Extraction of Methylmalonic Acid from Serum Using EVOLUTE® EXPRESS AX Prior to LC-MS/MS Analysis

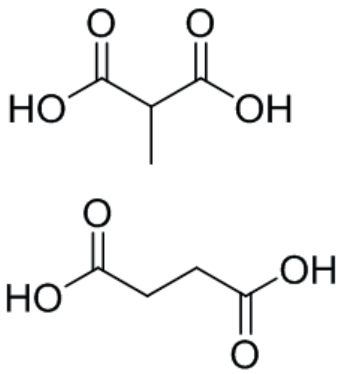

Figure 1. Structures of methylmalonic acid (MMA) and succinic acid (SA).

Introduction

Methylmalonic acid (MMA) in serum is measured to help diagnose a number of disorders, primarily Vitamin B12 deficiency. This application note describes a simple, effective protocol for the extraction of methylmalonic acid (MMA) from serum using EVOLUTE® EXPRESS AX solid phase extraction plates, demonstrating high, reproducible analyte recoveries with low protein and phospholipid content in the extracts. The well-known isobaric interference, succinic acid, is chromatographically separated to allow accurate quantitation of the MMA.

EVOLUTE EXPRESS AX plates contain a polymer-based mixed-mode sorbent with an optimized combination of non-polar (hydrophobic), polar (hydrophilic) and strong anion exchange interactions for extraction of acidic analytes such as MMA from aqueous samples. The mixed-mode retention mechanism allows a rigorous wash protocol to remove co-extracted endogenous interferences.

EVOLUTE EXPRESS solid phase extraction products combine powerful EVOLUTE sorbent chemistry with enhanced 'EXPRESS' components. EVOLUTE EXPRESS products dramatically improve flow characteristics, and enhance sample preparation productivity. By truly eliminating the need for conditioning and equilibration, samples can be prepared using a simple, fast load-wash-elute procedure.

Analytes

MMA and MMA-$^{13}C_4$ as internal standard.

Sample Preparation Procedure

Format:

EVOLUTE® EXPRESS AX 30 mg fixed well plate, part number 603-0030-PX01

Sample Pre-treatment

To serum (100 µL), add 10 µL of ISTD (10 ng/µL). Allow to stand for ~1 hour to allow binding to occur. Add HPLC grade water (290 µL) and vortex.

Sample Loading

Load pre-treated sample (400 µL) direct to the 96-well plate.

Wash 1

Elute interferences with HPLC grade water (1 mL).

Wash 2

Elute interferences with methanol (1 mL).

Analyte Elution

Elute analytes into a collection plate using 2% formic acid in acetonitrile (1 mL).

Post Extraction

Dry the extract in a stream of air or nitrogen using a SPE Dry (40 °C at 40 L/min) or TurboVap (40 °C at 1.0 bar).

Reconstitution

Add 100 µL of 0.4% formic acid (aq), seal with a plate mat and vortex for 30 seconds.

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UPLC Conditions

Instrument
Waters ACQUITY I Class UPLC equipped with a flow through needle (15 µL)

Column
Gemini 3 µm C18 (100 x 3 mm id)

Mobile Phase
A: 0.4% formic acid (aq)

B: 0.4% formic acid in methanol

Flow Rate
0.6 mL/min

Table 1. Gradient Conditions - numerical.

Step	%A	%B	Curve
0	100	0	1
1	100	0	6
2.5	98	2	6
3	100	0	11

Curve 6: Linear Gradient

Curve 11: Conditions in line initiated immediately once time reached. i.e. 0% B resumed at 3 minutes.

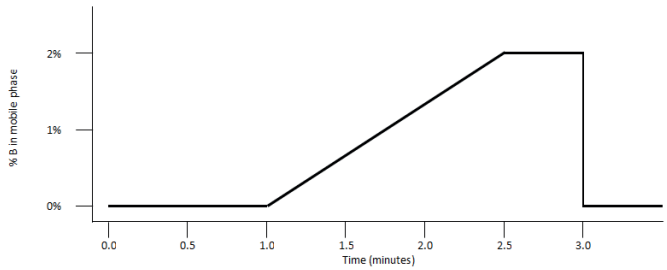


Figure 2. Gradient Conditions - graphical

Injection Volume
10 µL

Sample Temperature
20 °C

Column Temperature
50 °C

MS Conditions

Instrument
Waters XEVO TQS triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

Desolvation Temperature:
500 °C

Ion Source Temperature:
150 °C

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

Table 2. MRM Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
MMA	116.9 > 72.9	30	9
MMA-$^{13}C_4$	121.0 > 76.0	30	9

Results

Chromatography
Good separation was achieved between MMA and the isobaric interference succinic acid. **Figure 3.** shows a chromatogram of serum spiked with 10 ng/mL MMA and the baseline raised to 10:1 signal:noise, indicating an approximate lower limit of quantitation.

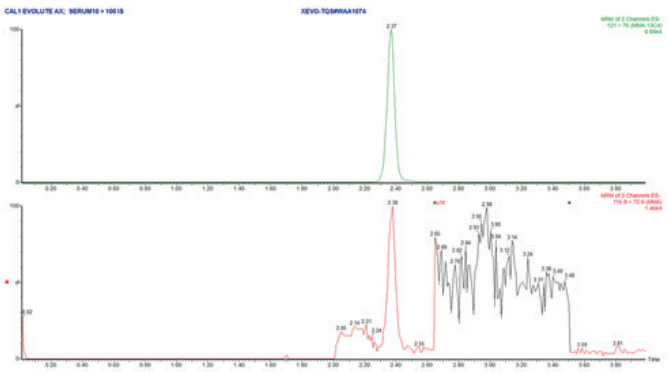


Figure 3. Chromatogram of $^{13}C_4$ MMA (top) at 100 ng/mL and MMA (bottom) at 10 ng/mL (~0.085 µMol/L) with x10 signal:noise indicator for the latter.

Recovery

Serum free of MMA was spiked at 250 ng/mL (~2.11 µMol/L). High reproducible recoveries >90% and corresponding RSDs of <10% were demonstrated. Typical recovery data is very comparable between protocols that include or exclude 1 mL steps of methanol and water to condition the plate, as shown in **Figure 4**.

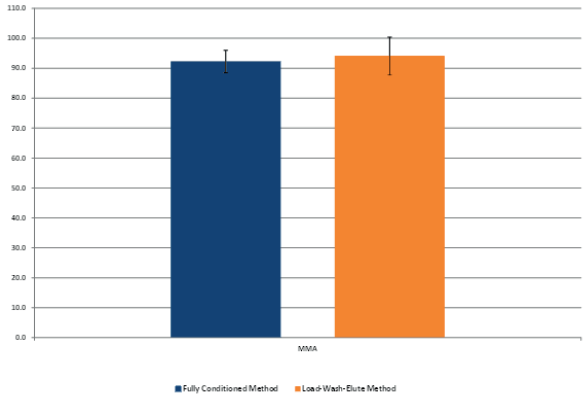


Figure 4. Chart demonstrating MMA recoveries from two extraction protocols.

Calibration Curves

Good linearity was observed over the range 10–2000 ng/mL (~0.085 - ~16.949 µMol/L). **Figure 5**. shows the coefficient of determination r^2 for the optimized method. In addition, commercial calibration samples from plasma matrix were extracted and their concentration values were evaluated against the in-house calibration line. Good agreement was reached and concentrations are summarized in **Table 3**.

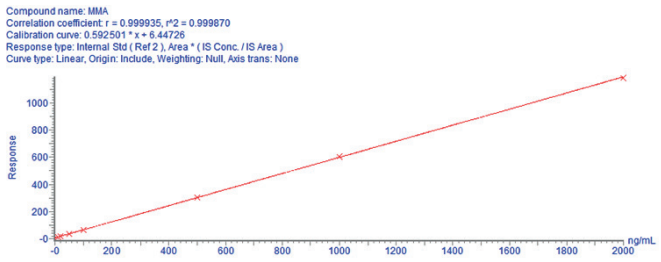


Figure 5. Calibration line of spiked serum extracted with the optimized protocol.

Table 3. Calculated MMA concentrations.

Chromsystems Calibration Level	Set Value (ng/mL)	Calculated Value (ng/mL)
Calibrator 1	13.7	10.4
Calibrator 2	28.4	27.3
Calibrator 3	51.3	53.3

Additional Notes

Processing Guidelines

- 96-well SPE plates were processed using a Biotage® PRESSURE+96 Positive Pressure Manifold at a pressure of 1–2 psi

Solvent Composition and Preparation Instructions

- All solvents were HPLC grade.
- 2% formic acid in acetonitrile: Add 200 µL concentrated formic acid to 9.8 mL of HPLC grade acetonitrile.
- 0.4% formic acid (aq): Add 200 µL concentrated formic acid to 49.8 mL of HPLC grade water.
- 0.4% formic acid in methanol: Add 200 µL concentrated formic acid to 49.8 mL of HPLC grade methanol.

Ordering Information

Part Number	Description	Quantity
603-0030-PX01	EVOLUTE® EXPRESS AX 30 mg Fixed Well Plate*	1
603-0003-AXG	EVOLUTE® EXPRESS AX 30 mg/ 1 mL (tabless)	100
121-5203	Collection plate, 2 mL, square	50
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1
C103264	TurboVap® 96, Evaporator 220/240V	1
C103263	TurboVap® 96, Evaporator 100/120V	1

*EVOLUTE EXPRESS AX is also available in tabless (or flangeless) column format. Up to 96 columns can populate a base plate for processing using Extrahera, Pressure+ or vacuum manifold, as a cost effective alternative to a 96-well plate.

Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™, using EVOLUTE® EXPRESS AX 30 mg SPE plates. Total time taken to process a full 96-well plate was 35 minutes. Method performance was comparable.

This appendix contains the software settings required to configure Extrahera to run this method.

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

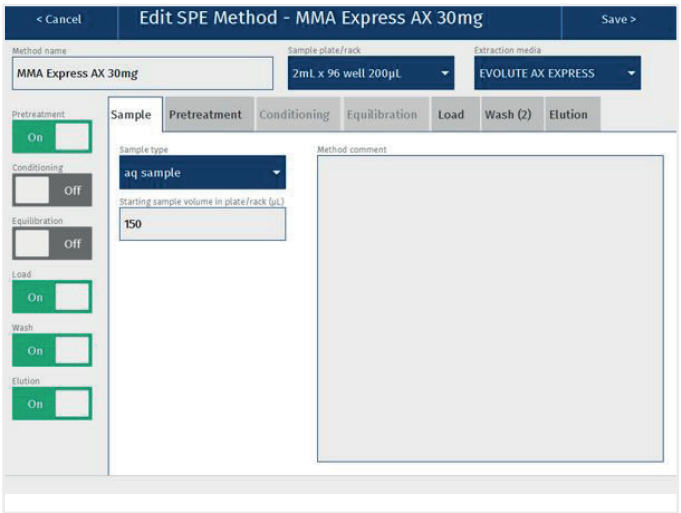
Biotage® Extrahera™ Data

Analyte	Methylmalonic Acid
Recovery (n=8) at 100 ng/mL	94.8
%RSD	1.5
Linearity (r^2)	0.994*
LLOQ	<10 ng/mL

*Note: Linearity experiments on Extrahera were run using 3PLUS1® Multilevel Plasma Calibrator Set Methylmalonic acid (Chromsystems Instruments and Chemicals GmbH). Manual processing using these standards gave linearity (r^2) of 0.990.

Data (manual processing) in the application note was generated using 'in house' spiked MMA free serum from Golden West Biologicals, Inc.

Sample Name: MMA EVOLUTE® Express AX
Sample Plate/Rack: 2 mL x 96 well 200 uL
Extraction Media: EVOLUTE® Express AX 30 mg

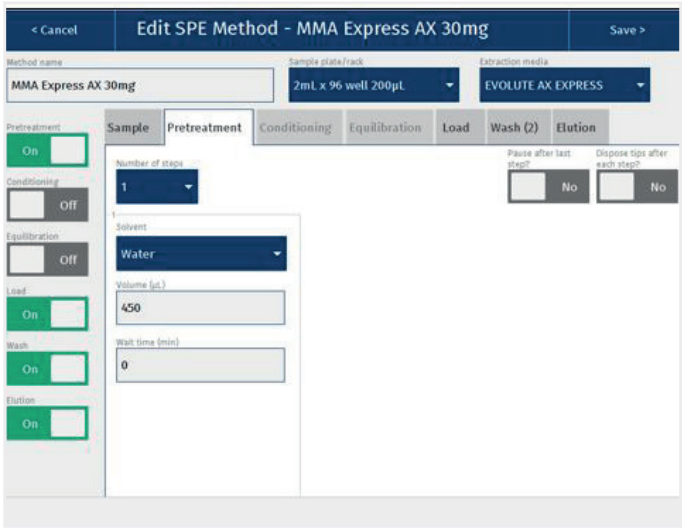


Settings

"Sample" Tab

Sample Type: Aqueous Sample
Starting Sample Volume (µL): 150
Method Comment:

Screenshot

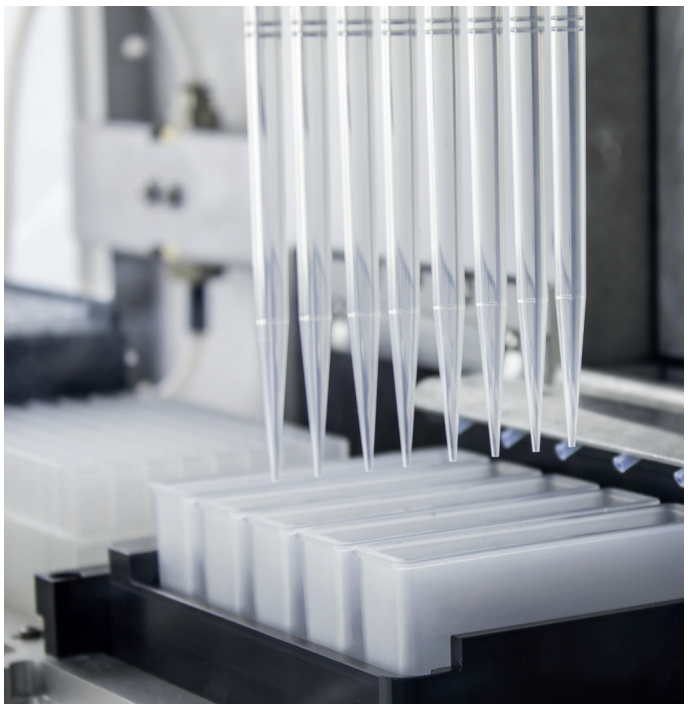


Settings

	Pre-treatment	Activated
	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)	450			
Wait Time (min)	0			



	Conditioning	Not Activated
	No. of steps	
	Pressure (Bar)	
	Dispose tips after this step	

	Solvent
1	
2	
3	
4	

	1	2	3	4
Volume (µL)				
Position				
Positive pressure time (s)				
Repeat				
Pause after this step				

	Advanced Settings	Not Activated

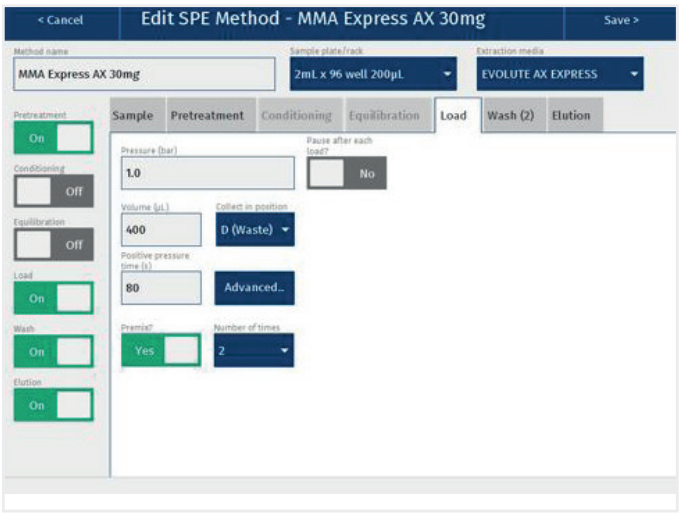
Biotage®

Equilibration	Not Activated
No. of steps	
Pressure (Bar)	
Dispose tips after this step	

Solvent
1
2
3
4

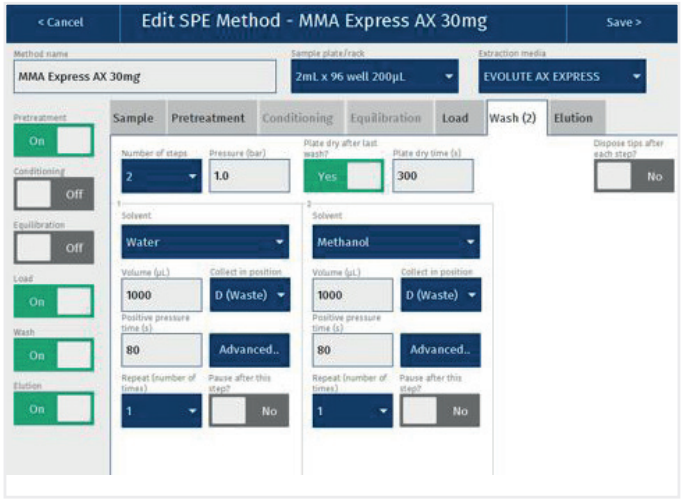
	1	2	3	4
Volume (µL)				
Position				
Positive Pressure time (s)				
Repeat				
Pause after this step				

Advanced Settings



Load	
Pressure (Bar)	1.0
Pause after each load	No
Volume (µL)	400
Collect in position	D
Positive pressure time (s)	80
Premix	Yes
Number of times	2

Advanced Settings

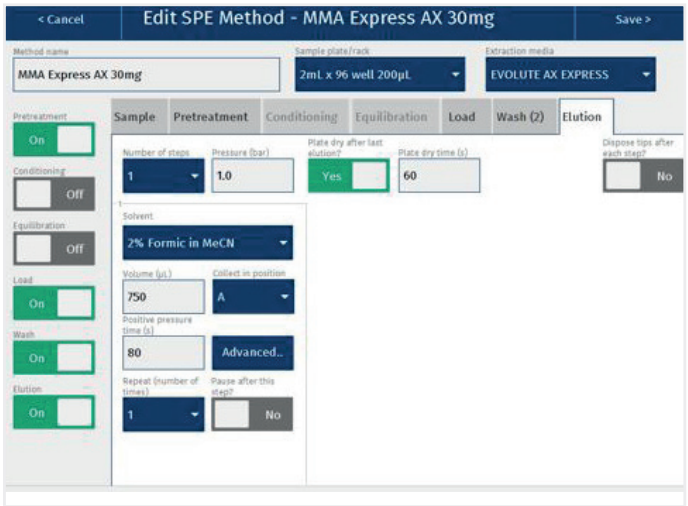


	Wash	
	No. of steps	2
	Pressure (Bar)	1.0
	Plate dry after last wash	Yes
	Plate dry time (s)	300
	Dispose tips after last step	No

	Solvent
1	Water
2	Methanol
3	
4	

	1	2	3	4
Volume (µL)	1000	1000		
Position	D	D		
Positive pressure time (s)	80	80		
Repeat	1	1		
Pause after this step	No	No		

	Advanced Settings



	Elution	Activated
	No. of steps	1
	Pressure (Bar)	1.0
	Plate dry after last elution	Yes
	Plate dry time(s)	60
	Dispose tips after each step	No

	Solvent
1	2% Formic in MeCN
2	
3	
4	

	1	2	3	4
Volume (µL)	750			
Position	A			
Positive pressure time (s)	80			
Repeat	1			
Pause after this step	No			

	Advanced Settings

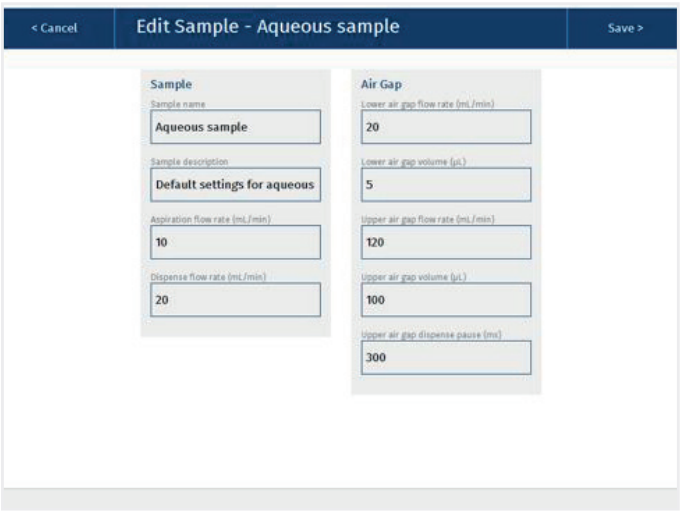
Biotage®

Solvent Properties

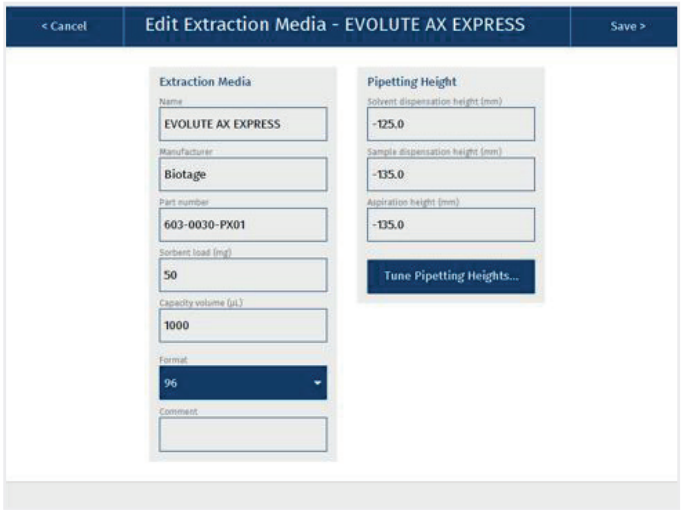
	Solvent Description
1	Water
2	Methanol
3	2% Formic in MeCN
4	
5	
6	
7	
8	
9	
10	

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							
Dispense flow rate (mL/min)	20	20	20							
Lower air gap flow rate (mL/min)	20	20	20							
Lower air gap volume (µL)	5	5	5							
Upper air gap flow rate (mL/min)	20	120	120							
Upper air gap volume (µL)	100	100	100							
Upper air gap dispense pause	300	300	300							
Conditioning?	Yes	Yes	Yes							
Conditioning number of times	2	3	3							
Conditioning flow rate (mL/min)	20	20	20							
Chlorinated	No	No	No							
Serial dispense	No	No	No							

Biotage®

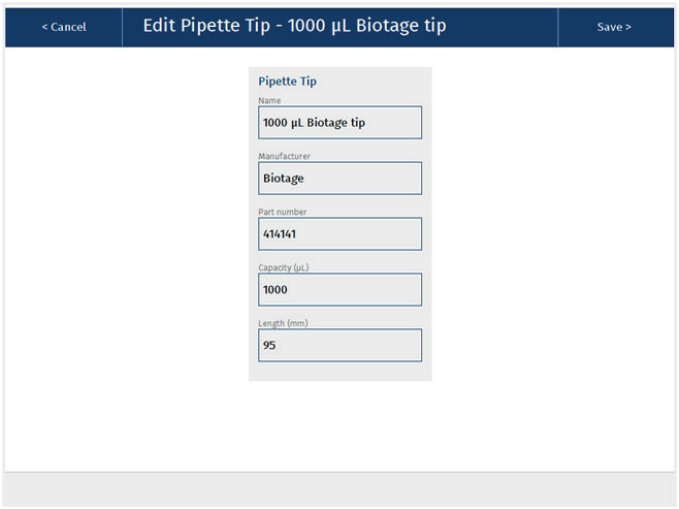


"Sample" Screen	
Sample name	Aqueous sample
Sample description	Aqueous sample
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	20
Lower air gap flow rate (mL/min)	20
Lower air gap volume (µL)	5
Upper air gap flow rate (mL/min)	120
Upper air gap volume (µL)	100
Upper air gap dispense pause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



"Extraction Media" Screen	
Name	EVOLUTE® Express AX 30mg
Manufacturer	Biotage
Part number	603-0030-PX01
Sorbent load (mg)	10
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 Sample x 96 well
Capacity volume (µL)	1800
Format	96
Aspiration height (mm)	-161.0
Pre-treatment dispensation height (mm)	-153.0



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

Additional Information

In this automated method, 150 µL of pre-spiked (IS) serum sample is mixed with 450 µL of water during the pre-treatment step. This gives a total volume of 600 µL, from which 400 µL is loaded.

Conditioning and equilibration steps are deactivated for this EVOLUTE® EXPRESS load-wash-elute SPE procedure.

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