# Laboratory Procedure Manual

*Analyte:* **Phthalates and Phthalate Alternative Metabolites**

*Matrix:* **Urine**

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

*Method No:* **6306.06**

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

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Director, Division of Laboratory Sciences

**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.

## Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

| Data File Name | Variable Name | SAS Label |
|---|---|---|
| PHTHTE_H | URXCNP | Mono(carboxyisononyl) phthalate (ng/mL) |
| | URXCOP | Mono(carboxyisoctyl) phthalate (ng/mL) |
| | URXECP | MECP phthalate (ng/mL) |
| | URXMBP | Mono-n-butyl phthalate (ng/mL) |
| | URXMC1 | Mono-(3-carboxypropyl) phthalate (ng/mL) |
| | URXMEP | Mono-ethyl phthalate (ng/mL) |
| | URXMHH | MEHP phthalate (ng/mL) |
| | URXMHNC | MHNCH (ng/mL) |
| | URXMHP | Mono-(2-ethyl)-hexyl phthalate (ng/mL) |
| | URXMIB | Mono-isobutyl phthalate (ng/mL) |
| | URXMNP | Mono-isononyl phthalate (ng/mL) |
| | URXMOH | MEOH phthalate (ng/mL) |
| | URXMZP | Mono-benzyl phthalate (ng/mL) |
| SSPHTE_H | URXHIBP | Mono-2-hydroxyisobutyl phthalate (ng/mL) |
| | URXMHBP | Mono-2-hydroxybutyl phthalate (ng/mL) |

# 1. Clinical Relevance and Summary of Test Principle

## a. Test Principle

The test principle utilizes high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) for the quantitative detection in urine of several metabolites of phthalates and phthalate alternatives [1] (Table 1). Urine samples are processed using enzymatic deconjugation of the glucuronidated analytes 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 target analytes. In addition, 4-methyl umbelliferyl glucuronide is used to monitor deconjugation efficiency. This selective method allows for rapid detection of metabolites of phthalate diesters or other alternative plasticizers in human urine with limits of detection in the low ng/mL range.

Table 1.List of phthalates and phthalate alternatives and their metabolites used as biomarkers of exposure

| Parent chemical | Metabolite |
|---|---|
| Di-n-octyl phthalate (DOP)<br>Di-n-butyl phthalate (DBP)<br>Other high molecular weight phthalates | Mono (3-carboxypropyl) phthalate (MCPP) |
| Di-ethyl phthalate (DEP) | Mono-ethyl phthalate (MEP) |
| Di-isobutyl phthalate (DiBP) | Mono-isobutyl phthalate (MiBP)<br>Mono-2-methyl-2-hydroxypropyl phthalate (MHiBP) |
| Di-n-butyl phthalate (DBP) | Mono-n-butyl phthalate (MBP)<br>Mono-3-hydroxybutyl phthalate (MHBP) |
| Benzylbutyl phthalate (BzBP) | Monobenzyl phthalate (MBzP) |
| Di(2-ethylhexyl) phthalate (DEHP) | Mono(2-ethylhexyl) phthalate (MEHP)<br>Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)<br>Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)<br>Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP) |
| Di-isononyl phthalate (DNP)* | *Mono-isononyl phthalate (MNP)<br>*Monocarboxyoctyl phthalate (MCOP) |
| Di-isodecyl phthalate (DDP)* | *Monocarboxy-isononly phthalate (MCNP) |

| Parent chemical | Metabolite |
|---|---|
| 1,2-Cyclohexane dicarboxylic acid, diisononyl ester (DINCH)* | *Cyclohexane-1,2-dicarboxylic acid-mono(hydroxy-isononyl) ester (MHINCH)<br>*Cyclohexane-1,2-dicarboxylic acid-mono(carboxyoctyl) ester (MCOCH) |

*isomeric mixtures

### b. Clinical Relevance

Phthalates, are a group of industrial chemicals widely used in consumer products and as solvents, additives, and plasticizers [2]. 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH) is a complex mixture of nine-carbon branched-chain isomers and is used as a replacement of some high molecular weight phthalates [3;4]. Humans are potentially exposed to many products containing phthalates and DINCH [5]. Phthalates are rapidly metabolized in humans to their respective monoesters, which depending on the phthalate can be further metabolized to their oxidative products [6;7]. Similarly, DINCH also forms oxidative metabolites in humans [1;8]. All of these metabolites may be glucuronidated, and excreted in the urine and feces [9-12]. Some of these metabolites can cause reproductive and developmental toxicities in animals [13-16], but little is known about the health effects of phthalates and DINCH exposure in people. Information on the concentration of metabolites of phthalates and phthalate alternatives in people is essential to understand human exposure.

## 2. 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 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. Laboratory personnel are advised to review the MSDS before using the chemicals and solvents.

### a. Radioactive Hazards

None.

### b. 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.

### c. 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.

### d. Protective Equipment

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

### e. 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.

### f. 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.

### g. 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 biological specimens 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 (Thermo Scientific, San Jose, CA, USA) on a Thermo Scientific Accela liquid chromatograph coupled with a Thermo Scientific TSQ Vantage triple

quadrupole 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 Thermo Scientific 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 a network drive. The Access database is located on an access-restricted 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

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 a network drive and 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 cups (150-250 mL) with caps.
(2) Pediatric urine collection bags
(3) Labels

(4) Cryovials
(5) Other sampling collection materials

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

(1) Preferably, urine specimens should be collected by using a pre-screened urine sampling collection device (e.g., cup, pediatric collection bag) to rule out external contamination with the target analytes from the sampling procedures.
(2) A minimum sample volume of 0.5 mL is preferred.
(3) Specimens may be stored frozen at temperatures at or below -40 °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 and in the DLS intranet). In the protocol, collection, transport, and special equipment required are discussed. In general, 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 [17]. Portions of urine that remain after the analytical aliquots are withdrawn should be frozen below -40 °C. All samples should be stored frozen until and after analysis. Specimens are rejected if tubes/vials leaked, are broken, appeared compromised or tampered with, or hold inadequate volume for 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 in-house deionized water. This solution is stored at room temperature in an amber bottle and can be used for 14 days from the day of preparation.

**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 can be used for one year from the day of preparation.

* 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).

**1.0 M Ammonium acetate buffer (pH 6.5)**. 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 of the solution reaches 6.5. The solution is transferred to a 500 mL volumetric flask and diluted to the 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.

**β-Glucuronidase solution.**
For a run of 50 samples; 1.5 mL of 1 M pH 6.5, ammonium acetate buffer is transferred into an autosampler tube. 90 µL of β-glucuronidase from E.coli K 12 (specific activity of approx. 140 U/mg at 37 °C or 80U/mg at 25 °C; with 4-nitrophenyl-β-D-glucuronide (4NPG) as substrate) is pipetted into the autosampler vial containing the ammonium acetate buffer. The solution is swirled to mix and placed in the sample preparation autosampler.

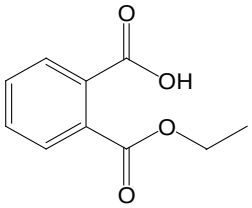
## b. Analytical Standards

### (1) Source

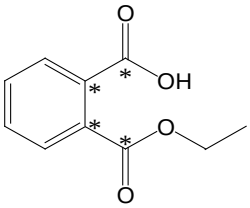
Metabolites of phthalates native and labeled standards are obtained from: Cambridge Isotope Laboratories Inc (Andover, MA), Los Alamos National Laboratory (Los Alamos, NM), SigmaAldrich, Professor Jurgen Angerer (Germany), Dr. Holger Koch (Germany), IDM (Germany) and Cansyn (Toronto, Canada). Metabolites of DINCH are obtained from Dr. Holger Koch (Germany).

$^{13}C_4$-4-methyl umbelliferone was purchased from Cambridge Isotope Laboratories Inc. 4-methyl umbelliferyl glucuronide is purchased from Sigma Aldrich Laboratories.

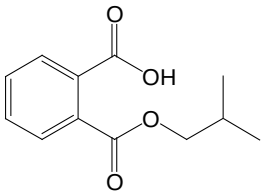
### (2) Native and labeled metabolites used in standards



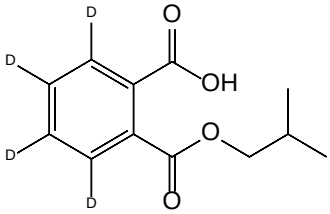

**MEP**
**monoethyl phthalate**



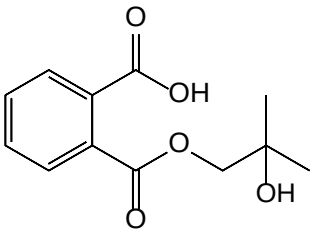

**MEP***
**monoethyl phthalate-$^{13}C_4$**



**MiBP**
**mono-isobutyl phthalate**



**MiBP***
**mono-isobutyl phthalate-D$_4$**



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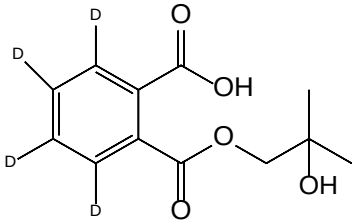
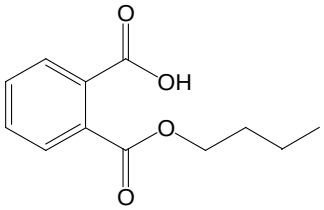
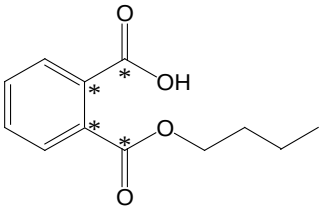
**mono-2-hydroxy-isobutyl phthalate**



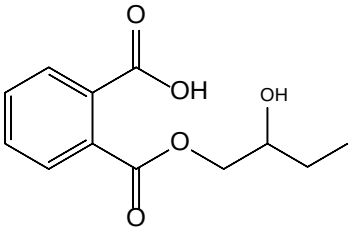
**MHiBP***
**mono-2-hydroxy-isobutyl phthalate-D$_4$**



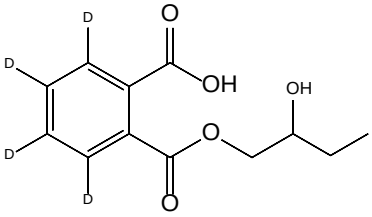
**MBP**
**mono-n-butyl phthalate**



**MBP***
**mono-n-butyl phthalate-$^{13}C_4$**



**MHBP**
**mono-2-hydroxy-n-butyl phthalate**



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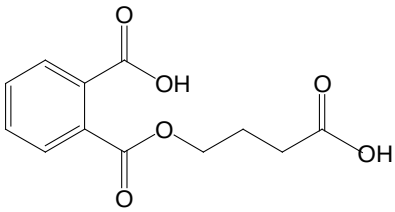
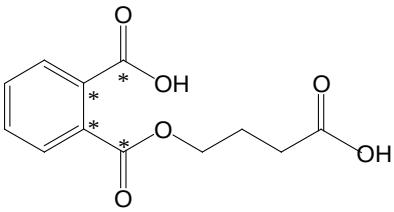


**MCPP**
**mono(3-carboxypropyl) phthalate**



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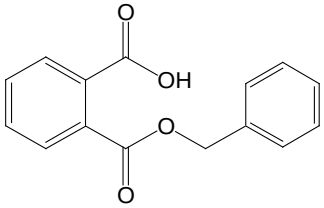
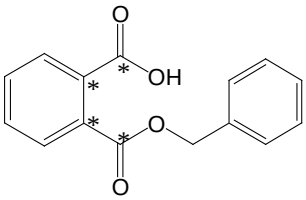
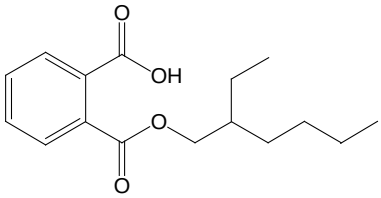
**mono(3-carboxypropyl) phthalate-$^{13}C_4$**



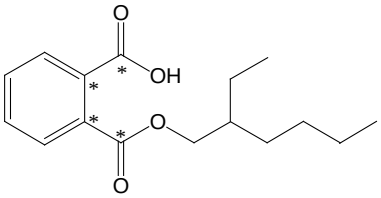
**MBzP**
**monobenzyl phthalate**



**MBzP***
**monobenzyl phthalate-$^{13}C_4$**



**MEHP**
**mono(2-ethylhexyl) phthalate**



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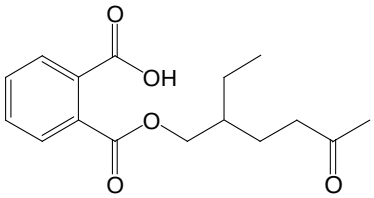
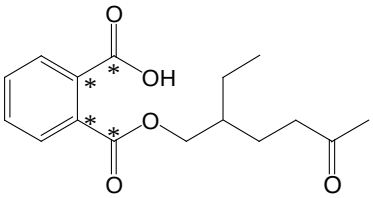
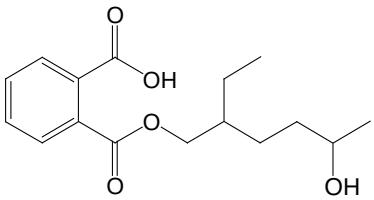
**mono(2-ethylhexyl) phthalate-$^{13}C_4$**



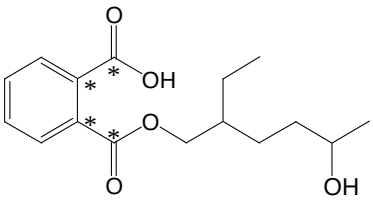
**MEOHP**
**mono(2-ethyl-5-oxohexyl) phthalate**



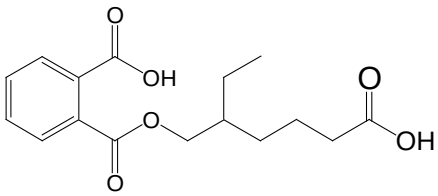
**MEOHP***
**mono(2-ethyl-5-oxohexyl) phthalate-$^{13}C_4$**

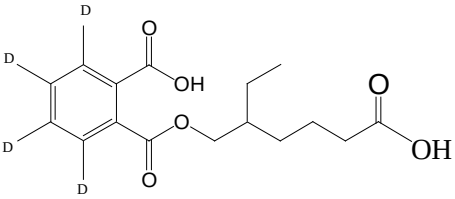


**MEHHP**
**mono(2-ethyl-5-hydroxyhexyl) phthalate**

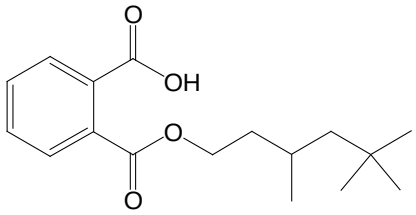


**MEHHP***
**mono(2-ethyl-5-hydroxyhexyl) phthalate-$^{13}C_4$**

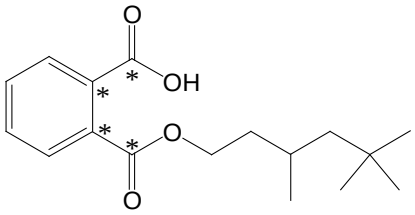




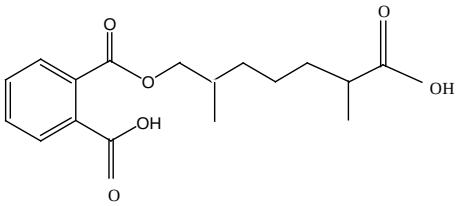
**MECPP**
**mono(2-ethyl-5-carboxypentyl)phthalate**



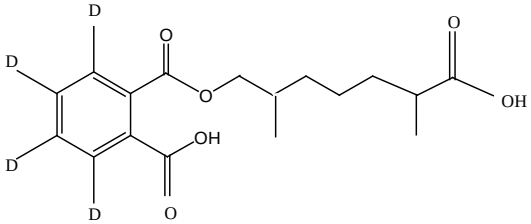
**MECPP***
**$D_4$-mono(2-ethyl-5-carboxypentyl)phthalate**



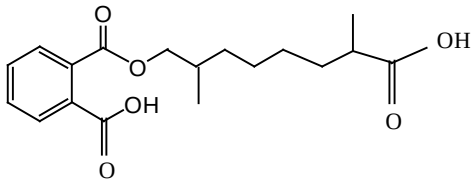
**MNP**
**mono(3,5,5-trimethyl-1-hexyl) phthalate**
**(mono-isononyl phthalate)**



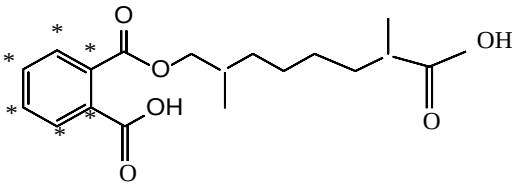
**MNP***
**mono(3,5,5-trimethyl-1-hexyl) phthalate-$^{13}C_4$**
**(mono-isononyl phthalate $^{13}C_4$)**



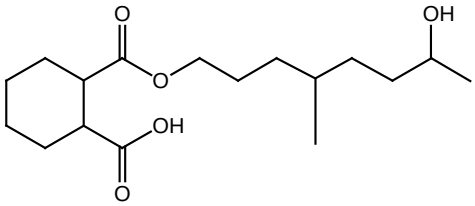
**MCOP**
**mono(2,6-methyl-6-carboxyhexyl)phthalate**
**(mono-carboxyisooctyl phthalate)**



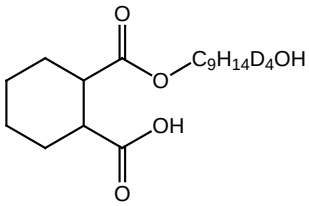
**MCOP***
**mono(2,6-methyl-6-carboxyhexyl)phthalate**
**($D_4$-mono-carboxyisooctyl phthalate)**



**MCNP**
**mono(2,7-methyl-7-carboxyheptyl)phthalate**
**(mono-carboxyisononyl phthalate)**

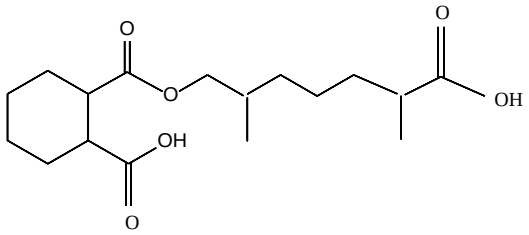


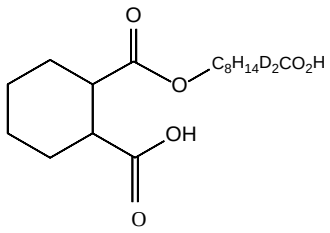
**MCNP***
**mono(2,7-methyl-7-carboxyheptyl)phthalate**
**(mono-carboxyisononyl phthalate$^{13}C_6$)**



**MHNCH**
**Cyclohexane-1,2-dicarboxylic**
**acid, mono(cis-hydroxy-isononyl) ester**

**$D_4$- MHNCH**
**Cyclohexane-1,2-dicarboxylic**
**acid, mono(cis-hydroxy-isononyl) ester-D4**




**MCOCH**
**Cyclohexane-1,2-dicarboxylic acid, mono(carboxyoctyl) ester**

**$D_2$- MCOCH**
**Cyclohexane-1,2-dicarboxylic acid, mono(carboxyoctyl) ester–$D_2$**

### (3) Standards Preparation

(a) Individual native standards of phthalate and DINCH metabolites. The stock solutions are prepared by accurately transferring approximately 5-25 mg of material onto a 50 mL volumetric flask. The metabolite is then dissolved in acetonitrile. This stock solution is stored at -20 °C in a methanol rinsed and air dried Teflon-capped amber glass bottle until use.

(b) Isototopically-labeled phthalate and DINCH metabolites and 4-methyl umbelliferone internal standards. These internal standards are prepared similarly to the native standards and stored at -20 °C until use.

(c) 4-methyl umbelliferyl glucuronide standard. The stock standard solution is prepared by transferring approximately 10 mg of the 4-methyl umbelliferyl 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 4-methyl umbelliferyl 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 -40 °C in a Teflon-capped glass bottle. The spiking solution, made in HPLC grade water by diluting the intermediate stock solution, is refrigerated and discarded after 3 month.

(d) Ten unique calibration standards with all native analytes and 4-methyl umbelliferone are prepared in 10% aqueous acetonitrile from the stock solutions and stored in the refrigerator.

(e) Internal standard spiking solution is prepared in 10% aqueous acetonitrile from the stock solutions of the isotopically-labeled internal standards and stored in the refrigerator.

### (4) 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 and remade as needed from the stock solutions. Stock standards and the working standard mixtures are remade, as necessary.

### (5) Proficiency Testing Standards

Variable volumes of each stock standard are added to 0.5L urine pools to produce 3 concentrations of proficiency testing (PT) standards. The spiked pools are mixed, aliquoted into cryovials, and frozen (at or below -40 ºC) until needed. The PT standards are characterized by at least 20 repeat determinations to characterize the mean and standard deviation for each analyte.

### (6) Materials

(1) Chromolith Flash RP-18e precolumn (4.6 mm x 5 mm, Merck KGaA, Germany).
(2) 1.5 mL silanized autosampler vials (ThermoFisher, USA) and pre-slit caps (caps with PTFE/Silicone).
(3) Thermo Scientific-Keystone Betasil phenyl HPLC column (3 µm, 150 mm × 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.

### (7) Equipment

(1) Pipettes (Rainin and Eppendorph)
(2) Balance (TR-203 Series Denver Instrument Company)
(3) Balance (Sartorius, Genius series)
(4) Sonicating waterbath (Branson 5210).
(5) Vortexer (Fisher, Genie 2 or eqivalent)
(6) Magnetic Stirrer (Corning or equivalent)
(7) Thermo Scientific Surveyor autosampler
(8) Fisher Scientific* accumet* AB15 University Meter Kit, pH/mV/°C

### (8) Analytical instrumentation

1) Thermo Scientific Accela High Pressure Liquid Chromatograph system
2) Thermo Scientific TSQ Vantage Triple Quadrupole Mass Spectrometer
3) Agilent LC pump with 10 port switching valve

## 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, 10 standards followed by unknowns, QC samples and blanks are normally analyzed. Typically two daily runs are combined and duplicate standards results are used to construct a 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 if necessary. The 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 ( http://www.cms.gov/Regulations-and-Guidance/Legislation/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)

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 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 (e.g.,MBP, MiBP, MBzP, MEHHP, MEOHP, 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/).

## 8. Operating Procedures; Calculations; Interpretation of Results

### a. Preliminaries

(1) The on-line solid phase extraction batch typically consists of 50 samples: 10 calibration standards, 4 Reagent Blanks (RB), 6 Quality Control (QC) materials of low (QCL; n=3) and high (QCH; n=3) concentration, and 30 unknown urine samples.
(2) The urine samples and QC materials are allowed to thaw completely at room temperature or 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 and the enzyme solution are prepared fresh just prior to addition to samples.

### b. Sample preparation

(1) 100 µL (or lesser amount when sample volume is limited) of calibration standard, urine, HPLC Grade water (for the reagent blank), QCH and QCL is transferred into a properly labeled autosampler vial (1.5 mL).
(2) The vial is caped with Teflon-lined screw cap.
(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 µL of 4-methylumbelliferyl glucuronide spiking solution, 100 µL Internal Standard (isotopically labeled mixture) spiking solution and 25 µL of β-glucuronidase/ammonium acetate buffer solution are added into the 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 at least 120 min of incubation at 37°C, 50 µL of glacial acetic acid is added and the sample is preserved by adding 150 µL 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.

### c. Instrumental Analysis

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

The analysis is performed using a Thermo Scientific LC pump, Thermo Scientific Accela liquid chromatograph coupled with a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer, equipped with an ESI interface. 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 urine sample after deconjugation 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 2). The 10 port valve is automatically switched to its alternate position, reversing the flow and allowing the analytes to be transferred from the SPE column on to HPLC column. The chromatographic

resolution is accomplished using a 3 µm, 150 mm × 2.1 mm Thermo Scientific 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;18].

Table 2. On-line SPE and HPLC solvent gradient programs

| | SPE pump | | | Switching valve | | HPLC pump | | | Switching valve |
|---|---|---|---|---|---|---|---|---|---|
| Time (min) | A (%) | B (%) | Flow (µL/min) | | | A (%) | B (%) | Flow (µL/min) | |
| | | | | | | | | | |
| 0 | 100 | 0 | 300 | SPE/Waste | | 77 | 23 | 300 | HPLC/Waste |
| 0.4 | 100 | 0 | 1800 | SPE/Waste | | | | | |
| 0.5 | 90 | 10 | 1800 | SPE/HPLC | | | | | |
| 1.1 | 90 | 10 | 1800 | SPE/HPLC | | | | | |
| 1.2 | 100 | 0 | 200 | SPE/Waste | | | | | |
| 3 | | | | | | 75 | 25 | 300 | HPLC-MS/MS |
| 3.5 | 100 | 0 | 200 | SPE/Waste | | | | | |
| 4 | 100 | 0 | 1500 | SPE/Waste | | | | | |
| 5 | | | | | | 75 | 25 | 300 | HPLC-MS/MS |
| 8.1 | 100 | 0 | 1500 | SPE/Waste | | | | | |
| 8.2 | 0 | 100 | 1500 | SPE/Waste | | | | | |
| 10 | | | | | | 67 | 33 | 350 | HPLC-MS/MS |
| 10.2 | 0 | 100 | 1500 | SPE/Waste | | | | | |
| 10.5 | 0 | 100 | 200 | SPE/Waste | | | | | |
| 15 | 0 | 100 | 500 | SPE/Waste | | | | | |
| 17 | | | | | | 70 | 30 | 325 | HPLC-MS/MS |
| 19.85 | | | | | | 66 | 34 | 350 | HPLC-MS/MS |
| 21.1 | 0 | 100 | 200 | SPE/Waste | | 60 | 40 | 350 | HPLC-MS/MS |
| 23.0 | 100 | 0 | 300 | SPE/Waste | | | | | |
| 23.1 | | | | | | 45 | 55 | 350 | |
| 25.1 | | | | | | 20 | 80 | 350 | |
| 25.2 | | | | | | 0 | 100 | 350 | |
| 25.6 | | | | | | 0 | 100 | 400 | |
| 26.6 | | | | | | 0 | 100 | 400 | |
| 26.7 | | | | | | 77 | 23 | 350 | |
| 27 | 100 | 0 | 300 | | | 77 | 23 | 350 | HPLC/Waste |

ESI in negative ion mode is used to ionize the analytes molecules and transfer the negatively charged ions into the gas phase.

During the analysis, the instrument is set in the multiple reaction monitoring mode so that precursor and the product ion combinations specific to the eluting analyte can be monitored. Reproducible chromatography allows 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 energy and the S lens voltage are specifically set for each ion (Table 3).

### (2) Multiple Reaction Monitoring Setup

Table 3. Analytes, 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 | |
| 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 | |
| MHiBP | 237/121 | 22 |
| $D_4$-MHiBP | 241/125 | |
| MBP | 221/77 | 26 |
| $^{13}C_4$-MBP | 225/79 | |
| MHBP | 237/121 | 22 |
| D4-MHBP | 241/125 | |
| 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 | |
| MCNP | 335/187 | 21 |
| $^{13}C_6$-MCNP | 341/187 | |
| MEHP | 277/134 | 21 |
| $^{13}C_4$-MEHP | 281/137 | |
| MNP | 291/121 | 27 |
| $^{13}C_4$-MNP | 295/124 | |
| MHNCH | 313/153 | 19 |
| $D_4$-MHNCH | 317/153 | |
| MCOCH | 327/173 | 19 |
| $D_2$-MCOCH | 329/175 | |

### d. Replacement and periodic maintenance of key components

The instrumentation used is serviced according to the manufacturer's guidance included in the instrument manuals or based on the recommendation of experienced analysts/operators after following appropriate procedures to determine that the instrument performs adequately for the intended purposes of the method.

## 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 limit of detection (LOD).

**b. Limit of detection (LOD)**

The method LODs are given in Table 4.

Table 4. Limits of detection (LODs) and calibration range of the method.

| Analyte | LOD (ng/mL) | Calibration range (ng/mL) |
|---|---|---|
| MCPP | 0.4 | 0.035-350 |
| MEP | 1.2 | 0.25-2500 |
| MiBP | 0.8 | 0.05-500 |
| MHiBP | 0.4 | 0.025-250 |
| MBP | 0.4 | 0.01-1000 |
| MHBP | 0.4 | 0.025-250 |
| MEHHP | 0.4 | 0.05-500 |
| MECPP | 0.4 | 0.1-1000 |
| MEOHP | 0.2 | 0.05-500 |
| MBzP | 0.3 | 0.05-500 |
| MEHP | 0.8 | 0.025-250 |
| MNP | 0.9 | 0.025-250 |
| MCOP | 0.3 | 0.05-500 |
| MCNP | 0.2 | 0.05-500 |
| MHNCH | 0.4 | 0.05-500 |
| MCOCH | 0.5 | 0.025-250 |

### c. Accuracy

The method accuracy is assessed through five replicate analyses of analytes spiked into synthetic or blank urine (Table 5).

Table 5. Accuracy of the method

| Analyte | Expected ng/mL | Observed ng/mL |
|---|---|---|
| MCPP | 3.5 | 3.6 |
| MEP | 50.0 | 48.0 |
| MEHHP | 12.5 | 12.7 |
| MiBP | 5.0 | 4.6 |
| MHiBP | 2.5 | 2.4 |
| MECPP | 12.5 | 12.7 |
| MBP | 13.1 | 11.8 |
| MHBP | 5.0 | 4.6 |
| MEOHP | 13.8 | 12.8 |
| MCOP | 5.0 | 5.0 |
| MBzP | 11.0 | 11.3 |
| MHNCH | 2.5 | 2.5 |
| MCOCH | 5.0 | 5.1 |
| MCNP | 5.0 | 5.2 |
| MEHP | 5.0 | 5.1 |
| MNP | 5.0 | 4.6 |

### 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 intra- and inter-day variability of the assay (Table 6).

Table 6. Precision at two concentration levels using urine QC pools

| Analyte | QCH | | QCL | |
|---|---|---|---|---|
| | CV (%) | Mean | CV (%) | Mean |
| MEP | 3.6 | 91.2 | 5.8 | 22.1 |
| MCPP | 4.4 | 13.1 | 8.4 | 2.2 |
| MiBP | 3.8 | 19.7 | 7.0 | 5.1 |
| MBP | 3.9 | 24.2 | 8.5 | 5.5 |
| MHBP | 10.5 | 16.4 | 10.2 | 4.2 |
| MHiBP | 10.9 | 11.4 | 12.6 | 3.9 |
| MBzP | 4.1 | 22.0 | 7.1 | 4.2 |
| MEHP | 5.5 | 14.0 | 8.5 | 5.0 |
| MNP | 5.2 | 13.0 | 8.6 | 4.4 |
| MEOHP | 3.7 | 18.3 | 6.8 | 5.1 |
| MEHHP | 4.2 | 19.7 | 6.5 | 4.9 |
| MECPP | 4.4 | 29.5 | 7.1 | 5.0 |
| MCNP | 3.3 | 14.2 | 5.9 | 4.5 |
| MCOP | 5.1 | 18.1 | 6.50 | 7.1 |
| MCOCH | 8.1 | 13.0 | 6.5 | 3.8 |
| MHNCH | 4.1 | 18.1 | 6.6 | 4.5 |

## 10. Quality control and quality assessment

### a. Individual sample quality control procedure

**1)** For each analyte, the relative retention time (ratio of $RT_{analyte}$ and $RT_{IS}$) of standards, unknowns, and QCs should be checked. If the relative RT falls outside of 0.90 to 1.10, both analyte and the internal standard peaks are manually reviewed to confirm that each peak is accurately identified.

2) For each analyte the total ion count (TIC) for the IS should meet minimum requirements (TIC of $1x10^3$). If the area count is below the minimum requirement, the ‘S’ lens, ion transfer tube, and the exit lens of the mass spectrometer should be cleaned and the affected sample should be re-extracted.

3) For each analyte, the calculated concentration of the reagent blanks (RB) should be less than three times the LOD.

4) For each analyte, if the analyte concentration in an unknown sample is more than the highest calibration standard, the sample is re-extracted with a smaller aliquot.

5) For each analyte, if the analyte concentration in an unknown sample is more than the highest calibration standard, the following sample is re-extracted for the potential carryover.

6) Unknown samples for which all of the analytes concentrations fall below the LOD are re-analyzed to confirm that urine was dispensed in the autosampler vial. Alternatively, one can measure the specific gravity of the sample to rule out that it is a field or solvent blank.

**b. Analytical batch quality control procedures**

**(1) QC Materials**

Quality control (QC) materials are prepared in urine. These QC samples are analyzed along with unknown samples to monitor for accuracy and precision throughout the analytical run.

**(2) QC Pools**

The QC pools are mixed uniformly, and divided into two sub-pools. The sub-pools are enriched with metabolites of phthalates and DINCH as needed to prepare low concentration (QCL) and high concentration (QCH) sub-pools. The pools are dispensed into 2mL cryovials and frozen until needed.

**(3) Characterization of QC Materials**

The QC pools are characterized to define the mean and the 95% and 99% control limits of metabolites of phthalates and DINCH concentrations from 60 QCL and 60 QCH runs over 3 weeks. In each run, one pair of QCL and one pair of QCH materials are analyzed and averaged. Using the pair average value from the 60 runs, the mean, and upper and lower 99% and 95% control limits are calculated.

**(4) Use of Quality Control Samples**

Each analytical run consists of 50 samples: 10 standards, 3 QCL, 3 QCH, 4 reagent blanks, and 30 unknowns. The concentrations of two randomly selected QCH and two QCL are averaged to obtain one measurement of QCH and QCL for each batch.

**(5) Final evaluation of Quality Control Results**

Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control [19].

**<u>Two QC pools *per run* with one QC result *per pool*</u>**

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:
   a) Extreme Outlier – Run result is beyond the characterization mean +/- $4S_i$
   b) 3S Rule - Run result is outside a $3S_i$ limit
   c) 2S Rule - Both run results are outside the same $2S_i$ limit
   d) 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
   e) R 4S Rule – Two consecutive standardized run results differ by more than $4S_i$. (Note: Since runs have a single result 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. Standardized results are used because different pools have different means.)

**Two QC pools *per run* with two or more QC results *per pool***

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:
   a) Extreme Outlier – Run mean is beyond the characterization mean +/- $4S_m$
   b) 3S Rule - Run mean is outside a $3S_m$ limit
   c) 2S Rule - Both run means are outside the same $2S_m$ limit
   d) 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:
   a) R 4S Rule – Within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit). Note: Since runs have multiple results 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 fail 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 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 the 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.

Inaccuracies in commercial standards can also affect accuracy [20]. Specifically, in 2012, after learning of inaccuracies affecting commercial phthalate metabolite analytical standards, CDC determined that a correction factor should be applied to the concentrations (including the limit of detection) of five metabolites as follows: Mono-ethyl phthalate, 0.66; Mono-benzyl phthalate, 0.72; Mono-n-octyl phthalate, 1.68; Mono-isononyl phthalate, 1.54; and Mono-cyclohexyl phthalate, 2.01. These correction factors have to be applied to all results—including the limits of detection for the affected analytes—reported by CDC through December 2011.

Contact with some plastics during specimen acquisition, storage, or sample analysis can result in interferences.

Prolonged exposure to room temperature during sample collection and/or transport may result in degradation of the urine specimen and/or certain metabolites. Care should be taken during sample collection and processing to prevent prolonged exposure to temperatures above freezing and the urine should be frozen as soon as possible after collection [17].

## 13. Reference Ranges (Normal Values)

The results from the National Health and Nutrition Examination Survey (NHANES) can be used as reference ranges to describe levels of exposure to plasticizers among the general US population [5].

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

The metabolites concentration values obtained using this method of analysis are only investigational markers of exposure to phthalates and DINCH; therefore critical values have not been determined.

## 15. Specimen Storage and Handling during Testing

Specimens are stored in the laboratory frozen prior to analysis. Prepared samples are kept at 10°C during the SPE-HPLC-MS/MS analysis. Frozen samples are allowed to thaw completely at room temperature or in a 25 °C sonicating water bath 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 Thermo Scientific Accela liquid chromatograph coupled with a Thermo Scientific TSQ Vantage Triple Quadrupole mass spectrometer. Alternative extraction techniques exist [21;22]., If the analytical system fails, prepared samples can be stored frozen in capped autosampler vials until the analytical system is restored. Otherwise, samples can be re-prepared. If the storage system fails, urine samples are transferred to an alternate freezer; if a freezer is not available, the urine samples can be temporarily stored refrigerated 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 reviewed first by the team lead and then by the laboratory supervisor. The supervisor provides feedback to the team lead 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. A Division statistician 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 are stored in electronic format in a database in the network drive.

g. Final hard copies of correspondence are maintained in the office of the Branch Chief and/or with the quality control officer.

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

A spreadsheet with information on specimens received and transferred is kept in a share folder. 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 samples are logged in to an MS access database. The shipping manifests for NHANES and other samples are kept in a binder in the Laboratory.

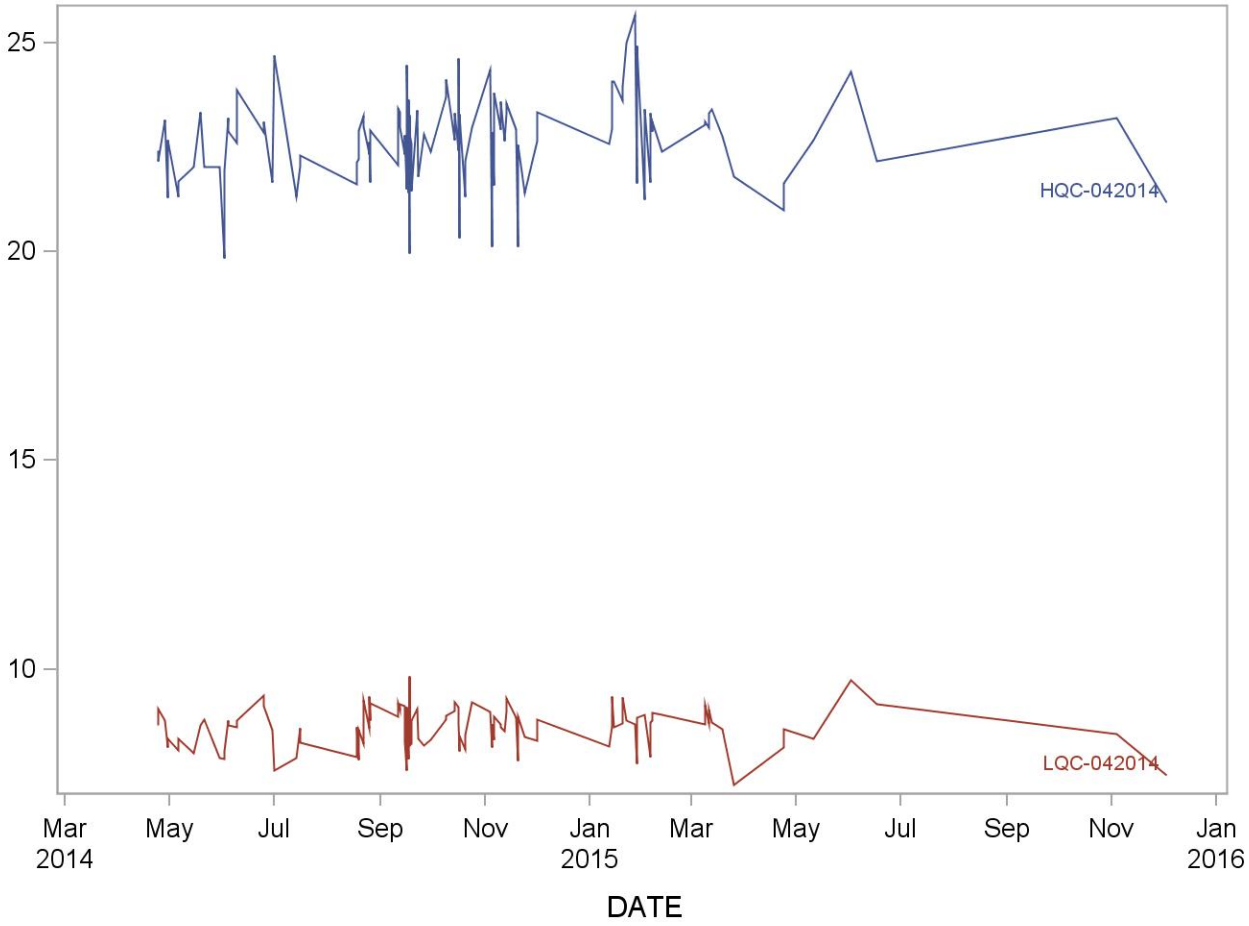
## 19. Summary Statistics and QC Graphs

See following pages.

*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.*

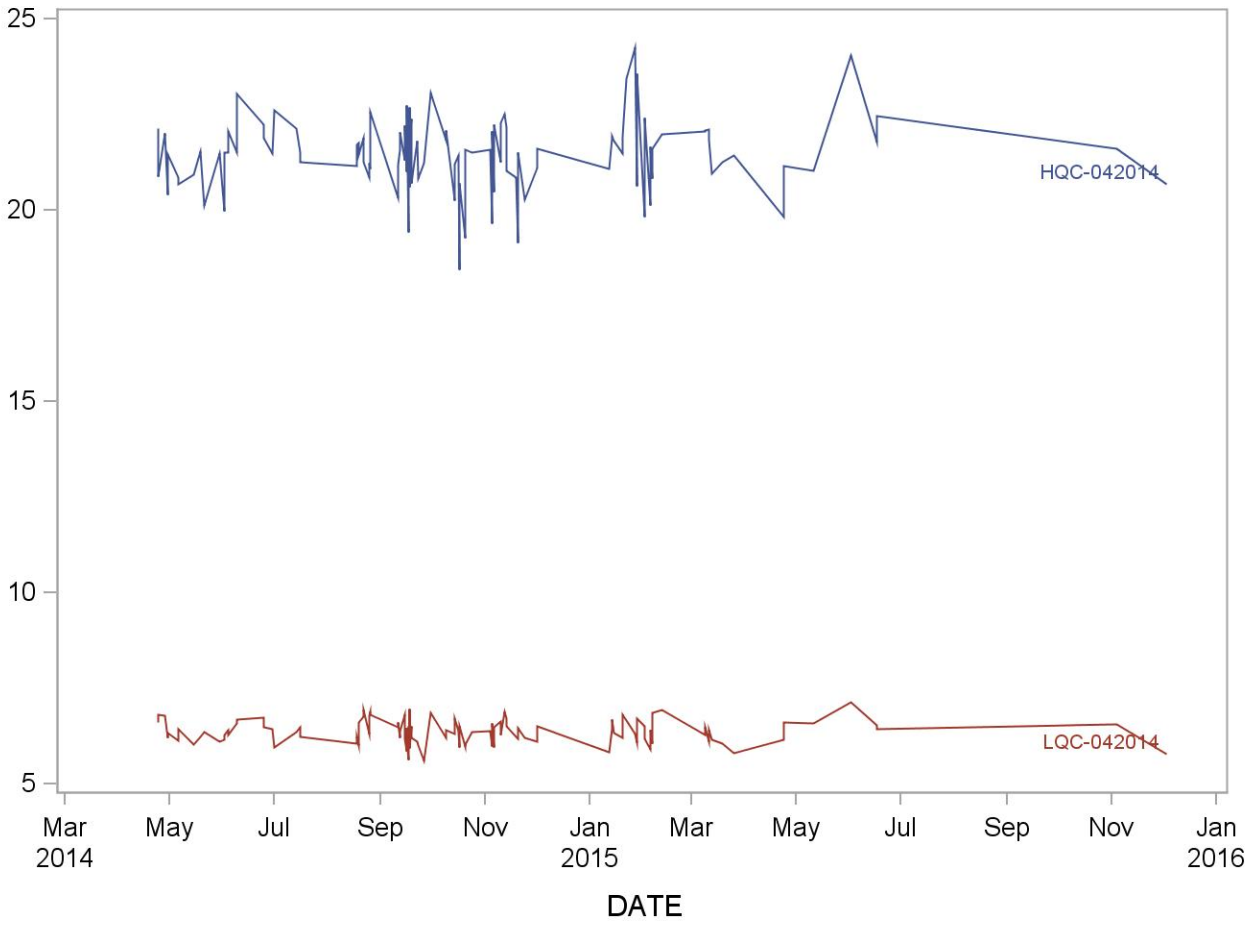
## 2013-2014 Summary Statistics and QC Chart for MECP phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 113 | 24APR14 | 03DEC15 | 22.667 | 1.072 | 4.7 |
| LQC-042014 | 113 | 24APR14 | 03DEC15 | 8.601 | 0.487 | 5.7 |



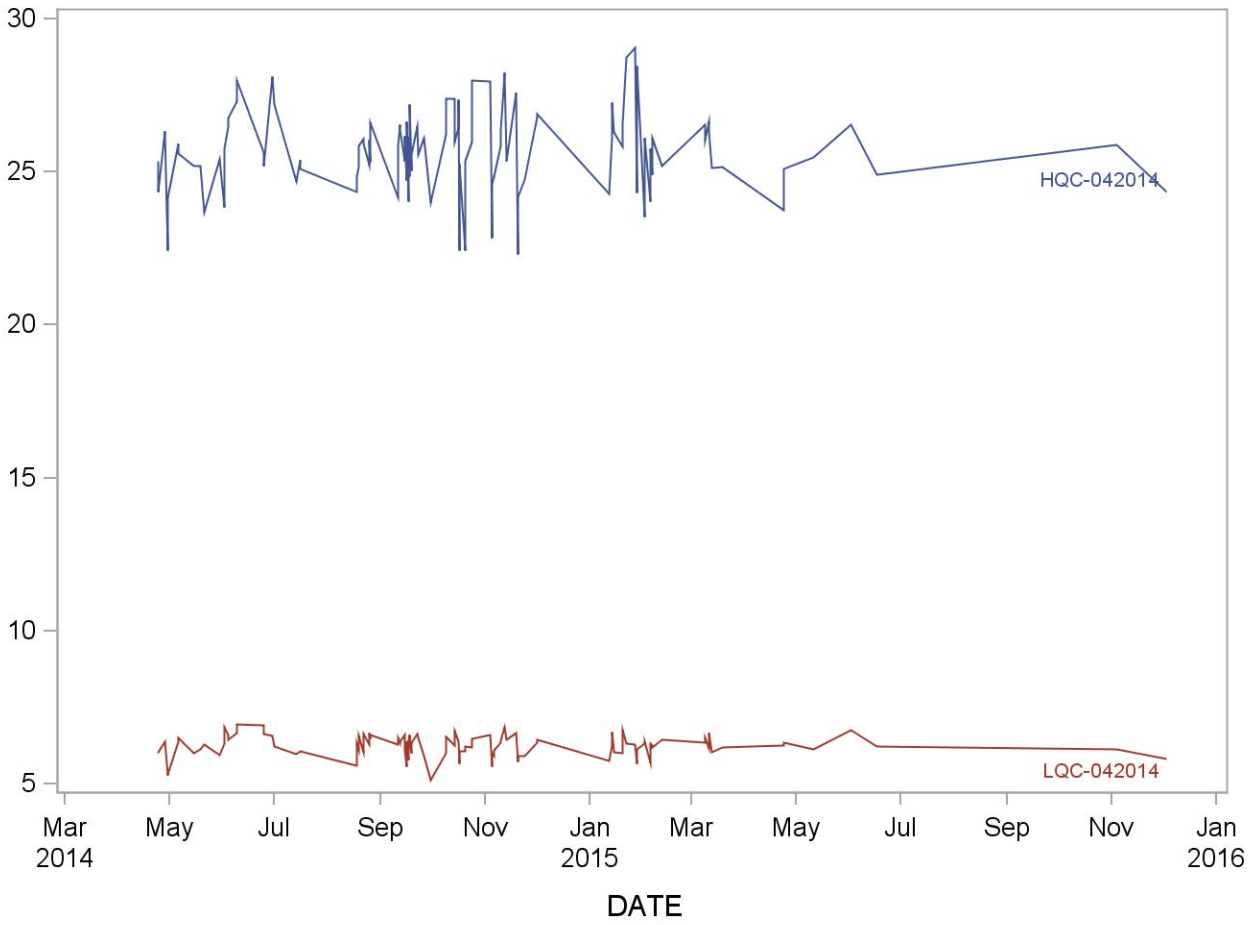
## 2013-2014 Summary Statistics and QC Chart for MEHP phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 114 | 24APR14 | 03DEC15 | 21.457 | 0.937 | 4.4 |
| LQC-042014 | 114 | 24APR14 | 03DEC15 | 6.371 | 0.307 | 4.8 |



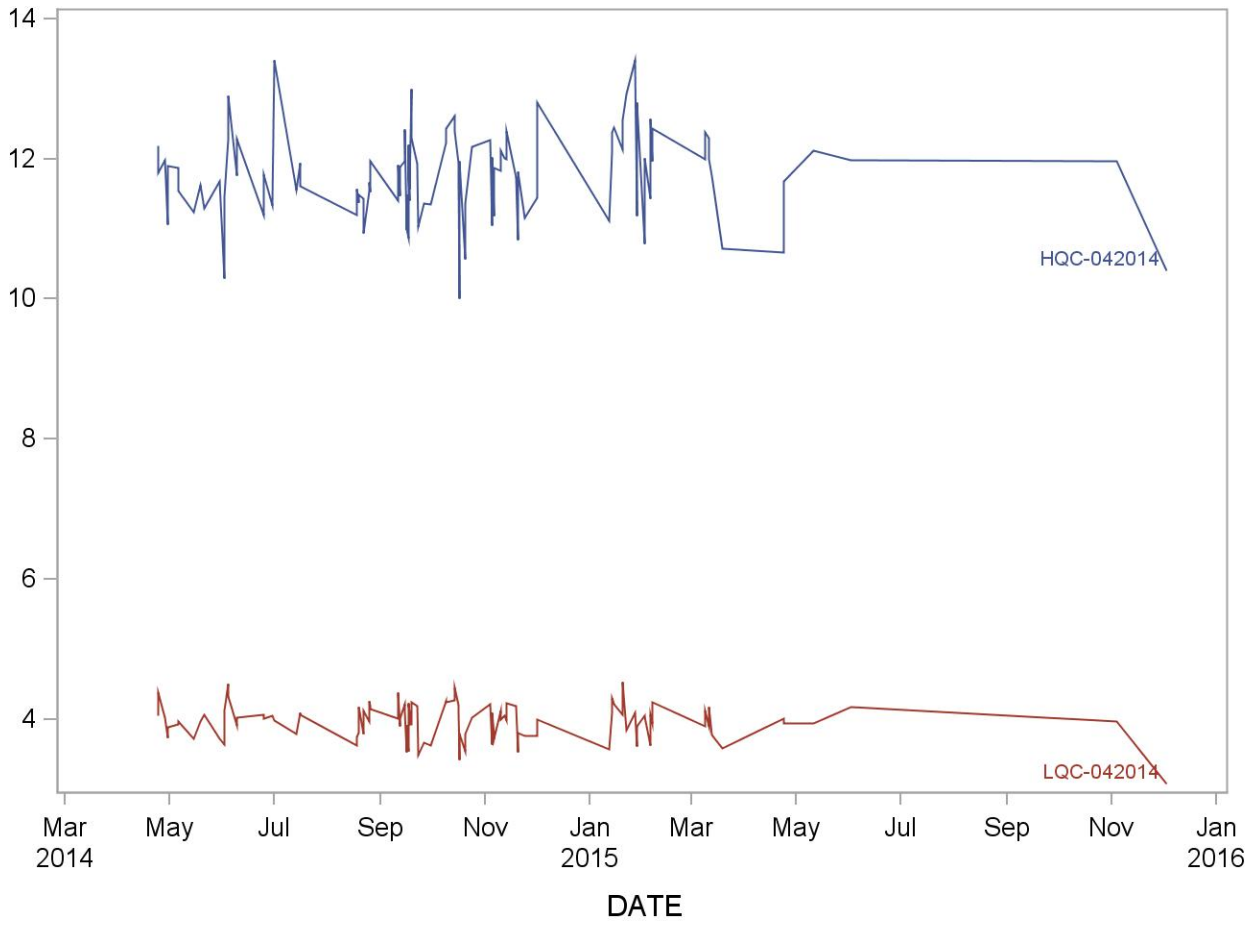
## 2013-2014 Summary Statistics and QC Chart for MEOH phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 113 | 24APR14 | 03DEC15 | 25.682 | 1.336 | 5.2 |
| LQC-042014 | 113 | 24APR14 | 03DEC15 | 6.257 | 0.346 | 5.5 |



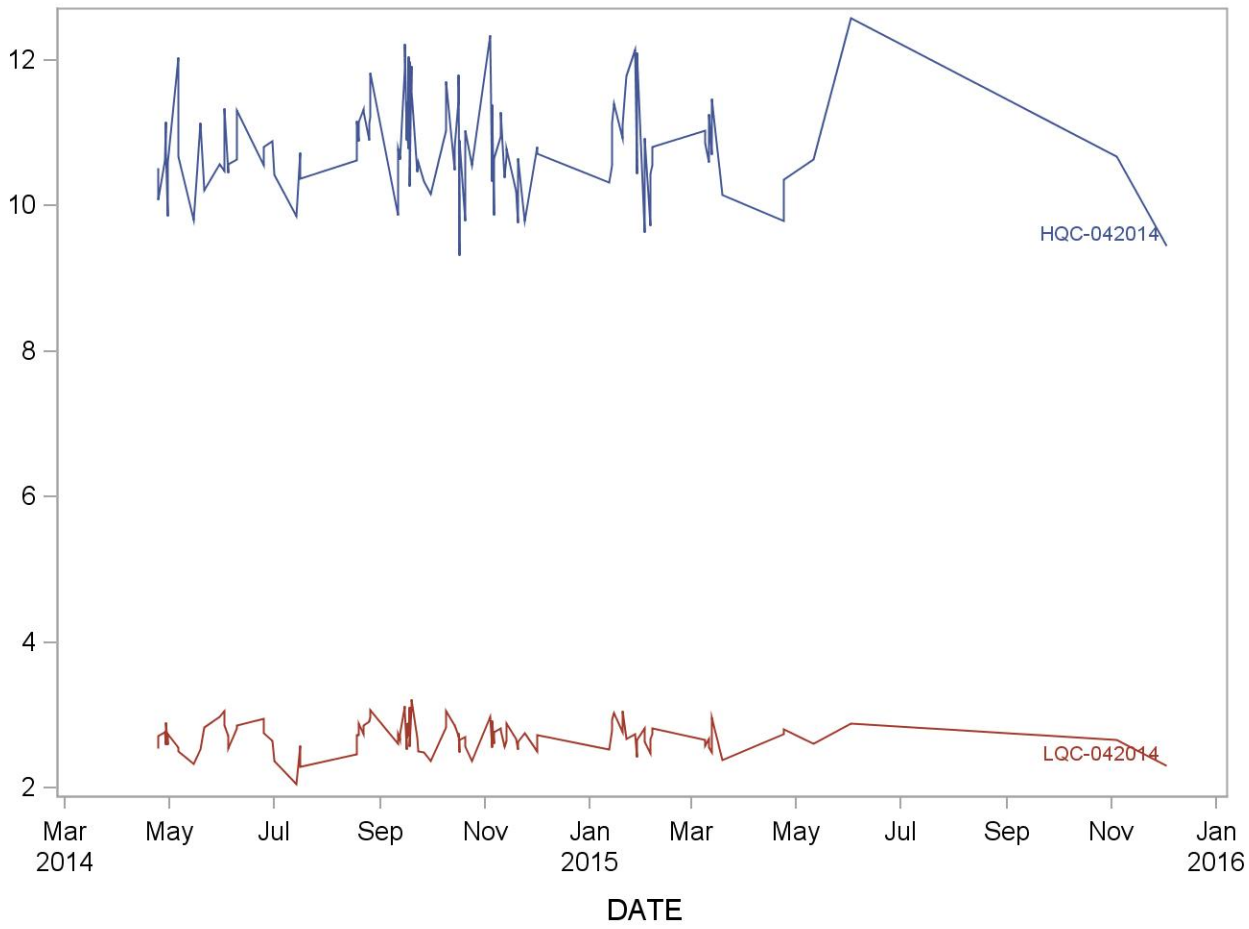
## 2013-2014 Summary Statistics and QC Chart for MHNCH (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 110 | 24APR14 | 03DEC15 | 11.766 | 0.622 | 5.3 |
| LQC-042014 | 110 | 24APR14 | 03DEC15 | 3.969 | 0.250 | 6.3 |



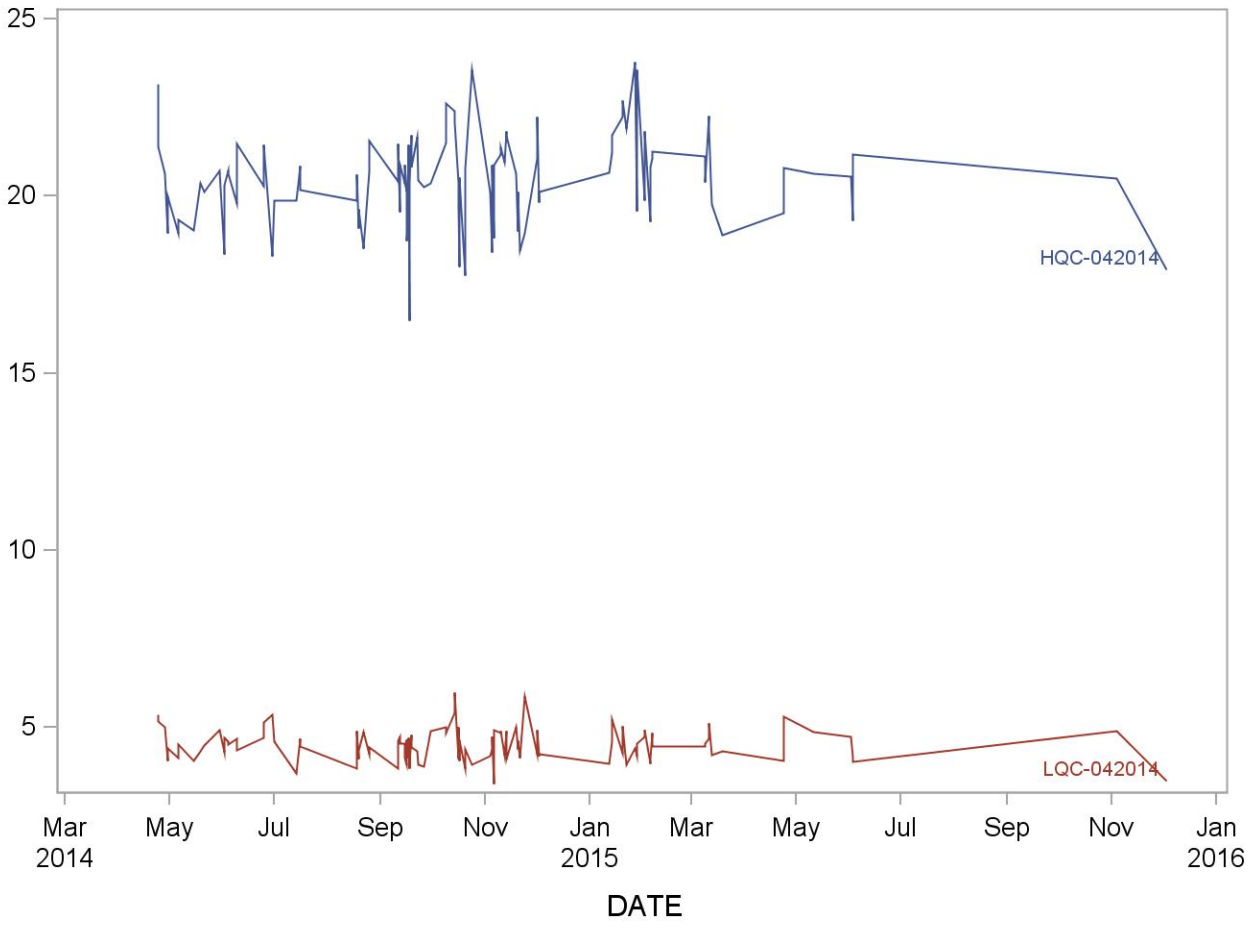
## 2013-2014 Summary Statistics and QC Chart for Mono-(3-carboxypropyl) phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 113 | 24APR14 | 03DEC15 | 10.816 | 0.652 | 6.0 |
| LQC-042014 | 113 | 24APR14 | 03DEC15 | 2.718 | 0.205 | 7.5 |



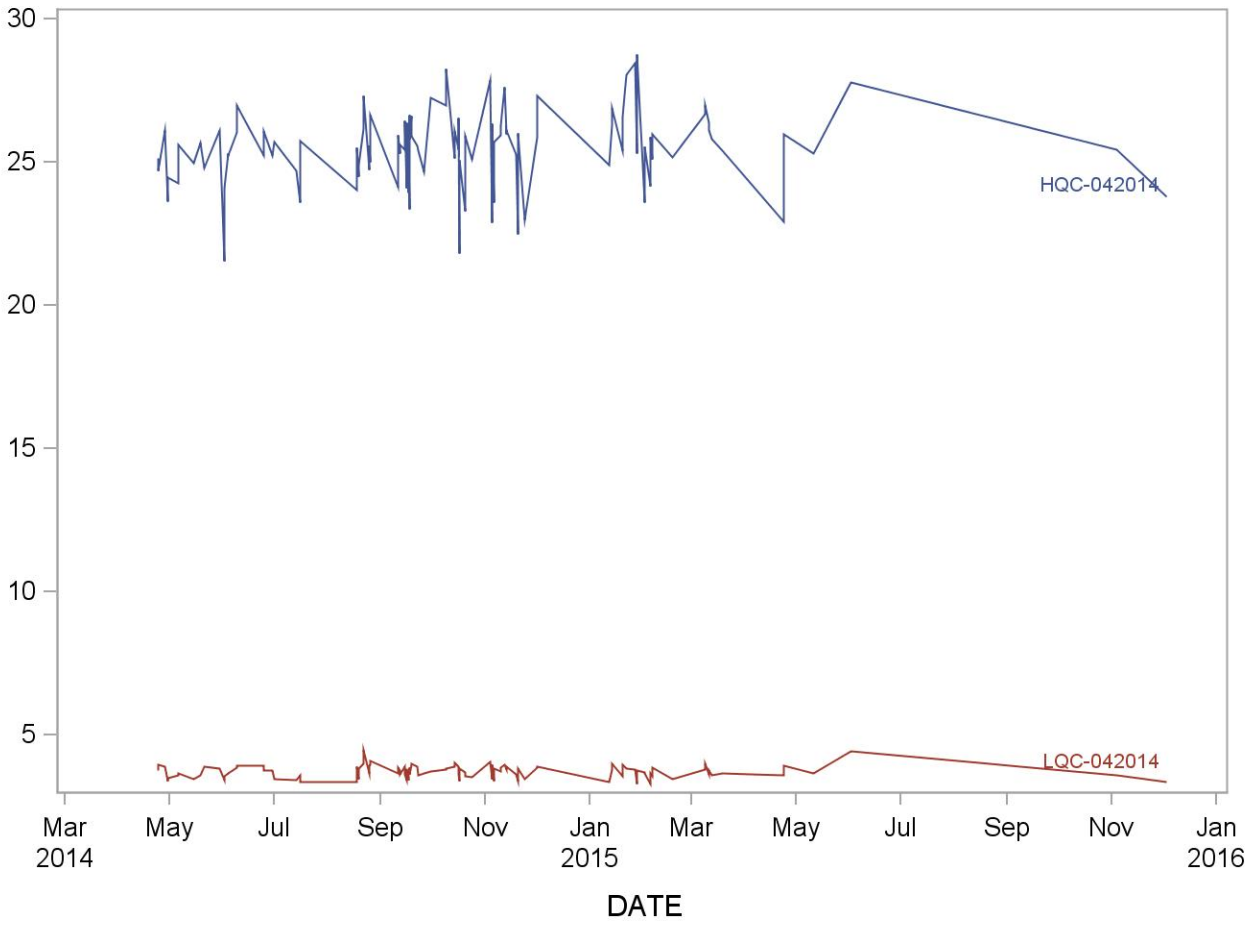
## 2013-2014 Summary Statistics and QC Chart for Mono-2-ethylhexyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 111 | 24APR14 | 03DEC15 | 20.452 | 1.316 | 6.4 |
| LQC-042014 | 111 | 24APR14 | 03DEC15 | 4.504 | 0.459 | 10.2 |



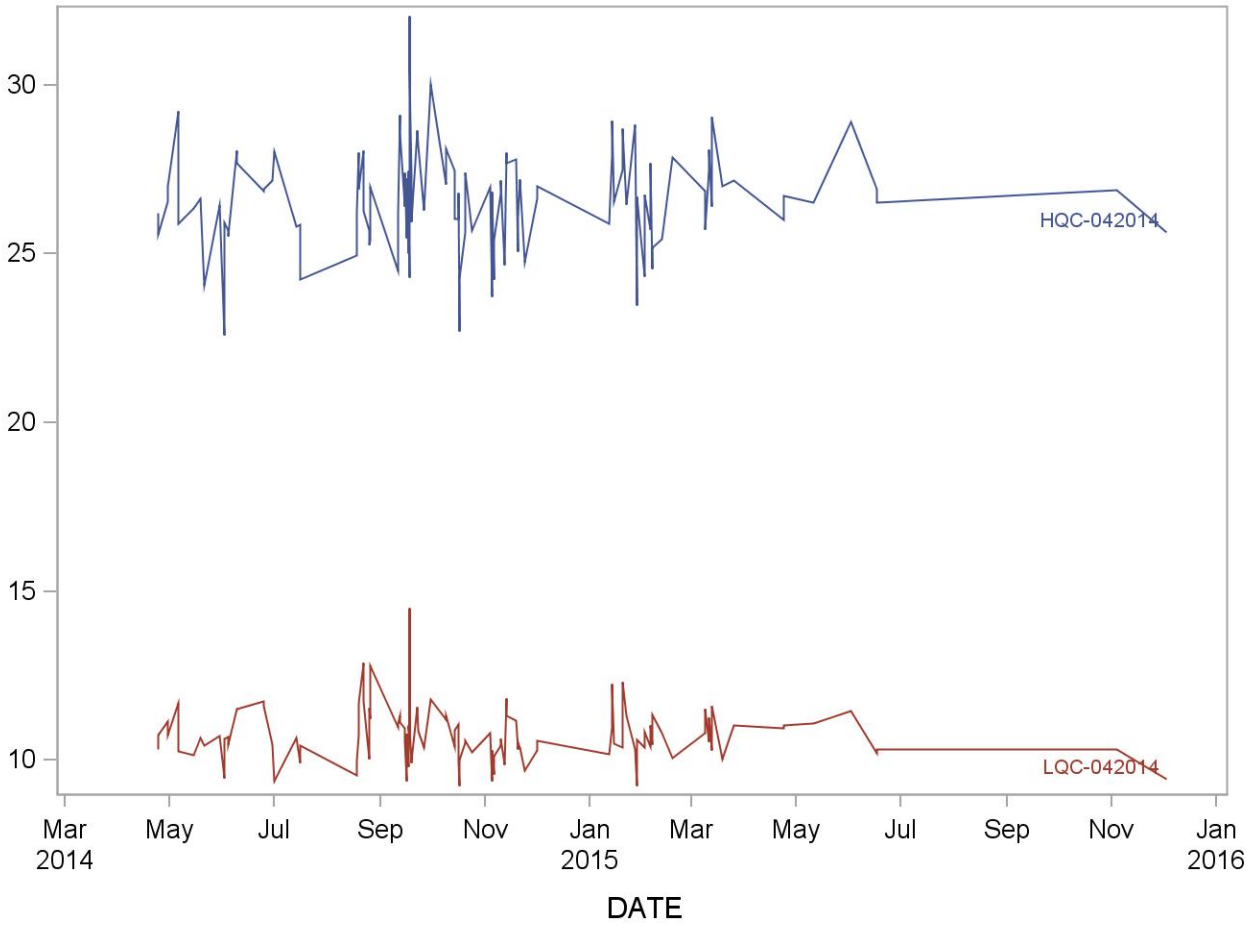
## 2013-2014 Summary Statistics and QC Chart for Mono-benzyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 110 | 24APR14 | 03DEC15 | 25.487 | 1.325 | 5.2 |
| LQC-042014 | 110 | 24APR14 | 03DEC15 | 3.732 | 0.219 | 5.9 |



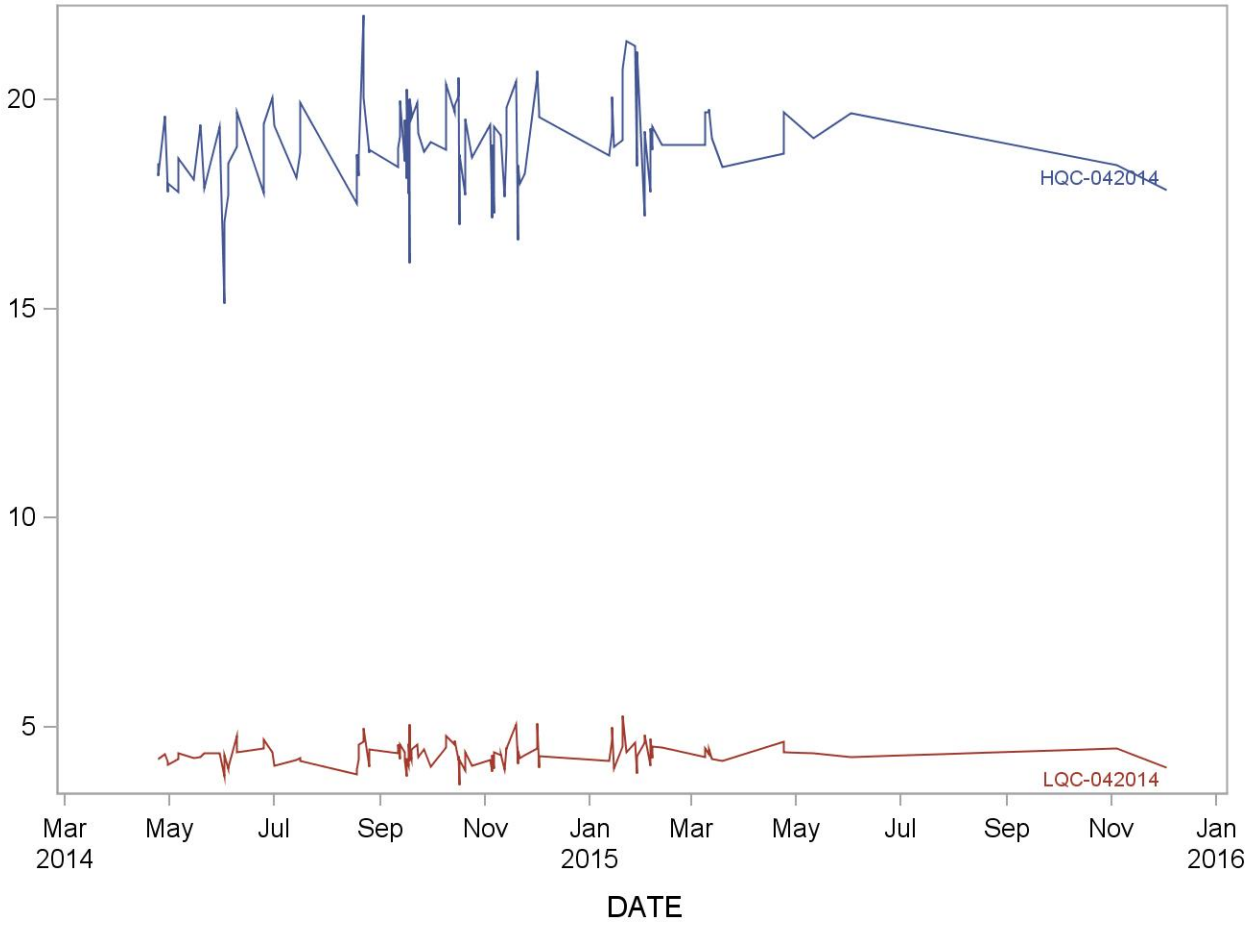
## 2013-2014 Summary Statistics and QC Chart for Mono-carboxyisoctyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 116 | 24APR14 | 03DEC15 | 26.600 | 1.520 | 5.7 |
| LQC-042014 | 116 | 24APR14 | 03DEC15 | 10.726 | 0.791 | 7.4 |



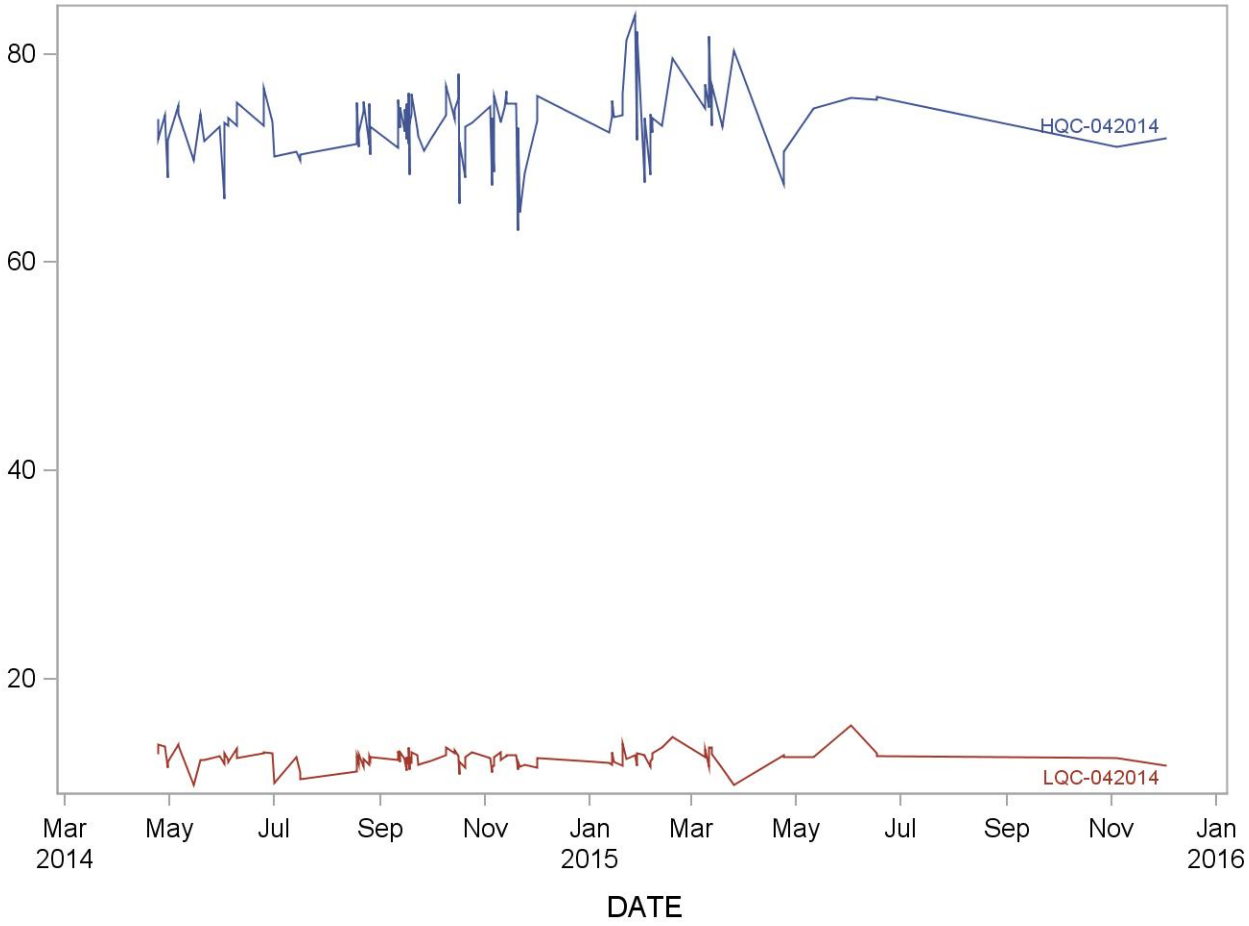
## 2013-2014 Summary Statistics and QC Chart for Mono-carboxyisononyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 112 | 24APR14 | 03DEC15 | 18.975 | 1.093 | 5.8 |
| LQC-042014 | 112 | 24APR14 | 03DEC15 | 4.363 | 0.286 | 6.6 |



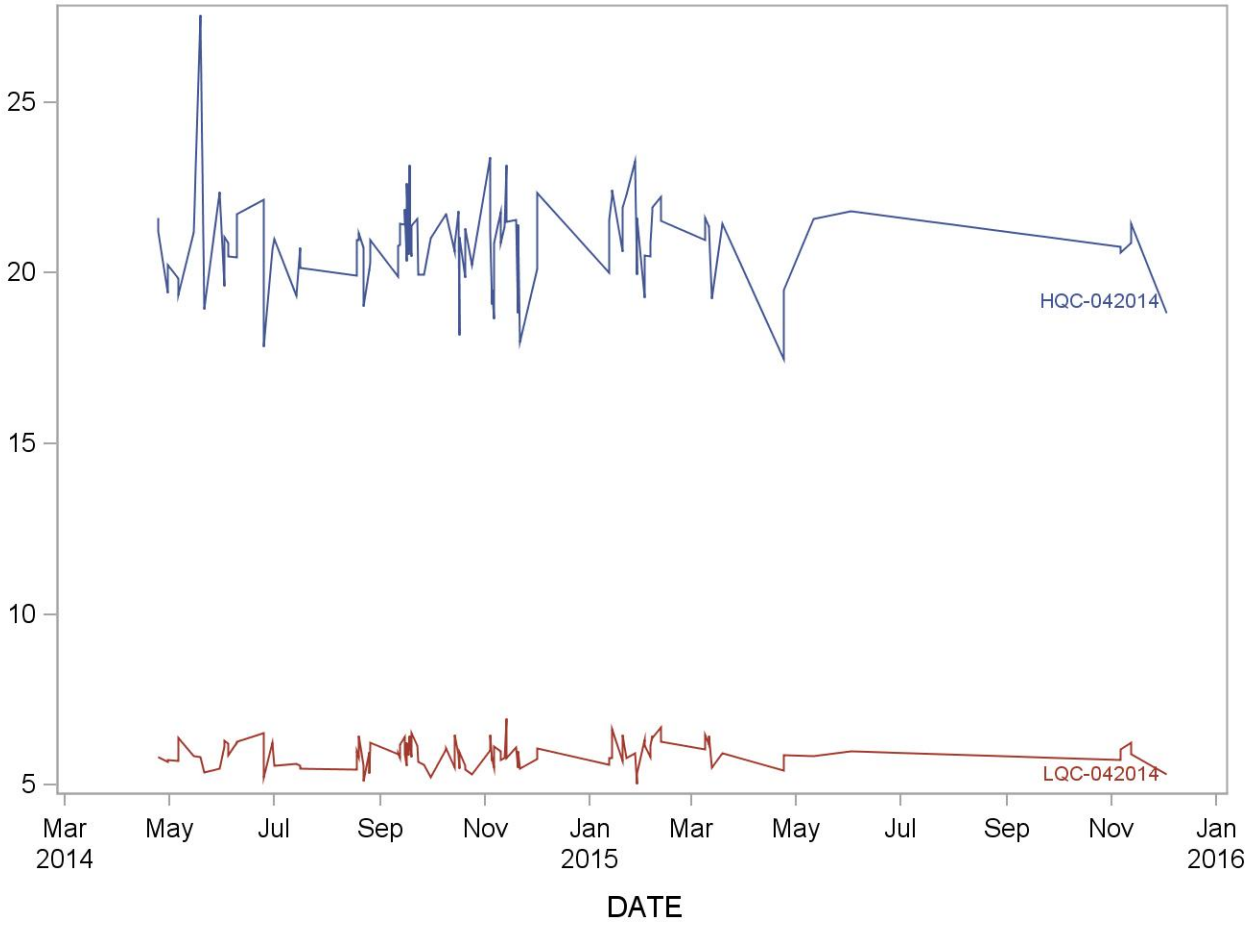
## 013-2014 Summary Statistics and QC Chart for Mono-ethyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 117 | 24APR14 | 03DEC15 | 73.366 | 3.333 | 4.5 |
| LQC-042014 | 117 | 24APR14 | 03DEC15 | 12.339 | 0.846 | 6.9 |



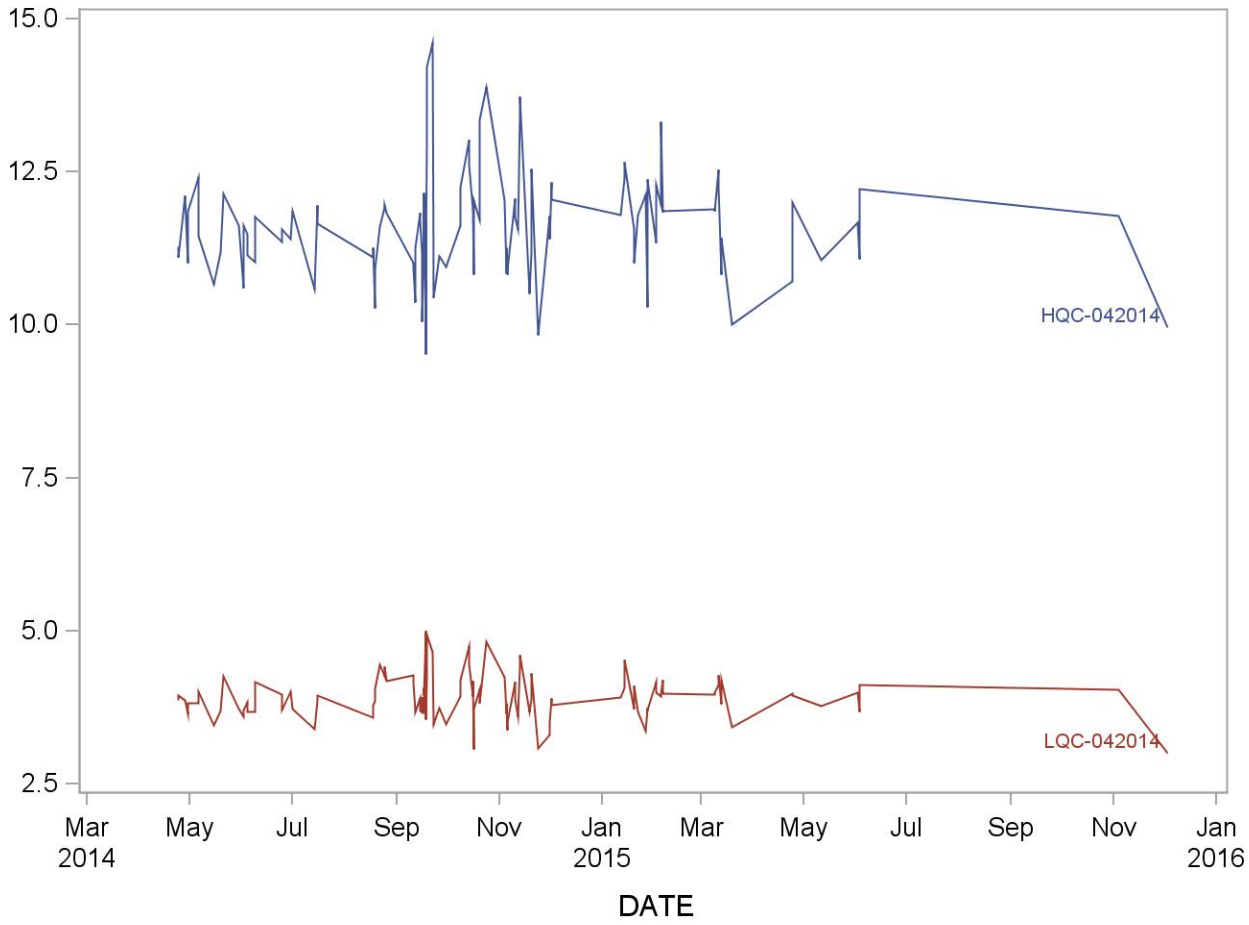
## 2013-2014 Summary Statistics and QC Chart for Mono-iso-butyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 115 | 24APR14 | 03DEC15 | 20.892 | 1.303 | 6.2 |
| LQC-042014 | 115 | 24APR14 | 03DEC15 | 5.921 | 0.371 | 6.3 |



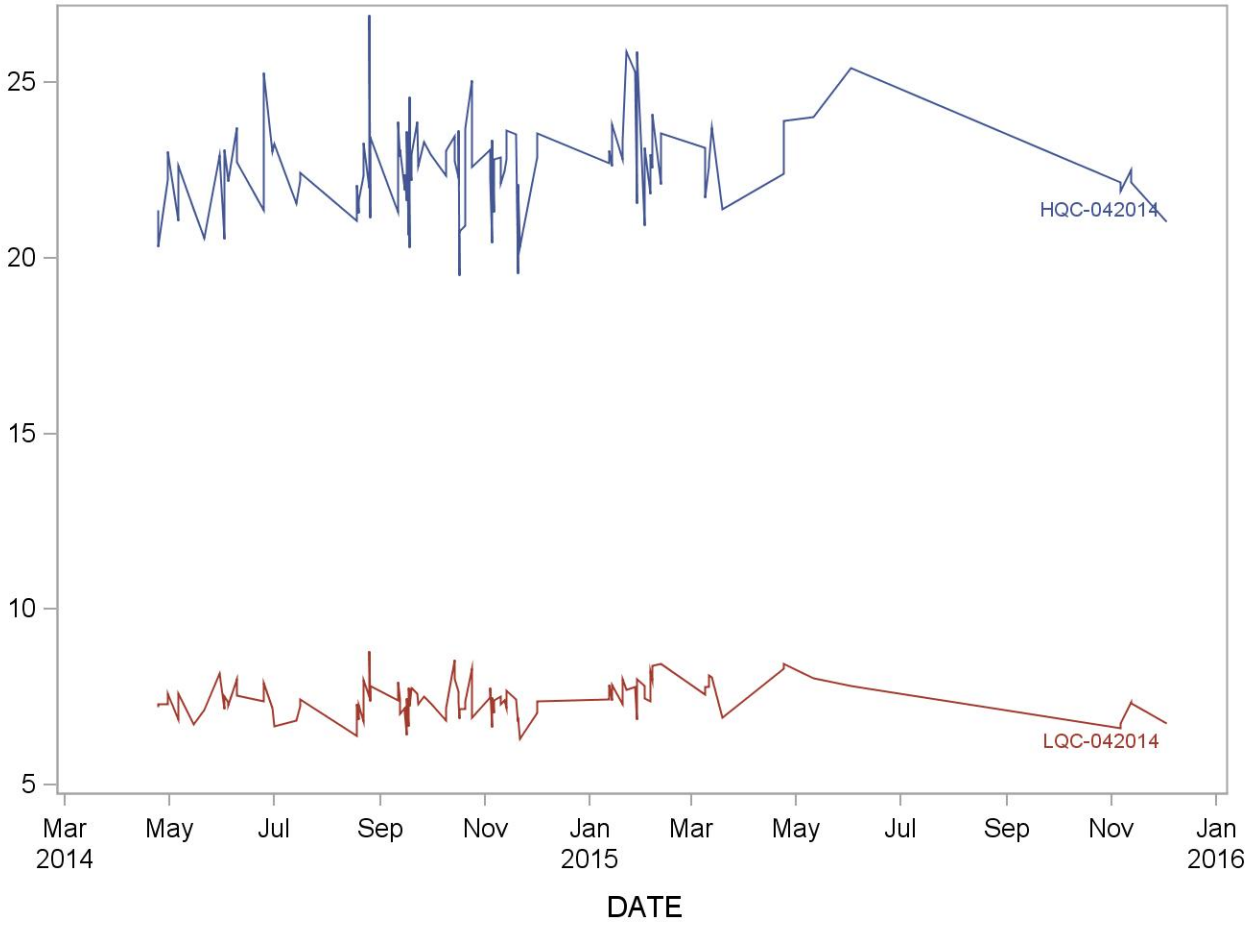
## 2013-2014 Summary Statistics and QC Chart for Mono-isononyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 112 | 24APR14 | 03DEC15 | 11.613 | 0.885 | 7.6 |
| LQC-042014 | 112 | 24APR14 | 03DEC15 | 3.915 | 0.359 | 9.2 |



## 2013-2014 Summary Statistics and QC Chart for Mono-n-butyl phthalate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| HQC-042014 | 115 | 24APR14 | 03DEC15 | 22.586 | 1.292 | 5.7 |
| LQC-042014 | 115 | 24APR14 | 03DEC15 | 7.453 | 0.497 | 6.7 |



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