Laboratory Procedure Manual

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

Matrix: **Urine**

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

Method No: **6306.07**

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

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Important Information for Users

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

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	Label
PHTHTE_I	URXCNP	Mono(carboxyisononyl) phthalate (ng/mL)
	URXCOP	Mono(carboxyisoctyl) phthalate (ng/mL)
	URXECP	Mono-2-ethyl-5-carboxypentyl phthalate (ng/mL)
	URXHIBP	MHIBP phthalate (ng/mL)
	URXMBP	Mono-n-butyl phthalate (ng/mL)
	URXMC1	Mono-(3-carboxypropyl) phthalate (ng/mL)
	URXMCOH	MCOCH phthalate (ng/mL)
	URXMEP	Mono-ethyl phthalate (ng/mL)
	URXMHBP	Mono-3-hydroxy-n-butyl phthalate (ng/mL)
	URXMHH	Mono-(2-ethyl-5-hydroxyhexyl) phthalate (ng/mL)
	URXMHNC	Cyclohexane 1,2-dicarboxylic acid monohydroxy isononyl ester (ng/mL)
	URXMHP	Mono-(2-ethyl)-hexyl phthalate (ng/mL)
	URXMIB	Mono-isobutyl phthalate (ng/mL)
	URXMNP	Mono-isononyl phthalate (ng/mL)
	URXMOH	Mono-(2-ethyl-5-oxohexyl) phthalate (ng/mL)
	URXMZP	Mono-benzyl phthalate (ng/mL)
SSMHHT_I	SSMHHT	Mono(2-ethyl-5-hydroxyhexyl) terephthalate (ng/mL)
	SSECPT	Mono(2-ethyl-5-carboxypentyl) terephthalate ((ng/mL)
	SSMONP	Mono-oxo-isononyl 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) 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) Mono-2-methyl-2-hydroxypropyl phthalate (MHiBP)
Di-n-butyl phthalate (DBP)	Mono-n-butyl phthalate (MBP) Mono-3-hydroxybutyl phthalate (MHBP)
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)
Di-isononyl phthalate (DNP)*	*Mono-isononyl phthalate (MNP) *Monocarboxyoctyl phthalate (MCOP) *Mono-oxo-isononyl phthalate (MONP)
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) *Cyclohexane-1,2-dicarboxylic acid-mono(carboxyoctyl) ester (MCOCH)
Di(2-ethylhexyl) terephthalate (DEHTP)	Mono(2-ethyl-5-hydroxyhexyl) terephthalate (MEHHTP) Mono(2-ethyl-5-carboxypentyl) terephthalate (MECPTP)

*isomeric mixtures

b. Clinical Relevance

Phthalates and terephthalates, are two groups 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, terephthalates and DINCH [5;6]. Phthalates are rapidly metabolized in humans to their respective monoesters, which depending on the phthalate can be further metabolized to their oxidative products [7;8]. Similarly, DINCH also forms oxidative metabolites in humans [1;9]. All of these metabolites may be glucuronidated, and excreted in the urine and feces [10-13]. Some of these metabolites can cause reproductive and developmental toxicities in animals [14-17], 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 https://www.msdsonline.com/msds-search. 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. 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 either 10% bleach, Lysol I.C., or Sporicidin. Contaminated analytical glassware is treated with bleach or Lysol I.C, 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 is processed by the Thermo Scientific Xcalibur software or Ascent Indigo Biosystems automated integration 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 or Ascent Indigo integration 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 [18]. 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 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.

* HPLC grade Acetonitrile HPLC grade Methanol, Acetic Acid (glacial) and HPLC grade water are purchased from Fisher Scientific (Pittsburgh, PA)
* Ammonium acetate is purchased from Sigma Aldrich Laboratories, Inc (St. Louis, MO)
* β-Glucuronidase (*Escherichia coli*-K12) is purchased from Sigma Aldrich Laboratories, Inc (St. Louis, MO).

1.0 M Ammonium acetate buffer (pH 6.5). To make 500 mL, 38.6 g of ammonium acetate is dissolved in ~496 mL deionized 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 deionized 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. 30 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.

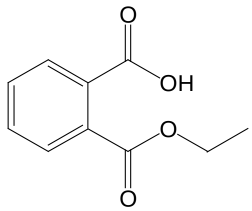
b. Analytical Standards

(1) Source

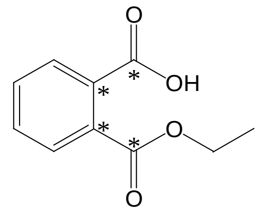
Metabolites of phthalates native and labeled standards are obtained from: Cambridge Isotope Laboratories Inc (Andover, MA), Los Alamos National Laboratory (Los Alamos, NM), SigmaAldrich, Professor Jurgen Angerer (Germany), Dr. Holger Koch (Germany), IDM (Germany) and Cansyn (Toronto, Canada). Metabolites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

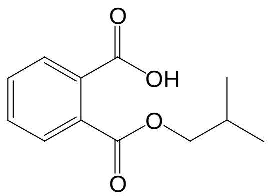
(2) Native and labeled metabolites used in standards



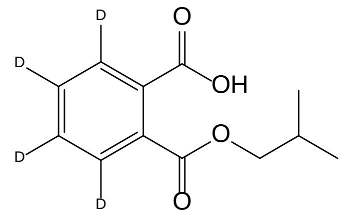
MEP
monoethyl phthalate



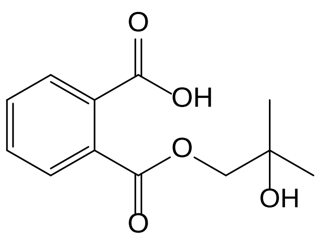
MEP*
monoethyl phthalate-$^{13}C_4$



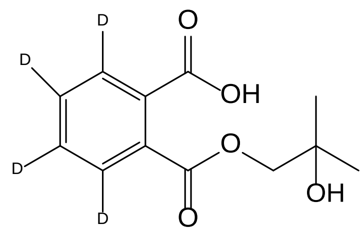
MiBP
mono-isobutyl phthalate



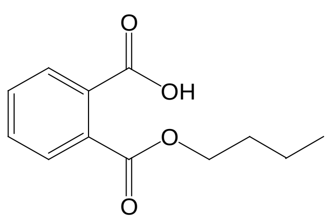
MiBP*
mono-isobutyl phthalate-D_4



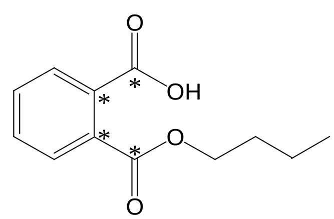
MHiBP
mono-2-hydroxy-isobutyl phthalate



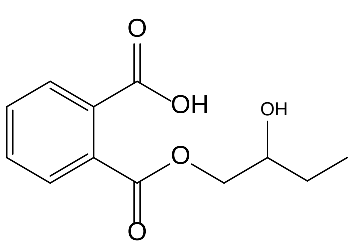
MHiBP*
mono-2-hydroxy-isobutyl phthalate-D_4



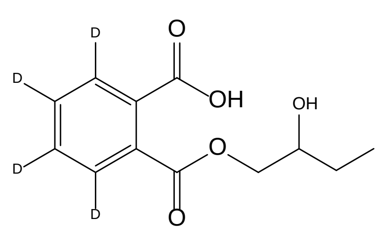
MBP
mono-n-butyl phthalate



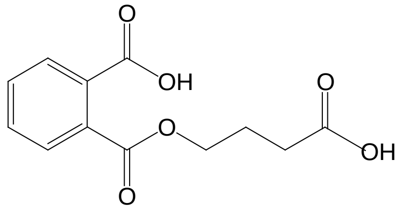
MBP*
mono-n-butyl phthalate-$^{13}C_4$



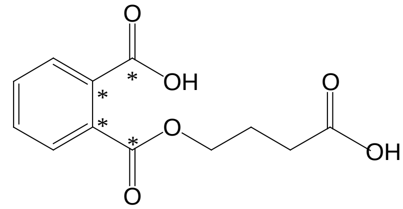
MHBP
mono-2-hydroxy-n-butyl phthalate



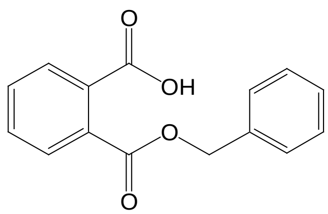
MHBP*
mono-2-hydroxy-n-butyl phthalate-D_4



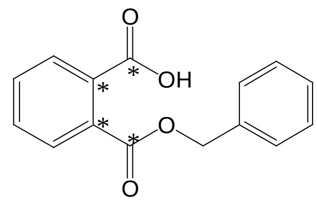
MCPP
mono(3-carboxypropyl) phthalate



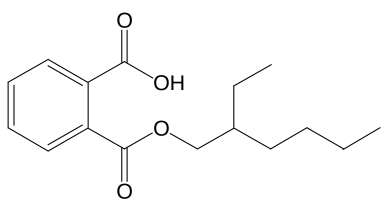
MCPP*
mono(3-carboxypropyl) phthalate-$^{13}C_4$



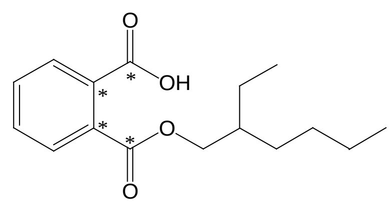
MBzP
monobenzyl phthalate



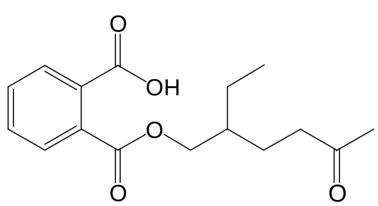
MBzP*
monobenzyl phthalate-$^{13}C_4$



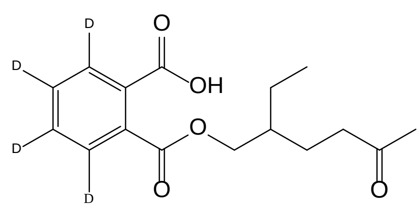
MEHP
mono(2-ethylhexyl) phthalate



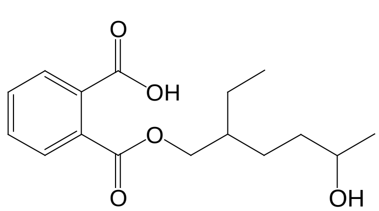
MEHP*
mono(2-ethylhexyl) phthalate-$^{13}C_4$

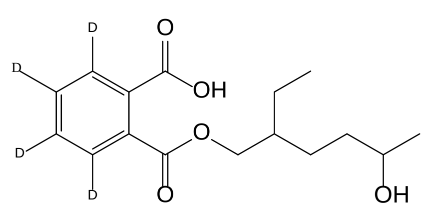


MEOHP
mono(2-ethyl-5-oxohexyl) phthalate



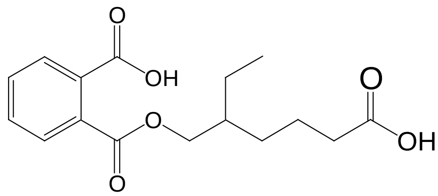
MEOHP*
mono(2-ethyl-5-oxohexyl) phthalate-D_4

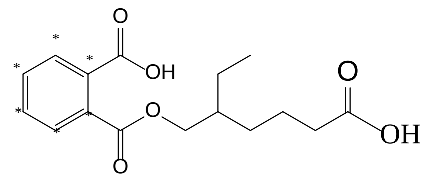




MEHHP
mono(2-ethyl-5-hydroxyhexyl) phthalate

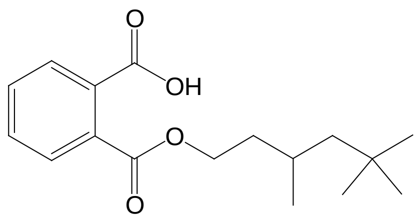
MEHHP*
mono(2-ethyl-5-hydroxyhexyl) phthalate-D_4

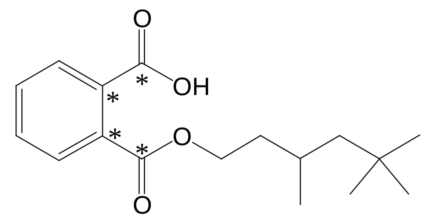




MECPP
mono(2-ethyl-5-carboxypentyl) phthalate

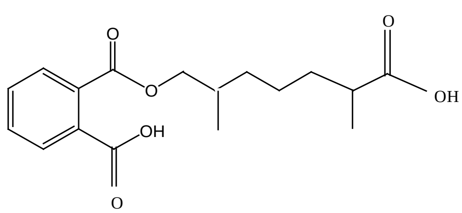
MECPP*
mono(2-ethyl-5-carboxypentyl) phthalate-$^{13}C_6$

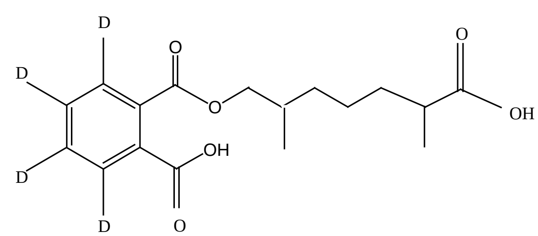




MNP
mono(3,5,5-trimethyl-1-hexyl) phthalate
(mono-isononyl phthalate)

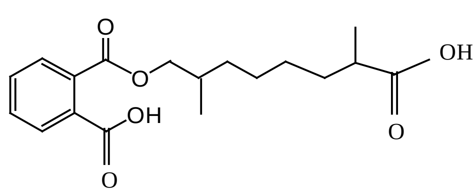
MNP*
mono(3,5,5-trimethyl-1-hexyl) phthalate-$^{13}C_4$
(mono-isononyl phthalate-$^{13}C_4$)

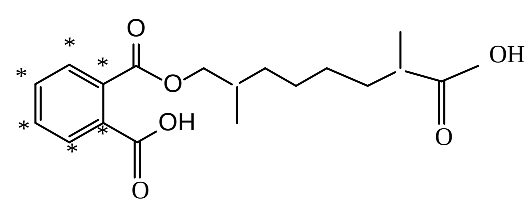




MCOP
mono(2,6-methyl-6-carboxyhexyl) phthalate
(mono-carboxyisooctyl phthalate)

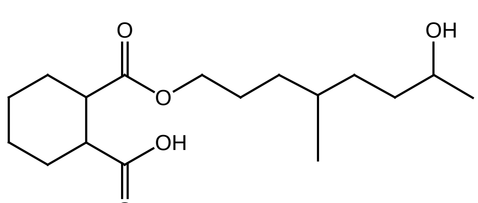
MCOP*
mono(2,6-methyl-6-carboxyhexyl) phthalate-D_4
(mono-carboxyisooctyl phthalate-D_4)

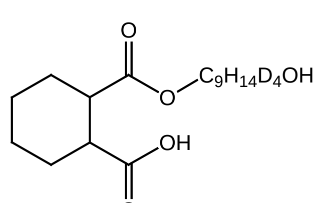




MCNP
mono(2,7-methyl-7-carboxyheptyl)phthalate
(mono-carboxy-isononyl phthalate)

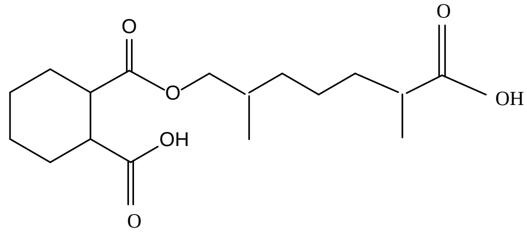
MCNP*
mono(2,7-methyl-7-carboxyheptyl)phthalate-$^{13}C_6$
(mono-carboxy-isononyl phthalate-$^{13}C_6$)

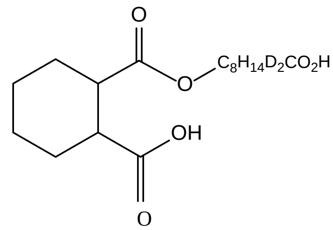




MHNCH
cyclohexane-1,2-dicarboxylic acid mono(cis-hydroxy-isononyl) ester

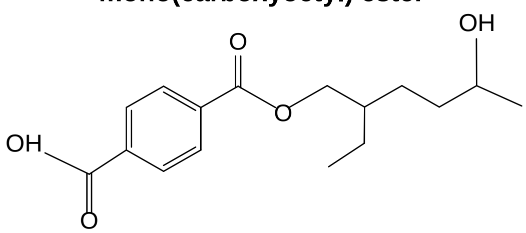
MHNCH*
cyclohexane-1,2-dicarboxylic acid mono(cis-hydroxy-isononyl) ester-D_4

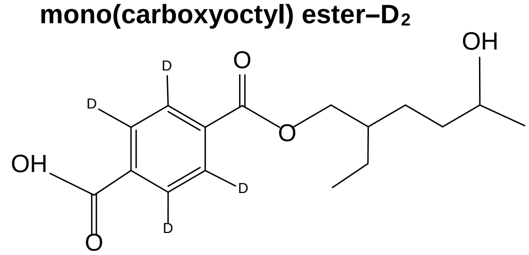




MCOCH
cyclohexane-1,2-dicarboxylic acid mono(carboxyoctyl) ester

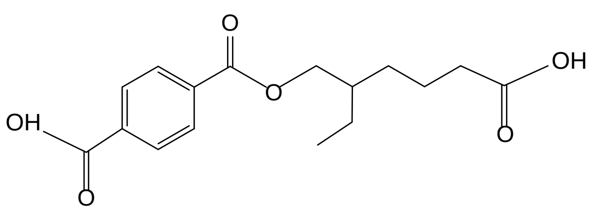
MCOCH*
cyclohexane-1,2-dicarboxylic acid mono(carboxyoctyl) ester–D_2

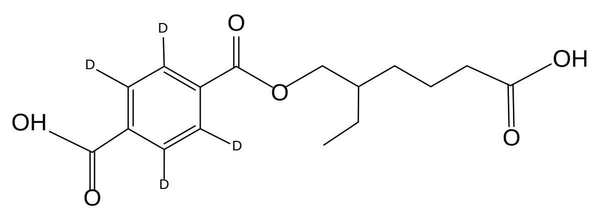




MEHHTP
mono(2-ethyl-5-hydroxyhexyl) terephthalate

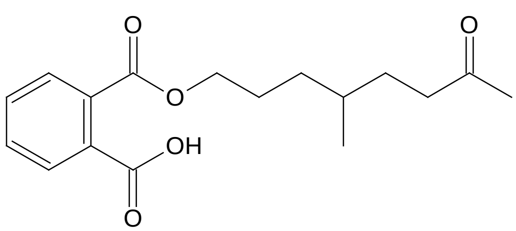
MEHHTP*
mono(2- ethyl-5-hydroxyhexyl) terephthalate-D_4

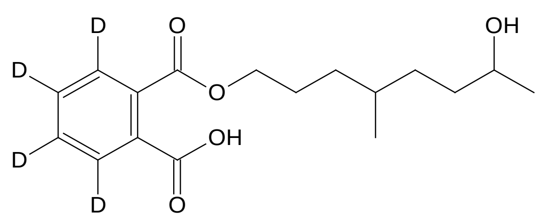




MECPTP
mono(2-ethyl-5-carboxypentyl) terephthalate

MECPTP*
mono(2-ethyl-5-carboxypentyl) terephthalate-D_4





MONP
mono(4-methyl-7-oxooctyl)phthalate

MONP*
mono(4-methyl-7-oxooctyl)phthalate-D_4

(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 or below -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 or below -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 deionized water. An intermediate 4-methyl umbelliferyl glucuronide stock solution is prepared by diluting the stock solution with deionized water. The stock solution and the intermediate stock solution are stored at or below -40 °C in a Teflon-capped glass bottle. The spiking solution, made in deionized water by diluting the intermediate stock solution, is then refrigerated.

(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 stock standards are kept in amber Boston round bottles with Teflon-lined screw caps at or below -40°C and remade as necessary. Working standard solutions are kept in 2 mL cryovials at or below -40 °C and remade as needed from the stock solutions.

(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 ^{o}C) 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 HighResolution RP-18 endcapped 5-4.6 mm Guard Cartridge, 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, 200 µL, and 20 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 or Accela autosampler
(8) Fisher Scientific* Orion star A111, or equivalent
(9) ThermoFisher Scientific Surveyor autosampler
(10) TECAN EVO automated sample handling system

(8) Analytical instrumentation

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

(9) Software

1) ThermoFisher Scientific Xcalibur software
2) Ascent Indigo Biosystems automated integration software

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, at least 2 Reagent Blanks (RB), at least 4 Quality Control (QC) materials of low (QCL; n$\geq$2) and high (QCH; n$\geq$2) 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 or a re-circulator.
(3) The samples are mixed well by vortexing.
(4) Each analytical sequence typically consists of two analytical runs.
(5) The β-glucuronidase enzyme solution is prepared fresh just prior to addition to samples.

b. Sample preparation

(1) 100 µL of calibration standard, urine, deionized 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 pre-slit 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 deionized water.
(7) The autosampler tray temperature is set to less than 5ºC after preparation of the whole set.
(8) The autosampler tray is moved to the HPLC/MS analytical system for analysis.

Alternatively, for automatic sample preparation by TECAN EVO liquid handling system:

(1) 100 µL of calibration standard, urine, deionized water (for the reagent blank), QCH and QCL is transferred into properly labeled pre-capped autosampler vial (1.5 mL) pre-placed on autosampler try by TECAN automated liquid handling system.
(2) 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.
(3) Autosampler tray is placed in an incubator at 37ºC for minimum of 120 min.
(4) After incubation 150 µL of glacial acetic acid in 5% acetonitrile in deionized water (1:3) is added by TECAN liquid handling system.
(5) 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 Accela liquid chromatograph coupled with a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer, equipped with an ESI interface. All three systems and the 10 port switching valve are controlled by the Xcalibur Software. The autosampler tray is set at 5ºC. 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 HighResolution 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 to the 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 needle 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;19].

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

	SPE pump				HPLC pump			Switching valve	
Time (min)	A (%)	B (%)	Flow (µL/min)		A (%)	B (%)	Flow (µL/min)	SPE	HPLC
0	100	0	300		77	23	300	SPE/Waste	HPLC/MS
0.4	100	0	1800					SPE/Waste	HPLC/MS
0.5	90	10	1800					SPE/Waste	HPLC/MS
1.1	90	10	1800		77	23	300	Waste	SPE/HPLC
1.2	100	0	200					Waste	SPE/HPLC
3					75	25	300	Waste	SPE/HPLC
3.5	100	0	200					SPE/Waste	HPLC/MS
4	100	0	1500					SPE/Waste	HPLC/MS
5					75	25	300	SPE/Waste	HPLC/MS
8.1	100	0	1500					SPE/Waste	HPLC/MS
8.2	0	100	1500					SPE/Waste	HPLC/MS
10					67	33	350	SPE/Waste	HPLC/MS
10.2	0	100	1500					SPE/Waste	HPLC/MS
10.5	0	100	200					SPE/Waste	HPLC/MS
15	0	100	500					SPE/Waste	HPLC/MS
17					70	30	325	SPE/Waste	HPLC/MS
19.85					66	34	350	SPE/Waste	HPLC/MS
21.1	0	100	200		60	40	350	SPE/Waste	HPLC/MS
23.0	100	0	300					SPE/Waste	HPLC/MS
23.1					45	55	350	SPE/Waste	HPLC/MS
25.1					20	80	350	SPE/Waste	HPLC/MS
25.2					0	100	350	SPE/Waste	HPLC/MS
25.6					0	100	400	SPE/Waste	HPLC/MS
26.0					0	100	400	SPE/Waste	HPLC/MS
26.7					77	23	350	SPE/Waste	HPLC/MS
27	100	0	300		77	23	350	SPE/Waste	HPLC/MS

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

During the analysis, the instrument is set in the multiple reaction monitoring mode to monitor product ion combinations specific to the eluting analytes. 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	
MEHHTP	293/121	22
D_4-MEHHTP	297/125	
MECPTP	307/165	20
D_4-MECPTP	311/169	
MONP	305/121	20
D_4-MONP	309/125	

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	0.05-1000
MHiBP	0.4	0.025-250
MBP	0.4	0. 1-1000
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	0.05-1000
MCNP	0.2	0.05-500
MHNCH	0.4	0.05-500
MCOCH	0.5	0.025-250
MEHHTP	0.4	0.025-250
MECPTP	0.2	0.025-250
MONP	0.4	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
MEHHTP	1.5	1.4
MECPTP	1.5	1.5
MONP	5.0	5.1

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			
MEP	5.4	81.2	7.3	20.7
MCPP	6.2	11.2	11.9	2.0
MiBP	9.2	15.6	11.2	8.1
MBP	7.8	20.1	10	9.5
MHBP	6.3	10.5	99.0	2.2
MHiBP	6.1	10.4	12.2	2.4
MBzP	5.4	12.5	7.2	3.1
MEHP	9.1	8.1	18.9	2.1
MNP	8.0	10.2	9.4	2.0
MEOHP	5.4	17.0	8.3	4.6
MEHHP	5.5	20.8	8.1	4.8
MECPP	5.7	24.4	6.8	9.3
MCNP	5.3	17.9	8.2	4.2
MCOP	5.0	35.8	5.9	8.5
MCOCH	4.7	10.1	9.6	2.3
MEHHTP	7.0	8.2	11.1	2.6
MECPTP	6.0	12.6	8.4	5.5
MHNCH	5.5	8.3	8.5	2.0
MONP	5.8	10.2	9.4	2.3

10. Quality control and quality assessment

a. Individual sample quality control procedure

1) For each analyte, the relative retention time (difference in $RT_{analyte}$ and RT_{IST}) of standards, unknowns, and QCs are monitored.

2) If the total ion count (TIC) of the internal standard of an analyte falls below $1x10^3$, the sample is repeated.

3) If the calculated concentration of the reagent blank (RB) is more than three times the LOD, the run is monitored for potential contamination. If no acceptable reasoning present (eg. carry over) the run is repeated.

4) 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) If the analyte concentration in an unknown sample is more than the highest calibration standard, the subsequent sample is re-extracted.

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 [20].

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 +/- $4S_i$
   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.)

<u>Two QC pools *per run* with two or more QC results *per pool*</u>

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 [21]. 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 [18].

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 [22].

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 at or below -40 ^{o}C prior to analysis. Frozen samples are allowed to thaw completely at room temperature or in a 25 °C sonicating water bath prior to aliquoting. Prepared samples are kept at or below -20 ^{o}C until analysis and kept at 5 °C during SPE-HPLC-MS/MS analysis.

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

The current analytical method utilizes a ThermoScientific Accela liquid chromatograph coupled with a ThermoScientific TSQ Vantage Triple Quadrupole mass spectrometer. Alternative extraction techniques exist [23;24]., If the analytical system fails, prepared samples are stored frozen in capped autosampler vials until the analytical system is restored or 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.

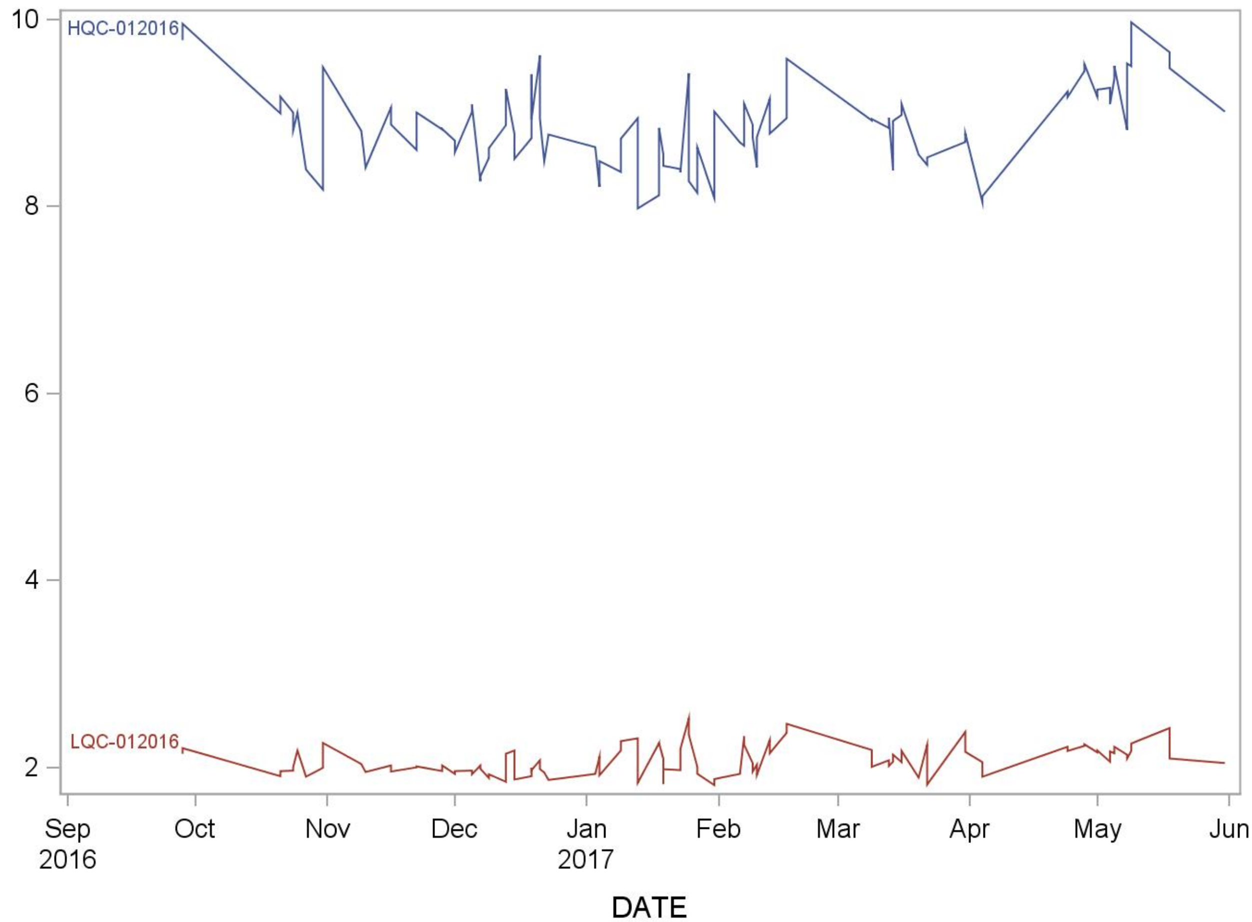
Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

19. Summary statistics and QC graphs:

Please see following pages.

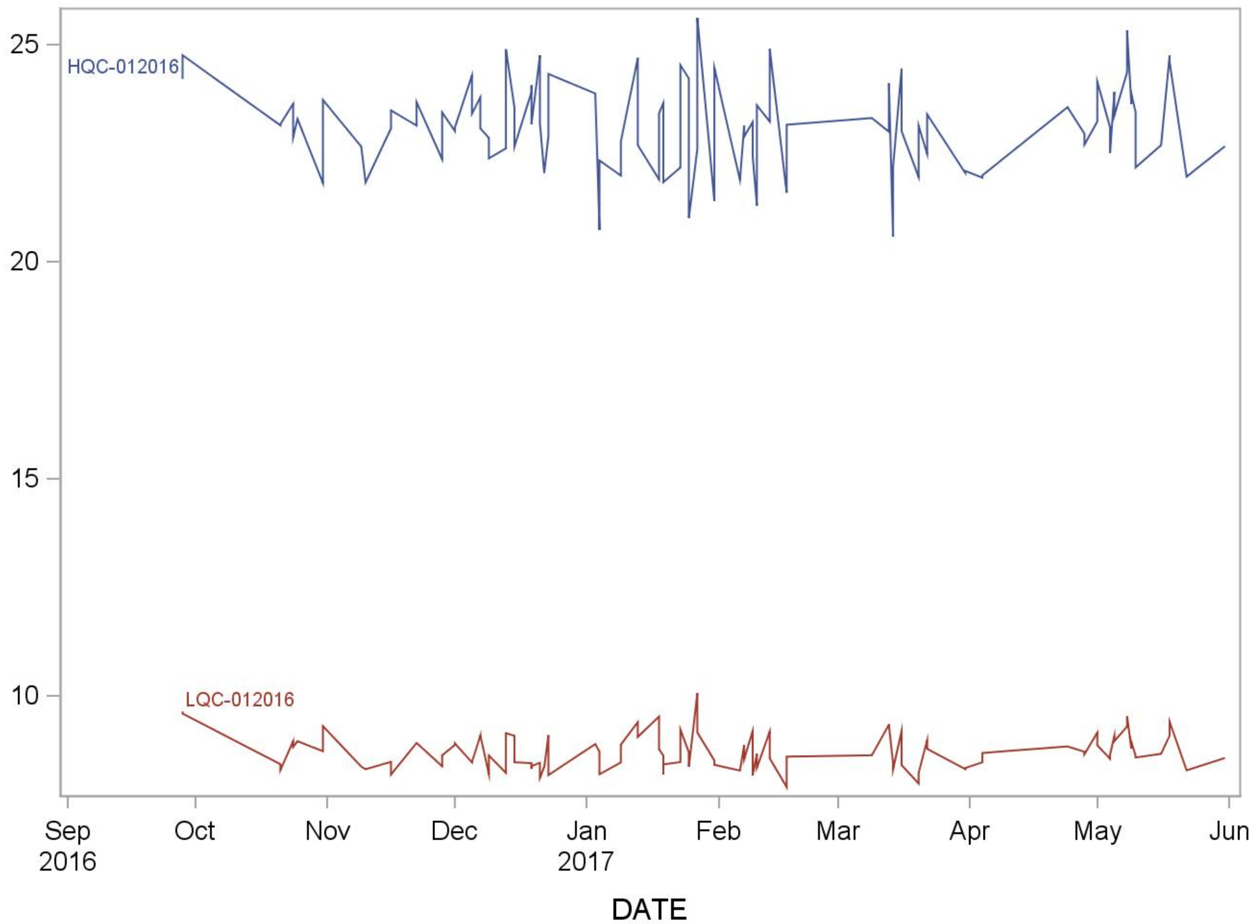
Summary Statistics and QC Chart for MCOCH phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	101	28SEP16	31MAY17	8.8552	0.4367	4.9
LQC-012016	101	28SEP16	31MAY17	2.0716	0.1572	7.6



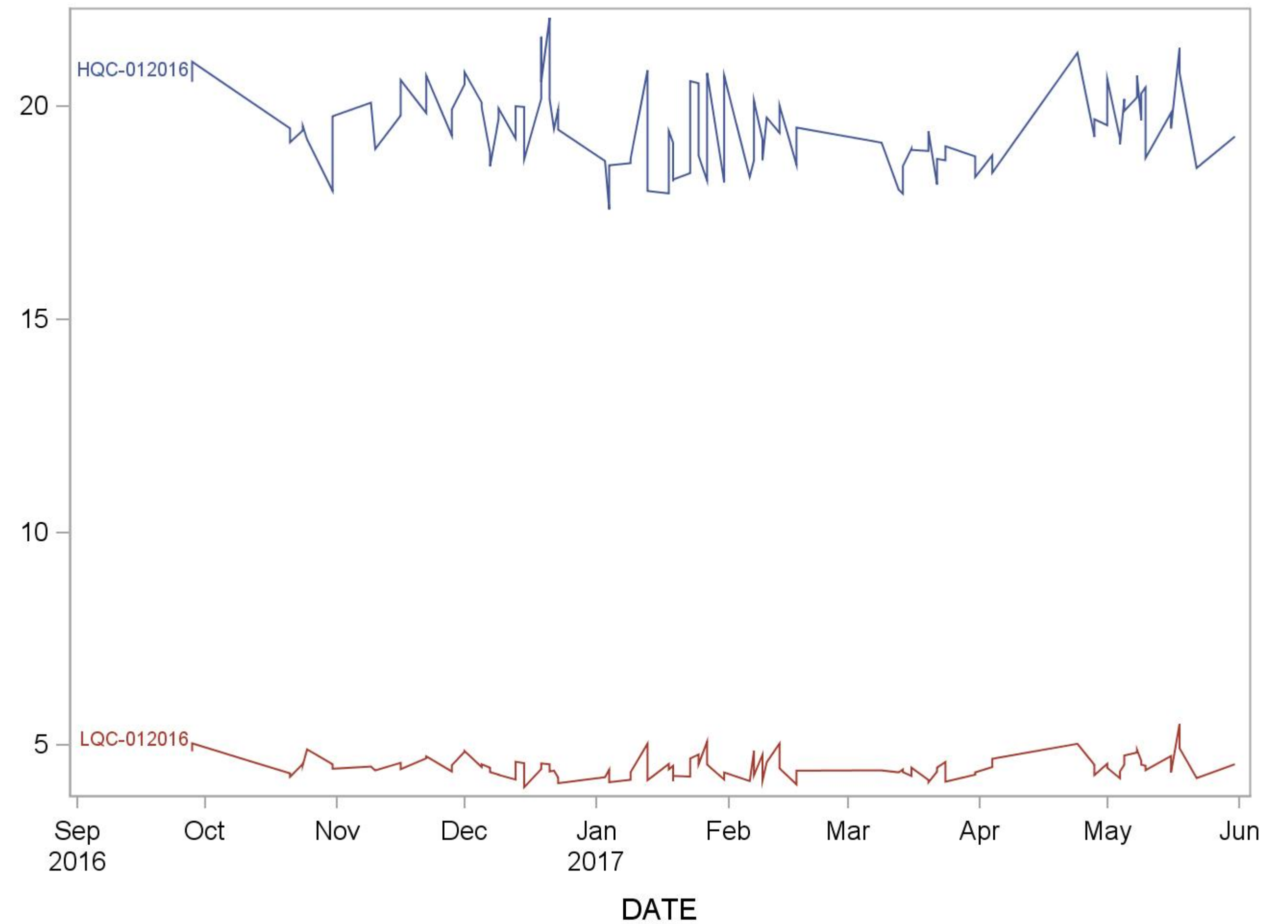
Summary Statistics and QC Chart for MECP phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	103	28SEP16	31MAY17	23.104	1.005	4.3
LQC-012016	103	28SEP16	31MAY17	8.712	0.410	4.7



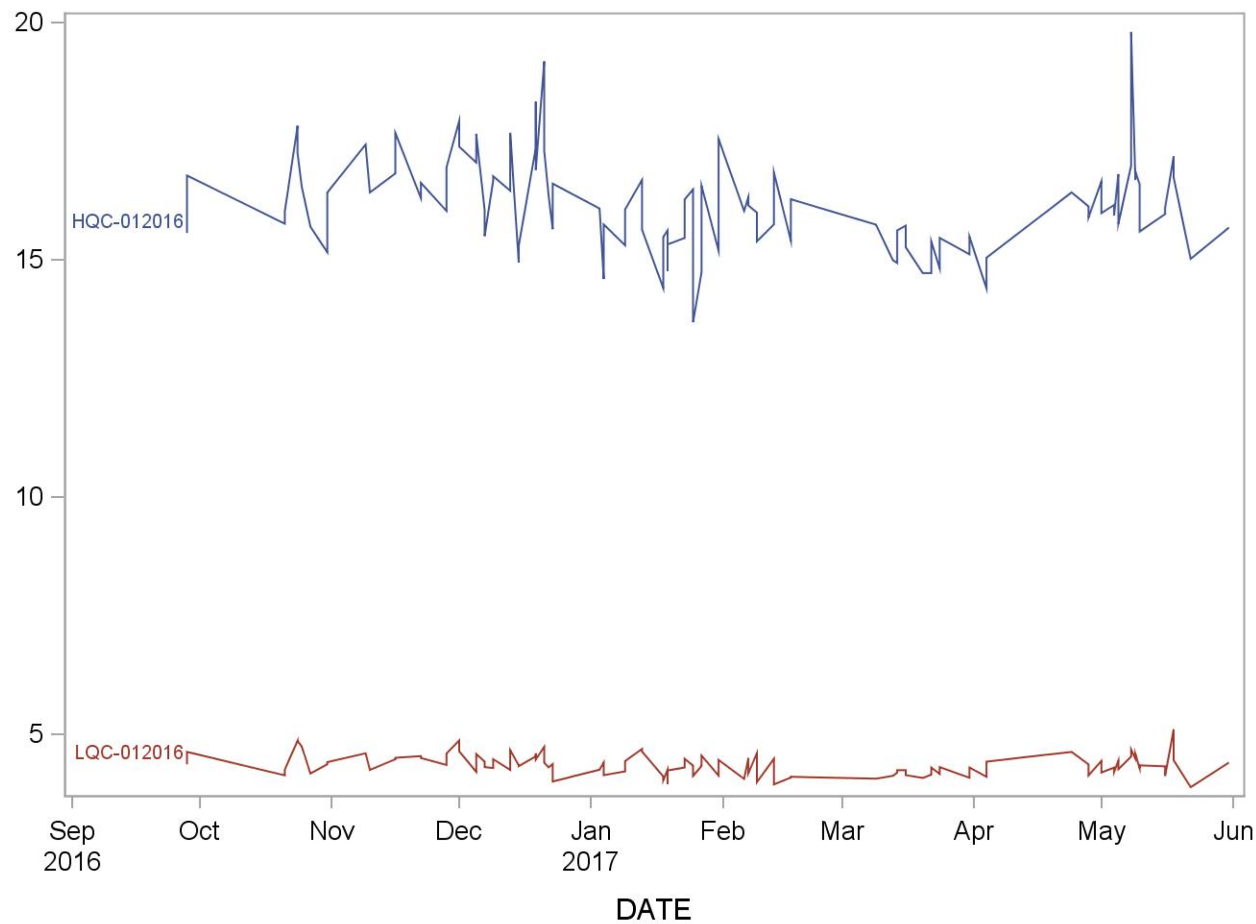
Summary Statistics and QC Chart for MEHP phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	104	28SEP16	31MAY17	19.486	0.922	4.7
LQC-012016	104	28SEP16	31MAY17	4.491	0.260	5.8



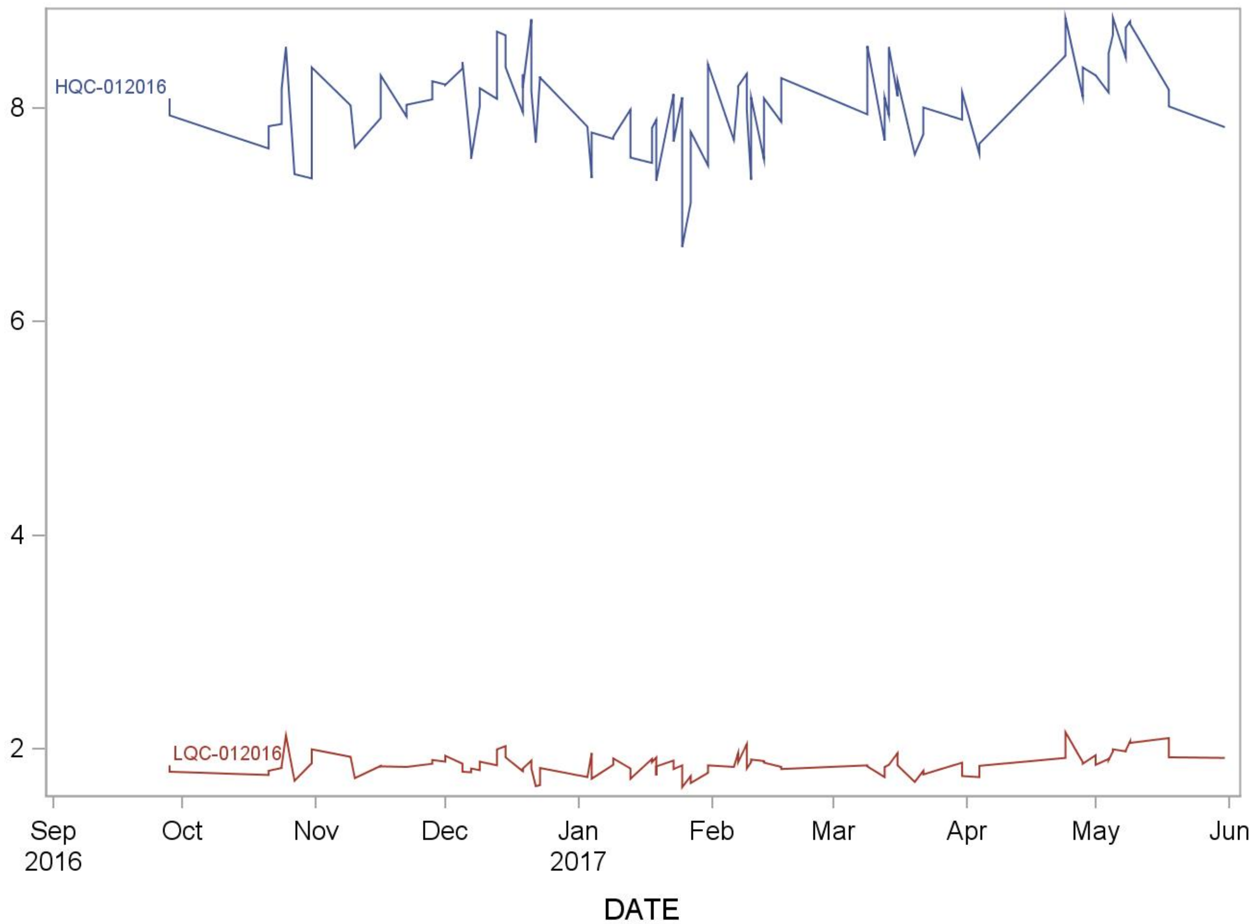
Summary Statistics and QC Chart for MEOH phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	103	28SEP16	31MAY17	16.139	1.004	6.2
LQC-012016	103	28SEP16	31MAY17	4.356	0.226	5.2



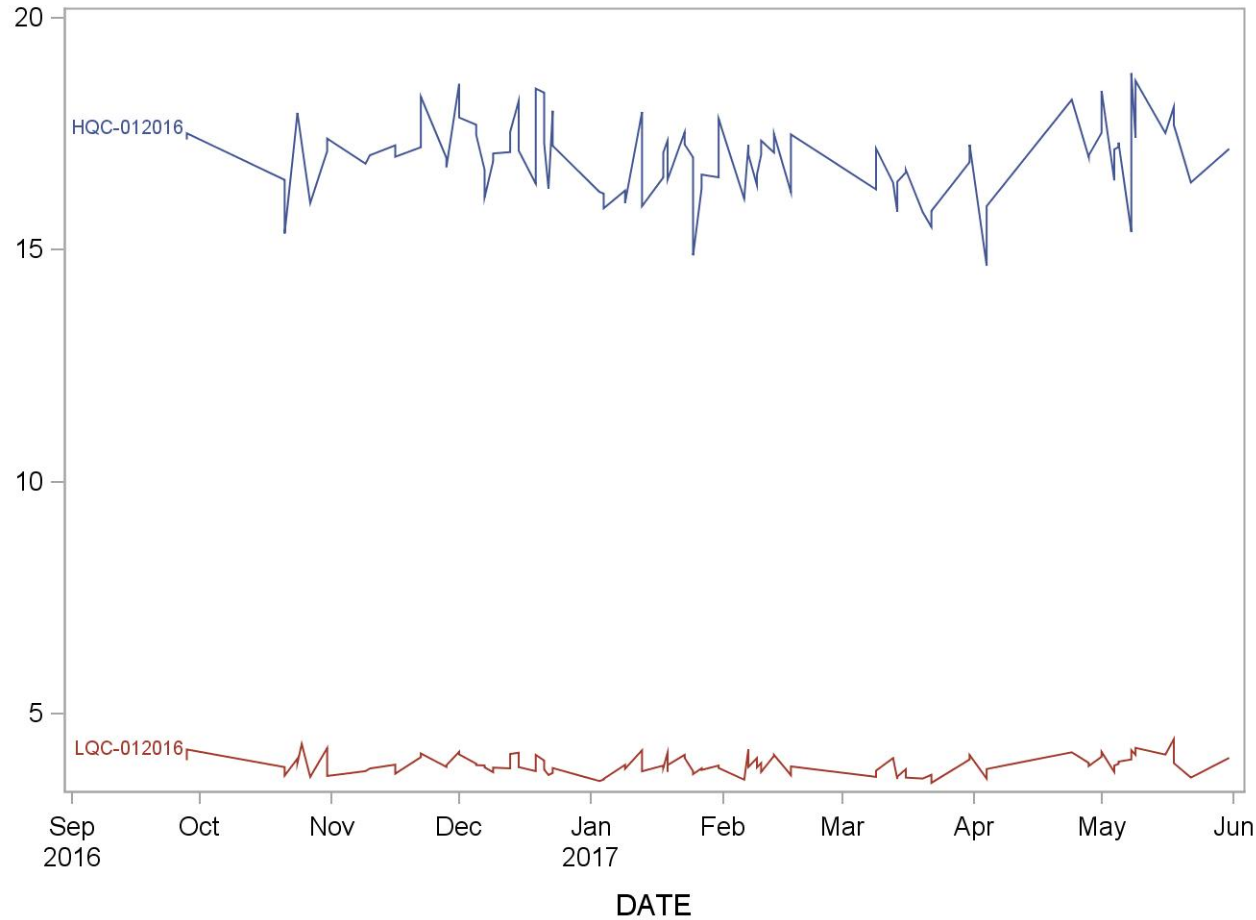
Summary Statistics and QC Chart for MHNCH (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	101	28SEP16	31MAY17	8.0370	0.4122	5.1
LQC-012016	101	28SEP16	31MAY17	1.8567	0.0994	5.4



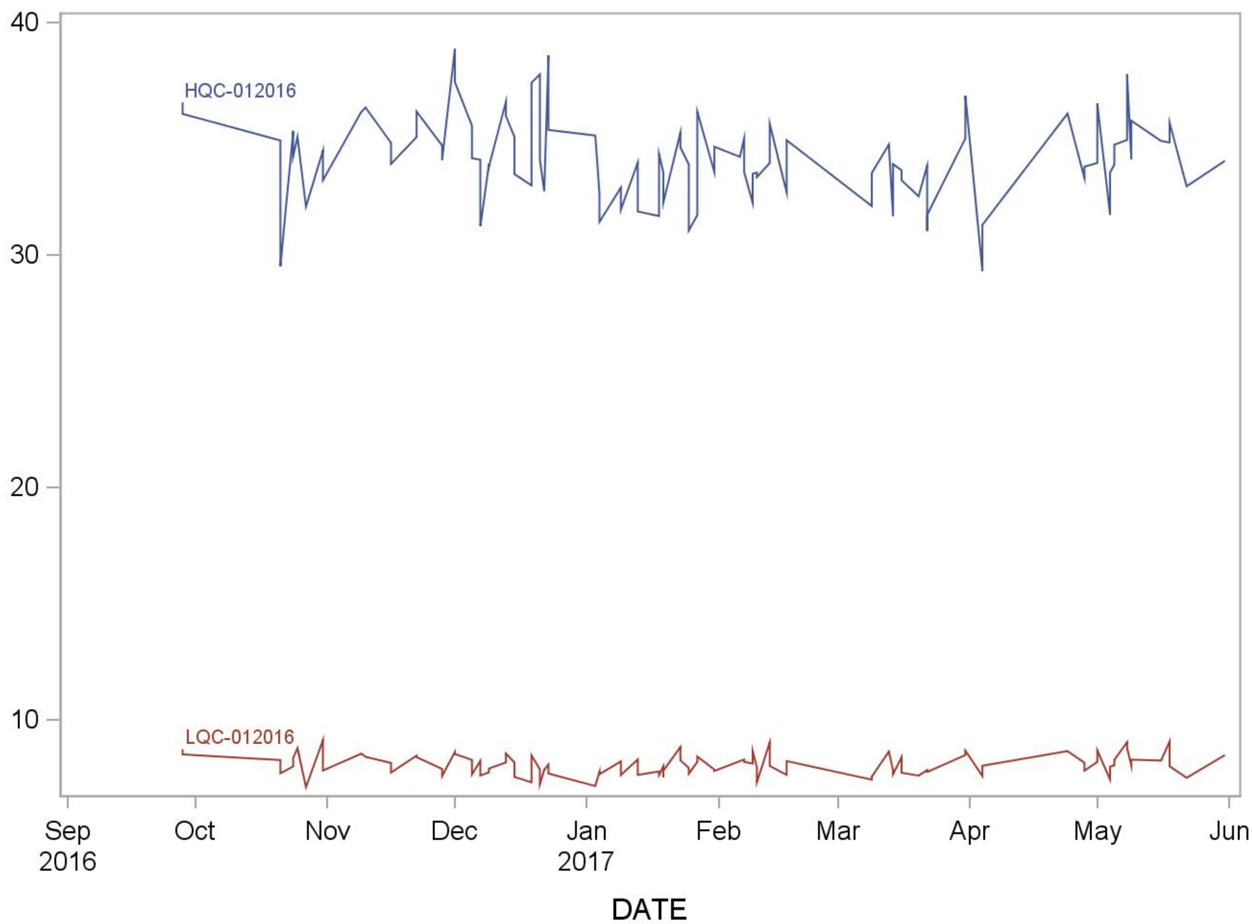
Summary Statistics and QC Chart for Mono(carboxynonyl) phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	101	28SEP16	31MAY17	16.999	0.825	4.9
LQC-012016	101	28SEP16	31MAY17	3.895	0.202	5.2



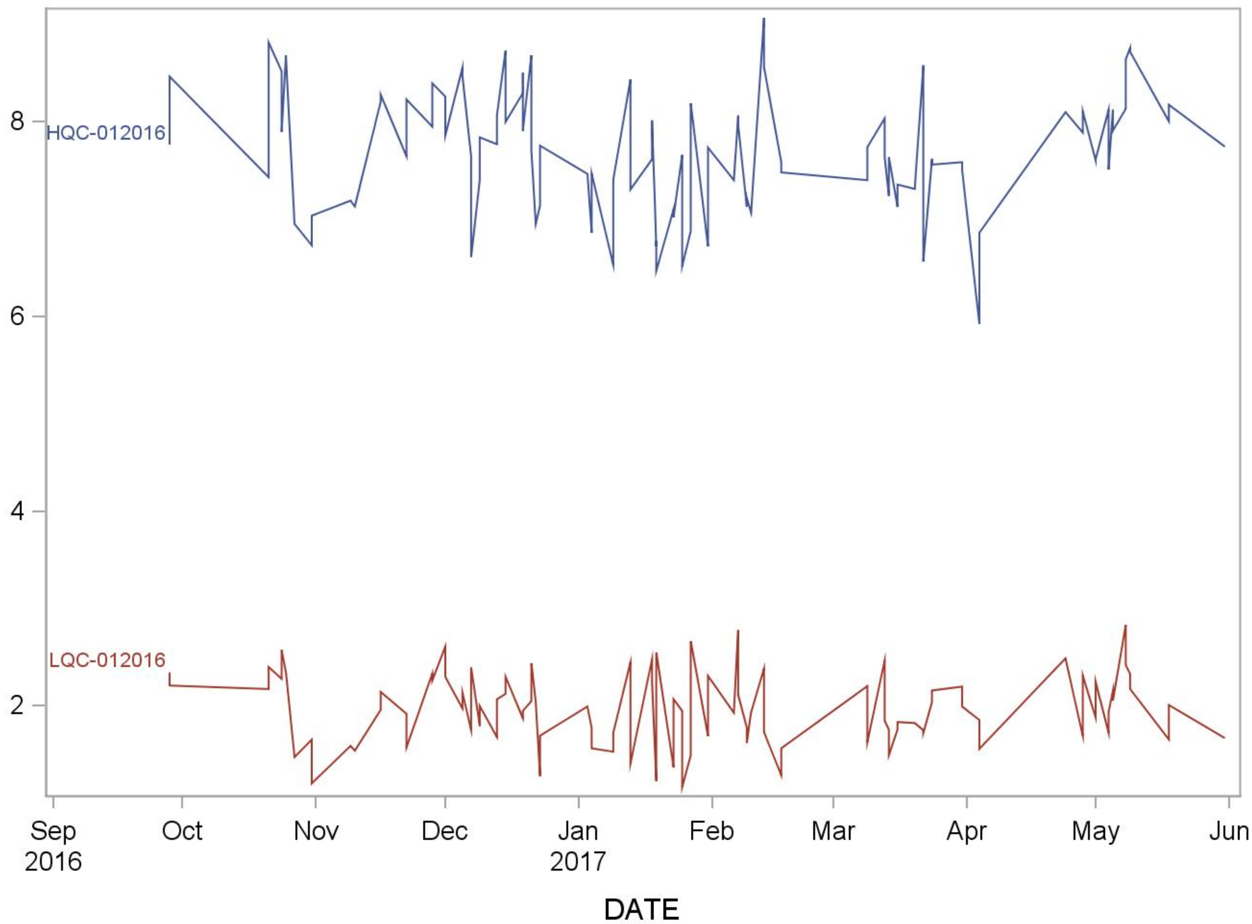
Summary Statistics and QC Chart for Mono(carboxyoctyl) phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	102	28SEP16	31MAY17	34.154	1.829	5.4
LQC-012016	102	28SEP16	31MAY17	8.064	0.450	5.6



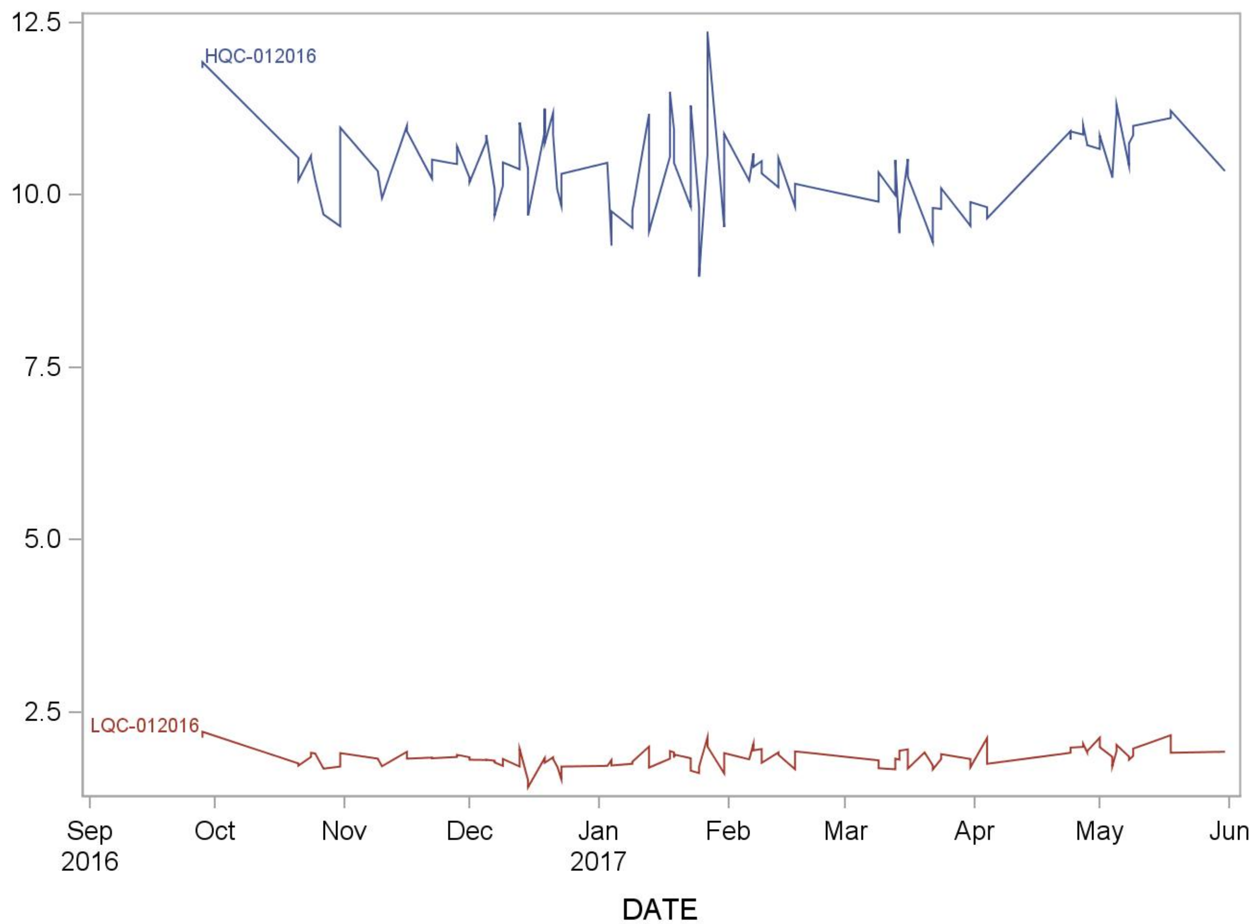
Summary Statistics and QC Chart for Mono-(2-ethyl)-hexyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	101	28SEP16	31MAY17	7.690	0.637	8.3
LQC-012016	101	28SEP16	31MAY17	1.960	0.371	18.9



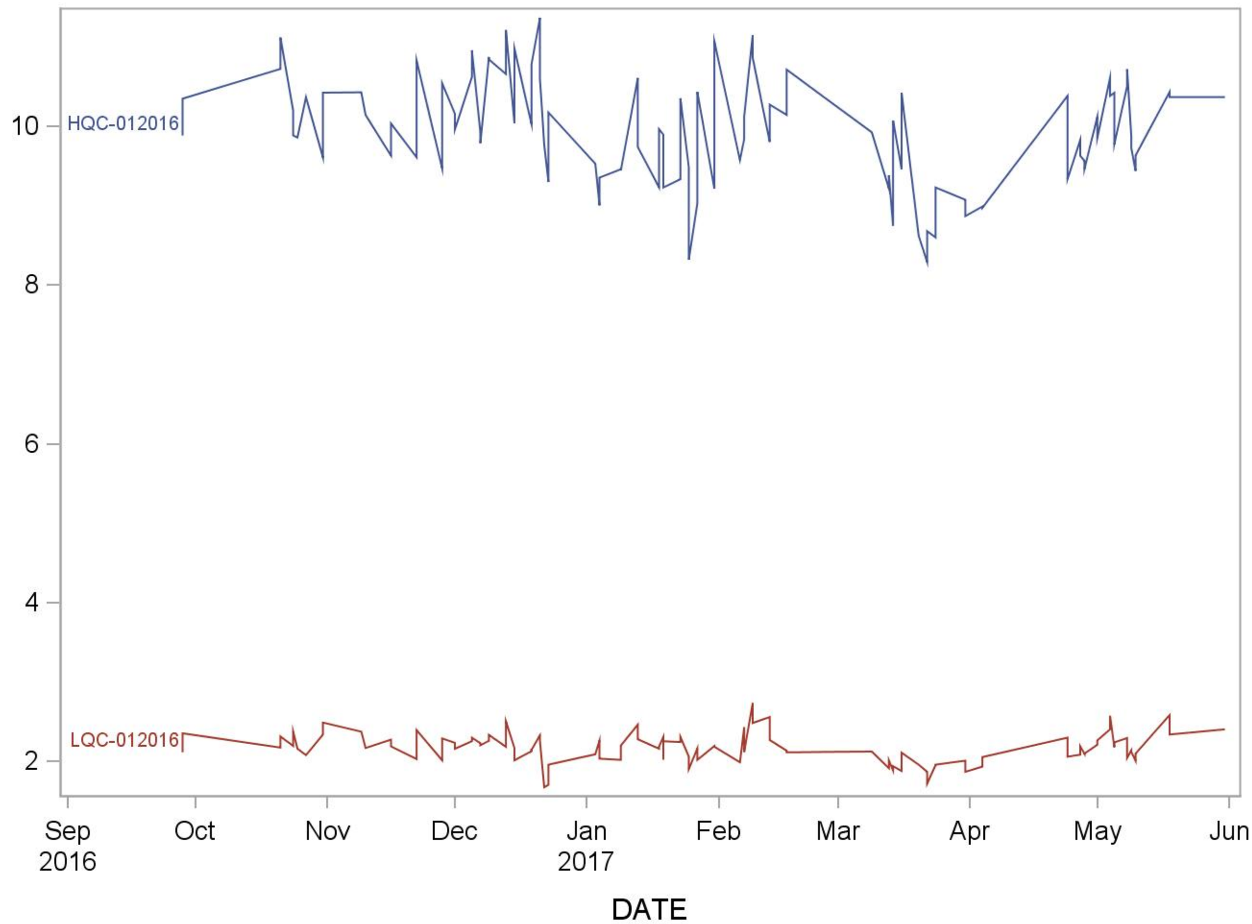
Summary Statistics and QC Chart for Mono-(3-carboxypropyl) phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	104	28SEP16	31MAY17	10.412	0.610	5.9
LQC-012016	104	28SEP16	31MAY17	1.835	0.139	7.6



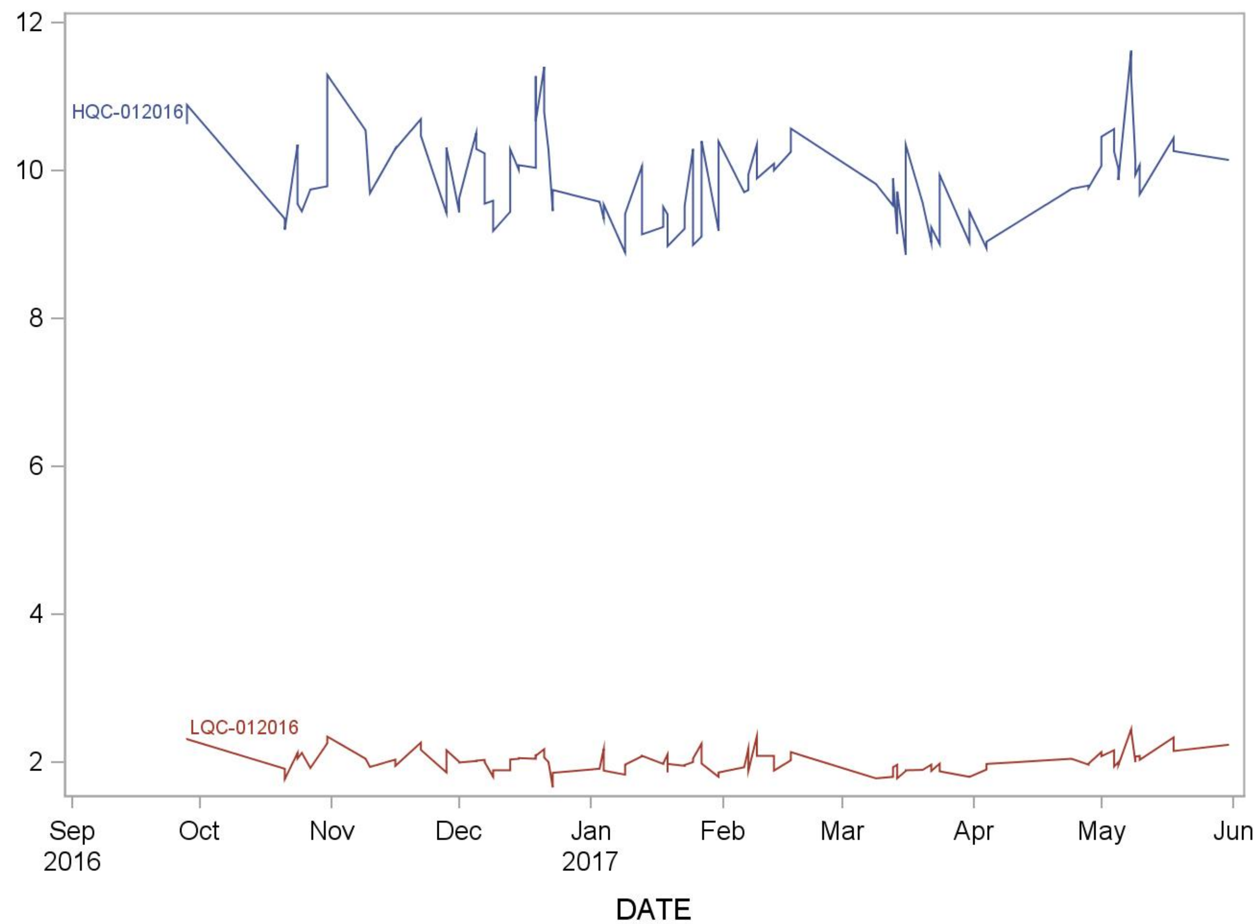
Summary Statistics and QC Chart for Mono-2-hydroxy-iso-butyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	104	28SEP16	31MAY17	9.9381	0.6783	6.8
LQC-012016	104	28SEP16	31MAY17	2.1655	0.1895	8.8



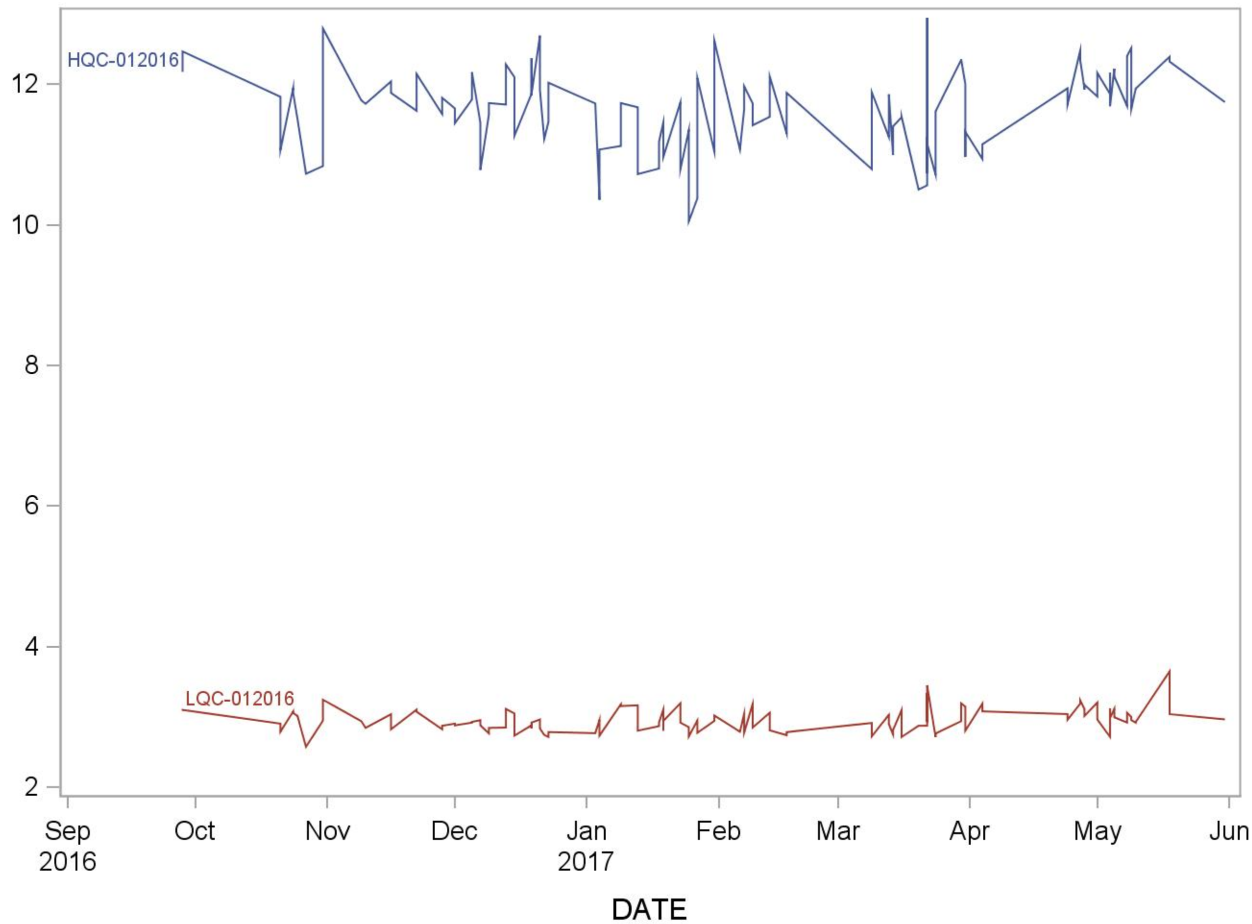
Summary Statistics and QC Chart for Mono-3-hydroxy-n-butyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	101	28SEP16	31MAY17	9.8819	0.6086	6.2
LQC-012016	101	28SEP16	31MAY17	2.0117	0.1510	7.5



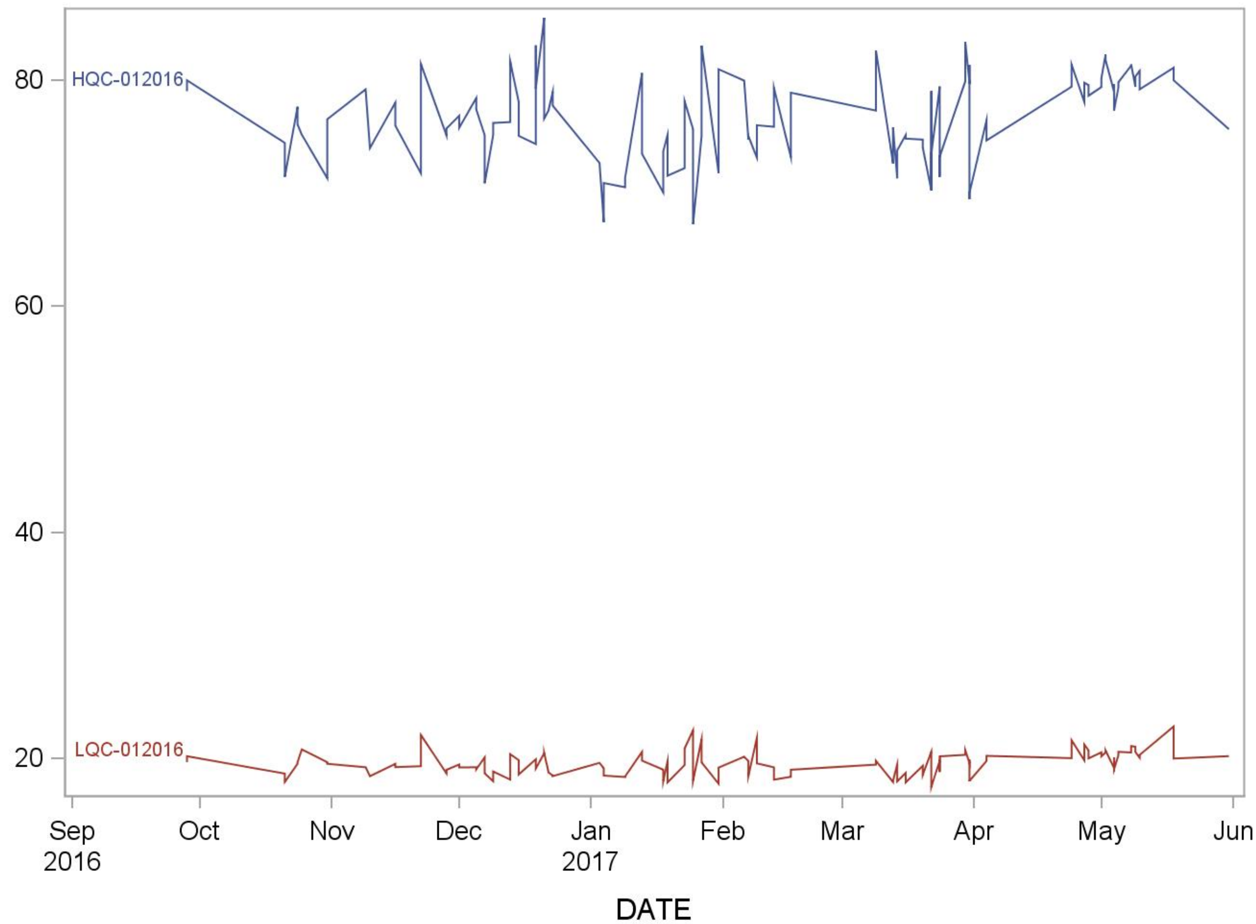
Summary Statistics and QC Chart for Mono-benzyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	113	28SEP16	31MAY17	11.642	0.569	4.9
LQC-012016	113	28SEP16	31MAY17	2.955	0.169	5.7



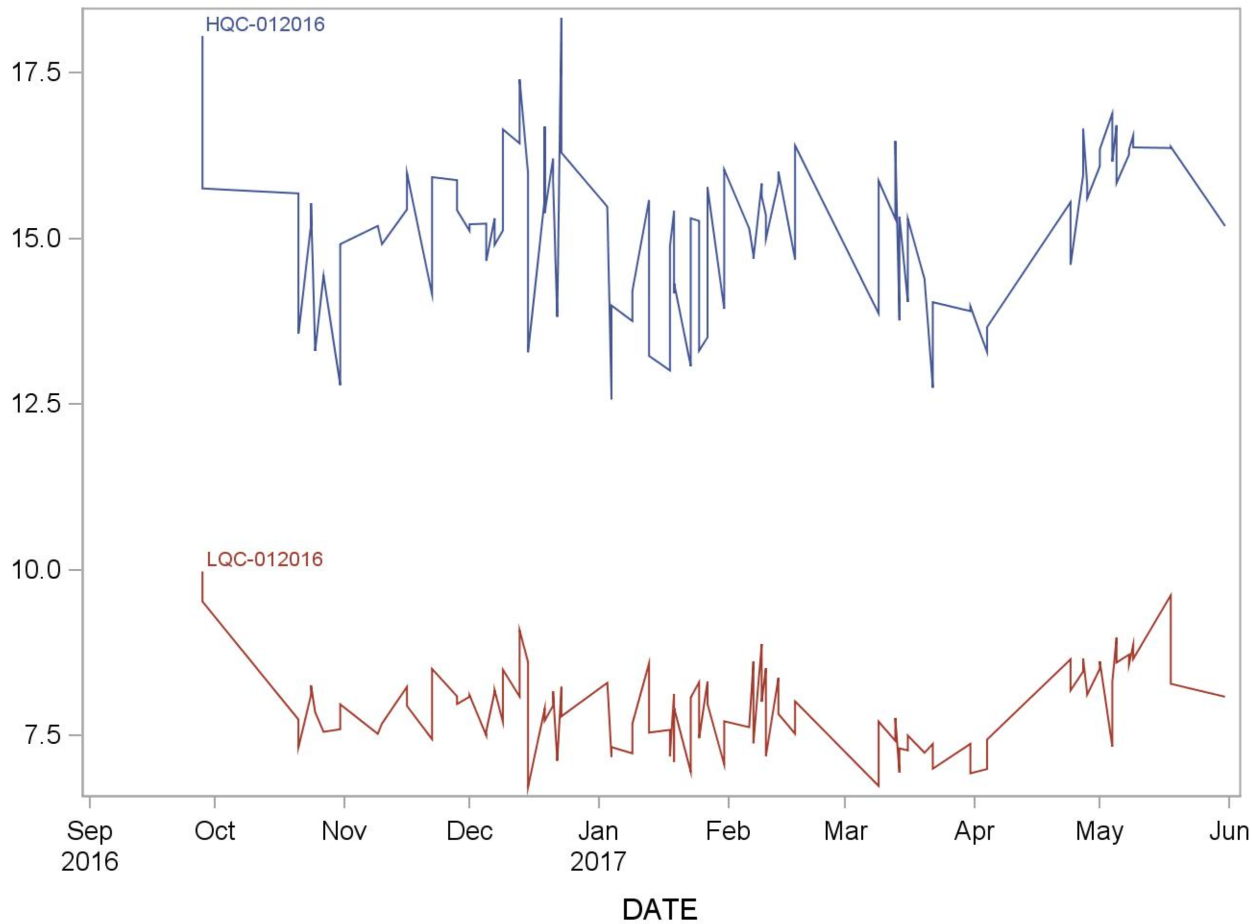
Summary Statistics and QC Chart for Mono-ethyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	117	28SEP16	31MAY17	76.550	3.773	4.9
LQC-012016	117	28SEP16	31MAY17	19.526	1.070	5.5



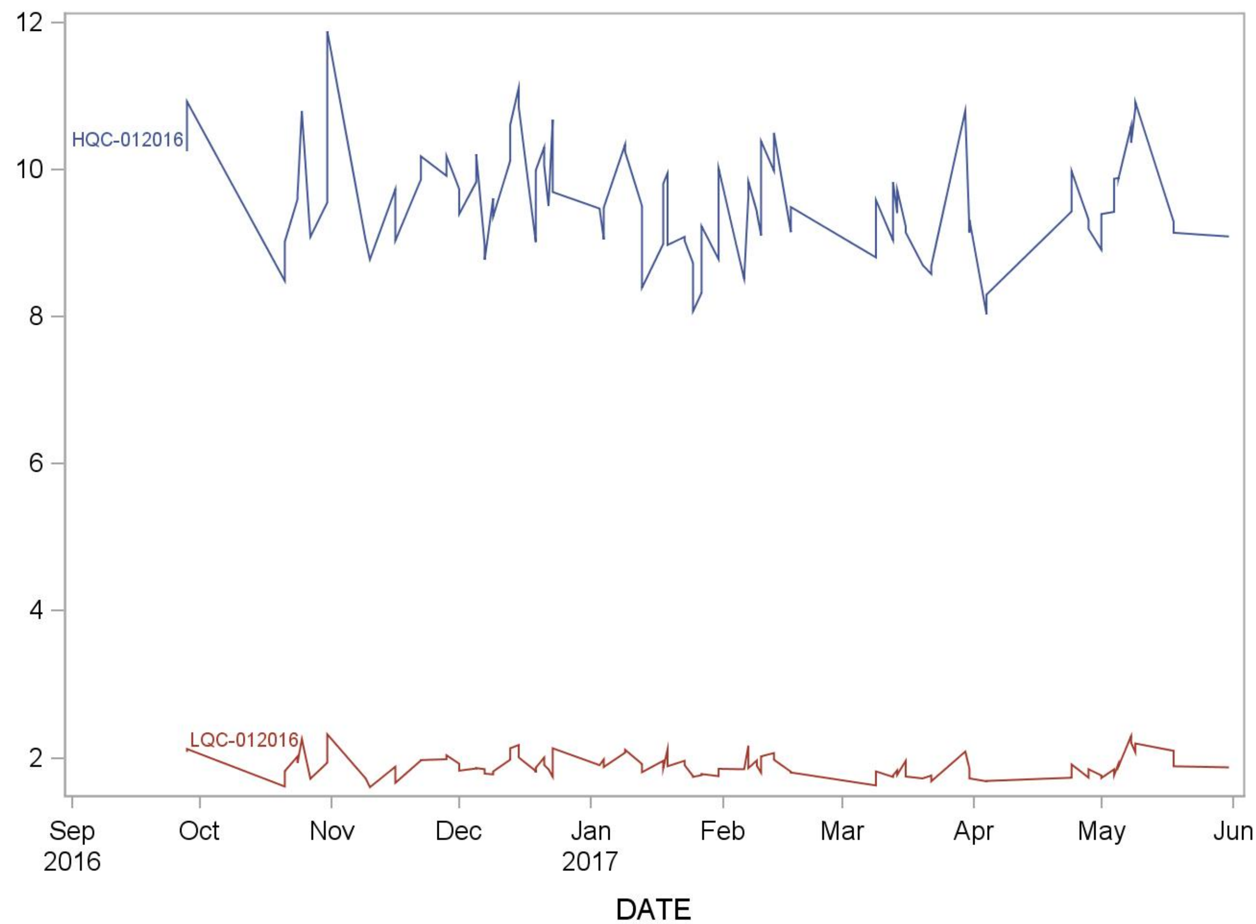
Summary Statistics and QC Chart for Mono-isobutyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	103	28SEP16	31MAY17	15.1746	1.1681	7.7
LQC-012016	103	28SEP16	31MAY17	7.9249	0.6255	7.9



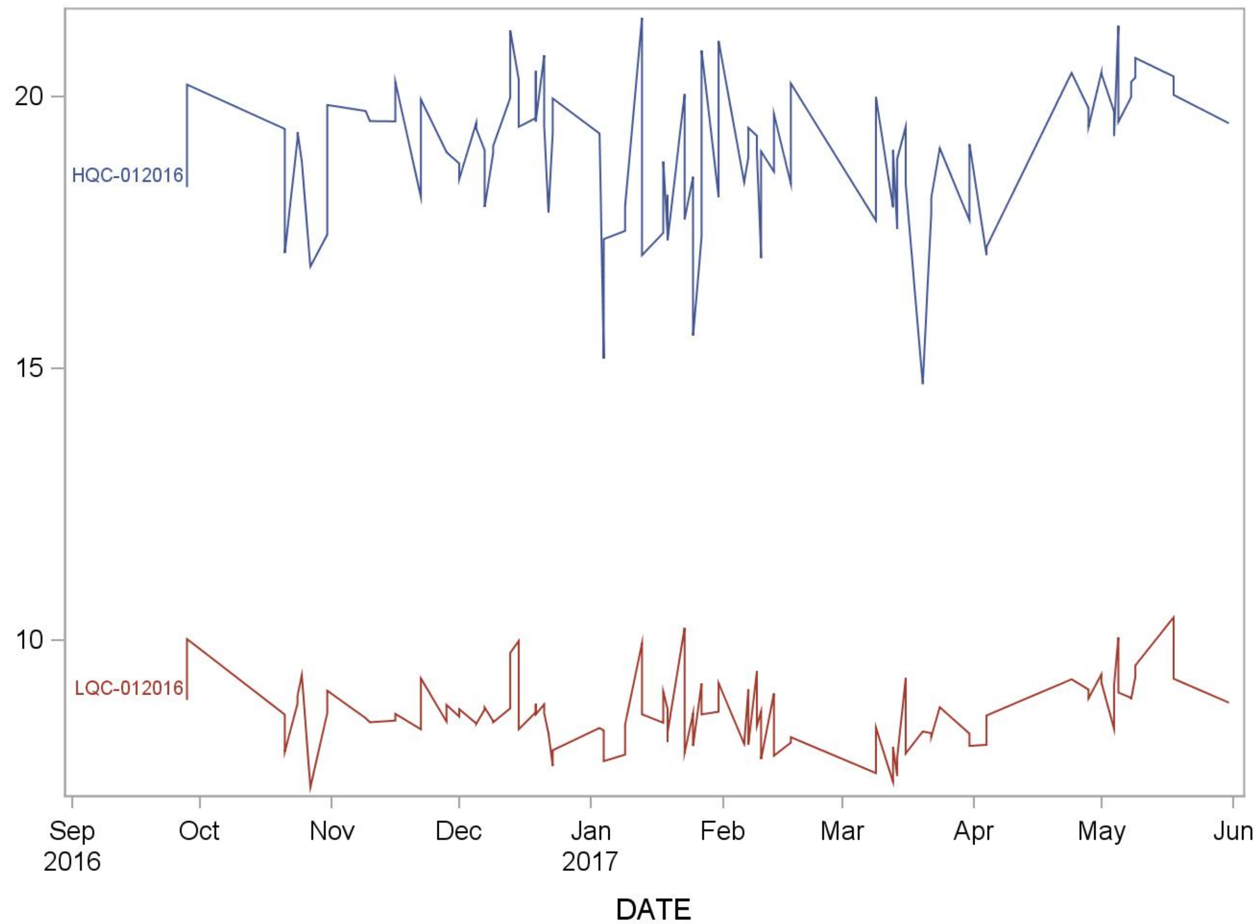
Summary Statistics and QC Chart for Mono-isononyl phthalate (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012016	102	28SEP16	31MAY17	9.566	0.716	7.5
LQC-012016	102	28SEP16	31MAY17	1.891	0.155	8.2



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-012016	101	28SEP16	31MAY17	18.956	1.284	6.8
LQC-012016	101	28SEP16	31MAY17	8.653	0.617	7.1



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