Laboratory Procedure Manual

Analyte: **Specific Organophosphorus Pesticides, Synthetic Pyrethroids, and Select Herbicides (Universal Pesticides)**

Matrix: **Urine**

Method **Solid Phase Extraction-High-Performance Liquid Chromatography-Heated Electrospray Ionization Tandem Mass Spectrometry**

Method No: 6103.05

as performed by:

Organic Analytical Toxicology Branch
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Important Information for Users

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	Analyte Name
UPHOPM_H	URX24D	2,4-dicholorphenoxyacetic acid
	URX4FP	4-fluoro-3-phenoxy-benzoic acid
	URXCPM	3,5,6-trichloropyridinol
	URXPAR	*para*-Nitrophenol
	URXOPM	3-phenoxybenzoic acid
	URXOXY	2-isopropyl-4-methyl-pyrimidinol
	URXTCC	*trans*-dichlorovinyl-dimethylcyclopropane carboxylic acid

1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

In 2007, an estimated 857 million pounds of conventional pesticides were applied in the United States (EPA 2012). Chlorpyrifos was the insecticide used the most in the agricultural market sector with 7-9 million pounds applied. 2,4-D was the most used herbicide in the home and garden sector and the commercial sector, and it was the second most used herbicide in the agricultural sector. Malathion was the most used insecticide in the commercial sector and the second most used insecticide in the home and garden sector. Pyrethroids were the most used insecticide in the home and garden sector. In 2007, the United States used 22% of all pesticides applied throughout the world. The widespread use of pesticides and the scientific interest in potential adverse health effect of pesticides exposure have increased the demand for fast and robust analytical methods for measuring biomarkers of pesticides.

This method is used to determine the urinary concentrations of three organophosphorous insecticide metabolites (2-isopropyl-6-methyl-4-pyrimidiol, 3,5,6-tricholor-2-pyridinol, and *para*-nitrophenol), three synthetic pyrethroid metabolites (3-phenoxybenzoic acid; 4-fluoro-3-phenoxybenzoic acid, and trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid) and one herbicide (2,4-dichlorophenoxyacetic acid)**.**

b. Test Principle

The approach followed is a modification of previous methodology (Davis et al. 2013). The target analytes are extracted and concentrated from the urine matrix using an automated solid phase extraction system. Selective separation of the analytes is achieved using high-performance liquid chromatography with a gradient elution program. Sensitive detection of the analytes is performed by a triple quadrupole mass spectrometer with a heated electrospray ionization source. Analytes are identified using the specific m/z ion transition, the retention time and the ion ratio of the quantification and confirmation m/z ion transitions. Isotopically labeled internal standards are used for precise and accurate quantification. This method can be used to assess human exposure to select non-persistent pesticides by measuring their metabolites in urine. It does not directly test for any disease.

Table1. Analytes Measured, their Parent and Class Pesticides

Analyte	Abbreviation	NHANES Code	Parent Pesticide	Pesticide Class
2-isopropyl-4-methyl-pyrimidinol	IMPY	OXY2	Diazinon	O
para-Nitrophenol	PNP	PAR	Methyl parathion, Parathion	O
2,4-dicholorphenoxyacetic acid	2,4-D	24D	2,4-D	H
3-phenoxybenzoic acid	3-PBA	OPM	Permethrin, Cypermethrin, Cyfluthrin, others	P
3,5,6-tricholor-2-pyridinol	TCPY	CPM	Chlorphrifos, Chlorpyrifos-methyl	O
4-fluoro-3-phenoxy-benzoic acid	4F-3PBA	4FP	Cyfluthrin	P
trans-dichlorovinyl-dimethylcyclopropane carboxylic acid	*trans*-DCCA	TCC	Permethrin, Cypermethrin	P

O – Organophosphorus Pesticide
H - Herbicide
P – Pyrethroid Insecticide

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents can be toxic and/or carcinogenic. Special care should be taken to avoid inhalation or dermal exposure to the acids and solvents necessary to carry out the procedure.

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

Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure can be found at http://www.ilpi.com/msds/index.html. Laboratory personnel must review the MSDS prior to 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 measures

to avoid contact with the specimen (see “Protective equipment” below). A hepatitis B vaccination series is usually recommended for health care and laboratory workers who are exposed to human fluids and tissues. Observe universal precautions.

d. Mechanical Hazards

Following standard safety practices while performing this procedure minimizes the risk for mechanical hazards. Avoid any direct contact with the electronic components of the mass spectrometer unless all power to the instrument has been shut off. Only qualified technicians should perform electronic maintenance and repairs.

e. Protective Equipment

Use standard personal protective equipment when performing this procedure. Wear a lab coat; safety glasses; and durable gloves. Use chemical fume hood for this procedure.

f. Training

Anyone performing this procedure must be trained and experienced in the use of a triple-quadrupole mass spectrometer. Formal training is not necessary; however, personnel should be trained appropriately by an experienced operator of the instrument.

g. Personal Hygiene

Be careful when handling any biological specimen. Use gloves and wash hands properly.

h. Disposal of Wastes

Always dispose of solvents and reagents in an appropriate container clearly marked for waste products, and temporarily store them in a flame-resistant cabinet (follow CDC’s guidelines entitled Hazardous Chemical Waste Management) containers, glassware, etc., that come in direct contact with the specimens. Autoclave or decontaminate appropriately. Wash the glassware and recycle or dispose it in an appropriate.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

A working knowledge of XCalibur, the software controlling the HPLC-MSMS system is required. In addition, a basic understanding of the Division approved database called STARLIMS is required. Personnel performing this method must be able to create a run, create and export a sequence, and import the instrument data into STARLIMS. Personnel should also have a working knowledge of the basics of chemistry, SPE, HPLC-MS/MS systems including troubleshooting, maintenance and operation, and a working knowledge of basic chemical separations and analytical chemistry.

b. Sample Information

Sample information related to the analysis of a given sample is tracked with a CDC-generated ID number. This number is used as a reference number to track the location and status of any sample.

c. Data Maintanance

Data stored in STARLIMS are backed up daily. Raw instrument data are temporarily backed up on a CDC-approved USB device until transferred to the CDC network which is also backed up regularly (e.g., twice daily).

4. Specimen Collection, Storage and Handling Procedures; Criteria for Specimen Rejection

a. Sample Handling

Urine can be collected in standard urine collection cups. Samples should be refrigerated as soon as possible and transferred to specimen vials within a few hours of collection. Specimen handling conditions are outlined in the Division protocol for urine collection and handling available on 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 dry ice. To minimize the potential degradation of the specimen, special care must be taken to avoid prolonged exposure of the urine to room temperature or refrigerator temperatures after collection. Freeze all samples until analysis. Portions of urine that remain after the analytical aliquots are withdrawn should be refrozen as soon as possible.

b. Sample Rejection

Reject specimens that have leaked, are broken, or otherwise appear to be compromised. Specimens with volumes less than 0.5 mL may be rejected if they cannot be reliably processed. Specimens that meet the rejection criteria may be analyzed if they are unique and difficult specimens to collect (i.e: infant urine). The results from these specimens should be flagged in the final data report.

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

Not applicable for this procedure.

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

NOTE: Other reagents and standard sources, materials, equipment and instrumentation can be used as long as they have comparable specifications

a. Reagents and Sources

All reagents were used without further purification. Other standards and reagents with similar specifications may be used.

Table 1. Compound Structures

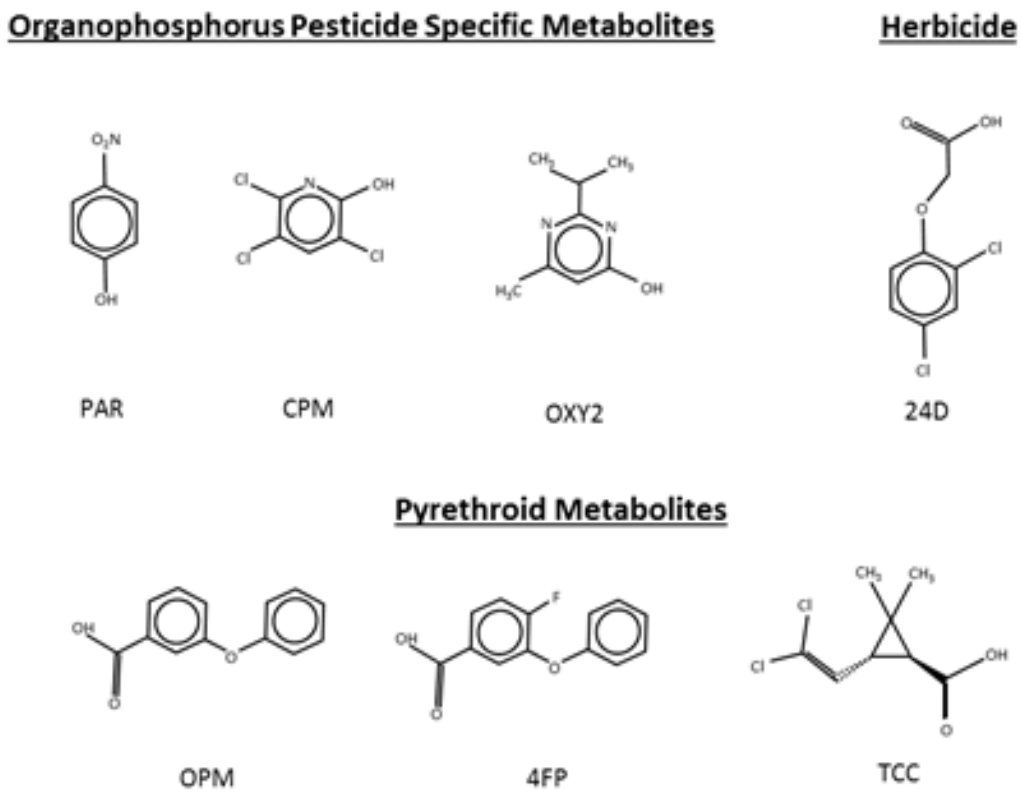


Table 2a. Reagents and Suggested Manufactures

Reagents	Suggested Manufacturers
Acetonitrile	Fisher Scientific Inc..
Acetone	Fisher Scientific Inc.
Methanol	Fisher Scientific Inc.
HPLC Grade Water	Fisher Scientific Inc.
Deionized water	NANOpure Infinity ultrapure water system
Glacial Acetic Acid	Sigma-Aldrich Co.
Sodium Acetate	Sigma-Aldrich Co.
β-glucuronidase type H-1 from *Helix pomatia*	Sigma-Aldrich Co.

Table 2b. Analytical Standards and Potential Sources

Analytes	Anayte Code	Source
Oxypyrimidine-methyl-4,5,6	OXY2	Cambridge Isotope Labs
$^{13}C_{4}$-Oxypyrimidine-methyl-4,5,6	OXY2_L	Cambridge Isotope Labs
para-Nitrophenol	PAR	Cambridge Isotope Labs
$^{13}C_{6}$-*para*-Nitrophenol	PARLL	Cambridge Isotope Labs
3,5,6-trichloro-2-pyridinol	CPM	Cambridge isotope Labs
$^{13}C_{5}$-3,5,6-tricholor-2-pyridinol	CPM_L	Cambridge Isotope Labs
2,4 Dichlorophenoxyacetic acid	24D	Cambridge Isotope Labs
$^{13}C_{6}$-2,4-Dichlorophenoxyacetic	24D_L	Cambridge Isotope Labs
3-phenoxybenzoic acid	OPM	Cambridge Isotope Labs
$^{13}C_{6}$-3-phenoxybenzoic acid	OPM_L	Cambridge Isotope Labs
4-fluoro-3-phenoxy benzoic acid	4FP	Cambridge Isotope Labs
$^{3}C_{6}$-4-fluoro-3-phenoxy benzoic acid	4FP_L	Cambridge Isotope Labs
trans-dichloro-dimethyl-cyclopropane carboxylic acid	TCC	Cambridge Isotope Labs
$^{13}C_{4}$-*trans*-dichlorovinyl-dimehtylcyclopropane carboxylic acid	TCC_L	Cambridge Isotope Labs
3-chloro-2-phenoxybenzoic acid.	3C2P	Aldrich Chemicals

All reagents were used without further purification. Other standards and reagents with similar specifications may be used.

b. Reagent Preparation

1) Liquid chromatography mobile phases

Mobile Phase A: 5% Methanol in 0.1% Acetic Acid in HPLC Grade Water. For a 1 L solution, pipette 0.95 mL of >99% glacial acetic acid in 950 mL of HPLC Grade Water. Then, add 50 mL of methanol optima grade to the 0.1% acetic acid solution. Mix thoroughly prior to use.

2) Buffer Solution

For a 700 mL solution, add 2.2 mL of >99% glacial acetic acid and 6.8 grams of sodium acetate to 700 mL of deionized water. Mix thoroughly until sodium acetate is fully dissolved. This solution should be stored refrigerated.

3) Enzyme Solution

This solution should be prepared at least 1 hour prior to use. For β-glucuronidase type H-1 from Helix pomatia with a specific activity of ~500 units/mg, 0.149 g should be added to 100 mL of 0.2M sodium acetate buffer in a 125 mL flask. Let enzyme buffer solution sit for 1 hour to fully dissolve. Do not swirl or mix the solution until the enzyme has fully dissolved.

4) Conditioning Solution - 1% acetic acid in water

For a 1 L solution, add 10 mL of >99% glacial acetic acid to 990 mL of deionized water. Mix thoroughly. This solution can be stored at room temperature.

5) Wash Solution - 25% methanol in 1% acetic acid in water solution

For a 1 L solution, add 250 mL of methanol optima grade to 750 mL of 1% glacial acetic acid solution. Mix thoroughly. This solution can be stored at room temperature.

6) Reconstitution Solution - 25% Methanol in HPLC grade water

For a 1 L solution, add 250 mL of methanol optima grade to 750 mL of HPLC grade water. Add enough solution of 3-chloro-2-phenoxybenzoic acid that the concentration will be approximately 3 ng/mL. Mix thoroughly. This solution can be stored at room temperature.

c. Standards and Quality Control Material Preparation

1) Individual Compound Stock Solutions

Individual stock solutions can be purchased from a commercial vendor, or prepared from neat material. When preparing from neat material, care should be taken to make a concentrated enough solution to cover the dilutions that will be needed. It is ideal for the solution to be prepared in one vial. To accomplish this, the neat material is weighed into a clean, screw capped vial (after noting its tare weight). The weight is recorded, tare the weight of the vial again, and the solvent is added. The weight of the solvent is recorded, and the density of the solvent is used to determine the final volume of the solution. A check of the density should be performed if the room is not at room temperature. Alternatively, the neat material can be weighed into a volumetric flask and the solvent added to the appropriate line on the flask. The solution is then transferred to another screw capped vial for storage. If the stock solution is purchased, it will most likely be received in a flame-sealed vial. The solution can be stored in this vial until use. The first time it is used, the solution should be transferred to a screw capped vial along with the vendor's label. All solutions should be stored at ≤-20 °C and allowed to come to room temperature before use. Acetonitrile is the typical solvent used to prepare stock solutions.

The receipt and preparation of every solution should be documented in the laboratory notebook. Each solution will be given a unique identifier comprised of the notebook number and page number of the solution. Minimum documentation

should also identify the source of each solution used in the preparation, the date prepared, expiration date, solvents used and the name of the person preparing the solution.

Every solution should be labeled with the unique identifying number, the name of the solution, the date prepared, the expiration date, the solvent and the name of the person preparing. The vial caps of solutions should never be labeled.

2) Internal Standard Spiking Solution

The concentration of each compound in the labeled spiking solution is dependent on the sensitivity of the instrumentation for that compound. Suggested concentration for labeled compounds in the spiking solution is 12 ng/mL, except for $^{13}C_5$ CPM and $^{13}C_4$ TCC, which is 24 ng/mL. This solution is prepared in two stages. First, a high level (e.g., ppm) solution is prepared that includes all the compounds. This solution is prepared at a volume that will fit in one vial for easy storage and tracking. From this high level combined solution, dilutions are made to bring the concentration to the appropriate spiking concentration. These dilutions are typically done at a larger volume to facilitate using the same solution for an entire project. Several dilutions can be made from the high level solution as needed. Because it is possible that the individual stocks may be in different solvents, which prevents an accurate determination of the overall solvent density, these solutions are typically made by aliquoting a given volume into a clean volumetric flask and adding additional solvent to bring the solution to the prescribed volume. For sample analysis, 50µL of this solution is aliquotted into every sample.

3) Calibration Standard Solutions

The range of concentrations for each analyte is based on instrument sensitivity and the concentrations typically found in an unexposed population based on previous research. The lowest concentrations of the calibration curve is meant to be below the detection limit and therefore, may not be used in the calibration plot. From the individual stocks, several high level combined solutions are made grouping compounds with similar concentrations in the same solution. The high level combined stock solutions are made by aliquoting the individual stocks solutions into a clean volumetric flask and bring the volume up with acetonitrile. From these high level solutions, aliquots are added to the calibration standard solutions. Several calibration curves can be prepared, as needed, from the high levels stocks, but each preparation should be documented separately as a new calibration curve. Calibration solutions are not made as dilutions of higher concentration calibration solutions. Due to the small volume of the calibration solutions, they are typically prepared in screw capped v-vials. The total volume alliquotted from the stocks is determined, and additional solvent is added by pipet to bring the volume to the prescribed final volume. For sample analysis, 50µL of each solution is aliquotted into separate aliquots of matrix blank urine so that the final suggested concentrations range from 0.02-50 ng/mL.

4) Injection Standard Solution

The injection standard 3-chloro-2-phenoxybenzoic acid (3C2P) is used for intra-run comparison; therefore, the precise concentration of the standard is not critical. The solution is purchased in small amounts (mg) as neat material. Upon receipt, a weighted amount of acetonitrile is added to the vial. The concentration is determined assuming the weight from the vendor is correct and using the density of acetonitrile. From this stock solution, an aliquot is added to the reconstitution solution such that the final concentration is approximately 3 ng/mL.

5) Instrument Standard Check Solution

The instrument standard check solution is prepared as standard 4 in the calibration standards. To produce the sample, 50µL of this solution is added to matrix blank material and prepared along with the other samples in the analytical run. This extract is used to verify the instrument performance before the analytical run begins.

6) Quality Control Materials

a. Quality Control and Proficiency Testing Pools

Quality Control and Proficiency Testing urine pools are produced from anonymous individual urine samples which are screened for the presence of endogenous material. Two quality control pools and three proficiency testing pools are produced. Depending on the amount of endogenous material found in the urine specimens used to produce the pool, additional material may need to be added in free form to produce the desired concentration. Whenever possible, this additional material should come from a secondary source from that used in the calibration standards. The concentration of the quality control pools is determined by procedures found in the Policies and Procedures manual available on the DLS intranet. Briefly, the pools are characterized with a minimum of 20 analytical runs with the target analyte mean and limits determined using the SAS program. Once the pool has been produced and the concentration of the target analytes determined through characterization, the pool can be aliquoted to small cryovials for long term storage.

b. Matrix Blank Material

Matrix blank material is produced from the same urine specimens that were previously screened. The selection of the urine specimens used is done to minimize the amount of endogenous material in the pool. Once the pool has been produced, it is diluted with HPLC grade water. The procedure for selecting the level of dilution is described in the Policies and Procedures manual available on the DLS intranet. Briefly, the pool is serially diluted with water and each level of dilution analyzed for all target analytes. The level

selected is the highest level of dilution which still produces slopes of the calibration curve similar to the undiluted urine.

c. Solvent Blank Material

The method does not use separate material for solvent blanks. The solvent blank sample in each analytical run includes all material added to a sample for sample preparation including internal standard and enzyme/buffer solution.

7) Calibration-Verification Materials

CLIA defines testing 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.

d. Materials

1) OASIS® HLB 30 mg/well 96-well Extraction Cartridge Plate *(Waters Corporation, Milford, MA,USA)*
2) Sample Vials, Clear Borosilicate Glass, Screw-Thread with Green Thermoset F217 & PTFE Lined Cap, assorted volumes *(VWR, Radnor, PA, USA)*
3) 2mL/well 96 square well collection plate *(Phenomenex, Torrance, CA, USA)*
4) 450µL pipet tips, 96-well plat format, 50 racks/carton *(Tomtec, Hamden, CT, USA)*
5) Argon gas *(Air Gas, Radnor Township, PA)*
6) Zero air and nitrogen from a PEAK generator *(PEAK Scientific, Billerica, MA, USA)*
7) 20 - 300 µL pipette tips (20 – 300 µL multi-channel pipette) 960/box *(Rainin Instrument LLC, Oakland, CA)*
8) 20 - 20 µL pre-sterilized filter pipette tips (20 - 20 µL electronic pipette) 960/box *(Rainin Instrument LLC, Oakland, CA)*
9) 100 – 1000 µL pre-sterilized filter pipette tips (100 - 1000 µL electronic pipette) 960/box *(Rainin Instrument LLC, Oakland, CA)*
10) 500 – 5000 µL pre-sterilized filter pipette tips (500 – 5000 µL electronic pipette) *(Rainin Instrument LLC, Oakland, CA)*
11) Glass vial silanized spring inserts (0.2 mL) 100/bag *(Sun-Sri, Rockwood, TN, USA)*
12) HPLC pre-column frits 0.250 OD x 0.062 in. thick, 2 µm pore, 10/pack *(Upchurch Scientific, Oak Harbor, WA)*
13) HPLC pre-column frits 0.250 OD x 0.062 in. thick, 0.5 µm pore, 10/pack *(Upchurch Scientific, Oak Harbor, WA)*
14) 2mL, clear glass I-D™, 12x32mm, flat base, target DP screw thread vials, 100/box *(National Scientific, Rockwood, TN, USA)*

15) Target DP screw caps with silicone septa (PTFE), assorted colors, 1000/case *(National Scientific, Rockwood, TN, USA)*
16) Pierceable sealing mats, 96 square well, ethylene vinyl acetate (EVA), 50/pack *(Phenomenex, Torrance, CA, USA)*
17) Anti-static, polystyrene, contour sides, EZ-pour, flexible 2 7/8 I.D. x 4 3/4L x 1 1/4in.D weigh boats *(Andwin Scientific, Tryon, NC)*
18) Bench Top Protector Sheets, case of 400 (green) *(Kimberly-Clark Professional, Roswell, GA, USA)*
19) Purification kit for low TOC systems 2000 Grains ion exchange capacity *(AQUA Solutions Inc., Jasper, Georgia)*
20) Replacement UV oxidizer lamp for UV2006X used on 12VDC system *(AQUA Solutions Inc., Jasper, Georgia*
21) 2 mL self-standing polypropylene Microtube *(Simport, Beloeil, Canada)*
22) Screw caps for microtubes with O-ring and flat tube, assorted colors, 1000/pack *(Simport, Beloeil, Canada)*
23) Automatic repeating pipette tip, 50 mL capacity, non-sterile *(Eppendorf, Hauppauge, NY, USA)*
24) Disposable SMS lab coats with knit collar and cuffs, assorted colors and sizes, 30/case *(LabSource, Romeoville, IL)*
25) High Five A+ Nitrile Exam Gloves, assorted sizes,1000/case *(High Five Company, Chicago, IL)*
26) Science Wipes 2-Ply Tissue, 14.7 in. x 16.6 in.,90/pack, 15/case *(Kimberly-Clark Professional, Roswell, GA, USA*
27) Safety glasses *(Thermo Fisher Scientific Inc., Waltham, MA)*
28) Polypropylene reagent reservoir with (3) baffles *(Tomtec, Hamden, CT, USA)*
29) HPLC analytical column; Betasil C18 100L x 2.1mm I.D., 3 µm particle size *(Thermo Fisher Scientific Inc., Waltham, MA)*
30) HPLC guard cartridge; Betasil C18 guard cartridge; 3µm particle size; 10L x 4.0mm I.D. *(Thermo Fisher Scientific Inc., Waltham, MA)*
31) Autoclave bag, PP, clear 31x38, 200/pk *(Thermo Fisher Scientific Inc., Waltham, MA)*
32) Sharps-a-Gator Sharps Container, 1 gallon (3.8 L), red *(Thermo Fisher Scientific Inc., Waltham, MA)*
33) Benchtop biohazard cup with lid; 40/pk *(Market Lab, Caledonia, MI)*
34) Purge valve assembly PTFE frit *(Agilent Technologies, Santa Clara, CA, USA)*
35) Needle seat capillary, 0.17mm id, 2.3 Ul *(Agilent Technologies, Santa Clara, CA, USA)*
36) Autosampler needle injector assembly, G1313A autosampler *(Agilent Technologies, Santa Clara, CA, USA)*
37) 100-place cryo boxes, assorted colors *(Thermo Fisher Scientific Inc., Waltham, MA)*
38) Ion transfer capillary removal tool *(Thermo Fisher Scientific Inc., Waltham, MA)*
39) Metal needle, 32-gauge, H-ESI *(Thermo Fisher Scientific Inc., Waltham, MA)*
40) Uniguard direct-connection guard cartridge holder *(Thermo Fisher Scientific Inc., Waltham, MA)*

41) Stainless steel pre-column inline filter holder for 2um and 0.5 um frits *(Upchurch Scientific, Oak Harbor, WA)*
42) 2 L media storage bottle with side neck and cap *(The Lab Depot, Inc., Dawsonville, GA)*

e. Equipment

1) Quadra 3 SPE Workstation *(Tomtec, Hamden, CT, USA)*
2) TurboVap® 96 Concentration Workstation *(Biotage LLC, Charlotte, NC, USA)*
3) Sartorius µLtramicro® Microbalance *(Sartorius AG, Goettingen, Germany)*
4) EDP2® electronic pipettes, assorted volumes (*Rainin Instrument LLC, Oakland, CA)*
5) E4 XLS Adjustable-spacer electronic multi-channel pipette 20 – 300 µL *(Rainin Instrument LLC, Oakland, CA)*
6) Brady 300MVP Plus label printer *(Brady Corporation, Milwaukee, WI)*
7) Positive-displacement electronic repeater pipette, 0.1 - 50 mL *(Rainin Instrument LLC, Oakland, CA)*
8) UV filter with built-in UV-oxidizer for house water *(AQUA Solutions Inc., Jasper, Georgia)*
9) Laboratory gas generator *(PEAK Scientific, Billerica, MA, USA)*
10) Isotemp 300 series incubator; Model 350D *(Thermo Fisher Scientific Inc., Waltham, MA)*
11) Flammables safety cabinet *(Thermo Fisher Scientific Inc., Waltham, MA)*
12) Digital Ultrasonic Cleaner; 4 qt. (3.8L) 5.5 x 9.5 x 6 in.*(Thermo Fisher Scientific Inc., Waltham, MA)*
13) Mini-Vortexer, 115 V *(VWR, Radnor, PA, USA)*
14) Large capacity mixer with pulsing 120V 100-2,000 rpms *(Glas-Col LLC, Terre Haute, IN)*
15) Special purpose under-counter freezer, flammable materials storage compatible, 5.6 cu ft. *(Thermo Fisher Scientific Inc., Waltham, MA)*
16) Stirrer/hot-plate; 4 x 5 in., 120V *(Corning Inc., Corning, NY)*

f. Instrumentation

1) High Pressure Liquid Chromotography system with two binary or quaternary pumps capable of delivering 0.5 mL.min flow rates, with column heating capabilities, and a separate 10-port switching valve.
2) ThermoFisher TSQ Quantum Ultra or Vantage triple-quadrupole mass spectrometer *(Thermo Fisher Scientific Inc., Waltham, MA)* equipped with a heated electrospray ionization (HESI) source.

7. Calibration and Calibration-Verification Procedures

a. Calibration Plot

1) A calibration plot is constructed using the instrument‘s Xcalibur software by performing a linear regression analysis of relative response factor (i.e., area native/area label) versus standard concentration with a 1/x weighting.

2) The lowest point on the calibration curve is at or below the measurable detection limits. If the signal does not meet the 3:1 signal-to-noise requirement, this point should not be used in the calibration plot. The highest point is above the expected range of results.
3) R-squared values for the curve must be greater than 0.98. Linearity of standard curves should extend over the entire standard range.
4) This calibration plot is used for the quantification of unknown and QC samples in the analytical run.

b. Verification of Calibration

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

c. Proficiency Testing

Proficiency testing should be performed once every 6 months.

For the pyrethroid metabolites, since 2010, the lab participates in the German External Quality Assessment Scheme (G-EQUAS) conducted by the Institute for Occupational, Environmental and Social Medicine at the Friedrich-Alexander University in Erlangen, Germany. Beginning in fall of 2014 and fall of 2015, 3,5,6-tricholor-2-pyridinol (CPM) and para-nitrophenol (PAR), respectively, were added to the G-EQUAS program. This assessment is conducted semi-annually. The two reference urine samples fortified with the pyrethroid metabolites 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/).

For the remaining metabolites, no formal PT testing program exists, and an in-house program is used. This in-house program currently includes pools prepared in-house or individually spiked but may also include independently prepared materials whose preparation was contracted out to an external laboratory. Once the in-house PT pools are characterized, their mean concentration and standard deviation 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 five randomly 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. The analytical results are evaluated by the PT administrator who is independent of the laboratory performing the analyses. 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 of its PT status (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 corrective action is taken, laboratory operations can resume.

8. Operating Procedures; Calculations; Interpretation of Results

a. Analytical Runs

An analytical run is comprised of 72 unknown samples, a solvent blank, a matrix blank, 12 calibration samples, two low-concentration QC samples, two high-concentration QC samples, and three instrument standard check samples. Specimens should be allowed to thaw and reach room temperature before aliquoting for analysis.

b. Sample Preparation

1. Sample Aliquoting and Incubation

a. A plate diagram is generated detailing the contents of all positions on the plate. This document is kept with the run sheet as raw data.
b. Before aliquoting samples, the pipette used will be checked for proper function by pipetting 1 mL of water into a tarred vial and recording the weight on the run sheet. If the result deviates by more than 0.2 grams from 1.0 grams, the pipette should not be used and should be noted for repair.
c. Fifty microliters (µL) of labeled spiking solution is added to every well
d. Fifty microliters of each calibration standard solution is added to the appropriate well designated on the plate diagram. Because the calibration curve consists of 12 points, these samples are usually kept on the same row on the plate.
e. Each thawed specimen and QC sample is vortexed, and 1 mL is aliquoted into the appropriate well on the plate.
f. For the calibration standards, 1 mL of matrix blank urine is aliquoted into each designated well. One more aliquot of the blank urine is designated as the matrix blank sample. Add 750 µL of enzyme solution to every well.
g. The entire plate is capped using a pierce able sealing mat.
h. The samples are placed in an incubator at 37°C for at least 6 hours, but typically overnight.

i. Samples are removed from the incubator and allowed to come to room temperature before beginning the SPE procedure.

2. Solid Phase Extraction (SPE) Procedure

a. The SPE part of the preparation is performed on the Quadra SPE system using the appropriate program. The process is semi-automated meaning the system does all the liquid handling, but considerable analyst intervention is required to maintain proper vacuum, empty waste, change solvent reservoirs and change collection plates. The pipette tips for the system have a total volume of 450µL. When the needed volume is larger than this, the transfer is complete in several repetitions.

b. The program completes the following procedure and includes steps not listed here to ensure proper pipetting and functioning:

1. The SPE plate is conditioned with 500µL of acetone
2. The SPE plate is conditioned with 500µL of conditioning solution described above.
3. The samples are loaded unto the SPE plate and allowed to elute. The pipet tips are discarded.
4. After the samples have eluted, the SPE plate is allowed to equilibrate for at least ten minutes without vacuum.
5. The extracts on the SPE plate are washed with 380µL of wash solution described above, and the pipet tips are discarded.
6. After the wash solution elutes, the vacuum is turned as high as possible and the plate is allowed to dry for at least 20 minutes.
7. The extracts are eluted from the SPE plate into a collection plate with 650µL of acetone.

c. Occasionally, a well on the SPE plate will become blocked due to precipitate or sediment in the samples. When this occurs, a small amount of positive pressure can be applied to the individual well using a pipet bulb.

3. Extract Concentration

a. Samples are concentrated to dryness in TurboVap® 96 Concentration Workstation at 40°C using pressurized nitrogen.

b. The nitrogen pressure should be adjusted low at the beginning to prevent cross-contamination. As the extract volume lowers, the pressure can be increased.

c. Occasionally, there are samples with a high aqueous content that will not concentrate. Add a small amount of acetone to these wells and continue concentrating. This can be repeated several times to drive off the aqueous portion.

4. Extract Reconstitution

a. Extracts are reconstituted on the Quadra 3 using the appropriate program, or the extracts can be reconstituted offline using appropriate pipettes.
b. 120µL of reconstitution solution is added to each well.
d. Since the autosampler used for analysis is capable of utilizing the 96-well plate. The extracts are left in the 96-well plate for analysis
e. The plate of extracts is capped with a pierce able silicone mat which seals each cell individually.
f. If the extracts are not going to be analyzed immediately, the plate is stored in a freezer.
g. Before analysis, the plate should be brought to room temperature.

5. Data Considerations

a. A run sheet is generated from the STARLIMS database that is used to track the samples through the analytical process.
b. Once a run of samples has been prepared, a sequence file for the mass spectrometer will be generated from the STARLIMS database.
c. The run sheet and the plate diagram for each run are considered raw data and will be kept in a project file.

c. Liquid Chromatography Conditions

a. The analytical method can be run in a single column mode where the analysis and column washing is done in the same analytical run, or an alternating column mode where the analysis is completed by one MS pump and the washing of the column is completed by a second MS pump. The advantage of the alternating column is the ability to analyze more field samples within the 24-hour window.
b. Instrumental conditions for liquid chromatography are specified in the Xcalibur instrument method file.
c. Pre-column setup: 2µm stainless steel frit, 0.5µm stainless steel frit, Betasil C-18 10x 4mm guard column.
d. A column switching valve is utilized to divert the mobile phase flow to the appropriate column.
e. HPLC Column: Betasil C-18, 2.1 x 100-mm, 3.0 µm particle size
f. Mobile Phase A: 95:5 0.1% Acetic Acid in HPLC-Grade Water: Methanol
g. Mobile Phase B: Acetonitrile
h. Single Column Analysis Gradient:
    1. Initial – 98% A, 2% B, hold for 2 minutes
    2. 4.0 minutes – 80% A, 20% B, hold for 2 minutes
    3. 7.0 minutes – 60% A, 40% B, hold for 1 minute
    4. 13.0 minutes – 40% A, 60% B, hold for 1 minute
    5. 14.5 minutes – 0% A, 100% B, hold for 2.5 minutes
    6. 17.01 minutes – 98% A, 2% B, hold for 3 minutes

i. Alternating Column Analysis
   a. Elution Pump Gradient
      1. Initial – 98% A, 2% B, hold for 2 minutes
      2. 4.0 minutes – 80% A, 20% B, hold for 2 minutes
      3. 7.0 minutes – 60% A, 40% B, hold for 1 minute
      4. 11.5 minutes – 46% A, 54% B,
      5. 11.51 minutes – 98% A, 2% B, hold for 3 minutes
   b. Washing Pump Gradient
      1. Initial – 98% A, 2% B,
      2. 1.0 minutes – 0% A, 100% B, hold for 7 minutes
      3. 7.0 minutes – 60% A, 40% B, hold for 1 minute
      4. 10.0 minutes – 98% A, 2% B, hold for 4.5 minutes
j. Flow Rate: 0.5mL/min
k. Injection Volume: 30µL
l. Column Temperature: 40ºC

d. Mass Spectrometry Conditions

a. Instrumental conditions for mass spectrometry are specified in the Xcalibur instrument method file.
b. A tune file and calibration file will have been generated by the field service engineer during the instrument's preventative maintenance. Table 3 list suggested tuning parameters that are optimized for this method.

Table 3. Tuning Parameters

MS Parameter	Setting
Ionization type	H-ESI
Ion polarity mode	Positive and Negative
Spray Voltage Positive Ion Mode	4000 mV
Spray Voltage Negative Ion Mode	3000 mV
Vaporizer temperature	300º C
Sheath Gas Pressure	60
Ion Sweep Gas Pressure	0
Aux Gas Pressure	20
Capillary Temperature	180º C
Tube Lens Offset	Set by mass with Tune file
Skimmer Offset	0
Collision Energy	Optimized per compound

Collision gas	Argon at 1.0 mTorr

c. It is suggested that the field service engineer use a tuning method specific for negative ions with either acetic acid clusters or phosphoric acid clusters.
d. The method is run in Selected Reaction Monitoring Mode.
e. The parent and product transitions for each target analyte are given in Table 5 along with the optimized collision energy and ion polarity mode.
f. The peak width for each compound is specified in Table 4. For CPM, TCC, and CCC, a higher mass resolution represented by a peak width of 0.2 is used in Q1 (the first quadrupole). All other compounds are run at the nominal (0.7) peak width. The peak width for all compounds in Q3 (third quadrupole) is 0.7.
g. Scan Time: optimized depending on the number of transitions in each window.
h. The data mode: Centroid.
i. A divert valve is used to divert mobile phase to waste during the first part of the analytical run

Table 4. Compound Specific Mass Spectrometry Conditions

Analyte	Ion Polarity Mode	Segment	Precursor (m/z)	Product (m/z)	Peak Width of Q1	Collision Energy (V)
OXY2	Positive	1	153	84	0.7	26
OXY2-C			153	70		25
OXY2-L			157	88		26
PAR	Negative	2	138	108	0.7	21
PAR-C			138	92		28
PAR-L			144	114		21
24D	Negative	3	219	160	0.7	15
24D-C			221	163		16
24D-L			225	167		31
TCC	Negative	3	206.97	207	0.2	5
TCC-C			208.97	209		5
TCC-L			210	210	0.7	6
CPM	Negative	3	195.91	196	0.2	7
CPM-C			197.91	198		7
CPM-L			202	202	0.7	7
4FP	Negative	3	231	93	0.7	42
4FP-C			231	187		19
4FP-L			237	99		42

OPM OPM-C OPM-L	Negative	3	213 213 219	93 169 99	0.7	42 17 42
3C-2PBA	Negative	3	247	93	0.7	21

e. Routine Operations

a. If needed, new pre-column frits and columns are replaced.
b. The mass spectrometer is set in the on position in the Xcalibur software tune page with the correct tune file loaded and the temperatures and pressures are allowed to equilibrate.
c. The HPLC system is also switched on with the initial conditions of the solvent gradient flowing and the column compartment heated coming to temperature.
d. Once the system has equilibrated, an instrument standard check sample is run to establish that the instrument is working properly. Each analyst will track the instrument check standard as they see fit and will be able to decide if the instrument is in proper working order. The critical demonstration of instrument sensitivity and operations is given with the calibration curve.
e. The analytical run is set up and run with the Xcalibur software.
f. After the analytical run, a column wash procedure may be run to clean the analytical columns.
g. At the end of analysis, the system is placed in standby mode until the next session.
h. It is recommended that the HPLC column be left in 100% acetonitrile when not being used.

f. Data Acquisition and Data Management Considerations

1) Xcalibur Sequence Setup
    1. Prior to running samples, a sequence file is produced in the Xcalibur software. This file can be imported from a Microsoft Excel file generated by the STARLIMS database, or it can be manually entered.
    2. The data filename for individual samples should follow the following format
        1. UP_Study Tracker Number_Instrument Letter_Analyst ID_Run Number_Sample Number.
        2. Example: UP_19990036-2007_O_guo4_S03_01
    3. All data for the analytical run will be kept in the same computer file , and the data path for project data will be similar to the data filename

1. UP_Study Tracker Number_Instrument Letter_Analyst ID_Run Number.

4. Additional samples are added to the sequence including instrument standard check samples at the beginning of the sequence, and a column wash procedure at the end of the run. These injections do not need to follow the sample file naming procedure above.

2) Data Management

1. Upon completion of an analytical run, the computer folder containing all the raw data from the run is transferred to a CDC-approved USB drive, and then to the CDC network. Once on the network, the files will be backed up on a regular basis.
2. For data reduction and review, the computer folder containing all the raw data is transferred to the hard drive of the computer where manipulation will take place. Once the manipulation has been completed, the entire computer file is again transferred to the CDC network and the old file is deleted.
3. Once the raw data has been processed and the results reviewed, a long report is generated in Microsoft Excel from the Xcalibur software. Using macros, the results included in the long report are imported into the STARLIMS database for further review and reporting.

g. Data Reduction and Review

1. The raw data from the run is processed using the Xcalibur software and a processing method
2. The processing method states the parameters for peak identification, integration and quantitation.
3. The Xcalibur software generates the least squares regression calibration curve for each compound based on the relative response ratio and the given concentrations of the calibration standards.
4. This calibration curve is used to quantify the unknown, blank and QC samples.
5. Specific compound and QC criteria are given below.
6. The analyst processes the data and manually verifies all integrations making changes to the automatic integration as necessary.
7. A second person, usually the Team Lead, then reviews all integrations in the file before the results are manipulated and imported into STARLIMS for reporting.

h. 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.

- **Daily**
  a. Change HPLC pre-column filters.
  b. Flush chromatography columns with copious amounts of mobile phase A and B at several concentrations. During this flush the temperature of the column can be raised to 50^{o}C to facilitate cleaning.
  c. The chromatography columns can also be reversed for the flushing and left in this position for the analytical run.
  d. Flush the probe with mobile phase at different concentrations. During this flush, the temperature of the probe can be raised to 450^{o}C, and the voltage of the instrument set to 0 to minimize the amount of material transferred to the instrument.
  e. Inject the instrument standard check extract and compare results to historical results for:
     1. Retention time of selected analytes.
     2. Sensitivity (area count of selected analytes)
     3. Chromatography (peak tailing, high background noise, anything odd)
  f. If problems are found with the first injection, inject the standard again. If problem still exist, do not continue until appropriate action is taken.
- **Weekly**
  a. Replace ion transfer tube while instrument is in stand-by.
  b. If several analytical runs have been completed in the week, the tube lens and skimmer of the instrument may need to be cleaned according to the instrument manufacturer's procedure.
  c. Clean the sweep cone, if needed. Clean ion transfer tubes using the manufacturer's suggested technique with nitric acid solution. Check for internal blockage and discard tubes which are blocked.
  d. Check the oil level in the rough pumps
     1. The closer to the top, the better but can be anywhere in between the two lines.
     2. The color should not look dark.
     3. It should not have black or other colored particles in it.
     4. If any of the factors indicate it needs to be changed, replenish as needed.
  e. Ballast the rough pumps on an as-needed basis. Do not leave open for longer than 30 minutes.

- **Quarterly**
  a. Flush IPA through the HPLC lines, A & B, (without column attached) for ~ 30 minutes each at 5 mL/min if pressure allows.
  b. Change the PTFE filter attached to the valve.

9. Method Performance Documentation

Method performance documentation for this method include accuracy, precision, sensitivity, specificity and stability. The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.

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) except for CBC3 which was spiked at 1 ng/mL, 2 ng/mL, and 3 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]. Recovery should be 85-115% except at 3*LOD where can be 80-120%. Any deviations from these ranges required approval from the Director. Current values are shown in Table 5.

Table 5. Accuracy of the Method

Analyte	Mean Accuracy (%)
OXY2	92
PAR	91.5
24D	93.2
CPM	95.1
OPM	97.1
4FP	97.6
TCC	97.7

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 required approval from the Director. Current values for total precision are shown in Table 6.

Table 6. Precision of the Method

Analyte	% RSD QC Low	% RSD QC High
OXY2	5.14	5.05
PAR	4.51	5.01
24D	6.94	6.85
CPM	13.91	8.68
OPM	5.3	9.61
4FP	7.1	6.09
TCC	13.54	11.28

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 $3S_0$, where S_0 is the estimated standard deviation (SD) at zero concentration and is determined by linear regression analysis of the absolute standard deviation (SD) versus concentration (Taylor 1987). 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 lowest detected 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. Current values are shown in Table 7 but LOD`s may vary over time.

Table 7. Analyte Detection Limits

Analyte	Low Standard (ng/mL)	$3S_0$ (ng/mL)
OXY2	0.2	0.01
PAR	0.2	0.07
24D	0.04	0.04
CPM	0.2	0.06
OPM	0.04	0.03
4FP	0.04	0.01
TCC	0.6	0.1

d. Analytical Specificity

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

This method requires that the analytes: 1) co-elute with the corresponding isotope labeled internal standard analog except for deuterated labeled internal standards

where minor retention time differences between labeled and native compounds are expected; 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 5.

e. Stability

Freeze and thaw stability of the analytes was determined by analyzing 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 after storing at room temperature for one day and comparing the values with the initial measurements (N=6). Stability of the processed samples was determined by analyzing processed samples from two quality control materials 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 after storing at or below -70C for two years (N=6). Results from stability testing are shown in Table 8.

Table 8. Stability Tests

	% Absolute difference from initial measurement		
Analyte	Freeze-thaw stability (3X)	Bench-top stability	Processed sample stability
OXY2	3.7	1.8	3.3
PAR	4.1	2.5	4.1
24D	9.2	1.9	12.6
CPM	6.3	1.6	8.1
OPM	6.8	4.2	10.9
4FP	8.1	2.5	5.8
TCC	6.4	3.2	4.9

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 eight 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 all compounds is from the LODs to 50 ppb.

10. Quality Control (QC) Procedures

Analytical batch quality control procedures

1) **QC Materials**
   - **a. Collection of Urine for QC Pools**
     1. Collect the urine and screen individual urine samples to determine the endogenous levels of pesticide residues.
     2. Select urine samples that contain very low levels of endogenous material and combine them to form the base pool for QC low and the blank urine used for calibration samples.
     3. Select urine samples with higher endogenous levels of metabolites and combine them to form the urine pool for QC high
   - **b. Urine Enrichment**
     1. Split the QC low pool into two separate pools. One pool will be used for the QC low pool and the other will be used for the blank urine used for the calibration standards. A larger volume of urine will be needed for the calibration standards pool than for the other pool.
     2. Giving consideration to the endogenous concentration of compound in the urine, fortify the QC low urine pool with analytical standards to produce concentrations around the third calibration standard level.
     3. Giving consideration to the endogenous concentration of compound in the urine, fortify the QC high urine pool with analytical standards to produce concentrations around the eighth calibration standard level.
     4. Homogenize all urine pools by mixing them overnight with a stir bar in a refrigerator.
   - **c. Characterization of QC Materials**
     1. Characterize the QC pools by at least 20 consecutive runs of each QC material as stated in the Division of Laboratory Sciences (DLS) Policy and Procedures Manual, Section 6. Quality Control.
     2. The data from the characterization runs is processed through the DLS SAS program to determine the confidence intervals for each pool. These intervals are then used for evaluating the QC samples analyzed with each analytical run of unknown samples.
     3. After a significant length of time, the QC pools can be re-characterized using the results from recent QC sample analysis.
2) **Use of QC Materials**

Each analytical run of unknown samples will also be comprised of two QC low aliquots and two QC high aliquots.

3) **Final Evaluation of Quality Control Results**

a. QC materials are evaluated using the Westgard multi-rule criterion that has been modified for use in the DLS (Caudill et al. 2008) as documented in the Policy and Procedures Manual, Section 6. Quality Control.

b. Two QC pools per run with two or more QC results per pool

1. If both QC run means are within 2Sm limits and individual results are within 2Si limits, then accept the run.
2. If 1 of the 2 QC run means is outside a 2Sm limit - reject run if:
    a. Extreme Outlier – Run mean is beyond the characterization mean +/- 4Sm
    b. 3S Rule - Run mean is outside a 3Sm limit
    c. 2S Rule - Both run means are outside the same 2Sm 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 2Si limit - reject run if:
    a. R 4S Rule – Within-run ranges for all pools in the same run exceed 4Sw (i.e., 95% range limit).
    b. Note: Since runs have multiple results per pool for 2 pools, the R 4S rule is applied within runs only.

c. Repeat out-of-control runs if residual sample is available. No data from runs considered out-of-control will be reported.

b. Individual samples (i.e., standards, unknown samples, and QC materials) QC procedures

The concentration measured in for the qualification ion transition should be within 25% of the concentration measured in the confirmation ion transition. If necessary, the extract can be re-injected. If the concentrations are not within this criterion, the result should be reported as “Interfering substance present.” This comparison is only applied when the concentration of the qualification ion transition is above the levels given in Table 9.

Table 9. Minimum Concentration of Qualifying Ion for Confirmation Comparison

Analyte	Minimum Concentration (ng/mL)
OXY2	0.4
PAR	1.5
24D	0.4

CPM	1.0
OPM	1.0
4FP	0.4
TCC	2.0

1) The relative retention time (RRT) of the native to label must fall within0.95 and 1.05 for all target analytes. If the RRT falls outside this range, the chromatogram should be examined closely to determine if the result is real. IF necessary, the extract can be re-injected with a different gradient to determine if the peak shifts with the label. No result should be reported outside the RRT criteria.
2) The ratio of the area of the internal standard to the area of the injection standard (3-chloro-2-phenoxybenzoic acid) should be within a defined. A low ratio could indicate ion suppression from sample matrix, a spiking error, or low recovery from the sample preparation. A high ratio could indicate a double spike.
3) The minimum signal-to-noise ratio for all native quantification ion peaks will be 3:1. This criterion is applied using the processing software but needs to be confirmed by the analyst.
4) The minimum signal-to-noise ratio for all labeled ion peaks will be 10:1 except for the labeled TCC peak which will be 3:1. As with the native ratios, the analyst must visually confirm the acceptable criteria.
5) If a valid result is found in the solvent blank sample from an analytical run, concentrations corresponding to three times the result will not be reported for the unknown samples associated with that blank.
6) The injection standard (3-chloro-2-phenoxybenzoic acid) is used to evaluate the instrument function during sample analysis. It is used as a diagnostic tool, and no specific criteria are required. If this peak cannot be found or is severely diminished in area compared to other injections, the injection of the sample may have been compromised and the extract may need to be reanalyzed.
7) If the measured target analyte ion ratio (native peak area/internal standard peak area) in an unknown sample is above the ratio of the highest calibration standard, the sample needs to be re-analyzed with a smaller amount of urine corresponding to a concentration that will be within the linear range of the ratios of the calibration curve.
8) When sample (A+1) run after a sample (A) which contained a high concentration of any given analyte, sample (A+1) might have to be repeated 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) may need to be reanalyzed.

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

If the calibration or QC systems fail to meet acceptable criteria, the extracts may be re-injected, or the samples can be re-extracted. If the failure continues, suspend all operations until the cause of failure is identified and corrected. 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 body fluids. By using high-resolution tandem mass spectrometry, you can eliminate most analytical interferences. Because of the matrix used in this procedure, occasional interfering, unknown substances have been encountered. 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. Reference Ranges (Normal Values)

The results from the National Health and Nutrition Examination Survey (NHANES) are used as the reference range to describe levels of exposure to these pesticides among the general US population (CDC 2019).

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

It is unlikely that any result would be a “critical call,” which would only occur with poisonings. Report test results in this laboratory in support of epidemiological studies, rather than clinical assessments. Data will help determine critical exposures.

15. Specimen Storage and Handling during Testing

Refrigerate urine samples overnight to expedite thawing prior to aliquotting the sample. Store the urine extracts in autosampler vials in a -20°C freezer after analysis. Stability studies suggest that the extracts remain stable at room temperature for up to 4 weeks.

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

The method is designed to run on a LC/MS/MS instrument and is not generally transferable to other instrumentation. If the system has failed, store sample refrigerated. You can store the extract samples for as long as 4 weeks. If you anticipate long-term interruption, store samples at or below -20°C.

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

Report data in support of epidemiological or health survey studies. At this time there is not protocol for reporting critical calls.

a. The data from analytical runs of unknowns are initially reviewed by the laboratory supervisor. The supervisor provides feedback to the analyst and/or his/her designee and requests confirmation of the data as needed.
b. One of the Division statisticians' reviews and approves the quality control charts pertinent to the results being reported.
c. 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 Division Director.
d. The data are sent (generally electronically by e-mail) to the person(s) that made the initial request.
e. All data (chromatograms, etc.) are stored in electronic format.
f. Final hard copies of correspondence are maintained in the office of the Branch Chief and/or his/her designee and with the quality control officer.

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

Use standard record-keeping systems (i.e., notebooks, sample logs, data files, creatinine logs, demographic logs) to keep track of all specimens. Transfer of refer to CLIA-specimens only certified laboratories. Any transfer of study samples is handled through the DLS special studies coordinator.

19. Notes

1. Special care should be taken to avoid loss of sample when thawing because cracks may occur in the sample containers. It is recommended to put samples in refrigerator overnight so that samples thaw slowly.

2. QC limits and means may vary over time as additional studies are completed and their QC data are added in the characterization.

3. Method specifications, including LOD and CV, are calculated for each study, so may vary slightly.

4. The analytical detection limits may vary from study to study as more data are available for statistical calculations. QC limits do not vary within studies, but remain constant.

5. The expiration time for the standard working solutions is determined by monitoring the peak intensity for each standard over time in the analytical runs.

6. The expiration time for the Quality Control material is determined by monitoring the concentration of each QC over time in the analytical runs.

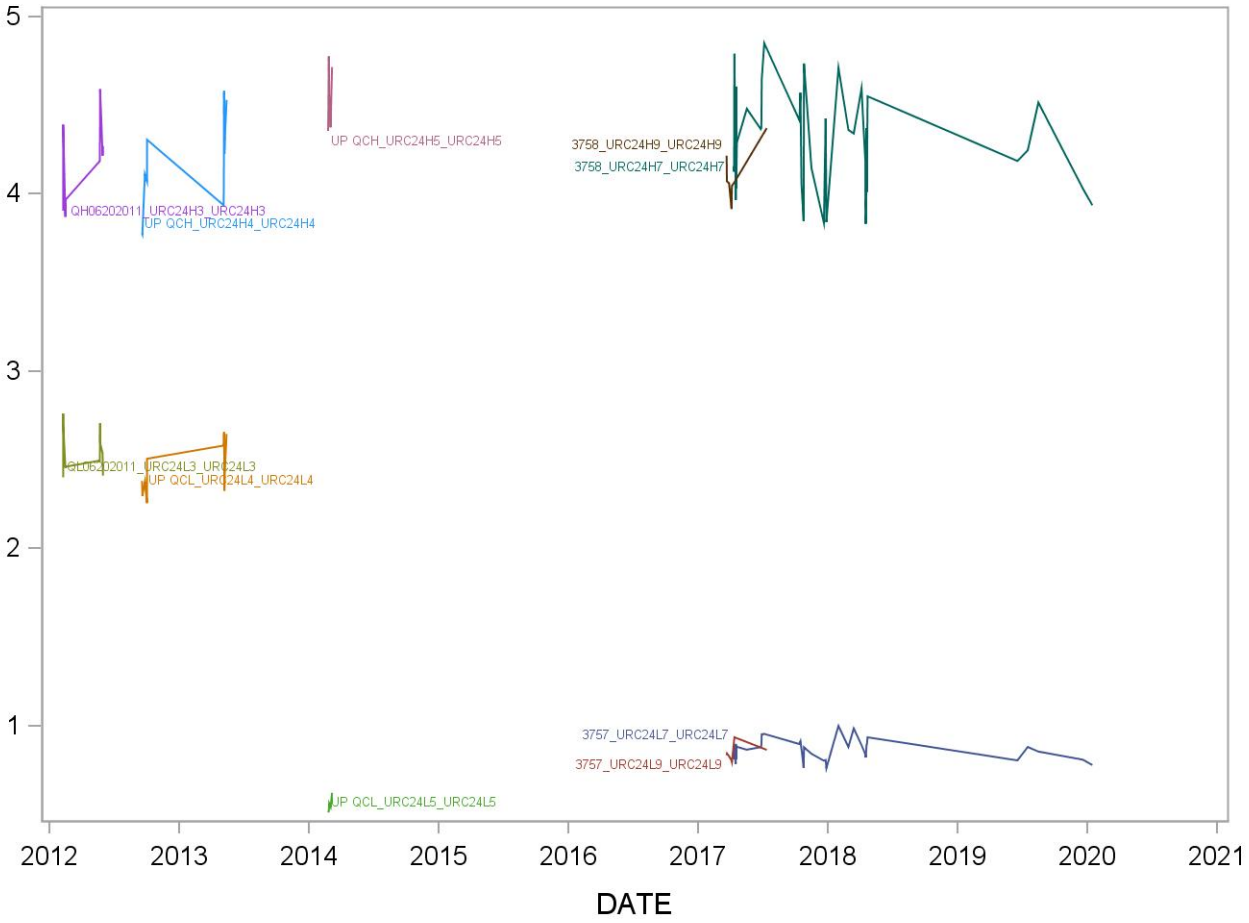
7. The frequency of cleaning the components of the mass spectrometer depends on the types and amounts of samples and solvents that are introduced into the instrument.

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.

20. Summary Statistics and QC Graphs

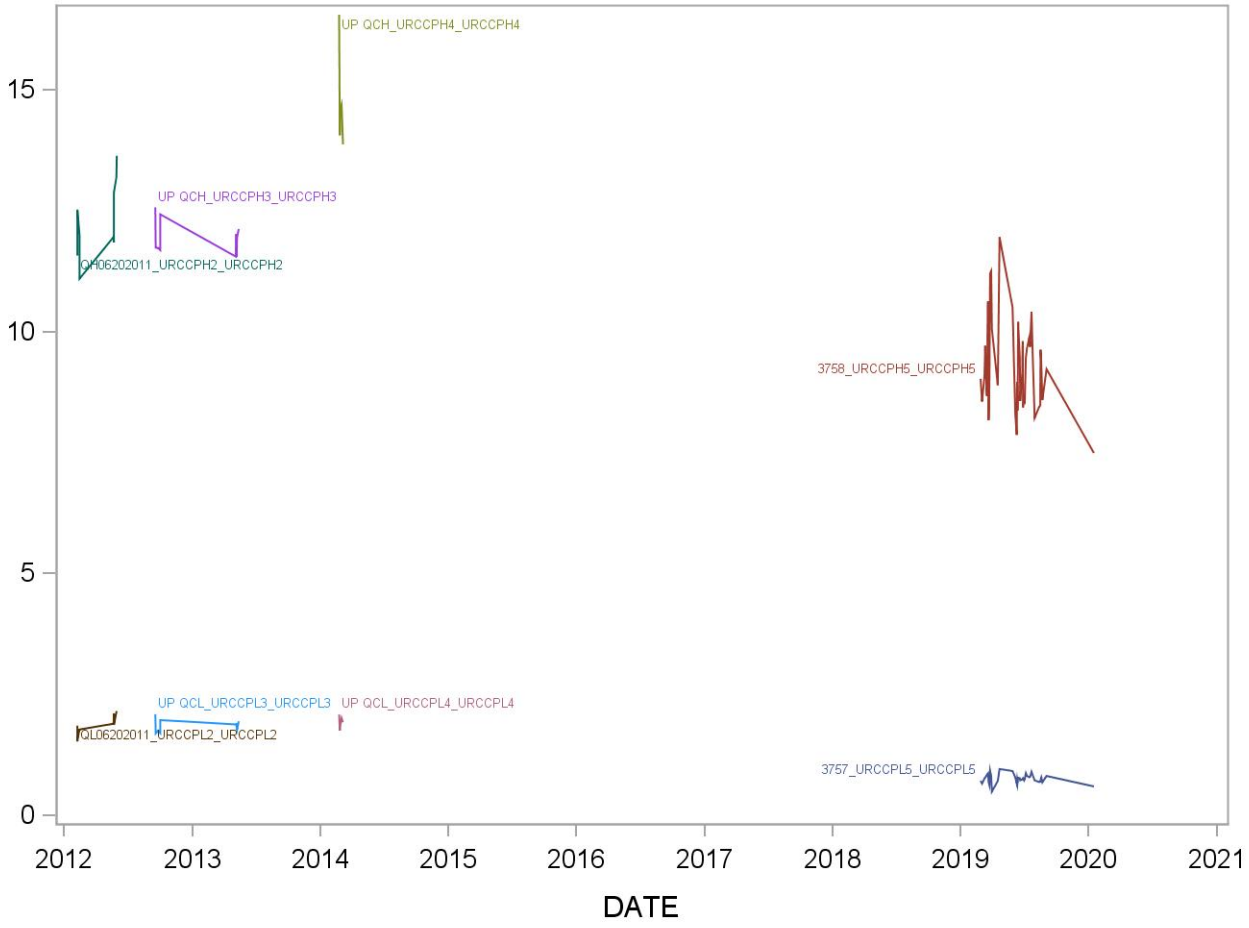
2013-2014 Summary Statistics and QC Chart for 2,4-D (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011_URC24H3_URC24H3	10	09FEB12	31MAY12	4.22050	0.24213	5.7
QL06202011_URC24L3_URC24L3	10	09FEB12	31MAY12	2.55150	0.13128	5.1
UP QCH_URC24H4_URC24H4	10	18SEP12	14MAY13	4.15900	0.28225	6.8
UP QCL_URC24L4_URC24L4	10	18SEP12	14MAY13	2.44400	0.14470	5.9
UP QCH_URC24H5_URC24H5	5	24FEB14	07MAR14	4.55700	0.19136	4.2
UP QCL_URC24L5_URC24L5	5	24FEB14	07MAR14	0.55000	0.04359	7.9
3758_URC24H9_URC24H9	7	22MAR17	13JUL17	4.10571	0.14541	3.5
3757_URC24L9_URC24L9	7	22MAR17	13JUL17	0.84229	0.04644	5.5
3758_URC24H7_URC24H7	37	11APR17	16JAN20	4.29865	0.29015	6.7
3757_URC24L7_URC24L7	37	11APR17	16JAN20	0.86394	0.05779	6.7



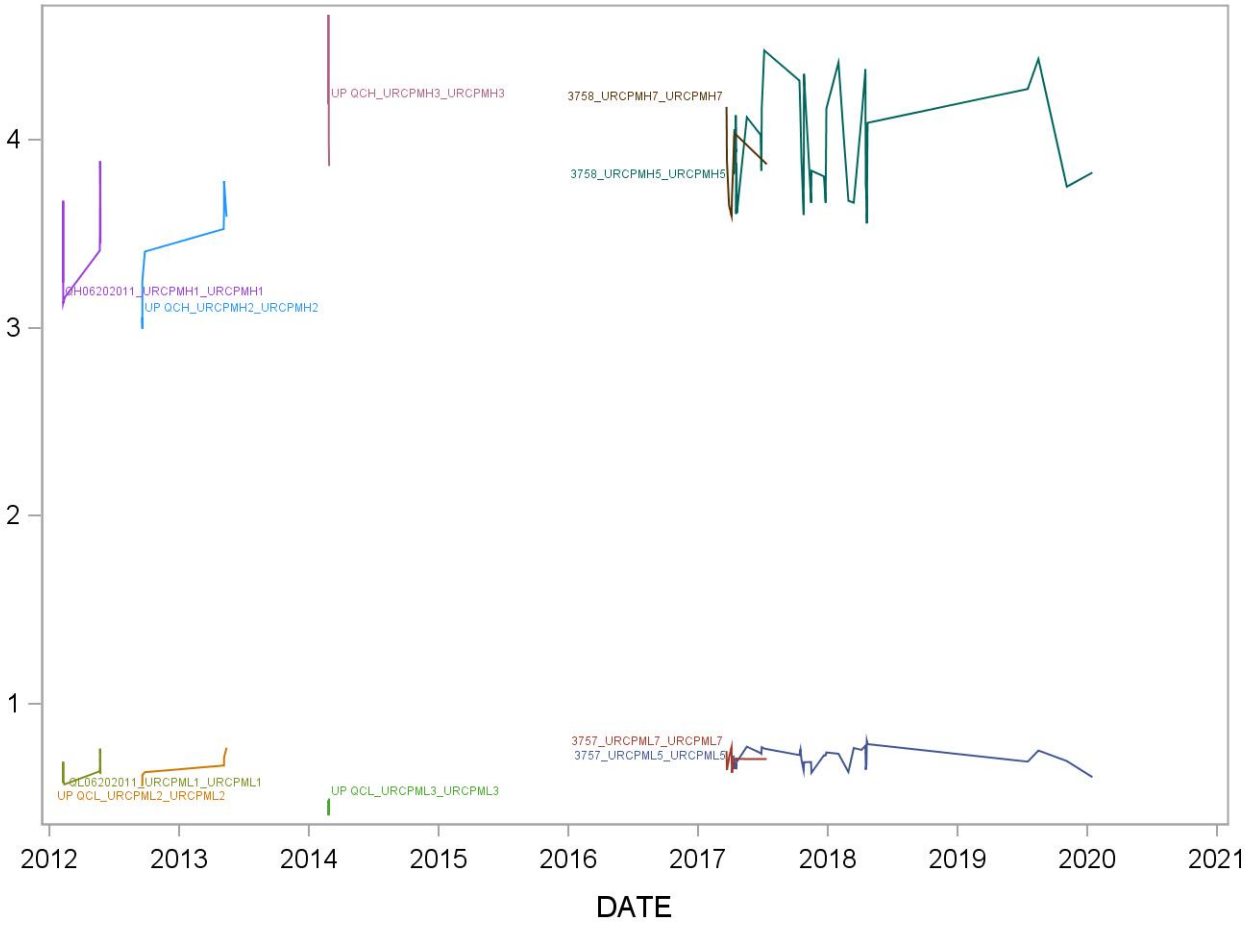
2013-2014 Summary Statistics and QC Chart for 3,5,6-trichloropyridinol (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011_URCCPH2_URCCPH2	10	09FEB12	31MAY12	12.2760	0.7740	6.3
QL06202011_URCCPL2_URCCPL2	10	09FEB12	31MAY12	1.8660	0.2193	11.8
UP QCH_URCCPH3_URCCPH3	10	18SEP12	14MAY13	11.9340	0.3524	3.0
UP QCL_URCCPL3_URCCPL3	10	18SEP12	14MAY13	1.8510	0.1274	6.9
UP QCH_URCCPH4_URCCPH4	5	24FEB14	07MAR14	14.7500	1.0634	7.2
UP QCL_URCCPL4_URCCPL4	5	24FEB14	07MAR14	1.9230	0.1313	6.8
3758_URCCPH5_URCCPH5	41	27FEB19	16JAN20	9.2456	0.9661	10.4
3757_URCCPL5_URCCPL5	41	27FEB19	16JAN20	0.7589	0.0915	12.1



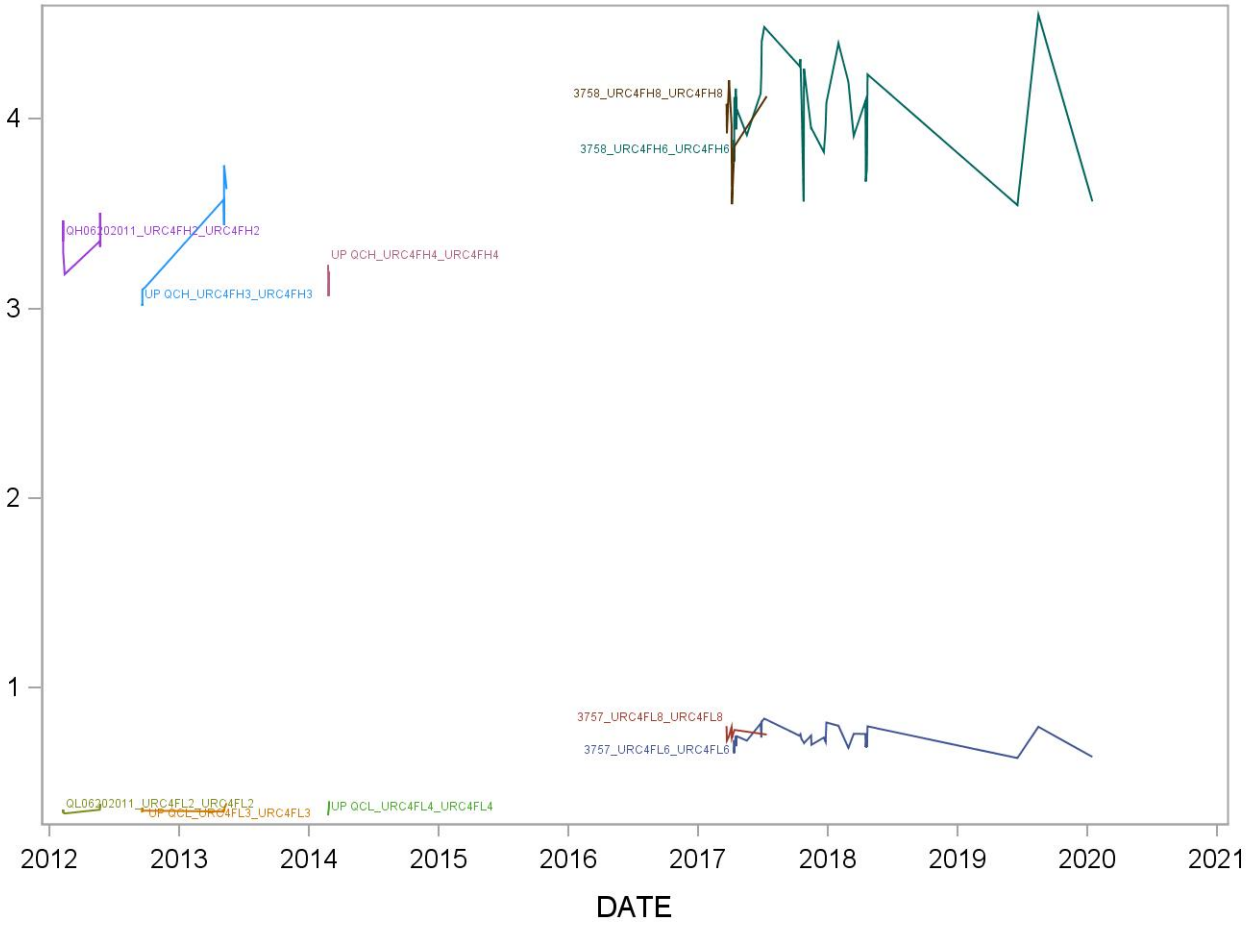
2013-2014 Summary Statistics and QC Chart for 3-phenoxybenzoic acid (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011_URCPMH1_URCPMH1	8	09FEB12	24MAY12	3.45750	0.27754	8.0
QL06202011_URCPML1_URCPML1	8	09FEB12	24MAY12	0.64750	0.07348	11.3
UP QCH_URCPMH2_URCPMH2	8	18SEP12	14MAY13	3.40438	0.28203	8.3
UP QCL_URCPML2_URCPML2	8	18SEP12	14MAY13	0.65063	0.06956	10.7
UP QCH_URCPMH3_URCPMH3	4	24FEB14	27FEB14	4.17375	0.35476	8.5
UP QCL_URCPML3_URCPML3	4	24FEB14	27FEB14	0.45250	0.04425	9.8
3758_URCPMH7_URCPMH7	7	22MAR17	13JUL17	3.84357	0.21299	5.5
3757_URCPML7_URCPML7	7	22MAR17	13JUL17	0.70236	0.04970	7.1
3758_URCPMH5_URCPMH5	37	11APR17	16JAN20	3.95297	0.27240	6.9
3757_URCPML5_URCPML5	37	11APR17	16JAN20	0.70986	0.04802	6.8



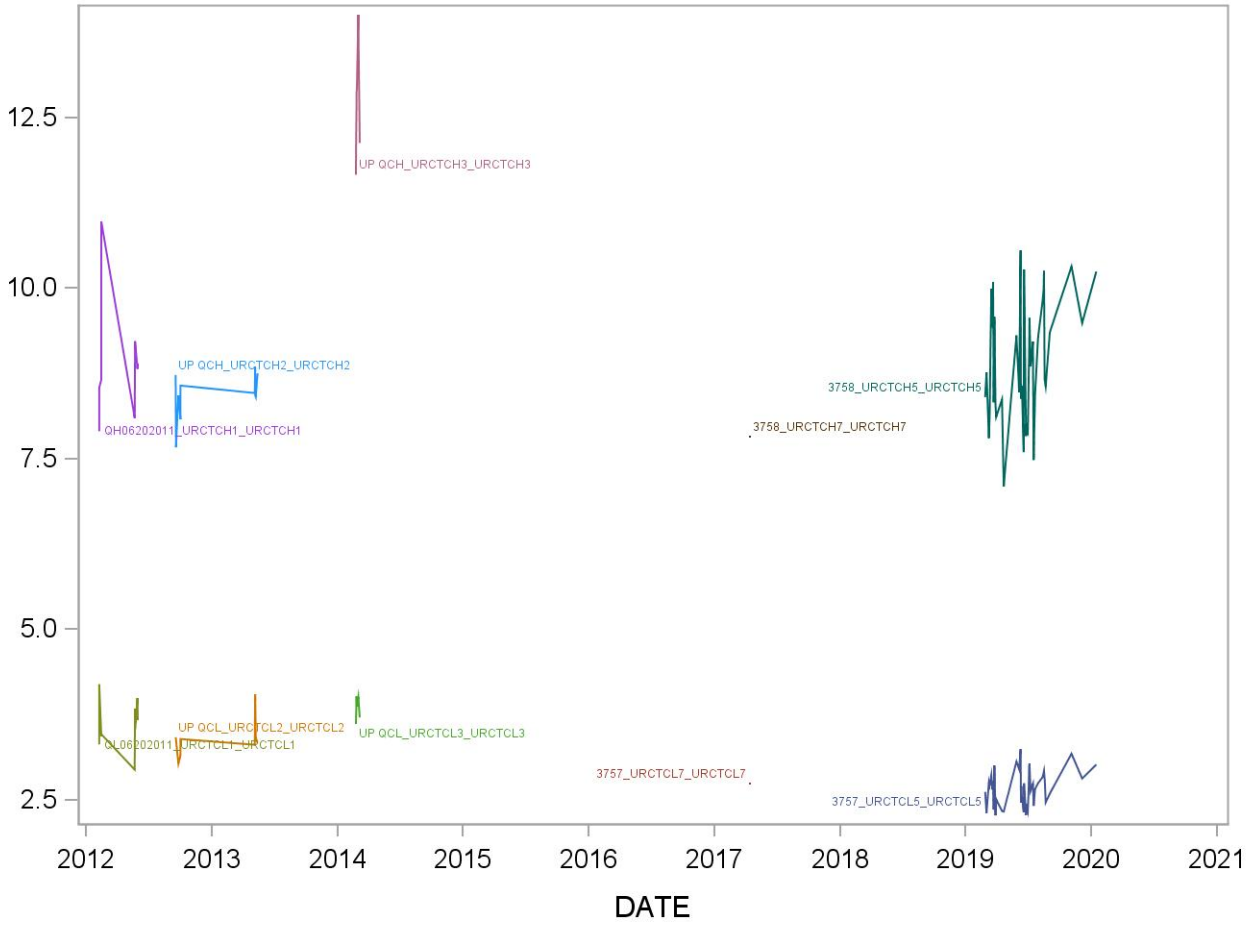
2013-2014 Summary Statistics and QC Chart for 4-fluoro-3-phenoxy-benzoic acid (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011_URC4FH2_URC4FH2	8	09FEB12	24MAY12	3.35125	0.10017	3.0
QL06202011_URC4FL2_URC4FL2	8	09FEB12	24MAY12	0.35563	0.01720	4.8
UP QCH_URC4FH3_URC4FH3	8	18SEP12	14MAY13	3.33125	0.30149	9.1
UP QCL_URC4FL3_URC4FL3	8	18SEP12	14MAY13	0.35688	0.01557	4.4
UP QCH_URC4FH4_URC4FH4	4	24FEB14	27FEB14	3.14250	0.08231	2.6
UP QCL_URC4FL4_URC4FL4	4	24FEB14	27FEB14	0.35875	0.03119	8.7
3758_URC4FH8_URC4FH8	7	22MAR17	13JUL17	3.95714	0.21616	5.5
3757_URC4FL8_URC4FL8	7	22MAR17	13JUL17	0.75864	0.02911	3.8
3758_URC4FH6_URC4FH6	35	11APR17	16JAN20	4.03171	0.25999	6.4
3757_URC4FL6_URC4FL6	35	11APR17	16JAN20	0.73178	0.05056	6.9



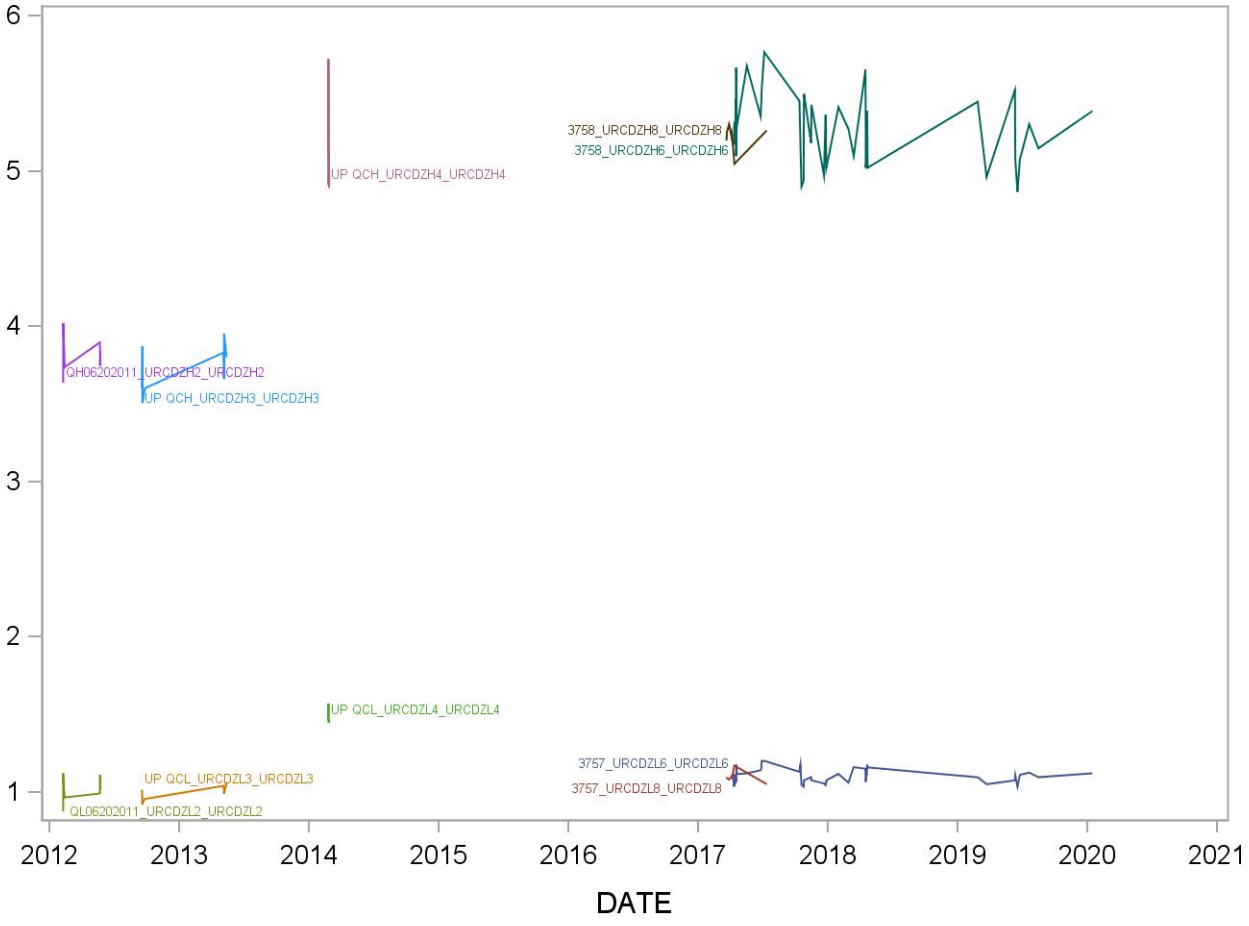
2013-2014 Summary Statistics and QC Chart for Dichlorovnl-dimeth prop carboacid (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011_URCTCH1_URCTCH1	9	09FEB12	31MAY12	8.90944	0.88628	9.9
QL06202011_URCTCL1_URCTCL1	9	09FEB12	31MAY12	3.59444	0.37609	10.5
UP QCH_URCTCH2_URCTCH2	10	18SEP12	14MAY13	8.43400	0.34865	4.1
UP QCL_URCTCL2_URCTCL2	10	18SEP12	14MAY13	3.43000	0.30076	8.8
UP QCH_URCTCH3_URCTCH3	5	24FEB14	07MAR14	12.71700	0.89391	7.0
UP QCL_URCTCL3_URCTCL3	5	24FEB14	07MAR14	3.84000	0.18000	4.7
3758_URCTCH5_URCTCH5	43	27FEB19	16JAN20	8.86512	0.88602	10.0
3757_URCTCL5_URCTCL5	43	27FEB19	16JAN20	2.66943	0.26267	9.8



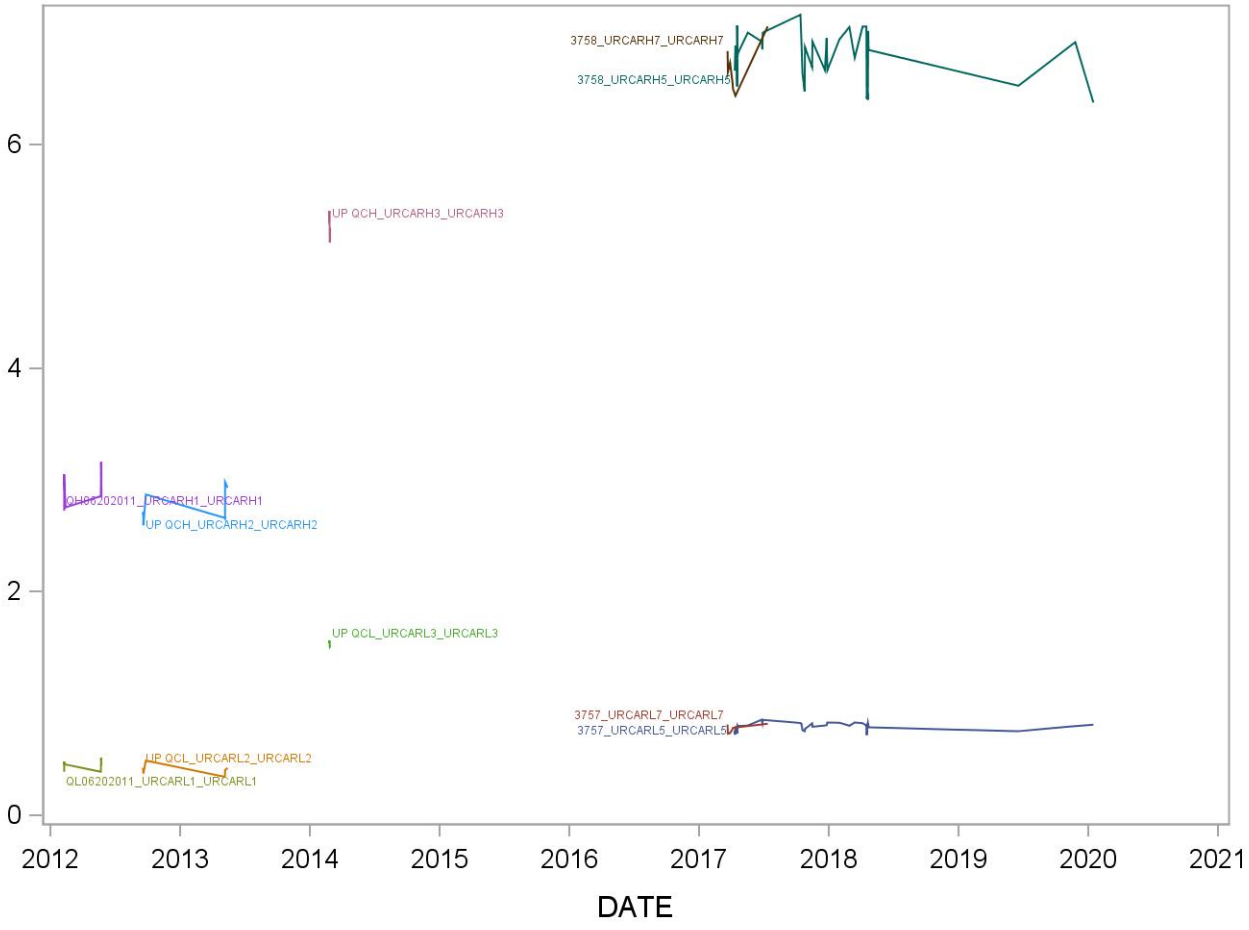
2013-2014 Summary Statistics and QC Chart for Oxypyrimidine (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011_URCDZH2_URCDZH2	8	09FEB12	24MAY12	3.81938	0.11626	3.0
QL06202011_URCDZL2_URCDZL2	8	09FEB12	24MAY12	1.03063	0.08504	8.3
UP QCH_URCDZH3_URCDZH3	8	18SEP12	14MAY13	3.72688	0.15682	4.2
UP QCL_URCDZL3_URCDZL3	8	18SEP12	14MAY13	0.98813	0.04949	5.0
UP QCH_URCDZH4_URCDZH4	4	24FEB14	27FEB14	5.16625	0.38690	7.5
UP QCL_URCDZL4_URCDZL4	4	24FEB14	27FEB14	1.48500	0.05730	3.9
3758_URCDZH8_URCDZH8	7	22MAR17	13JUL17	5.21500	0.08185	1.6
3757_URCDZL8_URCDZL8	7	22MAR17	13JUL17	1.09571	0.03656	3.3
3758_URCDZH6_URCDZH6	42	11APR17	16JAN20	5.26417	0.23041	4.4
3757_URCDZL6_URCDZL6	42	11APR17	16JAN20	1.10523	0.04527	4.1



2013-2014 Summary Statistics and QC Chart for para-Nitrophenol (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011_URCARH1_URCARH1	8	09FEB12	24MAY12	2.93000	0.15680	5.4
QL06202011_URCARL1_URCARL1	8	09FEB12	24MAY12	0.45250	0.04359	9.6
UP QCH_URCARH2_URCARH2	8	18SEP12	14MAY13	2.76125	0.14407	5.2
UP QCL_URCARL2_URCARL2	8	18SEP12	14MAY13	0.40250	0.04480	11.1
UP QCH_URCARH3_URCARH3	4	24FEB14	27FEB14	5.26875	0.11586	2.2
UP QCL_URCARL3_URCARL3	4	24FEB14	27FEB14	1.53250	0.02533	1.7
3758_URCARH7_URCARH7	7	22MAR17	13JUL17	6.67929	0.21501	3.2
3757_URCARL7_URCARL7	7	22MAR17	13JUL17	0.77700	0.03437	4.4
3758_URCARH5_URCARH5	36	11APR17	16JAN20	6.78986	0.21590	3.2
3757_URCARL5_URCARL5	36	11APR17	16JAN20	0.79570	0.03432	4.3



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