Comparison of Sample Preparation Strategies for the Extraction of Methylmalonic Acid Prior to LC-MS/MS Analysis

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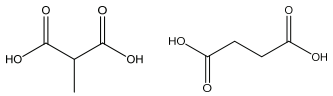
Introduction

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 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 and phospholipid removal.

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



Experimental

Reagents

Standards and MMA radiolabeled 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

All 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+	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: Various extraction strategies were evaluated, investigating effect of pH control, wash solvent and elution solvent optimization. The final and streamlined SPE protocols are detailed in ***Table 2.***

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

UPLC Conditons

Instrument: Waters Acquity IClass 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)
Mobile Phase 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 TQS 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, shown in ***Table 3.***
Desolvation Temperature: 500 °C
Ion Source Temperature: 150 °C
Collision Gas Pressure: 3.6 x 10^{-3} mbar

Table 3. *MRM parameters.*

Analyte	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

Chromatographic Optimization

MMA has an isobaric species in the form of Succinic acid (SA) with the mass spectrometer unable to discriminate (see **Figure 1.**). Therefore a chromatographic separation was achieved to ensure no interference from SA when quantifying MMA as demonstrated in **Figure 2.**

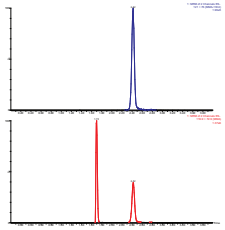
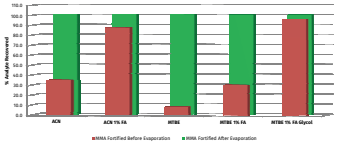


Figure 2*. Extraction of serum spiked with MMA (1000 ng/mL) + $^{13}C_4$ MMA (100 ng/mL) from EVOLUTE® EXPRESS AX.*

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 3.** During SLE+ extraction, however, there is no need for glycol, due to the particularly high concentration of acid in the aqueous pre-treatment step which pH controls the MTBE.

Figure 3*. Evaporative losses of MMA with varying solvents. Solvents either had no modifier, formic acid, ethylene glycol (10 µL) or a combination.*



MMA recovery was evaluate with phospholipid depletion (PLD+), protein precipitation (PPT+), supported liquid extraction (SLE+), and ion exchange solid phase extraction plates. **Figure 4.** demonstrates the recovery profile for the optimized protocols extracting serum at 100 ng/mL.

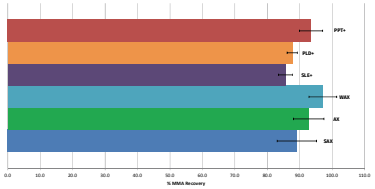


Figure 4*. Extraction recovery profile of MMA from serum using the various extraction techniques.*

Calibration curves were constructed using MMA free serum, from 10-2000 ng/mL. A typical calibration line is demonstrated in **Figure 5.**; with all method coefficient of determination (r^2) values summarized in **Table 4.**

Figure 5*. Calibration line for MMA at 10-2000 ng/mL from serum using ISOLUTE® SAX.*

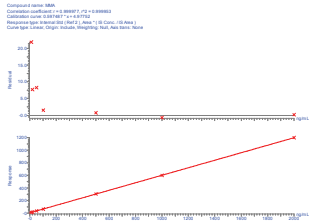


Table 4*. Summarized r^2 values*

	PPT+	PLD+	SLE+	WAX	AX	SAX
r^2	0.9992	0.9990	0.9996	0.9993	0.9998	0.9999

Alongside these calibration lines, commercially available MMA calibrators with known concentrations were extracted with results summarized in **Table 5.**

Table 5. *Summarized Chromsystems data.*

	Expected	PLD+	SLE+	WAX	AX	SAX
Level 1	13.7	11.8	10.0	9.8	10.4	12.4
Level 2	28.4	28.2	27.8	31.2	27.3	27.0
Level 3	51.3	51.5	56.5	55.4	53.3	52.8

For each approach, an extracted sample at 10 ng/mL gave signal to noise ratios of at least 10:1. **Figure 6.** demonstrates a typical baseline using EVOLUTE® EXPRESS AX.

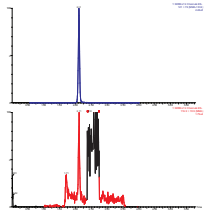
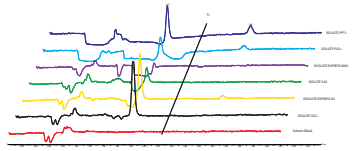


Figure 6*. Baseline noise elevated to demonstrate 10:1 signal:noise ratio in serum at 10 ng/mL following extraction by EVOLUTE AX Express.*

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 as demonstrated in **Figure 7**.

Figure 7*. Collated baselines of the various optimized extraction protocols, indicating suppression with negative intensity.*



Final extracts were also investigated for phospholipid content. **Figure 8.** demonstrates the total ion chromatograms (TICs) of typical phospholipid MRMs.

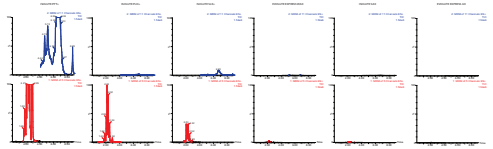


Figure 8*. Phospholipid MRM TICs for final serum extracts from each method*

Conclusion

- We demonstrate MMA extraction from serum using a variety of sample preparation approaches, with no succinic acid contribution.
- Good recoveries and excellent precision are demonstrated with r^2 values ≥ 0.999.
- All methods provided acceptable correlation to commercially available MMA calibrators.
- Extract cleanliness demonstrated good removal of endogenous matrix components leading to more reliable quantitation

MSACL, 2016, Palm Springs
Part Number: P142