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

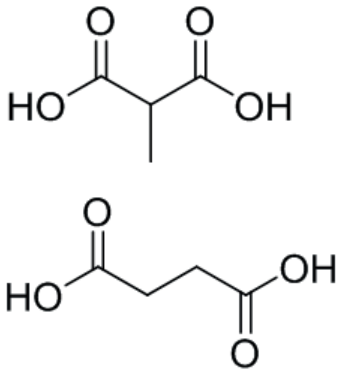


Figure 1. Structures of methylmalonic acid (MMA) and succinic acid (SA).

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

ISOLUTE® PLD+ Protein and Phospholipid Removal products offer a substantial improvement in extract cleanliness compared to traditional protein precipitation techniques for bioanalytical sample preparation. Requiring next to no method development, ISOLUTE PLD+ can be integrated quickly and easily into routine workflow, increasing productivity and reducing instrument downtime.

Analytes

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

Sample Preparation Procedure

Format:

ISOLUTE® PLD+ Protein and Phospholipid Removal plate, part number 918-0050-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® PLD+ 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 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.

Biotage®

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.

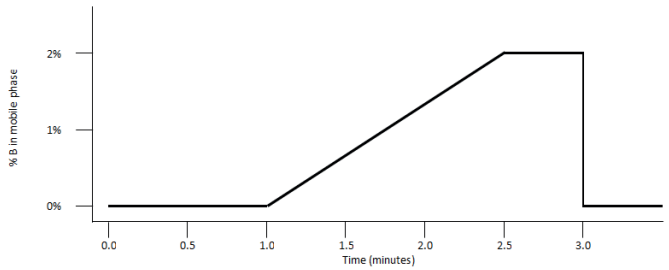


Figure 2. Gradient Conditions - graphical

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**. demonstrates the lower limit of quantitation (10:1 signal:noise) at 10 ng/mL of MMA.

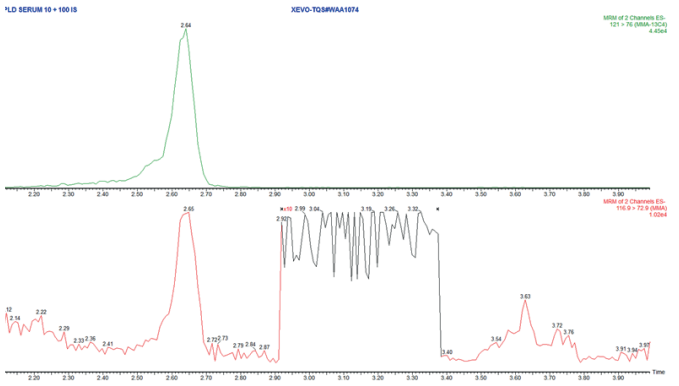


Figure 3. Chromatogram of $^{13}C_4$ MMA (top) at 100 ng/mL and MMA (bottom) at 10 ng/mL (~0.085 µMol/L)

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 shown in **Figure 4**.

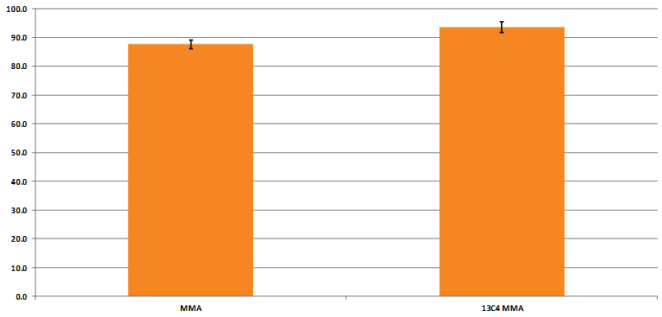


Figure 4. Chart demonstrating MMA and internal standard recoveries.

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**.

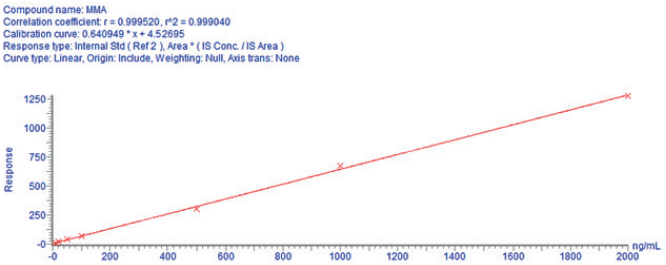


Figure 5. Calibration line of spiked serum extracted with the optimized protocol.

Table 3. Calculated MMA concentrations.

Chromsystems Calibration Level	Set Value (ng/mL)	Calculated Value (ng/mL)
Calibrator 1	13.7	11.8
Calibrator 2	28.4	28.2
Calibrator 3	51.3	51.5

Additional Notes

Processing Guidelines

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

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
918-0050-P01	ISOLUTE® PLD+ Protein and Phospholipid Removal Plate*	1
918-0005-AG	ISOLUTE PLD+ Columns, 50 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

*ISOLUTE® PLD+ 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 ISOLUTE® PLD+ Protein and Phospholipid Removal 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

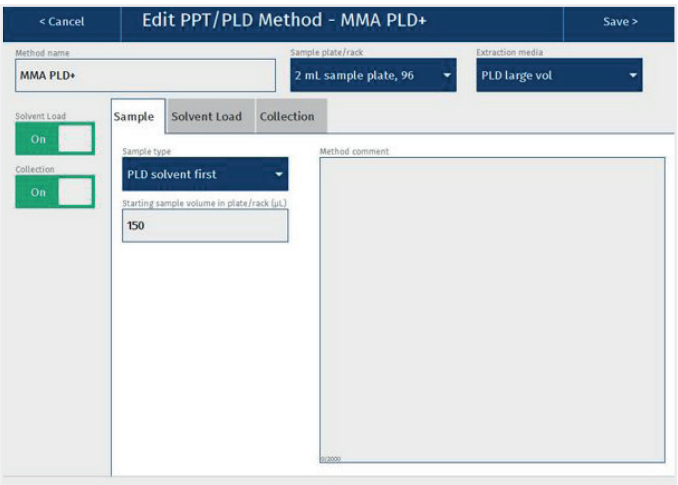
Biotage® Extrahera™ Data

Analyte	Methylmalonic Acid
Recovery (n=8) at 100 ng/mL	78.9%
%RSD	3.2
Linearity (r^2)	0.996*
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 0.992.

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® PLD+
Sample Plate/Rack: 2 mL sample plate, 96
Extraction Media: ISOLUTE PLD+ large volume

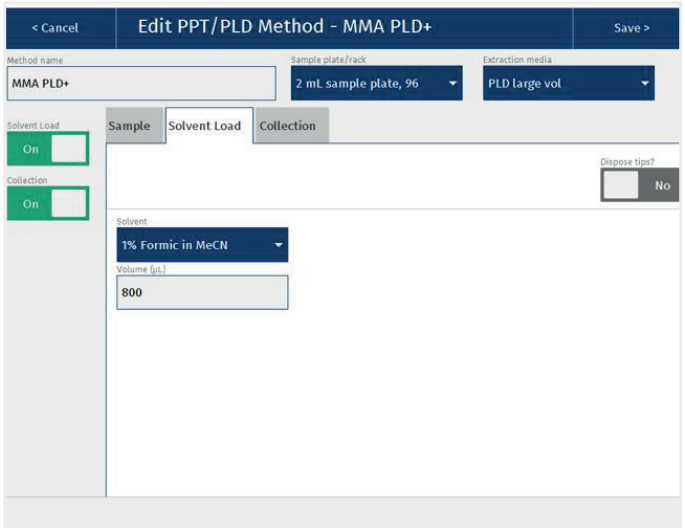


Settings

"Sample" Tab

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

Screenshot

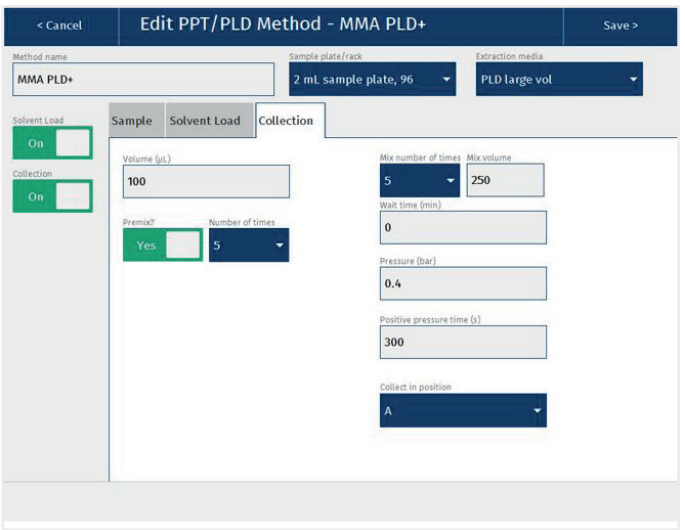


Settings

	Solvent Load	Activated
	Dispose tips	No

	Solvent
1	1% Formic in MeCN

	1
Volume (µL)	800

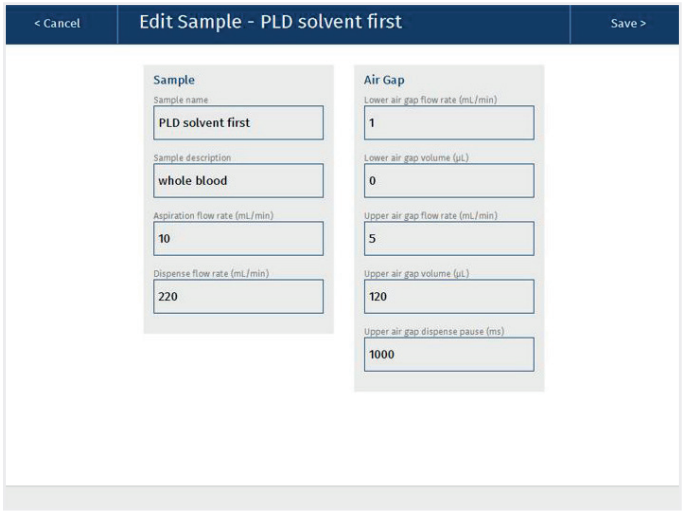


	Collection	Activated
	Volume (µL)	100
	Premix	Yes
	Number of times	5
	Mix number of times	5
	Mix Volume (µL)	250
	Wait time (min)	0
	Pressure (bar)	0.4
	Positive pressure time (s)	300
	Collect in position	A

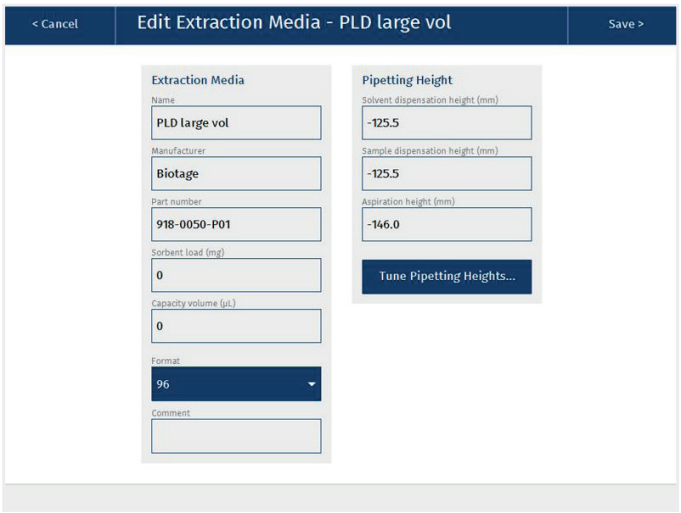
Solvent Properties

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

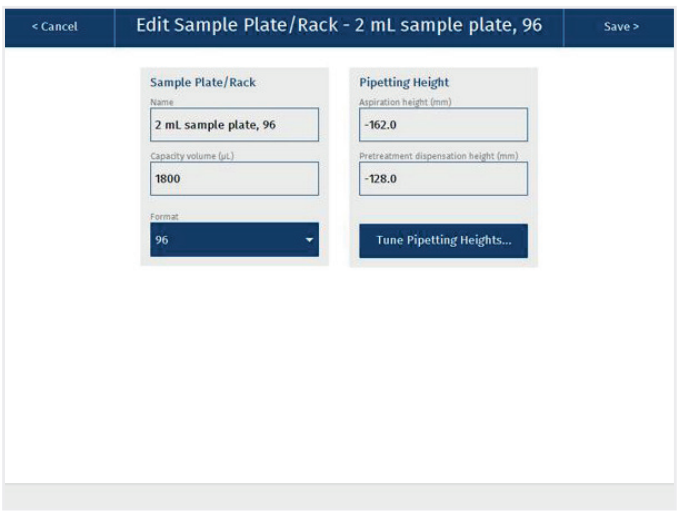
Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type		**Refillable**						**Non Refillable**		
Capacity	N/A									
Aspiration flow rate (mL/min)	10									
Dispense flow rate (mL/min)	20									
Lower air gap flow rate (mL/min)	20									
Lower air gap volume (µL)	5									
Upper air gap flow rate (mL/min)	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									



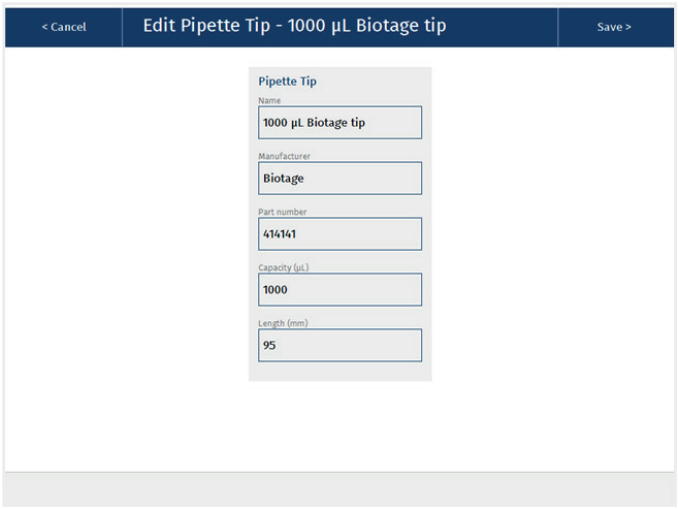
"Sample" Screen	
Sample name	PLD solvent first
Sample description	Whole blood
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	220
Lower air gap flow rate (mL/min)	1
Lower air gap volume (µL)	0
Upper air gap flow rate (mL/min)	5
Upper air gap volume (µL)	120
Upper air gap dispense pause	1000



"Extraction Media" Screen	
Name	ISOLUTE PLD+ large volume
Manufacturer	Biotage
Part number	918-0050-P01
Sorbent load (mg)	0
Capacity volume (µL)	0
Format	96
Comment	N/A
Solvent dispensation height (mm)	-125.5
Sample dispensation height (mm)	-125.5
Aspiration height (mm)	-146.0



"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

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