Laboratory Procedure Manual

Analyte:	**Methylmalonic Acid (MMA)**
Matrix:	**Serum**
Method:	**GC/MS**
Method No.:	
Revised:	
as performed by:	*Inorganic Toxicology and Nutrition Branch* *Division of Laboratory Sciences* *National Center for Environmental Health*
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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 NHANES 1999-2000 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label (and SI units)
lab06	LBXMMA	Methylmalonic acid (MMA)

1. Summary of Test Principle and Clinical Relevance

Methylmalonic acid (MMA) is extracted from plasma or serum along with an added internal standard using a commercially available strong anion-exchange resin (1). The extracted acid is then derivatized with cyclohexanol to form a dicyclohexyl ester. The derivatized samples are injected onto a gas chromatograph for separation from other constituents. The effluent from the gas chromatograph is monitored with a mass selective detector using selected ion monitoring. Results are quantitated by internal calibration using peak area ratios of MMA and the internal standard (d3MMA).

Increased concentrations of methylmalonic acid in plasma or serum and excessive urinary excretion of MMA are believed to be direct measures of tissue stores of cobalamin (vitamin B12) and to be the first indication of cobalamin deficiency (2). The concentration of MMA in plasma or serum was found to be a useful indicator of cobalamin deficiency, especially in patients with few or no hematological abnormalities, normal results for the Schilling test, or normal or only slightly depressed serum cobalamin concentrations (3). In folate deficiency, methylmalonic acid is normal. Methylmalonic acid may be elevated due to inborn errors of metabolism. The range of methylmalonic acid in plasma or serum from "healthy adults" is 0.05–0.26 µmol/L (4).

An international round robin performed in 1999 (5) demonstrated that this method is fully equivalent to the original method of Rasmussen (1), but also to the method of Marcell et al. (6).

2. Safety Precautions

Consider all plasma or serum specimens potentially positive for infectious agents including HIV and the hepatitis B virus. We recommend the hepatitis B vaccination series for all analysts working with whole blood and/or plasma. Observe universal precautions; wear protective gloves, laboratory coats, and safety glasses during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place disposable plastic, glass, and paper (pipet tips, autosampler vials, gloves, etc.) that contact plasma in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution when work is finished.

Handle acids and bases with extreme care; they are caustic and toxic. Handle organic solvents only in a well-ventilated area or, as required, under a chemical 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, MSDS for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs.

3. Computerization; Data System Management

a. Calculation and statistical evaluation of a given run is accomplished with the Microsoft Excel software installed on the PC. After a run is complete and any additional corrections by the analyst are made, the result file (containing the patient data as well as the QC data) is electronically transferred to the appropriate analyte-specific subfolder in Q:/ITN/Nutrition Lab/Import into Access on the NCEH/DLS Local Area Network (LAN). The analyst also gives a hardcopy of the result file to the reviewing supervisor. After the reviewing supervisor approves the final values for release by checking off the bench and blind QC values and signing the hardcopy, he/she sends an email to the computer support staff that the data has been released to be imported into the NHANES 1999+ database that is located in Microsoft Access; the computer support staff imports the data into the NHANES 1999+ database by using a macro. Data entry is verified by the computer support staff and the supervisor. Data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.

b. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a week onto a floppy disk or a CD-ROM using a CD writer.

c. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

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 plasma or serum. EDTA plasma is preferred, since homocysteine analysis is usually performed on the same sample.

c. A 1.0-mL sample of plasma or serum is preferable to allow for repeat analyses; a volume of 275 µL is required for analysis.

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

e. Specimens collected in the field are frozen, 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 5–10 freeze/thaw cycles (4).

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 Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q:/ITN/Nutrition Laboratory/CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, plasma should be transported and stored at no more than –20°C. Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalge cryovial labeled with the participant’s ID.

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 reagents using deionized water with resistance of at least 15 MΩ/cm.

1) 18 mol/L Formic acid

CAUTION! Formic acid is corrosive. Contact and inhalation hazard.

Dilute 190 mL of formic acid (99%) to 500 mL with deionized water and mix. This solution is stable for one year at room temperature.

2) 10 mol/L Formic acid

Dilute 190 mL of formic acid (99%) to 500 mL with deionized water and mix. This solution is stable for one year at room temperature.

3) 1.5 mol/L HCl in cyclohexanol

> **CAUTION!!! HCl gas is extremely toxic and corrosive.** Wear acid-resistant gloves, safety glasses (face shields are available if desired), lab coat and/or apron, and work only inside a properly operating fume hood with the sash placed between the operator and the reaction mix setup!
>
> **CAUTION! Cyclohexanol is an organ-specific toxin.** It is a contact and inhalation hazard.

(a) Set reagent-grade cyclohexanol bottle into warm water bath to melt the solvent. Weigh an empty 100-mL graduated cylinder with glass stopper. Decant approximately 85 mL of reagent-grade cyclohexanol into the cylinder, close the cylinder, and weigh again.

(b) SLOWLY and CAUTIOUSLY, in a fume hood, bubble technical-grade HCl gas through the cyclohexanol via a glass Pasteur pipette until the volume of the solution reaches 95–100 mL (~1.5 hours). At this point, the solution is saturated with HCl, resulting in a concentration of HCl greater than 1.5 mol/L (~4.5 mol/L).

(c) Close the graduated cylinder with the glass stopper, mix the content carefully by inverting a couple of times, and weigh the cylinder again.

(d) Calculate the HCl concentration in the cyclohexanol:

HCl conc. [mol/L] = ((End wt [g] – starting wt [g]) x 1000 [mL/L]) / (36.46 [g/mol] x end volume [mL])

(e) Calculate how much this solution has to be diluted to give an HCl concentration of 1.5 mol/L:

Volume of 1.5 mol/L HCl in cyclohexanol [mL] = (End volume [mL] x HCl conc. [mol/L]) / 1.5 [mol/L]

(f) Add the appropriate volume of untreated cyclohexanol to the HCl treated cyclohexanol so that the calculated volume of 1.5 mol/L HCl in cyclohexanol is reached.

(g) Mix solution cautiously, but thoroughly. Layer the top of the solution with nitrogen gas.

(h) Aliquot into 4-mL glass vials (fill only ~2/3 of the vial). Use black caps with teflon septum to close the vials.

(i) Freeze vials at −70°C (this preparation is stable for at least 6 months).

NOTE!!! If HCl gas is not available, this reagent can also be prepared by acidifying cyclohexanol with concentrated hydrochloric acid. Use 0.75 mL concentrated (smoking) hydrochloric acid for 5.25 mL cyclohexanol and mix vigorously before use. Prepare the reagent immediately before use.

b. Standards Preparation

1) Individual stock solutions

(a) Methylmalonic acid (MMA): Prepare a stock solution of ~1 mg/10 mL by dissolving 5 mg of MMA in a 50-mL volumetric flask, and diluting to volume with deionized water.

(b) Methyl-d3-malonic acid (d3MMA): Prepare a stock solution of ~1 mg/10 mL by dissolving 2.5 mg of d3MMA in a 25-mL volumetric flask, and diluting to volume with deionized water.

2) Formulas to calculate the molar concentration (µmol/L) of the stock solutions

(a) 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

(b) d3MMA: [a x 1000 x 1000] / [121.1 x b] = µmol/L d3MMA
where a = weight of d3MMA in mg, b = volume of stock solution in mL

3) Intermediate solutions

(a) Dilute the MMA stock solution properly with deionized water to obtain a 500 µmol/L intermediate solution (i.e., ~25 mL diluted to 50 mL). Aliquot this intermediate solution (0.25 mL/aliquot) into 1.5-mL microcaps. Prepare approximately 200 aliquots for one year's analyses. Store at –70°C. The solution is stable for at least one year.

(b) d3MMA: Dilute the d3MMA stock solution properly with deionized water to obtain a 100 µmol/L intermediate solution (i.e., ~10 mL diluted to 100 mL). Aliquot this intermediate d3MMA solution (0.5 mL/aliquot) into 1.5-mL microcaps. Prepare approximately 200 aliquots for one year's analyses. Store at –70°C. The solution is stable for at least one year.

4) Working Standard Solutions

(a) MMA: When a run is performed, thaw one vial MMA intermediate solution. Prepare a 100 µmol/L working standard solution by diluting 100 µL of the intermediate solution with 400 µL of deionized water (a 1:5 dilution).

(b) d3MMA: When a run is performed, thaw one vial of d3MMA intermediate solution. Prepare a 10 µmol/L working standard solution by diluting 100 µL of the intermediate solution with 900 µL of deionized water (a 1:10 dilution).

5) Injection standards for instrument testing

These are high concentration standards used only to check the performance of the GC/MS system. They are not used for calibration and they are also not used on a regular basis, but rather for troubleshooting, if necessary.

(1) Preparation of MMA and d3MMA injection standard stock solutions (~250 ppm).

(a) Weigh ~2.5 mg of MMA and place in a 1.0-mL Reacti-vial, weigh ~2.5 mg of d3MMA and place in another 1.0-mL Reacti-vial.

(b) Add 200 µL of 1.5 M HCl in cyclohexanol to each vial, cap the vials, and incubate them for 15 min at 115°C in a dry heating bath (Reacti-Therm) for derivatization.

(c) After allowing the vials to cool down for ~3 min, evaporate the cyclohexanol under a gentle flow of nitrogen in a Reacti-Therm at 70°C.

(d) Reconstitute each residue separately with methanol and dilute to a final volume of 10 mL.

(e) Aliquot these injection standard stock solutions (0.5 mL/aliquot) into 1.5-mL microcaps. Prepare approximately 20 aliquots for one year's analyses.

(f) Store at –70°C. The solution is stable for at least one year.

(2) Preparation of MMA and d3MMA injection standard working solution (~5 ppm).

(a) When needed, thaw one vial each of MMA and d3MMA injection standard stock solution.

(b) In a 12- x 32-mm autosampler vial, dilute 30 µL of injection standard stock solution with 1470 µL of methanol (a 1:50 dilution).

(c) Place these two injection standard working solutions into the GC autosampler tray, and inject 1 µL as part of the GC/MS instrument test.

(d) The residual injection standard stock solutions can be stored at 4°C for one month.

c. Preparation of Quality Control Materials

All EDTA-plasma pools are filtered through gauze before being dispensed to remove fibrin. Plasma (650 µL) is aliquoted into 2.0-mL Nalge cryovials, capped, and frozen. The QC pools are stored at –70°C and are stable for at least 3 years.

Means plus range limits for all pools are established by analyzing duplicates for at least 20 consecutive runs.

The low QC pool is prepared by selecting and pooling plasma that contains low levels of MMA (~0.2 µmol/L).

The medium QC pool is prepared by selecting and pooling plasma that contains MMA mostly at levels representing slightly elevated concentrations (~1 µmol/L). If no plasma with slightly elevated MMA concentrations is available, spiking the plasma with known amounts of synthetic MMA is a useful alternative.

The high QC pool is prepared by selecting and pooling plasma that contains MMA mostly at levels representing increased concentrations (~10 µmol/L). Patients with vitamin B12 deficiency or patients with renal insufficiency have often increased MMA plasma concentrations. If no plasma with increased MMA concentrations is available, spiking the plasma with known amounts of synthetic MMA is a useful alternative.

d. Other Materials

(1) DB-5MS capillary GC column, 0.25-mm x 30-m, 0.25-µm (J&W Scientific, Folsom, CA).

(2) Bond-Elut SAX solid-phase extraction columns, 100 mg bed, 1-mL capacity (Varian, Harbor City, CA).

(3) Disposable teflon valve liners (Supelco, Bellefonte, PA) (only necessary if solid-phase extraction is performed manually using a vacuum manifold).

(4) 12- x 75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY).

(5) 10- x 75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY).

(6) 2 dram (8 mL) glass vials for cyclohexanol/HCl storage (Wheaton #224884).

(7) 2.0-mL Polypropylene cryovials (Nalge Company, Rochester, NY).

(8) 5.75 inch disposable glass pasteur pipettes (Kimble, Toledo, OH).

(9) Blue tips (100-1000 µL) for Eppendorf pipette (Brinkmann).

(10) Yellow tips (10-100 µL) for Eppendorf pipette (Brinkmann).

(11) Combitip Plus (5 mL) for Eppendorf repeater pipette (Brinkmann).

(12) Positive displacement pipet tips (100 µL) for Rainin M-100 (Rainin CP100).

(13) Reacti-vials, 1-mL (Pierce).

(14) 12- x 32-mm Glass autosampler vials and 200-µL glass inserts (Kimble).

(15) Micro polypropylene tubes (microcaps), 1.5-mL (Sarstedt, Newton, NC).

(16) Nitrogen, ultrapure (>99.99% purity) (Air Products, Inc., Atlanta, GA).

(17) Helium, ultrapure (>99.99% purity) (Air Products, Inc., Atlanta, GA).

(18) Methanol, HPLC grade (Burdick & Jackson Laboratories, Muskegan, MI).

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

(20) Dichloromethane, HPLC grade (Burdick & and Jackson).

(21) Water, HPLC grade (Millipore, Bedford, MA).

(22) Formic acid, approx. 99% (Sigma Chemical Co., St. Louis, MO).

(23) Cyclohexanol, reagent grade (J.T. Baker, Phillipsburg, NJ).

(24) Hydrogen chloride 99+% (Aldrich Chemical Company, Inc., Milwaukee, WI).

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

(26) Methyl-d3-malonic acid (C/D/N Isotopes, Vaudreuil, Quebec, Canada).

(27) Malonic acid (Sigma Chemical Co., St. Louis, MO).

(28) Ethylmalonic acid (Sigma Chemical Co., St. Louis, MO).

(29) Succinic acid (Sigma Chemical Co., St. Louis, MO).

(30) Glutaric acid (Sigma Chemical Co., St. Louis, MO).

(31) Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware.

e. Instrumentation

(1) HP GC/MSD (Hewlett-Packard, San Fernando, CA).
  (a) Model 6890 GC
  (b) Model 6890 automatic liquid sampler (ALS)
  (c) Model 5973 mass selective detector (MSD)
  (d) Chem-Station workstation

(2) Aspec XL4 automatic solid-phase extraction system (Gilson, Middleton, WI).

(3) Visiprep DL manual solid-phase extraction vaccuum manifold (Supelco, Bellefonte, PA) (only needed if solid-phase extraction is performed manually).

(4) Reacti-Therm III Heating/Stirring module with nitrogen evaporation set-up (Pierce, Rockford, IL). Ideally, two of these are needed.

(5) Speedvac Plus SC110 A and UVS 400 (Savant Instrument Co., Farmingdale, NY).

(6) Daigger Vortex Genie 2 (VWR, Suwanee, GA).

(7) Magnetic stirrer (Baxter Scientific Products, Stone Mountain, GA).

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

(9) Eppendorf micropipette (Brinkmann Instruments Co., Westbury, NY).

(10) Gilson Pipetman micropipette (Rainin Instrument Co., Inc., Woburn, MA).

(11) Eppendorf repeator pipette (Brinkmann Instruments Co., Westbury, NY).

(12) Mettler PM400 balance (Mettler Instrument Corp., Hightstown, NJ).

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

7. Calibration and Calibration Verification Procedures

Results of in-house recovery studies showed a mean (± SD) recovery of 96.0% ± 1.9 for methylmalonic acid added externally to plasma at different levels (0.05-20 µmol/L MMA).

At the beginning of each run, prepare two calibrators (A and B) using the working standard solutions prepared as described in Section 6.d. of this document. Calibrator A contains only the internal standard (d3MMA), calibrator B contains MMA and the internal standard. Since the assay involves a 1:20 dilution of the working standard solutions (55 µL water or MMA working standard solution, 55 µL d3MMA working standard solution, and 990 µL water), the MMA and d3MMA concentrations in 1-mL assay volume used for solid-phase extraction are 0.05× the concentration of the working standard solution (Table 1, APPENDIX).

The remaining sample preparation (solid-phase extraction, derivatization) for these two calibrators is performed identically to the sample preparation of the unknown samples.

All necessary calculations are performed in an Excel spreadsheet after copying the summary tables containing the integrated areas from the ChemStation database into Excel. After calculating area ratios between MMA and the internal standard, a two-point linear standard curve, not forced through zero, is generated. MMA concentrations of unknown samples are calculated using the intercept and slope of the

obtained linear regression line. Since the added MMA concentration in calibrator B is per 1-mL assay volume, and only 250 µL of plasma are used, calculated MMA concentrations of unknown samples have to be multiplied by 4.

At the end of each run, the two calibrators are reanalyzed as unknowns. The measured concentrations of these calibrators must agree within 15% of their set values.

NIST reference materials are not available for MMA assays.

Since January 1999, this laboratory participates in DEKS external quality assessment program for methylmalonic acid run by Aarhus University Hospital, Denmark (2 specimens each, 6 times a year).

8. Procedure Operating Instructions; Calculations; Interpretation of Results

a. Preliminaries

(1) Build runs on the computer as described in Section 8.c. of this document. Participant ID numbers may be scanned into the computer if they are barcoded.

(2) Allow frozen plasma, quality control plasma, and standards to reach ambient temperature.

(3) Allow frozen 1.5 mol/L HCl in cyclohexanol solution to reach ambient temperature (this is typically done on the second day, when samples are derivatized).

b. Sample preparation for Solid-phase Extraction

A typical run consists of one reagent blank, 2 calibrators, 3 QC samples (first set), 18 patient samples, 3 QC samples (second set), and 21 further patient samples. If the sample throughput needs to be increased, the QC samples can be run in groups of 2 to accommodate 3 groups of patient samples: i.e., 2 calibrators, QC low, QC med, 19 patient samples, QC high, QC low, 22 patient samples, QC med, QC high, 22 patient samples.

Use positive displacement pipettes for the preparation of the working standard solutions, and the preparation of the preliminary assay volume (1-mL) used for the solid-phase extraction step. After this point the internal standard corrects for small variations due to pipetting.

(1) Prepare working standard solutions as described in Section 6.d. of this document.
(2) Set up and label one 10- x 75-mm glass tube and one 12- x 75-mm glass tube per sample.

(3) For automated solid-phase extraction, add to the bottom of the 10- x 75-mm glass tube (for manual solid-phase extraction, add to the bottom of the 12- x 75-mm glass tube) in the following order:

(a) Water (using a Digiflex diluter): 1300 µL for the reagent blank (vial 1), 1045 µL for calibrator A (vial 2), 990 µL for calibrator B (vial 3), and 770 µL for the plasma QCs and the patient samples.

(b) MMA working solution (using a positive displacement pipette): 55 µL for calibrator B (vial 3).

(c) d3MMA working solution (using an Eppendorf repeater pipet): 55 µL for all tubes except the reagent blank.

(d) Plasma or serum (using an Eppendorf micropipette): 275 µL for plasma QC and patient samples.

(e) Don't use vial 7 for patient plasma or serum, since there is the possibility of a small amount of MMA carry-over from vial 3 (calibrator B). To vial 7, add 275 µL of water instead of plasma or serum.

(4) Mix gently on a manual vortexer (position 5).

c. Automated Solid-phase Extraction using the Gilson Aspec XL4 system

(1) Preparation of the Gilson Aspec XL4 system.

(a) Prime pumps: On the keypad, press "Manual", select "Prime pumps", press "Enter", select all 4 pumps, press "Prime". After 2-3 syringe volumes have been primed, press "End".

(b) Prime solvents: On the keypad, select "Prime solvents", press "Enter", select all 4 solvents (A: methanol, B: water, C: 10 M formic acid, D: 18 M formic acid), press "Prime". The 4 solvents are primed sequentially in 3 cycles with 1.0 mL each. Press "Esc" to get back to "File MMA".

(2) Loading of the Gilson Aspec XL4 system.

(a) Load all 10- x 75-mm glass tubes containing 1.1 mL sample into rack 41 (first rack from the left) starting with position 1 (Maximum number of vials: 104).

(b) Load as many clean, pre-labeled 12- x 75-mm glass tubes into the aluminum collection racks as needed, starting with position 1 (second rack from the left, bottom). These tubes will be the containers for the cartridge effluent. Each rack has 36 spaces, and there are three racks.

(c) Load as many Bond Elut 1-mL SAX cartridges into the polypropylene mobile cartridge holder as needed, starting with position 1 (second rack from the left, top). Place a cap on top of each cartridge and push it down to be level.

(3) Running the Gilson Aspec XL4 system.

(a) On the keypad, press "Run".

(b) Enter the location of the first vial (usually "1").

(c) Enter the number of vials.

(d) Press "Enter" to start the run.

The Aspec XL4 system processes 4 vials simultaneously (this takes approximately 20 min). All steps, such as column conditioning, sample loading, column washing, and column elution, are performed automatically, similarly as described in Section 8.d. of this document.

d. Manual Solid-phase Extraction

(1) Place Bond Elut SAX columns on vacuum manifold (placed in a fume hood) and wash sequentially at a flow rate of 1-2 mL/min:

(a) 1 x 1.25 mL of MeOH using the wash bottle (1.25 mL / 1 full column volume).

(b) 2 x 1.25 mL of 10 M formic acid using the wash bottle.

(c) 1 x 1.25 mL of MeOH using the wash bottle.

(d) 3 x 1.25 mL of water using the wash bottle.

(2) Pipet 1.0 mL of sample (aqueous, plasma, or serum) prepared as described in Section 8.b.(3) of this document to the SAX column and allow to pass through at a flow rate of ~1 mL/min. Do not allow excessive air drying of the column bed in this step.

(3) Wash 3× with 1.25 mL of water. Do not allow excessive air drying of column bed in this step.

(4) Remove the SAX columns from the manifold and place them into the clean, labeled 10x75 mm glass tubes.

(5) Pipette 350 µL of 18.0 M formic acid into each column using an Eppendorf repeator pipettor.

(6) Elute column by centrifugation in the SpeedVac for 1-2 min.

e. Sample Preparation for GC/MS

(1) Dry down formic acid eluates in the SpeedVac (no heat). This takes approximately 1.5 hours and should not be stopped as long there is a liquid residue.

(2) Pipette 250 µL of MeOH into each tube and mix very gently to suspend the pellet (the pellet should remain intact).

(3) Using a glass pasteur pipette, transfer the methanol to a 1-mL pre-labeled Reacti-vial, leaving the pellet behind.

(4) Repeat process a second time (again 250 µL of MeOH), combining the MeOH with that from the first extraction.

(5) Dry down the MeOH in the SpeedVac (no heat). This takes approximately 20 min.

(6) During this drying step, turn on the Reacti-Therm dry bath #1 to 115°C (position 5½) and Reacti-Therm dry bath #2 to 70°C (position 2½). Allow the 1.5 mol/L HCl in cyclohexanol reagent to reach room temperature.

(7) Add 200 µL of 1.5 mol/L HCl in cyclohexanol reagent to each Reacti-vial, cap, and incubate for 15 min at 115°C in heating block #1.

(8) Remove Reacti-vials and allow to cool for ~3 min.

(9) Decap Reacti-vials and place them into the heating block #2.

(10) Dry down the 1.5 mol/L HCl in cyclohexanol reagent under a moderate stream of nitrogen at 70°C. This takes approximately 20 min. Try to stop the drying process as soon as the last drop of cyclohexanol is evaporated. Continuing the drying process any further will cause a decrease in mass abundances, since the derivatized MMA is volatile.

(11) Remove Reacti-vials and allow to cool for ~3 min.

(12) Reconstitute residue with 100 µL of MeOH. Vortex vigorously for 5 sec.

(13) Transfer the reconstituted sample using an Eppendorf micropipette to a labeled GC/MS autosampler vial containing a 200-µL glass insert, cap, and place on autosampler tray for injection.

The sample preparation procedure can be interrupted at several steps: the formic acid eluates can be stored overnight at room temperature, for 1 or 2 days at 4–8°C or for a few days at –20°C. After formic acid evaporation and reconstitution in methanol, the samples can be stored for 1 or 2 days at 4–8°C. After methanol evaporation, the cyclohexanol derivatization has to be performed without interruption. Once the cyclohexanol is evaporated and the samples are reconstituted in methanol, samples can be stored at 4–8°C for a few days.

f. Instrument Preparation

(1) Auto Tune the instrument.

At the start of every day, tune the GC/MS. In "Instrument Control", select "Instrument - Perform MS Autotune - Autotune - OK".

File the tune report in a log book. This way, changes in the MS tuning parameters and in the relative abundances of the tune masses can be monitored and used to detect the start of a tuning problem and to plan MS maintenance.

Although there is some variability from one Autotune to the next, there should not be drastic differences. One of the trends to watch for in the parameter settings reported at the top of the report is a gradual increase in the ion focus setting (EM voltage) accompanied by a decrease in the relative abundance of mass 502. This occurs as the ion source slowly becomes contaminated from repeated use. When the EM voltage approaches 2500 mV, the ion source needs to be cleaned.

The spectrum shown in the lower part of the tune report allows to detect any peaks that are not typical of PFTBA. For example, an air leak in the system can often be detected by the presence of unusual amounts of mass 18 (water) or 28 (nitrogen). The abundance of both masses should be less than 10% of the abundance of mass 69. Leaks of this sort should be fixed as soon as possible; they can cause premature contamination of the ion source, quadrupole, electron multiplier, and vacuum pumping system.

There are no target abundances for the tune masses 69, 219, and 502. In Auto Tune, also called Maximum Sensitivity Auto Tune, the system will optimize sensitivity across the entire mass range. The relative abundances of the tune masses and the ratios of the isotope masses to the tune masses listed below are generally accepted values.

Mass 69 abundance should be 200,000 but 400,000. It is normal at times to have a base peak (peak of highest intensity) of 219 instead of 69. Mass peak widths (PW50) should be 0.55 ± 0.1. Mass assignments should be 69.0 ± 0.2, 219.0 ± 0.2, and 502 ± 0.2.

(2) Clean injector parts.

(a) Clean the syringe with methanol (the plunger should not feel sticky or show any resistance if pulled up and down).

(b) Empty, wash, and refill the solvent bottles. Solvent A contains equal volumes of water and methanol (50:50), solvent B contains methanol only.

(c) Exchange the position 1 vial in the autosampler tray (methanol) with a new vial with fresh methanol.

(3) GC conditions (Method: MMA_SIM.m).

(a) Oven: 120–320°C at 18°C/min, hold at 320°C for 3 min.

(b) Front inlet: injector temperature: 280°C; initial pressure: 13.4 psi; pulse pressure: 40.0 psi at 0.52 min; purge flow: 51.0 mL/min at 0.50 min; total flow: 55.6 mL/min; gas saver: on at 5.0 min with a gas saver flow of 20.0 mL/min; gas type: Helium.

(c) Column: capillary column; J&W DB-5MS; maximum temperature: 325°C; nominal length: 30.0 m; nominal diameter: 250.0 µm; nominal film thickness: 0.25 µm; mode: constant flow; initial flow: 15.0 mL/min; nominal initial pressure: 13.4 psi; average velocity: 45 cm/sec; outlet: MSD; outlet pressure: vacuum.

(d) MSD transfer line: initial temperature: 310°C.

(e) Injector: sample washes: 1; sample pumps: 2; injection volume: 1.0 µL; syringe size: 5.0 µL; preinjection solvent A washes: 4; preinjection solvent B washes: 4; postinjection solvent A washes: 4; postinjection solvent B washes: 4; viscosity delay: 2 seconds; plunger speed: fast.

(4) MS acquisition parameters (Method: MMA_SIM.m).

(a) General information: tune file: atune.u; acquisition mode: SIM.

(b) MS information: solvent delay: 4.0 min; EM offset: 200; SIM parameters: group MMA, resolution high, monitor m/z 119.0 and m/z 122.0, dwell time 100 msec; MS quadrupole: 150°C, MS source 230°C.

(5) Test the performance of the instrument.

If the instrument was idle for several days, run a short sequence to test the instrument performance ("Insttest.s"). This sequence consists of the following injections: methanol, MMA injection standard, d3MMA injection standard, and methanol.

(a) Change from "Instrument Control" to "MS Top", by selecting "View - Top".

(b) Select "Sequence - Load". Load "Insttest.s" (d:\hpchem\1\sequence\insttest.s\).

(c) Select "Edit Sample Log Table". Make the necessary changes to the sample log table, then select "OK".

(d) Select "Sequence - Run". Make the necessary changes respective to the sequence description, the analyst, and the folder to which the chromatograms will be saved (d:\hpchem\1\data\current date\).

(e) Select "OK", not "Run"! If you select "Run", the sequence will start immediately. However, you want to first save the sequence and run a simulation of it.

(f) Select "Sequence - Save", and "OK".

(g) Select "Sequence - Simulate - Run Sequence". After the simulation of the sequence is finished the message "Sequence Verification Done!... View it?" appears. Select "Yes". A MultiVu window with the sequence data opens. Select "File - Print". Close the MultiVu window.

(h) Select “Sequence - Run - Run Sequence”.

(6) Review data files from instrument testing.

Once the sequence is completed, open “Data Analysis” to view the chromatograms of the injected samples. Load the desired files sequentially and check for the following:

- no interfering peaks when methanol is injected
- retention times and peak shapes of the MMA and d3MMA peak when the injection standards are injected

(7) Load the autosampler tray with the samples.

If your samples are ready to be injected, place them into the autosampler tray according to the following convention:

- position 1: reagent blank
- position 2: calibrator A
- position 3: calibrator B
- position 4: plasma QC low
- position 5: plasma QC medium
- position 6: plasma QC high
- position 7: water
- following positions: patient samples and second set of controls

Positions 98, 99, and 100 are reserved for methanol, the MMA injection standard, and the d3MMA injection standard.

(8) Run the samples.

(a) To start running your samples as a sequence, go to “MS Top” and select “Sequence - Load”. Load “Template.s” (c:\hpchem\1\sequence\template.s\).

(b) Follow the steps outlined in Section 8.f.(5) of this document to edit the Sample Log Table, save and simulate the sequence, and finally run the sequence.

g. Review of Data Files and Calculations

(1) Review the data files of the sequence.

On the next morning, or whenever the sequence is finished, you have to review the chromatograms and integrations before the custom reports database can be updated.

(a) Open “Data Analysis”.

(b) Check sequentially all files of the sequence for any “unusual looking” (i.e., no peaks, broad peaks, tailing peaks) chromatograms: “File - Load Data File - File - Next Data File”.

(c) Reload the first data file of the sequence.

(d) Select “View - QEdit Quant Result” to start quick editing the integrations. Evaluate the integration of the peaks, reintegrate if necessary.

(e) Check sequentially all files of the sequence for integration: “QEdit - Next File”. If you performed manual reintegration, save the changes made to the quant results file.

(f) Once the last file is reached, exit from QEdit and save the changes or abort the changes and exit. This brings you back to Data Analysis.

(2) Update the custom reports database.

(a) Select “Quantitate - Custom Reports”.

(b) Select “Charts/Edit Name of most recent custom reports database.crd”. Select “OK”. The message “There are no columns designated for charting” appears. Select “OK”.

(c) This is the custom reports database from the last run. It can be edited and updated for the recent run.

(d) Highlight all files from the last run and delete them.

(e) Put the cursor in the field where the first data file of the recent run will appear and select “File - Multiple File Select”.

(f) Mark all the data files from the recent run, and select “OK”. The files are loaded into the database, which contains the data file name, the sample name, the compound name, the compound target response, and the compound retention time.

(g) Save the custom reports database (“File - Save as”) under c:\hpchem\custrpt\mma\recent date.crd\. Select that the database be used for the MMA method. Select “OK”.

(h) Print the updated database: “File - Print - Page 1 and 2 - OK”.

(3) Calculate the results using Excel.

All calculations are performed by the computer. Calibration curves are linear, not forced through zero, and calculated on the basis of a single analysis of two standard concentrations according to the following formula:

MMA concentration in plasma (µM) = ((Area ratio MMA/IS - Intercept) / Slope) * 4

(a) Open Excel: “Start - Programs - Excel”.

(b) Open a template spreadsheet: “File - Open File - c:\chris\excel\mma results\template.xls”.

(c) Copy and paste the areas from the custom reports database into the template spreadsheet. The slope and intercept of the calibration curve are calculated automatically. Also, using the slope and intercept, the MMA concentrations of all controls and patient samples are calculated automatically.

(d) Save the file under c:\chris\excel\mma results\recent date.xls.

(e) Print the file.

(f) Close the file, exit from Excel and from the custom reports database.

h. System Maintenance

The system maintenance consists of following components:

(1) Daily cleaning of the syringe and of the solvent vials, daily replacement of the methanol vial in position 1 of the autosampler tray.

(2) Replacement of septum and liner after ~100–150 injections (~every 2-3 days).

(3) Trimming the head of the column as needed (~ every 2 months).

(4) Ion source cleaning as needed (~ every 6 weeks).

(5) Replacement of the helium tank as needed (~ every 3 months).

i. Special Method Notes

(1) The HP GC/MS system should always be "ON" and helium should be flowing through the system.

(2) Turn the system completely off only if it will be idle for several months.

(3) Have the gas saver mode on to save gas.

j. CDC Modifications

This method is based on the method described by Rasmussen et al. (1).

9. Reportable Range of Results

This method is linear for MMA in the range 0.05–20 µmol/L. Samples with results >0.4 µmol/L are re-analyzed for confirmation before results are released. Samples with MMA concentrations 20 µmol/L are diluted 10-fold with PBS and re-analyzed. This method has a total coefficient of variation in the range of 4–10%.

10. Quality Control (QC) Procedures

a. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Inorganic Toxicology and Nutrition Branch. These specimens are prepared at two levels so as to emulate the patient samples; the labels used are identical to those used for 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 plasma pools, which represent low, intermediate, and high levels of MMA in plasma. These pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run. The system is declared "in control" if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met – the run is then declared "out of control":

- 1_{3s} Any of the three QC results are outside the 3s limit
- 2_{2s} Two of the three QC results in the run are outside the 2s limit (same side of mean)
- R_{4s} Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean
- 10_x Ten sequential QC results (across pools and across runs) are on the same side of the mean

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 quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/Import into Access. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder on Q:/ITN/Nutrition Lab/Data handling/QC Results in Excel. A hardcopy of the QC results from each run is also kept by the analyst.

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

Check to make sure that the hardware is functioning properly. Make sure the MS is tuned properly, and the gas velocity is as required. Check the autosampler to make sure the injections are being made as programmed.

Look for sample preparation errors, i.e., analyst forgot to add internal standard, derivatizing agent.

Check the calibrations of the pipettes.

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. Do not report analytical results for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

Elevated plasma or serum methylmalonic acid is an indicator of functional cobalamin deficiency. However, in the very early stages of functional cobalamin deficiency, plasma or serum levels of methylmalonic acid may be normal.

In patients with serum cobalamin levels of less than 100 pg/mL, serum methylmalonic acid levels will be elevated in approximately 90% of this population.

In patients with serum cobalamin levels of 100–200 pg/mL, serum methylmalonic acid levels will be elevated in approximately 60% of this population.

Plasma or serum methylmalonic acid is rarely elevated in individuals with serum cobalamin greater than 200 pg/mL (<5%).

13. Reference Ranges (Normal Values)

Based on literature data (4), the current proposed normal and elevated ranges for this method are shown in Table 3 (APPENDIX).

14. Critical Call Results (“Panic Values”)

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX by the supervisor of any MMA result that is >2 µmol/L, which possibly represents a significant risk for cobalamin deficiency. Copies of FAXes sent concerning abnormal results are kept in a notebook by the supervisor for the duration of the study. For NHANES 1999+, Westat automatically notifies the NCHS survey physician because of several-times weekly electronic transmission of data.

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.

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

Because the analysis of plasma 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)

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX by the supervisor of any MMA result that is >2 µmol/L, which possibly represents a significant risk for cobalamin deficiency. Copies of FAXes sent concerning abnormal results are kept in a notebook by the supervisor for the duration of the study.

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, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically several times weekly to the Westat ISIS computer and then are transferred 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 Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If plasma or serum methylmalonic acid analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN.

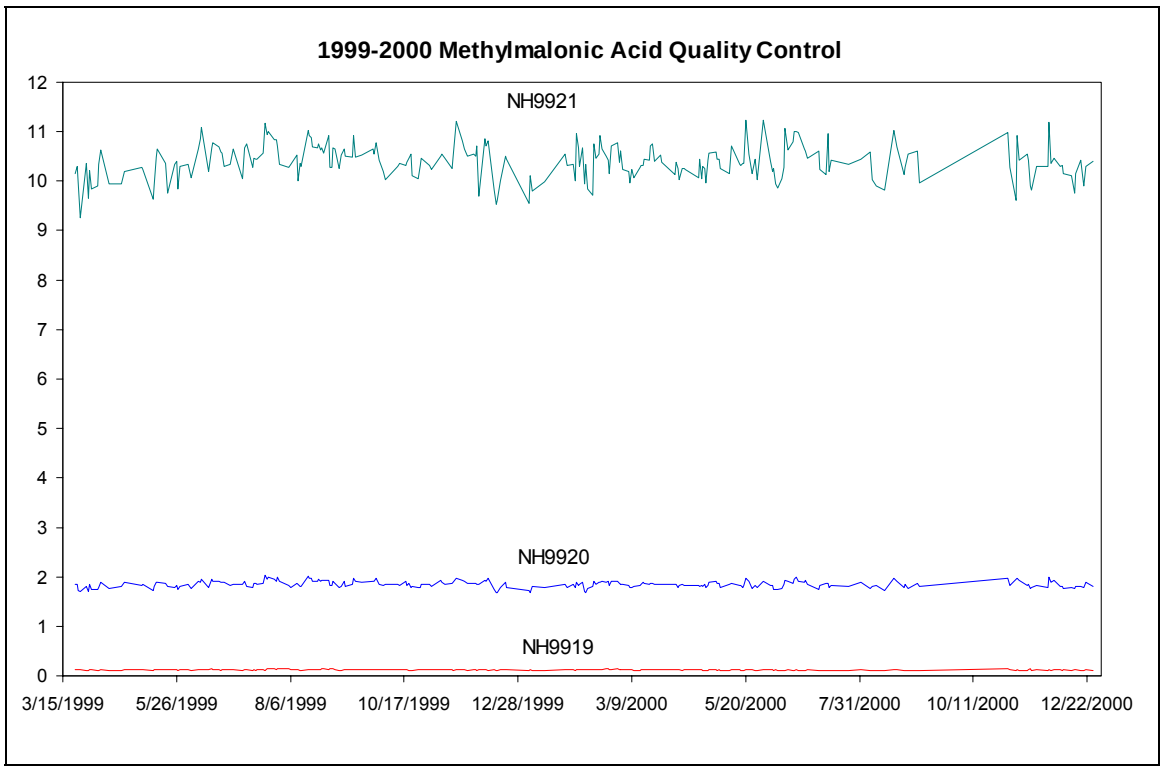
We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum 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 attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook 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

Summary Statistics for Methylmalonic Acid by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH9919	256	3/23/1999	12/26/2000	0.119	0.010	8.432
NH9920	256	3/23/1999	12/26/2000	1.848	0.067	3.622
NH9921	256	3/23/1999	12/26/2000	10.393	0.346	3.330



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APPENDIX (Tables 1-3)

Table 1. Concentrations of the working standard solutions and of the calibrators

Working standard solution	µmol/L MMA	µmol/L d3MMA	1-mL assay volume	µmol/L MMA	µmol/L d3MMA
MMA	100	---	Calibrator A	---	0.5
d3MMA	---	10	Calibrator B	5.0	0.5

Table 2. Relative ratios for prominent masses

m/z 69	100%
70/69	0.5% but 1.6%
219/69	70% but 250%
220/219	3.2% but 5.4%
502/69	3%
503/502	7.9% but 12.3%

Table 3. MMA Reference Ranges

	µmol/L MMA
Normal range	0.05 - 0.26
Cutoff point for diagnosing functional cobalamin deficiency	0.376