Extraction of Vitamin B7 (Biotin) from Serum Using EVOLUTE® EXPRESS ABN Prior to LC-MS/MS Analysis

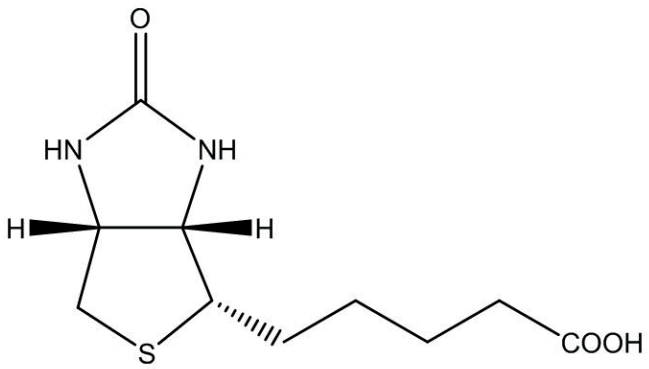


Figure 1. Structure of Vitamin B7.

Introduction

Vitamin B7 (Biotin) is a water soluble vitamin necessary for cell growth. The method described in this application note achieves high reproducible extraction recoveries of vitamin B7 from serum while minimizing co-extractable material in the form of proteins, lipids and phospholipids. Serum is extracted using the EVOLUTE® EXPRESS ABN 96-well plate.

EVOLUTE EXPRESS SPE products dramatically improve flow characteristics, and enhance sample preparation productivity providing clean, robust, sample preparation. This method can be automated using Biotage® Extrahera™ (see appendix for details).

Analytes

Biotin (with Biotin-[$^2H_2$] as internal standard).

Sample Preparation Procedure

Format

EVOLUTE® EXPRESS ABN 10 mg plate, part number 600-0010-PX01.

Sample Pretreatment

To 200 µL of serum add internal standard (Biotin-[$^2H_2$]) at 250 pg/mL and dilute using with 1% formic acid (aq) (200 µL). Mix.

Condition (Optional)

Condition each well with methanol (500 µL). This step is not required with the EVOLUTE EXPRESS Load-Wash-Elute procedure.

Equilibration (Optional)

Equilibrate each well with 1% formic acid (aq) (500 µL). This step is not required with the EVOLUTE EXPRESS Load-Wash-Elute procedure.

Sample Loading

Load 400 µL of pre-treated serum into each well.

Wash 1

Elute interferences with H_2O (500 µL).

Wash 2

Elute interferences with H_2O/MeOH (95/5, v/v, 500 µL).

Elution

Elute analytes with 0.1% NH_4OH in (H_2O/MeOH, 90/10, v/v, 200 µL).

Post Elution

Evaporate to dryness at 40 °C in a stream of air or nitrogen using a Biotage® SPE Dry.

Reconstitution

Reconstitute the extract with H_2O/ACN (90/10, v/v, 200 µL).

UPLC Conditions

Instrument

Waters ACQUITY I-Class

Column

ACE Excel 1.7 µ C18-PFP column (100 x 2.1 mm id)

Mobile Phase

A: 1 mM ammonium fluoride (aq).

B: Acetonitrile.

Flow Rate

0.4 mL/min.

Table 1. Gradient Conditions.

Time	% A	% B	Curve
0	90	10	1
1.50	82	18	6
2	20	80	1
2.6	90	10	1

Curve 6: Lineat Gradient

Injection Volume

10 µL

Sample Temperature

20 °C

Column Temperature

40 °C

Mass Spectrometry Conditions

Instrument

Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

Desolvation Temperature

500 °C

Ion Source Temperature

150 °C

Collision Cell Pressure Temperature

3.7 e^{-3} mbar

Negative ions acquired in multiple reaction monitoring (MRM) mode:

Table 2. MRM Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Vitamin B7 (Qual)	243.1 > 200.0	25	15
Vitamin B7 (Quant)	243.1 > 166.0	25	15
Vitamin B7-[$^2H_2$]	245.1 > 168.0	25	15

Results

Good retention and chromatographic peak shape was obtained using the C18-PFP column. Figure 2. demonstrates signal intensity and peak shape attained from serum spiked at 25 pg/mL with deuterated internal standard at 250 pg/mL.

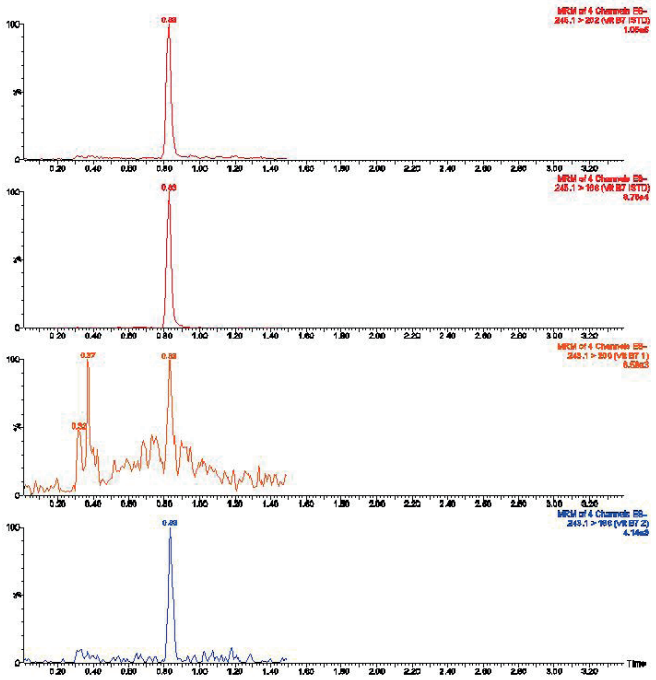


Figure 2. Chromatography obtained from serum spiked at 25 pg/mL. Retention time for vitamin B7 (biotin) is approximately 0.8 mins.

Recovery

Stripped serum was spiked at various concentrations from 25–5000 pg/mL for recovery determination. High reproducible recoveries > 80% with corresponding RSDs < 10% were demonstrated. Typical recovery data for full and Load-Wash-Elute methods from spiked serum at 2000 pg/mL is shown in figure 3.

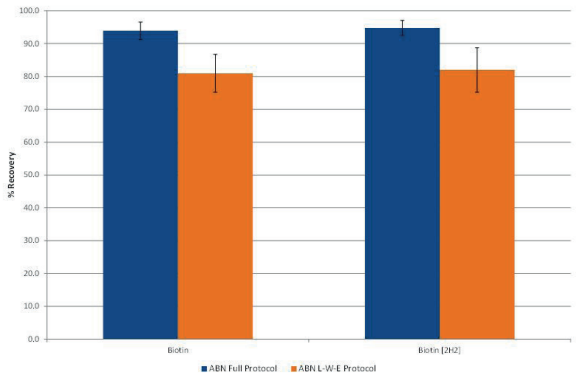


Figure 3. Spiked serum recovery profile for full and Load-Wash-Elute SPE protocols.

Calibration Curves

Calibration curves were generated using stripped serum spiked at concentrations from 25–1000 pg/mL. Good linearity, coefficients of determination ($r^2 > 0.99$) and sensitivity were obtained. Stripped serum matrix contained low residual endogenous levels of biotin which contributed to a slight intercept on the calibration curves. (see figure 4).

Extract Cleanliness

Phospholipid Removal

Post extraction residual phospholipid levels were investigated to provide an indication of extract cleanliness. The most abundant phospholipids in human serum (previously selected from full scan, SIR and precursor ion scanning experiments) were assessed using MRM transitions monitoring the common 184 product ion. Figure 5 demonstrates phospholipid content comparing 100 µL of protein precipitated serum with the final EVOLUTE EXPRESS ABN extraction protocol using 200 µL of matrix.

Post Column Infusion

Extract cleanliness was also investigated using post-column infusion (PCI) experiments. Mobile phase and blank serum extracts were injected onto the LC-MS/MS setup while simultaneously infusing Vitamin B7, to determine regions of suppression, as shown in Figure 6.

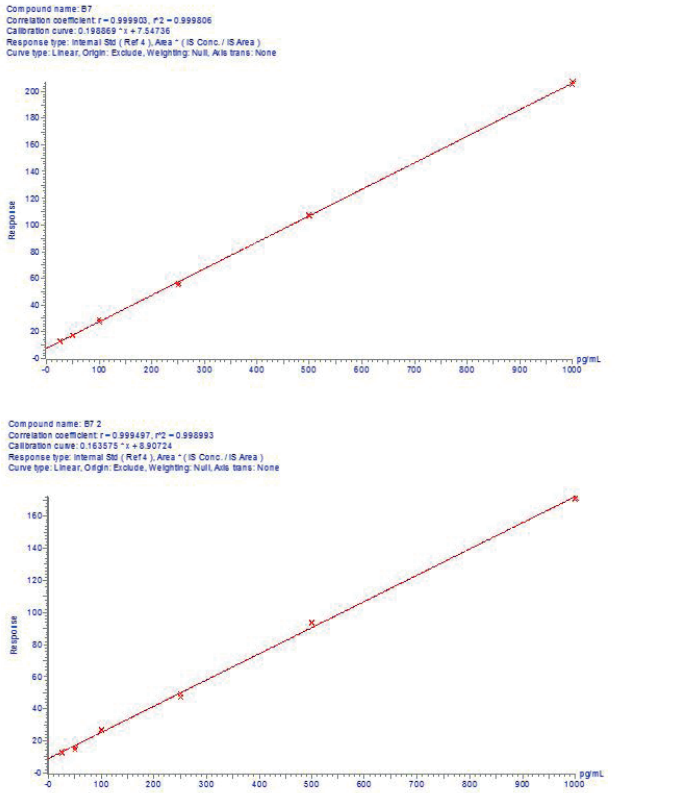


Figure 4. Serum quantifier and qualifier ion calibration curves spiked from 25–1000 pg/mL, extracted in duplicate.

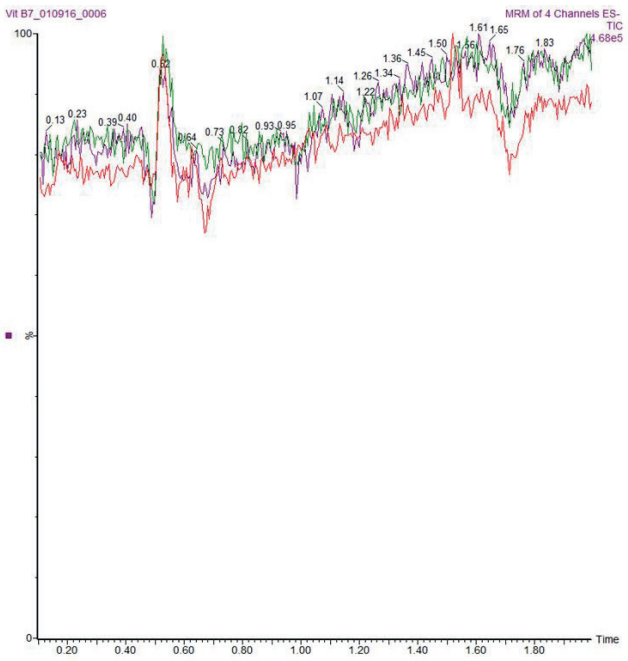


Figure 6. PCI baselines comparing blank solvent (red) to extracted blank serum using full SPE (green) or L-W-E procedures (purple). Minimal baseline disturbance, indicating low matrix effects, is evident at ~0.8 minutes (analyte retention time).

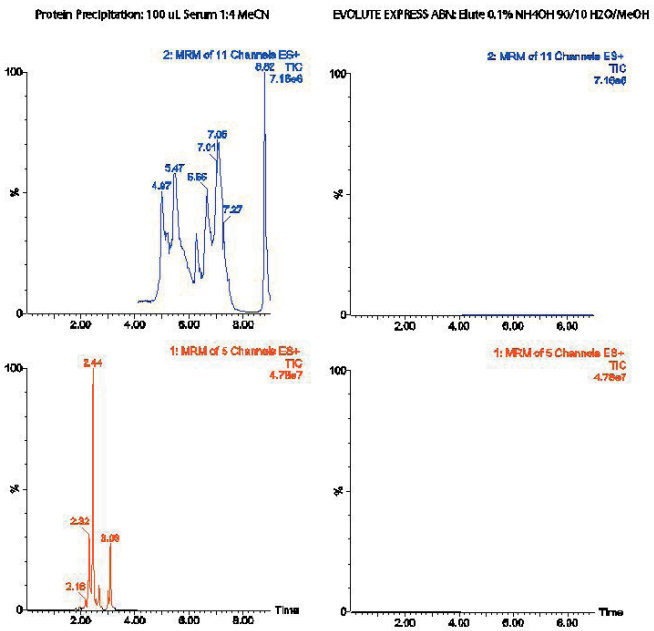


Figure 5. Phospholipid MRM TICs for final serum extraction protocol.

Additional Notes

Reagent Preparation

1. 1% formic acid aq: Measure 99 mL of H_2O and add 1 mL of formic acid (99% concentration).
2. H_2O/MeOH (95/5, v/v): Measure 95 mL of H_2O and add 5 mL of MeOH.
3. 0.1% NH_4OH in (90/10, v/v) H_2O/MeOH: Measure 89.9 mL of H_2O, add 100 µL of NH_4OH (aq, 28–32% concentration) followed by 10 mL of MeOH.
4. H_2O/MeCN (90/10, v/v): Measure 90 mL of H_2O and add 10 mL of MeCN.
5. 1 mM ammonium fluoride aq (mobile phase A): Weigh 0.03704 g and dissolve in H_2O. Dilute and make up to 1 L in H_2O.

Mobile Phase and Ionization Considerations

1. As a small polar carboxylic acid, ionization of vitamin B7 is possible using both + and – ion modes.
2. Negative ion mode provided better sensitivity due to the availability of more selective MRM transitions. Positive ion MRM showed a tendency for the water loss product ion $[M+H-H_2O]^+$.
3. Acidic mobile phase additives are typically used to provide extra retention of acid moieties during chromatography. However, due to the use of negative ionization and optimization of signal to noise these additives were omitted.
4. Ammonium fluoride was selected to scavenge baseline noise in negative ion electrospray. This resulted in better signal to noise than other additive such as ammonium acetate or formate.
5. The NH_4F resulted in shorter retention of the target analyte due to increased pH.
6. MeCN was selected for the organic eluent as a polar aprotic option for negative ionization.

SPE Considerations

1. EVOLUTE® EXPRESS ABN was compared to the mixed-mode strong and weak anion exchange sorbents. EVOLUTE EXPRESS AX also provided good recoveries and phospholipid removal. However, better cleanliness due to enhanced pigment removal was achieved using the optimized elution combinations with the ABN sorbent.
2. pH control using acidic additives for sorbent conditioning (optional) and sample pre-treatment was aimed at suppressing ionization of the analyte making it less polar for the initial retention.
3. Washing steps were kept highly aqueous (to avoid losses due to analyte polarity) but were also intended to remove residual acidic nature to allow easier elution.
4. Final elution conditions were optimized for highly aq elution conditions which resulted in massively reduced phospholipid content in the extracts. Elution with H_2O/MeOH (50/50, v/v) demonstrated good removal of phospholipids but the addition of small amounts of base resulted in the analyte being more ionized, and therefore more polar, allowing elution using 90% aqueous conditions. This provided excellent extract cleanliness.
5. Minimum elution volume was optimized at 200 µL for the highly aqueous elution solvent described above. A reduced volume (100–150 µL) with an increased organic content could be used. However, this may adversely impact extract cleanliness, due to increased co-elution of matrix components.
6. It was not possible to eliminate evaporation and move to direct injection using the selected chromatographic mobile phases. The use of a pH stable column may allow direct injection. Alternatively, a H_2O/MeOH (50/50, v/v) elution solvent could be used with aq dilution prior to injection.

Ordering Information

Part Number	Description	Quantity
600-0010-PX01	EVOLUTE® EXPRESS ABN 10 mg Fixed Well Plate	1
For Manual Processing		
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
For Automated Processing		
414001	Biotage® Extrahera	1
415040	Configuration Kit 96 Positions Dual Flow	1
414141	Extrahera Clear Tips	960

Part Number	Description	Quantity
Accessories/Consumables		
121-5203	Collection Plate, 2 mL, Square	50
121-5204	Piercable Sealing Cap	50
Evaporation		
SD-9600-DHS-EU	Biotage® SPE Dry 96 Sample Evaporator 220/240V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120V	1

Appendix

Biotage® Extrahera™ Settings

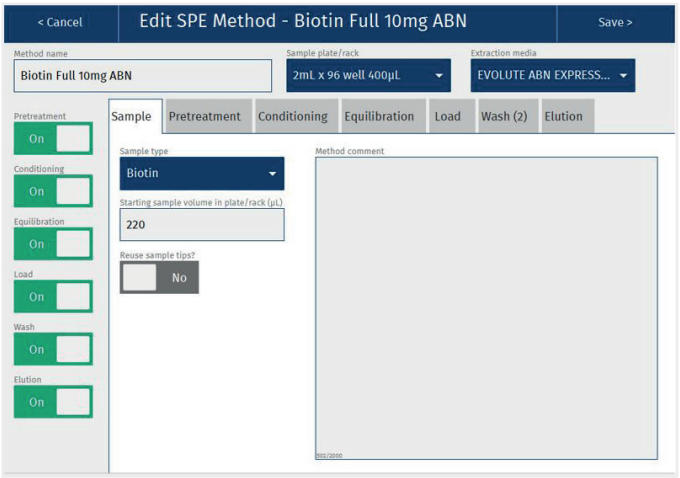
The methods described in this application note were automated on the Biotage® Extrahera™, using EVOLUTE® EXPRESS ABN 10 mg plates. Method performance was comparable.

Method	Total extraction time for 96 samples
EVOLUTE® EXPRESS Load-Wash-Elute method	30 mins 19 secs
EVOLUTE® EXPRESS 'full' method	38 mins 5 secs

This appendix contains the software settings required to configure Extrahera to run the method described in this application note.

An importable electronic copy of this method for Extrahera can be downloaded from www.biotage.com

Method Name: Biotin Full 10 mg ABN
Sample Plate/Rack: 2 mL x 96 well 400 µL
Extraction Media: EVOLUTE ABN EXPRESS 96 Well Plate

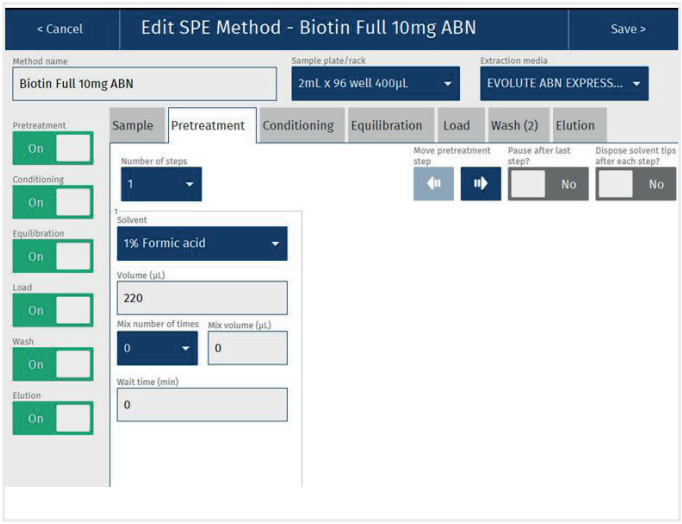


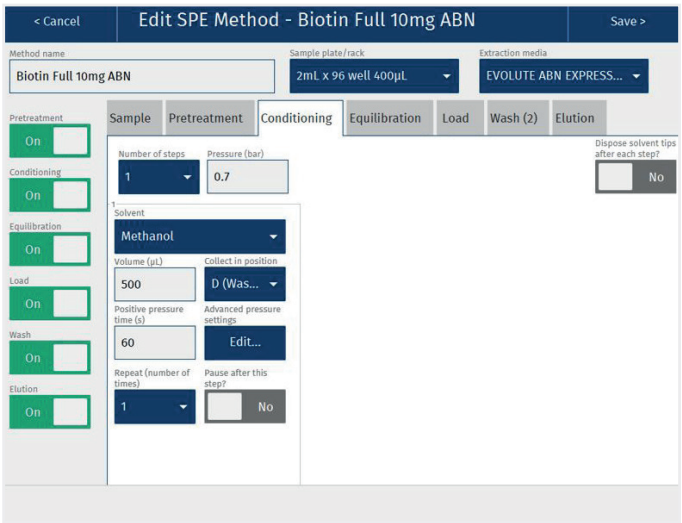
Settings

"Sample" Tab

Sample Type:	Biotin
Starting Sample Volume (µL)	220
Reuse sample tips?	No
Method comment:	

Screenshot





Settings

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

	Solvent
1	1% Formic Acid
2	
3	
4	

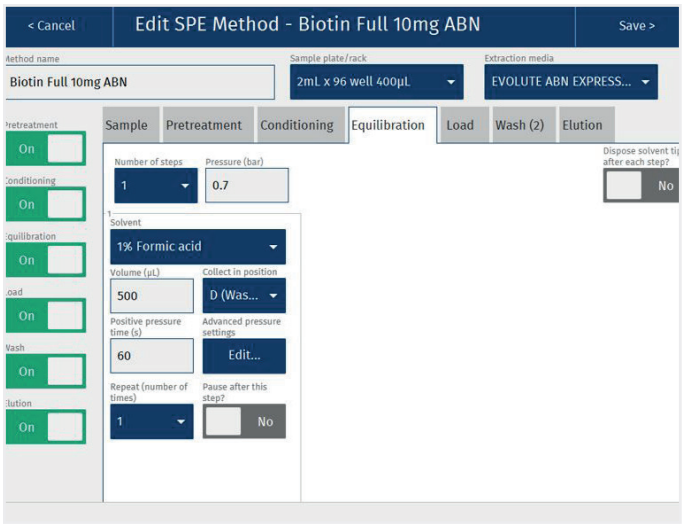
	1	2	3	4
Volume (µL)	220			
Mix number of times	0			
Mix volume (µL)	0			
Wait time (min)	0			

Conditioning	Activated
No. of steps	1
Pressure (Bar)	0.7
Dispose tips after each step	No

	Solvent
1	Methanol
2	
3	
4	

	1	2	3	4
Volume (µL)	500			
Collect in position	D			
Pressure time (s)	60			
Repeat	1			
Pause after this step	No			

'Advanced Settings'



	Equilibration	Activated
	No. of steps	1
	Pressure (Bar)	0.7
	Dispose tips after each step	No
	Dispose tips after each step	No

	Solvent
1	1% Formic acid
2	
3	
4	

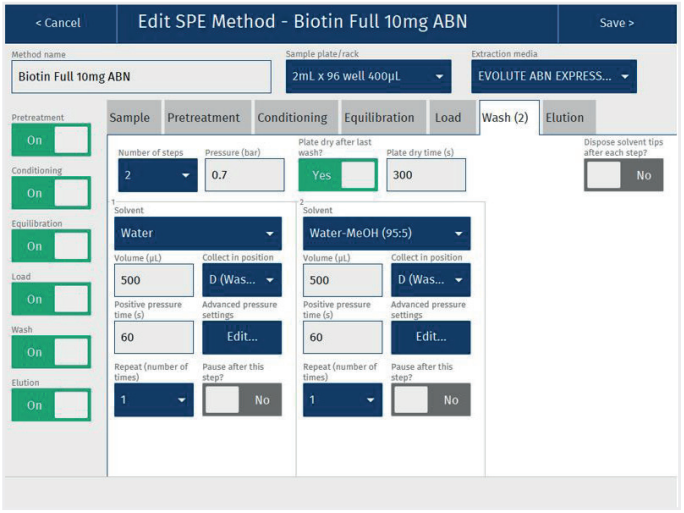
	1	2	3	4
Volume (µL)	500			
Collect in position	D			
Pressure time (s)	60			
Repeat	1			
Pause after this step	No			

'Advanced Settings'

	Load	Activated
	Pressure (Bar)	0.7
	Pause after each load	No
	Volume (µL)	400
	Collect in position	D
	Positive pressure time (s)	60
	Premix	Yes
	Number of times	3
	Rinsing	No
	Rinse volume (µL)	N/A
	Rinse solvent	N/A
	Tip Conditioning	No
	Conditioning solvent	N/A

'Advanced Settings'

Biotage®

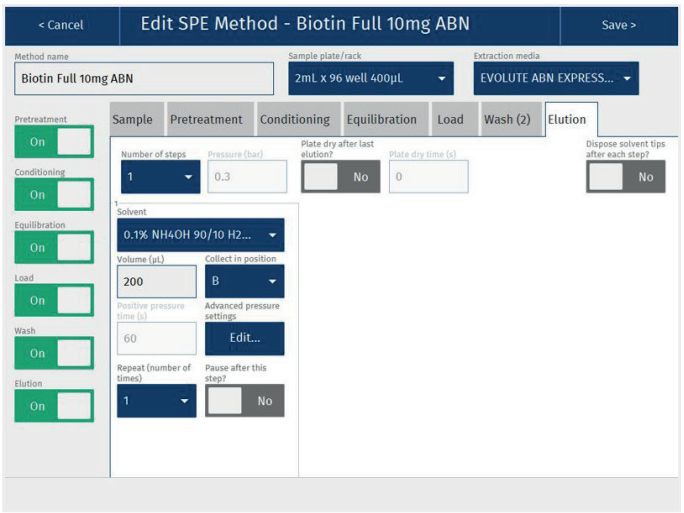


	Wash	Activated
	No. of steps	2
	Pressure (Bar)	0.7
	Plate dry after last wash	Yes
	Plate dry time (s)	300
	Dispose tips after each step	No

	Solvent
1	Water
2	Water-MeOH (95:5)
3	
4	

	1	2	3	4
Volume (µL)	500	500		
Collect in position	D	D		
Pressure time (s)	60	60		
Repeat	1	1		
Pause after this step	No	No		

‘Advanced Settings’



	Elution	Activated
	No. of steps	1
	Pressure (Bar)	Advanced
	Plate dry after last elution	No
	Plate dry time (s)	N/A
	Dispose tips after each step	No

	Solvent
1	Solvent
2	0.1% NH4OH 90/10 H20/MeOH
3	
4	

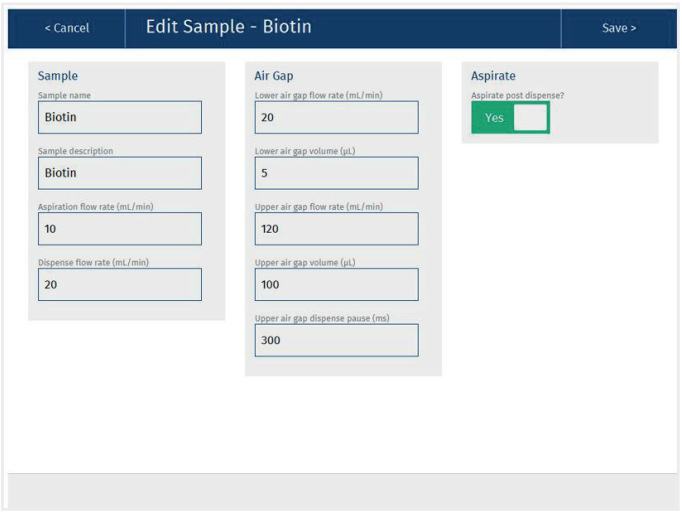
	1	2	3	4
Volume (µL)	200			
Position	B			
Pressure time (s)	Adv.			
Repeat	1			
Pause after this step	No			

‘Advanced Settings’
0.3 bar for 60 s then 1.0 bar for 40 s then 3.0 bar for 30 s

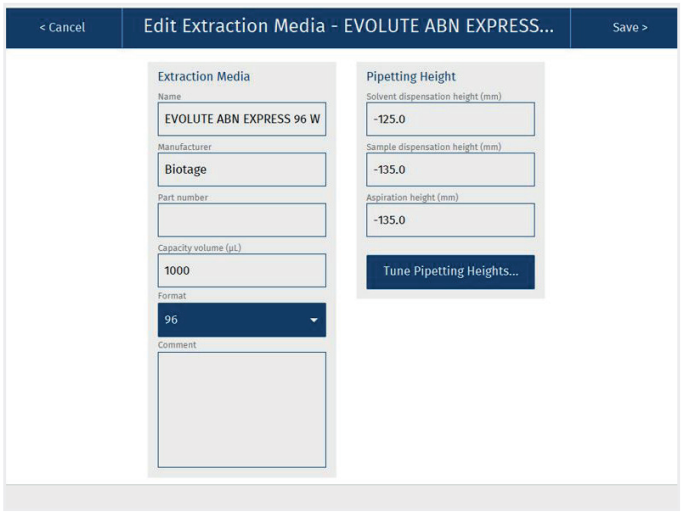
Solvent Properties

	Solvent Description
1	1% Formic acid
2	Methanol
3	Water
4	Water-MeOH (95:5)
5	0.1% NH4OH 90/10 H20/MeOH
6	
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 (mL/min)	10	10	10	10	10					
Dispense flow rate (mL/min)	20	20	20	20	20					
Lower air gap flow rate (mL/min)	20	20	20	20	20					
Lower air gap volume (µL)	5	5	5	5	5					
Upper air gap flow rate (mL/min)	20	120	20	20	120					
Upper air gap volume (µL)	100	100	100	100	100					
Upper air gap dispense pause	300	300	300	300	300					
Conditioning?	Yes	Yes	Yes	Yes	Yes					
Conditioning number of times	2	3	2	2	3					
Conditioning flow rate (mL/min)	20	20	20	20	20					
Conditioning volume (%)	100	100	100	100	100					
Aspirate post dispense	Yes	Yes	Yes	Yes	Yes					
Chlorinated	No	No	No	No	No					
Serial dispense	No	No	No	No	No					

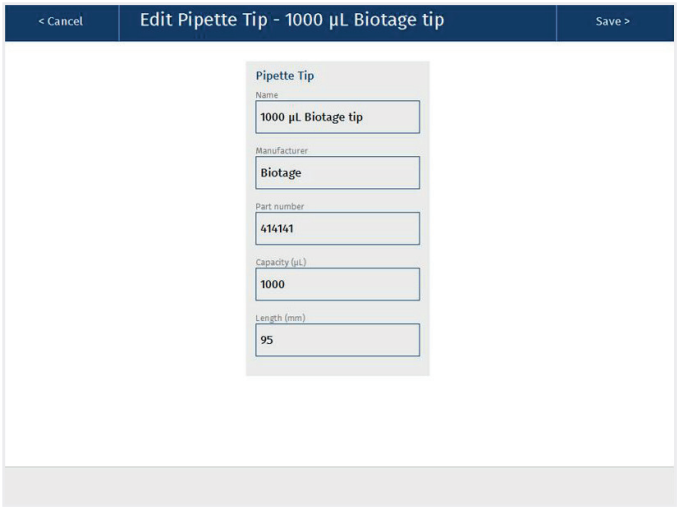


"Sample Screen"	
Sample name	Biotin
Sample description	Biotin
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	20
Lower air gap flow rate (mL/min)	20
Lower air gap volume (µL)	5
Upper air gap flow rate (mL/min)	120
Upper air gap volume (µL)	100
Upper air gap dispense pause	300
Aspirate post dispense	Yes



"Extraction Media Screen"	
Name	EVOLUTE EXPRESS ABN 96 Well Plate
Manufacturer	Biotage
Part number	600-0010-PX01
Capacity volume (µL)	1000
Format	96
Comment	
Solvent dispensation height (mm)	-125.0
Sample dispensation height (mm)	-135.0
Aspiration height (mm)	-135.0

"Sample Plate/Rack" Screen	
Name	2 mL x 96 well 400uL
Capacity volume (µL)	1800
Format	96
Aspiration height (mm)	-161.0
Pretreatment dispensation height (mm)	-135.0



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

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 2898 6655
Fax: +86 21 2898 6153
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

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