# Laboratory Procedure Manual

| | |
|---|---|
| *Analyte:* | **Methylmalonic acid (MMA)** |
| *Matrix:* | **Serum** |
| *Method:* | **LC-MS/MS** |
| *Method No:* | **4010.03** |
| *Revised:* | |
| *as performed by:* | Nutritional Biomarkers Branch (NBB)<br>Division of Laboratory Sciences<br>National Center for Environmental Health |
| *contact:* | James L. Pirkle, M.D., Ph.D.<br>Director, Division of Laboratory Sciences |

**Important Information for Users**

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

## Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table for data file **MMA_H**:

| Variable Name | Analyte Name |
|---|---|
| LBXMMA | Methylmalonic Acid (nmol/L) |

# 1. Summary of Test Principle and Clinical Relevance

## A. Clinical Relevance

Increased concentrations of methylmalonic acid in serum or plasma and excessive urinary excretion of MMA are believed to be direct measures of tissue stores of vitamin B12 (cobalamin) and to be the first indication of vitamin B12 deficiency **[1]**. The concentration of MMA in serum or plasma was found to be a useful indicator of vitamin B12 deficiency, especially in patients with few or no hematological abnormalities, normal results for the Schilling test, or normal or only slightly depressed serum vitamin B12 concentrations **[2]**. In folate deficiency, methylmalonic acid is normal. Methylmalonic acid may be elevated due to impaired renal function **[3]** and as a result of an inborn error of metabolism (methylmalonic aciduria). There is a lack of scientific consensus regarding cutoff values to determine low vitamin B12 status, particularly subclinical vitamin B12 deficiency **[4]**. A 2010 expert roundtable that advised CDC on vitamin B12 biomarkers and methods for future NHANES surveys recommended that due to problems with sensitivity and specificity of individual vitamin B12 biomarkers at least one biomarker of circulating vitamin B12 (serum vitamin B12 or holoTC) and one functional biomarker (MMA or tHcy) should be included in NHANES to assess population vitamin B12 status **[5]**.

## B. Test Principle

Methylmalonic acid (MMA) is analyzed by LC-MS/MS as dibutylester after liquid-liquid extraction using a modification of the method of Pedersen et al. **[6]**. MMA is extracted from serum (75 µL) along with an added internal standard ($d_3$-MMA) via liquid-liquid extraction with tert-butylmethylether/$H^+$ **[7]**. The extracted acid is then derivatized with butanol to form a dibutylester. The butanol is evaporated under vacuum and the derivatized sample is reconstituted in acetonitrile/water. The sample preparation takes ~4 h for a run containing 96 samples (calibrators, QC's and unknowns). MMA is chromatographically separated from other compounds as well as the isobaric succinic acid (same molecular mass as MMA), using isocratic mobile phase conditions within 5.9 min and measured by LC-MS/MS using multiple reaction monitoring. Quantitation is based on peak area ratios interpolated against a six-point aqueous calibration curve.

# 2. Safety Precautions

Consider all serum specimens as potentially positive for infectious agents including HIV, hepatitis B and hepatitis C. We recommend the hepatitis B vaccination series for all analysts working with serum. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place all disposable plastic, glassware, and paper (pipette tips, 96 well plate, gloves etc.) that contact serum in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Use disposable bench diapers during sample preparation and serum handling and discard after use. Also wipe down all contaminated work surface with 10% bleach solution or an appropriate disinfectant solution (e.g., 'ALL SAFE!', Momar, Inc., Atlanta, GA) when work is finished.

**o-Phosphoric Acid:** Handle with extreme care; caustic and harmful, avoid contact with skin and eyes.
**Glacial Acetic Acid:** Handle with extreme care; caustic and harmful, avoid contact with skin and eyes
**Hydrochloric Acid in Butanol:** toxic, caustic, avoid contact with skin and eyes. Handle under a fume hood.
**Organic solvents:** Handle only in well-ventilated areas or as required under a fume hood.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDSs) for these chemicals are readily accessible as hard copies in the lab. If needed, SDS for other

chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ossam/workplace-safety/safety-practices/chemical-safety/index.html.

## 3. Computerization; Data System Management

A. During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

B. The raw data file and respective batch file from the tandem mass spectrometer are collected using the instrument software and stored on the instrument workstation. The data file and batch file are transferred to the network where the data file is processed into a results file that is also saved on the CDC network. Results are typically generated by auto-integration, but may require in some cases manual integration. The results file (including analyte and internal standard names, peak areas, retention times, sample dilution factor, data file name, acquisition time, etc.) is imported into a LIMS database for data review and approval. See "**4010_SOP Computerization and Data System Management**" for a step-by-step description of data transfer, review, and approval.

C. For studies, data is transmitted electronically on a regular basis. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

D. The data file and results file from the instrument workstation are typically backed up to the CDC network after a run is completed and the data has been processed. This is a responsibility of the analyst under the guidance of the project leader. Files stored on the CDC network are automatically backed up nightly by ITSO support staff.

## 4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

A. For best results, a fasting sample should be obtained.

B. Specimens for methylmalonic acid analysis may be fresh or frozen serum. Plasma is also acceptable if serum is not available; citrate plasma is not preferred.

C. A 0.6-mL sample of serum is preferable to allow for repeat analyses; a volume of 75 µL is required for analysis.

D. The appropriate amount of serum is dispensed into a Nalgene 2.0-mL cryovial or other plastic screw-capped vial labeled with the participant's ID.

E. Specimens collected in the field are frozen and then shipped on dry ice by overnight carrier. Frozen samples are stored at -70°C. Samples are stable for at least 5 years if stored at -70°C. They can withstand 5 to 10 freeze/thaw cycles **[7, 8]**.

F. Specimens generally arrive frozen. Refrigerated samples may be used provided they are kept cold and brought promptly (within 2 hours) from the site of collection.

G. Specimens that have been through more than five freeze-thaw cycles, been refrigerated for more than one week, or undergone hemolysis may give inaccurate results.

H. Specimen handling conditions are outlined in the DLS Policies and Procedures Manual. The protocol discusses in general collection and transport of specimens and the special equipment required. If there is more than one test of interest in the specimen and it needs to be divided, the appropriate amount of

blood, serum or plasma should be transferred into a sterile Nalgene cryovial labeled with the participant's ID; avoid cross-contamination

## 5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

## 6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

### A. Reagent Preparation

Prepare all solutions, samples and standards with 0.45 µm filtered deionized water with a resistance of at least 18 MΩ/cm, and HPLC-grade solvents and reagents. Use Class A volumetric glassware in all cases. Perform all steps involving concentrated acids, bases, and organic solvents in a chemical fume-hood. Though each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare larger or smaller quantities if desired.

**1) 0.5 M o-Phosphoric acid in *tert*-Butylmethyl ether (TBME)**

To prepare extraction solvent for a full 96-well plate add 1.4 mL of 85% o-phosphoric acid (Sigma) to 41 mL of TBME and mix well (for other sample sizes adjust the volumes accordingly). This mixture is prepared immediately before use.

**2) Reconstitution solution - 70% Acetonitrile/30% DI water**

To prepare reconstitution solution for a full 96-well plate mix in a beaker 11.2 mL of acetonitrile with 4.8 mL of milliQ water (for other sample sizes adjust volumes accordingly). This mixture is prepared daily before use.

**3) HPLC Mobile Phase - 0.1% Acetic Acid in water**

Fill a 500 mL graduated cylinder with approximately 300 mL of deionized water, aliquot 500 µL of acetic acid, using a disposable volumetric glass pipette and fill to the 500 mL mark with deionized water.

Pour the contents of the graduated cylinder into the HPLC bottle, degas the mixture with stirring under a vacuum for 1-2 minutes (in the fume hood).

The mixture is stable for one week at room temperature.

**4) HPLC Wash Solution – 70% Methanol/30% DI water**

Add 350 mL of methanol and 150 mL of deionized water to the HPLC wash bottle.
Degas the solution under the fume hood with stirring, under low vacuum (make sure that the solution is not boiling, indication of strong vacuum) for 1minute. The mixture is stable for two weeks at room temperature.

### B. Standards Preparation

**1) Individual stock and intermediated solutions**

a) Stock methylmalonic Acid (MMA): Prepare a stock solution of ~1 mg/10 mL (~850 µmol/L) by dissolving 5 mg of MMA in a 50-mL volumetric flask, and diluting to volume with deionized water. Refer to formulas in **Appendix 1** for calculation of the stock concentration.

b) Stock methyl-$d_3$-malonic acid ($d_3$-MMA): Prepare a stock solution of ~1 mg/10mL (~826 µmol/L) by dissolving 2.5 mg of $d_3$-MMA in a 25-mL volumetric flask, and diluting to volume with deionized water. Refer to formulas in **Appendix 1** for calculation of the stock concentration.

c) Dilute the MMA stock solution properly with deionized water to obtain a 50 µmol/L intermediate solution. Aliquot this intermediate solution (5 mL/aliquot) into 10 mL vials. Store at -70°C. The solution is stable for at least 5-10 years.

d) $d_3$-MMA: Dilute the $d_3$-MMA stock solution properly with deionized water to obtain a 50 µmol/L intermediate solution. Aliquot this intermediate d3-MMA solution (0.3 mL/aliquot) into 1.5-mL microcaps. Store at -70°C. The solution is stable for at least 5-10 years.

**2) Working Calibration Standards**

All calibrators (1-6) are prepared in 100 mL volumetric flasks. Prepare all calibrators by adding the amounts of intermediate stock solution (50 µmol/L MMA) listed in the table below to the 100 mL volumetric flask and fill up to the mark with DIW.

| Calibration Level | Final MMA concentration, (nmol/L) | MMA stock solution (50 µmol/L) volume, (mL) |
|---|---|---|
| 6 | 2500 | 5.0 |
| 5 | 1000 | 2.0 |
| 4 | 400 | 0.8 |
| 3 | 150 | 0.3 |
| 2 | 50 | 0.1 |
| 1 | 25 | 0.05 |

Each calibration level was dispensed into cryovials (400 µL aliquots), and then placed in -70°C freezer until needed. These standards are used as working calibrators for daily analysis.
Stability of the prepared calibrators is at least 2 years at -70°C.

**3) Working internal standard solution (ISTD), 1 µmol/L of $d_3$-MMA**

To prepare ISTD for a full 96-well plate add 150 µL of intermediate stock ISTD solution (50 µmol/L $d_3$-MMA) to 7.35 mL of deionized water: methanol (50:50) mixture and mix well (for other sample sizes adjust volumes accordingly). The ISTD solution is prepared daily before the beginning of the sample preparation.

**4) Injection standards for instrument testing**

This solution is used only for check of the instrument (LC-MS/MS) performance. It is not used for calibration and is not used on a regular basis, but rather for troubleshooting, if necessary. The ~100 nmol/L MMA solution was prepared from MMA-dibutyl ester (purchased from Dr. Boykin's laboratory, Georgia State University) as follows: the neat oil was dissolved in acetonitrile to get a stock solution with approximate concentration of 935 µmol/L. The stock was used to prepare the instrument testing

solution ~100 nmol/L MMA (in 100% acetonitrile and in 70% ACN/water). The final two solutions were dispensed in 200 µL aliquots in Nalgene vials and stored at -70°C.

## C. Preparation of Quality Control Materials

Quality control materials for this assay are prepared in-house from blood products acquired from blood banks or from other volunteer blood donors. Approximate QC target values for serum MMA are ~100 (low), ~300 (medium), and above 500 nmol/L (high). If specimens don't contain the approximate target values for MMA, manipulation through spiking with standard compound is done.

The serum is pooled and pools are filtered through gauze before being dispensed to remove fibrin. Serum (usually 600 µL) is aliquoted into 2.0-mL Nalgene cryovials, capped, and frozen. The QC pools are stored at -70°C and are stable for at least 3 years.

Characterization limits are established for all three pools by analyzing duplicates of each pool for at least 20 runs.

## D. Other Materials

With some exceptions, a material listed herein may be substituted with an equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of standards, internal standards, chemicals and reagents, the chemical and/or isotopic purity of the substitute must meet or exceed that of the listed product. In the case of the HPLC column and guard cartridge, equivalent performance must be demonstrated experimentally in accordance with DLS Policies and Procedures.

1) C18 Hypersil Gold, 1.9 µm x 50 mm x 2.1mm (Thermo Electron, West Palm Beach, FL)
2) Guard column, 2.1 mm (Thermo Electron, West Palm Beach, FL)
3) Guard filter holder (Thermo Electron, West Palm Beach, FL)
4) 12 x 75 mm Disposable glass culture tubes (Corning Glassworks, Corning, NY)
5) Disposable volumetric glass pipette, 2 mL (Fisher Sci., Suwanee, GA)
6) Disposable volumetric glass pipette, 10 mL (Fisher Sci., Suwanee, GA
7) 2.0-mL Polypropylene cryovials (Nalgene Company, Rochester, NY)
8) Blue tips (100-1000 µL) for Rainin pipette (Rainin, Oakland, CA)
9) Green tips (10-100 µL) for Rainin pipette (Rainin, Oakland, CA)
10) Combitip Plus (0.5, 2.5 and 5.0 mL) for Eppendorf repeater pipette (Brinkmann)
11) Positive displacement pipette tips (100 µL) for Rainin M-100 (Rainin CP100)
12) Hamilton standard volume tips without filter (Hamilton, Reno, NV)
13) 96 well plate 0.7 mL, conical bottom (Waters, Milford, MA)
14) 96 well pre-slit silicon plate cover (Waters, Milford, MA)
15) CryoCool for Savant Vac. Pump, 1L (Fisher, Suwanee, GA)
16) Neutralization solution for Savant vac. Pump (Fisher, Suwanee, GA)
17) Argon, ultrapure (>99.99% purity) (Air Products, Inc., Atlanta, GA)
18) Methanol, HPLC grade (Burdick & Jackson Laboratories, Muskegan, MI)

19) Acetonitrile, HPLC grade (Burdick and Jackson Laboratories, Muskegan, MI)

20) *tert*-Butylmethyl ether, HPLC grade (Sigma Aldrich Chemical Co., St. Louis, MO)

21) Glacial Acetic acid, approx. 99% (Fisher Sci., Suwanee, GA)

22) 3M HCl in Butanol (Sigma Aldrich Chemical Co., St. Louis, MO)

23) o-Phosphoric acid, 85% (Fisher Sci., Suwanee, GA)

24) Methylmalonic acid (Sigma Chemical Co., St. Louis, MO)

25) Methyl-$d_3$-malonic acid (C/D/N Isotopes, Vaudreuil, Quebec, Canada)

26) Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware

## E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., UHPLC components, tandem quadrupole mass spectrometer) equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type.

1) Thermo-Electron HPLC System (Thermo Scientific, West Palm Beach, FL)

   a) Autosampler
   b) MS pump
   c) XCalibur software

2) Thermo-Electron TSQ triple quadrupole mass spectrometer (Thermo Scientific, West Palm Beach, FL)

   a) Heated electrospray ionization (HESI-II) probe
   b) XCalibur software
   c) Peak Scientific Nitrogen generator (Peak Scientific Instruments, Chicago, IL): connected to the in-house gas supply, it supplies source, auxiliary, and sheath gases to the instrument

3) Hamilton Liquid Handler Starlet 8-channel (Hamilton, Reno, NV)

   a) Various carriers (sample, reagent, tip, and 96-well plate)

4) Dry-bath incubator with 6 heating blocks (Fisher Sc., Suwanee, GA)

5) Multi-tube vortex mixer (Fisher Sc., Suwanee, GA)

6) SpeedVac Plus SC110 A (Savant Instrument Co., Farmingdale, NY)

7) Vortex Genie 2 (VWR, Suwanee, GA)

8) Magnetic stirrer (Baxter Scientific Products, Stone Mountain, GA)

9) Digiflex diluter (Micromedic Division, ICN Biomedical, Inc., Costa Mesa, CA)

10) Adjustable pipetters (Rainin Instrument Co., Oakland, CA)

11) Positive air displacement pipette (Gilson Inc, Middleton, WI)

12) Eppendorf repeater pipette (Brinkmann Instruments Co., Westbury, NY)

13) Sartorius analytical balance, model 1712 MP8 (Brinkmann Instruments Co., Westbury, NY)

# 7. Calibration and Calibration Verification Procedures

## A. Method Calibration

In-house studies showed that aqueous calibration provides equivalent results to calibration in serum and slopes for the two calibration curves (serum vs. water) were less than ± 5% different. Aqueous calibrators, prepared as described in section 6 subsection B, are carried through the entire procedure. Calculation of MMA in QC and unknown specimens is obtained from a six-point calibration curve. Area ratios of analyte to internal standard from single analysis of each calibration level are calculated and a linear calibration curve with 1/x weighting is generated. The measured concentrations of calibrators S2-S6 must agree within 15% of their target values. If a calibrator exceeds the 15% limit, it will be excluded and the curve will be reevaluated. At the end of each run, the calibrators are re-injected and analyzed as unknowns. The drift between the two injections is generally <15% for calibrators S2-S6.

The method is linear from 25 to 2500 nmol/L.

Calibration verification is conducted at least twice a year using NIST reference materials that we characterized in-house for MMA. This allows assessment of trends over time. Additionally, an extended calibration curve (<LOD to high cal) is analyzed twice a year. For details, see "**4010_SOP Calibration and Calibration Verification".**

NIST reference materials with certified MMA concentrations are currently not available.

Since January 2012, this method was used for laboratory participation in DEKS external quality assessment program for methylmalonic acid run by Aarhus University Hospital, Denmark (2 specimens each, 5 times a year). For general information on the handling, analysis, review, and reporting of proficiency testing materials see "**NBB_SOP Proficiency Testing Procedure".**

Method figures of merit are presented in **Appendix 2**.

Results from a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied are presented **Appendix 3.**

We have performed in-house comparison of the LC-MS/MS assay with CDC's GC/MS MMA method. We found excellent correlation ($r$ = 0.99) and agreement (no bias) between the two methods **[7]**.

## B. Instrument Calibration

### 1) Thermo TSQ mass spectrometer

The calibration of the mass spectrometer is scheduled on a semi-annual basis as part of a preventive maintenance program by service engineer from Thermo Scientific. If necessary the analyst can recalibrate with the calibration standard listed below by following the procedure listed in the TSQ Getting Started manual.
The tuning and mass calibration of the first and third quadrupoles of the TSQ is performed by using a solution of polytyrosine-1, 3, 6 by infusion, running the instrument in automatic tune and calibration mode and saving the data onto a file. The tuning and calibration procedure is done at the beginning of the study, upon venting and cleaning of the mass spectrometer.

### 2) Hamilton Microlab Starlet

Twice a year a Hamilton service engineer performs a preventative maintenance including volume verification at 10 µL and 1000 µL.

A volume verification of the various steps of the method can also be performed gravimetrically (e.g., using online gravimetric kit, Hamilton) by the user. Imprecision should be commensurate or exceed that obtained using manual pipettes. Daily and weekly instrument performance checks are performed (runs automatically by push of button).

**3) Pipettes (air and positive displacement)**

On site calibration is performed annually by a certified company. Every six months, calibration verification is performed by the analyst by weight, using a calibrated analytical balance.

**4) Balances**

On site calibration is performed annually by a certified company. Calibration verification is performed by the analyst as needed using certified weights.

## 8. Procedure Operating Instructions; Calculations; Interpretation of Results

A typical run is a full 96-well plate consists of injection of mobile phase, instrument testing solution, a reagent blank (double blank), a blank (containing internal standard); 6 calibrators starting from the lowest concentration, a mobile phase, 3 QCs (low, medium and high), ~80 patient samples, 1 blind QC samples for every 20 patient samples, 3 QCs (low, medium, and high), reinjection of calibrators and the blank, all followed by a shutdown method (this is the order in which the samples are injected into the LC-MS/MS).

### A. Preliminaries

1) Thaw frozen serum specimens (QCs and unknown patient samples), MMA standards and intermediate internal standard solution; it takes about 40 min for the samples to reach ambient temperature.
2) Prepare mobile phase and instrument wash solution (can be prepared ahead of time) as described in section 6A.
3) Prepare working internals standard solution, 1 µmol/L $d_3$-MMA in 50/50-MeOH/water as described in section 6B.
4) Vortex all thawed specimens thoroughly prior to pipetting and visually check for any unusual sample volume, specimen color or debris/precipitate.
5) Turn on the dry bath incubator to low heat (position 9): 60 ± 3°C.

### B. Automated Sample Pipetting using Liquid Handler to prepare for liquid-liquid extraction (routine runs)

1) The Hamilton Microlab Starlet is used for automated pipetting from cryovials into individual 12 x 75 borosilicate tubes that are then subject to liquid-liquid extraction and derivatization.
2) For a detailed step-by-step description, see "**4010_SOP Automated Sample Pipetting using Hamilton Microlab Starlet"**.
    a) Tip racks have to be checked and restocked.
    b) Labeled test tubes have to be put in place.
    c) Calibrators, QC samples, blanks and unknown patient samples have to be put in place.

d) Internal standard mixture has to be put in place.

e) The pipetting program is executed and dispenses according to the following scheme: all samples with the exception of the first one (a reagent blank) get 30 µL of working ISTD solution followed by 75 µL of sample.

3) Place the test tubes on a sample rack and mix on a multi-tube vortex for 1 minute.

## C. Manual Sample Aliquoting

Add to a labeled 12 x 75 borosilicate tube 75 µL of QC, calibration solution (1-6), water (blank) or unknown specimen. Add 30 µL of working ISTD to each tube with the exception to the reagent blank. Mix all tubes for 1 minute on a multi-tube vortex mixer.

## D. Liquid-Liquid Extraction and Derivatization

1) Prepare extraction mixture (0.5 M o-phosphoric acid in TBME) as described in section 6A.
2) Prepare dry ice/EtOH bath (approx. -50$^{o}$C).
3) Add 400 µL of the extraction mixture to each tube.
4) Mix the samples 2 x 1 minute with 2 minute break in between on multi-tube vortex mixer.
5) Place the test tubes in the dry ice/EtOH bath for 1 minute (12 at a time).
6) Gently pour off the top organic layer into a new pre-labeled borosilicate tube.
7) Dry down the organic layer in the SpeedVac (no heat) for 15 minutes.
8) Add 125 µL of 3M HCl in butanol reagent to all tubes, mix and incubate for 30 minute at 60 ± 3°C on the dry bath heating block.
9) Dry down the 3M HCl in butanol in Speed Vac at approx. 45$^{o}$C under vacuum (45 minutes/96 well plate). Drying time needs to be adjusted based on the sample size.
10) Allow the tubes to cool down to room temperature for ~2 minutes.

## E. Sample reconstitution and loading on the instrument

1) Prepare the reconstitution solution (70% ACN/water) as described in section 6A.
2) Add 150 µL of the reconstitution solution to each sample.
3) Vortex all tubes vigorously for 1 minute on multi tube vortex mixer.
4) Load all test tubes on the Hamilton Microlab Starlet for transfer to the 96 well plate.
5) For a detailed step-by-step description, see "**4010_SOP Automated Sample Pipetting using Hamilton Microlab Starlet"**.

a) Tip racks have to be checked and restocked.

b) Labeled test tubes with reconstituted sample have to be put in place.

c) 96-well plate has to be put in place.

d) The pipetting program is executed. It will transfer the entire content of each tube to an individual well of the 96-well plate.

## F. Instrument Preparation

1) The Thermo UHPLC system coupled to Thermo TSQ MS/MS system is used to quantitate MMA in extracted serum.
2) For a detailed step-by-step description, see "**4010_SOP LC-MS/MS Instrument Preparation"**.

a) Prior to every run, UHPLC lines are purged and the HPLC column is primed with a series of solvents; pressures are recorded:

- HPLC line purge: 3 mL 70% MeOH/30% water

b) HPLC column is conditioned with mobile phase (60% MeOH - 40% 0.1 % aq. AcOH) at a flow rate of 400µL/min for ≥15 min; pressure is recorded. HPLC system is ready for analysis.

c) Mass spec method parameters for MMA and the corresponding internal standard are listed in **Appendix 4**. The tandem mass spectrometer is prepared:

- Clean of sweep cone if necessary
- Replacing ion transfer tube if necessary
- Checking of ion spray needle for any blockage and cleaning if necessary

d) The appropriate instrument method is loaded and a new batch containing the sample sequence of the current run is created

e)Daily instrument checks are conducted:

- One sample solvent solution, followed by an instrument testing solution is injected before the actual run is started to verify that the system is working OK

f) The sample plate is loaded into the autosampler and the sequence is submitted

g) The HPLC shutdown method is loaded. It runs isocratically (50% MeOH/water for 5 minutes and 90% ACN:water after for total of 20 min run time).

## G. Processing and reporting a run

1) The Thermo Scientific Xcalibur software is used to process/integrate a run. A LIMS database is used for data review by the analyst, project leader, QA officer, and supervisor and for data reporting.
2) For a detailed step-by-step description, see "**4010_SOP Processing and Reporting a Run".**

a) Reviewing the run:
- When the batch run is finished acquiring the data, the data is reviewed in Xcalibur. Chromatograms for MMA and the ISTD are checked for retention time, peak shape, intensity and/or potential interferences.

b) Quantitation and integration of the completed data file:

- Generate a results table using auto integration.
- Review integrations and make any necessary integration corrections either using the manual or auto integration option. Auto integration is preferred over manual integration.
- Save the results as Excel to allow future review and documentation.
- Import the results file into the LIMS database for r data review.

## H.Exporting a run

The procedure how a run is exported to the LIMS database is described in section 3.

### I. System Maintenance

The system maintenance consists mainly of the different prime, purge and wash cycles described in Section 8 of this document, and performed before and after each run. Column connections are checked for leaks daily, and are wiped with a water-moistened tissue if any residues have built up. Solvent bottles are refilled as needed, and cleaned on a monthly basis. The guard column filters are replaced as needed. The ion sweep cone is cleaned as needed with deionized water, followed by methanol than air dried. The ion transfer tube is replaces as needed. Preventative maintenance is performed by service engineers on all major equipment (MS/MS, HPLC, Hamilton) at least once a year.

### J. CDC Modifications

This method **[7]** is based on the method described by Pedersen *et al.* **[6].**

## 9. Reportable Range of Results

This MMA method is linear over the range of 25-2500 nmol/L. Samples with MMA concentrations >376 nmol/L are re-analyzed for confirmation before results are released. Samples with MMA concentrations exceeding the highest calibrator (≥2500 nmol/L) are diluted with deionized water and re-analyzed. There is no known maximum acceptable dilution. When possible, avoid small volume pipetting and minimize use of serial dilutions when generating diluted samples.

## 10. Quality Control (QC) Procedures

### A. Blind Quality Controls

Blind QC specimens can be inserted into the mix of patient specimens. These QC specimens are generally prepared at two levels that would be encountered in patient samples; the labels used are identical to those used for patient samples. Alternatively, open label blind QC specimens can be used where the analyst knows that the sample is blind QC, but they do not know what pool the sample is from. Open label blind QCs are only used if one can choose from at least 6 different pools and the analyte concentrations are similar to those found in patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

### B. Bench Quality Controls

Bench QC specimens are prepared from three serum pools, which represent low, intermediate, and high levels of MMA in serum. These pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

Three QC pools per run with two or more QC results (replicates) per pool:

1) If all three QC run means are within 2Sm limits and individual results are within 2Si limits, accept the run

2) If 1 of the 3 QC run means is outside a 2Sm limit – reject run if:

   a. $1_{3s}$: Any of the three QC results are outside the 3s limit

   b. $2_{2s}$: Two of the three QC results in the run are outside the 2s limit (same side of mean)

   c. $10_x$: Ten sequential QC results (across pools and across runs) are on the same side of the mean.

3) If one of the six QC individual results is outside a 2 Si limit – reject run if:

   a. Outlier – One individual result is beyond the characterization mean $\pm$ 4 Si or

   b. $R_{4s}$: Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean

*Si = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).*
*Sm = Standard deviation of the run means (the limits are shown on the chart).*
*Sw = Within-run standard deviation (the limits are not shown on the chart).*

The QC results are checked after each run using of a multi-rule quality control program **[9]**. A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC. The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated periodically. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in a LIMS database. For runs that are not imported into the database (i.e., R&D, troubleshooting, research-type runs), QC results are stored electronically in the analyte-specific folder on the DLS network.

### C. Sample QC Criteria

Each individual sample result is checked against established sample QC criteria limits to assure data quality. The method uses the following sample QC criteria:

- Relative retention time (retention time quantitation ion/retention time ISTD)
- Confirmation ion ratio (confirmation ion area/quantitation ion area)
- ISTD minimum area

For details, see "**4010_SOP Sample QC Criteria**".

## 11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

The following steps are provided as a general guideline for identifying possible problems resulting in "out of control" values for QC materials. The troubleshooting process should be done in consultation with the supervisor and may involve additional experiments beyond what is indicated below. Analytical results for runs not in statistical control should not be reported.

1) Check the chromatography to ensure the internal standard response is consistent and peaks are correctly integrated and identified.

2) Look for sample preparation errors, i.e., if the analyst forgot to add internal standard, specimen, BuOH-HCl was stored properly.

3) Check the autosampler for proper sample injections.

4) Check the performance of the HPLC column and the pre-column filter (and replace if necessary).

5) Check the gas flow for sheath and auxiliary gas.
6) Check the Vaporizer temperature and the spray voltage of the HESI probe.
7) Verify the turbo speed is 750 Hz.
8) Check the vacuum pressures and see if they are appropriate for analytical conditions
    - Foreline pressure: 0.85 – 0.97 torr
    - Collision Cell pressure: 1.0 mtorr
    - Ion Gauge pressure ~ 1.8 x $10^{-6}$ torr
9) Replace the Ion Transfer tube.
10) Verify the response of polytyrosine in a Q1 scan.
11) Check the calibrations of the pipettes including Hamilton Microlab Starlet. Calibration verification of the Starlet can be done using a yellow dye test.
12) If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions.
13) Do not report analytical results for runs not in statistical control.

## 12. Limitations of Method; Interfering Substances and Conditions

1) The most common causes of imprecision are intermittently inaccurate micropipettors and pipetting errors.
2) Stock standards, internal standards and specimens should be mixed thoroughly by vortexing before pipetting.
3) Handling stocks and internal standards in step wise sequential manner will minimize the chances of cross-contaminations.
4) Also change of gloves after preparations of stock and working standards and internal standards are recommended to avoid any contamination.
5) HPLC system (lines and column) should be purged and primed properly.

## 13. Reference Ranges (Normal Values)

Originally, normal MMA values in healthy subjects were reported as mean ± 3 SD, but more recently investigators have been using the mean ± 2 SD to define normal. Normal MMA range: 53–376 nmol/L (mean ± 3 SD); 73–271 nmol/L (mean ± 2 SD) **[10]**. MMA values exceeding 2,000 nmol/L are considered to be representative of vitamin B12 deficiency **[11]**. Elevated MMA values should be interpreted with caution, as they could also be a result of impaired renal function **[3]**, particularly in older persons. There is a lack of scientific consensus regarding cutoff values to determine low vitamin B12 status, particularly subclinical vitamin B12 deficiency **[4]**. Carmel *et al.* have developed criteria for clinical and subclinical vitamin B12 deficiency based on a combination of biochemical tests that have often been used by investigators **[12]**:

"Clinical" deficiency:

- Vitamin B12 levels low (<200 ng/L; <148 pmol/L) and often very low (<100 ng/L; <74 pmol/L) in the majority of cases
- Metabolic abnormalities present in majority of cases; often severe (MMA >1000 nmol/L; tHcy >50 µmol/L); all metabolic tests usually abnormal

"Subclinical" deficiency:

- Vitamin B12 levels usually low, but can be low-normal (250–350 ng/L; 185–258 pmol/L)

- At least one metabolic abnormality present; usually mild (MMA 300–800 nmol/L; tHcy 15–25 µmol/L); some metabolic tests may be normal

The newest reference ranges for the U.S. population generated with the MMA GC-MS method for NHANES 2003-2004 have been published in CDC's Second Nutrition Report **[13]**. The Second Nutrition Report also shows reference ranges by population subgroups. In our hands, the current MMA LC-MS/MS method and the previous MMA GC-MS method give very comparable results.

Serum MMA (3 y and older): 63.2–387 nmol/L ($2.5^{th}$ -$97.5^{th}$ percentile; *n* = 7,544)

## 14. Critical Call Results ("Panic Values")

Any NHANES samples with serum MMA levels >2,000 nmol/L may represent a significant risk for vitamin B12 deficiency, but MMA is not considered a reportable result for NHANES. For smaller, non-NHANES studies, abnormal values may be identified to the study principal investigator, depending on individual study arrangements. Emails sent concerning abnormal results are maintained by the supervisor for the duration of the study. Most of these studies are epidemiological in nature.

## 15. Specimen Storage and Handling During Testing

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to run, the prepared samples are placed into the autosampler tray. The unused portion of the patient specimen is returned to the freezer (typically at ≤-70°C) as soon as possible.

## 16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

Because the analysis of serum MMA is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, we recommend that the extracted specimens be stored at ≤ -20°C until the analytical system is restored to functionality.

## 17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results that are not abnormal are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, generally through electronic mail or via ftp site.

For NHANES 1999+, all data are reported electronically on a periodic basis to Westat who then transfer the results to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

## 18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

The LIMS database is used to keep records and track specimens for NHANES 1999+. If plasma or serum MMA analyses are used for smaller, non-NHANES studies, records may be kept in Excel files on the network.

We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum from these analyses for non-NHANES studies may be discarded at the request of the principal investigator, or may be transferred to the CDC

CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

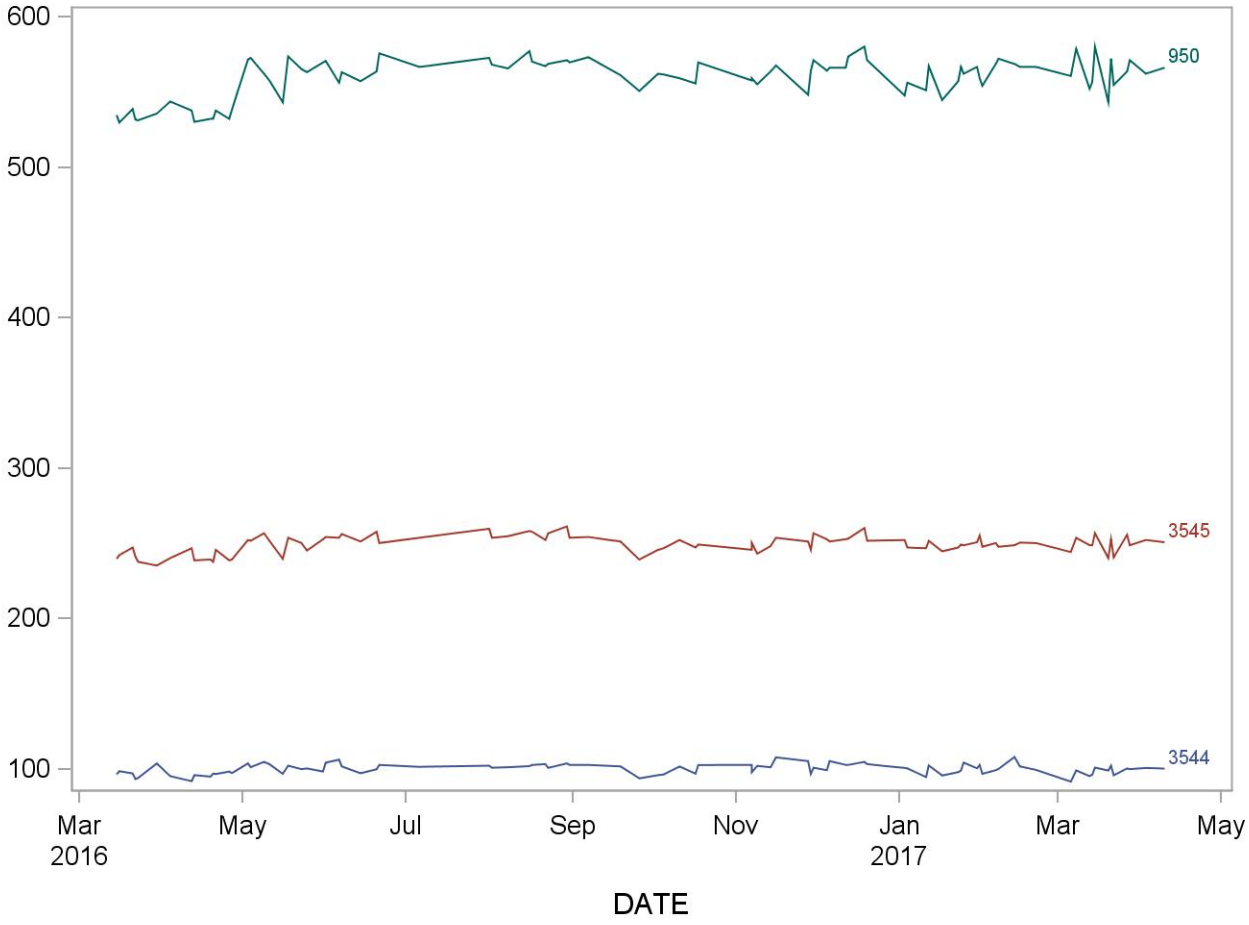
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70°C. The specimen ID is read off of the vial by a barcode reader and used to prepare the electronic specimen table for the analytical system. When the analyses are completed the result file is loaded into the database. The analyst is responsible for keeping a record containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

## 19. Summary Statistics and QC Graphs

See following pages

## 2013-2014 Summary Statistics and QC Chart for Methylmalonic Acid (nmol/L)

| Lot | N | Start | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |
| | | | | | | |



## References


1. Moelby L, Rasmussen K, Jensen MK and Pedersen KO. The relationship between clinically confirmed cobalamin deficiency and serum methylmalonic acid. J Intern Med 1990; 228:373-78.
2. Rasmussen K, Moller J, Lyngbak M, Holm Pedersen A-M, Dybkjaer L. Age- and gender-specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation. Clin Chem 1996; 42(4):630-6.
3. Lindgren A. Elevated serum methylmalonic acid: how much comes from cobalamin deficiency and how much comes from the kidneys? Scand J Clin Lab Invest. 2002; 62:15-20.
4. Bailey RL, Carmel R, Green C, Pfeiffer CM, Cogswell ME, Osterloh JD, Sempos CT, Yetley EA. Monitoring of vitamin B-12 nutritional status in the United States by using plasma methylmalonic acid and serum vitamin B-12. Am J Clin Nutr. 2011; 94:552-61.
5. Yetley EA, Pfeiffer CM, Phinney KW, Bailey RL, Blackmore S, Bock JL, Brody LC, et al. Biomarkers of vitamin B-12 status in the National Health and Nutrition Examination Survey (NHANES): a roundtable summary. Am J Clin Nutr. 2011; 94:313S-21S.
6. Pedersen TL, Keyes WR, Shahab-Ferdows S, Allen LH, Newman JM. Methylmalonic acid quantitation in low serum volumes by UPLC-MS/MS. J Chrom B. 2011; 879(19):1502-6.
7. Mineva EM, Zhang M, Rabinowitz DJ, Phinney KW, Pfeiffer CM. An LC-MS/MS method for serum methylmalonic acid suitable for monitoring vitamin B12 status in populations surveys. Anal Bional Chem 2015; 407:29555-64.
8. Holleland G, Schneede J, Ueland PM, Lund PK, Refsum H, Sandberg S. Cobalamin deficiency in general practice. Assessment of the diagnostic utility and cost benefit analysis of methlymalonic acid determination in relation to current diagnostic strategies. Clin Chem 1999; 45(2):189-98.
9. Caudill SP, Schleicher RL, Pirkle JL. 2008. Multi-rule quality control for the age-related eye disease study. Stat Med 27:4094-4106.
10. Allen RH, Stabler SP, Savage DG, Lindenbaum J. Diagnosis of cobalamin deficiency I: usefulness of serum methylmalonic acid and total homocysteine concentrations. Am J Hematol. 1990;34:90-8.
11. Rasmussen K. Solid-phase extraction for rapid determination of methylmalonic acid in serum and urine by a stable-isotope-dilution method. Clin Chem 1989; 35(2):260-4.
12. Carmel R, Green R, Rosenblatt DS, Watkins D. Update on cobalamin, folate, and homocysteine. Hematology Am Soc Hematol Educ Program 2003; 62-81.
13. U.S. Centers for Disease Control and Prevention. Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 2012. Atlanta (GA): National Center for Environmental Health; April 2012.


## Acknowledgements


We gratefully acknowledge the contributions of Ekaterina M. Mineva, Ph.D., Mindy Zhang, and Christine M. Pfeiffer, Ph.D., who assisted in developing the methodology and performing the analyses for serum methylmalonic acid.

## Appendix 1 - Formulas to calculate the molar concentration (μmol/L) of the stock solution

**MMA**: [a x 1000 x 1000] / [118.1 x b] = μmol/L MMA
where a = weight of MMA in mg, b = volume of stock solution in mL

**$d_3$-MMA**: [a x 1000 x 1000] / [121.1 x b] = μmol/L $d_3$-MMA
where a = weight of $d_3$-MMA in mg, b = volume of stock solution in mL

## Appendix 2 - Method Figures of Merit

### Accuracy:

Results of in-house recovery studies showed a mean ± SD recovery of 94.0% ± 2.1% for MMA added externally to serum at different levels (50-1000 nmol/L MMA).

Recoveries of spiked MMA in serum based on area ratios (analyte/IS) were 99% ± 4.

The liquid-liquid-extraction efficiency (independent of the IS) for MMA extraction from serum was 81% ± 12%.

### Precision:

Total method imprecision was 5.7 % (*n* = 22 runs).

### Limit of detection:

Determination of the limit of detection (LOD) was conducted by serially diluting a "low" QC pool with charcoal stripped human serum and by estimating the SD at a concentration of zero ($\sigma_0$) by extrapolating repeat analyte measurements (*n* = 9) made near the detection limit in these dilutions (LOD defined as 3 $\sigma_0$). The calculated method LOD was 22.1 nmol/L.

## Appendix 3 - Ruggedness Testing

This method has undergone a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. A total of five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at a value substantially lower and higher than that specified in this method while holding all other experimental variables constant. Please, refer to 2012 DLS Policies and Procedures Manual for further information on ruggedness testing.

An increased concentration of serum MMA is believed to be a functional indicator of Vitamin B12 deficiency. We use an isotope-dilution tandem mass spectrometric method in multiple reaction-monitoring mode (MRM) coupled with liquid chromatography (LC-MS/MS) for quantitative measurements of MMA.

1. **Sample Preparation**

   a. **Principle:** MMA is extracted from serum by LLE, followed by derivatization to MMA-dibutyl ester. To assure good sensitivity some of the most important steps of the sample preparations include: optimum extraction of the analyte from the aqueous layer and separation of the organic, containing the MMA, optimum completion of the derivatization reaction, evaporation of the derivatization reagent under vacuum and heat. Each one of these important steps can affect analyte sensitivity and potentially affect the results.

   b. **Proposal:** To vary and test the conditions of the following important sample preparation steps:

      1. Concentration of o-phosphoric acid in the extraction solvent
      2. Time in the dry ice/EtOH batch after LLE
      3. Time needed for derivatization at 60$^{o}$C
      4. Time in the vacuum centrifuge after derivatization

   c. **Findings:**

      1. Varying the amount of o-phosphoric acid in the extraction solvent seems to affect the extraction of the MMA from the aqueous layer.
      2. Varying the amount of time the samples sit in the dry ice/EtOH bath doesn't appear to affect the results of MMA in serum.
      3. Leaving the derivatized samples in the vacuum centrifuge beyond the time specified in the CLIA method doesn't seem to be affected the MMA results, when the concentration is taking into consideration. Comparing the area of the analyte, however shows significant drop (approx. 30%) when left even 10 minutes longer possibly due to loss or decomposition of MMA-dibutyl ester.
      4. Varying the amount of time the samples are left to react with the derivatization reagent doesn't seem to affect the results of MMA.

2. **HPLC conditions**

   a. **Principle:** MMA is analyzed in positive ESI mode. Acid is used to enhance the ionization. The concentration of the acid can enhance or reduce the sensitivity, and potentially affect the MMA results.

   b. **Proposal:** To vary the concentration of the acetic acid in the MP

   c. **Findings:**

1. Varying the amount of AcOH in the MP seems to affect the sensitivity of MMA. Increasing the amount of acid in the MP significantly reduces the area of the analyte.

## Table 1: Ruggedness testing for MMA by LC-MS/MS

| Factor | Method specifies | Results[a] (nmol/L) | Lower level | Results[a] (nmol/L) | Higher level | Results[a] (nmol/L) |
|---|---|---|---|---|---|---|
| **1.** Amount of phosphoric acid in the extraction solvent | 0.5 M | 94.6 (24716 area) | 0.025 M | 95.2 (12090 area) | --- | --- |
| **2.** Time in the dry ice/EtOH bath | 1 min | 96.8 | --- | --- | 2 min | 104 |
| **3.** Time for derivatization | 30 min | 96.5 | 20 min | 95.4 | 40 min | 101 |
| **4.** Time the derivatized samples left in the vacuum centrifuge | 50 min | 96.5 (12946 area) | --- | --- | 60 min | 90.2 (9896 area) |
| **5.** Acetic acid concentration in mobile phase | 0.1% | 99.2 (16433 area) | 0.05% | 107 (13684 area) | 0.5% | 128 (11332 area) |

[a]Results are shown for the low QC sample.

## Appendix 4 - Typical MRM Method Parameters (analysis in positive electrospray ion mode)*

| Analyte (Transition) | *CE |
|---|---|
| MMA (*m/z* 231.1 - > 119.1) | 12 |
| MMA (*m/z* 231.1 - > *m/z* 175.1) | 9 |
| $d_3$-MMA(*m/z* 234.13 - > *m/z* 122.1) | 12 |
| $d_3$-MMA(*m/z* 234.13 - > *m/z* 178.1) | 9 |

*CE, collision energy; S-lens: 49 V (analyte specific). The general instrument parameters used for LC-MS/MS detection and quantitation of all analytes in multiple reaction mode (MRM) were as follows: mass resolution in Q1 and Q3: 0.7 FWHM, scan time: 280 ms; scan width: 0.1 Da, declustering potential: 0, Ar collision gas pressure in Q2: 1.0 mTor; Spray voltage:4500 V; nitrogen sheath gas pressure and auxiliary gas flow were 30 and 5 arbitrary units resp.; vaporizer temp.: 250 $^oC$; ion transfer capillary tube temperature:290 $^oC$ .*

*Method specific parameters are developed during method validation. They should be verified because they may vary between different instruments.