# Extraction of Methylmalonic Acid from Serum Using ISOLUTE<sup>®</sup> SAX 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 ISOLUTE<sup>®</sup> SAX strong anion exchange 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.

ISOLUTE SAX is a silica-based strong anion exchange sorbent used to extract acidic analytes, such as MMA, from aqueous samples. Very clean extracts are possible due to the purely anion exchange retention mechanism available using this silica based sorbent. This minimizes the co-extraction of endogenous interferences through hydrophobic (or non-polar) retention.

## Analytes

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

## Sample Preparation Procedure

## Format:

ISOLUTE<sup>®</sup> SAX 25 mg fixed well plate, part number 500-0025-P01

## Sample Pre-treatment

To serum (100  $\mu$ L), add 10  $\mu$ L of internal standard (10 ng/ $\mu$ L). Allow to stand for ~1 hour to allow binding to occur. Add HPLC grade water (190  $\mu$ L) and vortex.

## Conditioning

Condition each well with methanol (500 µL).

## Equilibration

Equilibrate each well with HPLC grade water (500  $\mu$ L).

## Sample Loading

Load 300  $\mu L$  of pre-treated sample.

## Wash 1

Elute interferences with HPLC grade water (500 µL).

## Wash 2

Elute interferences with methanol (500  $\mu$ L).

## **Analyte Elution**

Elute analytes with 2% formic acid in acetonitrile (600  $\mu L)$  into a collection plate.

## **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  $\mu L$  of 0.4% formic acid (aq), seal with a plate mat and vortex for 30 seconds.



## **UPLC Conditions**

#### Instrument

Waters ACQUITY I Class UPLC equipped with a flow through needle (15  $\mu L)$ 

#### 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**

o.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

 $\mbox{Curve 11:}$  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-13C4	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  $\mu$ Mol/L) with x10 signal:noise indicator for the latter.



## Recovery

Serum free of MMA was spiked at 250 ng/mL (~2.11  $\mu$ Mol/L). High reproducible recoveries >90% 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  ${\rm ISOLUTE}^*$  SAX method.

#### Table 3. Calculated MMA concentrations.

Chromsystems Calibration Level	Set Value (ng/mL)	Calculated Value (ng/mL)
Calibrator 1	13.7	12.4
Calibrator 2	28.4	27.0
Calibrator 3	51.3	52.8

## **Additional Notes**

#### **Processing Guidelines**

» 96-well SPE plates were processed using a Biotage<sup>®</sup> 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. Mix.
- » 0.4% formic acid (aq): Add 200 µL concentrated formic acid to 49.8 mL of HPLC grade water. Mix.
- » 0.4% formic acid in methanol: Add 200 µL concentrated formic acid to 49.8 mL of HPLC grade methanol. Mix.

## **Ordering Information**

Part Number	Description	Quantity
500-0025-P01	ISOLUTE <sup>®</sup> SAX 25 mg Fixed Well Plate	1
121-5203	Collection plate, 2 mL, square	50
PPM-96	Biotage <sup>®</sup> PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage <sup>®</sup> SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage <sup>®</sup> SPE Dry Sample Concentrator System 100/120 V	1
C103264	TurboVap <sup>®</sup> 96, Evaporator 220/240V	1
C103263	TurboVap® 96, Evaporator 100/120V	1

## **Calibration Curves**

Good linearity was observed over the range 10 – 2000 ng/mL ( $\sim$ 0.085 -  $\sim$ 16.949 µMol/L). **Figure 5**. shows the coefficient of determination r<sup>2</sup> for the optimized method. In addition.



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



# Appendix Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage<sup>®</sup> Extrahera<sup>™</sup>, using ISOLUTE<sup>®</sup> SAX 25 mg SPE plates. Total time taken to process a full 96-well plate was 43 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<sup>®</sup> Extrahera<sup>™</sup> Data

Analyte	Methylmalonic Acid
Recovery (n=8) at 100 ng/mL	92.4%
%RSD	2.5
Linearity (r <sup>2</sup> )	0.999*
LLOQ	<10 ng/ mL

\*Note: Linearity experiments on Extrahera were run using 3PLUS1 $^{\circ}$  Multilevel Plasma Calibrator Set Methylmalonic acid (Chromsystems Instruments and Chemicals GmbH). Manual processing using these standards gave linearity (r<sup>2</sup>) 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: Sample Plate/Rack: Extraction Media: MMA ISOLUTE° SAX 2 mL x 96 well 200 µL ISOLUTE SAX 96 Well Plate





## Settings

"Sample" Tab Sample Type: Starting Sample Volume (µL): Method Comment:

Aqueous Sample 150



## Screenshot

< Cancel	Ed	it SPE Met	nod - MMA	ISOLUTE S	AX		Save >
lethod name			Sample plat	e/rack		Extraction media	
MMA ISOLUTE	SAX		2mL x 9	5 well 200µL	-	ISOLUTE SA	X 96 Well Plate 👻
retreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number o	f steps				Pause aft step?	er last Dispose tips aft each step?
onditioning	1	-					No N
On	1 Solvent						
On	Water	6	-				
and	Volume (p	L)					
On 👘	300						
lash	Wait time	(min)					
On	0						
ution							
On							

## 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
Volur	ne (µL)	300			
Wait	Time (min)	0			

< Cancel	Ed	it SPE Meth	nod - MMA	ISOLUTE S	AX		Save >
tethod name			Sample plat	e/rack		Extraction media	3
MMA ISOLUTE	SAX		2mL x 90	5 well 200µL	*	ISOLUTE SA	AX 96 Well Plate 👻
retreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number o	f steps Pressure (b	ar)				Dispose tips : each step?
On	- 1 Solvent						
On	Metha	nol	-				
oad On	500	D (Was	te) -				
lash	Positive p time (s)	ressure	cod				
On	Repeat (ni	umber of Pause after step?	this				
On 📃	1	•	No				

	Conditioning		ACL	Ivateu	
	No. of steps		1		
	Pressure (Bar)		1.0		
	Dispose tips aft	er this step	No		
	Solvent				
1	Methanol				
2					
3					
4					
		1	2	3	4
Volum	ne (μL)	500			

Position	D		
Positive pressure time (s)	40		
Repeat	1		
Pause after this step	No		

Advanced Settings



< Cancel	Ed	it SPE Metl	hod - MMA	ISOLUTE S	AX		Save >
Method name			Sample plat	e/rack	_	Extraction media	
MMA ISOLUTE S	SAX		2mL x 9	5 well 200µL	•	ISOLUTE SA	X 96 Well Plate 🔻
Pretreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number of	f steps Pressure (t	par)				Dispose tips after each step?
Conditioning	1	- 1.0					No
On	1						
Equilibration	Solvent						
On	Water	2					
Load	Volume (u	L) Collect in p	position				
On	500	D (Was	te) 🔻				
Wash	Positive pr time (s)	ressure					
On	40	Advan	iced				
Elution	Repeat (ni	umber of Pause after	r this				
On	1	-	No				

Equ	uilibration			Not Acti	vated	
No.	of steps			1		
Pre	ssure (Bar)			1.0		
Dis	pose tips aft	er this step		No		
So	vent					
1 Wa	ter					
2						
3						
4						
		1	2		3	4
Volume (µ	ıL)	500				
Position		D				
Positive						
Pressure t	time (s)	40				
Repeat		1				
Pause aft	er					
this step		No				
٨d	anced Sett	inas				
Au	rancea Sett	ings				

< Cancel	Ed	it SPE Met	hod - MMA	ISOLUTE S	AX		Save >	
Method name			Sample plat	e/rack		Extraction media		
MMA ISOLUTE	SAX		2mL x 96	5 well 200µL	*	ISOLUTE SA	X 96 Well Plate 👻	
retreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution	
On	Pressure (	bar)	Pause al load?	lter each				
anditioning	1.0			No				
On	Volume (p	L) Collect in	position					
On	300	D (Wa	ste) 👻					
	Positive p time (s)	ressure						
On	80	Adva	nced					
lash	Premix?	Number	of times					
On	Yes	2	-					
lution								
On								

Load	
Pressure (Bar)	1.0
Pause after each load	No
Volume (µL)	300
Collect in position	D
Positive pressure time (s)	80
Premix	Yes
Number of times	2

Advanced Settings



Method name			Sample plat	e/rack		Extraction media	
MMA ISOLUT	e sax		2mL x 9	6 well 200µL	-	ISOLUTE SA	XX 96 Well Plate 👻
retreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number o	f steps Pressure (t	Plate dry var) wash?	after last Plate dry	time (s)		Dispose tips aft each step?
Inditioning	2	- 1.0	Yes	300			N
On	- 1 Solvent		2 Solver	1			
uilibration	Water	Í	✓ Met	hanol	-	1	
On	) Volume (v	() Collect in r	antition Melum	e (vil) Collect	in position		
ad	500	D (Was	te) - 500	D (W	Vaste) 👻		
	Positive p	ressure	Positiv	re pressure	i sama na sa		
On	60	Advan	ced 60	Adv	vanced		
rtion	Repeat (n	umber of Pause after	this Repea	t (number of Pause :	after this		
On	1	stepr	No 1	- stepr	No	r I	

	Wash			Activated		
	No. of steps			2		
	Pressure (Ba	r)		1.0		
	Plate dry afte	er last was	sh	Yes		
	Plate dry tim	e (s)		300		
	Dispose tips	after last	step	No		
		_	_		_	
	Solvent					
1	Water					
2	Methanol					
3						
4						
		1	2	3		4
Volum	ie (µL)	500	500			
Positio	on	D	D			
Positiv	/e					
pressu	ure time (s)	60	60			
Repea	t	1	1			
Pause	after this					
step		No	No			

Advanced Settings

4

IMA ISOLUTE	SAX		Sample plat	e/rack 5 well 200µL	-	Extraction media	X 96 Well Plate 👻
treatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number of	steps Pressure ()	par) Plate dry elution? Yes	after last Plate dry 30	time (s)		Dispose tips at each step?
On	-1 Solvent						
On	2% For Volume (µl	mic in MeCN	position				
On h	600 Positive pr time (s)	A					
On	80 Repeat (nu	Advar	r this				
On	times)	step?	No				

Elution	Activated
No. of steps	1
Pressure (Bar)	1.0
Plate dry after last elution	Yes
Plate dry time (s)	30
Dispose tips after each step	No

	Solvent
1	2% Formic in MeCN
2	
3	
4	

	1	2	3	4
Volume (µL)	600			
Position	A			
Positive pressure time (s)	80			
Repeat	1			
Pause after this step	No			

Advanced Settings



## 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		Refil	able				N	on Refillabl	e	
Capacity	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							



Sample	Air Gap	
Sample name	Lower air gap flow rate (mL/min)	
Aqueous sample	20	
Sample description	Lower air gap volume (µL)	
Default settings for aqueous	5	
Aspiration flow rate (mL/min)	Upper air gap flow rate (mL/min)	
10	120	
Dispense flow rate (mL/min)	Upper air gap volume (µL)	
20	100	
	Upper air gap dispense pause (ms)	
	300	
	Sup. As	

"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



Sample Plate/Rack	Pipetting Height Aspiration height (mm)	
2mL x 96 well 200µL	-161.0	
Capadity volume (µL)	Pretreatment dispensation height (mm)	
1800	-153.0	
Format		
96	Tune Pipetting Heights	

## "Extraction Media" Screen

Name	ISOLUTE SAX
Manufacturer	Biotage
Part number	500-0025-P01
Sorbent load (mg)	25
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 200 µL
Capacity volume (µL)	1800
Format	96
Aspiration height (mm)	-161.0
Pre-treatment dispensation height (mm)	-153.0



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

"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 300  $\mu$ L of water during the pre-treatment step. This gives a total volume of 450  $\mu$ L, from which 300  $\mu$ L is loaded.

#### 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

To locate a distributor, please visit our website www.biotage.com



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