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

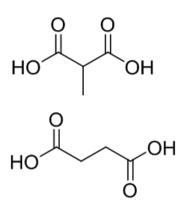


Figure 1. Structures of methylmalonic acid (MMA) and succinic acid (SA).

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:

 $\mathsf{EVOLUTE}^\circ\,\mathsf{EXPRESS}\,\mathsf{AX}$ 30 mg fixed well plate, part number 603-0030-PX01

Sample Pre-treatment

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

Sample Loading

Load pre-treated sample (400 μ L) 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.



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.

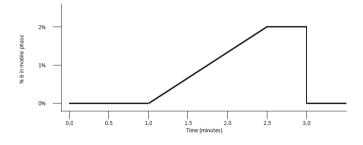


Figure 2. Gradient Conditions - graphical

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.

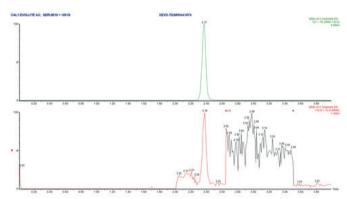
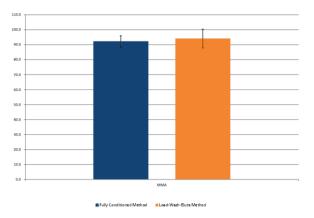


Figure 3. Chromatogram of $^{13}C_4$ MMA (top) at 100 ng/mL and MMA (bottom) at 10 ng/mL (~0.085 μ Mol/L) with x10 signal:noise indicator for the latter.



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**.



 $\ensuremath{\mbox{Figure 4.}}$ 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 μ Mol/L). **Figure 5**. shows the coefficient of determination r² 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**.

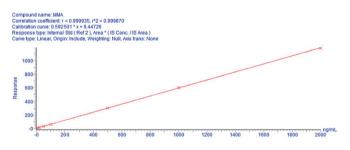


Figure 5. Calibration line of spiked serum extracted with the optimized protocol.

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.
- » o.4% formic acid (aq): Add 200 µL concentrated formic acid to 49.8 mL of HPLC grade water.
- » o.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 $^{\circ}$ Multilevel Plasma Calibrator Set Methylmalonic acid (Chromsystems Instruments and Chemicals GmbH). Manual processing using these standards gave linearity (r²) 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: Sample Plate/Rack: Extraction Media: MMA EVOLUTE[®] Express AX 2 mL x 96 well 200 uL EVOLUTE[®] Express AX 30 mg

od name			Sample plate	e/rack		Extraction media	1	
IA Express A	X 30mg		2mL x 96	i well 200µL	-	EVOLUTE A	X EXPRESS	•
eatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution	
Dn 🗾	Sample ty	pe	Meth	od comment				
itioning	aq sar	nple	-					
Off	Starting s	ample volume in plate/	rack (µL)					
bration	150							
Off								
_								
n								
n								
n 1								
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n 1								



Settings

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

Aqueous Sample 150



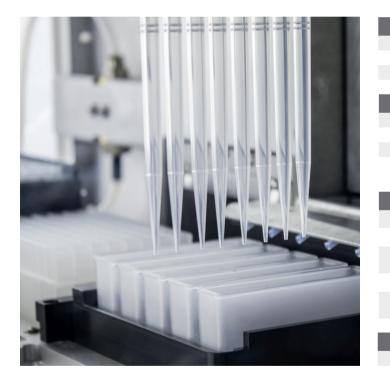
Screenshot

Method name			Sample plate	a/rack		Extraction media	
MMA Express	AX 30mg		2mL x 90	5 well 200µL	•	EVOLUTE A	X EXPRESS 👻
retreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number o	f steps				Pause aft step?	er last Dispose tips af each step?
oditioning	1						No N
ullbration	1 Solvent						
off	Water		-				
and .	Volume (µ	4.)					
On	450						
ush	Wait time	(min)					
On	0						
ution							
On:	14 C						

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
Volu	me (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
Volum	e (µL)		
Positic	on		
Positiv	e		
pressu	ıre time (s)		
Repea	t		
Pause			
this st	ер		
_			
	Advanced Settings	Not Activated	





E	quilibration	Not Activated	
Ν	lo. of steps		
F	Pressure (Bar)		
C	Dispose tips after this step		
5	Solvent		
1			
2			
3			
4			
_			_
	1 2	3	4
Volume	e (µL)		
Positio	n		
Positive	2		
Pressur	re time (s)		
Repeat			
Pause a			
this ste	ep		
A	Advanced Settings		

Aethod name			Sample plat	e/rack	-	Extraction media		
MMA Express	AX 30mg		2mL x 9	5 well 200µL	-	EVOLUTE A	K EXPRESS	•
retreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution	
On	Pressure	(bar)	Pause a load?	fter each	-			
onditioning	1.0			No				
Off	Volume &	L) Collect in	position					
off	400	D (Wa	iste) 👻					
	Positive p time (s)	ressure						
on On	80	Adva	nced					
lash	Premix7	Number	times					
On	Yes	2	-					
ution		_	-					
On								

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



ethod name			Sample plat	R/rack	_	Estraction media	
MMA Express A	XX 30mg		2mL x 9	6 well 200µL	•	EVOLUTE A	X EXPRESS 👻
etreatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution
On	Number of	etaps Pressure (Plate dry wash?	vafter last Plate dr	y time (a)		Dispose tips a each step?
off	2	- 1.0	Yes	300			
ullibration	Solvent		2 Solver	¢.			
off	Water		• Met	hanol			
să .	Velume (µ) Collect in (position Volum	e (pL) Collec	t in position		
On	1000	D (Was	te) 🔻 100	D ()	Vaste) 🔻		
sh	Positive pr time (s)	455078	Pasitin time (ve pressure s)			
On	80	Advan	nced 80	Ad	vanced		
tion	Repeat (nu timas)			t (number of Pause step?	after this		
On	1	-	No 1		No		
			_				

Wash							
No. of step	No. of steps						
Pressure (E	Bar)		1.0				
Plate dry a	fter last was	h	Yes				
Plate dry ti	me (s)		300				
Dispose tip	s after last s	tep	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

MA Express	AX 30mg		Sample plat	6 well 200µL	-	Extraction media	Service Section 201	-
treatment	Sample	Pretreatment	Conditioning	Equilibration	Load	Wash (2)	Elution	
On	Number o	of steps Presbure ()	Plate dry elution?	after last Place dry	sime (s)			ose tips aft step?
Off	1	- 1.0	Yes	60				N
libration	Solvent							
Off	2% Fo	rmic in MeCN	-					
	Volume () 750	A) Collect in ;	naitian					
On	Positive p	and the second se						
On	80	Advan	iced					
ipe	Repeat (n times)	umber of Pause after step?	rithia					
On	1	-	No					
-								

No. of steps	No. of steps				
Pressure (Ba	Pressure (Bar)				
Plate dry aft	Plate dry after last elution				
Plate dry tin	ne(s)		60		
Dispose tips	after each s	step	No		
			_		
Solvent					
1 2% Formic	in MeCN				
2					
3					
4					
	1	2		3	4
Volume (µL)	750				
Position	А				
Positive					
pressure time (s)	80				
Repeat	1				
Pause after this					

Activated

Advanced Settings

step

No



Solvent Properties

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



Solvent	1	2	3	4	5	6	7	8	9	10
Reservoir Type		Refil	lable				N	on Refillab	le	
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 (μ L)	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 (pL)	
Default settings for aqueous	5	
Aspiration flow rate (mL/min)	Lipper air gap flow rate (mL/min)	
10	120	
Dispense flow rate (mt/min)	Upper air gap volume (µL).	
20	100	
	Upper air gap dispense pause (ms)	
	300	
	- 6	

"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	Pipetting Height	
Name	Solvent dispensation height (mm)	
EVOLUTE AX EXPRESS	-125.0	
Manufacturer	Sample dispensation height (mm)	
Biotage	-135.0	
Part number	Aspiration height (mm)	
603-0030-PX01	-135.0	
Sorbert load (mg)		
50	Tune Pipetting Heights	
Capacity volume (µL)		
1000		
Format		
96 👻		
Comment		

96	• · · · · · · · · · · · · · · · · · · ·	
Comment		Solve
		Samp
		Aspira
Edit Sample Plate/Ra	ack - 2mL x 96 well 200µL	Save> "Sam
Sample Plate/Rack	Pipetting Height Aspiratian height (mm)	Name
2mL x 96 well 200µL	-161.0	
Capacity volume (µL) 1800	Pretreatment dispensation height (mm)	Сара
1000		Form

Tune Pipetting Heights.

"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

nple 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



< Cancel	Edit Pipette Tip - 1000 µL Biotage tip	Save >
	Pipette Tip Name 1000 µL Biotage tip Manufacturer Biotage Part number 414111 Capadity (gL) 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 450 µL of water during the pre-treatment step. This gives a total volume of 600 μ L, from which 400 μ L is loaded.

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

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