



Laboratory Procedure Manual

Analyte: **Phthalate Metabolites**

Matrix: **Urine**

Method: **HPLC/ESI-MS/MS**

Method No: **6306.03**

Revised: **August 18, 2009**

as performed by: Personal Care Products Laboratory
Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

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Dr. Eric J. Sampson, Director
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Important Information for Users

The Centers for Disease Control and Prevention (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.

**Phthalate Metabolites in Urine
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Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table for NHANES 2005—2006 data:

Data File Name	Variable Name	SAS Label
PHTHTE_D	URXECP	Mono-2-ethyl-5-carboxypentyl phthalate
	URXCNP	Mono(carboxynonyl) phthalate (ng/mL)
	URXCOP	Mono(carboxyoctyl) phthalate(ng/mL)
	URXMBP	Mono-n-butyl phthalate (ng/mL)
	URXMC1	Mono-(3-carboxypropyl) phthalate (ng/mL)
	URXMCP	Mono-cyclohexyl Phthalate (ng/mL)
	URXMEP	Mono-ethyl phthalate (ng/mL)
	URXMHH	Mono-(2-ethyl-5-hydroxyhexyl) (ng/mL)
	URXMHP	Mono-(2-ethyl)-hexyl phthalate (ng/mL)
	URXMIB	Mono-isobutyl phthalate (ng/mL)
	URXMNM	Mono-n-methyl phthalate (ng/mL)
	URXMNP	Mono-isononyl phthalate (ng/mL)
	URXMOH	Mono-(2-ethyl-5-oxohexyl) (ng/mL)
	URXMOP	Mono-n-octyl phthalate (ng/mL)
	URXMZP	Mono-benzyl phthalate (ng/mL)

1. Clinical Relevance and Summary of Test Principle

a. Test Principles

The test principle utilizes high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) for the quantitative detection in urine of the following phthalate metabolites: monomethyl phthalate (mMP), monoethyl phthalate (mEP), monobutyl phthalate (mBP), mono-isobutyl phthalate (miBP), mono (3-carboxypropyl) phthalate (mCPP), monocyclohexyl phthalate (mCHP), mono(2-ethylhexyl) phthalate (mEHP), monoethyl phthalate (mOP), monobenzyl phthalate (mBzP), monoisononyl phthalate (mNP), mono(2-ethyl-5-oxohexyl) phthalate (mEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP), mono(2-ethyl-5-carboxypentyl) phthalate (mECP), monocarboxyethyl phthalate (MCOP), and monocarboxynonyl phthalate (MCNP)[1,2]. Urine samples are processed using enzymatic deconjugation of the glucuronidated phthalate monoesters followed by on-line solid phase extraction (SPE) coupled with reversed phase HPLC-ESI-MS/MS. Assay precision is improved by incorporating isotopically-labeled internal standards of the phthalate metabolites. In addition, 4-methyl umbelliferone glucuronide is used to monitor deconjugation efficiency. This selective method allows for rapid detection of monoester metabolites of commonly used phthalate diesters in human urine with limits of detection in the low ng/mL range.

b. Clinical Relevance

Phthalates, are a group of industrial chemicals widely used in consumer products and as solvents, additives, and plasticizers [3]. Humans are potentially exposed to many products containing phthalates. Phthalates are rapidly metabolized in humans to their respective monoesters, which depending on the phthalate can be further metabolized to their oxidative products. Monoesters and the oxidative metabolites of phthalates may be glucuronidated, and excreted in the urine and feces [4-8]. Some phthalates and their monoester metabolites can cause reproductive and developmental toxicities in animals [9-12], but little is known about the effects of phthalate exposure on humans. Information on the concentration of phthalates in people is essential to understand the human exposure to phthalates. Measurement of an internal dose, or biomarker of exposure, is a key aspect of assessing exposure [13].

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents used are toxic. Special care should be taken to avoid inhalation, eye or skin contact to the reagents used throughout the procedure. Avoid use of the organic solvents in the vicinity of an open flame, and use

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solvents only in well-ventilated areas. Care should be exercised in handling of all chemical standards.

β -Glucuronidase is a known sensitizer. Prolonged or repeated exposure to the sensitizer may cause allergic reactions in certain sensitive individuals.

Note: Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure can be found at

www.actiocms.com/msdsxchange/english/index.cfm

The hard copy may be found in the binder in the laboratory. Laboratory personnel are advised to review the MSDS before using chemicals.

b. Radioactive Hazards

None.

c. Microbiological Hazards

The possibility of being exposed to various microbiological hazards exists. Appropriate measures should be taken to avoid any direct contact with the specimens (i.e., utilize gloves, chemical and/or biological hoods). A Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues. Laboratory personnel handling human fluids and tissues are required to take the "Bloodborne Pathogens Training" course and subsequent refresher courses offered at CDC to insure proper compliance with CDC safe work place requirements.

d. Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should avoid any direct contact with the electronics of the mass spectrometer, unless all power to the instrument is off. Generally, only qualified technicians should perform the electronic maintenance and repair of the mass spectrometer. Contact with the heated surfaces of the mass spectrometer should be avoided.

e. Protective Equipment

Standard safety protective equipment should be utilized when performing this procedure. This includes lab coat, safety glasses, and nitrile or latex gloves.

f. Training

Training in the use of an HPLC system and a triple quadrupole mass spectrometer should be obtained by anyone using this procedure. Operators are required to read the laboratory standard operating procedures manual. Formal

training is not necessary; however, an experienced user should train all of the operators.

g. Personal Hygiene

Care should be taken in handling any biological specimen. Routine use of gloves, lab coats and proper hand washing should be practiced. No food or drink is allowed in laboratory areas.

h. Disposal of Wastes

Solvents and reagents are disposed of in an appropriate container clearly marked for waste products. Containers, glassware, etc., that come in direct contact with the specimen are either autoclaved or decontaminated with 10% bleach. Contaminated analytical glassware is treated with bleach, washed and reused; disposable lab ware is autoclaved prior to disposal. To insure proper compliance with CDC requirements, laboratory personnel are required to take annual hazardous waste disposal courses.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

All samples queued for analyses are entered in a database created using Microsoft Access. Mass spectrometry data are collected using the Xcalibur software (ThermoFinnigan, San Jose, CA, USA) on a ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Quantum mass spectrometer equipped with an electrospray ionization (ESI) interface. During sample preparation and analysis, samples are identified by their External Sample Name and Sample number. The External Sample Name is a number that is unique to each sample. Sample number is given to identify each specimen, the date of sample preparation and the preparer. In case of repeated measurements, the sample can have more than one Sample number, but only one Sample name in the database. The Sample name links the laboratory information with the demographic data recorded by the sample takers. All raw mass spectral data are archived for future reference. Data analysis is also controlled by the ThermoFinnigan Xcalibur software. The software selects the appropriate peak based on the precursor/product ion combination and chromatographic retention time and subsequently integrates the peak area. The chromatographic peaks are manually inspected and integrated if necessary. All data are exported from the Xcalibur Quan software as an Excel spreadsheet report and imported into a relational database (Microsoft Access, Redmond, WA) using an automated, custom - written Visual Basic module. Further manipulation of the data, including QC evaluation, reagent blank subtraction, and statistical analyses of the data, programming, and reporting, are performed using the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). Raw files are regularly backed up onto an external hard drive. The Access database is located on an access-restricted

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network drive as well as in several archive locations. Knowledge and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

b. **Sample Information**

External Sample Names, Sample numbers, sample volume and project number are entered into the Access database before sample preparation. If possible, for QCs and unknown samples, the sample IDs are read in by a barcode reader directly from the sample vials. The Sample Log Sheet containing Sample Names and Sample IDs is printed from the Access database and is used to record information during the sample preparation. After MS data collection and peak integration, the data are exported into the Access database.

c. **Data Maintenance**

All sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The database is routinely backed up onto a computer hard drive and onto a network drive. Data from completed studies are saved on CD-ROM and/or on an external hard drive. Additionally, final reports are saved as paper copy as an official government record.

4. **Procedures for Collecting, Storing, and Handling Specimens; Criteria for Specimen Rejection**

a. **Materials needed for urine collection and storage**

- (1) Urine collection cup (150-250 mL plastic, sterile, pre-screened for phthalate metabolites) with cap.
- (2) Pediatric urine collection bag (pre-screened)
- (3) Labels
- (4) Cryovials (pre-screened)
- (5) Other sampling collection materials (pre-screened)

b. **Urine collection, storage and handling**

- (1) Preferably, urine specimens for phthalate analysis should be collected by using a pre-screened urine sampling collection device (e.g., cup, pediatric collection bag).
- (2) A minimum sample volume of 0.5 mL is required.
- (3) Specimens may be stored frozen at temperatures at or below -20 °C for several years prior to analysis.
- (4) Specimen handling conditions are outlined in the Division protocol for urine collection and handling (copies are available in the laboratory). In the protocol, collection, transport, and special equipment required are discussed. In general,

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urine specimens should be shipped in cryovials packed in boxes frozen and securely packed in dry ice. To minimize the potential degradation of the specimen, special care must be taken to avoid prolonged exposure of the urine to room or refrigerator temperatures after collection [14]. Portions of urine that remain after the analytical aliquots are withdrawn should be frozen below -20 °C. All samples should be stored frozen until and after analysis.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

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

Note: Class A glassware such as volumetric flasks are used unless otherwise stated.

a. Reagent Preparation

Mobile phase A (0.1% acetic acid in water). To make 1L, 1.0 mL of acetic acid is added to 1000 mL graduated cylinder and filled to the mark with HPLC grade water or inhouse deionized water. This solution is stored at room temperature in an amber bottle and kept for a maximum of seven days.

Mobile phase B (0.1% acetic acid in acetonitrile). To make 1L, 1.0 mL of acetic acid is added to 1000 mL HPLC grade acetonitrile. This solution is stored at room in an amber bottle temperature and discarded after one month.

* Acetonitrile, methanol, and HPLC grade water are purchased from Honeywell Burdick & Jackson (Muskegon, MI)

* Acetic acid (glacial) and ammonium acetate are purchased from Sigma Aldrich Laboratories, Inc (St. Louis, MO)

* β -Glucuronidase (*Escherichia coli*-K12) is purchased from Roche Biomedical (Mannheim, Germany).

pH 6.5 Ammonium acetate buffer. To make 500 mL, 38.6 g of ammonium acetate is dissolved in ~496 mL water in a 1L beaker on a magnetic stirrer. Glacial acetic acid is then added drop wise to the ammonium acetate solution until pH reaches 6.5. The solution is transferred to a 500 mL volumetric flask and adjusted to 500 mL mark with water. The contents in the volumetric flask are mixed well and transferred to a glass bottle and stored in the refrigerator. The pH meter is calibrated using pH 4, 7 and 10 calibrators before use.

b. Analytical Standards

(1) Source

Phthalate metabolite native and labeled standards were obtained from: Cambridge Isotope Laboratories Inc (Andover, MA), Los Alamos National Laboratory (Los Alamos, NM), Professor Jurgen Angerer (Germany), and Cansyn (Toronto, Canada).

$^{13}\text{C}_4$ -4-methyl umbelliferone was purchased from Cambridge Isotope Laboratories Inc. 4-methyl umbelliferone glucuronide was purchased from Sigma Chemicals (Cat. M5664)

(2) Standards Preparation

- (a) Individual native standards of phthalate metabolites. The stock solutions are prepared by transferring approximately 5 mg of material accurately to a 50 mL volumetric flask. The phthalate metabolite is then dissolved in acetonitrile. This stock solution is stored at $-20\text{ }^{\circ}\text{C}$ in a methanol rinsed and air dried Teflon-capped amber glass bottle until use.
- (b) Internal standards of isotopically-labeled phthalate metabolites and $^{13}\text{C}_4$ -4-methyl umbelliferone. These internal standards are prepared similarly to the native standards and stored at $-20\text{ }^{\circ}\text{C}$ until use. The isotopic purity of each internal standard, confirmed empirically by tandem mass spectral analysis, was determined to contain less than 1% of the native compound.
- (c) 4-methyl umbelliferone glucuronide standard. A 25 mL volume of stock standard solution is prepared by transferring approximately 10 mg of the 4-methyl umbelliferone glucuronide accurately to a 25 mL volumetric flask (methanol rinsed) and then adding 2.5 mL of acetonitrile and 22.5 mL of HPLC grade water. An intermediate 40 ppm 4-methyl umbelliferone glucuronide stock solution is prepared by diluting the stock solution with HPLC grade water. The stock solution and the intermediate stock solution are stored at $-20\text{ }^{\circ}\text{C}$ in a Teflon-capped glass bottle (methanol rinsed). The 0.16 ppm spiking solution is made in HPLC grade water by diluting the intermediate stock solution and stored in the refrigerator and discarded after 3 month.
- (d) Eleven unique intermediate stock standards with all analytes and 4-methyl umbelliferone are prepared in 10% aqueous acetonitrile from the stock solutions of native and the isotopically-labeled internal standards for each analyte. Intermediate stock standards are stored in the refrigerator.
- (e) Eleven unique working standards are prepared from the intermediate stock standards in 0.1% acetic acid in water to construct calibration curves and stored in the refrigerator at $4\text{ }^{\circ}\text{C}$.

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- (f) Internal standard spiking solution is prepared in 1:9 acetonitrile:water from the stock solutions of the isotopically-labeled internal standards and stored in the refrigerator.

(3) Storage and Stability

All standards are kept in amber Boston round bottles with Teflon-lined screw caps. Working standard solutions are kept in the refrigerator (4 °C) and remade as needed from the stock solutions. Stock standard mixtures are kept in the refrigerator. Stock standard mixtures are remade, as necessary.

c. Proficiency Testing Standards

Aliquots of each stock standard were added to 1L urine pools. The volume of each standard varied, as in the table below, to produce 3 concentrations of proficiency testing (PT) standards. The spiked pools were mixed overnight and are aliquoted into cryovials and frozen (<-20°C) until needed. The PT standards are characterized by at least 20 repeat determinations to characterize the mean and standard deviation for evaluation.

d. Materials

- (1) Chromolith Flash RP-18e precolumn (4.6 mm x 25 mm, Merck KGaA, Germany).
- (2) 1.5 mL autosampler vials (Agilent, USA) and pre-slit caps (caps with PTFE/Silicone).
- (3) ThermoFinnigan-Keystone Betasil phenyl HPLC column (3 µm, 150 mm x 2.1 mm).
- (4) Inline filters (2 µm and 0.5 µm, Upchurch).
- (5) Pipette tips: 5 ml, 1 mL, 100 µL, 50 µL, 20 µL and 10 µL sizes.

e. Equipment

- (1) Repipettors (Rainin and Eppendorph) and Reference Pipettes (Rainin)
- (2) Balance (TR-203 Series Denver Instrument Company)
- (3) Balance (Sartorius, Genius series)
- (4) Sonicating waterbath (Branson 5210).
- (5) Fisher Isotemp Incubator (300 Series Model 350D).
- (6) Vortexer (Fisher, Genie 2).
- (7) Magnetic Stirrer (Corning).
- (8) ThermoFinnigan Surveyor autosampler
- (9) 6 port switching valve (Rheodyne)
- (10) Corning pH meter (Fisher Scientific)

f. Analytical instrumentation

- 1) ThermoFinnigan Surveyor High Pressure Liquid Chromatograph system
- 2) ThermoFinnigan LC pump
- 3) ThermoFinnigan Quantum Classic Triple Quadrupole Mass Spectrometer

7. Calibration and Calibration-Verification Procedures

Before mass spectral analysis of unknown samples, a known standard is injected to confirm acceptable chromatographic resolution and mass spectral sensitivity. If the instrument yields acceptable performance, a full set of 11 standards followed by the unknowns, QC samples and blanks are analyzed. The analysis is completed by another set of eleven standards. The duplicate standards results are used to construct a daily calibration curve for each analyte (known concentration versus analyte/internal standard area ratio). Each point in the calibration curve is weighted ($1/x$); correlation coefficients are typically > 0.99 . Concentrations are adjusted based on the purity of the analytical standards. The daily calibration curve is used by the Xcalibur data analysis software for all unknowns, QC samples and blanks analyzed on that day.

a. Calibration Verification

- 1) Calibration verification is not required by the manufacturer. However, it should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.
- 2) Calibration verification must be performed at least once every 6 months.
- 3) All calibration verification runs and results shall be appropriately documented.
- 4) According to the updated CLIA regulations from 2003 www.cms.hhs.gov/CLIA/downloads/6065bk.pdf, the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months.
- 5) All of the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.

b. Proficiency testing

PT samples are prepared in-house as described in the standard preparation section. These PT samples encompass the entire linear range of the method and are characterized in our laboratory. The characterization data are forwarded to a CDC's Division of Laboratory Sciences (DLS) PT administrator in charge of executing the PT program. The PT administrator establishes the mean and confidence limits for each analyte concentration.

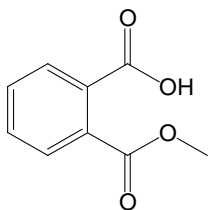
Proficiency testing is performed a minimum of once every 6 months. The PT administrator will randomly select five PT materials for analysis. The PT samples are treated as unknown samples and the analytical results are forwarded directly to the PT

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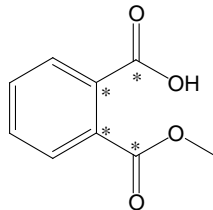
administrator for interpretation. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the administrator. The PT administrator will notify the laboratory of its PT status (i.e. pass/fail).

All proficiency test results are appropriately documented.

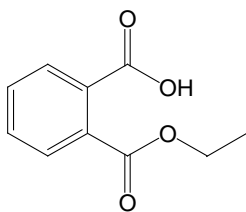
In addition to the in-house PT program, a minimum of once per year, two reference urine samples fortified with several phthalate metabolites (MBP, MEHHP, MEOHP, and MECPP) are received from the German External Quality Assessment Scheme (G-EQUAS) organized and managed by the Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (Erlangen, Germany). The PT samples are analyzed and the data are reported for evaluation. The program, evaluation, and certification are based on the guidelines of the German Federal Medical Council (<http://www.g-equas.de/>).



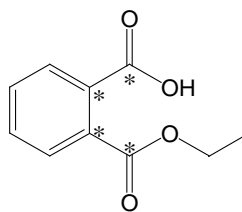
mMP
monomethyl phthalate



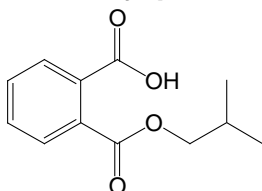
mMP*
monomethyl phthalate-¹³C₄



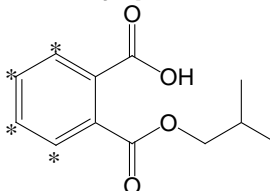
mEP
monoethyl phthalate



mEP*
monoethyl phthalate-¹³C₄

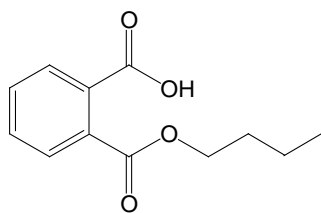


miBP
mono(2-methylpropyl) phthalate
mono-isobutyl phthalate

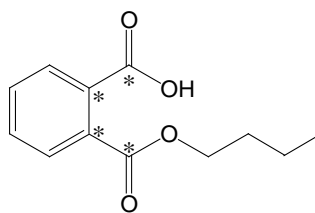


miBP*
mono(2-methylpropyl) phthalate-D₄
mono-isobutyl phthalate-D₄

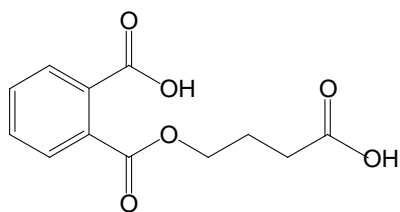
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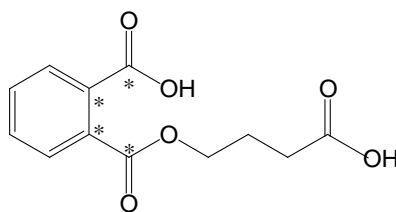
mBP
mono-n-butyl phthalate



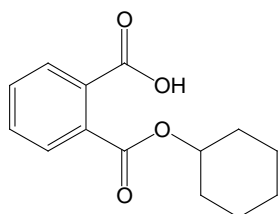
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mono-n-butyl phthalate-¹³C₄



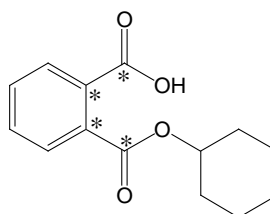
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mono(3-carboxypropyl) phthalate



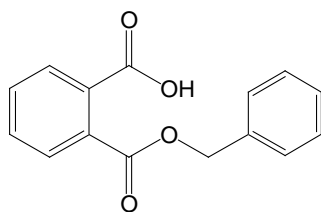
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mono(3-carboxypropyl) phthalate-¹³C₄



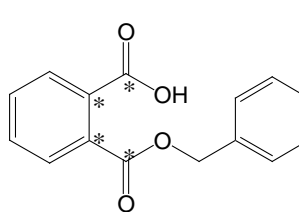
mCHP
monocyclohexyl phthalate



mCHP*
monocyclohexyl phthalate -¹³C₄

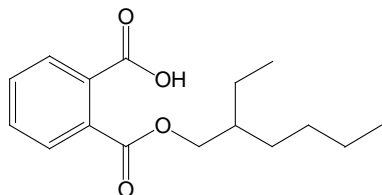


mBzP
monobenzyl phthalate

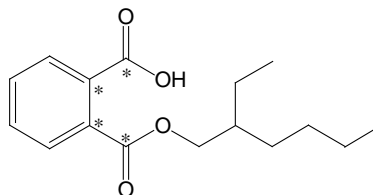


mBzP*
monobenzyl phthalate-¹³C₄

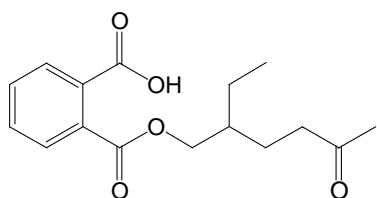
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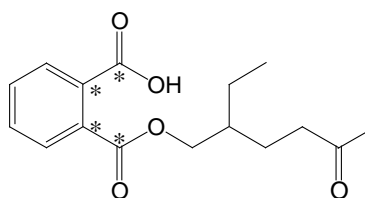
mEHP
mono(2-ethylhexyl) phthalate



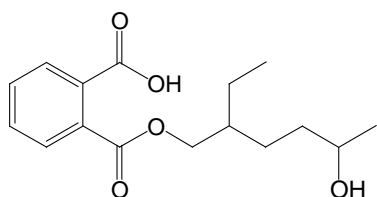
mEHP*
mono(2-ethylhexyl) phthalate-¹³C₄



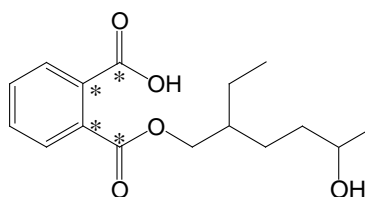
mEOHP
mono(2-ethyl-5-oxohexyl) phthalate



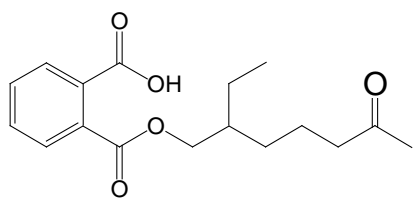
mEOHP*
mono(2-ethyl-5-oxohexyl) phthalate-¹³C₄



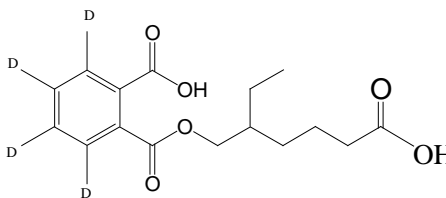
mEHHP
mono(2-ethyl-5-hydroxyhexyl) phthalate



mEHHP*
mono(2-ethyl-5-hydroxyhexyl) phthalate-¹³C₄

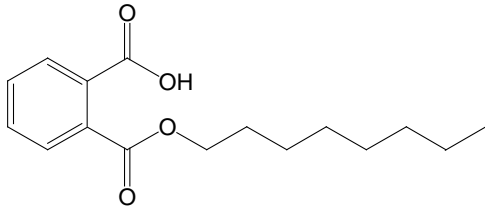


MECPP
mono(2-ethyl-5-carboxypentyl)phthalate

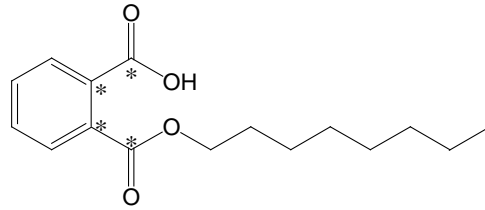


mECPP*
D₄-mono(2-ethyl-5-carboxypentyl)phthalate

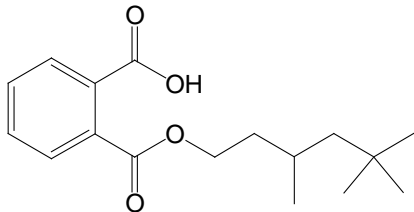
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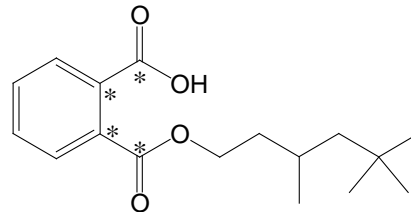
mOP
mono(2,6-methyl-6-carboxyhexyl)phthalate



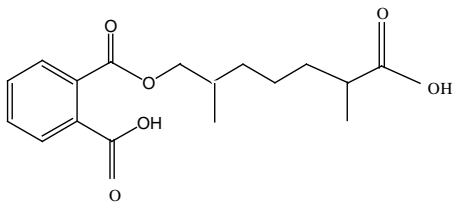
mOP*
mono(2,6-methyl-6-carboxyhexyl)phthalate-¹³C₄



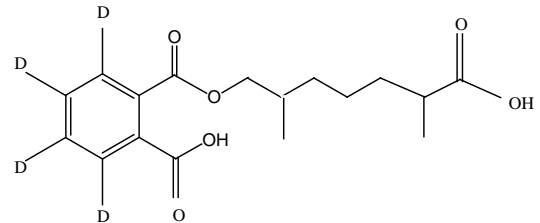
mNP
mono(3,5,5-trimethyl-1-hexyl)phthalate
(mono-isononyl phthalate)



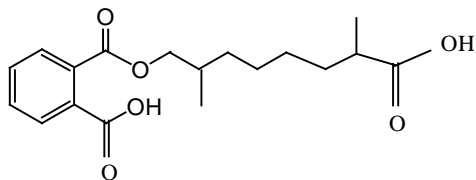
mNP*
mono(3,5,5-trimethyl-1-hexyl)phthalate-¹³C₄
(mono-isononyl phthalate ¹³C₄)



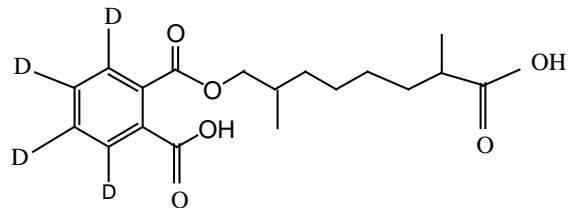
mCOP
mono(2,6-methyl-6-carboxyhexyl)phthalate
(mono-carboxyisooctyl phthalate)



mCOP
mono(2,6-methyl-6-carboxyhexyl)phthalate
(D₄-mono-carboxyisooctyl phthalate)



mCNP
mono(2,7-methyl-7-carboxyheptyl)phthalate
(mono-carboxyisooctyl phthalate)



mCNP*
mono(2,7-methyl-7-carboxyheptyl)phthalate
(D₄-mono-carboxyisooctyl phthalate)

8. Operating Procedures; Calculations; Interpretation of Results

a. Preliminaries

- (1) The on-line solid phase extraction batch typically consists of: 4 Reagent Blanks (RB), 2 Quality Control materials of low (QCL) and 2 of high (QCH) concentration, and 40 unknown urine samples.
- (2) The urine samples and QC materials are allowed to thaw completely at room temperature in a sonicating water bath.
- (3) The samples are mixed well by vortexing.
- (4) Each analytical sequence typically consists of two analytical runs.
- (5) The β -glucuronidase solution (mix fresh just prior to addition to sample) is prepared as follows.
 - (a) For a run of 50 samples; 1.5 mL of 1 M, pH 6.5, ammonium acetate buffer is transferred accurately into an autosampler tube. 30 μ L of β -glucuronidase is pipetted into the beaker containing the ammonium acetate buffer.
 - (b) The solution is swirled to mix and placed in the sample preparation autosampler.

b. Sample preparation

- (1) 100 μ L of urine, 100 μ L mL HPLC Grade water (for the reagent blank), or 100 μ L mL of QCH or QCL are transferred manually into properly labeled autosampler vials (1.5 mL).
- (2) The vial is capped with Teflon-lined screw caps.
- (3) The vial is placed in the sample tray in the sample preparation autosampler for automated sample preparation.
- (4) The autosampler tray is set at 37°C for incubation of samples.
- (5) 25 μ L of 0.16 ng/mL 4-Methylumbelliferone glucuronide spiking solution, 100 μ L Internal Standard (isotopically labeled mixture) spiking solution and 25 μ L of β -glucuronidase/ammonium acetate buffer solution are added into each vial and mixed. For analysis of free unconjugated phthalates β -glucuronidase/ammonium acetate buffer solution is replaced with the ammonium acetate buffer without β -glucuronidase.
- (6) After minimum of 90 min incubation, 50 μ L of glacial acetic acid is added and the enzyme activity is stopped by adding and 200 μ L of 5% acetonitrile in water.
- (7) The autosampler tray temperature is set to 0°C after preparation of the whole set.
- (8) The autosampler tray is moved to the HPLC/MS analytical system for analysis.
- (9) The standards do not undergo the automated sample preparation procedure as they are prepared using native standards which are not in their conjugated form.

c. Instrumental Analysis

(1) On-line SPE-HPLC/MS analysis

The analysis is performed using a ThermoFinnigan LC pump, ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer, equipped with an ESI (Electrospray Ionization) interface [1,2]. All three systems and the six port Reodyne switching valve are controlled by the Xcalibur Software. The autosampler tray is set at 10°C. With the LC pump in the sample loading position, 450 µL of the deconjugated urine sample is injected using the Surveyor autosampler. The sample is loaded onto a Chromolith Flash RP-18e SPE column and rinsed using 0.1% acetic acid in water: 0.1% acetic acid in acetonitrile at 1.8 mL per min (Table 1). The Reodyne valve is automatically switched to its alternate position, reversing the flow and allowing the [1,2] analytes to be transferred from the SPE column on to HPLC column. The chromatographic resolution is accomplished using a 3 µm, 150 mm x 2.1 mm Keystone Betasil phenyl column and a solvent gradient (Table 1). Each sample (450 µL) is injected using the liquid chromatograph autosampler, configured with syringe washes between injections to minimize carryover. Inline filters are used to remove particulate materials from the injected samples and to extend the lifetime of the SPE column and the analytical column [1].

Table 1. On-line SPE and HPLC solvent gradient programs

Time (min)	SPE pump			Switching valve	HPLC pump			Switching valve
	A (%)	B (%)	Flow (mL/min)		A (%)	B (%)	Flow (mL/min)	
0	100	0	0.3	SPE/Waste	77	23	0.3	HPLC/Waste
0.4	100	0	1.8	SPE/Waste				
0.5	85	15	1.8	SPE/HPLC				
1.1	85	15	1.8	SPE/HPLC				
1.2	100	0	0.2	SPE/Waste				
3					75	25	0.30	HPLC-MS/MS
3.5	100	0	0.2	SPE/Waste				
4	100	0	1.5	SPE/Waste				
5					75	25	0.3	HPLC-MS/MS
8.1	100	0	1.5	SPE/Waste				
8.2	0	100	1.5	SPE/Waste				
10					67	33	0.35	HPLC-MS/MS
10.2	0	100	1.5	SPE/Waste				
10.5	0	100	0.2	SPE/Waste				
17					70	30	0.325	HPLC-MS/MS
19.85					66	34	0.35	HPLC-MS/MS
20.1	0	100	0.2	SPE/Waste	60	40	0.35	HPLC-MS/MS
20.2	100	0	0.3	SPE/Waste				
23.1					45	55	0.35	
25.1					20	80	0.35	
25.2					0	100	0.35	
25.6					0	100	0.4	
26.6					0	100	0.4	

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26.7					77	23	0.35	
28	100	0	0.3		77	23	0.35	HPLC/Waste

Electrospray ionization (ESI) in negative ion mode was used to ionize the analyte molecules and transfer the negatively charged analyte ions into the gas phase. The source collision induced dissociation voltage was set to 10 V to break down acetate clusters.

During the analysis, the instrument was set in the multiple reaction monitoring mode so that precursor and the product ion combinations specific to the eluting analyte could be monitored. Reproducible chromatography allowed for the use of different data acquisition windows for different analyte groups. Product ions are formed in the collision cell using argon at ~1.5 mTorr. The collision offset is specifically set for each ion (Table 2).

(2) Multiple Reaction Monitoring Setup

Table 2. Phthalate metabolites and their native and labeled precursor and product ion transitions and collision energies.

Analyte	Precursor/ Product ion	Collision energy (V)
MCPP	251/103	10
¹³ C ₄ -MCPP	255/103	
MMP	179/77	24
¹³ C ₄ -MMP	183/79	
MEP	193/77	25
¹³ C ₄ -MEP	197/79	
MECPP	307/159	22
D ₄ -MECPP	311/159	
MiBP	221/77	26
D ₄ -MiBP	225/81	
MBP	221/77	26
¹³ C ₄ -MBP	225/79	
MEOHP	291/121	27
¹³ C ₄ -MEOHP	295/124	
MEHHP	293/121	27
¹³ C ₄ -MEHHP	297/124	
MCOP	321/173	19
D ₄ -MCOP	325/173	
MBzP	255/183	14
¹³ C ₄ -MBzP	259/186	
MCHP	247/77	27
¹³ C ₄ -MCHP	251/79	
MCNP	335/187	21
D ₄ -MCNP	339/187	
MEHP	277/134	21
¹³ C ₄ -MEHP	281/137	
MOP	277/125	23

¹³ C ₄ -MOP	281/127	
MNP	291/121	27
¹³ C ₄ -MNP	295/124	

d. Calculations

The concentration of the individual analytes in each sample is calculated using the calibration curve derived from the known standard mixtures.

All analyte concentrations are corrected for the concentrations of the same analytes present in the reagent blanks. The concentrations in the blanks in each run are averaged; the average blank concentration is subtracted from the concentration of each sample and the QC.

The final urinary concentrations of phthalate metabolites are normally adjusted for creatinine.

e. Interpretation of Results

The urinary concentrations of phthalate monoesters obtained using this analytical method can be used to estimate recent exposure to phthalates. However, the metabolism of each phthalate is unique and the proportion of monoester metabolite and oxidative metabolites is different for each phthalate. Therefore similar metabolite concentrations from different phthalates may not reflect similar exposure levels.

9. Reportable Range of Results

The linear range of the standard calibration curves and the method limit of detection (LOD) determine the reportable range of results. The reportable range must be within the range of the calibration curves.

a. Linearity Limits

The calibration curve is linear for all analytes ($R^2 > 0.98$) over three orders of magnitude. The limit on the linearity is determined by the highest standard analyzed in the method. Unknown urine samples with concentrations exceeding the upper calibration standard are reanalyzed using a smaller aliquot. The low end of the linear range is limited by the method LOD. Samples with concentrations below the method LOD are reported as non-detectable.

b. Limit of Detection (LOD)

The formal LOD for each analyte was calculated as $3S_0$, where S_0 is the standard deviation value as the concentration approaches zero [15]. S_0 was determined from the replicate analysis of low-level standards. The functional LOD is equal to the formal LOD unless the lowest point in the calibration curve is higher, then the functional LOD is defined as the lowest standard concentration used in the calibration curve (Table 3).

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Table 3. Limits of detection (LODs) and calibration range of the method.

Analyte	LOD(ng/mL)	Calibration range (ng/mL)
MCPPP	0.2	0.1-400
MMP	1.1	0.2-800
MEP	0.8	0.2-4100
MiBP	0.3	0.1-520
MBP	0.6	0.3-1040
MEHHP	0.7	0.2-672
MECPP	0.6	0.1-2050
MEOHP	0.7	0.2-640
MCHP	0.3	0.1-240
MBzP	0.3	0.2-720
MEHP	1.2	0.2-720
MOP	1.1	0.2-600
MNP	0.8	0.1-400
MCOP	0.7	0.1-400
MCNP	0.6	0.1-400

c. Accuracy

The method accuracy was assessed through 5 replicate analyses of analytes spiked at three different concentrations (0.25-1.3 ng/mL, 9.6-42 ng/mL, and 96-416 ng/mL) (Table 4).

Table 4. Accuracy of the method.

Analyte	Expected Conc. (ng/mL)	Measured Conc. (ng/mL)	STD	Expected Conc. (ng/mL)	Measured Conc. (ng/mL)	STD	Expected Conc. (ng/mL)	Measured Conc. (ng/mL)	STD
MCPPP	0.6	0.54	0.04	19.8	18.9	0.1	192	191.8	1.8
MMP	1	0.91	0.11	32	31	0.8	320	318.9	10.7
MEP	1.2	0.98	0.64	38.4	37.7	1.3	384	384.4	11
MiBP	0.65	0.42	0.16	20.8	20.6	0.8	208	207.4	7.4
MBP	1.3	1	0.09	41.6	40.4	2	416	413.2	6.8
MEHHP	0.7	0.5	0.06	22.4	24	3	224	225.6	6.1
MECPP	0.5	0.38	0.09	16	15.7	0.5	160	160	1.8
MEOHP	0.8	0.67	0.04	25.6	24.7	0.9	256	257.3	5.1
MCHP	0.25	0.18	0.03	9.6	9.3	0.3	96	95.1	2.4
MBzP	0.9	0.68	0.17	28.8	28.3	1.4	288	289.3	6.4
MEHP	0.9	1.13	0.18	28.8	28.4	0.5	288	284.2	4.8
MOP	0.75	0.65	0.1	24	23.3	2.5	240	238.2	13.6
MNP	0.5	0.36	0.02	16	15.8	1.2	160	162.1	2.7
MCOP	0.5	0.43	0.02	16	15.6	0.3	160	158.8	5.1
MCNP	0.5	0.45	0.03	16	16	0.3	160	160.2	1.2

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d. Precision

The precision of the method is determined by calculating the coefficient of variation (CV) of repeated measurements of the QC materials over time. This value reflects both the intraday and interday variability of the assay (Table 5).

Table 5. Precision at two concentration levels using urine QC pools

Analyte	QCH		QCL	
	CV (%)	Mean	CV (%)	Mean
mMP	4.0	48.3	17.8	5.7
mEP	2.9	485.6	5.2	62.1
mCPP	14.0	26.3	15.5	4.1
miBP	7.3	63.6	13.2	9.0
mBP	7.5	68.6	17.4	13.9
mCHP	7.5	35.8	6.9	4.0
mBzP	6.8	72.9	8.9	8.4
mEHP	6.3	75.6	12.0	13.9
mOP	22.6	80.3	23.2	14.1
mNP	6.9	36.1	8.2	10.1
mEOHP	3.3	51.2	4.4	12.9
mEHHP	3.8	57.1	5.7	14.0
mECPP	5.3	43.0	7.2	12.9
MCNP	10.2	57.9	8.7	9.2
MCOP	6.2	40.6	7.1	8.0

10. QC Procedures

Quality control (QC) materials are prepared from urine pools collected from several anonymous donors. Preliminary human quantification is used to set target ranges for baseline levels (QC Low: 5-20 ppb), and higher levels (QC High: 30-500 ppb). The human urine pool is spiked with additional phthalate monoester analytes as needed. The urine is then thoroughly mixed and dispensed into labeled cryovials. The vials are tightly capped and stored at or below -20 °C until used. The QC pools are characterized to determine the mean concentration and the 95th and 99th confidence intervals for both means and

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variance. QC characterization involved at least 100 discrete measurements spanned over at least one month, prior to analysis of unknown samples. Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control [16].

When using 2 QC pool levels (1QCL and 1 QCH) per run, the rules are:

- 1) If both QC run results are within $2S_i$ limits, then accept the run.
- 2) If 1 of the 2 QC run results is outside a $2S_i$ limit - reject run if:
 - Extreme Outlier – Run result is beyond the characterization mean $\pm 4S_i$
 - 1. 3S Rule – Run result is outside a $3S_i$ limit
 - 2. 2S Rule – Both run results are outside the same $2S_i$ limit
 - 3. 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
 - 4. R 4S Rule – Two consecutive standardized run results differ by more than $4S_i$ (standardized results are used because different pools have different means). Since runs have single measurements per pool for 2 pools, comparison of results for the R 4S rule will be with the previous result within run or the last result of the previous run.

When using 2 QCs per QC pool levels (2QCL and 2 QCH) per run, the rules are:

- 1) If both QC run means are within $2S_m$ limits and individual results are within $2S_i$ limits, then accept the run.
- 2) If 1 of the 2 QC run means is outside a $2S_m$ limit - reject run if:
 - Extreme Outlier – Run mean is beyond the characterization mean $\pm 4S_m$
 - 1 3S Rule – Run mean is outside a $3S_m$ limit
 - 2 2S Rule – Both run means are outside the same $2S_m$ limit
 - 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean
- 3) If one of the 4 QC individual results is outside a $2S_i$ limit - reject run if:
 - R 4S Rule – Within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit). Since runs have multiple measurements per pool for 2 pools, the R 4S rule is applied within runs only.

Abbreviations:

S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).

S_m = Standard deviation of the run means (the limits are shown on the chart).

S_w = Within-run standard deviation (the limits are not shown on the chart).

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

If the QC systems or the calibrations failed to meet acceptable criteria, all operations are suspended until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for instance, failure of the mass spectrometer or a pipetting

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error, the problem is immediately corrected. Otherwise, problem is further investigated and corrective measures are implemented. Before beginning another analytical run, several QC materials and calibration standards are reanalyzed. After calibration and quality control have been reestablished, analytical runs are resumed.

12. Limitations of Method; Interfering Substances and Conditions

The procedure requires expensive instrumentation.

Sources of imprecision in the procedure may occur due to intermittently imprecise pipetting and/or phthalate contamination in extraction materials and contaminated solvents.

Any contact with some plastics during specimen acquisition, storage, or sample analysis can result in interference.

Prolonged exposure to room temperature during sample collection and/or transport may result in degradation of the urine specimen and/or phthalate metabolites. Care should be taken during sample collection and processing to prevent prolonged exposure to temperatures above freezing and the sample should be frozen within a short time after collection.

13. Reference Ranges (Normal Values)

The results from the National Health and Nutrition Examination Survey (NHANES) 1999-2004 will be used as the reference range to describe levels of phthalate exposure among the general US population (Table 6).

Table 6. Creatinine adjusted phthalate metabolite concentrations in urine ($\mu\text{g/g}$ creatinine) from NHANES 1999-2000, 2001-2002, and 2003-2004.

Metabolite	Survey years	N	LOD (ng/mL)	Geometric mean	Median	75 th percentile	90 th percentile	95 th percentile
mMP	01-02	2772	0.2	1.08	1.33	2.62	5.0	7.97
	03-04	2605	1.0	*	1.53	3.45	7.95	13.5
mEP	99-00	2536	1.2	163	141	360	898	1950
	01-02	2772	0.9	167	147	388	975	1860
	03-04	2605	0.4	181	153	452	1110	2040
mBP	99-00	2541	0.9	22.4	21.9	38.9	68.3	97.5
	01-02	2772	1.1	17.8	17.4	30.4	52.4	81.3
	03-04	2605	0.4	19.8	19.3	33.9	58.9	91.6
miBP	01-02	2772	1.0	2.53	2.44	4.50	8.02	12.0
	03-04	2605	0.3	3.57	3.57	6.18	10.9	15.3

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mBzP	99-00	2541	0.8	14.0	13.3	25.1	50.1	77.4
	01-02	2772	0.3	14.1	13.5	26.6	55.1	90.4
	03-04	2605	0.11	12.9	12.5	24.6	45.9	70.0
mCPP	01-02	2772	0.4	2.57	2.45	4.07	7.25	11.4
	03-04	2605	0.16	2.74	2.59	4.39	7.70	10.7
mCHP	99-00	2541	0.9	*	<LOD	<LOD	<LOD	3.0
	01-02	2782	0.3	*	<LOD	<LOD	0.588	0.854
	03-04	2605	0.2	*	<LOD	<LOD	<LOD	0.45
mEHP	99-00	2541	1.2	3.12	3.08	5.88	10.8	18.5
	01-02	2772	1.1	3.99	3.89	7.94	18.2	32.8
	03-04	2605	0.9	2.20	1.89	4.31	10.7	24.9
mEHHP	01-02	2772	1.0	18.8	16.6	32.3	70.8	147
	03-04	2605	0.32	20.4	17.7	35.8	93.4	176
mEOHP	01-02	2772	1.0	12.6	11.2	21.3	45.1	87.5
	03-04	2605	0.45	13.6	12.1	24.3	63.0	118
mECPP	03-04	2605	0.25	32.6	27.0	54.6	139	248
mNP	99-00	2541	0.8	*	< LOD	< LOD	< LOD	4.29
	01-02	2772	0.8	*	< LOD	< LOD	< LOD	<LOD
	03-04	2605	1.0		<LOD	<LOD	<LOD	<LOD
mOP	99-00	2541	0.9	*	< LOD	< LOD	2.40	3.51
	01-02	2772	1.0	*	< LOD	< LOD	< LOD	< LOD
	03-04	2605	1.0		<LOD	<LOD	<LOD	<LOD

14. Critical-Call Results (“Panic” Values)

Reported urine levels of some phthalate monoesters can approach the mg/L range. The phthalate monoester values obtained using this method of analysis are investigational markers of phthalate exposure only; therefore critical values have not been determined.

15. Specimen Storage and Handling during Testing

Specimens are stored in the laboratory frozen (≤ -20 °C) prior to analysis. Prepared samples are kept at 10 °C during analysis. Frozen samples are allowed to thaw completely at room temperature prior to the initiation of the analytical procedure.

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

The current analytical method utilizes a ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Classic Quantum mass spectrometer. The solid phase extraction can also be done manually [17,18] or automated [19]. If the analytical system fails, prepared samples can be stored (≤ -20 °C) in capped autosampler vials until the analytical system is restored. Otherwise, samples can be re-prepared. If storage system fails, urine samples are transferred to an alternate freezer; if a freezer is not available, the urine samples can be temporarily stored in the refrigerator (≤ 5 °C) for a maximum of 24 hours.

17. Test-Result Reporting System; Protocol for Reporting Critical Calls (if Applicable)

- a. The data from analytical runs of unknowns are initially reviewed by the laboratory supervisor. The supervisor provides feedback to the analyst and/or his/her designee and requests confirmation of the data as needed.
- b. The Quality Control officer reviews each analytical run and identifies the quality control samples within each analytical run and determines whether the analytical run is performed under acceptable control conditions.
- c. One of the Division statisticians reviews and approves the quality control charts pertinent to the results being reported
- d. If the quality control data are acceptable, the laboratory supervisor or his/her designee generates a memorandum to the Branch Chief, and a letter from the Division Director to the person(s) who requested the analyses reporting the analytical results.
- e. These data are then sent to the person(s) that made the initial request.
- f. All data (chromatograms, etc.,) are stored in electronic format in the laboratory.
- g. Final hard copies of correspondence are maintained in the office of the Branch Chief and with the quality control officer.

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

One spreadsheet form (CLIA Specimen Tracking Records) with information for receiving/transferring specimens is kept in the laboratory. In this form, the samples received are logged in when received and when stored/transferred after analysis. For NHANES samples, the person receiving the specimens signs and dates the shipping manifests. The shipping manifests for NHANES and other samples are kept in a binder in the Laboratory.

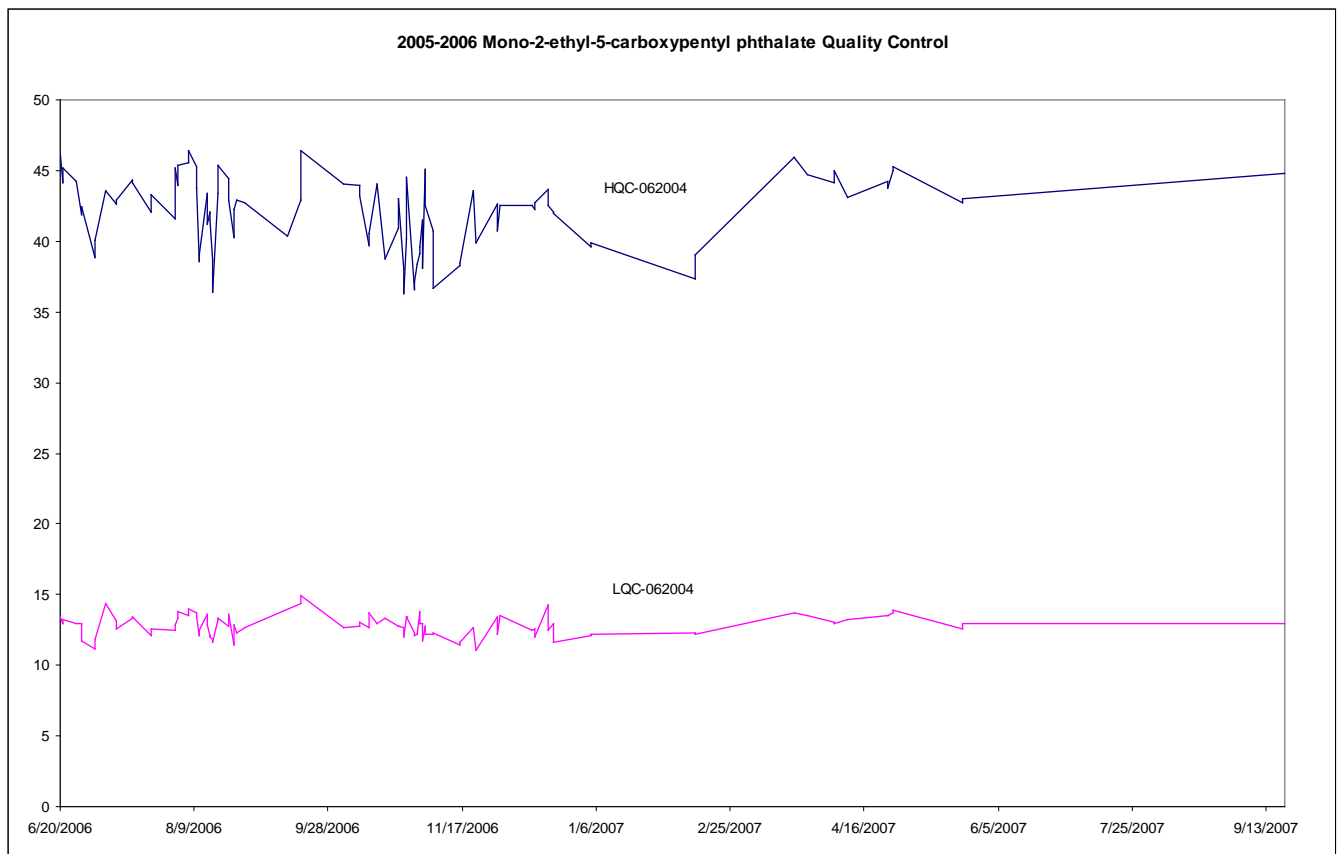
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19. Summary Statistics and QC Graphs

a. Mono-2-ethyl-5-carboxypentyl phthalate (URXECP)

Summary Statistics for Urinary Mono-2-ethyl-5-carboxypentyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	95	6/20/2006	9/20/2007	12.811	0.753	5.9
HQC-062004	96	6/20/2006	9/20/2007	42.101	2.586	6.1

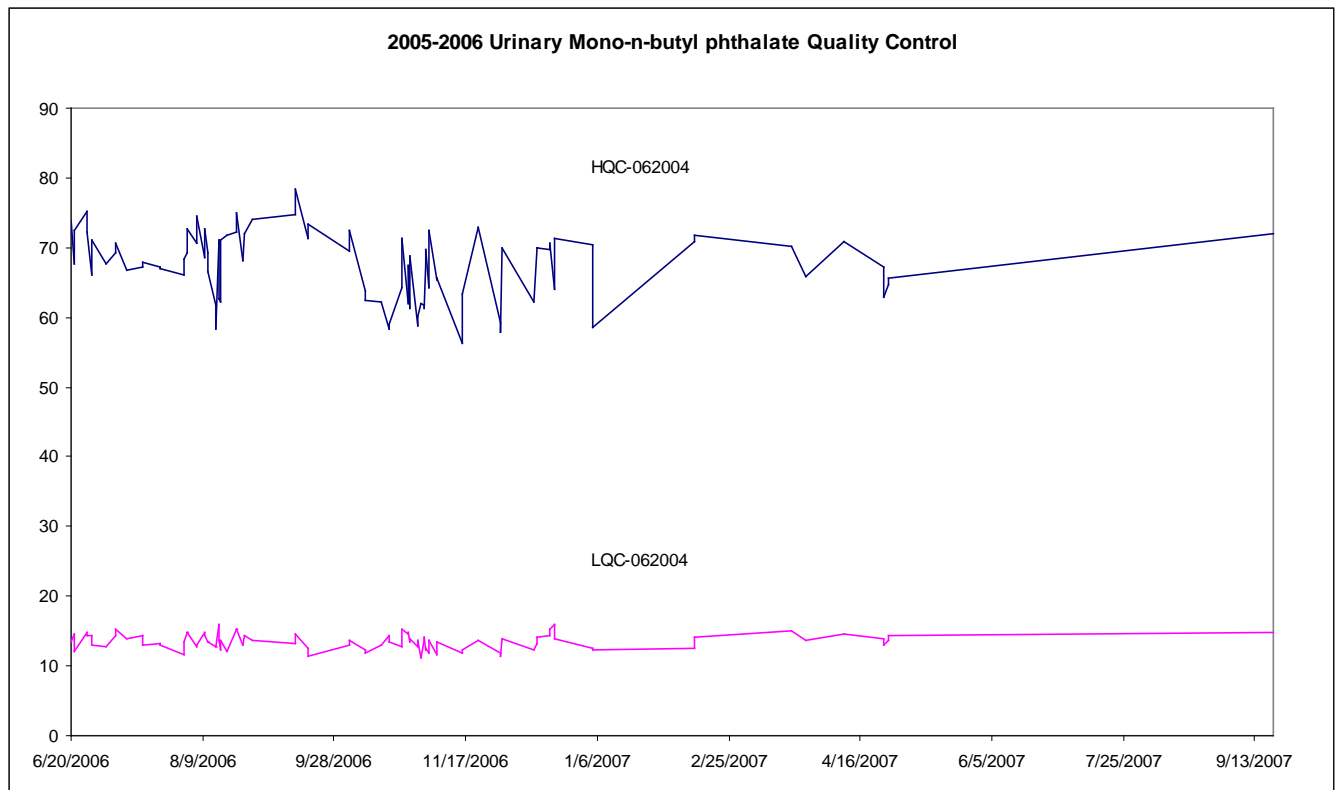


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b. Mono-n-butyl phthalate (URXMBP)

Summary Statistics for Urinary Mono-benzyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	88	6/21/2006	9/20/2007	8.308	0.733	8.8
HQC-062004	88	6/21/2006	9/20/2007	72.198	4.778	6.6

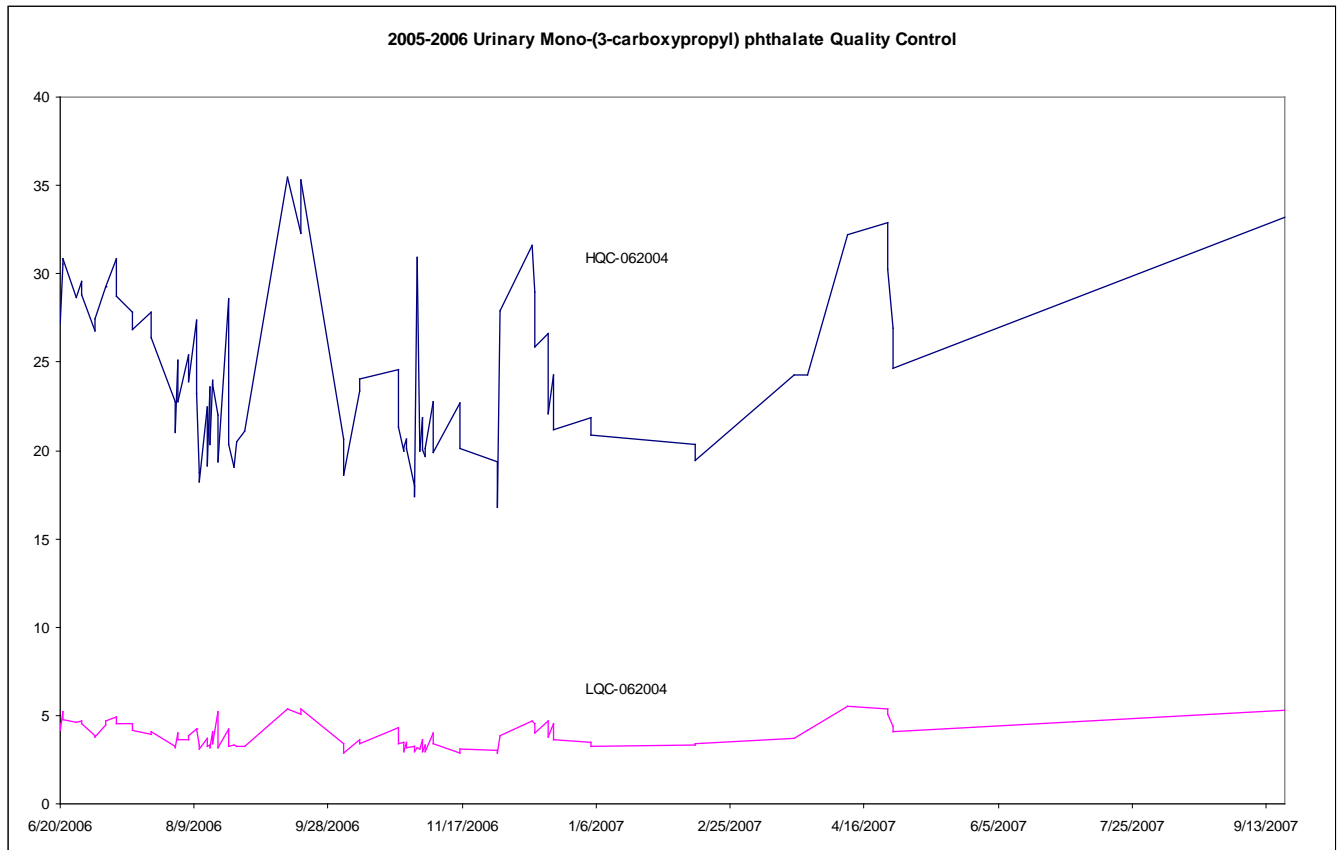


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c. Mono-(3-carboxypropyl) phthalate (URXMC1)

Summary Statistics for Urinary Mono-(3-carboxypropyl) phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	87	6/20/2006	9/20/2007	3.884	0.719	18.5
HQC-062004	87	6/20/2006	9/20/2007	24.395	4.548	18.6

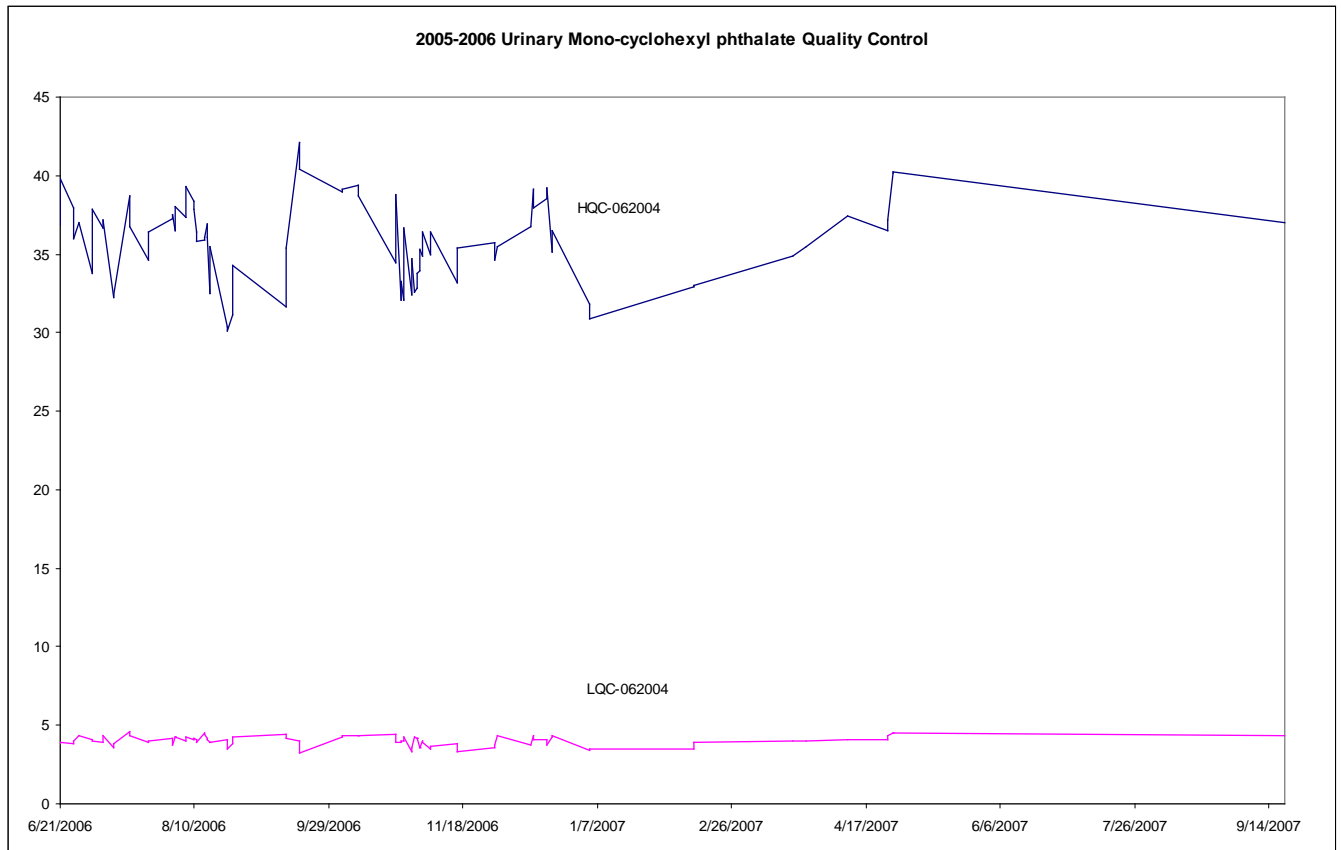


**Phthalate Metabolites in Urine
NHANES 2005-2006**

d. Mono-cyclohexyl Phthalate (URXMCP)

Summary Statistics for Urinary Mono-cyclohexyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	84	6/21/2006	9/20/2007	4.013	0.314	7.8
HQC-062004	84	6/21/2006	9/20/2007	35.872	2.597	7.2

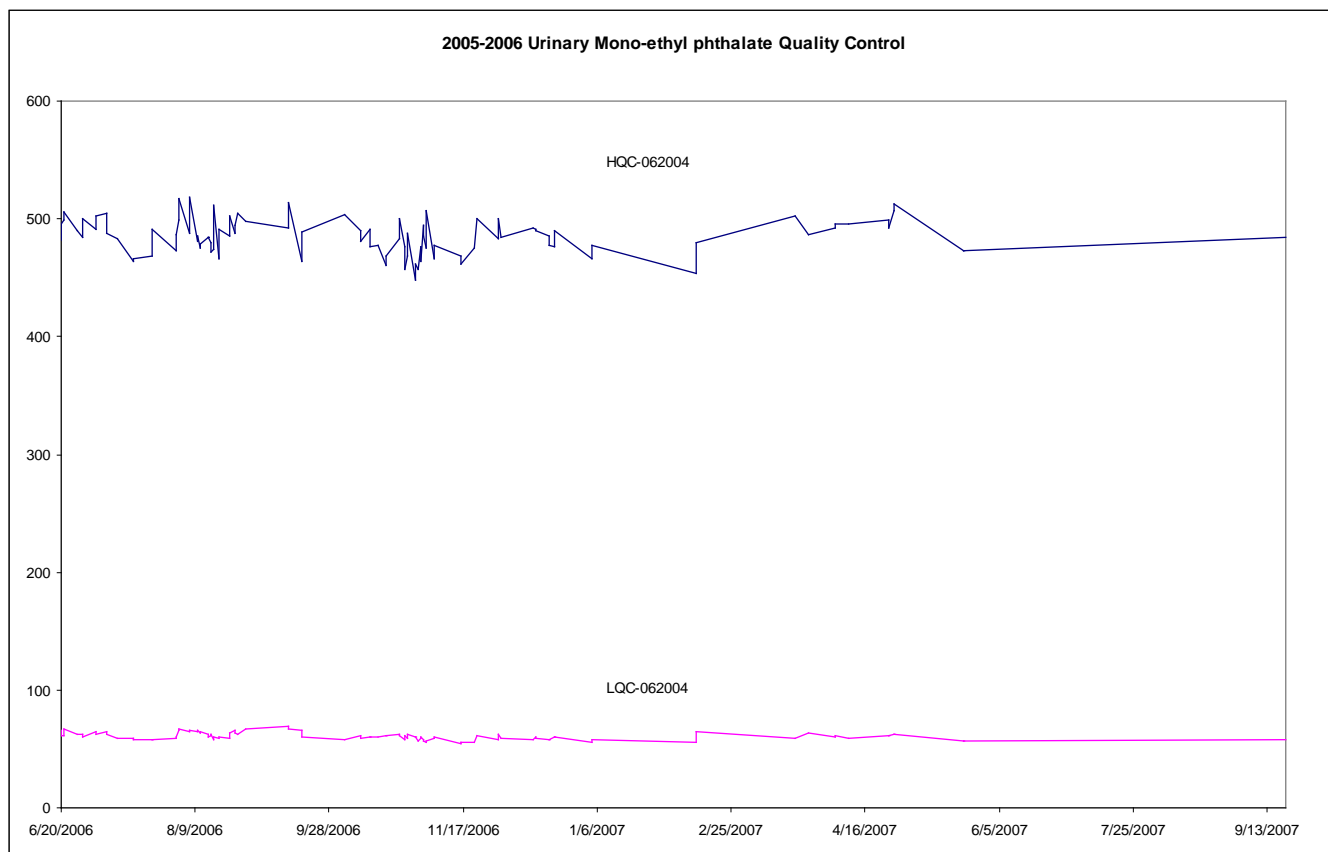


Phthalate Metabolites in Urine NHANES 2005-2006

e. Mono-ethyl phthalate (URXMEP)

Summary Statistics for Urinary Mono-ethyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	99	6/20/2006	9/20/2007	60.745	3.092	5.1
HQC-062004	99	6/20/2006	9/20/2007	484.970	14.910	3.1

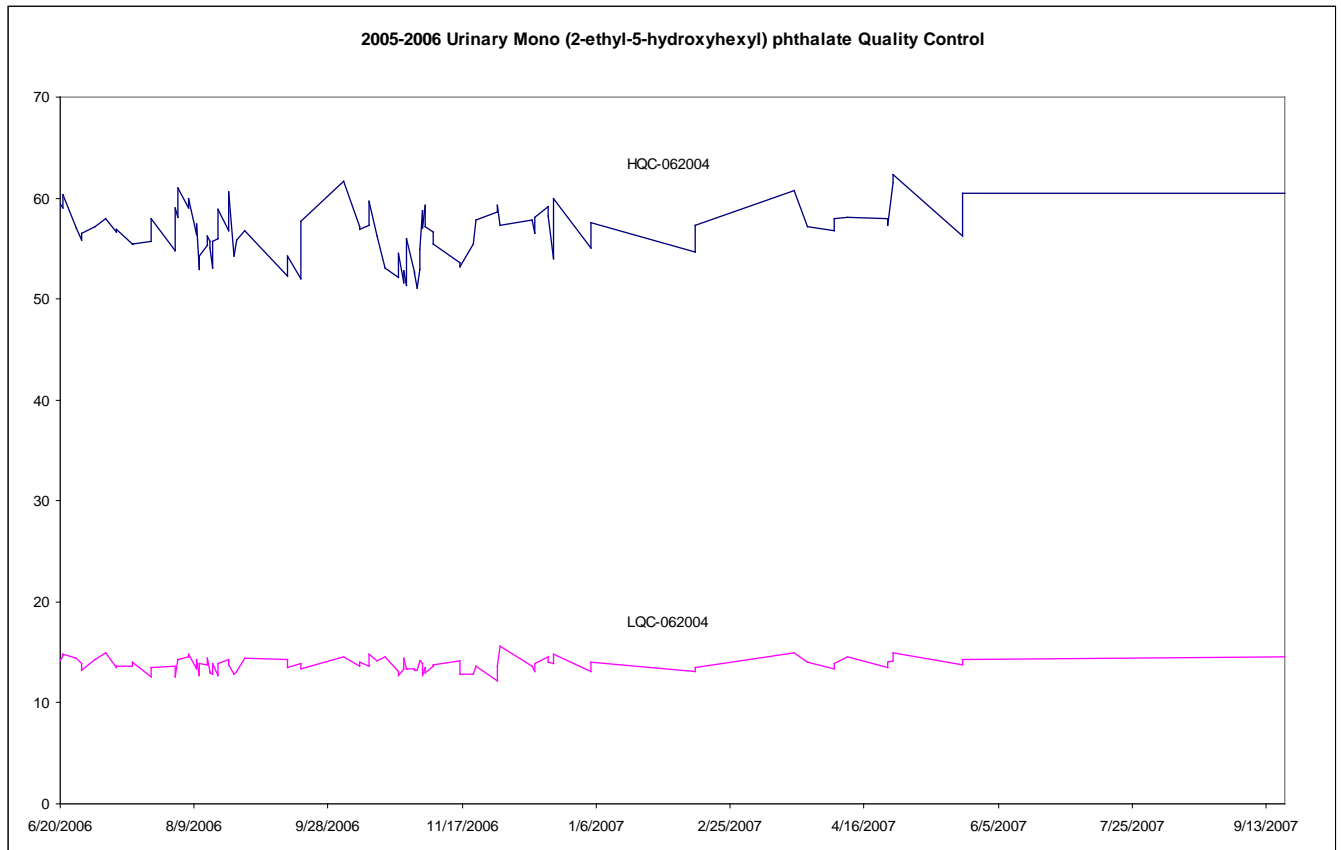


**Phthalate Metabolites in Urine
NHANES 2005-2006**

f. Mono-(2-ethyl-5-hydroxyhexyl) (URXMHH)

Summary Statistics for Urinary Mono (2-ethyl-5-hydroxyhexyl) phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	95	6/20/2006	9/20/2007	13.780	0.661	4.8
HQC-062004	95	6/20/2006	9/20/2007	56.634	2.548	4.5

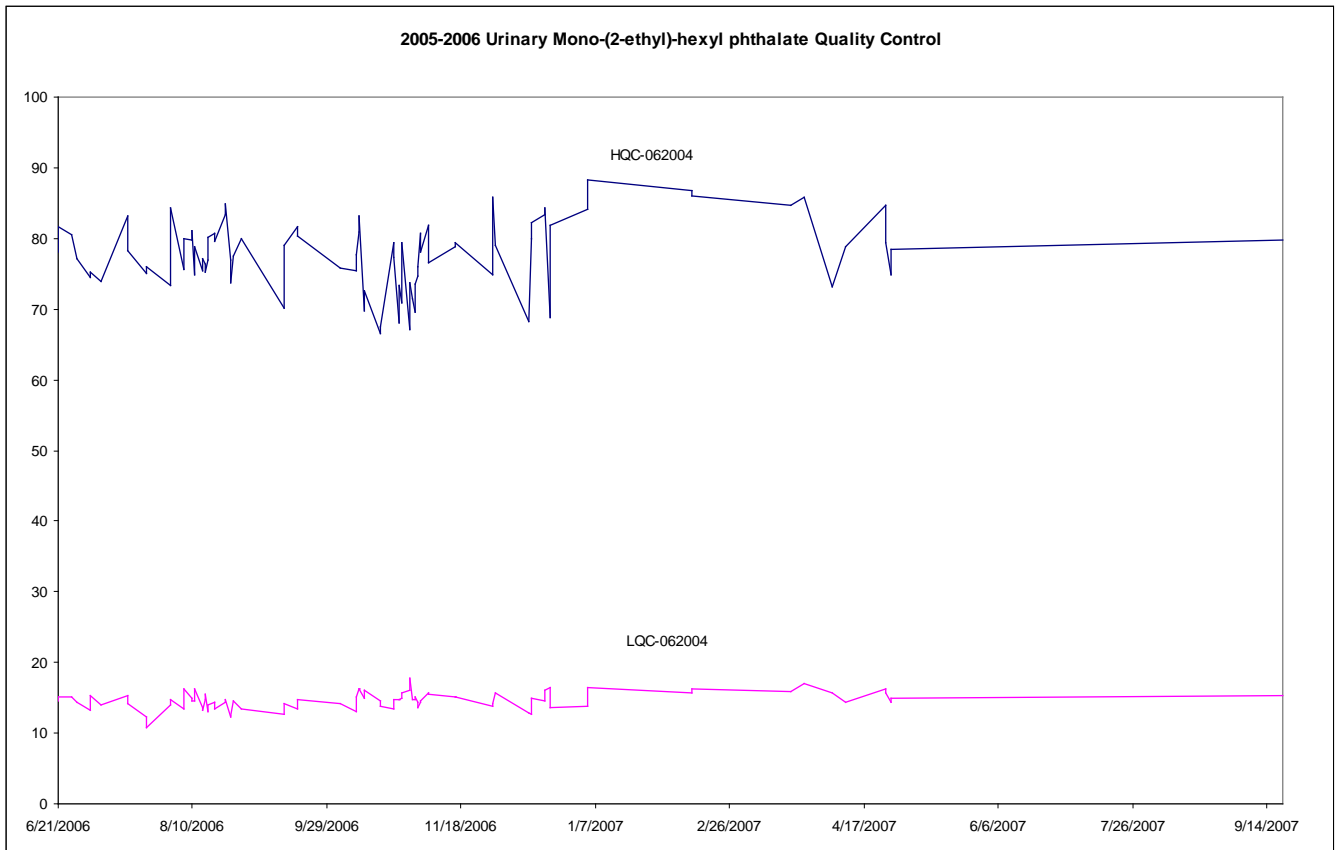


**Phthalate Metabolites in Urine
NHANES 2005-2006**

g. Mono-(2-ethyl)-hexyl phthalate (URXMHP)

Summary Statistics for Urinary Mono-(2-ethyl)-hexyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	88	6/21/2006	9/20/2007	14.633	1.186	8.1
HQC-062004	88	6/21/2006	9/20/2007	77.757	4.926	6.3

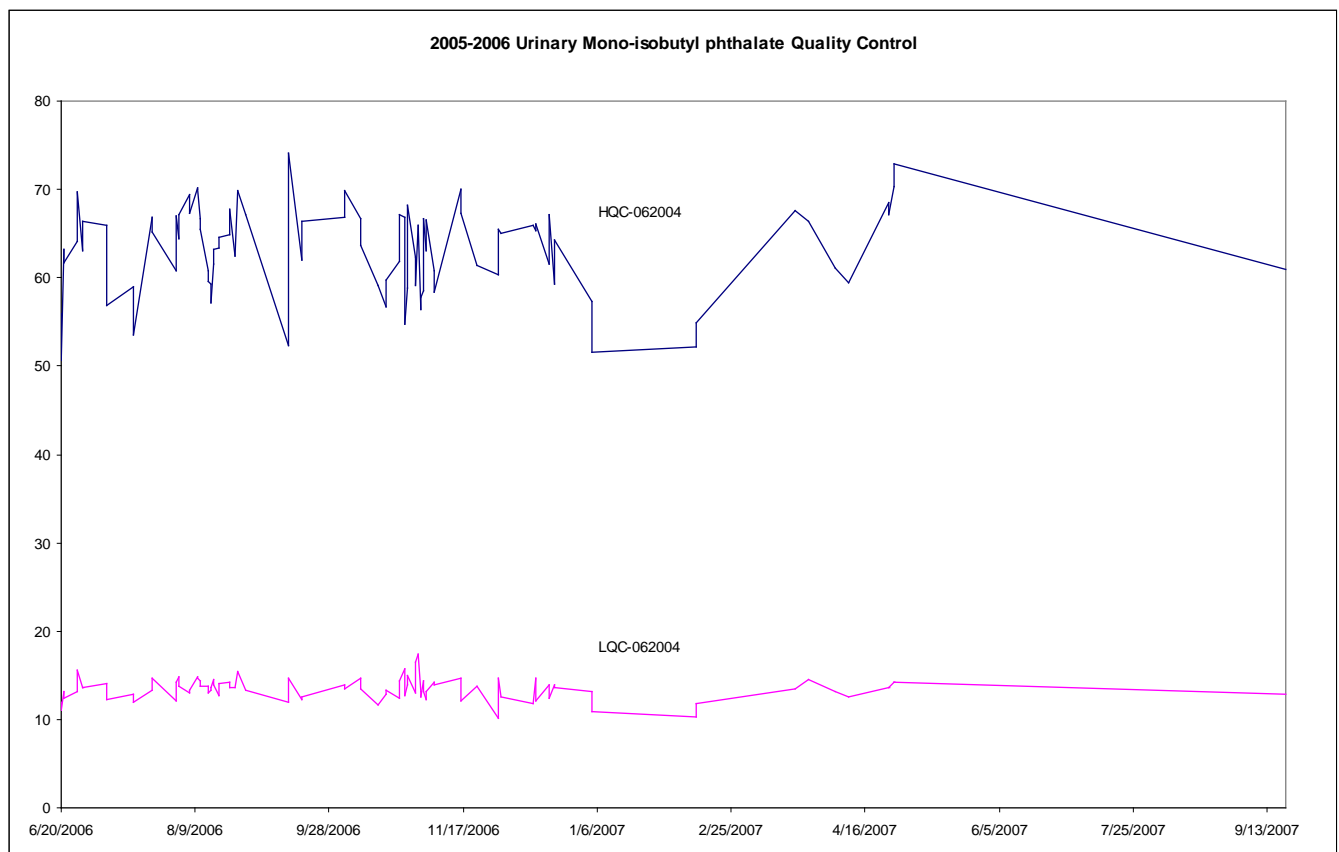


Phthalate Metabolites in Urine NHANES 2005-2006

h. Mono-isobutyl phthalate (URXMIB)

Summary Statistics for Urinary Mono-isobutyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	91	6/20/2006	9/20/2007	13.433	1.212	9.0
HQC-062004	91	6/20/2006	9/20/2007	63.209	5.011	7.9

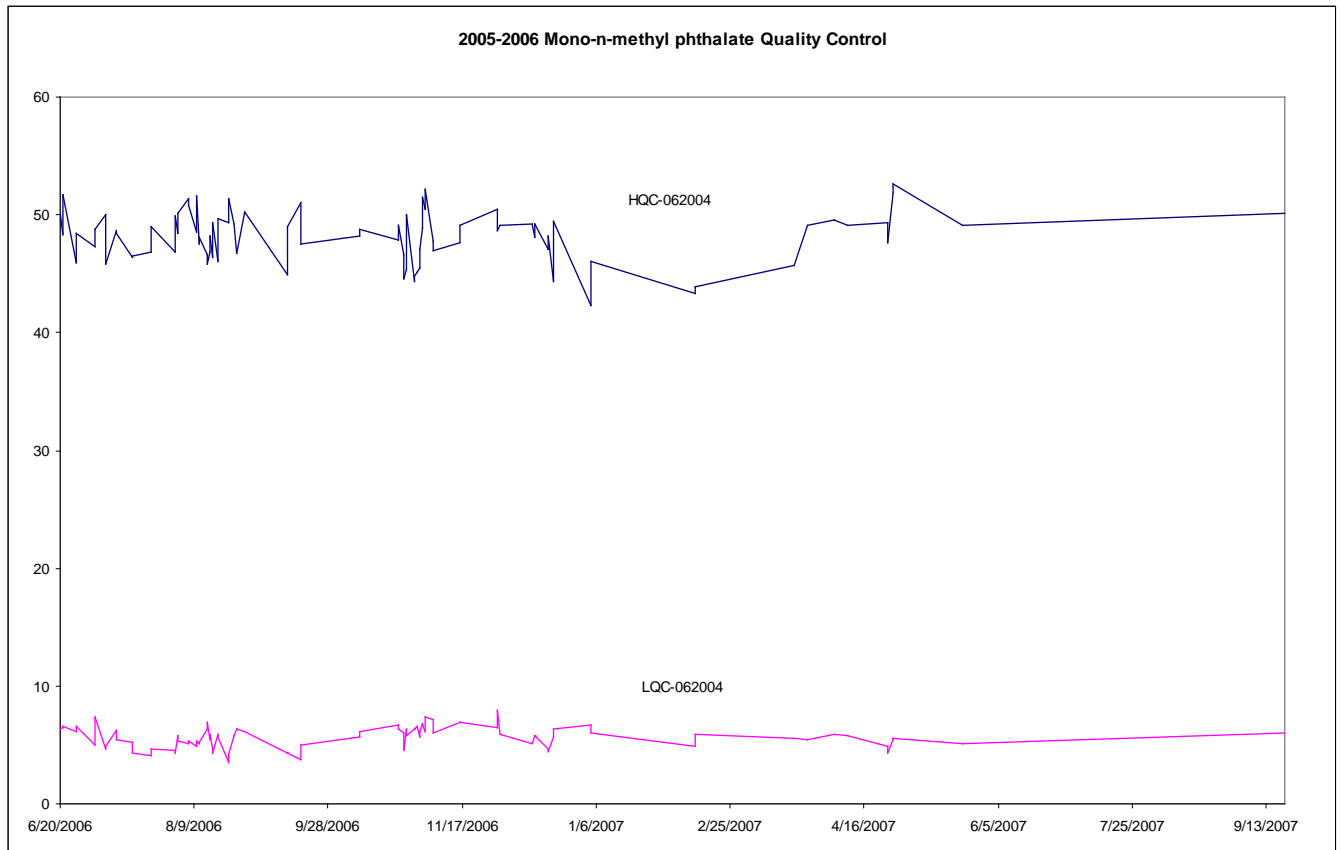


**Phthalate Metabolites in Urine
NHANES 2005-2006**

i. Mono-n-methyl phthalate (URXMNM)

Summary Statistics for Urinary Mono-n-methyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	87	6/20/2006	9/20/2007	5.649	0.905	16.0
HQC-062004	87	6/20/2006	9/20/2007	48.155	2.161	4.5

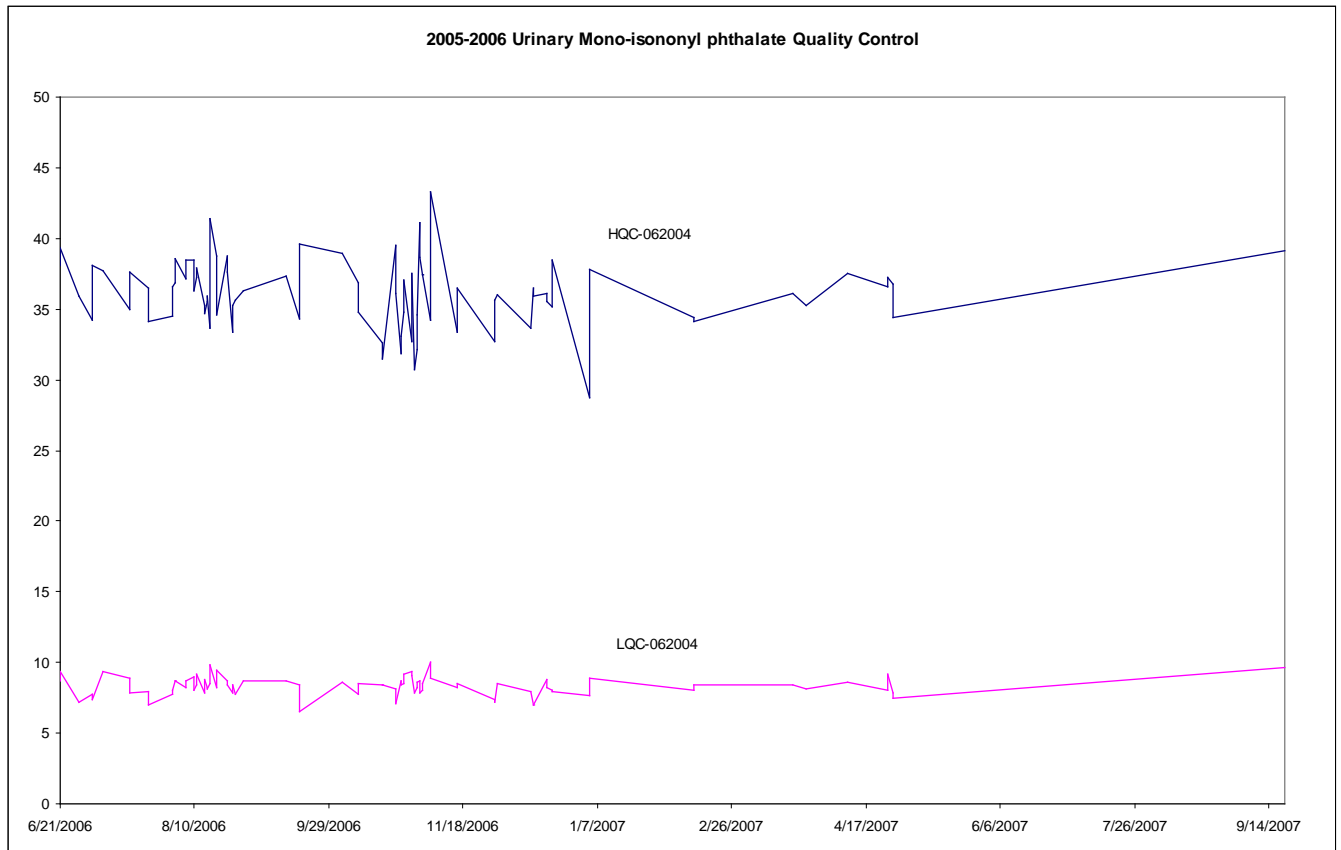


**Phthalate Metabolites in Urine
NHANES 2005-2006**

j. Mono-isononyl phthalate URXMNP)

Summary Statistics for Urinary Mono-isononyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	83	6/21/2006	9/20/2007	8.313	0.678	8.2
HQC-062004	83	6/21/2006	9/20/2007	36.105	2.440	6.8

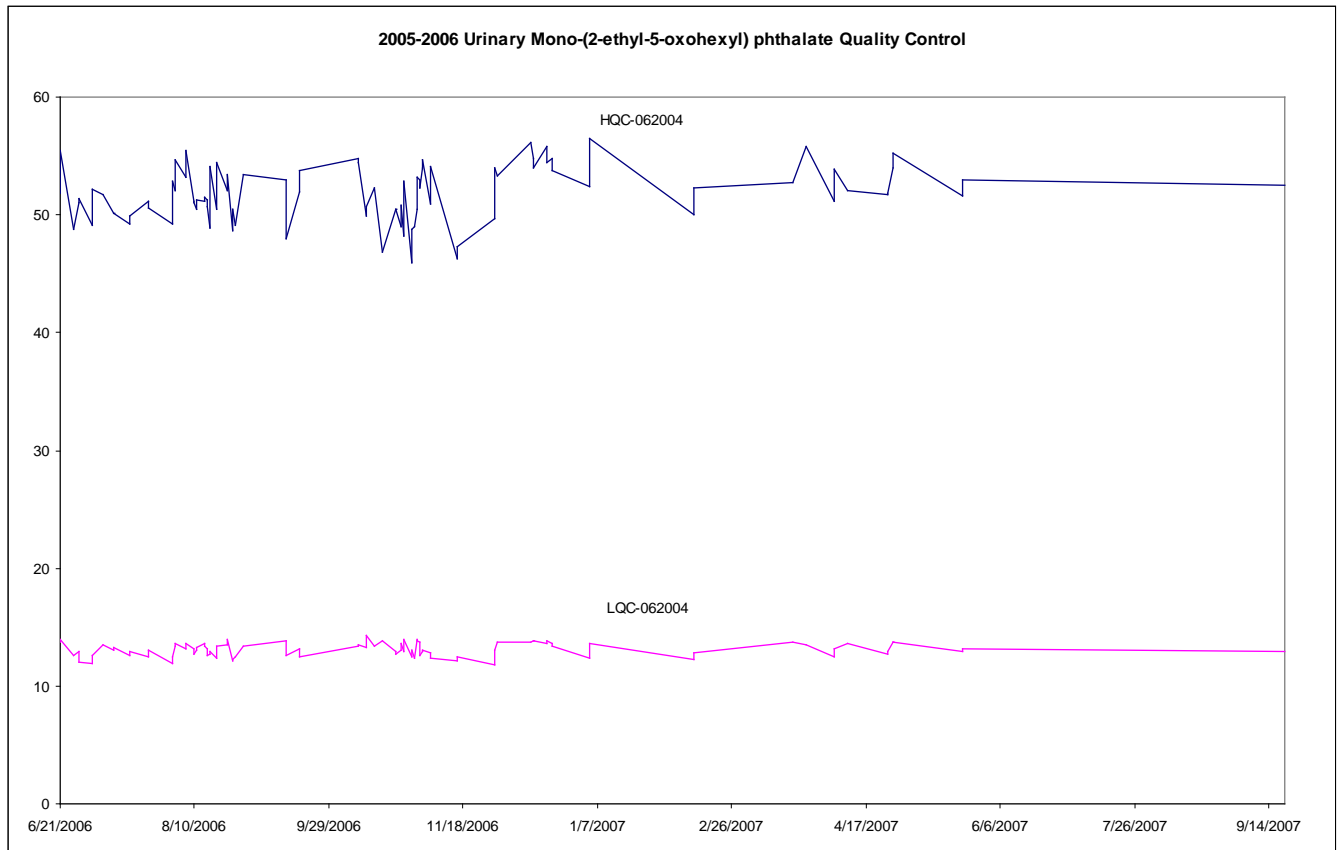


**Phthalate Metabolites in Urine
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k. Mono-(2-ethyl-5-oxohexyl) (URXMOH)

Summary Statistics for Urinary Mono- (2-ethyl-5-oxohexyl) phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	93	6/21/2006	9/20/2007	13.087	0.573	4.4
HQC-062004	93	6/21/2006	9/20/2007	51.806	2.321	4.5

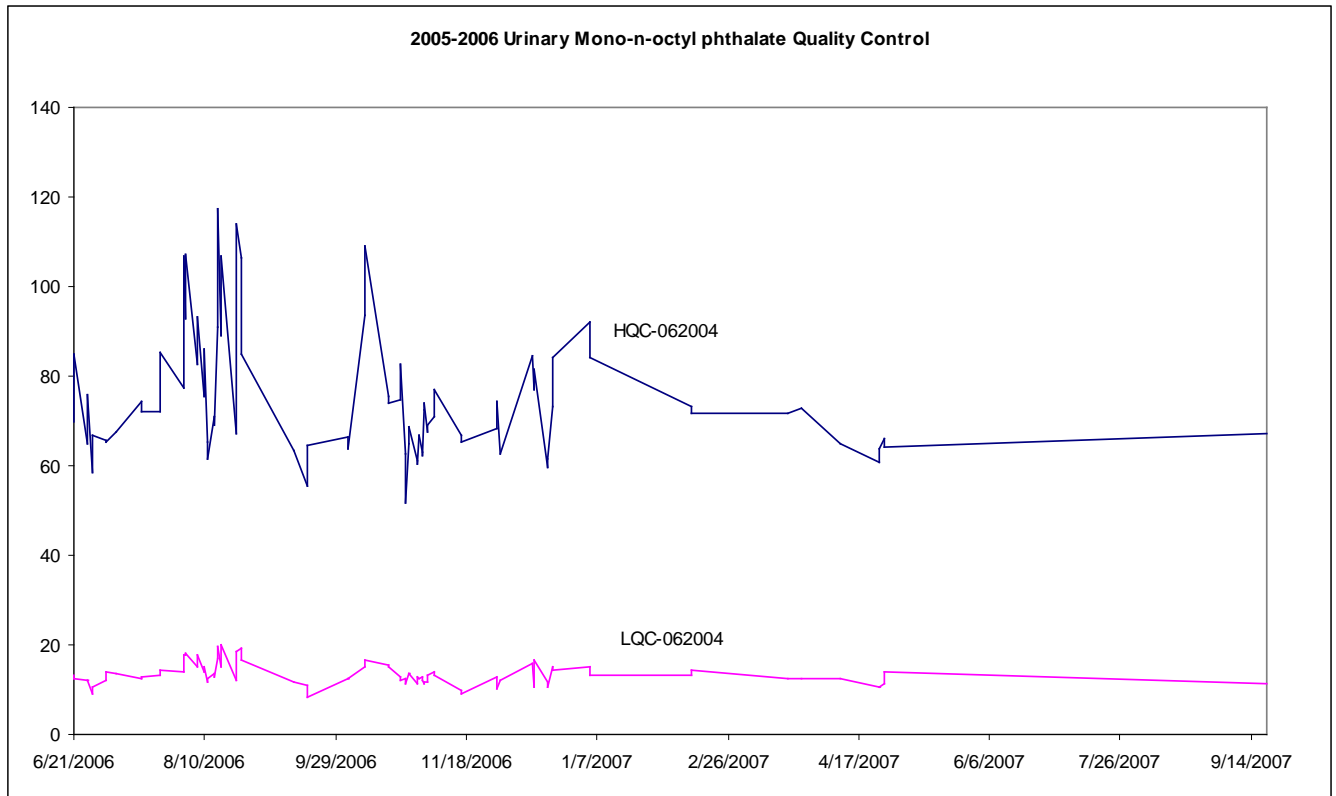


Phthalate Metabolites in Urine NHANES 2005-2006

I. Mono-n-octyl phthalate (URXMOP)

Summary Statistics for Urinary Mono-n-octyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	83	6/21/2006	9/20/2007	13.360	2.453	18.4
HQC-062004	83	6/21/2006	9/20/2007	74.857	14.079	18.8

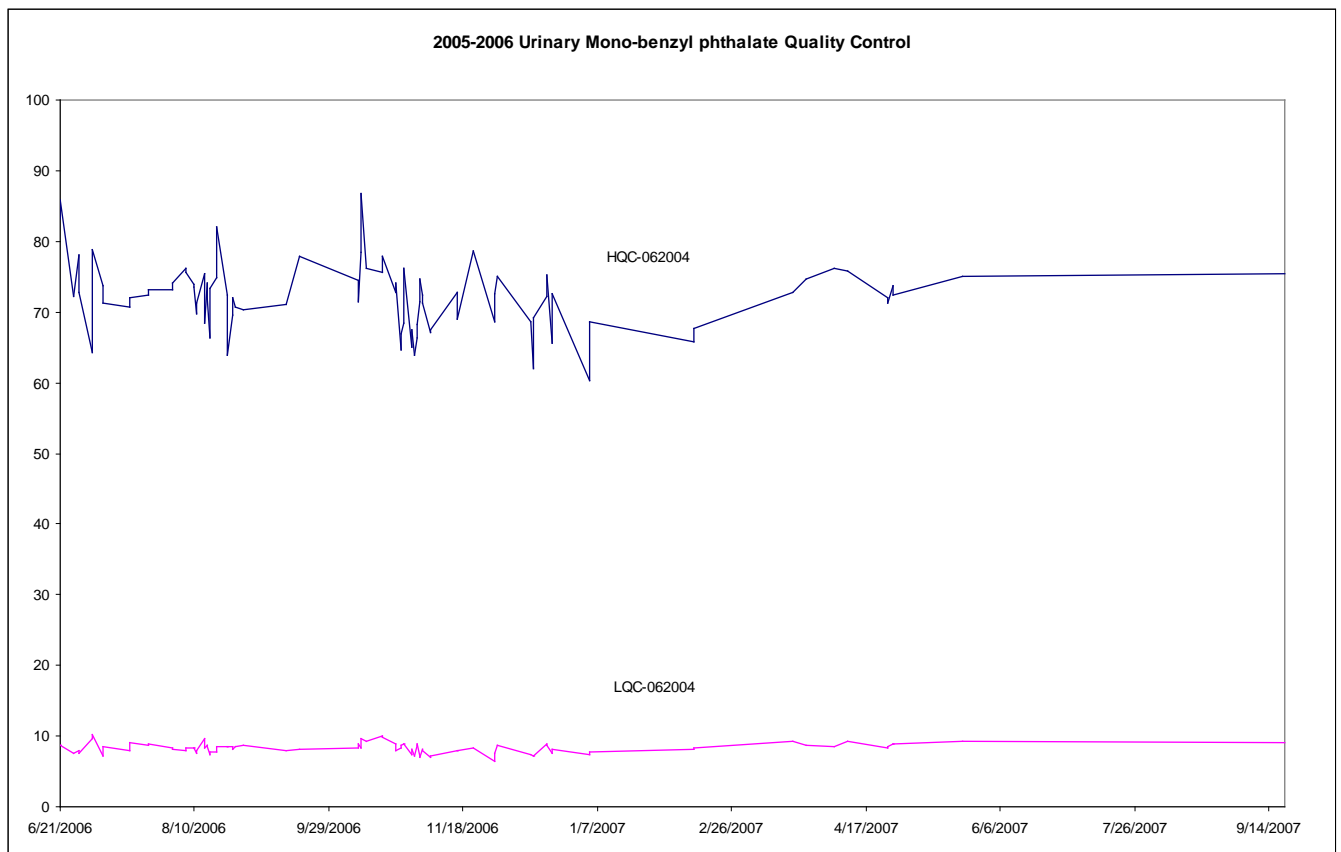


**Phthalate Metabolites in Urine
NHANES 2005-2006**

j. Mono-benzyl phthalate (URXMZP)

Summary Statistics for Urinary Mono-benzyl phthalate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-062004	87	6/20/2006	9/20/2007	5.649	0.905	16.0
HQC-062004	87	6/20/2006	9/20/2007	48.155	2.161	4.5



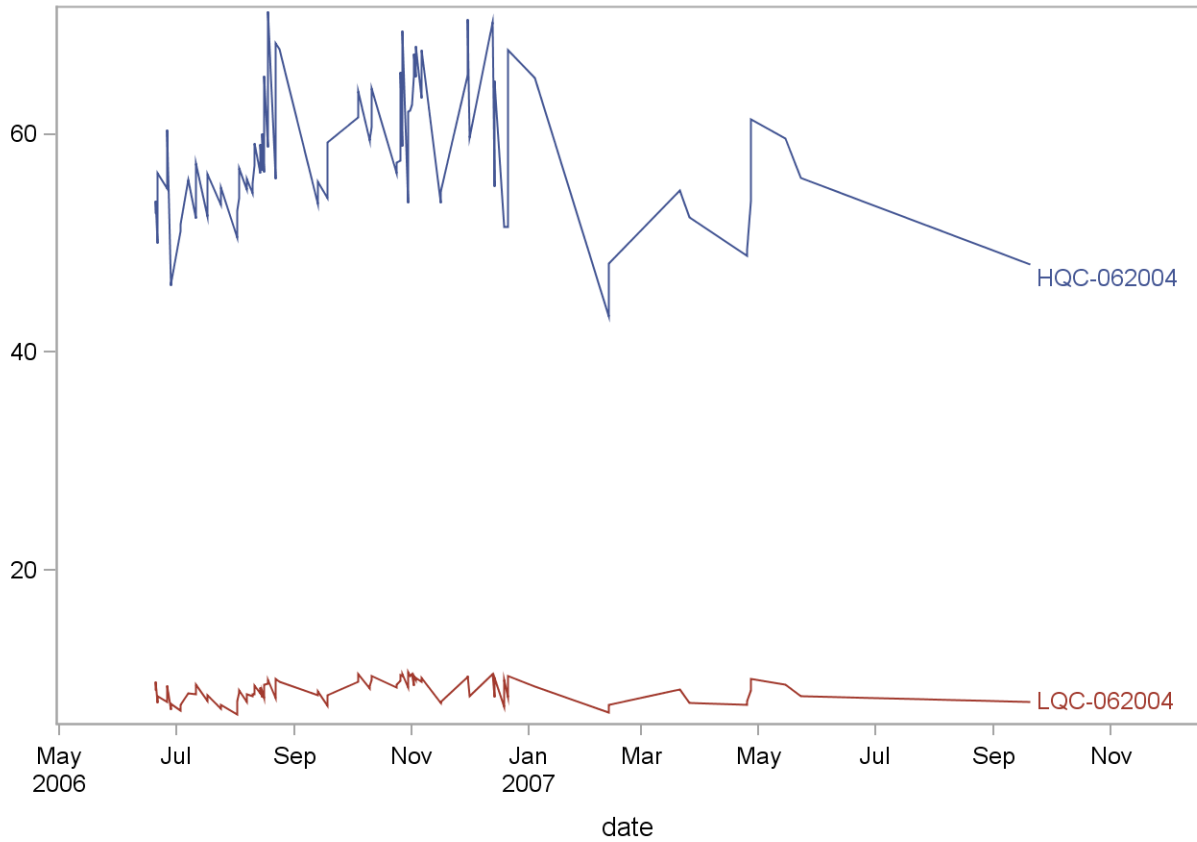
**Phthalate Metabolites in Urine
NHANES 2005-2006**

i. Mono(carboxynonyl) phthalate (ng/mL)

Summary Statistics for Mono(carboxynonyl) phthalate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-062004	87	20JUN06	20SEP07	57.89	6.22	10.7
LQC-062004	88	20JUN06	20SEP07	8.98	1.01	11.2

2005-2006 Mono(carboxynonyl) phthalate (ng/mL) Quality Control



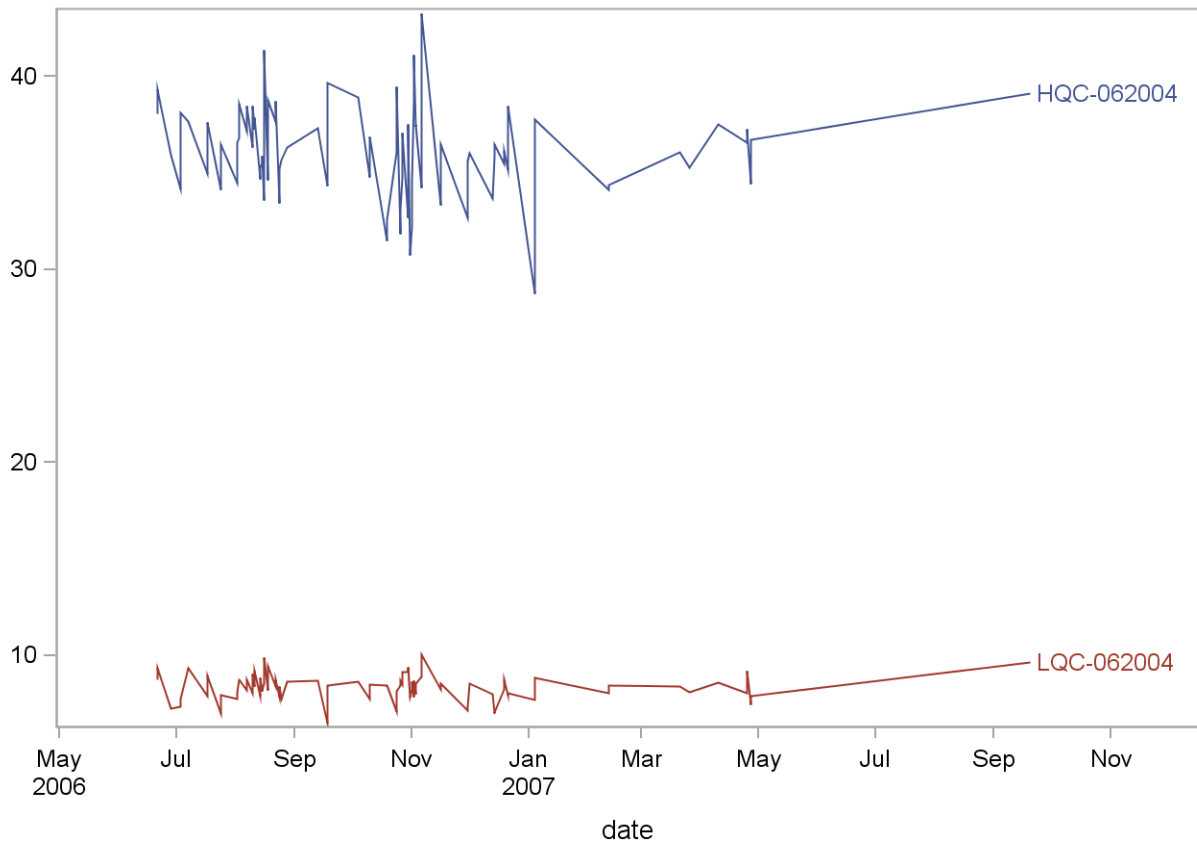
**Phthalate Metabolites in Urine
NHANES 2005-2006**

ii. Mono(carboxyoctyl) phthalate(ng/mL)

Summary Statistics for Mono(carboxyoctyl) phthalate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-062004	83	21JUN06	20SEP07	36.11	2.44	6.8
LQC-062004	83	21JUN06	20SEP07	8.31	0.68	8.2

2005-2006 Mono-isonyl phthalate (ng/mL) Quality Control



Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

**Phthalate Metabolites in Urine
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