Extraction of THC and its Metabolites from Human Hair Using ISOLUTE® SLE+ Prior to UPLC-MS/MS Analysis

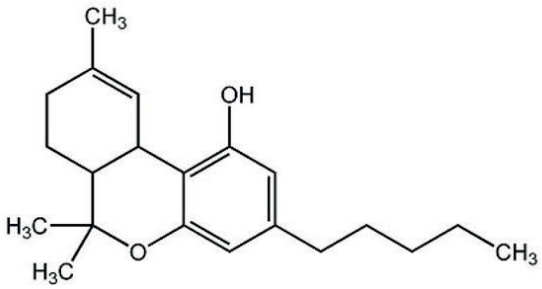

Figure 1. Structure of Δ⁹-tetrahydrocannabinol (THC).

Introduction

This application note describes a procedure for sample pre-treatment and extraction of THC and metabolites from human hair, using Biotage® Lysera for matrix pulverization of the sample prior to clean up using ISOLUTE® SLE+ supported liquid extraction.

Manual processing protocols were developed using the Biotage® PRESSURE+ 96 (plate format) or 48 (column format) positive pressure manifolds. For automated processing, protocols were developed using Biotage® Extrahera™.

The application note contains procedures optimized for both individual column format and 96-well plate format for higher throughput applications. The methodology delivers clean extracts with analyte recoveries ›75% (plate format) or ›60% (column format) with %RSD ‹10% for most analytes and LLOQ from 200 fg/mg of hair.

Both manual and automated procedures gave comparable results.

ISOLUTE® SLE+ Supported Liquid Extraction columns and 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 preparation time.

Analytes

Tetrahydrocannabinol (THC), 11-Nor-9-carboxy-Δ⁹-tetrahydrocannabinol (THC-COOH), 11-Hydroxy-Δ⁹-tetrahydrocannabinol (THC-OH), Δ⁹-tetrahydrocannabinolic acid-A (THCAA), cannabidiol (CBD), and cannabinol (CBN)

Internal Standards

Tetrahydrocannabinol –D_3 (THC- D_3), 11-Nor-9-carboxy-Δ⁹-tetrahydrocannabinol-D_3 (THC- COOH-D_3) and 11-Hydroxy-Δ⁹-tetrahydrocannabinol- D_3 (THC-OH- D3)

Sample Preparation Procedure

Format

ISOLUTE® SLE+ 400 µL capacity columns (p/n 820-0055-B) or

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

Matrix Preparation

Weigh 20 mg of hair into 2 mL Biotage® Lysera tubes containing 4 x 2.4 mm stainless steel beads. Add 1 mL methanol to each hair sample. Also add 10 µL of a 100 pg/mL internal standard solution making a 50 pg/mg spike.

Micropulverization Procedure

Grind to a fine powder using Biotage® Lysera: 3 x 5.3 m/sec for 3 minutes with a 20 sec dwell.

Centrifuge tubes for 10 minutes at 13,300 rpm (Heraeus Pico 17 Microcentrifuge (Thermo Scientific) with 24 position, 2 mL rotor).

Post Micropulverization

Transfer 200 µL of supernatant into 12 x 75 mm glass tubes or 2 mL collection plates and evaporate extracts using a TurboVap® LV at 20 °C or Biotage®SPE Dry 96 depending on the format being used.

Reconstitute in methanol:water (70:30, v/v. 200 µL).

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Supported Liquid Extraction Conditions

	ISOLUTE® SLE+ 400 µL Columns Part Number 820-0055-B	ISOLUTE® SLE+ 400 µL Plate Part Number 820-0400-P01
Sample Loading	Load 200 µL of reconstituted extract onto the ISOLUTE®SLE+ column. A pulse of pressure is not needed as the methanolic extract flows straight onto the bed. Allow the sample to absorb for 5 minutes.	Load 200 µL of reconstituted extract onto the ISOLUTE®SLE+ well. A pulse of pressure is not needed as the methanolic extract flows straight onto the bed. Allow the sample to absorb for 5 minutes.
Analyte Extraction	Apply MTBE (600 µL) allow to flow under gravity for 5 minutes. Apply a further aliquot of MTBE (600 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).	Apply MTBE (600 µL) allow to flow under gravity for 5 minutes. Apply a further aliquot of MTBE (600 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).
Collection Vessels	Collect extract in 12 x 75 mm glass tubes.	Collect extract in 96-well collection plates.
Post Elution	Evaporate extracts to dryness at 40 °C, for 30 minutes at a flow rate of 1.5 L/min using a TurboVap® LV.	Evaporate extracts to dryness at 40 °C, for 30 minutes at a flow rate of 20–40 L/min using a Biotage® SPE Dry 96.
Reconstitute	Reconstitute extracts in a mix of mobile phase A/mobile phase B (70:30, v/v, 200 µL). Vortex mix and transfer to a 96-well format plate and cover with a sealing mat prior to injection.	Reconstitute extracts in a mix of mobile phase A/mobile phase B (80:20, v/v, 200 µL). Vortex mix. Cover plate with a sealing mat prior to injection.

UHPLC Conditions

Instrument
Shimadzu Nexera X2 UHPLC

Column
ACE Excel 2 C18 (50 x 2.1 mm) with a Restek EXP holder and Restek C18 guard column

Mobile Phase
A: 0.01% Acetic Acid (aq)

B: 0.01% Acetic Acid in MeOH

Flow Rate
0.3 mL/min

Column Oven Temperature
50 °C

Injection Volume
5 µL

Table 1. UHPLC Gradient.

Time (min)	%A	%B
0	50	50
0.5	20	80
2.00	10	90
4.00	10	90
4.01	50	50

Mass Spectrometry Conditions

Instrument
Shimadzu 8060 Triple Quadrupole MS using ES interface

Nebulizing Gas Flow
3 L/min

Drying Gas Flow
5 L/min

Heating Gas Flow
15 L/min

Interface Temperature
400 °C

DL Temperature
300 °C

Heat Block Temperature
500 °C

CID Gas Flow
270 kPa

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

Analytes	MRM Transition	Collision Energy
THC-D_3	318.0 > 196.15 318.0 > 123.2	-24 -32
THC	315.0 > 193.10 315.0 > 123.2	-23 -32
OH-THC-D_3	334.0 > 316.15 334.0 > 196.25	-15 -25
OH-THC	331.0 > 313.3 331.0 > 193.25	-15 -26
THC-COOH-D_3	346.3 > 302.3 346.3 > 248.30	22 28
THC-COOH	343.3 > 299.3 343.3 > 245.25	22 30
CBN	311.0 > 223.0 311.0 > 241.2	-22 -17
CBD	313.2 > 245.15 313.2 > 179.25	24 20

Results

This simple sample preparation method delivers clean extracts and analyte recoveries mostly greater than 75% with RSDs lower than 10% for all analytes (see fig 2), and LLOQs below 10 pg/mg and as low as 200 fg/mg for THC-COOH and (see table 3) for all ISOLUTE® SLE+ formats used.

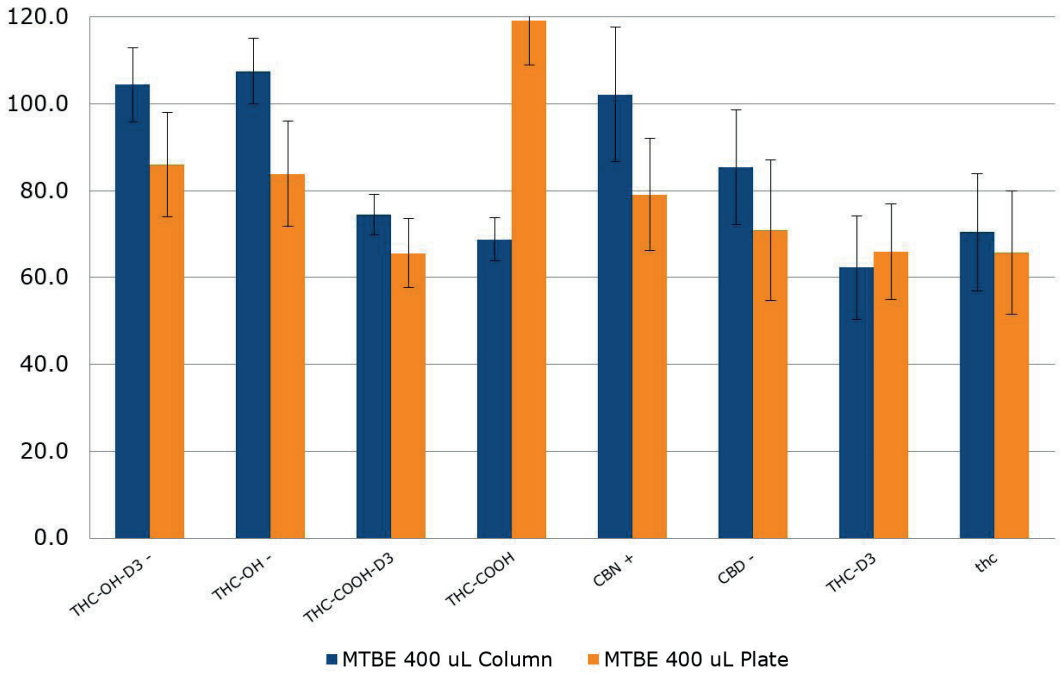


Figure 2. Average analyte recoveries and %RSD (n=7) for ISOLUTE® SLE+ column and plate formats.

Calibration curve performance was investigated from hair samples spiked between 0.1–200 pg/mg of hair. Good linearity was observed for all analytes typically delivering r^2 values greater than 0.99. Table 3. details linearity performance and associated LOQ for each analyte using the ISOLUTE® SLE+ column format. Similar results were achieved using the 96-well plate format.

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

Analytes	Column Format		Plate Format	
	r^2	LLOQ (pg/mg)	r^2	LLOQ (pg/mg)
THC	0.997	10	0.998	10
OH-THC	0.997	10	0.998	10
THC-COOH	0.997	0.2	0.997	0.2
CBN	0.997	10	0.997	10
CBD	0.997	1	0.995	0.5

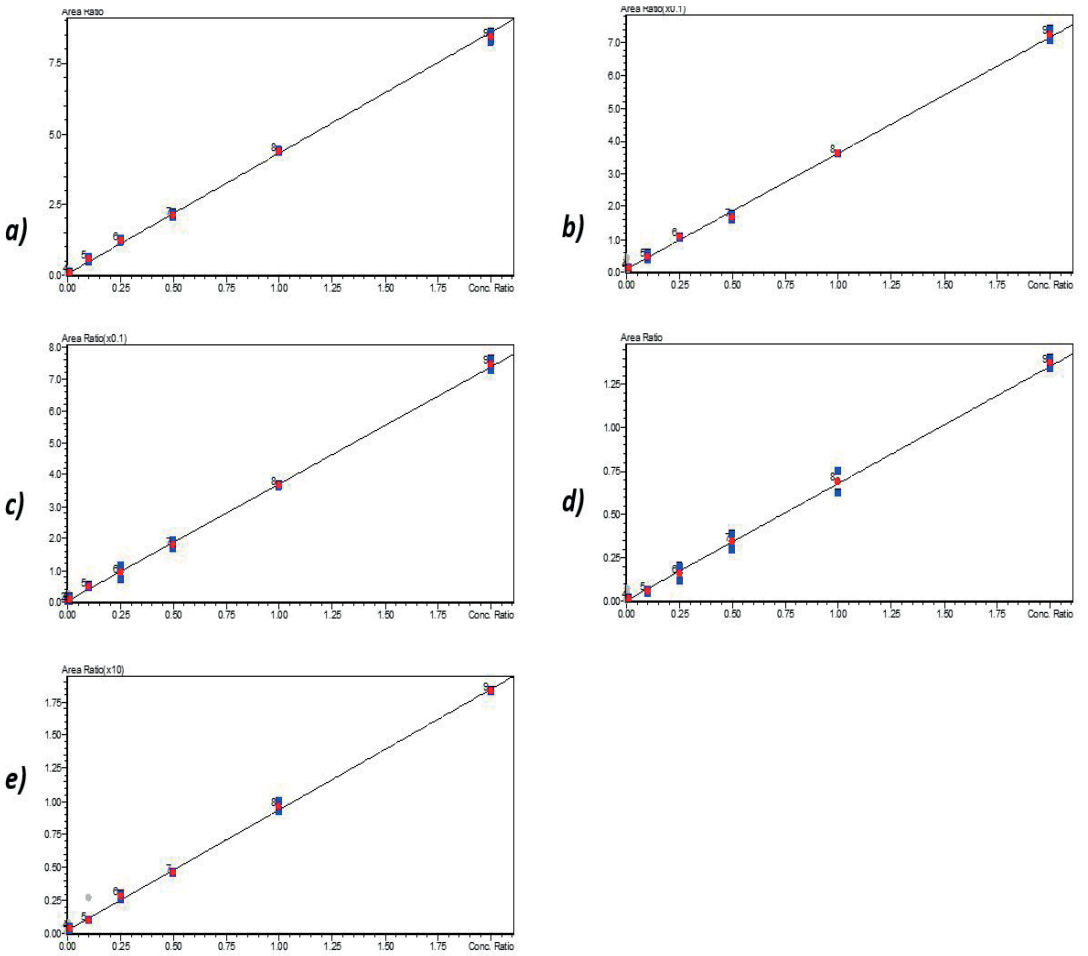


Figure 5. Calibration curves for THC (a), OH-THC (b), THC-COOH (c), CBD (d) and CBN (e) using human hair with 400 µL capacity column format (loading 200 µL of extracted sample as described).

Chemicals and Reagents

- Methanol (LC-MS grade), Ultra-Pure Methanol (Gradient MS), dichloromethane (99.8%), isopropanol (99.9%), MTBE (99%) and formic acid (98%) were purchased from Honeywell Research Chemicals (Bucharest, Romania).
- All analyte standards and deuterated internal standards, and acetic acid (LC-MS grade) were purchased from Sigma- Aldrich Company Ltd. (Gillingham, UK).
- Water used was 18.2 MOhm-cm, drawn daily from a Direct-Q5 water purifier.
- 0.1% NH_4OH was prepared by adding 100 µL of ammonium hydroxide to 99.9 mL of methanol
- Mobile phase A (0.01% Acetic acid (aq)) was prepared by adding 50 µL to 500 mL of purified water.
- Mobile phase B (0.01% Acetic acid (aq)) was prepared by adding 50 µL to 500 mL of HPLC grade methanol.
- 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.

Additional Information

- All data shown in this application note was generated using real hair samples, both dyed and natural, provided by healthy human volunteers. All hair types gave similar analyte recovery and extract cleanliness.
- Biotage® Lysera hints and tips
  - A minimum of four tubes must be loaded in the tube carriage to ensure balance during processing
  - Ensure vial caps and Lysera head are firmly tightened and Lysera locking mechanism is fully engaged
  - 2 mL Lysera tubes were placed directly into the centrifuge, no transfer to centrifuge vials was needed.

Ordering Information

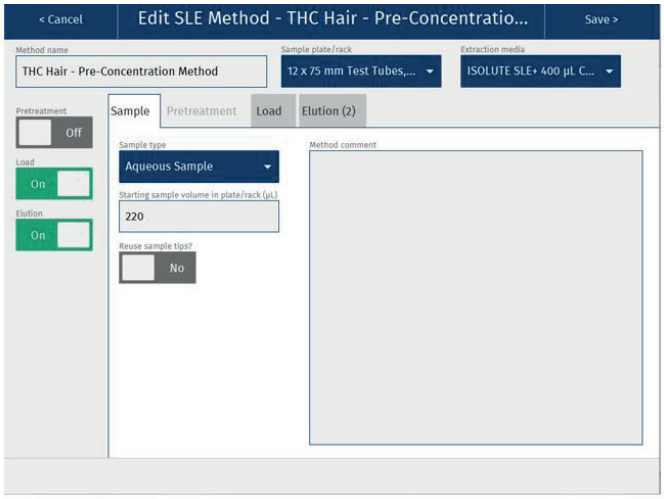
Part Number	Description	Quantity
19-060	Biotage® Lysera	1
19-649	2 mL Reinforced Tubes with screw caps (Bulk pack)	1000
19-640	2.4 mm Metal Beads - 500 grams	1
820-0055-B	ISOLUTE® SLE+ 400 µL Sample Volume Columns	50
820-0400-P01	ISOLUTE® SLE+ 400 µL Capacity Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
PPM-48	Biotage® PRESSURE+ 48 Positive Pressure Manifold	1
415000	TurboVap® LV	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
C44651	Test Tubes (12 x 75 mm, Uncapped)	1000
414001	Biotage® Extrahera™	1

Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™ using ISOLUTE® SLE+ 400 µL capacity columns and 96-well plates. This appendix contains the software settings required to configure Extrahera to run the column format method. As described in the main body of the application note, analyte recoveries, %RSDs, linearities and LOQs were comparable for both manually processed and automated methods, for both extraction formats.

Sample Name:	THC Hair – Pre concentration Method
Sample Plate/Rack:	12 x 75 mm Test Tubes, 24
Extraction Media:	ISOLUTE® SLE+ 400 µL Columns



Settings

"Sample" Tab

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

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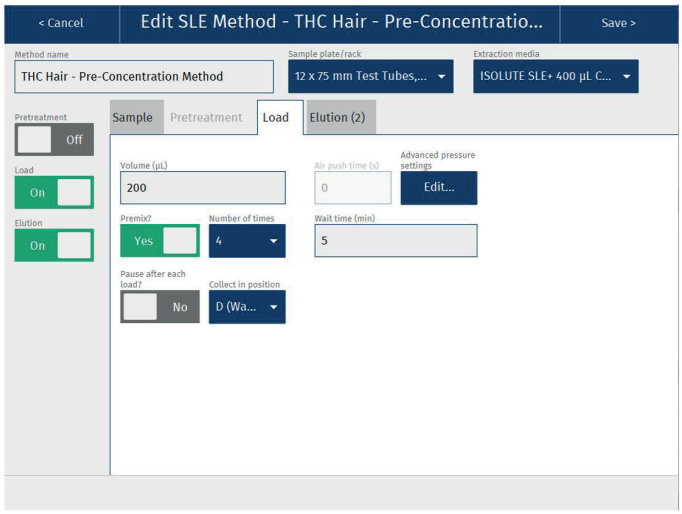
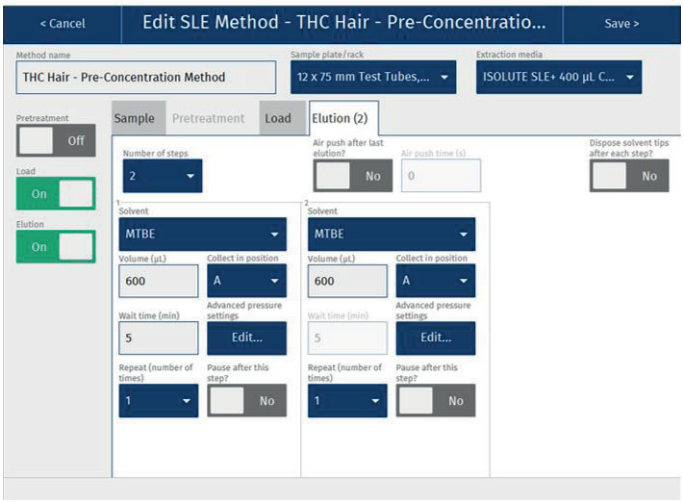


Load	
Pressure (Bar)	1.0 for 10 sec
Pause after each load	No
Volume	200
Collect in position	D
Positive pressure time	0
Premix	Yes
Number of times	4
Wait time (min)	5

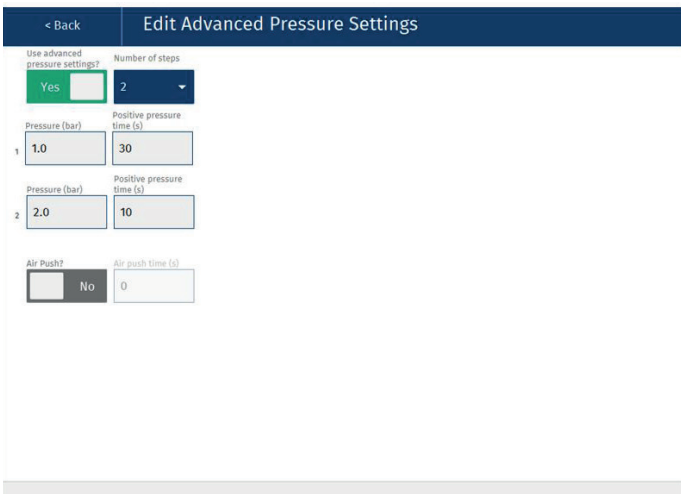
'Advanced Settings'
Pressure for 10 seconds at 1 bar



Elution	Activated
No. of steps	2
Pressure (Bar)	
Plate Dry	No
Dry time	0
Pause	5

Solvent	
1	MTBE
2	
3	
4	

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

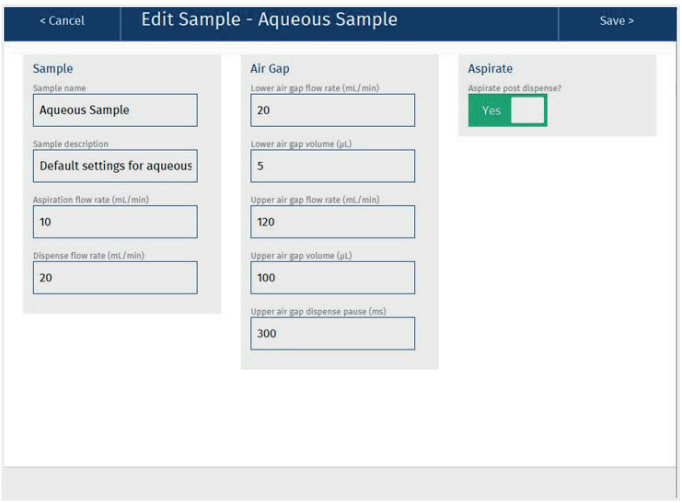


'Advanced Settings'
Advanced Pressure:
2 Steps; 1.0 Bar for 30 seconds; 2.0 bar for 10 seconds

Solvent Properties

	Solvent Description
1	MTBE
2	
3	
4	
5	
6	
7	
8	
9	
10	

Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type	**Refillable**					**Non Refillable**				
Capacity										
Aspiration flow rate (mL/min)	10									
Dispense flow rate (mL/min)	10									
Lower air gap flow rate (mL/min)	10									
Lower air gap volume (µL)	5									
Upper air gap flow rate (mL/min)	120									
Upper air gap volume (µL)	100									
Upper air gap dispense pause	300									
Conditioning?	Yes									
Conditioning number of times	2									
Conditioning flow rate (mL/min)	10									
Chlorinated	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

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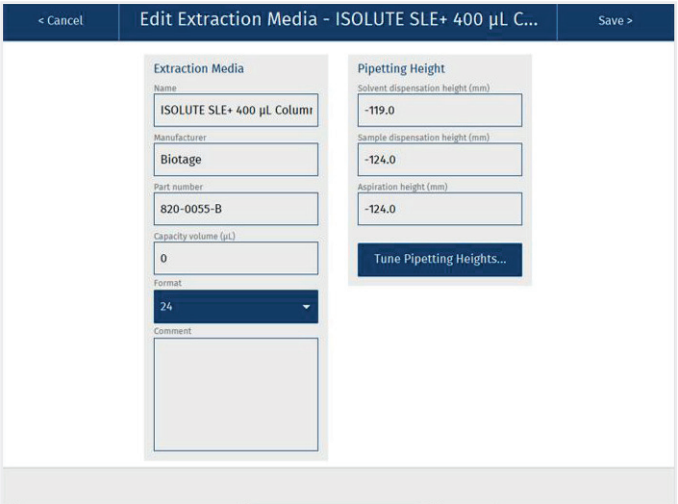
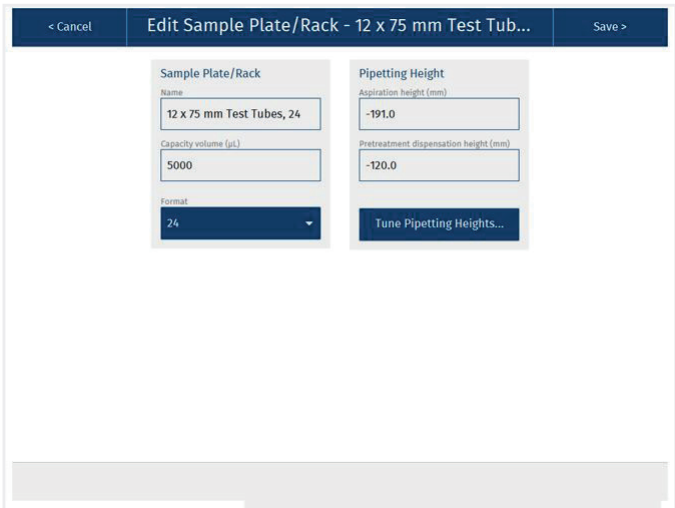
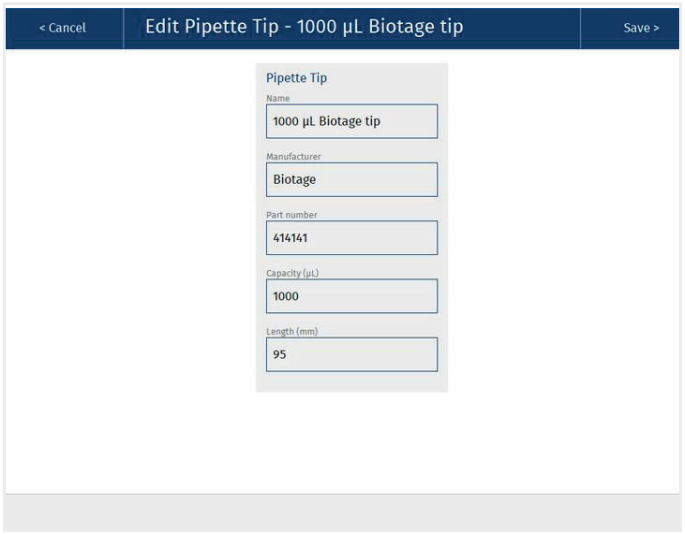


"Extraction Media" Screen	
Name	ISOLUTE® SLE+ 400 µL Column
Manufacturer	Biotage
Part number	820-0055-B
Capacity volume	400
Format	24
Comment	
Solvent dispensation height	-119
Sample dispensation height	-124
Aspiration height	-124



"Sample Plate/Rack" Screen	
Name	12 x 75 mm Test Tubes, 24
Capacity volume	5000
Format	24
Aspiration height	-191
Pretreatment dispensation height	-120



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

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