APPLICATION NOTE No. 21172

Fast and reliable method for the analysis of methylmalonic acid from human plasma

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Keywords:

Methylmalonic acid (MMA), succinic acid (SA), SOLAμ WAX, Acclaim Surfactant Plus, LC-MS/MS, SPE, mixed-mode ion-exchange, micro-elution, vitamin B12 deficiency, cobalamin deficiency

Goal

To describe an accurate and precise high-throughput analytical technique for the analysis of methylmalonic acid (MMA) utilizing mixed-mode ion-exchange micro-scale solid phase extraction (SPE), followed by liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS). This technique further separates MMA from the known endogenous interference succinic acid (SA), a compound that has the same molecular weight as MMA and demonstrates a similar fragmentation pattern, which can cause issues with selectivity in mass spectrometry (MS) detection. Deuterated MMA (MMA-d3) was used as an internal standard.

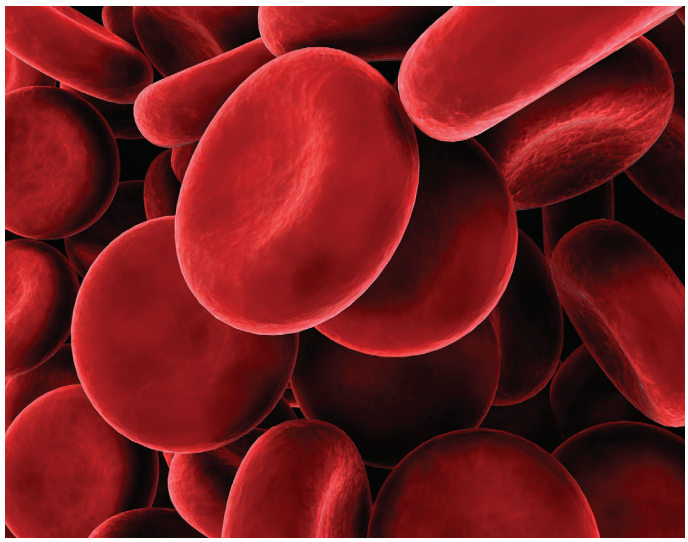
Introduction

Typical analysis for MMA is performed by gas chromatography-mass spectrometry (GC-MS) with lengthy chromatography and time consuming derivitization steps. By utilizing Thermo Scientific™ SOLAμ™ WAX solid phase extraction (SPE) plates, a fast, reproducible, and reliable method can be created to accurately measure levels of MMA from plasma samples. High levels of recovery for MMA can be achieved with minimal matrix effects in a high-throughput workflow.

SOLAμ products provide reproducibility, robustness, and ease-of-use at low elution volumes by utilizing the revolutionary SOLA SPE technology. This removes the need for frits delivering a robust, reproducible format, which ensures highly consistent results at low elution volumes.

SOLAμ products deliver:

- Lower sample failures due to high reproducibility at low elution volumes
- Increased sensitivity due to lower elution volumes
- The ability to process samples that are limited in volume
- Improved stability of biomolecules by reduction of adsorption and solvation issues



Measuring MMA presents issues to the bioanalyst due to the high endogenous levels; obtaining blank matrices for matched standards can present a problem. Phosphate buffer saline (PBS) is regularly used as a surrogate matrix because of its low cost and availability; however, it is often not a close match for the sample requiring analysis. Treated or stripped plasma can be used as a surrogate matrix, but in order to remove trace levels of any particular compound, the level of processing required can be so high that the surrogate is no longer a close matrix match.

Mixed-mode ion-exchange SPE can be employed as a technique to achieve high levels of recovery and low levels of matrix effects. Provided monitoring of matrix to a surrogate is carried out on a batch-by-batch basis; samples can be extracted alongside calibration standards prepared in a surrogate matrix with confidence in the accuracy of the data.

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The Thermo Scientific™ Acclaim™ Surfactant Plus column, based on novel mixed-mode chromatography technology and advanced surface chemistry, provides both reversed-phase and anion-exchange mechanisms. The result of which is a specific elution order of cationic, nonionic, amphoteric, and anionic surfactants and, therefore, a highly selective stationary phase.

MMA analysis can also suffer from interference by succinic acid (Figure 2) due to the similarity in structure, molecular weight and fragmentation pattern observed by MS/MS. Excellent liquid chromatographic separation can be achieved in just 3.5 minutes using an Acclaim Surfactant Plus analytical column prior to detection by a Thermo Scientific™ TSQ Vantage™ MS/MS.

No derivitization steps are used prior to injection onto the chromatographic system, so 96 samples can be processed and ready for analysis within 1 to 2 hours.

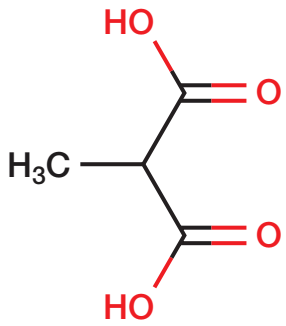

Figure 1. Methylmalonic acid (MMA)

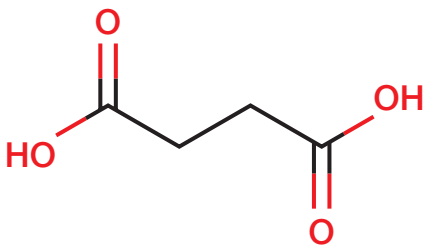

Figure 2. Succinic acid (SA).

Experimental

Consumables

- SOLAμ WAX plate (P/N 60209-005)
- Acclaim Surfactant Plus 3 µm, 150 × 2.1 mm id (P/N 078951)
- Acclaim Surfactant Plus 5 µm, 3.0 × 10 mm drop-in guard (P/N 078959)
- 96-well square well microplate (P/N 60180-P212)
- Thermo Scientific™ Webseal™ mat (P/N 60180-M120)
- Fisher Scientific™ LC-MS grade water (P/N 10095164)
- Fisher Scientific LC-MS grade methanol (P/N 10636545)
- Fisher Scientific LC-MS grade acetonitrile (P/N 10055454)
- Fisher Scientific analytical grade formic acid (P/N 10063427)
- Phosphate buffer saline (PBS) (P/N 10151570)

Sample handling equipment

SPE hardware

- Thermo Scientific™ HyperSep™ 96 vacuum manifold (P/N 60103-351)
- Vacuum pump, European version (P/N 60104-241)
- Vacuum pump, North American version (P/N 60104-243)

Compounds and matrix

Compounds

- Methylmalonic acid (MMA)

Internal standards

- d_3-methylmalonic acid (MMA-d3)

Matrix

- Human plasma and phosphate buffer solution

Calibration and quality control (QC) preparation

Working solutions of 5 µL of MMA prepared in methanol were added to 95 µL of PBS to give concentrations in the range of 15 to 1200 ng/mL. QC samples were prepared in PBS and control human plasma with lithium heparin anti-coagulant at 25, 200, and 1000 ng/mL levels.

Endogenous level investigation samples

Blank plasma was spiked with internal standard to measure endogenous levels of MMA.

Sample pre-treatment

First, 10 µL of 500 ng/mL methanol stock solution of internal standard (MMA-d3) was added to each PBS or plasma calibration and QC sample. Then, 10 µL of methanol was added to each blank PBS or plasma sample. Finally, 300 µL of 15 mM ammonium acetate (pH 4 with formic acid) was added to each PBS or plasma sample and vortexed.

Sample preparation

SPE plate type	SOLAμ WAX
Condition	Apply 200 µL of methanol
Equilibrate	Apply 200 µL 15 mM ammonium acetate (pH 4 with formic acid)
Load	Load the entire pretreated sample
Wash 1	Apply 500 µL of 15 mM ammonium acetate (pH 4 with formic acid)
Wash 2	Apply 200 µL of methanol
Elution	2 × 50 µL of 5% ammonia solution in water

During development it was found that the extracts could either be directly injected onto the chromatographic system or blown down under nitrogen at 50 °C and reconstituted in 50 µL of the starting mobile phase conditions for a more concentrated extract.

Separation conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system
Column	Acclaim Surfactant Plus 3 µm, 150 × 2.1 mm
Guard column	Thermo Scientific™ Hypersil GOLD™ 5 µm, 10 × 4 mm drop-in guard
Mobile phase A	15 mM ammonium acetate pH 4 (with formic acid)
Mobile phase B	Acetonitrile
Gradient	See Table 1
Flow rate	0.450 mL/min
Column temperature	30 °C
Injection details	15 µL
Injection wash	Mobile phase A solvent 1
Injection wash	30:30:30:10 methanol/IPA/water/acetone solvent 2

Table 1. LC gradient conditions

Time (min)	A	B
0.0	80	20
3.5	30	70
3.6	5	95
4.0	5	95
4.1	80	20
5.5	80	20

MS conditions

Instrumentation	Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer
Ionization source	Heated electrospray ionization (HESI)
Polarity	Negative
Scan time	0.02 s
Q1 (FWHM)	0.75
Q3 (FWHM)	0.75
Compound transition details	See Table 2

Table 2. Compound transition details

Compound	Methylmalonic acid	d_3-Methylmalonic acid	Succinic acid	Succinic acid (confirmatory ion)
Precursor (m/z)	117.0	120.1	117.1	117.1
Products (m/z)	73.1	76.1	73.1	99.1
Collision energy (V)	11	11	11	12
S-lens (V)	35	35	35	45

Data processing

Thermo Scientific™ LCQUAN™ quantitative software was used for data processing.

Results and discussion

Due to the high levels of MMA present in control human plasma normally used in preparation of calibration and quality control samples, a surrogate matrix of phosphate buffer solution was used. Human plasma was used to spike additional QC samples in order to confirm that accuracy, precision, and matrix effects are still in acceptable limits when compared to PBS samples. Excellent levels of accuracy and precision were observed for MMA using the SOLAμ WAX SPE plate. High levels of recovery were also seen, as well as low levels of matrix effects, on both PBS and plasma samples giving a high degree of confidence in the analytical results.

Chromatography

Excellent separation of MMA and SA was achieved using the Acclaim Surfactant Plus analytical column as shown in Figure 3.

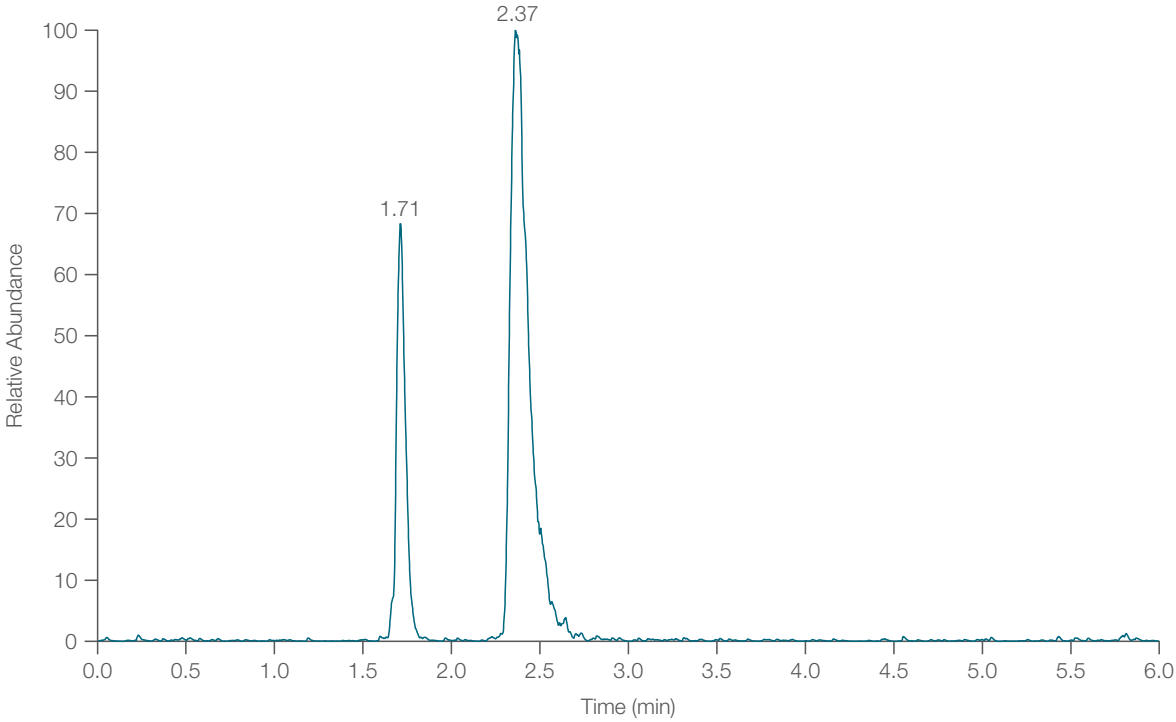

Figure 3. Separation of succinic acid (SA) at 1.71 min RT and methylmalonic acid (MMA) at 2.37 min RT

Linearity

Extracted MMA standards from PBS gave a linear calibration curve over the dynamic range of 15 to 1200 ng/mL with an R^2 of 0.9953 (Figure 4 and Table 3). A linear $1/x^2$ weighting was applied to the calibration curve.

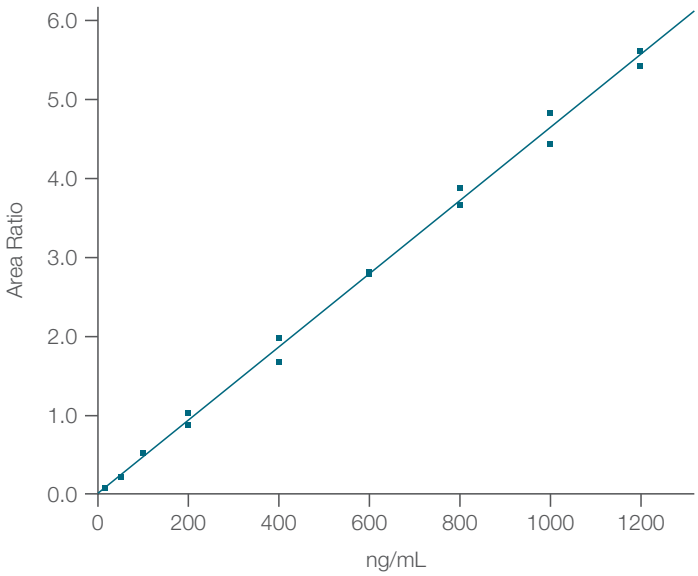

Figure 4. MMA linearity over a dynamic range of 15 to 1200 ng/mL

Table 3. Accuracy data for calibration standards of MMA from PBS over a range of 15 to 1200 ng/mL, n=2

Sample Name	Nominal Concentration (ng/mL)	Average Calculated Concentration (ng/mL)	% Bias from nominal
S1	15	14.8	-1.31%
S2	25	25.0	0.00%
S3	50	49.8	-0.46%
S4	100	111	10.9%
S5	200	201	0.510%
S6	400	386	-3.55%
S7	600	593	-1.18%
S8	800	798	-0.29%
S9	1000	980	-1.98%
S10	1200	1170	-2.65%
R^2 value	**-**	**0.9951**	**-**

Accuracy and precision

QC samples were run in replicates of six at concentrations of 25, 200, and 1000 ng/mL. The precision and accuracy of the QC extracted from PBS is presented in Table 4. The mean endogenous levels of MMA in human plasma is shown in Table 5. The precision and accuracy of the QC extracted from plasma is presented in Table 6.

Table 4. Accuracy data for QC samples of MMA from PBS, n=6

Sample Name	Nominal Concentration (ng/mL)	Average Calculated Concentration (ng/mL)	% Bias from nominal	% RSD
Low QC	25	24.3	-2.66%	9.18%
Mid QC	200	178	-11.0%	4.54%
High QC	1000	897	-10.3%	3.44%

Table 5. Mean endogenous levels of MMA in human plasma, n=6

Blank Plasma with Internal Standard	Mean Measured Endogenous Levels from Plasma (ng/mL)	% RSD
n=6	12.2	12

Table 6. Accuracy data for QC samples of MMA from plasma, n=6

Sample Name	Nominal Concentration (ng/mL)	Measured MMA Concentration from Blank Plasma (ng/mL)	Adjusted Nominal (ng/mL)	Calculated Concentration	% Bias from Adjusted Nominal	% RSD
Low QC	25	12.2	37.2	39.2	5.20%	3.7
Mid QC	200	12.2	212	233	9.8%	4.9
High QC	1000	12.2	1012	1014	0.153%	2.0

Recovery and matrix effects

Recovery and matrix effects from both matrices are presented in Table 7.

Table 7. Recovery and matrix effects data for QC samples of MMA from PBS and plasma, n=6

Sample Name	Recovery[i] from PBS Samples	Matrix Effects[ii]	Recovery[i] from Plasma Samples	Matrix Effects[ii]
Low QC	91	-8	81	-10
Mid QC	92	-4	105	-12
High QC	96	-10	81	-22

[i] $\text{Recovery} = \frac{\text{Average Response of Sample}}{\text{Average Response of Overspike}} \times 100$

[ii] $\text{Absolute \% Matrix Suppression} = 100 - \frac{\text{Average Response of Overspike}}{\text{Average Response of Solution Standard}} \times 100$

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Conclusion

- The use of SOLAμ WAX mixed-mode ion-exchange SPE allows for fast, reliable extraction of MMA from human plasma.
- The cleanliness of the final extraction allows the use of a surrogate matrix in preparation of calibration standards enabling analysis of normal and abnormal levels of MMA.
- Excellent levels of accuracy and precision were observed on all compounds using the SOLAμ WAX SPE plate. High levels of recovery as well as low levels of matrix effects were also seen on both PBS and plasma samples, giving a high degree of confidence in the analytical results.
- Known interference from succinic acid can easily be avoided via chromatographic separation within 3 minutes by using an Acclaim Surfactant Plus analytical column.

Useful links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:
appslab.thermoscientific.com

Find out more at
thermofisher.com/solaspe

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