# Laboratory Procedure Manual

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|---|---|
| *Analyte*: | **Diphenyl phosphate (DPhP), Bis(1,3-dichloro-2-propyl) phosphate (BDCPP), Bis-(1-chloro-2-propyl) phosphate (BCPP), Bis-2-chloroethyl phosphate (BCEtP), Dibutyl phosphate (DBuP), 2,3,4,5-tetrabromobenzoic acid (TBBA), Dimethylphosphate (DMP), Diethylphosphate (DEP), Dimethylthiophosphate (DMTP), Dimethyldithiophosphate (DMDP), Diethylthiophosphate (DETP), and Diethyldithiophosphate (DEDP)** |
| Matrix: | **Urine** |
| Method: | **Flame Retardants and OP Pesticides in Urine** |
| Method No: | **6121** |
| As performed by: | Organic Analytical Toxicology Branch<br>Division of Laboratory Sciences<br>National Center for Environmental Health |
| Contact: | Maria Ospina, Ph.D.<br>Phone: 770-488-7407<br>Email: meo3@cdc.gov<br><br>James L. Pirkle, M.D., Ph.D.<br>CLIA Laboratory Director<br>Centers for Disease Control and Prevention |

**Important Information for Users**

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

## Public Release Data Set Information

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

| Data File Name | Variable Name | SAS Label |
|---|---|---|
| **OPD_K_R** | URXOP1 | Dimethylphosphate (ng/mL) |
| | URXOP2 | Diethylphosphate (ng/mL) |
| | URXOP3 | Dimethylthiophosphate (ng/mL) |
| | URXOP4 | Diethylthiophosphate (ng/mL) |
| | URXOP5 | Dimethyldithiophosphate (ng/mL) |
| | URXOP6 | Diethyldithiophosphate (ng/mL) |

## 1. Clinical Relevance and Summary of Test Principle

Polybrominated diphenyl ethers (PBDEs) were produced as flame retardants in the U.S. market until their phase-out in 2004 and 2013. Alternative chemicals, such as chlorinated alkyl and non-chlorinated aryl organophosphate flame retardants (OPFRs) and non-PBDE brominated flame retardant (FR) compounds such as 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) have been introduced as replacements for PBDE technical mixtures, and used as additives in furniture foam and other products.[1,2] EH-TBB is used in combination with OPFRs in non-PBDE flame retardants such as Firemaster 550, Firemaster BZ-54, and CN-2065.[2,3,4] Urinary metabolites of several FRs have been used as biomarkers of exposure to FR compounds, and detected in U.S. adults and children.[5,6] However, exposure to these FRs among the U.S. general population is yet unknown.

Organophosphate (OP) pesticides are used in both residential and agricultural settings in the United States. OP pesticides include malathion, diazinon, chlorpyrifos, Guthion® (azinphosmethyl), malathion, parathion, and many others. All OP pesticides have a common mode of toxicity because they are competitive inhibitors of acetylcholinesterase, the enzyme responsible for deacetylation of the neurotransmitter acetylcholine.[7,8] Unfortunately, the toxic effects of OP pesticides are not unique to insects; high doses can similarly affect wildlife and people. Because of their potential adversely affect especially vulnerable populations, such as children, and because of their common mode of toxicity, OP pesticides were among the first of the U.S. Environmental Protection Agency (EPA)-registered pesticides whose food tolerances were reassessed by EPA.[9]

Most of the organophosphates pesticides registered for use in the United States by the EPA are O, O-dimethyl or O, O-diethyl substituted that metabolize to dialkylphosphate (DAP) metabolites. The six common DAP metabolites do not retain any of the structure unique to the pesticides from which they were derived, so it is impossible to identify individual pesticides from these metabolites. However, because these metabolites are common to the majority of OP pesticides, they can provide invaluable information about cumulative exposure to the OP class.

We have developed a method to quantify eleven FR metabolites and six DAP metabolites (Figure 1) in urine to provide population exposure data in support of epidemiological studies. This method does not test for any disease. This analytical method quantifies: Diphenyl phosphate (DPhP), Bis(1,3-dichloro-2-propyl) phosphate (BDCPP), Bis-(1-chloro-2-propyl) phosphate (BCPP), Bis-2-chloroethyl phosphate (BCEtP), Dibutyl phosphate (DBuP), 2,3,4,5-tetrabromobenzoic acid (TBBA), Dimethylphosphate (DMP), Diethylphosphate (DEP), Dimethylthiophosphate (DMTP), Dimethyldithiophosphate (DMDP), Diethylthiophosphate (DETP), and Diethyldithiophosphate (DEDP).

The method uses solid phase extraction (SPE) coupled with isotope dilution-ultrahigh performance liquid chromatography (UHPLC)-tandem mass spectrometry.[10] The method relies on an enzymatic hydrolysis of urinary conjugates, automated off-line SPE to pre-concentrate the target compounds while minimizing urine matrix potential interferences to increase the overall sensitivity and specificity. The deconjugated target analytes in the urine extract are separated on an UHPLC system with reversed phase chromatography and quantified by isotope dilution tandem mass spectrometry.

## 2. Safety Precautions

### a. Reagent Toxicity or Carcinogenicity

Some reagents used in this procedure are toxic. Special care should be taken to avoid inhalation, eye or dermal 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/sulfatase and formic acid are known sensitizers. Prolonged or repeated exposure to sensitizers can cause allergic reactions in certain sensitive individuals.

**Note:** Safety Data Sheets should be obtained from the same manufacturer or supplier of the specific chemical that you have. Chemical concentrations in purchased solutions can vary, some chemicals may or may not have inhibitors or stabilizers added, so it is best that the source of the chemical and the SDS is the same. Safety Data Sheets (SDS) for the chemicals and solvents used in this procedure can also be found on the internet at http://www.ilpi.com/msds/. Laboratory personnel must review the SDS before using chemicals.

### b. Radioactive Hazards

None

### c. Microbiological Hazards

Although urine is generally regarded as less infectious than serum, the possibility of exposure to various microbiological hazards exists. Take appropriate protective measures to avoid contact with biological specimens (see “Protective equipment” below). Laboratory personnel handling human fluids and tissues are required to take the “Bloodborne Pathogens Training” course offered at CDC to ensure proper compliance with CDC safe work place requirements.

**d. Mechanical Hazards**

Follow standard safety practice while performing this procedure to minimize the risk for mechanical hazard. Avoid any direct contact with the electronic components of the mass spectrometer unless all power to the instrument has been shut off. Generally, only qualified technicians perform electronic maintenance and repair of the mass spectrometer. Contact with the heated surfaces of the mass spectrometer should be avoided.

**e. Protective Equipment**

Use standard safety personal protective equipment when performing this procedure. Wear a lab coat; safety glasses; and appropriate gloves. Use a chemical fume hood when preparing the reagents.

**f. Training**

Anyone performing this procedure should be trained and experienced in the use of automated SPE and UHPLC systems, and triple-quadrupole mass spectrometers. Although formal training is not necessary, personnel are appropriately trained by an experienced operator of the instrument or untrained personnel must work under the supervision of a trained person. All personnel who operate the instrument must also read all operation manuals and the laboratory standard operating procedures manual.

**g. Personal Hygiene**

Use caution when handling any biological specimen. Be sure to use gloves and wash hands properly. No food or drink is allowed in laboratory areas.

**h. Disposal of Wastes**

Always dispose of solvents and reagents in an appropriate container clearly marked for waste products, and temporarily store the containers in flame-resistant cabinets or equivalent storage space (follow CDC's guidelines entitled Hazardous Chemical Waste Management). Use caution when handling containers, glassware, etc. that come in direct contact with the specimens. Decontaminate sample preparation surfaces with 10% bleach. Wash the glassware or dispose of it in an appropriately labeled autoclave pan. Contaminated analytical glassware can be treated with bleach, washed and reused; disposable labware is autoclaved prior to disposal. To ensure 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**

A working knowledge of Analyst® and MultiQuant®, the software packages used for data acquisition and integration in the HPLC-MS/MS, is required.

A database named STARLIMS, developed on the CDC internal network, is used to store, retrieve, and analyze data. Personnel performing this method must be able to create a sequence, export it into the instrument and import instrument data into STARLIMS. The Statistical Analysis System (SAS)® software package is used to maintain the data-management structure. Knowledge and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

**b. Sample Information**

Sample information can be retrieved from the database (STARLIMS). This includes sample-identification (ID) code, notebook number associated with the sample preparation, sample type, standard number, and any other information not associated with the mass-spectral analysis. All sample information will be electronically transferred onto mass spectrometer-related-software upon sample analysis.

**c. Data Maintanance**

Copy raw instrument data to a CDC-approved jump drive and transfer to the CDC network for processing and integration. Upload sample and analytical data into the database, check for errors and overall validity. The database is on the CDC network which is backed up regularly.

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

**a. Sample Handling**

Use standard urine collection cups to collect urine specimens from participants. To minimize the potential degradation of the specimen, samples should be refrigerated as soon as possible. Preferably, the urine (2-5 mL) should be transferred into appropriate polypropylene or polyethylene containers within 4 hours of collection; borosilicate glass containers may also be used. The specimens are then labeled, frozen, and stored on dry ice for shipping. Special care must be taken in packing to protect urine containers from breakage during shipment. In general, urine specimens should be shipped in cryovials packed in boxes frozen and securely packed in dry ice. Store all samples at controlled temperatures (at or below -20˚C) until analysis.

**b. Sample Rejection**

Specimens are rejected if tubes/vials leaked, are broken, appeared compromised or tampered with, or hold inadequate volume for analysis. Also, generally reject samples with volume less than 0.2-mL because they cannot be reliably processed.

## 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

### a. Reagents and Sources

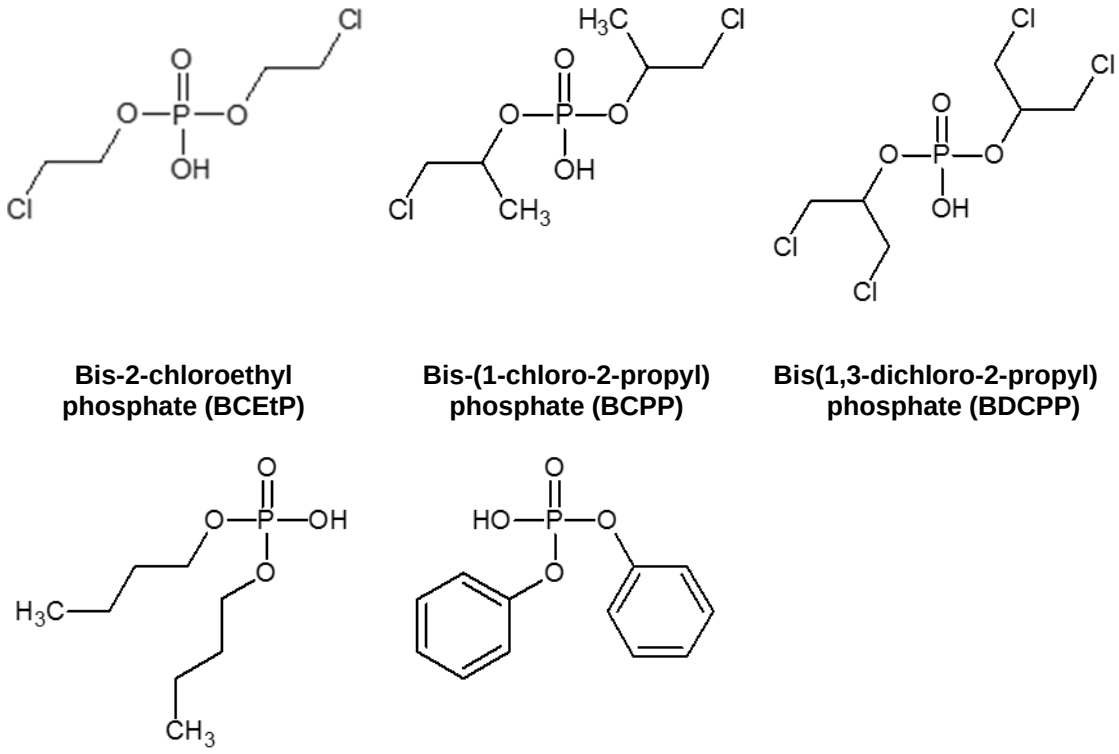
**Table 1. Calibration Standards, Reagents and their Manufacturers**

| Reagents | Manufacturers* |
|---|---|
| Dibutyl phosphate (DBuP) | Sigma-Aldrich (St. Louis, MO, USA) |
| Dibutyl phosphate-d18 (DBuP_L) | Sigma-Aldrich (St. Louis, MO, USA) |
| Diphenyl phosphate (DPhP) | TRC (Toronto, ON, Canada) |
| Diphenyl phosphate-d10 (DPhP_L) | TRC (Toronto, ON, Canada) |
| Bis(1,3-dichloro-2-propyl) phosphate (BDCPP) | Wellington Laboratories (Guelph, ON, Canada) |
| Bis(1,3-dichloro-2-propyl) phosphate-d10 (BDCPP_L) | Wellington Laboratories (Guelph, ON, Canada) |
| 2,3,4,5-tetrabromobenzoic acid (TBBA) | Wellington Laboratories (Guelph, ON, Canada) |
| 2,3,4,5-tetrabromobenzoic acid-$^{13}C_6$ ring (TBBA_L) | Wellington Laboratories (Guelph, ON, Canada) |
| Bis-(1-chloro-2-propyl) phosphate (BCPP) | TRC (Toronto, ON, Canada) |
| Bis-(1-chloro-2-propyl) phosphate-d12 (BCPP_L) | TRC (Toronto, ON, Canada) |
| Bis-2-chloroethyl phosphate (BCEtP) | TRC (Toronto, ON, Canada) |
| Bis-2-chloroethyl phosphate-d8 (BCEtP_L) | TRC (Toronto, ON, Canada) |
| O,O-Diethylphosphorodithioate – d10 (DEDP_L) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Diethylphosphorodithioate (DEDP) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Diethylphosphoric acid – d10 (DEP_L) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Diethylphosphoric acid (DEP) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Diethylphosphorothioate – d10 (DETP_L) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Diethylphosphorothioate (DETP) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Dimethylphosphoric acid – d6 (DMP_L) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Dimethylphosphoric acid (DMP) | Cambridge Isotope Laboratories (Andover, MA, USA) |

| | |
|---|---|
| O,O-Dimethylphophorodithioate – d6 (DMDP_L) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Dimethylphophorodithioate (DMDP) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Dimethylphosphorothioate – d6 (DMTP_L) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| O,O-Dimethylphosphorothioate (DMTP) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| 4-Methylumbelliferyl β-D-glucuronide hydrate (UMB) | Sigma-Aldrich (St. Louis, MO, USA) |
| 4-Methylumbelliferone (2,3,4,Methyl-$^{13}C_4$) (UMB_L) | Cambridge Isotope Laboratories (Andover, MA, USA) |
| Acetonitrile | Fisher Scientific (Pittsburg, PA, USA) |
| Methanol | Fisher Scientific (Pittsburg, PA, USA) |
| Glacial Acetic Acid | Sigma-Aldrich (St. Louis, MO, USA) |
| Formic Acid | Sigma-Aldrich (St. Louis, MO, USA) |
| Ammonium Hydroxide (28-30%) | Fisher Scientific (Pittsburg, PA, USA) |
| Deionized (DI) water | NANO pure Infinity ultrapure water system |
| β-glucuronidase Type H-1 from Helix pomatia | Sigma-Aldrich (St. Louis, MO, USA) |

*Standards and reagents with similar specifications from other manufacturers may be used

**Figure 1. Analyte abbreviations and structures**

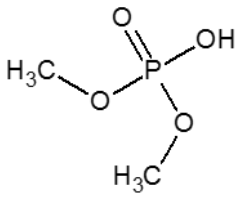


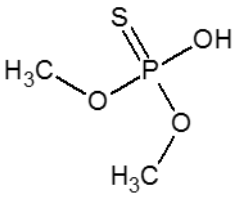

**Bis-2-chloroethyl phosphate (BCEtP)**

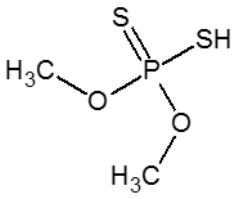
**Bis-(1-chloro-2-propyl) phosphate (BCPP)**

**Bis(1,3-dichloro-2-propyl) phosphate (BDCPP)**

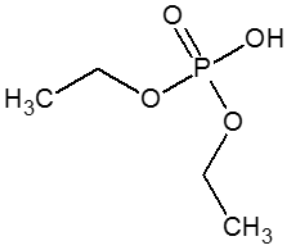
**Dibutyl phosphate (DBuP)** **Diphenyl phosphate (DPhP)**

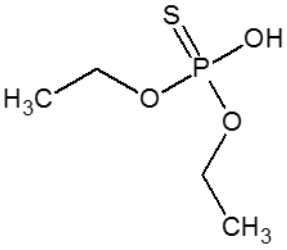


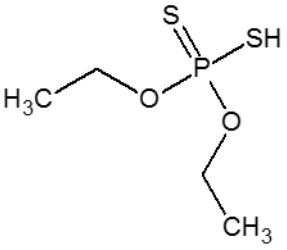




**Dimethyl phosphate (DMP)** **Dimethyl thiophosphate (DMTP)** **Dimethyl dithiophosphate (DMDTP)**







**Diethyl phosphate (DEP)** **Diethyl thiophosphate (DETP)** **Diethyl dithiophosphate (DEDTP)**

## b. Reagent Preparation

### 1) Liquid Chromatography Mobile Phases

Mobile Phase A = 0.1% Acetic acid in Water (v/v). Mix 1000mL of deionized water with 1mL of Acetic acid.

Mobile Phase B = 1:1 Acetonitrile: Methanol. Add equal volumes of acetonitrile and methanol, and mix well.

### 2) Sample Clean-up Reagents

0.2M Sodium Acetate Buffer solution: Add 6.8 grams sodium acetate and 2.2mL glacial acetic acid into 700mL of DI water and mix well.

Enzyme Solution: This solution should be made immediately before use. To 50mL of 0.2M sodium acetate buffer, add 0.025 grams of β-glucuronidase. Add the enzyme to the buffer at room temperature in a

flask and let it sit for approximately an hour to dissolve. Do not vortex or mix rigorously to dissolve the enzyme.

2% formic acid in water (v/v) aqueous solution: Add 20 mL of formic acid into 980 mL of DI water and mix well.

2% formic acid in methanol (v/v): Add 20 mL of formic acid into 980 mL of methanol and mix well.

~2% ammonium hydroxide in methanol (v/v): Add 3 mL of ammonium hydroxide into 150 mL of methanol.

### c. Standards Preparation

#### 1) Stock Solutions of Analytes

Prepare the individual stock solutions of analytes by dissolving a known amount of commercially available standards in appropriate solvent (check solubility data). Store the stock solutions in freezer-safe amber vials below 0˚C.

#### 2) Stock Solutions of Labeled Isotope Standards

Prepare the individual stock solutions of labeled analytes by dissolving a known amount of commercially available labeled standards in appropriate solvent (check solubility data). Store the stock solutions in freezer-safe amber vials below 0˚C.

#### 3) Deconjugation Standard Solution

4-methylumbelliferyl glucuronide and $^{13}C_4$-4-methylumbelliferone are used as deconjugation standards to monitor the extent of the enzymatic reaction. Prepare an individual stock solution of 4-methylumbelliferyl glucuronide by dissolving a known amount of commercially available 4-methylumbelliferyl glucuronide powder in MeOH. Use commercially available $^{13}C_4$-4-methylumbelliferone solution as the labeled standard.

#### 4) Labeled Isotope (ISTD) Spiking Solution with deconjugation standards

Prepare the spiking solution of isotope-labeled standards and deconjugation standards in 1:1 methanol:water, giving approximate concentrations of the individual labeled compounds of 40 ng/mL, 4-methylumbelliferyl glucuronide of 3000 ng/mL, and $^{13}C_4$-4-methylumbelliferone of 150 ng/mL.

#### 5) Native Spiking Standard Solutions

Prepare an intermediate stock solution of DMP, DMT, DEP, BCEtP, BCPP, DPhP, and DBCPP in 1:1 methanol: water from their individual

stock solutions, giving a concentration of individual compounds of 1000ng/mL. Prepare another intermediate stock solution with the rest of the analytes in 1:1 methanol: water from their individual stock solutions, giving a concentration of individual compounds of 1000ng/mL. Prepare two more intermediate stock solutions of all target analytes in 1:1 methanol: water from the individual stock solutions, giving a concentration of individual compounds of 500ng/mL, and 50ng/mL. Ten working standard solutions containing all target analytes are prepared in 1:4 methanol:water as shown in Figure 2. The final concentrations in urine from a 50-µL spike would cover a range of 0.05ng/mL to 40ng/mL for DMP, DMT, DEP, BCEtP, BCPP, DPhP and BDCPP and 0.05ng/mL to 20ng/mL for the rest of the analytes.

**6) Calibration Verification Materials**

CLIA defines calibration materials as "a solution which has a known amount of analyte weighed in or has a value determined by repetitive testing using a reference or definitive test method." According to this definition, our quality control (QC) materials qualify as calibration verification materials.

**7) Proficiency Testing (PT) Materials**

Proficiency testing materials are matrix-based samples (typically spiked samples) with a known or characterized concentration. These samples may be spiked or have endogenous levels of the target analytes. The PT materials are characterized by at least 20 repeat measurements to determine the mean and standard deviation for each analyte and kept frozen until needed.

## d. Equipment/Supplies

**1)** Sartorius Ultramicro® Microbalance (Westbury, NY)
**2)** Electronic 12-channel pipette (Rainin Instrument Company, Oakland, CA)
**3)** 450 µL pipette tips (TOMTEC, Hamden, CT)
**4)** EDP2® pipettes (Rainin Instrument Company, Oakland, CA)
**5)** Presterilized filter pipette tips (Rainin Instrument Company, Oakland, CA)
**6)** Vortex Genie® vortex mixer (Scientific Industries Inc., Springfield, MA)
**7)** 96-well plates (2 ml square well, Varian, Lake Forest, CA)
**8)** 96-well format cartridges 60 mg Strata-X-AW polymeric SPE (Phenomenex, Torrance, CA)
**9)** Hypersil GOLD aQ, 150 x 4.6 mm, 3 µm (Thermoscientific, San Jose, CA)
**10)** TOMTEC Quadra 4 automated SPE station (Hamden, CT)
**11)** Turbovap 96 concentration workstation (Caliper Life Sciences, Hopkinton, MA)
**12)** Acqua Solutions ultrapure water system
**13)** Qorpak bottles (Lab Depot, Inc., Cumming, GA)

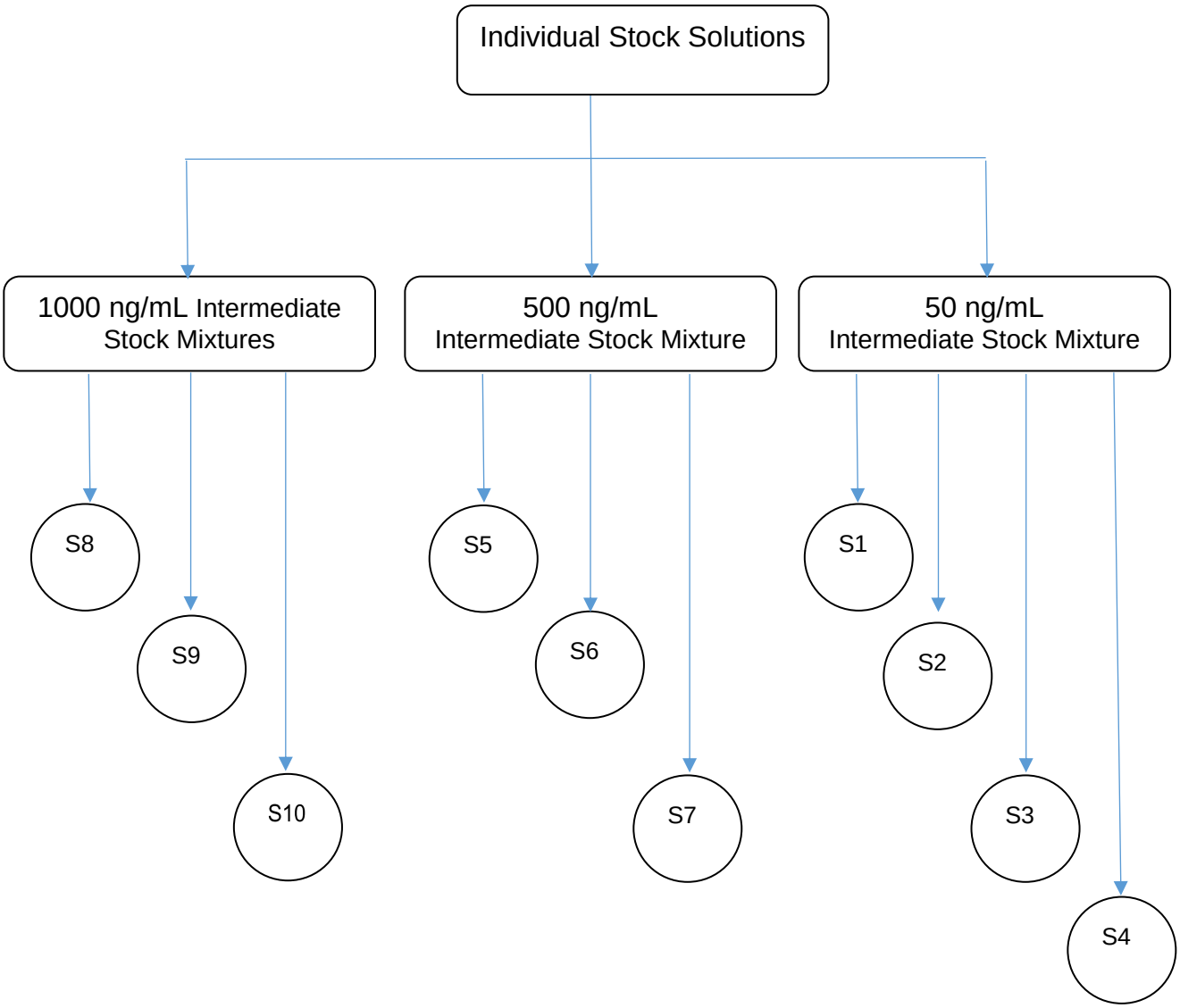



**Figure 2.** Scheme for the preparation of calibration standards. Each arrow represents one dilution step.

## e. Instrumentation

### 1) Mass Spectrometer – ABSciex 5500 & ABSciex 6500+

The Sciex 5500 and Sciex 6500+ mass spectrometers (Applied Biosystems/ MDS Sciex, Foster City, CA, USA) are equipped with a turbo spray (ESI) atmospheric pressure ionization source operated in the negative ion mode and controlled by Analyst® software. For this method, the instruments operate in the Scheduled MRM (sMRM) mode. Both instruments produced

comparable results for the quantification of flame retardants and OP pesticide metabolites.

**2) Agilent LC System**

Each mass spectrometer is coupled to an Agilent 1290 ultrahigh-performance liquid chromatography (UHPLC) system. The UHPLC system is composed of a binary pump, a temperature-controlled autosampler, and a temperature-controlled column compartment.

## 7. Calibration and Calibration Verification Procedures

### a. Calibration Plot

**1)** Construct a calibration plot by performing a linear regression analysis of relative response ratio factor (i.e., area native/area label) versus standard concentration.

**2)** The lowest point on the calibration curve is at or below the calculated method limit of detection (LOD) and the highest point is above the expected range of results.

**3)** Determination of the slope and intercept of this curve is by linear least squares fit using Analyst® software.

**4)** $R^2$ values for the curve must be greater than 0.98. Linearity of standard curves should extend over the entire standard range. Intercepts, calculated from the least squares fit of the data, should not be significantly different from 0; if they are, the sources of this bias need to be identified.

**5)** Periodically or when needed, re-establish the standard curve to incorporate the newest data points.

### b. Calibration Verification

1) Calibration verification is not required by the manufacturer(s) of the UHPLC-MS/MS system. 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.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 the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.

## c. Proficiency Testing (PT)

Proficiency testing should be performed a minimum of once every 6 months. Where applicable, NIST matrix-based certified reference materials may be included as PT materials. Because no formal PT testing program exists for most of the target analytes of this method, an in-house program is used. This in-house program currently includes pools prepared in-house but could also include independently prepared materials whose preparation was contracted out to an external laboratory. Once the in-house PT pools are characterized, the data are forwarded to a DLS representative responsible for executing the PT program (PT administrator). These PT samples are blind-coded by the PT administrator and returned to the laboratory staff for storage. Approximately every six months, the laboratory supervisor or his/her designee will notify the PT administrator who will randomly select five PT materials for analysis. The selected PT materials will be analyzed in the same manner as unknown samples. These PT materials will be selected from among three different concentration ranges spanning the linear range of the method. The concentration range for each sample will be blinded to all analysts. Following analysis, the results will be forwarded directly to the PT administrator for evaluation. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the PT administrator. The PT administrator will notify the laboratory chief or his/her designee of the PT results (i.e. pass/fail). If a PT challenge is failed, a second attempt to demonstrate proficiency by analyzing a second set of PT samples is undertaken. If the second attempt fails, laboratory operations will cease until an appropriate corrective action is taken. After correction action is taken, laboratory operations can resume. All proficiency results shall be appropriately documented.

In addition to the in-house PT program, for six DAPs analytes, we participate in the ongoing German External Quality Assessment Scheme (G-EQUAS) organized and managed by the Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine of the Friedrich-Alexander-University in Erlangen-Nuremberg, Germany. A minimum of once per year, two reference urine samples fortified with the six DAPs metabolites are analyzed. The program, evaluation, and certification are based on the guidelines of the German Federal Medical Council (http://www.g-equas.de/).

Also for DPhP and BDCPP, we participate in Quebec External Quality Assessment Scheme for Organic Substances in Urine program (OSEQAS). A minimum of once per year, three reference urine samples fortified with DPhP and BDCPP are analyzed. The program, evaluation, and certification are based on the guidelines of the Centre de Toxicologie du Québec (CTQ), Institut National du Santé Public du Quebec (INSPQ) in Canada.

(https://www.inspq.qc.ca/sites/default/files/documents/ctq/ipaqe-participants-guide.pdf ).

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

### a. Analytical Runs

A typical analytical run consists of 80 unknown/study urine samples, four quality control (QC) samples (two low-concentration QC [QCL] and two high-concentration QC [QCH], a blank, and up to 10 calibration standard samples (in one 96-well plate).

### b. Sample Preparation

Remove unknown samples and QC materials from storage freezer and allow them to come to room temperature. In a 96-well plate (2 ml square well, Varian, Lake Forest, CA), 200 μL aliquots of urine samples and QCs are spiked with 50 μL of internal standard solution. Reagent blank is prepared by adding 50 μL of internal standard, and 200 μL of water. Calibration standards are prepared by adding 50 μL of internal standard, 50 μL of the calibration solutions and 150 μL of water to each well. All samples are spiked with 400 μL of enzyme buffer solution and incubated at least for six hours (typically overnight) at 37 ºC. Next morning, 1000 μL of 2% formic acid in water is added to each sample. Then the 96-well plate is placed on a TOMTEC Quadra4 automated SPE station (Hamden, CT). Urine samples, reagent blank, and calibration standards are processed by use of the same automated procedure.

A 96-well format cartridge bed (60 mg Strata XAW polymeric SPE packing with 1.5 mL liquid space, Phenomenex, Torrance, CA) is cleaned with 430 μL of water with 3×400 μL of freshly prepared ~2% NH4OH in methanol followed by 430 μL of methanol to eliminate the background interferences from the SPE sorbent. After initial cleaning, the SPE plate is conditioned with 2×430 μL of HPLC-grade 2% formic acid in methanol and 2×430 μL of 2% formic acid in water. All samples are pipette-mixed twice right before loading onto the SPE cartridge. After sample loading (6×300 μL), the cartridges are washed with 2×430 μL of 2% formic acid in water followed by 2% formic acid in methanol. The target analytes are eluted with 2×400 μL of freshly prepared ~2% $NH_4OH$ in methanol. The eluted samples are evaporated to ~20 μL under a stream of dry nitrogen (UHP grade) at ~40 ºC in a Turbovap 96 concentration workstation (Caliper Life Sciences, Hopkinton, MA). After 40 minutes of evaporation, 300 μL of acetonitrile are added to each well and mixed slowly, and evaporation is continued in the Turbovap 96. The evaporated SPE extracts are reconstituted with 100 μL of 95:2.5:2.5 Water: ACN: MeOH solution. The sample plate is transferred to the autosampler and kept at ~4ºC.

### c. Liquid Chromatography Conditions

The HPLC separation uses a gradient elution as shown in Table 2.

**Table 2. HPLC Separation Conditions**

| Time (min) | Flow Rate (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.700 | 95.0 | 5.0 |
| 0.50 | 0.700 | 95.0 | 5.0 |
| 4.50 | 0.700 | 50.0 | 50.0 |
| 14.00 | 0.700 | 0.0 | 100.0 |
| 15.50 | 0.800 | 0.0 | 100.0 |
| 16.00 | 0.700 | 95.0 | 5.0 |
| 20.00 | 0.700 | 95.0 | 5.0 |

### d. Mass Spectrometer Conditions

Configuration parameters for this instrument are listed in Table 3. The optimized precursor/product ion pairs, and the retention time for the target compounds, are summarized in Table 4.

**Table 3. Mass Spectrometer Ion Source Conditions**

| MS Parameter | Settings for Sciex 5500 | Settings for Sciex 6500+ |
|---|---|---|
| Ionization Type | Turbo Spray (ESI) | Turbo Spray (ESI) |
| Ion Polarity Mode | Negative ion | Negative ion |
| Curtain Gas (CUR) | 20 | 30 |
| Collision Gas (CAD) | Medium | 8 |
| Ion Spray Voltage (IS) | -4500 | -3500 |
| Temperature (TEM) | 450˚C | 450˚C |
| Ion Source Gas 1 (GS1) | 45 | 50 |
| Ion Source Gas 2 (GS2) | 45 | 50 |

## Table 4: Compound Specific HPLC-Mass Spectrometry Parameters

| Analyte | Precursor ion (m/z) | Product ion (Confirmation ion) (m/z) | Retention time* (min) |
|---|---|---|---|
| BCEtP | 221 (223) | 35 (37) | 4.5 |
| BCEtP_L | 229 | 35 | 4.5 |
| BCPP | 249 (251) | 35 (37) | 5.4 |
| BCPP_L | 261 | 35 | 5.4 |
| BDCPP | 317 (321) | 35 (37) | 6.3 |
| BDCPP_L | 329 | 35 | 6.3 |
| DBuP | 209 | 153 (79) | 6.2 |
| DBuP_L | 227 | 79 | 6.2 |
| DPhP | 249 | 155 (93) | 5.9 |
| DPhP_L | 259 | 98 | 5.9 |
| TBBA | 436.7 (434.7) | 392.7 (390.7) | 9.3 |
| TBBA_L | 442.7 | 398.7 | 9.3 |
| UMB | 175 | 103 | 7.3 |
| UMB_L | 179 | 21 | 7.3 |
| DMP | 125 | 63 (110) | 2.6 |
| DMP_L | 130 | 63 | 2.6 |
| DEP | 153 | 125 (79) | 3.1 |
| DEP_L | 163 | 79 | 3.1 |
| DMT | 141 | 126 (96) | 2.7 |
| DMT_L | 147 | 97 | 2.7 |
| DMD | 157 | 112 (142) | 2.9 |
| DMD_L | 163 | 145 | 2.9 |
| DTP | 169 | 95 (141) | 4.1 |
| DTP_L | 179 | 95 | 4.1 |
| DED | 185 | 111 (157) | 4.5 |
| DED_L | 195 | 111 | 4.4 |

** RT may shift but within an acceptable range*

### 1) Sequence Setup

- Open Analyst® Software.
- Choose correct method from drop down menu at the top of the screen.
- To import a sequence: Double click on the 'Build Acquisition Batch' Icon.
- The 'Batch Editor' box will open. From the 'sample' tab, right click in the empty space, and choose 'import from file'.
- Select the .xls file with the correct sequence.
- Choose the correct 'Quantitation' and 'Acquisition' files from the respective drop-down menus. Fill in tray number and vial position.
- Choose the 'Quantitation' tab and fill in all concentration levels for each analyte.
- Save sequence by choosing "File, Save as" and filling in the appropriate file name.

### 2) Run the sequence

- For more detailed information about this topic, please see Analyst Software Operator's Manual-Creating Batches, Submitting Batches and Controlling the Acquisition Queue.
- In Analyst Software, click on the 'equilibrate' icon located on the top tool bar.
- Choose the correct 'Acquisition Method' and press the 'OK' button.
- Allow this method to equilibrate for about 30 minutes prior to running the sequence. Also carefully monitor the icons in the bottom right-hand corner of the Analyst Software to ensure that they are green.
- In order to run the standard check sequence, open 'Test" batch file.
- Input the correct date, acquisition method, and sample position and save the sequence.
- Click on the 'Submit' tab, and choose the correct sample, then click on the submit button.
- Ensure that a sample is in the correct vial position and click on the icon 'start sample'.
- Once the sample has finished running, click on the 'Open Data File'. Select the correct data file and sample and review. If sensitivity and counts appear to be in the appropriate range, proceed to running sequence for real samples.
- Open batch file for appropriate sequence.
- Ensure that samples are in the correct well position.
- Click on the 'Submit' tab, and choose all samples, then click on the submit button.

## e. Processing data

- For more detailed information about this topic, please see MultiQuant Software Operator's Manual-Viewing and Organizing Data.

- To process a batch of samples:
    - Double-click on the ‘Quantitation Wizard’ icon and choose the correct data file and samples.
    - Then follow the prompts from the ‘Wizard’ and choose your correct quantitation method, and then click ‘Finish’.
    - The ‘Wizard’ will process the samples and create a results table.

### f. Quantification

- For more detailed information about this topic, please see MultiQuant Software Operator’s Manual-Analyzing Data.
- After processing the samples, manually evaluate the chromatograms for correct peak detection and baseline selection. This is most easily done by opening the ‘Display the peak review’, and the ‘Display the calibration curve’, and monitoring each peak of each analyte for correct peak choice and integration.
- After each analyte peak has been checked and all work has been saved, the results can be exported.
- To export results, click the ‘File’ drop down menu from the top left corner, and click on ‘Export’.
- Name the file appropriately and click ‘Save’. A text file has been created that can be opened in Microsoft ‘Excel’.

### g. Rearrangement of Data Files

Data are automatically rearranged into a multiple worksheet (Excel format) ® file that is compatible with our division’s database, STARLIMS, using an Excel® macro. This macro also allows for analyst evaluation of quantification and confirmation ions.

### h. Transfer of Data

Upon completion of an analytical run, the computer folder containing all the raw data from the run is automatically transferred to the CDC network, or alternatively, transferred to a CDC-approved USB drive, and then to the CDC network where the data will be backed up on a regular basis.

All data manipulation such as integration and review are undertaken in a shared network drive within the CDC network. Once the raw data has been processed and the results reviewed, a result table is generated as a text document from the MultiQuant software. Using macros, the results included in the result table are converted into Microsoft Excel document and imported into the STARLIMS database for further review and reporting.

### i. Importation of Data into Database

Select “Upload data” option in STARLIMS database. A password is required to access the database.

**j. Statistical Analysis and Interpretation of Data**

Data are exported from the STARLIMS database into SAS. SAS programs for QC evaluation have been created by the Division statisticians and may be executed in SAS when this information is needed.

**k. Routine 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.

***Mass Spectrometer***

- Preventive maintenance is required every 6 months and it is performed by an ABSciex Service Engineer. Instrument inspections, testing, cleaning and part replacements are done according to the manufacture guidelines. All records for this maintenance are kept electronically in the instrument computer.

- In general, the maintenance procedures of calibration and cleaning curtain plate are performed by the operator if there is a decrease in the system performance (sensitivity or S/N ratio) without any other apparent technical reasons.

- The frequency of cleaning the components of the mass spectrometer depends on the type and amounts of samples and solvents that are introduced into the instrument.

***Agilent 1290 HPLC-Systems***

Systems undergo annual routine maintenance by service technicians. All records for this maintenance are kept electronically in the instrument computer.

The HPLC systems require only a few simple maintenance procedures to keep them in optimum working condition.

- Check the solvent tubing and connections for leaks
- Back-flush or change analytical column as necessary (usually every 1000 injections or before, if peak tailing or high pressure is observed).
- Replace pre-column filters after each batch.
- Check needle position into vials & sample wells.
- Ensure that the solvent reservoir contains sufficient running solvent for all samples.

## 9. METHOD PERFORMANCE DOCUMENTATION

### a. Accuracy

The accuracy of this method is determined by recovery analysis after spiking two biological materials with the analytes of interest at zero concentration and 3 different concentrations in the reportable range (10 ng/mL, 20 ng/mL, and 30 ng/mL). Three replicates were spiked per concentration level resulting in a total of 12 samples which were analyzed in two analytical runs on 2 separate days. Recovery of the added analyte is calculated as [(final concentration - initial concentration) / added concentration] *100. Recovery should be 85-115% except at 3*LOD where can be 80-120%. Any deviations from these ranges require approval from the CLIA Laboratory Director.

**Table 5. Accuracy**

| | Accuracy (%) | | |
|---|---|---|---|
| Analyte | 10 ng/mL | 20 ng/mL | 30 ng/mL |
| DBuP | 103.0 | 102.5 | 97.9 |
| BCPP | 99.1 | 98.9 | 98.8 |
| DPhP | 100.5 | 101.8 | 102.5 |
| BDCPP | 98.4 | 97.9 | 97.2 |
| TBBA | 102.3 | 103.1 | 101.7 |
| BCEtP | 106.4 | 108.3 | 106.4 |
| DMP | 92.2 | 89.0 | 91.0 |
| DMTP | 98.9 | 100.0 | 97.0 |
| DMDP | 110.9 | 116.3 | 114.1 |
| DEP | 103.0 | 102.9 | 98.8 |
| DETP | 111.9 | 111.7 | 106.6 |
| DEDP | 116.2 | 118.3 | 118.7 |

### b. Precision

Precision is determined by calculating the relative standard deviation (RSD) of repeat measurements (N=20) of quality control materials at two concentrations, performed in duplicates during 10 different analytical runs. Within-run, between-run and total precision are calculated from these data. Relative standard deviation should not exceed 15%. Any deviations from these ranges require approval from the CLIA Laboratory Director.

**Table 6. Precision**

| Analyte | Precision (%RSD) | |
|---|---|---|
| | QCL 4 ng/mL | QCH 15 ng/mL |
| DBuP | 5.9 | 5.6 |
| BCPP | 4.1 | 4.2 |
| DPhP | 3.2 | 3.2 |
| BDCPP | 4.9 | 3.7 |
| TBBA | 3.0 | 2.6 |
| BCEtP | 6.0 | 5.1 |
| DMP | 8.5 | 6.9 |
| DMTP | 9.5 | 9.1 |
| DMDP | 6.1 | 6.8 |
| DEP | 8.0 | 9.5 |
| DETP | 5.1 | 5.1 |
| DEDP | 5.7 | 6.3 |

### c. Analytical Sensitivity

Analytical sensitivity is the lowest analyte concentration that can be measured with acceptable accuracy and precision, and it is expressed as the limit of detection (LOD). LODs are calculated as 3S0, where S0 is the estimated standard deviation (SD) at zero concentration and is determined by linear regression analysis of the absolute standard deviation (SD) versus concentration [(11)]. The detection limits vary based on the current operating precision and the cleanliness of the analytical system. The method detection limits for each compound can also be set by the concentration of the lowest detected calibration standard. For many analytes, the concentration of S1 is given as the detected lowest standard. The reported LOD can be higher than these values if the team lead feels this is necessary, but the value cannot be below these values. LOD`s may vary over time.

**Table 7. Analyte Detection Limits**

| Analyte | ng/mL |
|---|---|
| DBuP | 0.1 |
| BCPP | 0.1 |
| DPhP | 0.1 |
| BDCPP | 0.1 |
| TBBA | 0.05 |
| BCEtP | 0.1 |
| DMP | 0.1 |
| DMTP | 0.1 |
| DMDP | 0.1 |
| DEP | 0.1 |
| DETP | 0.1 |
| DEDP | 0.1 |

### d. Analytical Specificity

Potential interfering substances are evaluated during method development using samples from approximately 50 persons.

This method requires that the analytes: 1) co-elute with the corresponding isotope labeled internal standard analog; 2) elute at a specific retention time; 3) have precursor ions with specific mass/charge ratios; and 4) have two specific product ions formed from the precursor ion with specific mass/charge ratios. The quantitation and confirmation ions for each analyte are listed in Table 4.

### e. Stability

Freeze and thaw stability of the analytes was determined by analyzing three replicates of two quality control materials after three freeze-thaw cycles and comparing the values with the initial measurements (N=6). Bench-top stability was assessed by analyzing two quality control materials in triplicates after storing at room temperature for one day and comparing the values with the initial measurements (N=6). Stability of the processed samples were determined by analyzing processed samples from two quality control materials in triplicates after storing them at room temperature for one day and comparing the values with initial measurements (N=6). Long term stability of the analytes will be assessed by analyzing two quality control samples in triplicates after storing at or below -70 °C for two years (N=6).

**Table 8. Stability Test Results**

| Analyte | Stability Tests (%absolute difference from initial measurement) | | |
|---|---|---|---|
| | Free-thaw Stability (3 cycles) | Bench-top Stability | Processed Sample Stability |
| DBuP | 3.4 | 1.8 | 2.1 |
| BCPP | 1.4 | 2.1 | 3.1 |
| DPhP | 1.1 | 3.2 | 3.5 |
| BDCPP | 2.4 | 4.5 | 0.9 |
| TBBA | 0.9 | 0.7 | 0.1 |
| BCEtP | 1.6 | 0.3 | 0.4 |
| DMP | 0.7 | 4.1 | 2.9 |
| DMTP | 0.5 | 4.5 | 0.6 |
| DMDP | 0.4 | 0.1 | 0.6 |
| DEP | 1.6 | 0.6 | 0.9 |
| DETP | 1.0 | 0.2 | 1.8 |
| DEDP | 0.9 | 1.8 | 0.1 |

### f. 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. However, samples with analytical data values exceeding the highest reportable limit may be

diluted, re-extracted, and reanalyzed so that the measured value will be within the range of the calibration.

**g. Linearity Limits**

The high linearity limit is determined by the highest standard analyzed in the method. The low end of the linear range is limited by the method LOD. Concentrations which are below the method LOD are flagged as non-detectable. Calibration curves with a minimum of 10 standard points are calculated for each batch from the area ratios ([analyte peak area]/ [internal standard peak area]) from freshly analyzed standards and linear regression analysis where each concentration is weighed by 1/[measured concentration]. Acceptable calibration curves have correlation coefficients normally greater than 0.98; the lowest calibration point should be at or below the LOD and the highest calibration point has to be above the expected range of results for most samples. Samples with concentrations exceeding the highest calibration standard are re-extracted using less urine. The linear range for DMP, DMTP, DMDP, and DEP is from LOD to 40 ng/mL, and that for rest of the analytes is from LOD to 20 ng/mL.

**h. Reference Range (normal range)**

The results from the National Health and Nutrition Examination Survey (NHANES) can be used as reference ranges to describe exposure to these chemicals (or their precursors) among the general U.S. population[12]. Reference values for the urinary flame retardant metabolites and six DAPs are available at https://www.cdc.gov/exposurereport/.

## 10. Quality Control (QC) Procedures

### a. Individual samples (i.e., standards, unknown samples, and quality control (QC) materials) QC procedures

i. The relative retention time (RT) (ratio of $RT_{analyte}$ and $RT_{IS}$) of standards, unknowns, and QCs should be within 0.90 ~ 1.10. If the relative RT falls outside the range, check the RT(s) of the peaks of analyte and IS to make sure the program picked the correct signals for integration.

ii. The area counts of IS for each analyte should meet minimum requirements (e.g., not deviate more than 75% from the mean area counts of IS for standards within the same batch). Low IS area count could indicate strong ion suppression from sample matrix, or simply missing of IS (i.e., error in spiking). Depending on the findings, either re-extract the original sample or dilute the sample first and re-extract it.

iii. The calculated concentration of the reagent blank should be less than three times the LOD. Using the current method, all standards, blanks and

unknown samples are prepared by the same procedure, thus the background is automatically subtracted from unknown levels and represented as the intercept of the calibration curve. If the background levels are consistently high, the reagents used for sample preparation and (or) mobile phases need to be checked for potential contamination.

iv. The ratio of the calculated concentration of quantitation ion (Q1) and the calculated concentration of confirmation ion (Q2) for a given analyte should be within 30% of the ratio obtained for standards and QCs for the same analyte (e.g., be 0.7-1.3), when the Q1 concentration is greater than 10 times the LOD).

v. When sample (A+1) runs after a sample (A) which contained a high concentration of any given analyte (e.g., ~ ppm levels), sample (A+1) might have to be repeated (or reinjected) to eliminate the possibility of carryover. If the calculated carryover amount (0.05 % x concentration of sample A) is greater than 30% of the calculated concentration of sample (A+1), sample (A+1) needs to be reanalyzed.

vi. If a given analyte concentration in an unknown sample is above the highest calibration standard, the sample needs to be re-analyzed with a smaller amount of urine or a dilution.

vii. Unknown samples, for which all analytes concentrations fall below the LOD, may be re-analyzed to confirm that urine was dispensed in the plate.

## b. Analytical batch quality control procedures

### a. QC Materials

Urine pools enriched with known amounts of target analytes are used as the quality control materials. (QC) materials and are analyzed in each run with unknowns, blanks and standards.

### b. Collection of Urine for QC Pools

Collect the urine samples from multiple anonymous donors and screen the urine to ensure that the endogenous levels of target analytes are low or non-detectable. Combine and homogenize the urine samples (~24 hrs.) to form a base pool. The protocol for anonymous collection of urine to prepare QC pools was reviewed and approved by CDC's Institutional Review Board.

### c. Filtration and Dispensing

The urine pool may be pressure filtered with a 0.45-μm filter capsule and homogenized well for ~24 hrs. Filtration of urine is not essential for this method.

**d. Urine Enrichment**

Take two equal pools (~250 ml) from the base pool and reserve the rest for later use at or below -10°C. Enrich one of the pools with an appropriate amount of the stock solution of each target analyte to yield an approximate concentration of 4.0ng/mL (QCL) and enrich the other pool to yield an approximate concentration of 15ng/mL (QCH). Mix the individual pools thoroughly (~24hrs), aliquot in to 1.0mL portions and store in polypropylene vials at or below -20°C until use.

**e. Characterization of QC Materials**

Characterize the QC pools by analyzing them at least on 20 separate runs per instrument (2 from each level per run for 40 measurements). Use the data from these runs to establish the mean and both upper- and lower- 99% and 95% control limits.

**f. Use of QC Samples**

During each analytical run, four QC materials (two from each QCL & QCH levels) are analyzed together with unknown samples. The average value of the paired QCH and QCL samples is evaluated to determine a run either in-control or out-of-control.

**g. Final Evaluation of Quality Control Results**

The results of the QC materials analyzed with the study samples are evaluated according to standard Westgard multi-rule criteria[13] to determine a run either in-control or out-of-control. No data from runs considered out-of-control will be reported.

When using 2 QC pool levels per run, the rules are:

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

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

Abbreviations:

$S_i$ = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).
$S_m$ = Standard deviation of the run means (the limits are shown on the chart).
$S_w$ = Within-run standard deviation (the limits are not shown on the chart).

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

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until the source or cause of failure is identified and corrected. Check for any irregularities (e.g., low calibration curve regression coefficient, change in calibration curve slope or intercept, high reagent blank concentration, low internal standard sensitivity). If the source of failure is easily identifiable, for example, a pipetting error, correct the problem immediately. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure). After re-establishing calibration or QC, resume analytical runs.

## 12. Limitations of Method; Interfering Substances and Conditions

This method is an isotope-dilution mass spectrometry method, which is widely regarded as the definitive method for the measurement of organic toxicants in human specimens. By using tandem mass spectrometry, a large number of analytical interferences are eliminated. However, endogenous matrix components may occasionally interfere with quantification. Interferences with the internal standards result in rejection of that analysis. If repeat analysis still

results in an interference with the internal standard do not report the results for that analyte.

## 13. Critical-Call Results ("Panic Values")

Insufficient data exist to correlate urinary concentrations of these environmental chemicals with serious health effects in humans. Therefore, critical call values have not been established.

## 14. Specimen Storage and Handling during Testing

All samples and standards must remain frozen prior to use. Unnecessary storage of samples at room temperature during sample preparation should be avoided. The LC autosampler should keep extracts at about 10°C or colder.

## 15. Alternate Methods for Performing Test and Storing Specimens If Test System Fails

The method is designed to run on an LC-MS/MS instrument and is not transferable to other instrumentation. If the analytical system fails, prepared samples can be stored frozen in covered 96-well plate until the analytical system is restored for up to one week. Otherwise, samples can be re-prepared. If the storage system fails, urine samples are transferred to an alternate freezer; if a freezer is not available, the urine samples can be temporarily stored refrigerated for a maximum of 24 hours.

## 16. 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 and/or his/her designee. The supervisor provides feedback to the analyst and/or his/her designee and requests confirmation of the data as needed.
b. The person serving as 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 reporting the analytical results to the person(s) who requested the analyses to be signed by the CLIA Laboratory Director.
e. The data are sent (generally electronically by e-mail) to the person(s) that made the initial request.
f. All data (chromatograms, etc.) are stored in electronic format.

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

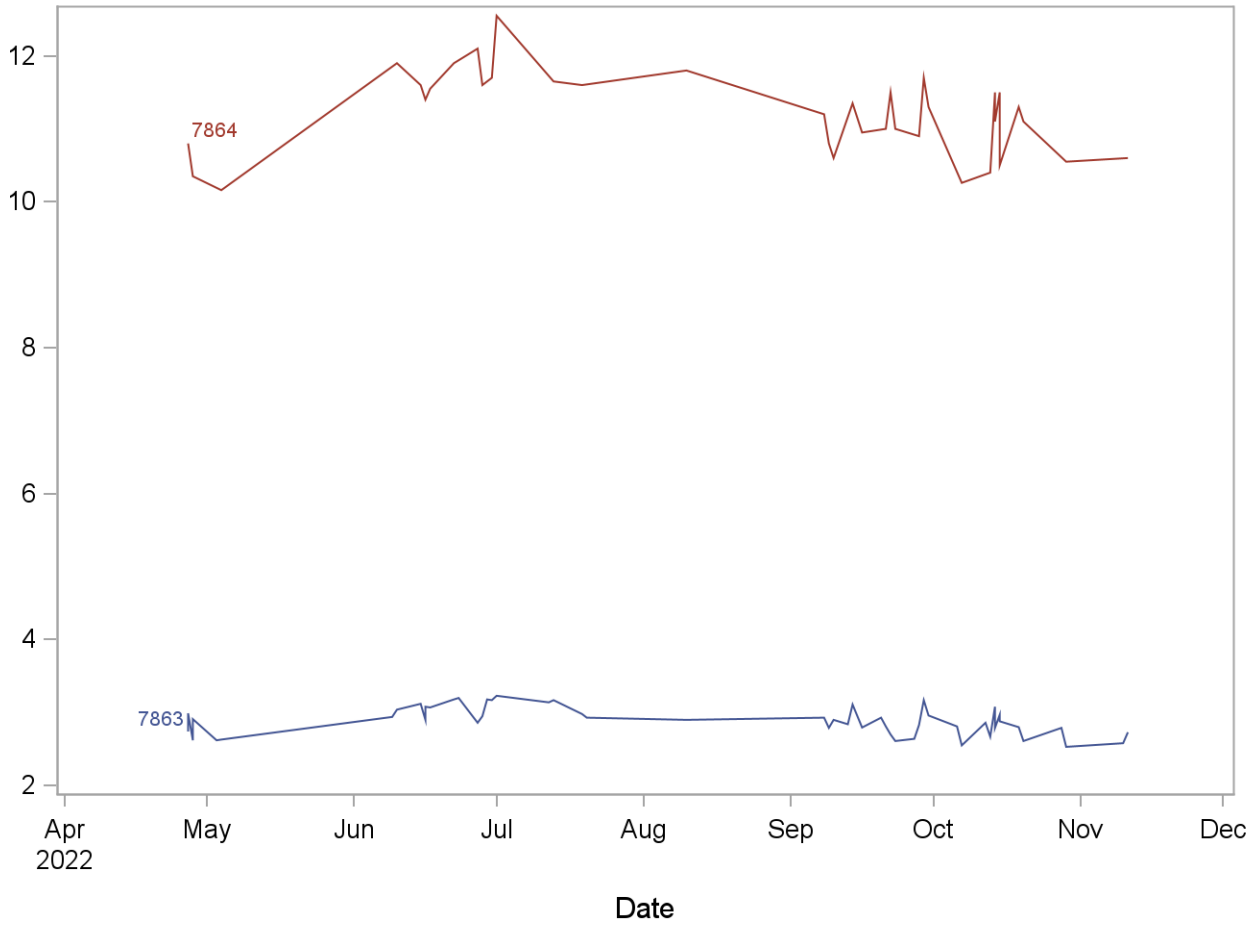
Use standard record-keeping systems (i.e., notebooks, sample logs, data files,) to keep track of the biological specimens. Transfer of CLIA-specimens is only permitted to certified laboratories. Any transfer of study samples is handled through the DLS special studies coordinator. One spreadsheet electronic file with information for receiving/transferring specimens is kept in the pesticides laboratory share drive folder. In this file, the samples received are logged in when received and when stored/transferred after analysis.

## 18. Summary Statistics and QC Graphs

Please see following pages.

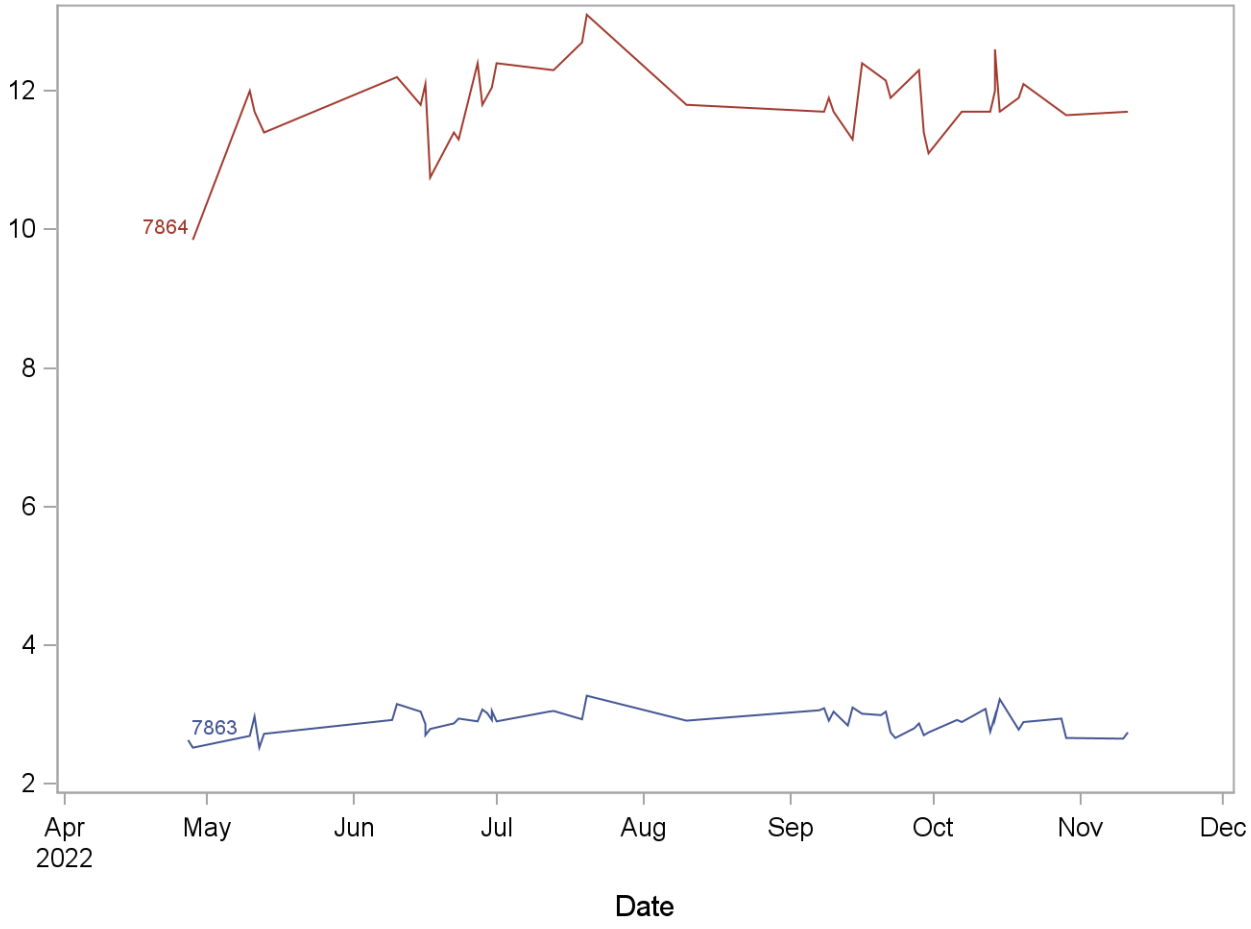
## Summary Statistics and QC Chart
## URXOP1 (Dimethylphosphate (ng/mL))

| Lot | N | Start Date | End Date | MEAN | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 7863 | 53 | 27APR22 | 11NOV22 | 2.89406 | 0.19607 | 6.8 |
| 7864 | 36 | 27APR22 | 11NOV22 | 11.21583 | 0.56451 | 5.0 |



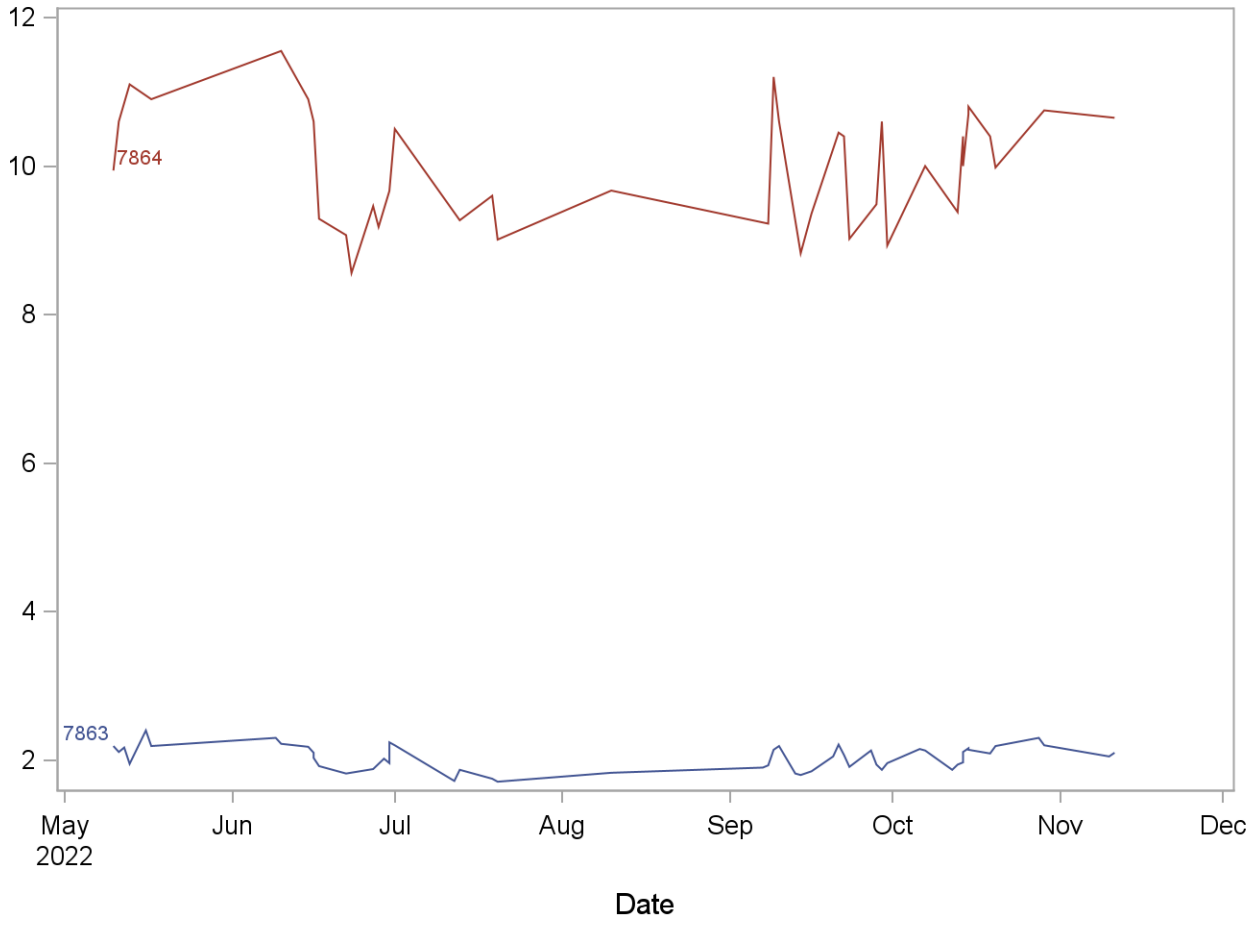
## Summary Statistics and QC Chart
## URXOP2 (Diethylphosphate (ng/mL))

| Lot | N | Start Date | End Date | MEAN | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 7863 | 53 | 27APR22 | 11NOV22 | 2.89453 | 0.16862 | 5.8 |
| 7864 | 38 | 28APR22 | 11NOV22 | 11.83289 | 0.56452 | 4.8 |



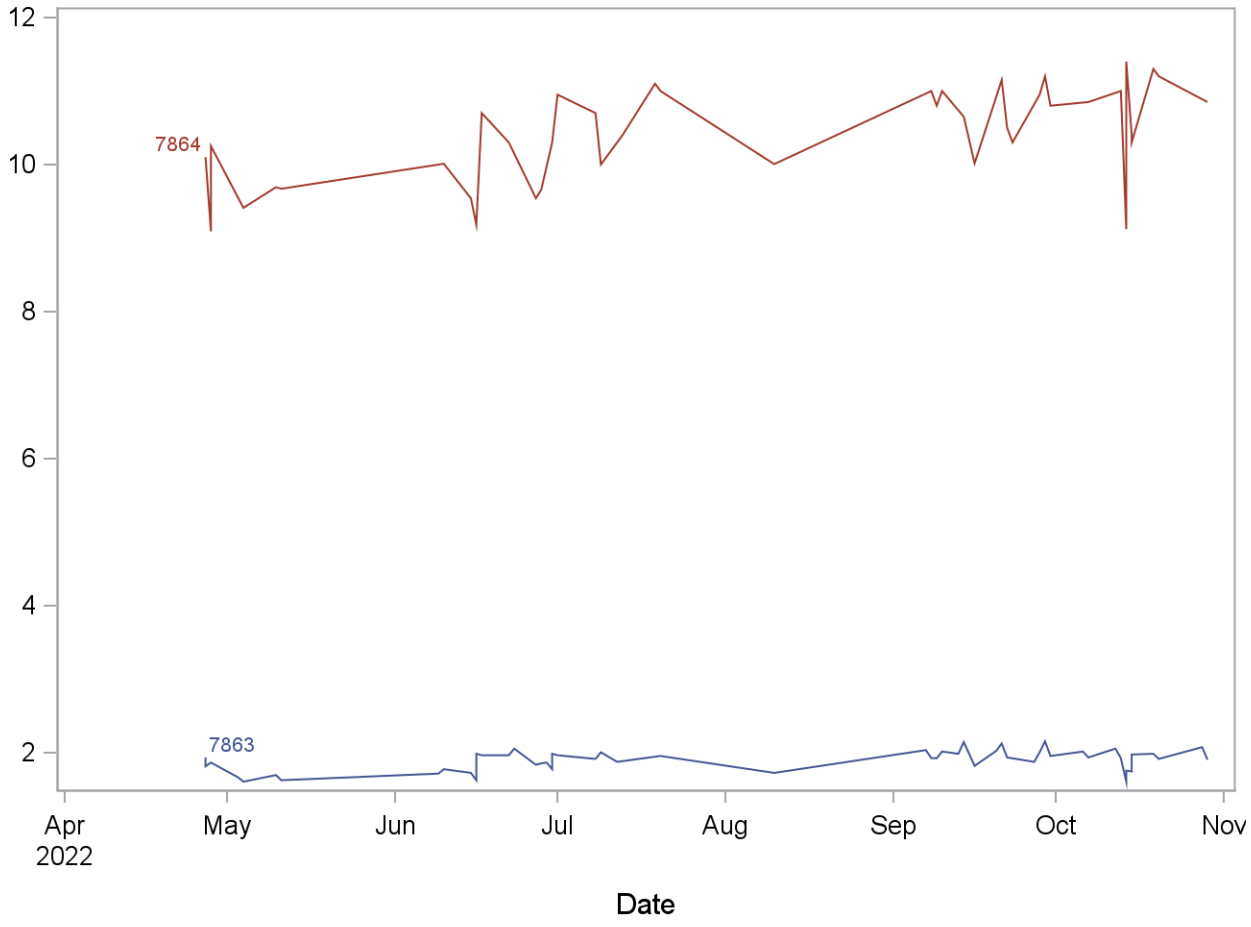
## Summary Statistics and QC Chart
## URXOP3 (Dimethylthiophosphate (ng/mL))

| Lot | N | Start Date | End Date | MEAN | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 7863 | 52 | 10MAY22 | 11NOV22 | 2.03712 | 0.16439 | 8.1 |
| 7864 | 39 | 10MAY22 | 11NOV22 | 10.00051 | 0.77137 | 7.7 |



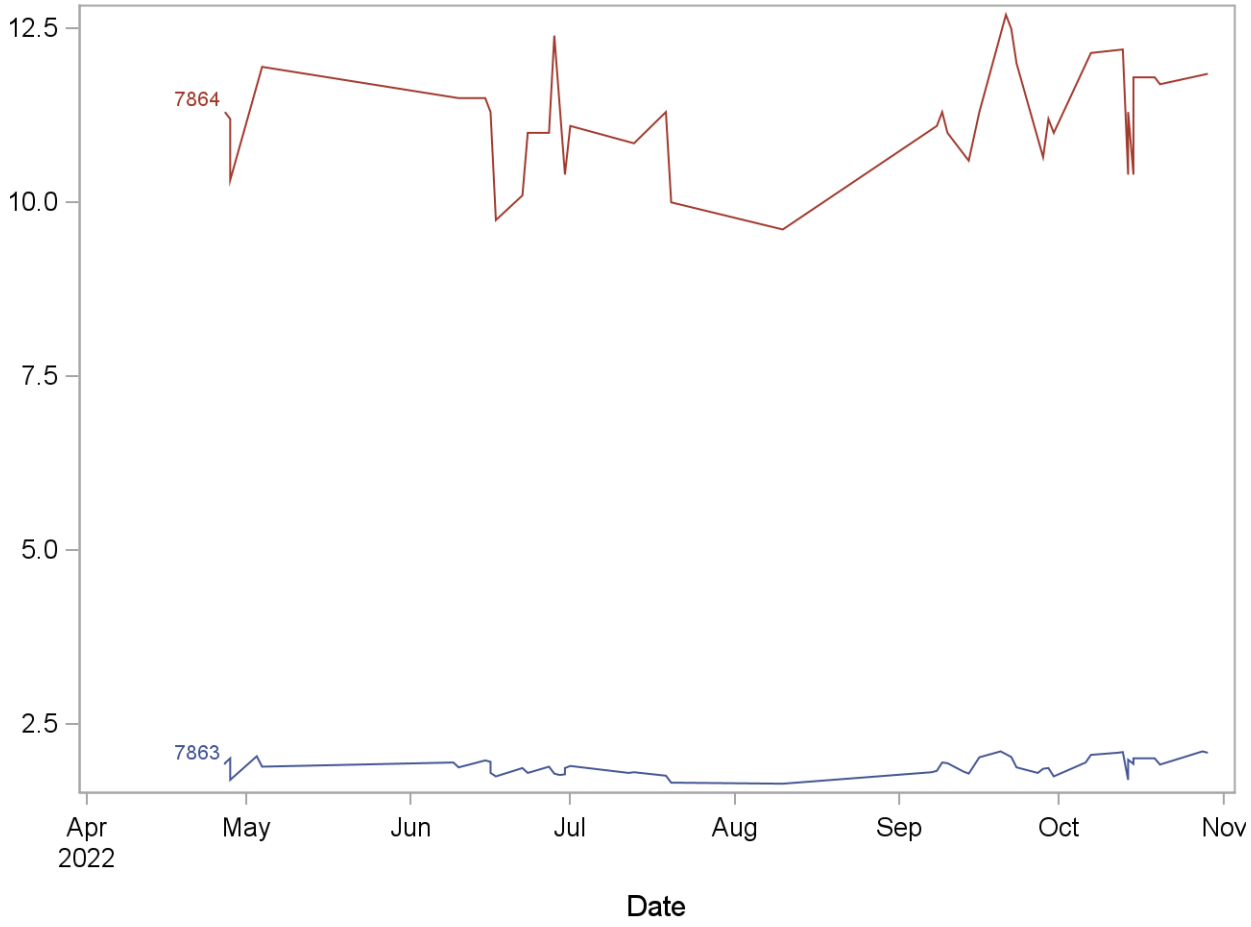
## Summary Statistics and QC Chart
## URXOP4 (Diethylthiophosphate (ng/mL))

| Lot | N | Start Date | End Date | MEAN | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 7863 | 53 | 27APR22 | 29OCT22 | 1.89179 | 0.13974 | 7.4 |
| 7864 | 41 | 27APR22 | 29OCT22 | 10.39951 | 0.64847 | 6.2 |



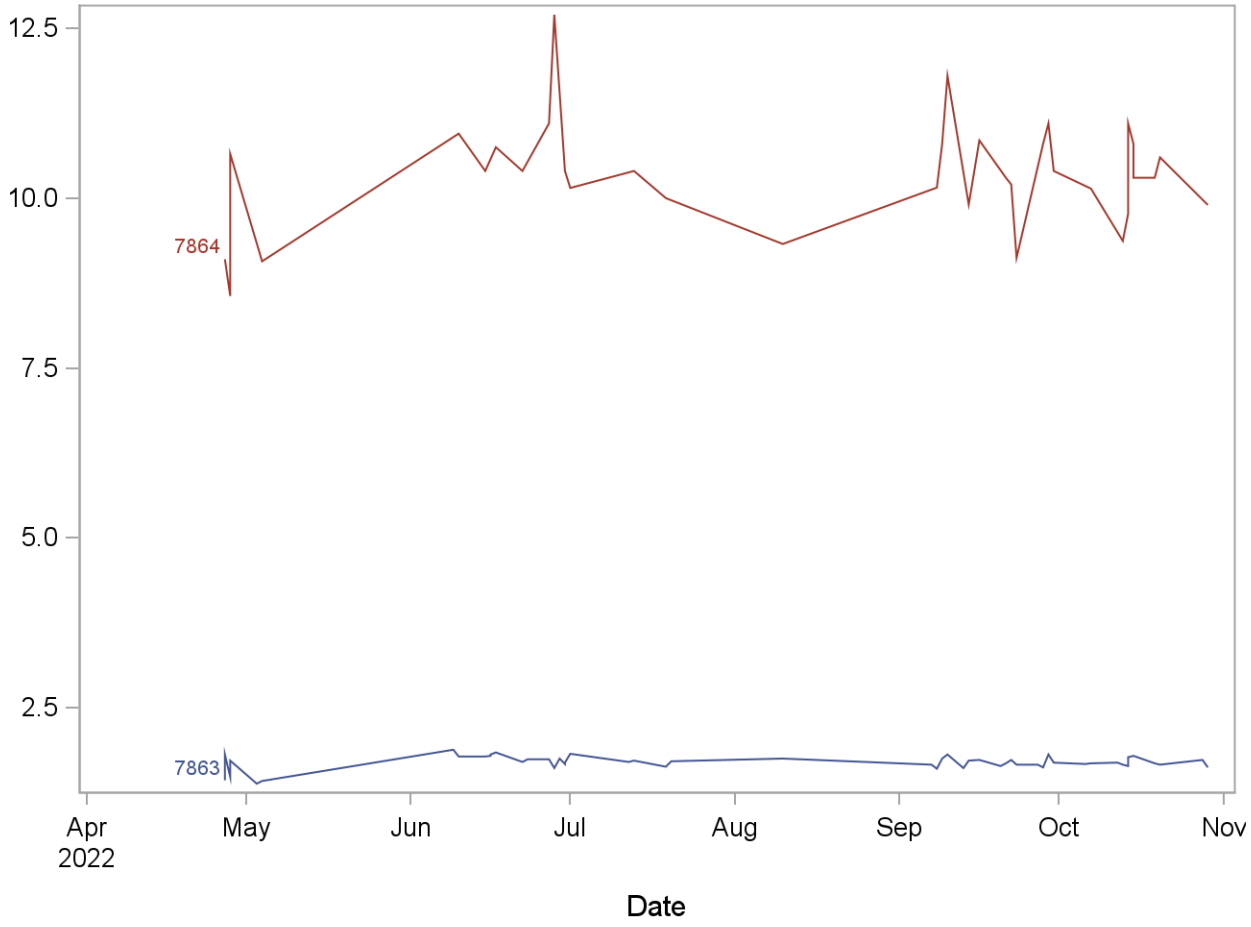
## Summary Statistics and QC Chart
## URXOP5 (Dimethyldithiophosphate (ng/mL))

| Lot | N | Start Date | End Date | MEAN | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 7863 | 52 | 27APR22 | 29OCT22 | 1.88731 | 0.12279 | 6.5 |
| 7864 | 38 | 27APR22 | 29OCT22 | 11.19803 | 0.75466 | 6.7 |



## Summary Statistics and QC Chart
## URXOP6 (Diethyldithiophosphate (ng/mL))

| Lot | N | Start Date | End Date | MEAN | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 7863 | 52 | 27APR22 | 29OCT22 | 1.69423 | 0.10235 | 6.0 |
| 7864 | 35 | 27APR22 | 29OCT22 | 10.33329 | 0.80047 | 7.7 |



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