upper air gap flow rate	24
Upper air gap volume	250
Upper air gap dispense pause	1000



"Extraction Media" Screen	
Name	ISOLUTE® HAX 3mL 200mg
Manufacturer	Biotage
Part number	903-0020-B
Capacity volume	3001
Format	24 positions, columns
Comment	
Solvent dispensation height	29.0
Sample dispensation height	19.0
Aspiration height	11.0

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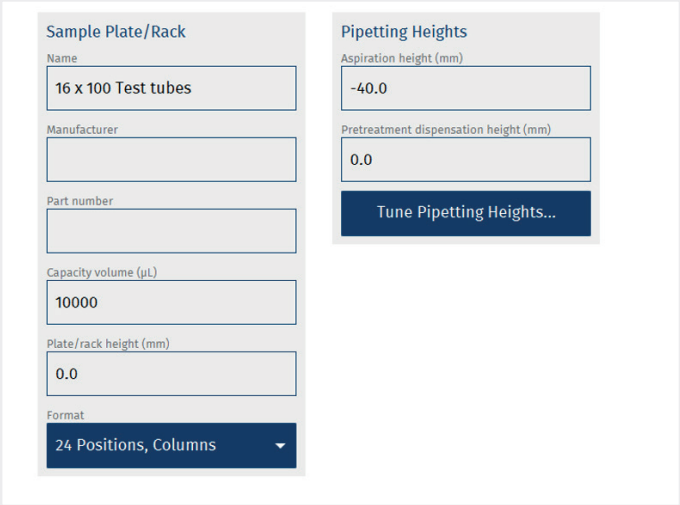

"Sample Plate/Rack" Screen	
Capacity volume	10000
Format	24 Positions, columns
Aspiration height	-40.0
Pretreatment dispensation height	0.0

"Pipette tip" Screen	
Name	5000 µL Biotage tip
Manufacturer	Biotage
Part number	417007
Capacity	5000
Length	120.0

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