Extraction of Methylmalonic Acid from Serum Using ISOLUTE® SLE+ Prior to LC-MS/MS Analysis

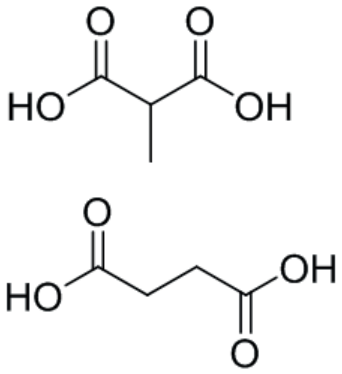

Figure 1. Structures of methylmalonic acid (MMA) and succinic acid (SA).

Introduction

Methylmalonic acid (MMA) in serum is measured to help diagnose a number of disorders, primarily Vitamin B12 deficiency. This application note describes the extraction of methylmalonic acid from serum, prior to LC-MS/MS analysis. An effective and efficient ISOLUTE® SLE+ protocol optimized for the 200 µL capacity 96-well plate format is used. The simple sample preparation procedure delivers clean extracts and analyte recoveries greater than 80% with RSDs below 10%.

ISOLUTE® SLE+ Supported Liquid Extraction plates and columns offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation.

Analytes

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

Sample Preparation Procedure

Format:

ISOLUTE® SLE+ 200 µL Supported Liquid Extraction plate, part number 820-0200-P01

Sample Pre-treatment

To serum (100 µL), add 10 µL of ISTD (10 ng/µL). Allow to equilibrate and add 4.6M formic acid (aq) (100 µL). Mix.

Sample Loading

Load the pre-treated serum (200 µL) onto the plate and apply a pulse of vacuum or positive pressure (3–5 seconds) to initiate flow. Allow the sample to absorb for 5 minutes.

Analyte Extraction

Apply MTBE (750 µL) and allow to flow under gravity for 5 minutes. Apply vacuum or positive pressure (5–10 seconds) to pull through any remaining extraction solvent.

Post Elution

Evaporate the extract in a stream of air or nitrogen using a SPE Dry (40 °C, 20 to 40 L/min). Addition of 2 µL of ethylene glycol prior to evaporation can help reduce analyte losses due to volatility (optional).

Reconstitution

Reconstitute extracts with 100 µL of 0.4% formic acid (aq).

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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. 100% resumed at 3 minutes.

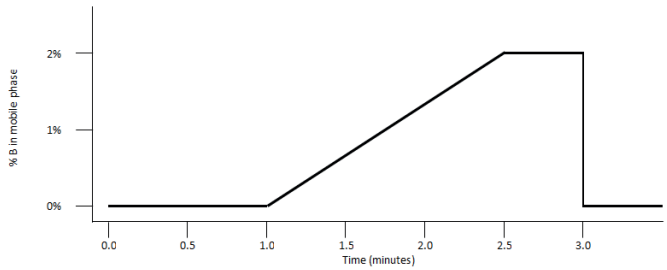

Figure 2. Gradient Conditions - graphical

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**. demonstrates 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.

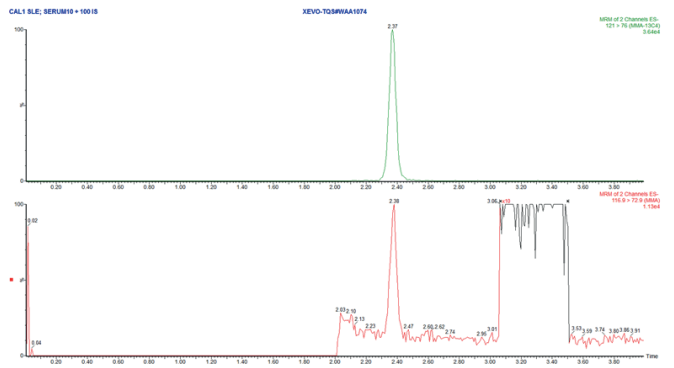

Figure 3. Chromatogram of $^{13}C_4$ MMA (top) at 100 ng/mL and MMA (bottom) at 10 ng/mL (~0.085 µMol/L) with x10 signal:noise indicator for the latter.

Recovery

Serum free of MMA was spiked at 250 ng/mL (~2.11 µMol/L). High reproducible recoveries ›80% and corresponding RSDs of ‹10% were demonstrated. Typical recovery data is shown in **Figure 4**.

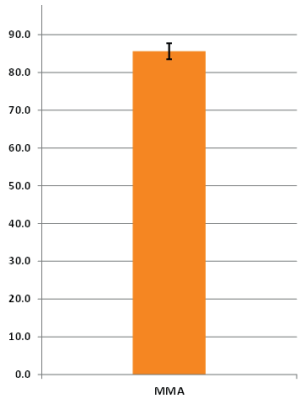

Figure 4. Chart demonstrating typical MMA recovery using the optimized ISOLUTE® SLE+ method.

Calibration Curves

Good linearity was observed over the range 10 – 2000 ng/mL (~0.085 - ~16.949 µMol/L). **Figure 5**. demonstrates 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**.

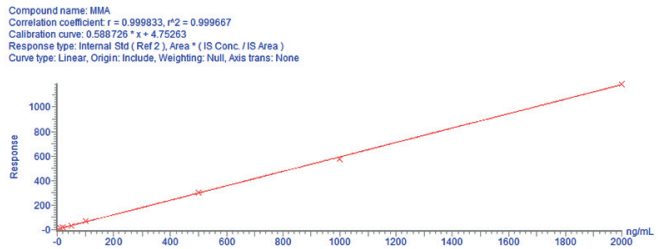

Figure 5. Calibration line of spiked serum extracted with the optimized protocol.

Table 3. Calculated MMA concentrations.

Chromsystems Calibration Level	Set Value (ng/mL)	Calculated Value (ng/mL)
Calibrator 1	13.7	10.0
Calibrator 2	28.4	27.8
Calibrator 3	51.3	56.5

Additional Notes

Solvent composition and preparation instructions

- All solvents were HPLC grade.
- 4.6M (17.7%) formic acid (aq): Add 8.85 mL of concentrated formic acid to 41.15 mL of HPLC grade water.
- 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
820-0200-P01	ISOLUTE® 200 µL Supported Liquid Extraction Plate	1
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

Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™, using ISOLUTE® SLE+ Supported Liquid Extraction plates. Total time taken to process a full 96-well plate was 27 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	74.8%
%RSD	3.2
Linearity (r^2)	0.999*
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 0.999.

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

Method Name: MMA ISOLUTE® SLE+ 200
Sample Plate/Rack: 2 mL sample plate, 96
Extraction Media: ISOLUTE SLE+ 200 µL 96 Well Plate

Settings

"Sample" Tab

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

SLE method for MMA in serum. Start with 0.15 mL of serum in a 2 mL collection block and run on an ISOLUTE SLE+ 200 plate with MTBE as the elution solvent. The method uses a highly acidic pretreatment solvent, as a result it is recommended to use this in one of the non-refillable front reservoirs and that this is safely washed after use.

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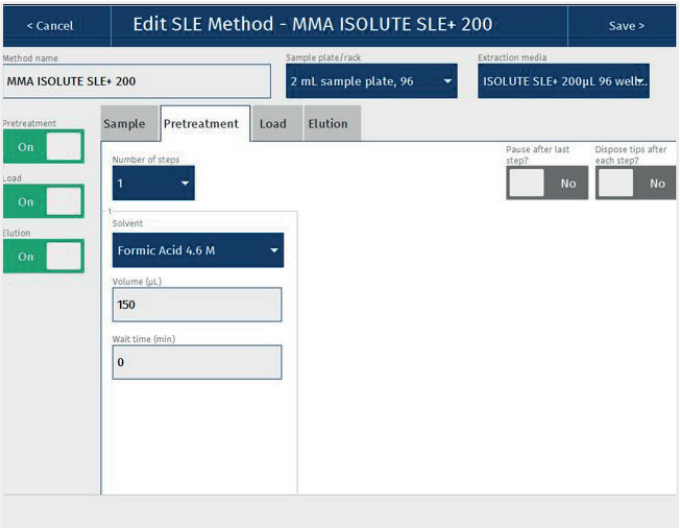
Screenshot

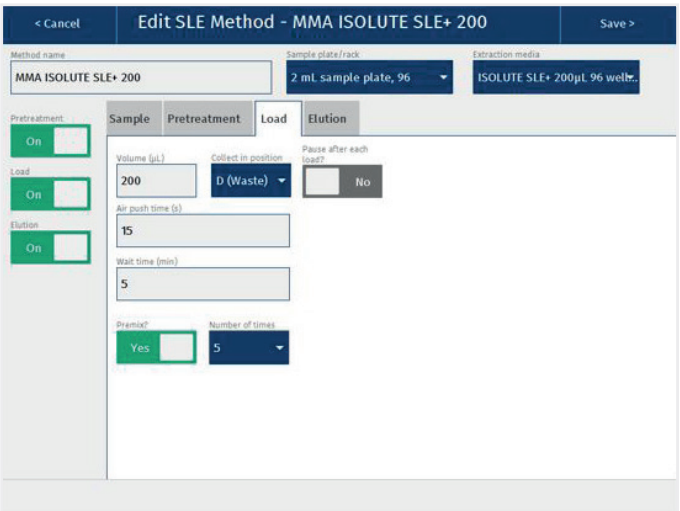


Settings

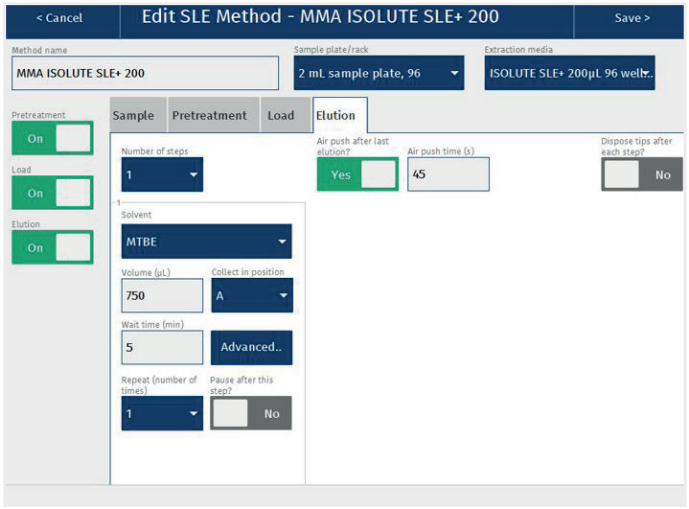
	Pre-treatment	Activated
	No. of steps	1
	Pause after last step	Yes
	Dispose tips after last step	No

	Solvent
1	Formic Acid 4.6 M
2	
3	
4	

	1	2	3	4
Volume (µL)	150			
Wait Time (min)	0			



	Load	
	Air Push Time(s)	15
	Pause after each load	No
	Volume (µL)	200
	Collect in position	D
	Air Push Time (s)	15
	Wait time (min)	5
	Premix	Yes
	Number of times	5

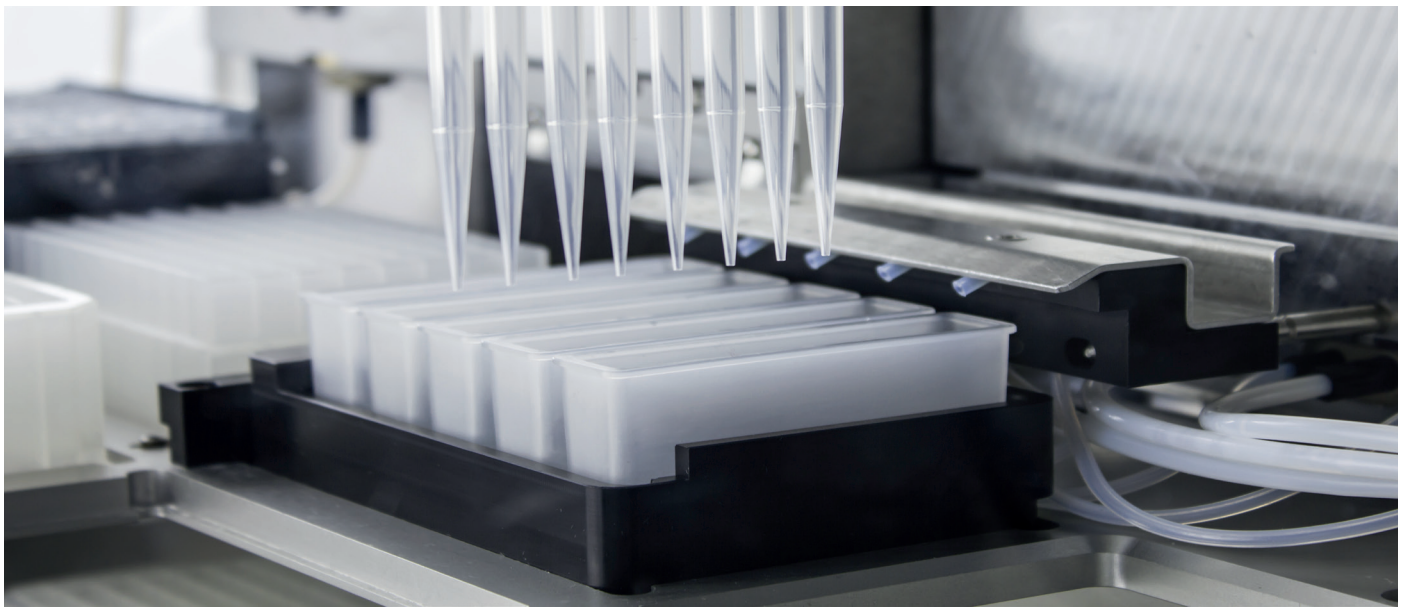


	Elution	Activated
	No. of steps	1
	Pause after last step	No
	Air push after last elution	Yes
	Air push time (s)	45
	Dispose tips after each step	No

	Solvent
1	MTBE
2	
3	
4	

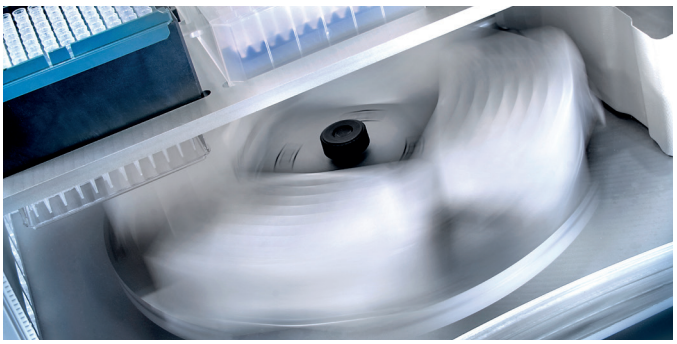
	1	2	3	4
Volume (µL)	750			
Collect in position	A			
Wait time (min)	5			
Repeat	1			
Pause	No			

Advanced Settings
No

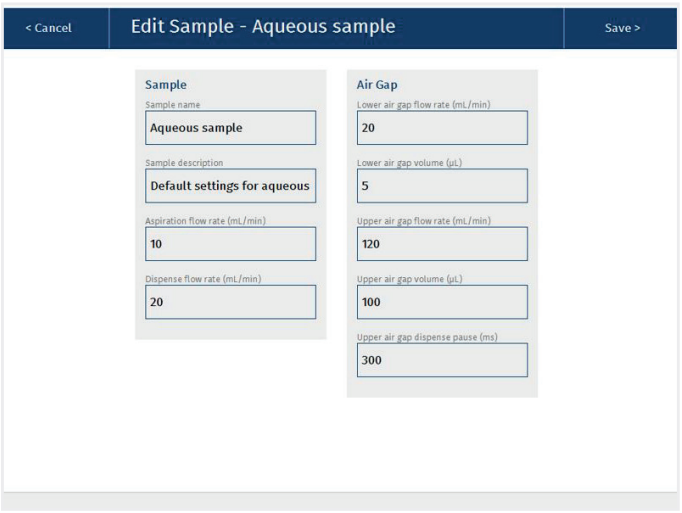


Solvent Properties

	Solvent Description
1	Methyl Tert Butyl Ether
2	
3	
4	
5	
6	4.6 M formic acid in water
7	
8	
9	
10	

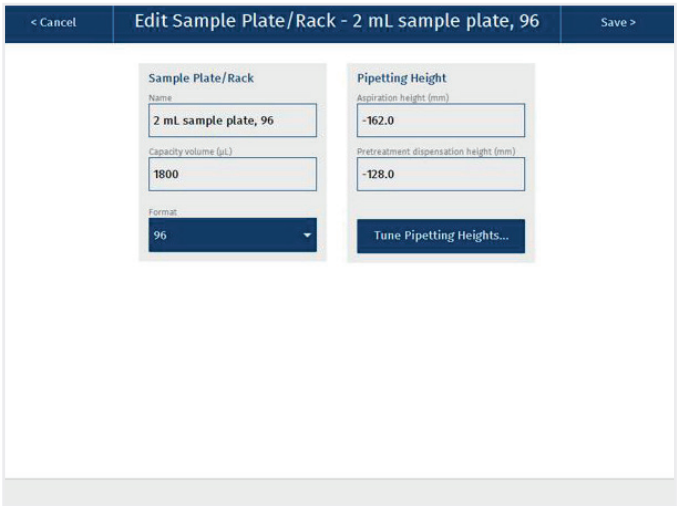


Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type	**Refillable**					**Non Refillable**				
Capacity	N/A					N/A				
Aspiration flow rate (mL/min)	10					10				
Dispense flow rate (mL/min)	10					20				
Lower air gap flow rate (mL/min)	10					20				
Lower air gap volume (µL)	5					5				
Upper air gap flow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

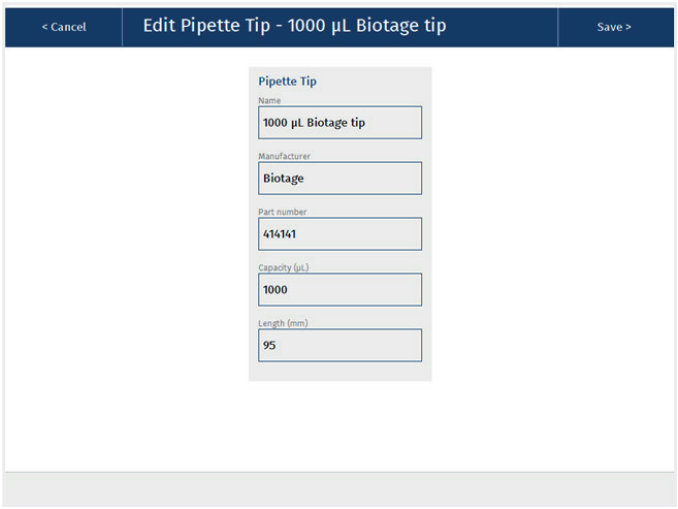


"Sample" Screen	
Sample name	Aqueous sample
Sample description	Aqueous sample
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	20
Lower air gap flow rate (mL/min)	20
Lower air gap volume (µL)	5
Upper air gap flow rate (mL/min)	120
Upper air gap volume (µL)	100
Upper air gap dispense pause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

"Extraction Media" Screen	
Name	ISOLUTE® SLE+ 200 µL 96 well plate
Manufacturer	Biotage
Part number	820-0200-P01
Sorbent load (mg)	0
Capacity volume (µL)	0
Format	96
Comment	N/A
Solvent dispensation height (mm)	-125.0
Sample dispensation height (mm)	-130.5
Aspiration height (mm)	-148.5



"Sample Plate/Rack" Screen	
Name	2 mL Sample plate, 96
Capacity volume (µL)	1800
Format	96
Aspiration height (mm)	-162.0
Pre-treatment dispensation height (mm)	-128.0



"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 150 µL of water during the pre-treatment step. This gives a total volume of 300 µL, from which 200 µL is loaded.

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