Methylmalonic Acid: Evaluation of Sample Preparation and Simplicity of Method Implementation Using Automated Sample Preparation Prior to LC-MS/MS Analysis

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Introduction

The screening for elevated levels of methylmalonic acid (MMA) in serum is commonly used as a clinical diagnostic indicator of Cobalamin (Vitamin B12) deficiency in humans. MMA is commonly analyzed using LC-MS/MS with or without prior derivatization. This poster summarizes various sample preparation strategies for the extraction of MMA from serum without the necessity for derivatization, prior to LC-MS/MS analysis. A range of extraction techniques of varying complexity were evaluated: protein precipitation, phospholipid depletion, supported liquid extraction and solid phase extraction using both silica and polymer-based mixed-mode anion exchange chemistries. Method performance was evaluated for evaporative effects, assay recovery, ion suppression, phospholipid removal and simplicity of transfer to an automated sample preparation platform.

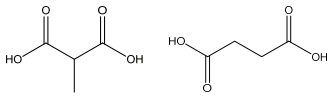

Figure 1. *Structures of MMA (left) and SA (right)*

Experimental

Reagents

MMA, MMA $^{13}C_4$ ISTD, formic acid, ammonium hydroxide, ammonium acetate, ethylene glycol and LC/MS grade solvents were obtained from Sigma-Aldrich Chemical Co. (Poole, UK). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Human serum and stripped serum was kindly donated by Golden West Biologicals Inc. (Ca, USA.) and serum calibrators purchased from Chromsystems (Munich, Germany).

Sample Preparation

Extractions were performed using the 96-well plate format. ***Table 1.*** demonstrates the required processing steps for each technique.

MMA-$^{13}C_4$ ISTD: 100 µL of serum spiked with ISTD at 100 ng/mL.

Table 1*. Summary of sample processing steps.*

Step	PPT+	PLD+	SLE+ 200µL	Standard Processing: SAX, WAX and AX	Load-Wash-Elute EXPRESS AX
Condition	-	-	-	√	-
Equilibration	-	-	-	√	-
Pre-treatment	√	√	√	√	√
Sample load	√	√	√	√	√
Mixing	1 to 8 ratio		-	-	-
Wash 1	-	-	-	√	√
Wash 2	-	-	-	√	√
Elution	√	√	√	√	√

Extraction Optimization: The final and streamlined SPE protocols are detailed in ***Table 2.*** SPE was performed in 25 mg format for ISOLUTE® SAX and 30 mg format for EVOLUTE® chemistries. ISOLUTE® PPT+ and PLD+ were processed using serum (100 µL) precipitation 1:8 with 1 % formic acid in ACN. ISOLUTE® SLE+ 200 µL capacity plates used serum (100 µL) pretreatment 1:1 with 4.6 M formic acid (aq), 200 µL loads and extraction with 750 µL of MTBE.

Post extraction: All extracts were evaporated to dryness using a SPE Dry unit at 40 °C and reconstituted in 100 µL 0.4% formic acid (aq).

Table 2. *Optimized SPE procedures.*

Step	ISOLUTE® SAX	EVOLUTE® EXPRESS WAX	EVOLUTE® EXPRESS AX	EVOLUTE® EXPRESS AX L-W-E
Condition	MeOH	MeOH	MeOH	-
Equilibration	H_2O	NH_4OAc	H_2O	-
Pre-treatment	1:2 H_2O	1:5 NH_4OAc	1:3 H_2O	1:3 H_2O
Sample load	300 µL	600 µL	400 µL	400 µL
Wash 1	H_2O	NH_4OAc	H_2O	H_2O
Wash 2	MeOH	MeOH	MeOH	MeOH
Elution	2% Formic ACN	2% NH_4OH MeOH	2% Formic ACN	2% Formic ACN

Biotage® Extrahera™ Automated Sample Preparation Platform

The optimized extraction protocols were transferred to an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. The Extrahera™ platform is shown in ***Figure 2***.

Processing: Full processing conditions for each technique are available on request.

Figure 2. *Biotage® Extrahera™ automated sample preparation platform.*

UPLC Conditions

Instrument: Waters Acquity I-Class UPLC equipped with a 15 µL flow through needle (Waters Assoc., Milford, MA, USA)
Column: Gemini C18: 100 mm x 3.0 mm id, 3 µm, (Phenomenex UK.)
Mobile Phase: A: 0.4% formic acid (aq): **B:** 0.4% formic acid in MeOH
Flow Rate: 0.6 mL/min
Gradient: 100 % A for 1 min; linear ramp to 2 % B at 2.5 min; hold 0.5 min; resume initial starting conditions
Column Temperature: 50 °C : **Injection Volume:** 10 µL

Mass Spectrometry

Instrument: Xevo TQ-S triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface. Negative ions were acquired in the multiple reaction monitoring (MRM) mode using the deprotonated precursor ion for each analyte: MMA 116.9 > 72.9; MMA $^{13}C_4$ 121.0 > 76.0 using a cone voltage of 30V.
Desolvation Temperature: 500 °C: **Ion Source Temperature:** 150 °C
Collision Gas Pressure: 3.6 x 10^{-3} mbar: **Collision energy:** 9 eV

Results

Chromatographic Optimization

MMA has an isobaric species in the form of Succinic acid (SA) as shown in ***Figure 1***. Therefore a chromatographic separation was achieved to ensure no interference from SA when quantifying MMA, as illustrated in ***Figure 3.***

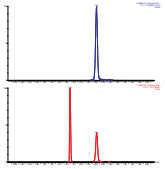

Figure 3*. Extraction of serum spiked with MMA (1000 ng/mL) + $^{13}C_4$ MMA (100 ng/mL) using EVOLUTE® EXPRESS AX.*

Extraction Optimization

Due to the small polar nature of the analyte, volatility during evaporation was an issue. Acidification or the addition of ethylene glycol helped towards eliminating evaporative losses as demonstrated in ***Figure 4.*** The use of acidic elution solvents from an extraction standpoint was therefore preferred. The high concentration of acid in the aqueous pre-treatment step for ISOLUTE® SLE+ extraction provided sufficient pH control to avoid losses.

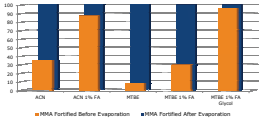

Figure 4*. Evaporative losses of MMA with varying solvents. Solvents either had no modifier, formic acid, ethylene glycol (10 µL) or a combination.*

MMA recovery was optimized for each of the techniques and ultimately transferred to the Extrahera™ automated sample preparation platform. ***Figure 5.*** demonstrates the recovery profile for the optimized protocols extracting serum at 100 ng/mL using manual and Extrahera™ processing.

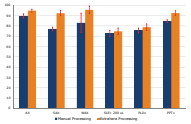

Figure 5*. Extraction recovery profile of MMA from serum using various extraction techniques.*

Calibration curves were constructed using MMA free serum, spiked from 10-2000 ng/mL. Typical calibration lines are demonstrated for EVOLUTE® EXPRESS AX and ISOLUTE® SAX in ***Figure 6.***

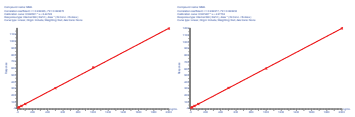

Figure 6. *Serum calibration curves extracted using EVOLUTE EXPRESS® AX and ISOLUTE® SAX.*

Calibrated serum lines (concentrations: 13.7, 28.4 and 51.3 ng/mL) extracted with ISOLUTE® SLE+ using manual and Extrahera™ processing are shown in ***Figure 7.*** All method coefficients of determination (r^2) are summarized in ***Table 3.***

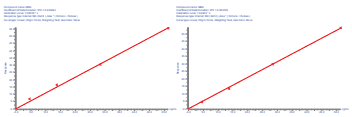

Figure 7. *Calibrated serum lines comparing manual and Extrahera™ processing using ISOLUTE® SLE+.*

Table 3. *Summarized r^2 values.*

	PPT+	PLD+	SLE+	WAX	AX	SAX
Manual 10-2000 ng/mL	0.999	0.999	0.999	0.999	0.999	0.999
Manual Calibrated	0.998	0.992	0.995	0.997	0.991	0.999
Extrahera™ Calibrated	0.998	0.995	0.999	0.997	0.994	0.999

Extract cleanliness was investigated using post-column infusion (PCI) experiments. Blank extracts injected into MMA infused mobile phase was used to determine regions of suppression for each technique, as demonstrated in ***Figure 8***.

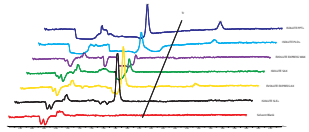

Figure 8. *PCI baselines of the various optimized extraction protocols, indicating suppression with negative intensity.*

Final extracts were also investigated for phospholipid content. ***Figure 9.*** demonstrates the total ion chromatograms (TICs) of typical phospholipid MRMs.

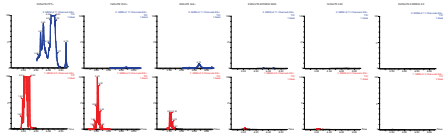

Figure 9. *Phospholipid MRM TICs for final serum extracts from each method.*

For each approach, an extracted sample spiked at 10 ng/mL gave signal to noise ratios of at least 10:1. ***Figure 10.*** demonstrates a typical serum baseline extracted using EVOLUTE® EXPRESS AX.

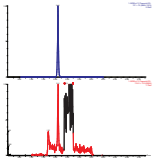

Figure 10. *Chromatogram showing S/N for serum spiked at 10 ng/mL and extracted using EVOLUTE® EXPRESS AX.*

All techniques were easy to implement using the Extrahera™, but involved optimization of various parameters, illustrated in ***Table 4***:

Table 4. *Parameters for optimization using the Biotage® Extrahera™.*

Step	PPT+/PLD+	SLE+ 200µL	Standard: SAX, WAX and AX	Load-Wash-Elute EXPRESS AX
Solvent ASP/DSP speed	Crash	Elution	All steps	L-W-E
Sample ASP/DSP speed		√		
Pipetting Heights: Sample/Solvent		√		
Mixing	On plate crash	Pre-treatment prior to loading		
PP Flow rates	Elution	Load/Elution	All Steps	L-W-E
Processing Time (min/96)	22	27	~ 45	35

Conclusion

- We demonstrate MMA extraction from serum using a variety of sample preparation approaches, with no SA contribution.
- Good recoveries and excellent precision are demonstrated with r^2 values ≥ 0.999 using both manual and processing using the Extrahera™ platform.
- All methods provided acceptable correlation to commercially available MMA calibrators.
- Extract cleanliness demonstrated good removal of endogenous matrix components leading to more reliable quantitation.
- All techniques were easily transferred to the Biotage® Extrahera™ automated sample preparation platform.

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