



# Laboratory Procedure Manual

*Analyte:* **Metabolites of phthalates and phthalate alternatives**

*Matrix:* **Urine**

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

*Method No:* **6306.04**

*Revised:* **April, 2013**

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

*Contact:* Antonia Calafat, PhD  
Phone: 770-488-7891  
Email: ACalafat@cdc.gov

James L. Pirkle, M.D., Ph.D.  
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.

**Metabolites of phthalates and phthalate alternatives in urine NHANES 2011-2012****Public Release Data Set Information**

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

<b>Data File Name</b>	<b>Variable Name</b>	<b>SAS Label</b>
PHTHTE_G	URXECP	Mono-(2-ethyl-5-carboxypentyl) phthalate (ng/mL)
	URXCNP	Mono(carboxyisononyl) phthalate (ng/mL)
	URXCOP	Mono(carboxyisooctyl) phthalate (ng/mL)
	URXMBP	Mono-n-butyl phthalate (ng/mL)
	URXMC1	Mono-(3-carboxypropyl) phthalate (ng/mL)
	URXMEP	Mono-ethyl phthalate (ng/mL)
	URXMHH	Mono-(2-ethyl-5-hydroxyhexyl) phthalate (ng/mL)
	URXMHP	Mono-(2-ethylhexyl) phthalate (ng/mL)
	URXMIB	Mono-isobutyl phthalate (ng/mL)
	URXMNM	Mono-methyl phthalate (ng/mL)
	URXMNP	Mono-isononyl phthalate (ng/mL)
	URXMOH	Mono-(2-ethyl-5-oxohexyl) phthalate (ng/mL)
	URXMZP	Mono-benzyl phthalate (ng/mL)
	URXMHN	Cyclohexane-1,2-dicarboxylic acid monohydroxy isononyl ester (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 the following phthalate metabolites and phthalate alternatives (Table 1)[1;2]. 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

<b>Parent chemical</b>	<b>Metabolite</b>
Di-methyl phthalate(DMP)	Mono-methyl phthalate (MMP)
Di-n-octyl phthalate (DOP) Di-n-butyl phthalate (DBP) 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)
Di-n-butyl phthalate (DBP)	Mono-n-butyl phthalate (MBP)
Benzylbutyl phthalate (BzBP)	Monobenzyl phthalate (MBzP)
Di(2-ethylhexyl) phthalate (DEHP)	Mono(2-ethylhexyl) phthalate (MEHP) Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) Mono(2-ethyl-5-oxohexyl) phthalate (MEOHP)

## Metabolites of phthalates and phthalate alternatives in urine NHANES 2011-2012

Parent chemical	Metabolite
Di-isononyl phthalate (DNP)	Mono-isononyl phthalate (MNP) Monocarboxy-octyl phthalate (MCOP)
Di-isodecyl phthalate (DDP)	Monocarboxy-isononyl phthalate (MCNP)
1,2-Cyclohexane dicarboxylic acid, diisononyl ester (DINCH)	Cyclohexane-1,2-dicarboxylic acid-mono(hydroxy-isononyl) ester (MHINCH)

### b. Clinical Relevance

Phthalates, are a group of industrial chemicals widely used in consumer products and as solvents, additives, and plasticizers [3]. 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. Humans are potentially exposed to many products containing phthalates and DINCH. Phthalates are rapidly metabolized in humans to their respective monoesters, which depending on the phthalate can be further metabolized to their oxidative products. Similarly, oxidative metabolites of DINCH are identified in the urine of humans dosed with DINCH [4]. Monoester and the oxidative metabolites may be glucuronidated, and excreted in the urine and feces [5-8]. Some of these metabolites can cause reproductive and developmental toxicities in animals [9-12], but little is known about the effects of phthalates and DINCH exposure on humans. Information on the concentration of phthalates and phthalate alternatives in people is essential to understand the human exposure. Measurement of an internal dose, or biomarker of exposure, is a key aspect of assessing 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.

$\beta$ -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](http://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.

**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 nitrile or latex 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 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 (Thermo Scientific, San Jose, CA, USA) on a Thermo Scientific Surveyor 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**

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 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 required.
- (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). 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 [13]. 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.

**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 discarded after 14 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 year.

\* 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)

\*  $\beta$ -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 of the solution 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.

**$\beta$ -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  $\mu$ L of  $\beta$ -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- $\beta$ -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**

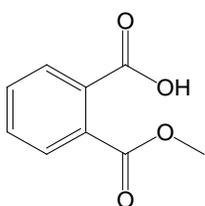
Phthalate and DINCH metabolite native and labeled standards are obtained from: Cambridge Isotope Laboratories Inc (Andover, MA), Los Alamos National

## Metabolites of phthalates and phthalate alternatives in urine NHANES 2011-2012

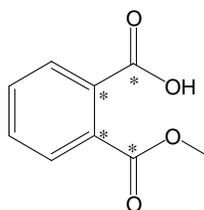
Laboratory (Los Alamos, NM), SigmaAldrich, Professor Jurgen Angerer (Germany), Dr. Holger Koch (Germany) and Cansyn (Toronto, Canada).

$^{13}\text{C}_4$ -4-methyl umbelliferone was purchased from Cambridge Isotope Laboratories Inc. 4-methyl umbelliferyl glucuronide is purchased from Sigma Aldrich Laboratories.

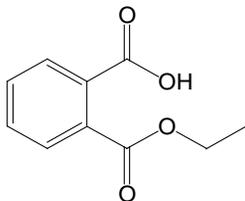
### (2) Native and labeled metabolites used in standards



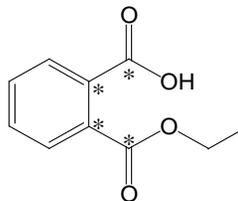
**MMP**  
monomethyl phthalate



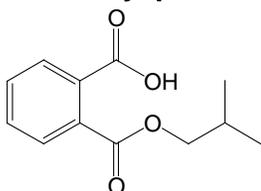
**MMP\***  
monomethyl phthalate- $^{13}\text{C}_4$



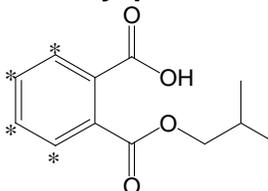
**MEP**  
monoethyl phthalate



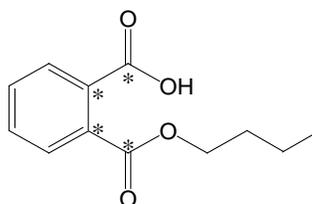
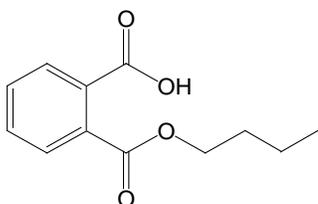
**MEP\***  
monoethyl phthalate- $^{13}\text{C}_4$



**MiBP**  
mono(2-methylpropyl) phthalate  
mono-isobutyl phthalate

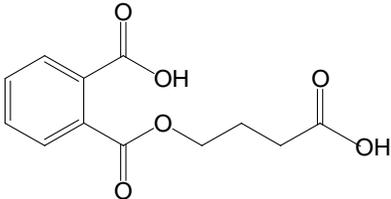


**MiBP\***  
mono(2-methylpropyl) phthalate- $\text{D}_4$   
mono-isobutyl phthalate- $\text{D}_4$

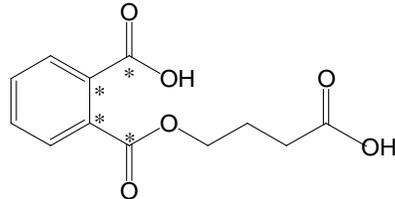


**Metabolites of phthalates and phthalate alternatives in urine NHANES 2011-2012**

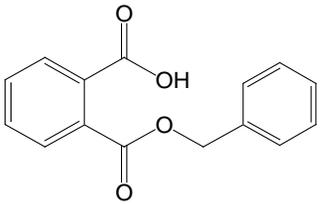
**MBP**  
mono-n-butyl phthalate



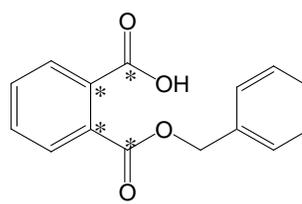
**MBP\***  
mono-n-butyl phthalate-<sup>13</sup>C<sub>4</sub>



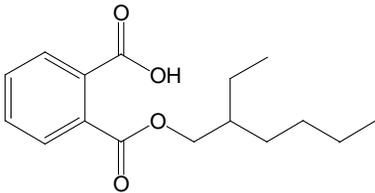
**MCPP**  
mono(3-carboxypropyl) phthalate



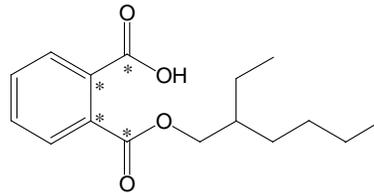
**MCPP\***  
mono(3-carboxypropyl) phthalate-<sup>13</sup>C<sub>4</sub>



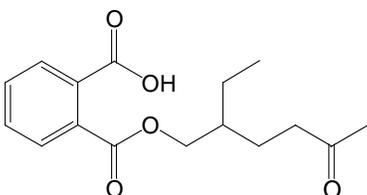
**MBzP**  
monobenzyl phthalate



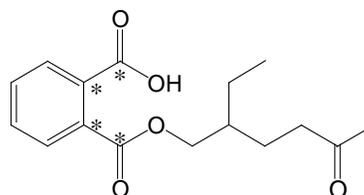
**MBzP\***  
monobenzyl phthalate-<sup>13</sup>C<sub>4</sub>



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



**MEHP\***  
mono(2-ethylhexyl) phthalate-<sup>13</sup>C<sub>4</sub>

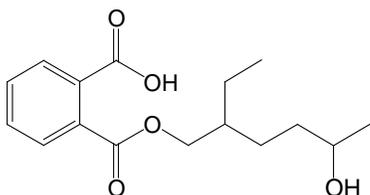


**MEOHP**

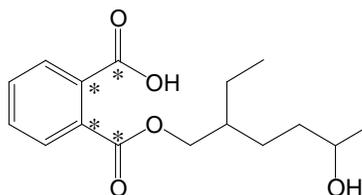
**MEOHP\***

**Metabolites of phthalates and phthalate alternatives in urine NHANES 2011-2012**

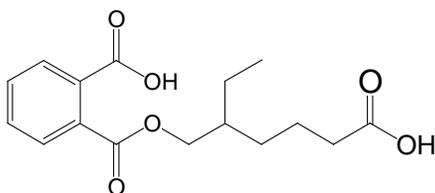
**mono(2-ethyl-5-oxohexyl) phthalate**



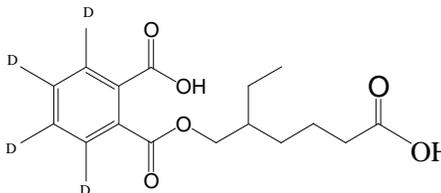
**mono(2-ethyl-5-oxohexyl) phthalate-<sup>13</sup>C<sub>4</sub>**



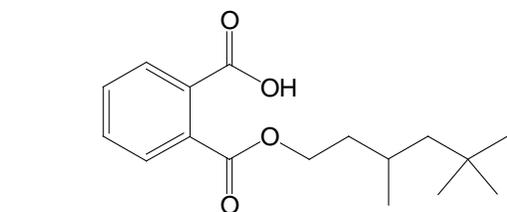
**MEHHP  
mono(2-ethyl-5-hydroxyhexyl) phthalate**



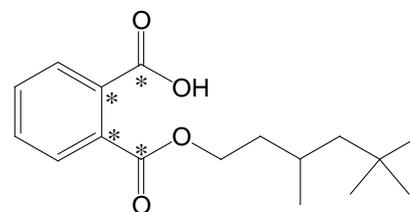
**MEHHP\*  
mono(2-ethyl-5-hydroxyhexyl) phthalate-<sup>13</sup>C<sub>4</sub>**



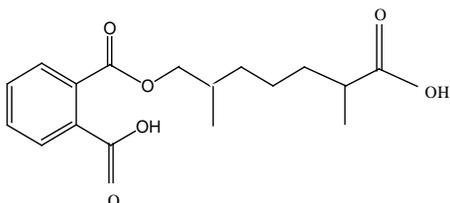
**MECPP  
mono(2-ethyl-5-carboxypentyl)phthalate**



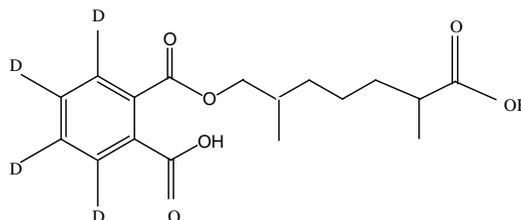
**MECPP\*  
D<sub>4</sub>-mono(2-ethyl-5-carboxypentyl)phthalate**



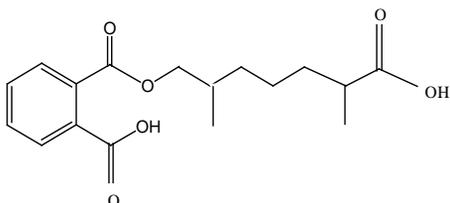
**MNP  
mono(3,5,5-trimethyl-1-hexyl) phthalate  
(mono-isononyl phthalate)**



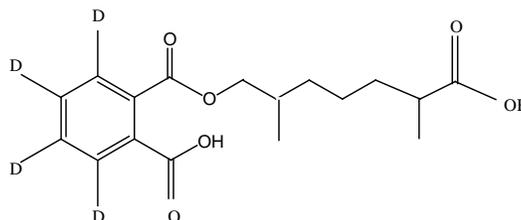
**MNP\*  
mono(3,5,5-trimethyl-1-hexyl) phthalate-<sup>13</sup>C<sub>4</sub>  
(mono-isononyl phthalate <sup>13</sup>C<sub>4</sub>)**



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

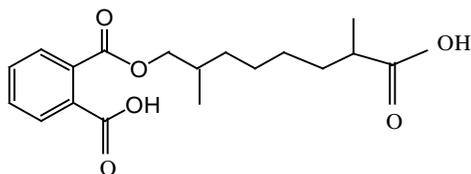


**MCOP\*  
mono(2,6-methyl-6-carboxyhexyl)phthalate**



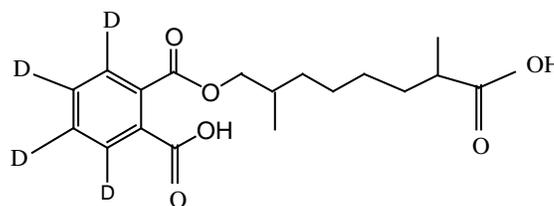
## Metabolites of phthalates and phthalate alternatives in urine NHANES 2011-2012

(mono-carboxyisooctyl phthalate)

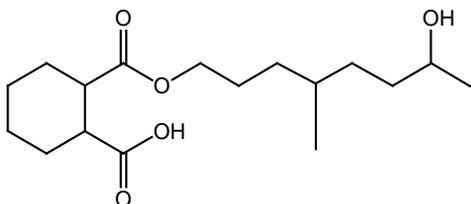


**MCNP**  
mono(2,7-methyl-7-carboxyheptyl)phthalate  
(mono-carboxyisooctyl phthalate)

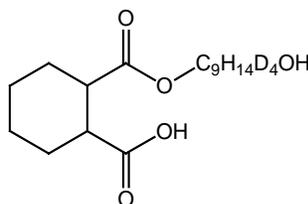
(D<sub>4</sub>-mono-carboxyisooctyl phthalate)



**MCNP\***  
mono(2,7-methyl-7-carboxyheptyl)phthalate  
(D<sub>4</sub>-mono-carboxyisooctyl phthalate)



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



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

### (3) Standards Preparation

- Individual native standards of phthalate metabolites. The stock solutions are prepared by accurately transferring approximately 5-25 mg of material onto a 50 mL volumetric flask. The phthalate 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.
- Isotopically-labeled phthalate metabolites and 4-methyl umbelliferone internal standards. These internal standards are prepared similarly to the native standards and stored at -20 °C until use. The isotopic purity of each internal standard, confirmed empirically by tandem mass spectral analysis, is determined to contain less than 1% of the native compound.
- 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 is made in HPLC grade water by

diluting the intermediate stock solution and stored in the refrigerator 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 (4 °C) and remade as needed from the stock solutions. Stock standards and the working standard mixtures are remade, as necessary.

**(5) Proficiency Testing Standards**

Aliquots of each stock standard were added to 1L urine pools. The volume of each standard varied, to produce 3 concentrations of proficiency testing (PT) standards. The spiked pools were mixed overnight and are aliquoted into cryovials and frozen (<-40°C) until needed. The PT standards are characterized by at least 20 repeat determinations to characterize the mean and standard deviation for evaluation.

**(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 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.

**(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)
- (6) Magnetic Stirrer (Corning)
- (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, a minimum of 8 standards followed by unknowns, QC samples and blanks are 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 [www.cms.hhs.gov/CLIA/downloads/6065bk.pdf](http://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)**

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 (MBP, MiBP, MBzP, 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/>).

## **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 materials of 3 of low (QCL) and 3 of high (QCH) 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  $\beta$ -glucuronidase solution and the enzyme solution are prepared fresh just prior to addition to samples.

### **b. Sample preparation**

- (1) 100  $\mu$ L of calibration standard, urine, HPLC Grade water (for the reagent blank), QCH and QCL is transferred manually into a properly labeled autosampler vial (1.5 mL).
- (2) The vial is capped 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 minimum of 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 Surveyor liquid chromatograph coupled with a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer, equipped with an ESI (Electrospray Ionization) 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 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 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].

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

Time (min)	SPE pump			Switching valve	HPLC pump			Switching valve
	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

Electrospray ionization (ESI) in negative ion mode is used to ionize the analyte molecules and transfer the negatively charged analyte ions into the gas phase..

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 energy and the S lens voltage are specifically set for each ion (Table 3).

**(2) Multiple Reaction Monitoring Setup**

Table 3. Phthalate metabolites, their native and labeled precursor and product ion transitions and collision energies.

Analyte	Precursor/ Product ion	Collision energy (V)
MCPP	251/103	10
<sup>13</sup> C <sub>4</sub> -MCPP	255/103	
MMP	179/77	24
<sup>13</sup> C <sub>4</sub> -MMP	183/79	
MEP	193/77	25
<sup>13</sup> C <sub>4</sub> -MEP	197/79	
MECPP	307/159	22
D <sub>4</sub> -MECPP	311/159	
MiBP	221/77	26
D <sub>4</sub> -MiBP	225/81	
MBP	221/77	26
<sup>13</sup> C <sub>4</sub> -MBP	225/79	
MEOHP	291/121	27
<sup>13</sup> C <sub>4</sub> -MEOHP	295/124	
MEHHP	293/121	27
<sup>13</sup> C <sub>4</sub> -MEHHP	297/124	
MCOP	321/173	19
D <sub>4</sub> -MCOP	325/173	
MBzP	255/183	14
<sup>13</sup> C <sub>4</sub> -MBzP	259/186	
MCNP	335/187	21
D <sub>4</sub> -MCNP	339/187	
MEHP	277/134	21
<sup>13</sup> C <sub>4</sub> -MEHP	281/137	
MNP	291/121	27
<sup>13</sup> C <sub>4</sub> -MNP	295/124	
MHNCH	313/153	19
D <sub>4</sub> -MHNCH	317/153	

**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**

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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). 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 [14].  $S_0$  was determined from the replicate analysis of low-level standards in synthetic urine.

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 4).

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

Analyte	LOD (ng/mL)	Calibration range (ng/mL)
MCPPP	0.2	0.035-350
MMP	0.5	0.08-800
MEP	0.6	0.5-5000
MiBP	0.2	0.05-500
MBP	0.4	0.013-1313
MEHHP	0.2	0.125-1250
MECPP	0.2	0.125-1250
MEOHP	0.2	0.138-1375
MBzP	0.3	0.113-1125
MEHP	0.5	0.05-500
MNP	0.5	0.058-575
MCOP	0.2	0.05-500
MCNP	0.2	0.05-500
MHNCH	0.4	0.025-250

### c. Accuracy

The method accuracy was assessed through 5 replicate analyses of analytes spiked into synthetic urine (Table 5).

Table 5. Accuracy of the method

Analyte	Expected ng/mL	Observed ng/mL
MMP	7.0	7.2
MCPP	3.5	3.6
MEP	50.0	48.0
MEHHP	12.5	12.7
MiBP	5.0	4.6
MECPP	12.5	12.7
MBP	13.1	11.8
MEOHP	13.8	12.8
MCOP	5.0	5.0
MBzP	11.0	11.3
MHNCH	2.5	2.5
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 intraday and inter-day variability of the assay (Table 6).

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

Analyte	QCH		QCL	
	CV (%)	Mean	CV (%)	Mean
MMP	4.2	12.6	7.3	4.8
MEP	3.6	91.2	5.8	22.1
M CPP	4.4	13.1	8.4	2.2
MiBP	3.8	19.7	7	5.1
MBP	3.9	24.2	8.5	5.5
MBzP	4.1	22	7.1	4.2
MEHP	5.5	14	8.5	5
MNP	5.2	13	8.6	4.4
MEOHP	3.7	18.3	6.8	5.1
MEHHP	4.2	19.7	6.5	4.9
M E CPP	4.4	29.5	7.1	5
MCNP	3.3	14.2	5.9	4.5
MCOP	5.1	18.1	6.5	7.1
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  $RT_{\text{analyte}}$  and  $RT_{\text{IS}}$ ) of standards, unknowns, and QCs should be within 0.90 ~ 1.10. If the relative RT falls outside this range, both analyte and the internal standard peaks are manually reviewed to confirm each peak is accurately identified.
- 2) For each analyte the total ion count (TIC) for the IS should exceed  $1.0 \times 10^3$ . If the area count is below the minimum requirement the 'S' lens, ion transfer tube and the exit lens of the mass spectrometer are cleaned and the affected sample is 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 concentration of the preceding sample is more than the highest calibration standard, the sample is re-extracted to eliminate potential carryover.
- 5) 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.

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

**(3) Characterization of QC Materials**

The QC pools were characterized to define the mean and the 95% and 99% control limits of phthalate and MHNCH concentrations from 60 QCL and 60 QCH runs over 3 weeks. In each run, one pair of QCL and one pair of QCH materials were 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 [15].

**Two QC pools per run with one QC result per pool**

- 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  $\pm 4S_i$
  - 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  $\pm 4S_m$
  - 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 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 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.

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].

### **13. Reference Ranges (Normal Values)**

The results from the National Health and Nutrition Examination Survey (NHANES) 1999-2010 can be used as the reference range to describe levels of phthalate exposure among the general US population [16].

After learning of inaccuracies affecting commercial phthalate metabolite analytical standards [17], 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. In 2012, these correction factors were applied to these five analytes for NHANES survey periods 1999-2000, 2001-2002, 2003-2004, 2005-2006, and 2007-2008 and published in the Updated tables of the Exposure Report. Consequently, since 2012, the concentrations of these phthalate metabolites in the *Updated Tables* are slightly different from the concentrations presented in the *Fourth Report on Human Exposure to Environmental Chemicals* and in all of the previous *Reports*.

### **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.

### **11. Specimen Storage and Handling during Testing**

Specimens are stored in the laboratory frozen ( $\leq -20$  °C) prior to analysis. Prepared samples are kept at 10°C during analysis. Frozen samples are allowed to thaw completely at room temperature or in a 25 °C sonicating bath prior to the initiation of the analytical procedure.

### **12. Alternate Methods for Performing Test and Storing Specimens if Analytical System Fails**

The current analytical method utilizes a Thermo Scientific Surveyor/Accela liquid chromatograph coupled with a Thermo ScientificTSQ Vantage Triple Quadrupole mass

spectrometer. Although alternative extraction techniques exist [18;19], if the analytical system fails, prepared samples can be stored ( $\leq -40$  °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 ( $\leq 5$  °C) for a maximum of 24 hours.

**13. 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 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 with the quality control officer.

**14. 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.

**15. Summary Statistics and QC Graphs**

See next page(s).

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*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.*

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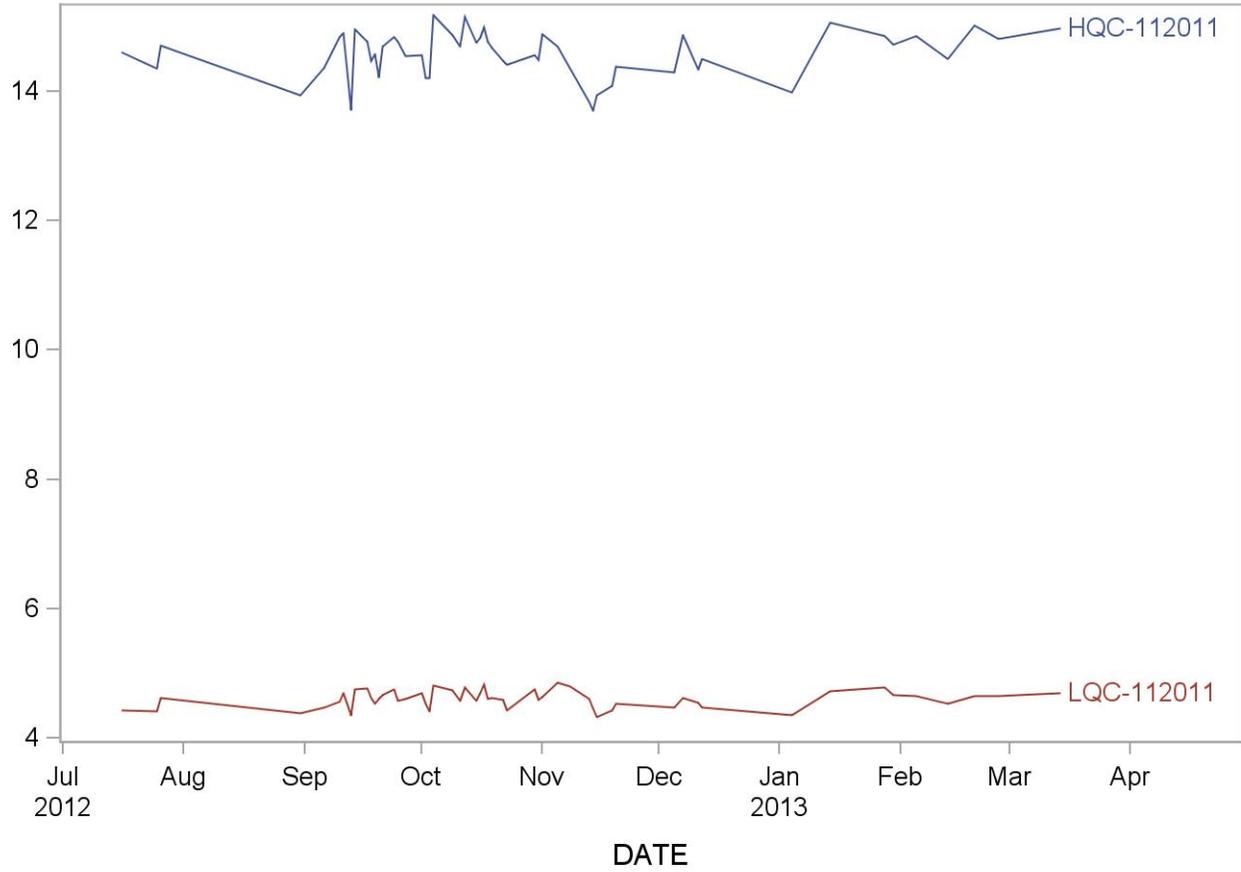
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### Summary Statistics for Mono(carboxynonyl) phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	89	16JUL12	14MAR13	14.560	0.417	2.9
LQC-112011	89	16JUL12	14MAR13	4.593	0.150	3.3

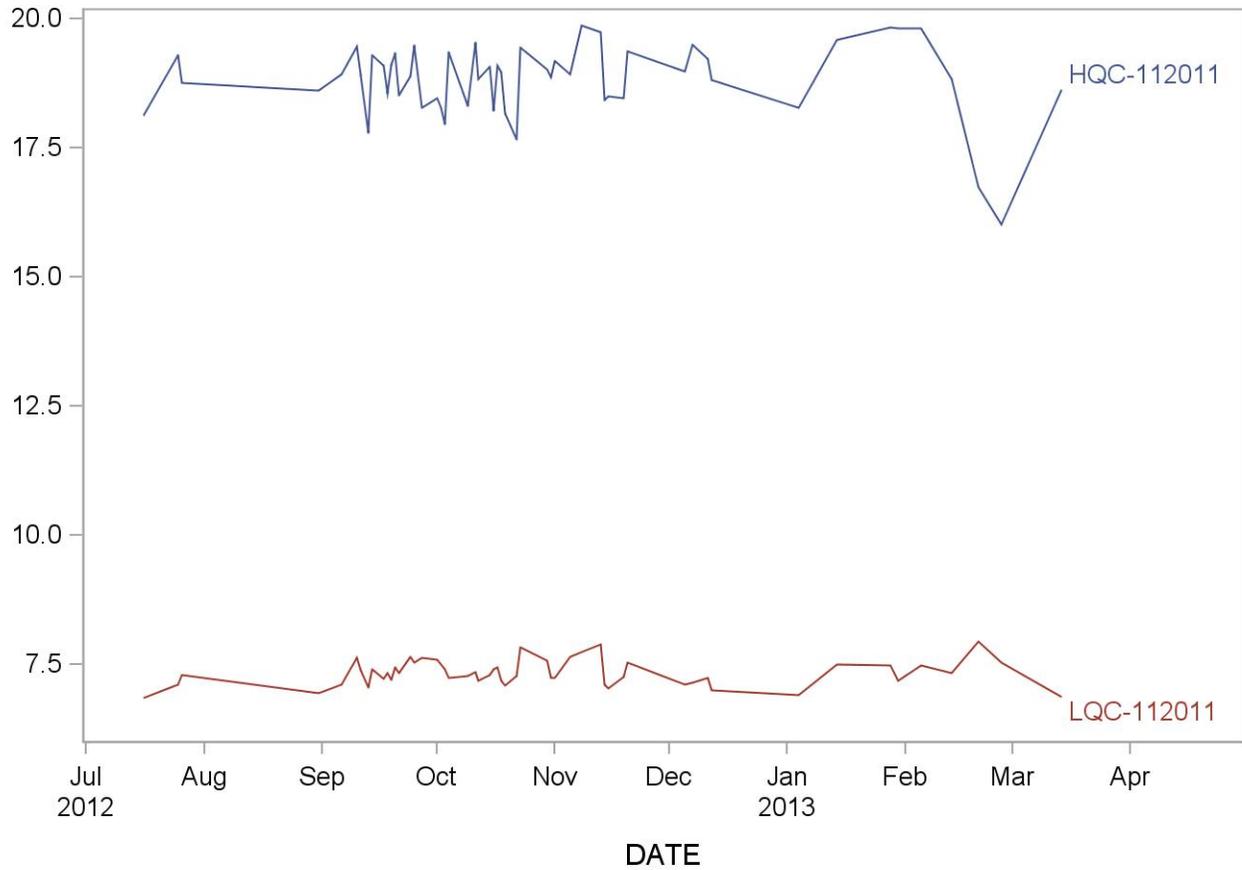
### 2011-2012 Mono(carboxynonyl) phthalate (ng/mL) Quality Control



**Summary Statistics for Mono(carboxyoctyl) phthalate(ng/mL)**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	90	16JUL12	14MAR13	18.823	0.741	3.9
LQC-112011	90	16JUL12	14MAR13	7.314	0.265	3.6

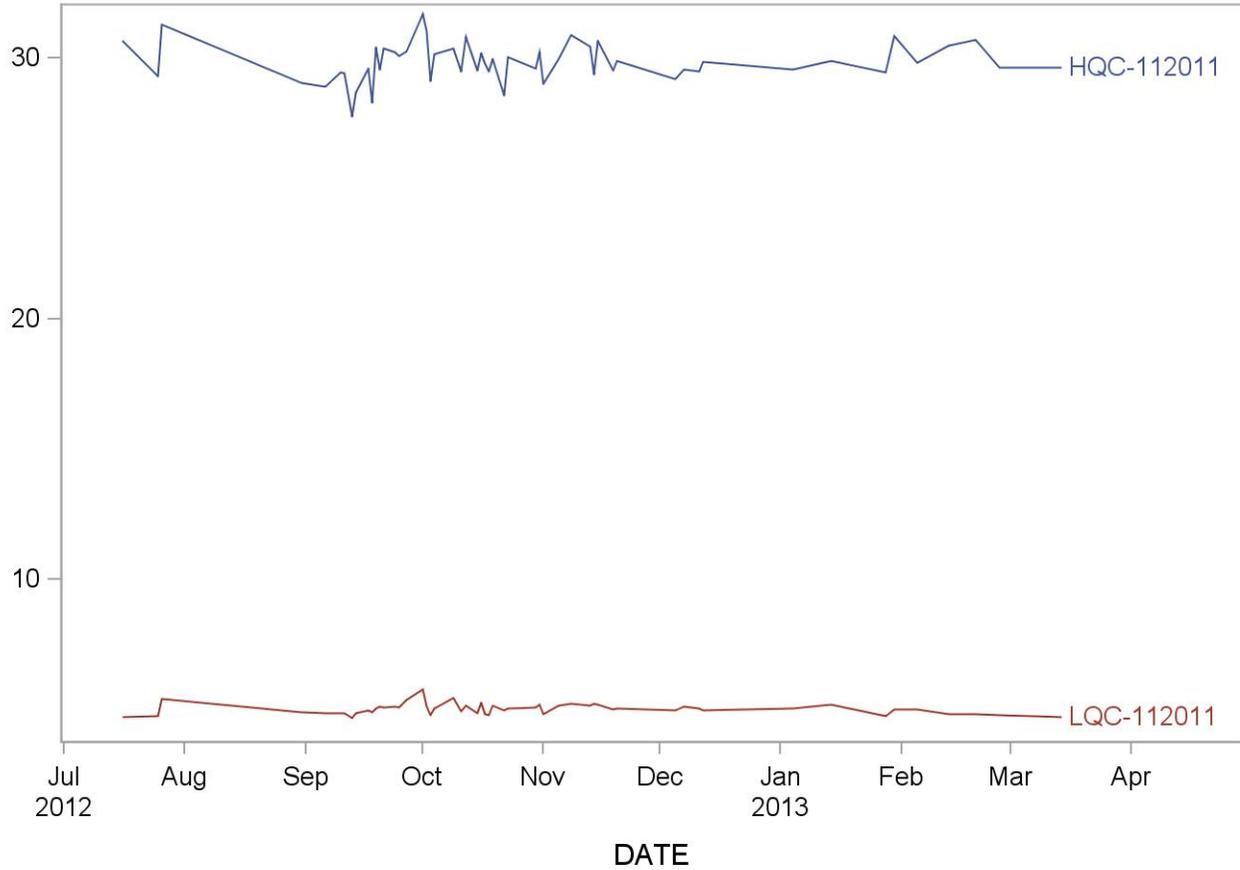
**2011-2012 Mono(carboxyoctyl) phthalate(ng/mL) Quality Control**



## Summary Statistics for Mono-2-ethyl-5-carboxypentyl phthalate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	89	16JUL12	14MAR13	29.798	0.835	2.8
LQC-112011	89	16JUL12	14MAR13	5.010	0.220	4.4

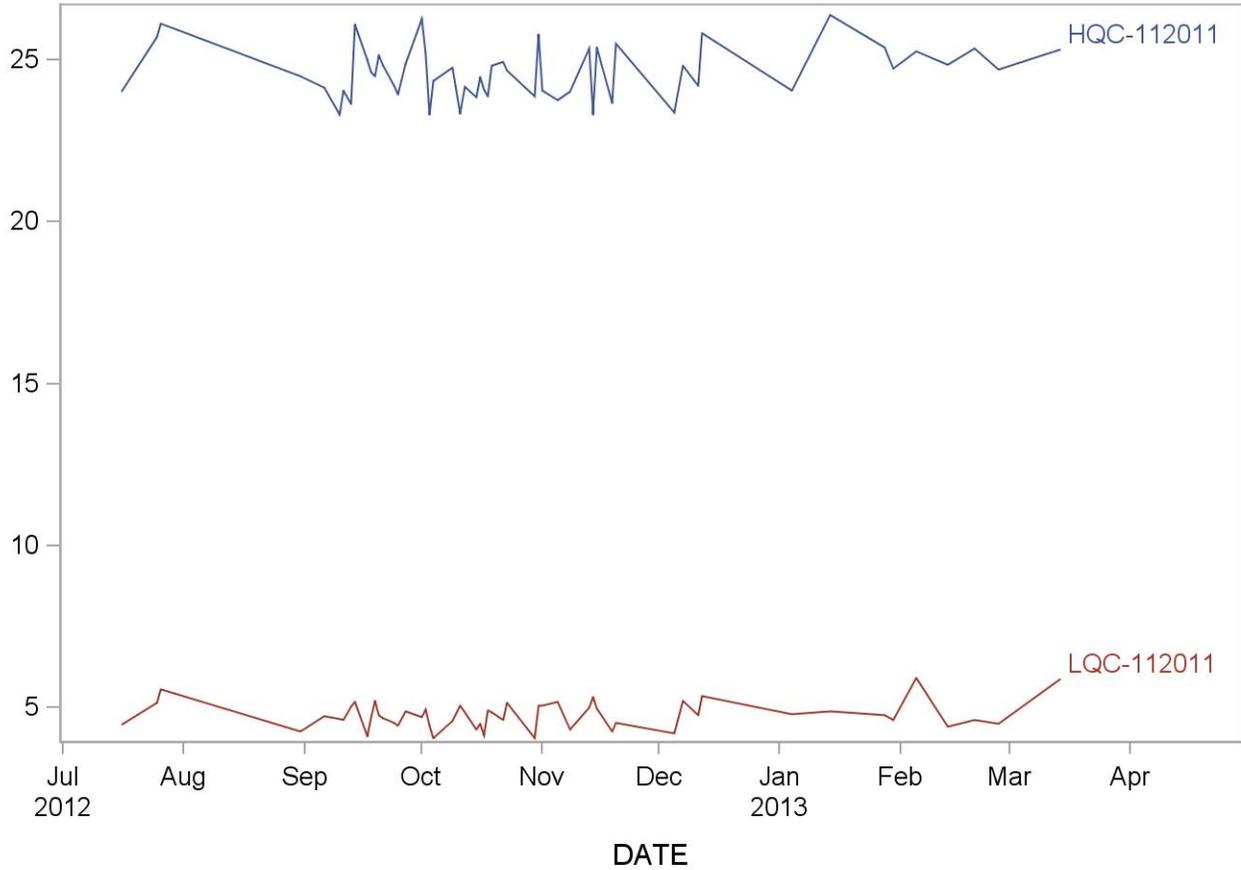
### 2011-2012 Mono-2-ethyl-5-carboxypentyl phthalate Quality Control



Summary Statistics for Mono-n-butyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	90	16JUL12	14MAR13	24.602	0.855	3.5
LQC-112011	90	16JUL12	14MAR13	4.790	0.461	9.6

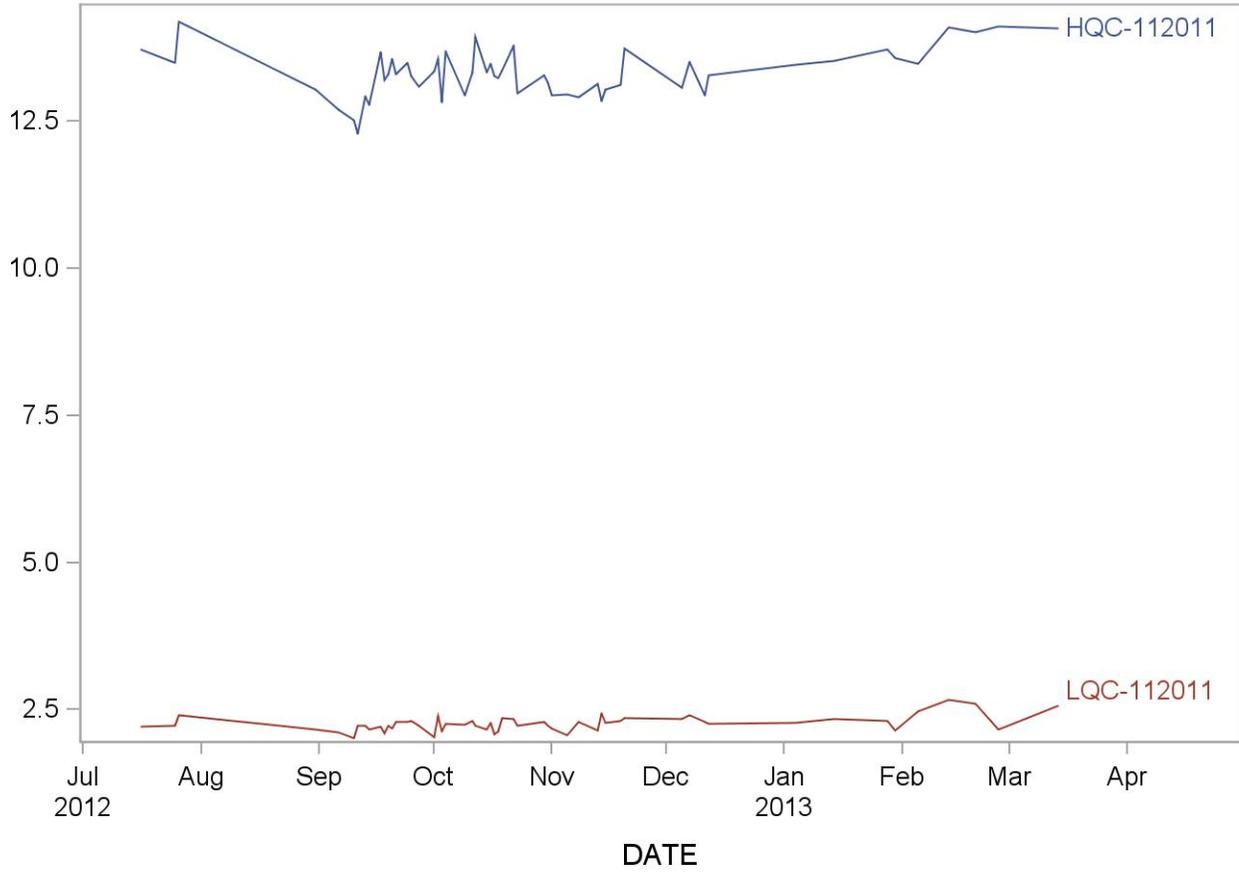
2011-2012 Mono-n-butyl phthalate (ng/mL) Quality Control



Summary Statistics for Mono-(3-carboxypropyl) phthalate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	90	16JUL12	14MAR13	13.310	0.474	3.6
LQC-112011	90	16JUL12	14MAR13	2.268	0.133	5.9

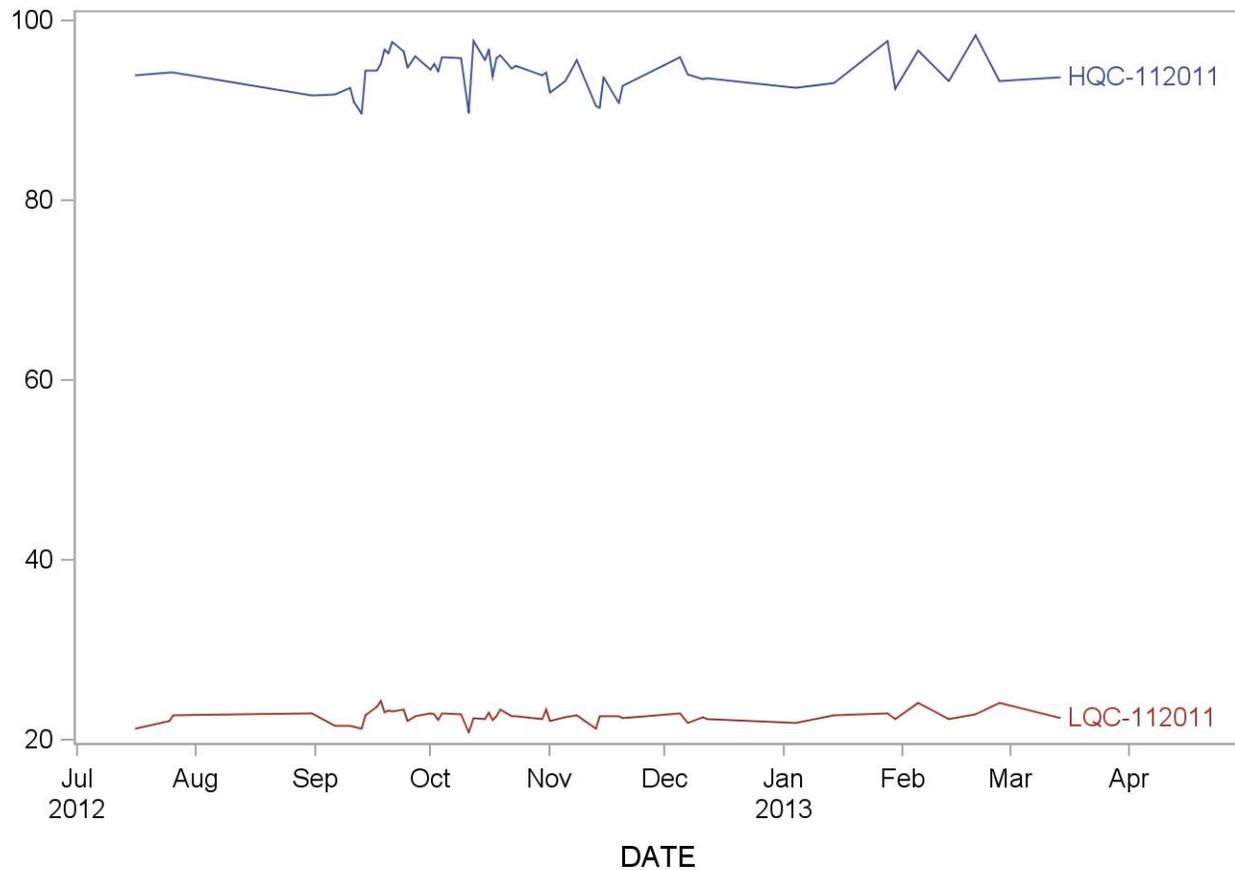
2011-2012 Mono-(3-carboxypropyl) phthalate Quality Control



### Summary Statistics for Mono-ethyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	90	16JUL12	14MAR13	94.283	2.303	2.4
LQC-112011	90	16JUL12	14MAR13	22.607	0.758	3.4

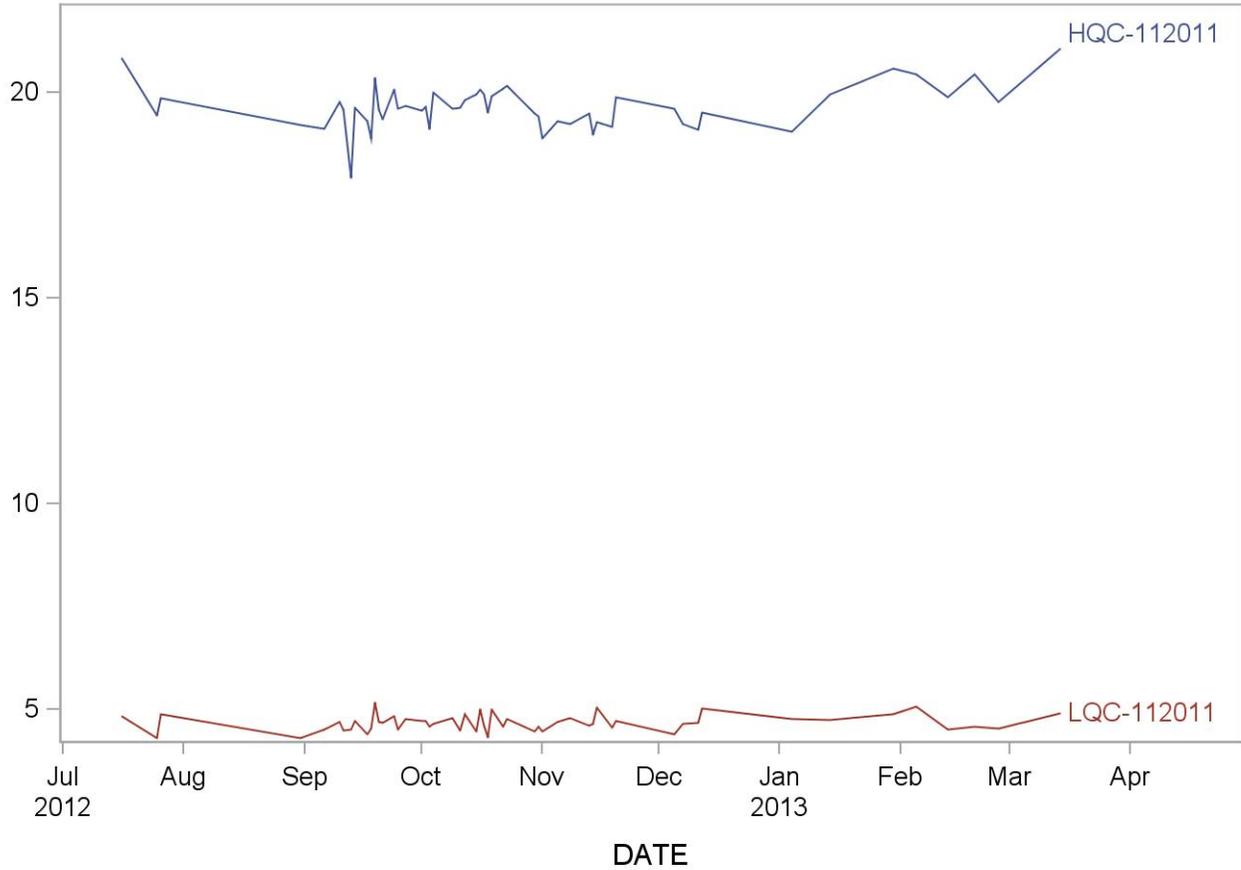
### 2011-2012 Mono-ethyl phthalate (ng/mL) Quality Control



## Summary Statistics for Mono-(2-ethyl-5-hydroxyhexyl) phthalate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	88	16JUL12	14MAR13	19.628	0.598	3.0
LQC-112011	88	16JUL12	14MAR13	4.668	0.241	5.2

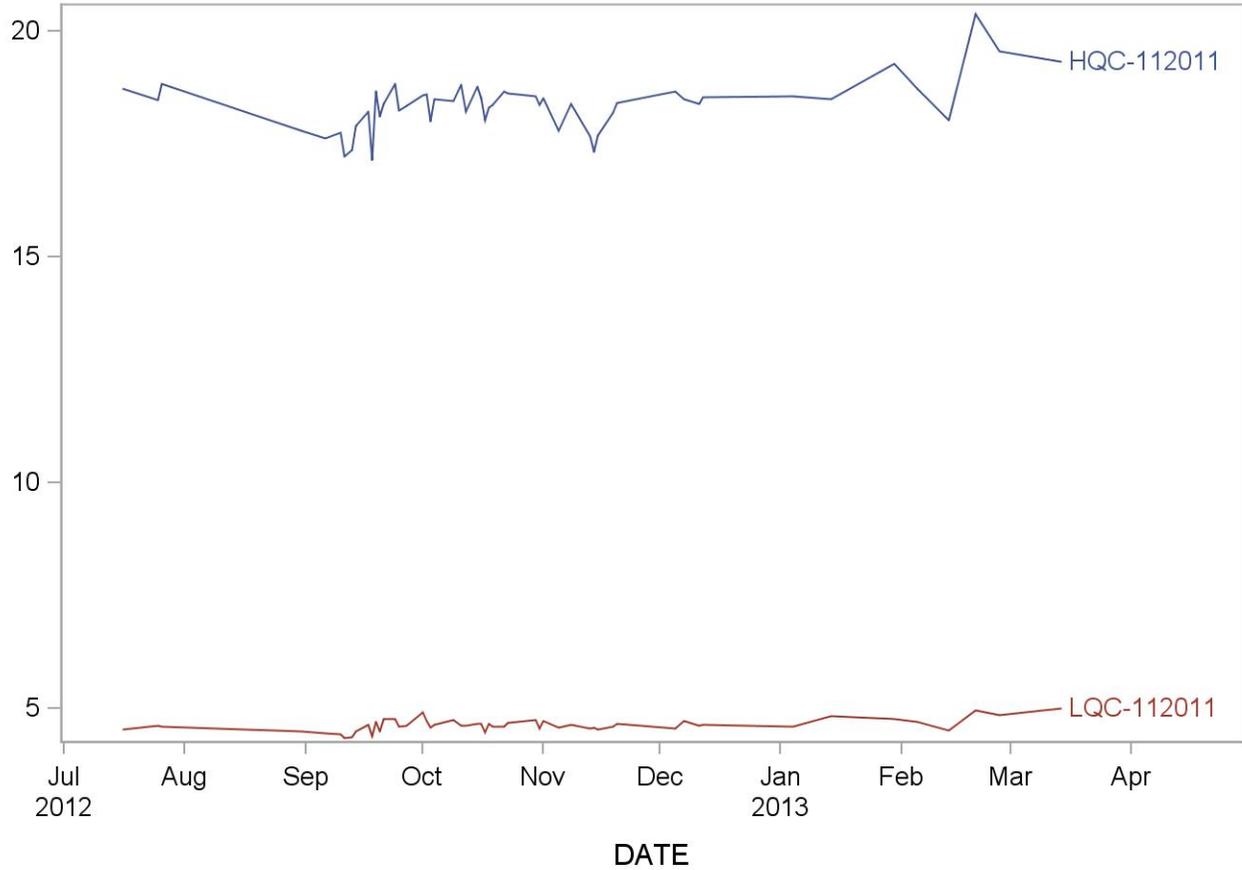
### 2011-2012 Mono-(2-ethyl-5-hydroxyhexyl) phthalate Quality Control



**Summary Statistics for Cyclohexane1,2dicarbox acid,monohyd ison**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	88	16JUL12	14MAR13	18.336	0.563	3.1
LQC-112011	88	16JUL12	14MAR13	4.631	0.140	3.0

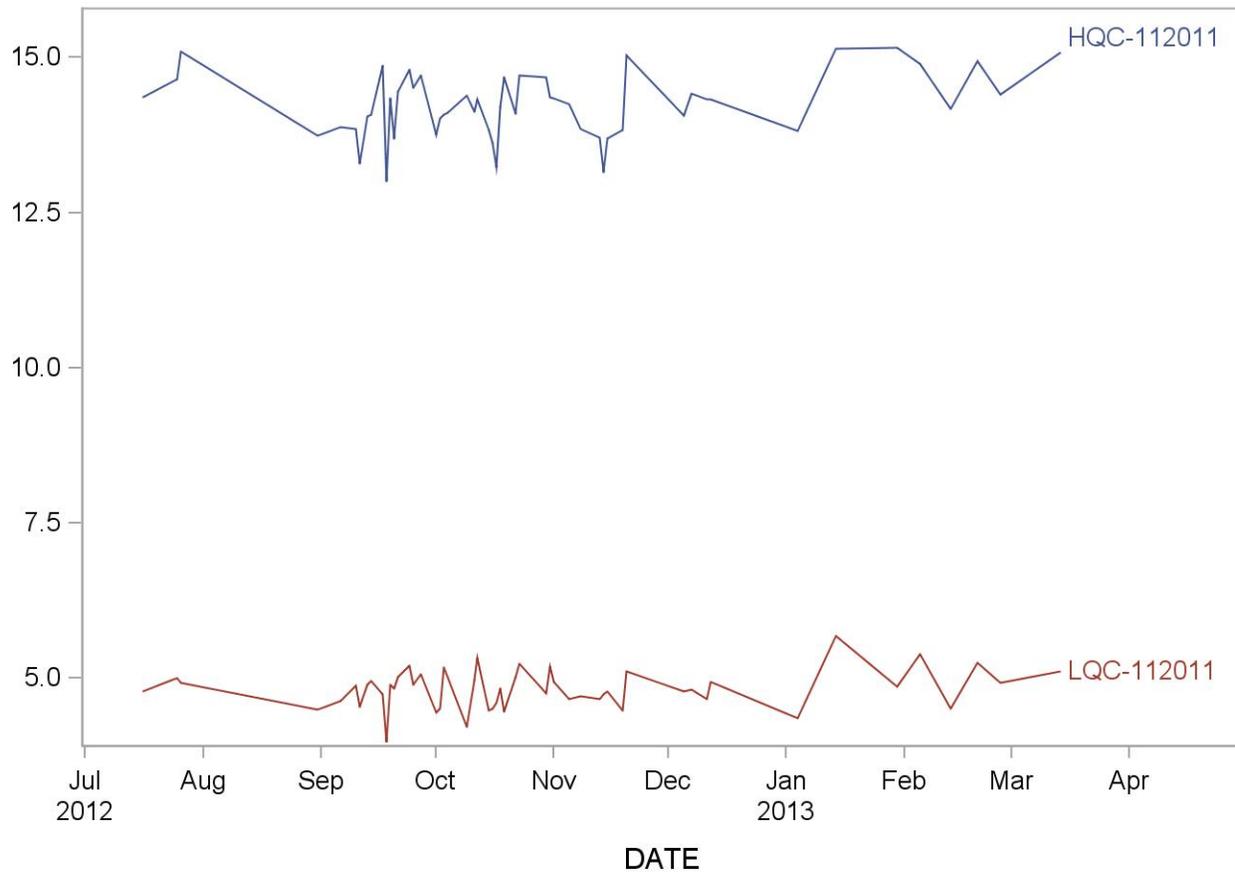
**2011-2012 Cyclohexane1,2dicarbox acid,monohyd ison Quality Control**



### Summary Statistics for Mono-(2-ethyl)-hexyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	88	16JUL12	14MAR13	14.2203	0.5783	4.1
LQC-112011	88	16JUL12	14MAR13	4.8249	0.3297	6.8

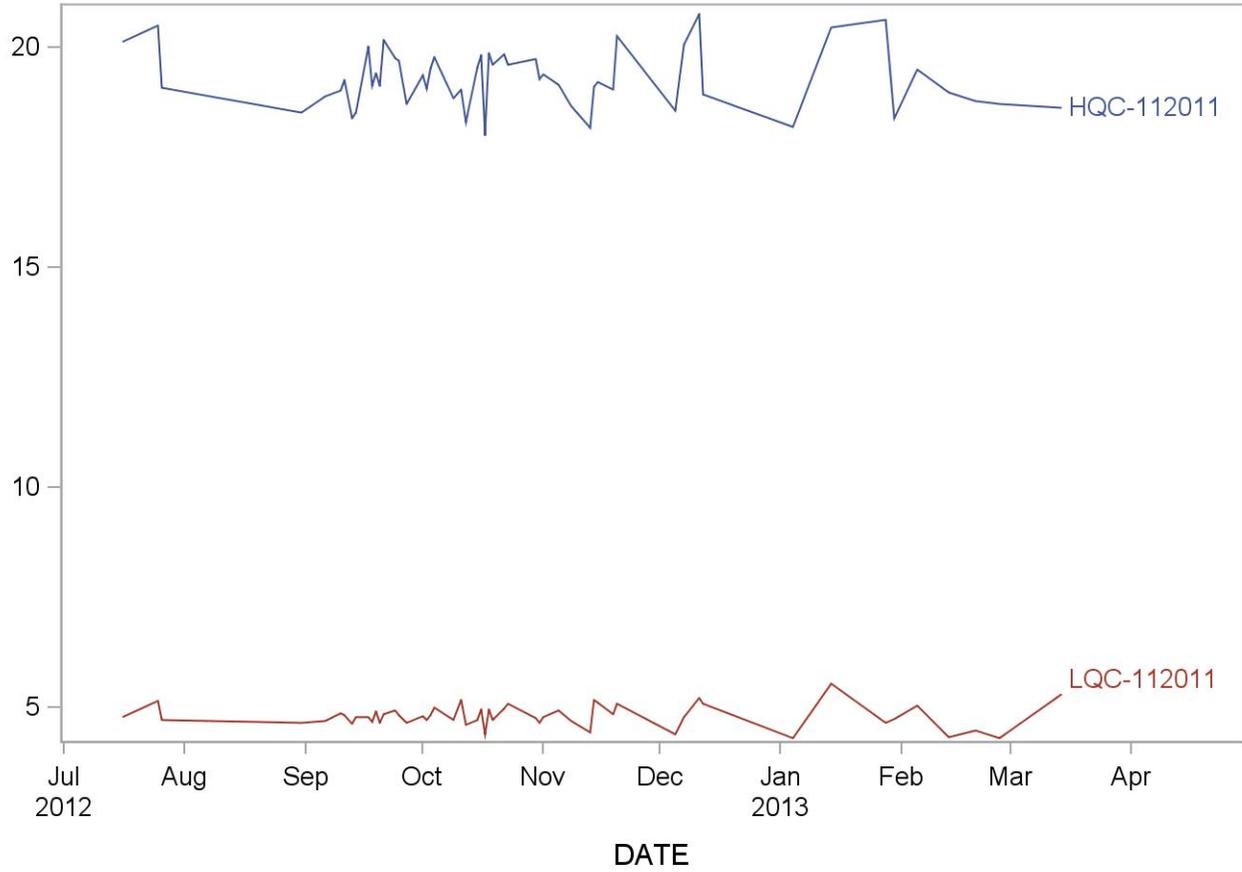
### 2011-2012 Mono-(2-ethyl)-hexyl phthalate (ng/mL) Quality Control



### Summary Statistics for Mono-isobutyl pthalate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	89	16JUL12	14MAR13	19.3504	0.7620	3.9
LQC-112011	89	16JUL12	14MAR13	4.8247	0.2765	5.7

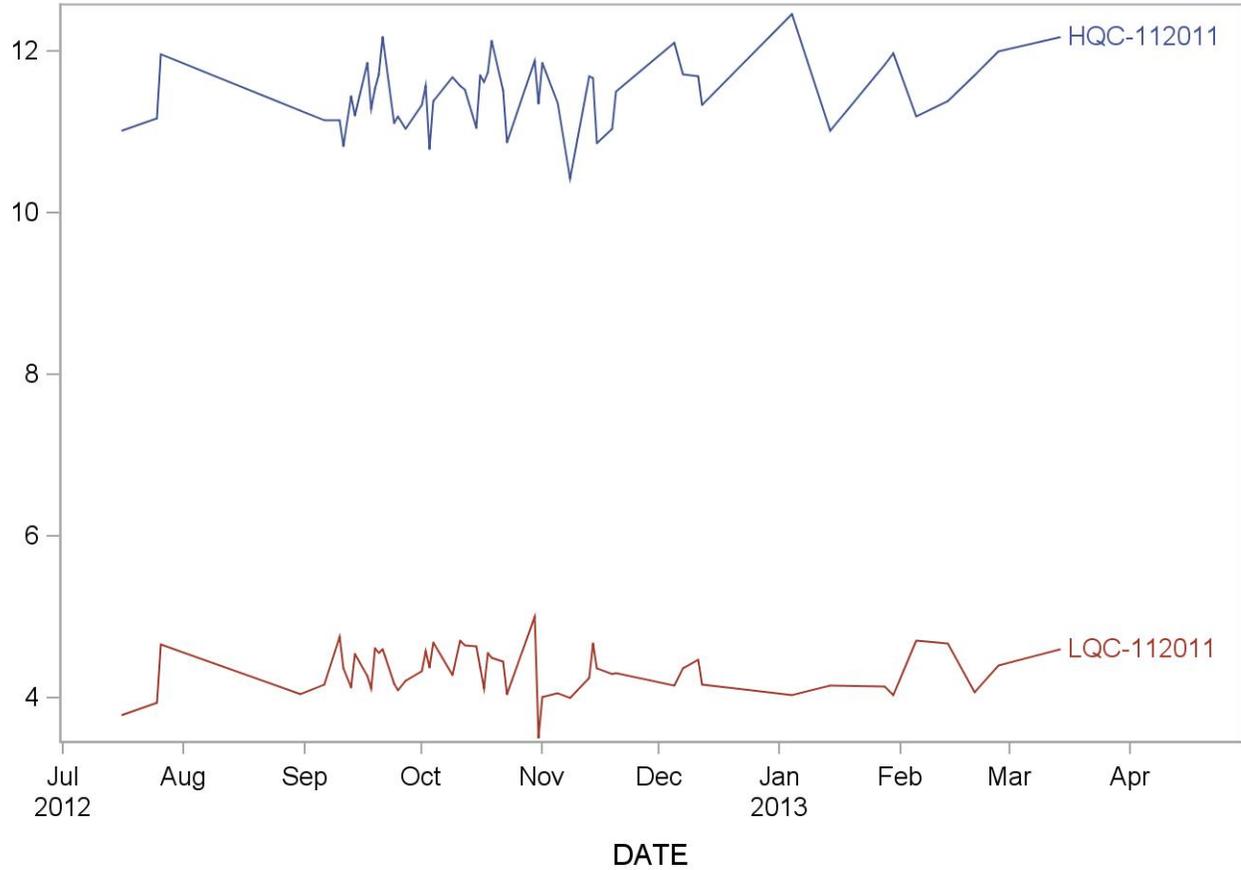
### 2011-2012 Mono-isobutyl pthalate Quality Control



### Summary Statistics for Mono-n-methyl phthalate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	90	16JUL12	14MAR13	11.457	0.448	3.9
LQC-112011	90	16JUL12	14MAR13	4.342	0.324	7.5

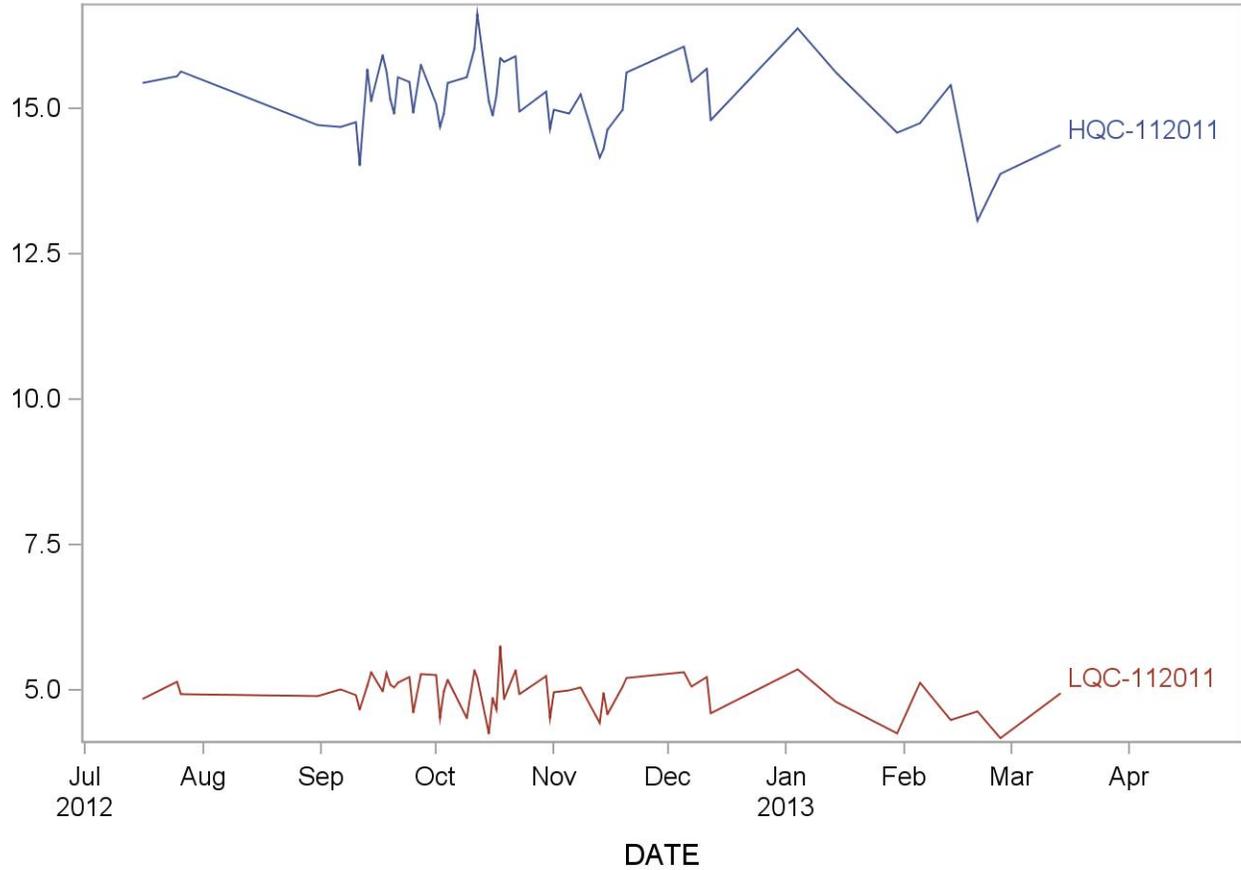
### 2011-2012 Mono-n-methyl phthalate Quality Control



### Summary Statistics for Mono-isobutyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	88	16JUL12	14MAR13	15.189	0.620	4.1
LQC-112011	88	16JUL12	14MAR13	4.960	0.338	6.8

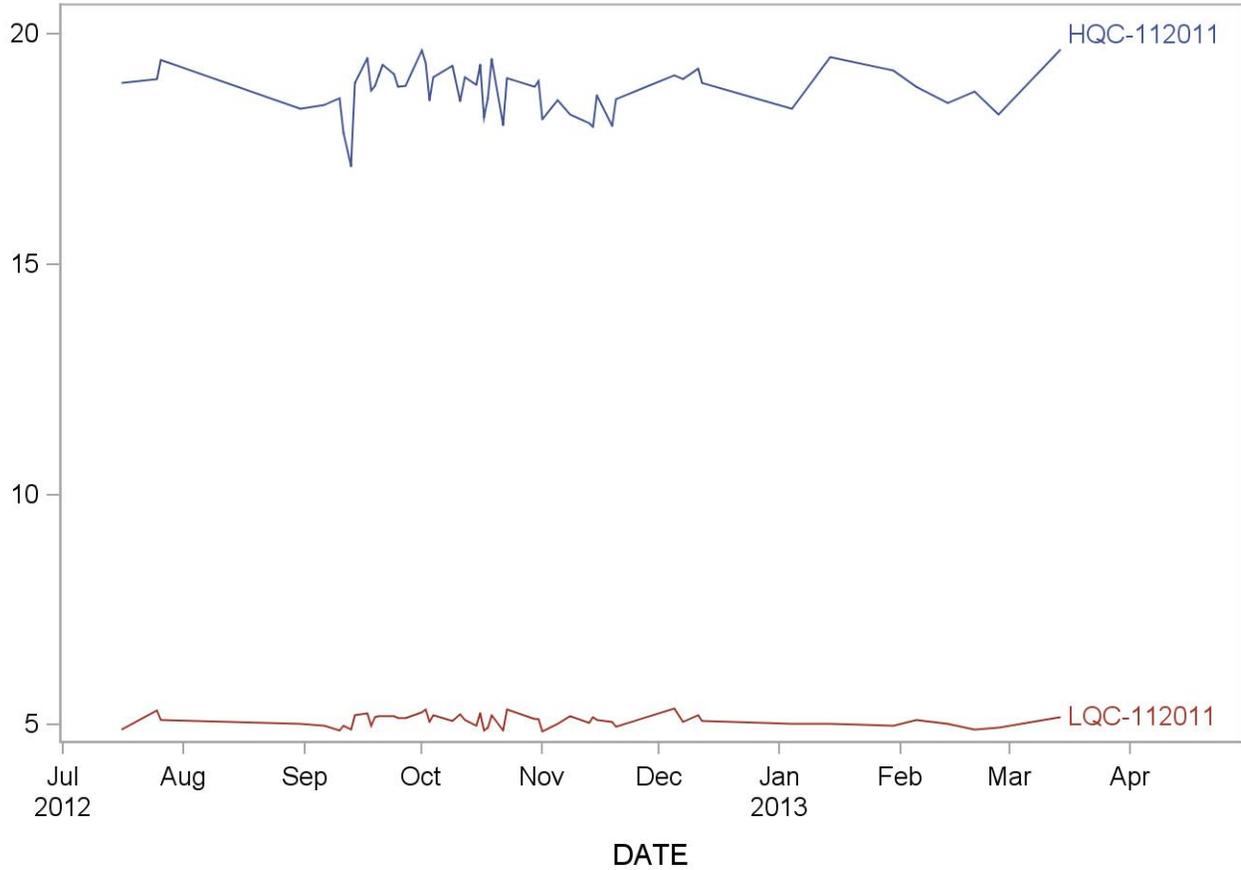
### 2011-2012 Mono-isobutyl phthalate (ng/mL) Quality Control



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

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	88	16JUL12	14MAR13	18.788	0.561	3.0
LQC-112011	88	16JUL12	14MAR13	5.105	0.153	3.0

2011-2012 Mono-(2-ethyl-5-oxohexyl) phthalate Quality Control



### Summary Statistics for Mono-benzyl phthalate (ng/mL )

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-112011	88	16JUL12	14MAR13	22.963	0.750	3.3
LQC-112011	88	16JUL12	14MAR13	4.197	0.173	4.1

### 2011-2012 Mono-benzyl phthalate (ng/mL ) Quality Control

