Extraction of Methylmalonic Acid from Serum Using ISOLUTE® PPT+ Protein Precipitation Plates Prior to LC-MS/MS Analysis

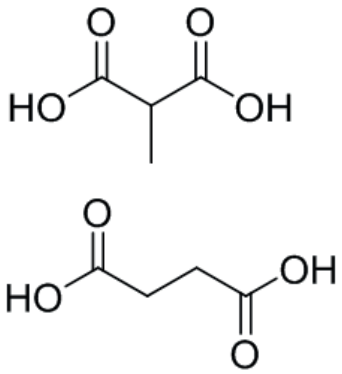


Figure 1. Structures of methylmalonic acid (MMA) and succinic acid (SA).

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 ISOLUTE® PPT+ protocol for the extraction of methylmalonic acid (MMA) from serum, demonstrating high, reproducible analyte recoveries with low protein content in the extracts.

ISOLUTE PPT+ Protein Precipitation plates offer a fast and extremely simple sample preparation approach. Serum proteins are efficiently removed from the sample using an in-plate, 'solvent first', protein crash and filter procedure, which is easily automated.

Analytes

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

Sample Preparation Procedure

Format:

ISOLUTE® PPT+ Protein Precipitation plate, part number 120-2040-P01

Sample Pre-treatment

To serum (100 µL), add 10 µL of ISTD (10 ng/µL). Mix. Allow to stand for ~1 hour to allow binding to occur.

Solvent Application

Apply 800 µL of 1% (v/v) formic acid in acetonitrile (MeCN) to each well of the ISOLUTE® PPT+ plate.

Sample Application

Add 100 µL of serum with ISTD to each well and mix thoroughly via repeat aspirate/dispense steps.

Analyte Elution

Apply vacuum (-0.2 bar) or positive pressure (3 psi) for approximately 5 minutes. For highly particulate laden or viscous samples, increased pressure or vacuum conditions may be required.

Post Extraction

Dry the extract in a stream of air or nitrogen using a Biotage® 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) and vortex for 30 seconds.

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.

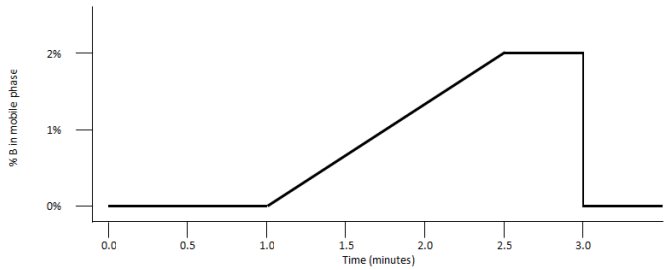


Figure 2. Gradient Conditions - graphical

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

Recovery
Serum free of MMA was spiked at 250 ng/mL (~2.11 µMol/L). High reproducible recoveries (ave 84.7%, n=8) and corresponding RSDs of 1.3% were achieved.

Calibration Curves
Calibration curves were generated using a commercially available methylmalonic acid calibrator set. Good linearity was observed over the range 5–50 ng/mL. Figure 2 shows the coefficient of determination r^2 for the optimized method.

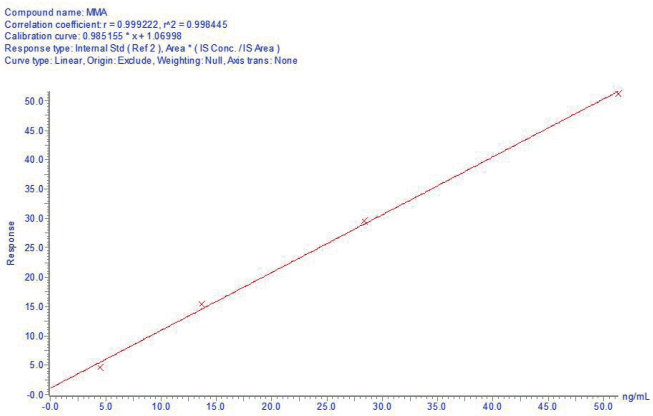


Figure 3. Calibration line of spiked serum extracted with the optimized protocol.

Additional Notes

Processing Guidelines

- Positive Pressure: Process at approximately 3 psi.
- Vacuum Processing: Process at approximately -0.2 bar.

For maximum protein removal, do not exceed these vacuum/pressure conditions. For very viscous samples, a slight increase in pressure or vacuum may be required. The use of high vacuum/pressure conditions may lead to breakthrough of matrix components.

Solvent Composition and Preparation Instructions

- All solvents were HPLC grade.
- 1% formic acid in acetonitrile: Add 100 µL concentrated formic acid to 9.9 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
120-2040-P01	ISOLUTE® PPT+ Protein Precipitation Plate	1
121-5203	Collection plate, 2 mL, square	50
121-9600	Biotage® VacMaster™-96 Sample Processing Manifold	1
121-9602	VacMaster VCU-2 Vacuum Control and Generation Unit	1
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
C103263	TurboVap® 96, Evaporator 100/120V	1
C103264	TurboVap® 96, Evaporator 220/240V	1

Appendix

Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™, using ISOLUTE® PPT+ Protein Precipitation plates. Total time taken to process a full 96-well plate was 22 minutes. Method performance was comparable.

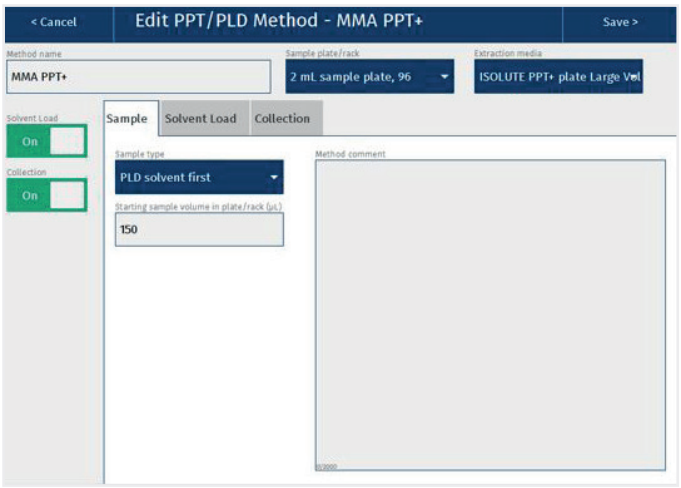
This appendix contains the software settings required to configure Extrahera to run this method. An importable electronic copy of this method for Extrahera can be downloaded from www.biotage.com

Biotage® Extrahera™ Data

Analyte	Methylmalonic Acid
Recovery (n=8) at 100 ng/mL	92.5%
%RSD	2.2
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 0.998.

Method Name: MMA PPT+
Sample Plate/Rack: 2 mL sample plate, 96
Extraction Media: ISOLUTE® PPT+ plate large volume

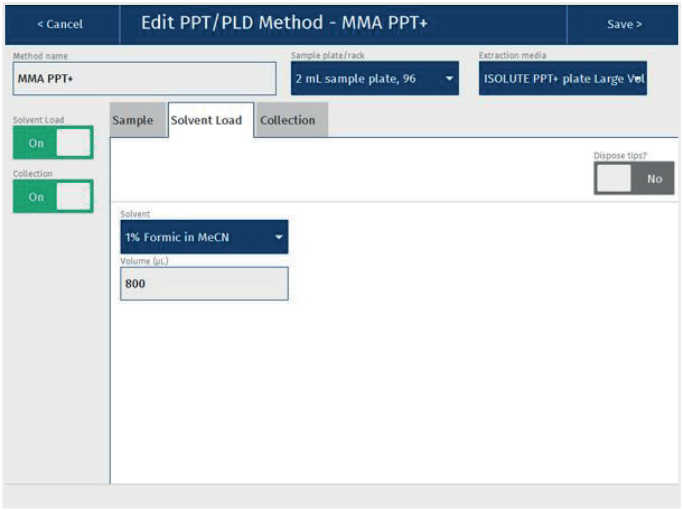


Settings

"Sample" Tab

Sample Type: PLD solvent first
Starting Sample Volume (µL): 150
Method Comment:

Screenshot

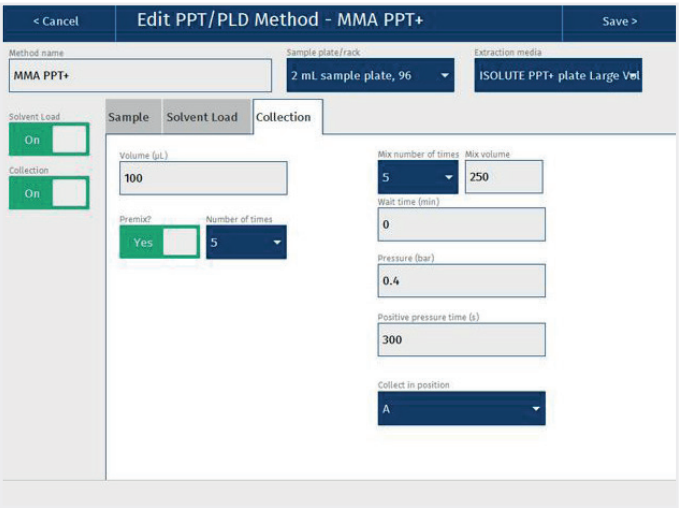


Settings

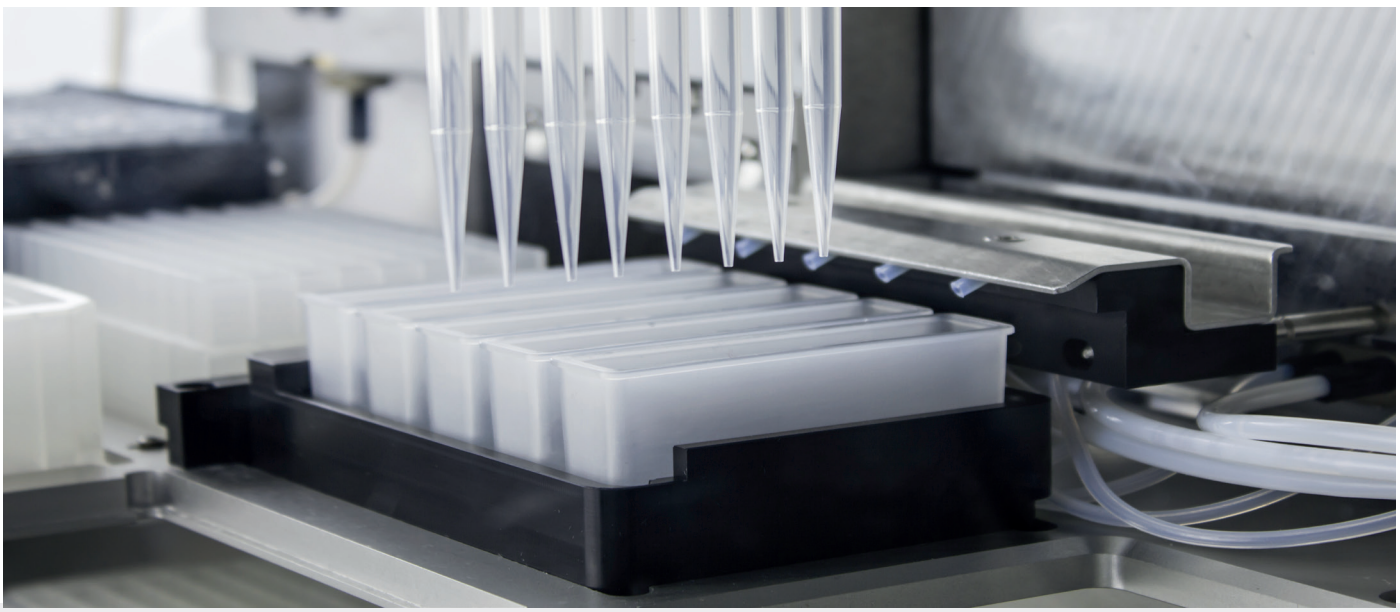
	Solvent Load	Activated
	Dispose tips	No

	Solvent
1	1% Formic in MeCN

	1	2	3	4
Volume (µL)	800			



	Collection	Not Activated
	Volume (µL)	100
	Premix	Yes
	Number of times	5
	Mix number of times	5
	Mix Volume (µL)	250
	Wait time (min)	0
	Pressure (bar)	0.4
	Positive pressure time	300
	Collect in position	A

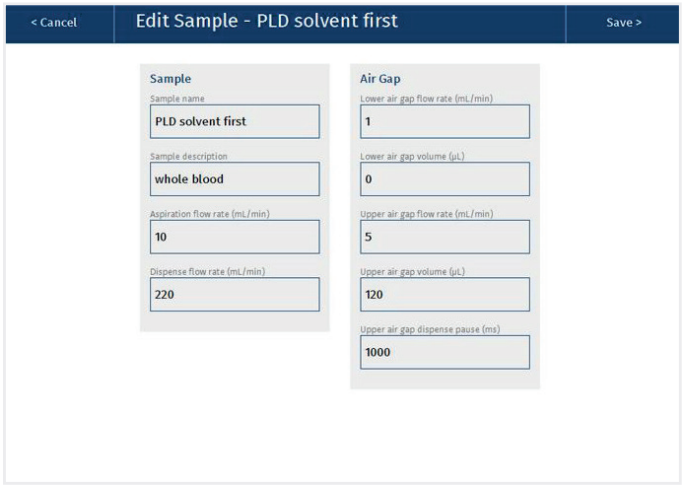


Biotage®

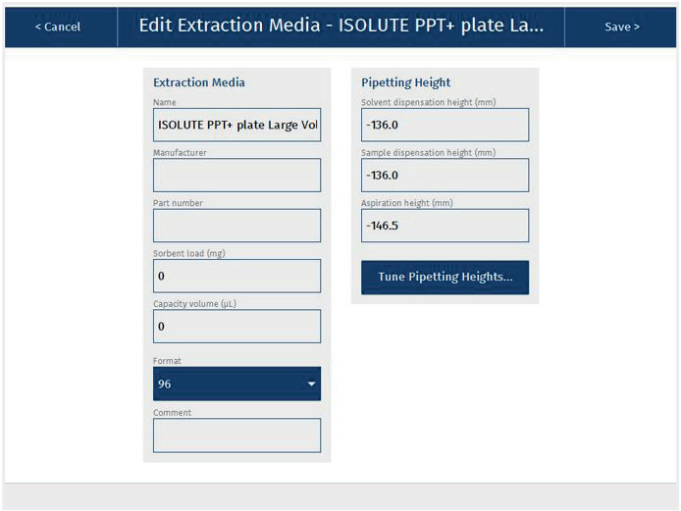
Solvent Properties

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

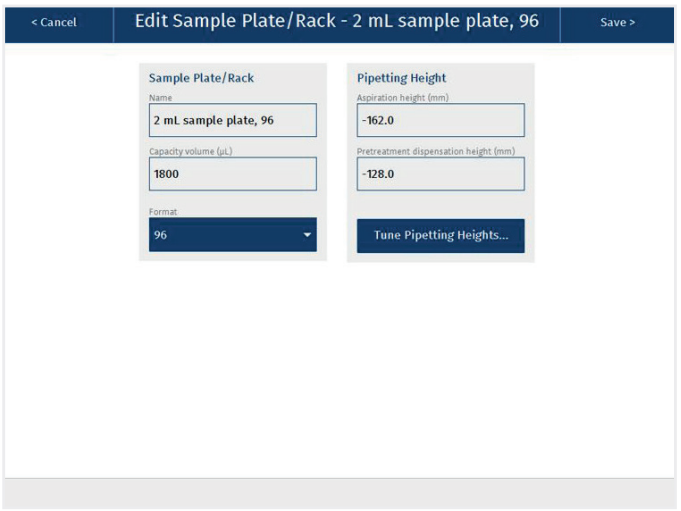
Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type		**Refillable**						**Non Refillable**		
Capacity	N/A									
Aspiration flow rate (mL/min)	10									
Dispense flow rate (mL/min)	20									
Lower air gap flow rate (mL/min)	20									
Lower air gap volume (µL)	5									
Upper air gap flow rate (mL/min)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



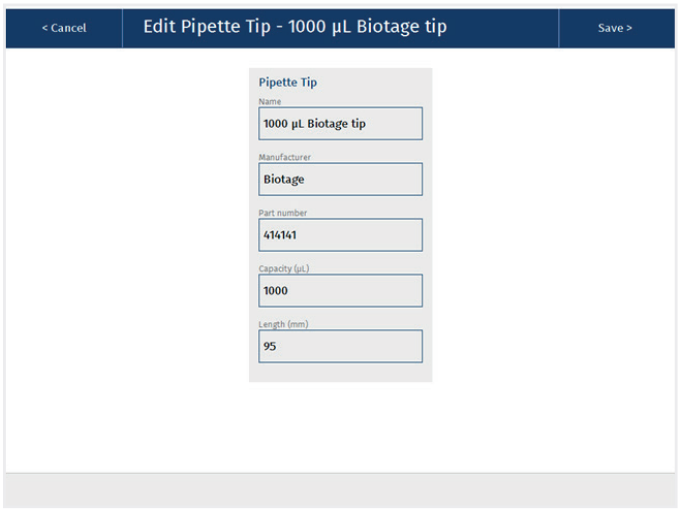
"Sample" Screen	
Sample name	PLD solvent first
Sample description	Whole blood
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	220
Lower air gap flow rate (mL/min)	1
Lower air gap volume (µL)	0
Upper air gap flow rate (mL/min)	5
Upper air gap volume (µL)	120
Upper air gap dispense pause	1000



"Extraction Media" Screen	
Name	ISOLUTE PPT+ plate large volume
Manufacturer	Biotage
Part number	120-2040-P01
Sorbent load (mg)	0
Capacity volume (µL)	0
Format	96
Comment	
Solvent dispensation height (mm)	-136.0
Sample dispensation height (mm)	-136.0
Aspiration height (mm)	-146.5



"Sample Plate/Rack" Screen	
Name	2 mL Sample x 96 well
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

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