Laboratory Procedure Manual

Analyte:	**Specific Organophosphorus Pesticides, Synthetic Pyrethroids, and Select Herbicides (i.e., Universal Pesticides Panel)**
Matrix:	**Urine**
Method:	**Online Extraction-High Performance Liquid Chromatography-Heated Electrospray Ionization Tandem Mass Spectrometry**
Method No:	**6123.02**
Revised:	**01/04/2023**
As performed by:	Organic Analytical Toxicology Branch Division of Laboratory Sciences National Center for Environmental Health
Contact:	Dr. Maria Ospina Phone: 770-488-7407 Email: meo3@cdc.gov James L. Pirkle, M.D., Ph.D. CLIA Laboratory Director 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
UPHOPM_I	URX24D	2,4-D (ug/L)
	URXCPM	3,5,6-trichloropyridinol (ug/L)
	URX4FP	4-fluoro-3-phenoxy-benzoic acid (ug/L)
	URXOPM	3-phenoxybenzoic acid (ug/L)
	URXOXY	Oxypyrimidine (ug/L)
	URXPAR	para-Nitrophenol (ug/L)
	URXTCC	Dichlorovnl-dimeth prop carboacid (ug/L)
	URXMAL	Malathion diacid

1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

Pesticides are used by commercial pesticide applicators, farmers, and homeowners to kill or control pests, plant diseases and weeds. In 2012, an estimated 1.1 billion pounds of pesticides were used in the USA, which accounted for approximately 22% of all pesticide usage in the world (Atwood & Paisley-Jones, 2017). The most popular types of pesticides are insecticides (for example: organophosphorus [OPs] and pyrethroids) and herbicides. In 2012, the three most used OP insecticides were chlorpyrifos, acephate and malathion. Although the estimated usage of OP insecticides declined from 70 million pounds in 2000 to 20 million pounds in 2012 (more than 70%) due to toxicological concerns, chlorpyrifos, was the insecticide used the most in the agricultural market sector with 4-8 million pounds applied. Malathion was the 4th most used in the garden market with approx. 1-3 million pounds applied in 2012. Pyrethroids were the third most used insecticides in the home market sector with approximately 1-3 million pounds applied. 2,4-Dichlorophenoxyacetic acid (2,4-D) was the most used herbicide in the home and garden sector, and it was the fourth most used herbicide with 30-40 million pounds overall applied in 2012. Widespread use of pesticides and scientific interest in potential adverse health effect of pesticides exposure have increased the demand for robust analytical methods for measuring pesticides biomarkers.

The method presented here is used to determine the urinary concentrations of four organophosphorus insecticide metabolites (i.e., 2-isopropyl-6-methyl-4-pyrimidiol; 2-[(dimethoxyphosphorothioyl) sulfanyl] succinic acid; 3,5,6-trichloro-2-pyridinol; 4-nitrophenol), five synthetic pyrethroid metabolites (3-phenoxybenzoic acid; 4-fluoro-3-phenoxybenzoic acid; cis-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid; trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid; cis-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid)) and one herbicide (2,4-D)**.**

b. Test Principle

This method replaces a previously described method (Davis et al., 2013) and incorporates changes in sample volume, solid phase extraction and mass spectrometry detection. In brief, 0.2 mL of urine are hydrolyzed, and automated sample cleanup is achieved using an online solid phase extraction column. The analytes are backflushed into an analytical column and 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. 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 concentrations of 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
2-[(dimethoxyphosphorothioyl) sulfanyl] succinic acid	MDA	MAL	Malathion	O
para-Nitrophenol	PNP	PAR	Methyl parathion, Parathion	O
3,5,6-trichloro-2-pyridinol	TCPY	CPM	Chlorpyrifos- methyl	O
2,4-dichlorophenoxyacetic acid	2,4-D	24D	2,4-D	H
3-phenoxybenzoic acid	3-PBA	OPM	Permethrin, Cypermethrin, Cyfluthrin, others	P
4-fluoro-3-phenoxy-benzoic acid	4F-3PBA	4FP	Cyfluthrin	P
cis-dichlorovinyl-dimethylcyclopropane carboxylic acid	*cis*-DCCA	CCC	Permethrin, Cypermethrin	P
trans-dichlorovinyl-dimethylcyclopropane carboxylic acid	*trans*-DCCA	TCC	Permethrin, Cypermethrin	P
cis-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid))	*cis*-DBCA	CBC3	Deltamethrin	P

O – Organophosphorus Insecticide
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.

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

Safety Data Sheets (SDS) 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 SDS 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 microbiological hazards still exists. Take appropriate measures to avoid contact with the specimen (see “Protective equipment” below). 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. At a minimum, 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.

g. Personal Hygiene

Be careful when handling any biological specimen. Use gloves and other PPE, 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 manner.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

A working knowledge of XCalibur, the software controlling the HPLC-MS/MS system is required. In addition, a basic understanding of the Division 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, 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 automatically transferred to and backed up on the CDC network, in the case of automatic data transfer failure, this process is accomplished using the ISLE - a Lab Computer network protected from the CDC network by a firewall managed by Information Technology Services Office (ITSO), or by using CDC approved USB devices.

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 preferably within 4 hours of collection. Specimen handling conditions are outlined in the Division protocol for urine collection and handling available on the DLS intranet and at CDC's Collection and Management of Human Samples (available at https://www.cdc.gov/biomonitoring/human_samples.html). In these generic protocols, collection, transport, and special equipment required are discussed. In general, urine specimens should be shipped overnight in cryovials packed in enough 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. For long-term storage, samples should be kept at or below -20°C.

b. Sample Rejection

Reject specimens that have leaked, are broken, or otherwise appear to be compromised. Also, reject specimens with insufficient volume because they cannot be reliably processed. Specimens that meet the rejection criteria may be analyzed if they are unique and difficult specimens to collect (e.g.: infant urine). The results from these specimens will be flagged in the final data report.

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

Not applicable for this procedure.

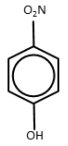
6. Preparation of Reagents, Calibrators (Standards), Controls, and All Other Materials; Equipment and Instrumentation

a. Reagents and Sources

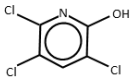
All reagents are used without further purification. Other standards and reagents with similar specifications may be used.

Table 1. Compound Structures and Abbreviations

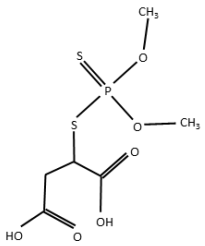
Organophosphorus Pesticide Specific Metabolites



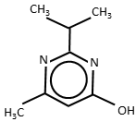
PAR



CPM

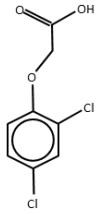


MAL



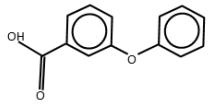
OXY2

Herbicide

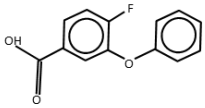


24D

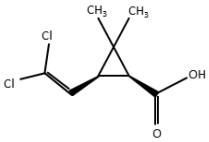
Pyrethroid Metabolites



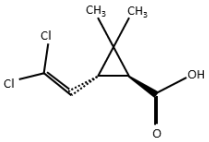
OPM



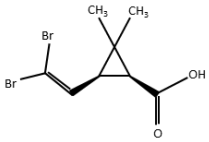
4FP



CCC



TCC



CBC3

Table 2a. Reagents and Suggested Manufactures

Reagents	Suggested Manufacturers
Acetonitrile	Fisher Scientific Inc.
Methanol, Optima Grade	Fisher Scientific Inc.
LC/MS Grade Water	The Lab Depot/LabSolv
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

Compound	Analyte Code	Source
2,4-dichlorophenoxyacetic acid, 100µg/mL in acetonitrile	24D	Cambridge Isotopes
2,4-dichlorophenoxyacetic acid (ring $^{13}C_6$ 99%)	24D_L	Cambridge Isotopes
4-Flouro-3-phenoxybenzoic acid	4FP	Cambridge Isotopes
4-flouro-3-phenoxybenzoic acid (phenoxy-$^{13}C_6$)	4FP_L	Cambridge Isotopes
cis-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid)) (Metabolite of deltamethrin)	CBC3	Cambridge Isotopes
cis-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid)) (1, carboxyl-$^{13}C_2$, 99%, 1-D, 96%)	CBC3_L	Cambridge Isotopes
cis--3-(2,2-dichlorovinyl)-2,2-dimethyl-1 – cyclopropane carboxylic acid	CCC	Cambridge Isotopes
cis--3-(2,2-dichlorovinyl)-2,2-dimethyl-1 – cyclopropane carboxylic acid (1, carboxyl-$^{13}C_2$, 1-D, 98%)	CCC_L	Cambridge Isotopes
3,5,6-trichloro-2-pyridinol	CPM	Cambridge Isotopes
3,5,6-trichloro-2-pyridinol (4,5,6-$^{13}C_3$)	CPM_L	Cambridge Isotopes
2-[(dimethoxyphosphorothioyl) sulfanyl] succinic acid (Malathion)	MAL	Cambridge Isotopes
2-[(dimethoxyphosphorothioyl) sulfanyl] succinic acid (Malathion) ($^{13}C_4$, 99%)	MAL_L	Cambridge Isotopes
3-phenoxybenzoic acid	OPM	Cambridge Isotopes
3-phenoxybenzoic acid (phenoxy-$^{13}C_6$)	OPM_L	Cambridge Isotopes
2-isopropyl-6-methyl-4-pyrimidinol (Oxypyrimidine)	OXY	Cambridge Isotopes
2-isopropyl-6-methyl-4-pyrimidinol (Oxypyrimidine) (methyl-4,5,6-$^{13}C_4$, 99%)	OXY_L	Cambridge Isotopes
para-Nitrophenol (Methyl parathion)	PAR	Cambridge Isotopes
Para-Nitrophenol (Methyl parathion) ($^{13}C_6$, 99%)	PAR_L	Cambridge Isotopes
trans--3-(2,2-dichlorovinyl)-2,2-dimethyl-1 – cyclopropane carboxylic acid	TCC	Cambridge Isotopes
trans---3-(2,2-dichlorovinyl)-2,2-dimethyl-1 – cyclopropane carboxylic acid (1, carboxyl-$_{13}C_2$, 1-D)	TCC_L	Cambridge Isotopes
cis-dibromovinyl-dimethylcyclopropane carboxylic acid	CBC3	Cambridge Isotopes
cis-dibromovinyl-dimethylcyclopropane carboxylic acid (1, carboxyl-$^{13}C_2$, 99%; 1-D, 96%)	CBC3_L	Cambridge Isotopes

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

b. Reagent Preparation

Liquid chromatography mobile phases

Mobile Phase A: 0.1% Acetic Acid in LC/MS Grade Water. For a 4 L solution, pipette 4 mL of >99% glacial acetic acid in 4L bottle of LC/MS Grade Water. Mix thoroughly prior to use.
Mobile Phase B: 100% LC-MS Grade Methanol.

Sodium Acetate Buffer Solution

Add 2.2 mL of >99% glacial acetic acid and 6.8 grams of sodium acetate to 140 mL of LC/MS grade water. Mix thoroughly until sodium acetate is fully dissolved. This solution should be stored refrigerated.

Enzyme Solution

This solution should be prepared within one hour prior to use. 74.5 mg (0.0745g) of β-glucuronidase type H-1 from *Helix pomatia* enzyme with a specific activity of ~500 units/mg is dissolved in 10 mL of sodium acetate enzyme buffer above in a 20 mL beaker. Let enzyme buffer solution sit for 1 hour to fully dissolve. This is enough enzyme for a 96 well plate.

Enzyme Quenching solution

The enzyme quenching solution contains acetic acid for enzyme inactivation and 3-chloro-2-phenoxybenzoic acid (3C-2PBA) as a sample injection standard solution. Add enough 3C-2PBA to 1 L of LC/MS grade water to yield an approximate 3 ng/mL concentration. This solution may be stored at room temperature for repeated use. To create working enzyme quenching solution, add 1 mL of Glacial Acetic Acid to 9 mL of ~3 ng/mL 3C-2PBA solution to create 10% acetic acid solution. Mix thoroughly. Only create acidified working enzyme solution for immediate use.

c. Standards and Quality Control Material Preparation

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. The vial caps of solutions should never be labeled.

Internal Standard Spiking Solution

The concentration of each analyte in the labeled spiking solution is dependent on the sensitivity of the instrumentation for that analyte. Table 3a shows the concentration of each compound in the spiking solution. 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, 50 µL of this solution is aliquotted into every sample.

Table 3a. Internal Standard Spiking Solution Concentrations

ISTD Abbreviation	Conc of spiking stock (ng/mL)
24D_L	24
4FP_L	24
CBC3_L	48
CCC_L	96
CPM_L	48
MAL_L	48
OPM_L	24
OXY_L	48
PAR_L	24
TCC_L	48

Calibration Standard Solutions

The concentrations of the individual compounds in the calibration solutions are given in Table 3b. The range of concentrations for all analytes are 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. Individual stock solutions were received or prepared at a concentration of 100 µg/mL in acetonitrile or the solvent in which they were received. A solution containing 200 ng/mL concentration of all analytes was prepared from the individual stock solutions. This solution concentration corresponds to the highest calibrant. This high level combined stock solution was made by aliquoting the individual stocks solutions into a clean 25 mL volumetric flask and bringing the volume up with a solution of 25% acetonitrile in LC-MS grade water. From this high level solution, eleven other solutions were prepared using no more than three serial dilutions to create twelve working solutions ranging from 0.1-200 ng/mL. 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 to create a 12 point calibration curve ranging from 0.025-50 ng/mL.

Table 3b. Calibration Standard Solution Concentrations

Analyte Code	CS12	CS9	CS11	CS9	CS8	CS7	CS6	CS5	CS4	CS3	CS2	CS1
24D	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
4FP	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
CBC3	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
CCC	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
CPM	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
MAL	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
OPM	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
OXY	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
PAR	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025
TCC	50	25	12.5	6	3	1.5	0.75	0.3	0.2	0.1	0.05	0.025

Concentrations in ng/mL.

Injection Standard Solution

The injection standard 3-chloro-2-phenoxybenzoic acid (3C-2PBA) 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 enzyme quenching solution such that the final concentration is approximately 3 ng/mL.

Instrument Standard Check Solution

The instrument standard check solution is prepared as standard 3 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 check is used to verify the instrument performance before the analytical run begins.

Quality Control Materials

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 target analytes. Two quality control pools and three proficiency testing pools are produced. Depending on the amount of endogenous target analytes detected in the urine specimens used to produce the pool, additional target analytes may need to be added in free form to produce the desired concentration. Whenever possible, this additional concentration of target analytes should come from a secondary source from what is 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 a Division-wide 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.

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 target analytes in the pool. Alternatively, if it is determined that there is no significant difference in calibration curves prepared in matrix and solvent, a solvent curve can be used.

Solvent Blank Material

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

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) ISOLUTE FILTER+ Plate 25 µm/0.2 µm (*Biotage LLC, Charlotte, NC, USA)*
2) 2mL/well 96 square well collection plate *(Phenomenex, Torrance, CA, USA)*
3) 450µL pipet tips, 96-well plate format, 50 racks/carton *(Tomtec, Hamden, CT, USA)*
4) Argon gas *(Air Gas, Radnor Township, PA)*
5) 20 - 300 µL pipette tips (20 – 300 µL multi-channel pipette) 960/box *(Rainin Instrument LLC, Oakland, CA)*
6) 20 - 20 µL pre-sterilized filter pipette tips (20 - 20 µL electronic pipette) 960/box *(Rainin Instrument LLC, Oakland, CA)*
7) 100 – 1000 µL pre-sterilized filter pipette tips (100 - 1000 µL electronic pipette) 960/box *(Rainin Instrument LLC, Oakland, CA)*
8) 500 – 5000 µL pre-sterilized filter pipette tips (500 – 5000 µL electronic pipette) *(Rainin Instrument LLC, Oakland, CA)*
9) HPLC pre-column frits 0.250 OD x 0.062 in. thick, 0.5 µm pore, 10/pack *(Upchurch Scientific, Oak Harbor, WA)*
10) 2mL, clear glass I-D™, 12x32mm, flat base, target DP screw thread vials, 100/box *(National Scientific, Rockwood, TN, USA)*
11) Target DP screw caps with silicone septa (PTFE), assorted colors, 1000/case *(National Scientific, Rockwood, TN, USA)*
12) Pierceable sealing mats, 96 square well, silicone, 50/pack *(Phenomenex, Torrance, CA, USA)*
13) 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)*

14) Bench Top Protector Sheets, case of 400 (green) *(Kimberly-Clark Professional, Roswell, GA, USA)*
15) 2 mL self-standing polypropylene Microtube *(Simport, Beloeil, Canada)*
16) Screw caps for microtubes with O-ring and flat tube, assorted colors, 1000/pack *(Simport, Beloeil, Canada)*
17) Automatic repeating pipette tip, 50 mL capacity, non-sterile *(Eppendorf, Hauppauge, NY, USA)*
18) Disposable SMS lab coats with knit collar and cuffs, assorted colors and sizes, 30/case *(LabSource, Romeoville, IL)*
19) High Five A+ Nitrile Exam Gloves, assorted sizes,1000/case *(High Five Company, Chicago, IL)*
20) Science Wipes 2-Ply Tissue, 14.7 in. x 16.6 in.,90/pack, 15/case *(Kimberly-Clark Professional, Roswell, GA, USA*
21) Safety glasses *(Thermo Fisher Scientific Inc., Waltham, MA)*
22) Polypropylene reagent reservoir with (3) baffles *(Tomtec, Hamden, CT, USA)*
23) HPLC analytical column; Hypersil GOLD™ aQ C18 Polar Endcapped HPLC column; 150mm x 4.6mm, 3 µm particle size *(Thermo Fisher Scientific Inc., Waltham, MA, USA)*
24) HPLC guard cartridge; Hypersil GOLD™ aQ C18 guard cartridge; 10mm x 4mm, 5 µm particle size *(Thermo Fisher Scientific Inc., Waltham, MA, USA)*
25) HPLC online SPE column; Chromolith™ Flash RP-18 Endcapped HPLC column; 25mm x 4.6mm, 2µm particle size *(Millipore Sigma, Burlington, MA, USA)*
26) Autoclave bag, PP, clear 31x38, 200/pk *(Thermo Fisher Scientific Inc., Waltham, MA)*
27) Sharps-a-Gator Sharps Container, 1 gallon (3.8 L), red *(Thermo Fisher Scientific Inc., Waltham, MA)*
28) Benchtop biohazard cup with lid; 40/pk *(Market Lab, Caledonia, MI)*
29) Uniguard™ Direct-Connection Guard Cartridge Holder; 4mm-4.6mm *(Thermo Fisher Scientific Inc., Waltham, MA, USA)*
30) Stainless steel pre-column inline filter holder for 2um and 0.5 um frits *(Upchurch Scientific, Oak Harbor, WA)*
31) 2 L media storage bottle with side neck and cap *(The Lab Depot, Inc., Dawsonville, GA)*

e. Equipment

1) OHAUS balance (Parsippany, NJ)
2) UCT 96 Well Plate Positive Pressure Extraction Manifold (United Chemical Technologies, Inc., Bristol, PA, USA)
3) EDP2® electronic pipettes, assorted volumes (*Rainin Instrument LLC, Oakland, CA)*
4) E4 XLS Adjustable-spacer electronic multi-channel pipette 20 – 300 µL *(Rainin Instrument LLC, Oakland, CA)*
5) Brady 300MVP Plus label printer *(Brady Corporation, Milwaukee, WI)*
6) Positive-displacement electronic repeater pipette, 0.1 - 50 mL *(Rainin Instrument LLC, Oakland, CA)*
7) Laboratory gas generator *(PEAK Scientific, Billerica, MA, USA)*
8) Isotemp 300 series incubator; Model 350D *(Thermo Fisher Scientific Inc., Waltham, MA)*

9) Flammables safety cabinet *(Thermo Fisher Scientific Inc., Waltham, MA)*
10) Digital Ultrasonic Cleaner; 4 qt. (3.8L) 5.5 x 9.5 x 6 in*. (Thermo Fisher Scientific Inc., Waltham, MA)*
11) Mini-Vortexer, 115 V *(VWR, Radnor, PA, USA)*
12) Large capacity mixer with pulsing 120V 100-2,000 rpms *(Glas-Col LLC, Terre Haute, IN)*
13) Special purpose under-counter freezer, flammable materials storage compatible, 5.6 cu ft. *(Thermo Fisher Scientific Inc., Waltham, MA)*
14) Stirrer/hot plate; 4 x 5 in., 120V *(Corning Inc., Corning, NY)*

f. Instrumentation

1) UltiMate 3000 Ultra High-Pressure Liquid Chromatography system *(Thermo Fisher Scientific Inc., San Jose, CA)* with two binary or quaternary pumps capable of delivering 2.0 mL/min flow rates, with column heating capabilities, and a separate 10-port switching valve.
2) ThermoFisher TSQ Altis™ triple-quadrupole mass spectrometer *(Thermo Fisher Scientific Inc., San Jose, CA)* equipped with a heated electrospray ionization (HESI) source or an equivalent mass spectrometer.

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 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. Since 2010, the lab participates in the German External Quality Assessment Scheme (GEQUAS) conducted by the Institute for Occupational, Environmental and Social Medicine at the Friedrich-Alexander University in Erlangen, Germany for the pyrethroid metabolites, OPM, 4FP, TCC, CCC and CBC3. Beginning in the fall of 2014 and in the fall of 2015, 3,5,6-tricholo-2-pyridinol (CPM), and para-nitrophenol (PAR), respectively, were added to the G-EQUAS program. This assessment is conducted semi-annually on two reference urine samples fortified with the pyrethroid metabolites which 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 2,4D, we started participating in the External Quality Assessment Scheme for Organic Substances in Urine program (OSEQAS) in June 2021. A minimum of once per year, three reference urine samples fortified with 2,4D will be 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)

For the remaining target analytes, 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 fails, 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, 2 solvent blanks, 12 calibration standards, two low-concentration QC samples, two high-concentration QC samples, and two instrument standard check samples. Specimens should be allowed to thaw and reach room temperature before aliquoting for analysis.

b. Sample Preparation

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 200 µL pipette used will be checked for proper function by pipetting 200 µL of water into a tared vial and recording the weight on the run sheet. If the result deviates by more than 5% from 200 mg, 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 are 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 first row on the plate.
e. Two 200 µL aliquots of LC/MS grade water designated as solvent blanks are pipetted into the two wells. One solvent blank is placed in the second row of the plate below one of the high calibrants. This helps in identifying any splashes from calibrants. The other blank is placed in a random well in the plate.
f. Each thawed specimen and QC sample is vortexed, and 200 µL is aliquoted into the appropriate well on the plate.
g. For the calibration standards, 150 µL of LC/MS grade water is aliquoted into each designated well.
h. 50 µL of freshly prepared β-glucuronidase enzyme solution is added to every well.
i. The entire plate is capped using a pierceable silicone sealing mat.
j. The samples are placed in an incubator at 37°C for at least 6 hours, but typically overnight.
k. Samples are removed from the incubator and allowed to come to room temperature before beginning filtration and enzyme quenching procedure.

c. Filtration and Enzyme Quenching Procedure

a. Incubated samples from the 96 well plate are transferred to a Biotage ISOLUTE + filtration plate using a multi-channel pipette or other liquid handling equipment. Alternatively, an appropriate liquid handling system can be used for aliquoting purposes.
b. The transferred samples are filtered through the filtration plate into a 96 well collection plate using the UCT PPM by incremental increases of regulated air flow for approximately 30 minutes. The entire 300 µL of filtrate per sample should be present in collection plate before proceeding. Refer to Appendix B for UCT instrumentation operation and maintenance.
c. After filtration, 10 mL of 10% acetic acid enzyme quenching solution is created from the 3C-2PBA in LC/MS Grade Water. 100 µL of the acidified enzyme quenching solution is aliquoted into each well of the collection plate. The plate is sealed using a pierceable silicone mat and ready for an analytical run. The prepared plate is stored at -20°C if not used immediately.

d. 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 folder.

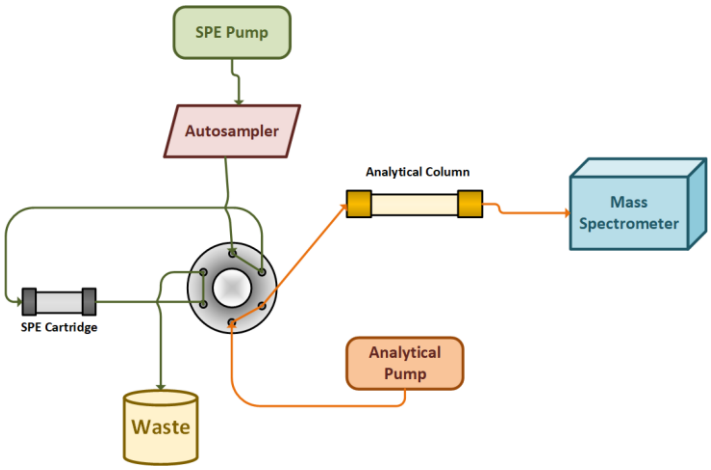
e. Liquid Chromatography Conditions

a. The method is run in an alternating column mode using an UHPLC system containing dual, independently operating Ternary Separation Pumps. An alternative column injection mode is necessary to facilitate an online SPE method whereby the initial sample injection is loaded onto a SPE column and then back eluted onto an analytical column. This is accomplished by separately designated “SPE” and “Analytical” pumps.
b. Samples are first loaded onto the SPE column via the SPE designated pump and washed isocratically with mobile phase A to remove matrix. The designated analytical pump then back elutes the sample from the SPE column onto the analytical column by utilizing a six-port valve for changing flow direction. Once the analytes are fully transferred to the analytical column, flow from the analytical pump is switched from the SPE column to the analytical column and chromatographic separation begins with a gradient.
c. During the chromatographic separation, the SPE pump is used to wash and equilibrate the SPE column for the next injection.
d. Instrument conditions for liquid chromatography are created and stored with the Xcalibur software.
e. Column configuration: Precolumn filter using 0.5 µm frit installed between autosampler and six port valve; Chromolith Flash RP-18e monolithic 25x4.6 mm column used as SPE column; Thermo Scientific™ Hypersil Gold aQ 150x4.6 mm, 3 µm particle size used as analytical column with guard column holder and frit of same packing material
f. Mobile Phase A: 0.1% Glacial Acetic Acid in LC/MS Grade Water
g. Mobile Phase B: 100% Methanol
h. Column Temperature: 40^{o}C
i. Injection Volume: 300 µL

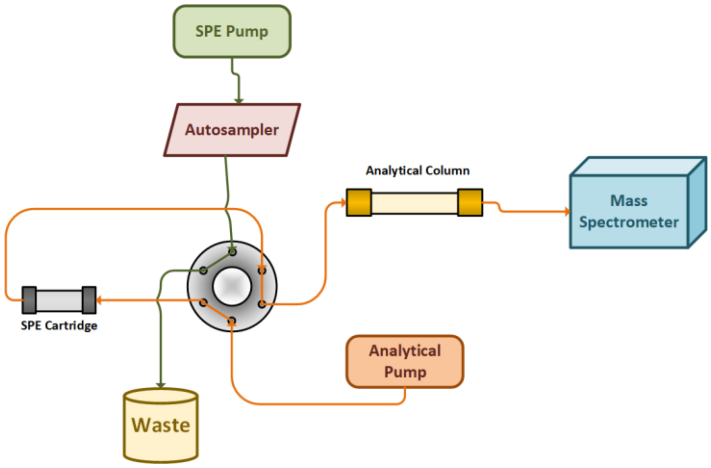
Table 4. Online SPE and Analytical Chromatographic Program

SPE pump			Analytical pump		
Time (min)	**Flow Rate (mL/min)**	**%B**	**Time (min)**	**Flow Rate (mL/min)**	**%B**
0.0	2.00	1.0	0.0	0.75	20.0
3.1	2.00	1.0	6.1	0.75	20.0
3.1	0.20	1.0	6.1	0.75	20.0
5.0	0.20	1.0	8.0	0.75	70.0
7.0	0.20	1.0	10.0	0.40	70.0
8.0	0.75	100.0	20.0	0.40	70.0
13.0	0.75	100.0	20.0	0.40	100.0
14.0	0.50	1.0	23.0	0.40	100.0
25.0	0.50	1.0	23.5	0.75	20.0
27.0	2.00	1.0	27.0	0.75	20.0

Step 1. Sample loading and cleanup, analytical column equilibration.



Step 2. Sample back elution onto analytical column, analytical column equilibration.



Step 3. Chromatographic separation, SPE column equilibration.

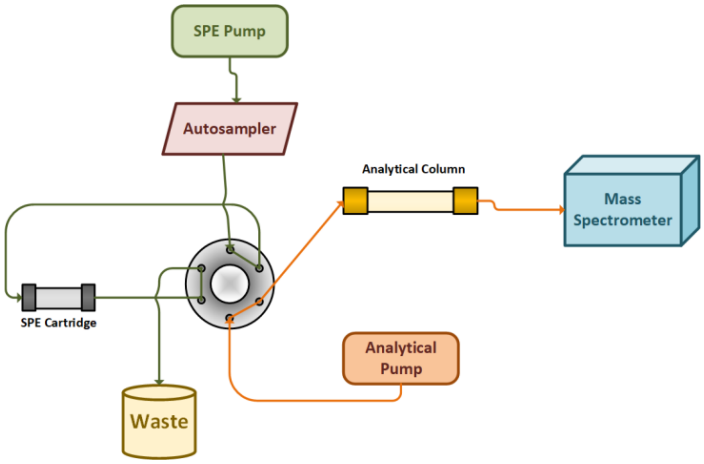


Figure 1. On-line dual pump switching scheme with flow paths and mobile phase gradient profiles.

f. Mass Spectrometry Conditions

1) Instrumental conditions for mass spectrometry are specified in the Xcalibur instrument method file.
2) A tune file and calibration file will have been generated by the field service engineer during the instrument's preventative maintenance or by the operator during periodical instrument tune and calibration exercises. Table 5 list suggested optimized parameters for this method. It is important to note that instrument specific differences do exist due to engineering variations and therefore it is important to optimize each instrument for the method for parameters such as RF lens values.
3) Tuning is performed using Pierce™ Triple Quadrupole Calibration Solution Extended Mass Range
4) The method is run in Selected Reaction Monitoring Mode.
5) The parent and product transitions for each target analyte are given in Table 5 along with the optimized collision energy and ion polarity mode.
6) The peak width for each compound is specified in Table 6. 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.
7) Scan Time: optimized depending on the number of transitions in each window.
8) The data mode: Centroid.
9) A divert valve is used to divert mobile phase to waste during the first part of the analytical run

Table 5. Mass Spectrometer Parameters

MS Parameter	Setting
Ionization type	H-ESI
Ion polarity mode	Positive and Negative
Spray Voltage Positive Ion Mode	3500 mV
Spray Voltage Negative Ion Mode	2500 mV
Vaporizer temperature	350º C
Sheath Gas Pressure	50
Ion Sweep Gas Pressure	1
Aux Gas Pressure	10
Ion Transfer Tube Temperature	325º C
Tube Lens Offset	Set by mass with Tune file
Skimmer Offset	0
Collision Energy	Optimized per compound
Collision gas	Argon at 1.0 mT
Q1 and Q2 Resolution (FWHM)	0.7

Table 6. Compound Specific Mass Spectrometry Conditions

Analyte	Ion Polarity Mode	Segment	Precursor (m/z)	Product (m/z)	Collision Energy (V)
OXY2	Positive	1	153	84	18
OXY2-C			153	70	20
OXY2-L			157	88	18
MAL	Negative	2	273	141	10
MAL-C			273	157	19
MAL-L			277	141	10
PAR	Negative	2	138	108	17
PAR-C			138	92	24
PAR-L			144	114	18
24D	Negative	3	219	161	15
24D-C			219	125	27
24D-L			225	167	15
CPM	Negative	3	198	35	15
CPM-C			198	37	17
CPM-L			199	35	16
TCC & CCC	Negative	3	207	35	12
TCC-C & CCC-C			209	35	12
TCC-L & CCC-L			212	37	10
4FP	Negative	3	231	93	26
4FP-C			231	187	14
4FP-L			237	193	14
OPM	Negative	3	213	93	22
OPM-C			213	169	13
OPM-L			219	99	27
CBC3	Negative	3	297	79	10
CBC3-C			297	81	10
CBC3-L			300	79	15

g. Routine Operations

1. If needed, new pre-column frits and columns are replaced.
2. 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.
3. The UHPLC system is also switched on with the mobile phase flowing at the initial conditions and the column compartment heated to predetermined temperature.
4. 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 condition. The critical demonstration of instrument sensitivity and operation is given with the calibration curve.
5. The analytical run is set up and run with the Xcalibur software.

6. After the analytical run, a column wash procedure may be run to clean the analytical columns.
7. At the end of analysis, the system is placed in standby mode until the next session.
8. It is recommended that the HPLC column be left in 100% methanol when not being used.

h. Data Acquisition and Data Management Considerations

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:
   UP_Study Tracker Number Instrument Letter Analyst ID Run Number_Sample Number.
   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:
   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.

Data Management

1. 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.
2. All data manipulation such as integration and review is undertaken in a shared network drive within the CDC network. 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.

i. 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 imported into STARLIMS for reporting.

j. 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**

1. Change UHPLC pre-column filters and guard column cartridges.
2. Flush analytical and SPE columns with mobile phase A and B at several concentrations. During this flush the temperature of the column can be raised to 50oC to facilitate cleaning.
3. The analytical and SPE columns can also be reversed for the flushing and left in this position for the analytical run.
4. Flush the probe with mobile phase at different concentrations. During this flush, the temperature of the probe can be raised to 400oC, and the voltage of the instrument set to 0 to minimize the amount of material transferred to the instrument.
5. Inject the instrument standard check extract and compare results to historical results for:
   a. Retention time of selected analytes.
   b. Sensitivity (area count of selected analytes)
   c. Chromatography (peak tailing, high background noise, anything odd)
6. 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**

1. Replace ion transfer tube while instrument is in stand-by.
2. 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.
3. 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.
4. Check the oil level in the rough pumps
   a. Oil level should be in between designated capacity markings. Replenish if below the line.
   b. Oil color should appear light and somewhat transparent/free of debris and particles. Call the manufacturer service line if it needs to be changed.
5. Ballast the rough pumps on an as-needed basis. Do not leave open for longer than 30 minutes.

- **Quarterly**

1. Flush isopropyl alcohol through the UHPLC lines, A & B without columns attached for ~ 30 minutes each at 5 mL/min if pressure allows.

9. Method Performance Documentation

a. Accuracy

The accuracy of this method was determined by recovery analysis after spiking two biological materials with target analytes at three levels spread throughout the linear range, i.e., low, medium, and high, and comparing to the corresponding non-spiked material. Three replicates were prepared per level, including the non-spiked material, resulting in a total of 12 samples per material per analytical run. Recovery of the analytes was calculated as [(final concentration – initial concentration)/spiked concentration]. Acceptable recovery should be 85-115% except at 3*LOD where it can be 80-120%.

Table 7. Accuracy

Analyte	Accuracy (%)		
	10 ng/mL	20 ng/mL	30 ng/mL
24D	97	99	101
OPM	96	99	101
4FP	96	99	102
CCC	97	96	99
OXY2	97	100	102
MAL	94	94	95
PAR	95	96	98
CPM	99	100	101
TCC	100	99	102
CBC3	95	95	99

b. Precision

Precision is determined by calculating the relative standard deviation (RSD) of repeat measurements (minimum N=20) of quality control materials at two concentrations, performed in duplicate through different analytical runs. Within-run, between-run and total precision are calculated from these data. Relative standard deviation should not exceed 15%.

Table 8. Precision

Analyte	Precision (%RSD)	
	QCL (ng/mL)	QCH (ng/mL)
24D	6.9	6.8
OPM	13.9	8.7
4FP	7.1	6.1
CCC	13.3	10.7
OXY2	5.1	5.1
MAL	9.9	11.8
PAR	4.5	5
CPM	13.5	11.3
TCC	5.3	9.6
CBC3	12.9	9.5

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 S_1 is given as the concentration of the lowest detected standard. The reported LOD can be higher than these values but cannot be below. LODs may vary over time.

Table 9. Analyte Detection Limits

Analyte	ng/mL
24D	0.15
OPM	0.1
4FP	0.1
CCC	0.5
OXY2	0.1
MAL	0.5
PAR	0.1
CPM	0.1
TCC	0.6
CBC3	0.5

d. Analytical Specificity

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 6.

e. Stability (Freeze-Thaw, Bench Top and Long-Term)

Freeze-thaw stability was determined by comparing analyte concentrations before and after three subsequent freeze-thaw cycles for two quality control materials. For each quality control material, n=3 measurements were made initially and then n=3 measurements were made after three freeze-thaw cycles. Bench-top stability was assessed by comparing analyte concentrations in two quality control materials before and after materials were stored at room temperature for one day. For each pool, n=3 measurements were made initially and n=3 measurements were made after materials were stored at room temperature for one day. Long term stability of the analytes should be assessed by analyzing two quality control samples after freezer storage for an extended time. This method has not been active long enough for this assessment. Long

term stability of the analytes will be assessed by analyzing two quality control samples after storing at or below -70 °C for two years (N=6).

Table 10. Stability results expressed as percent relative standard deviations of the concentrations of the materials used for the test; n=3 for all measurements

Analyte	Material 1			Material 2		
	Three freeze-thaw cycles	Bench-top stability	Processed sample stability	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
24D	12.8	12.8	0.0	7.0	7.6	-1.9
OPM	13.5	12.5	-5.2	6.9	5.7	-4.4
4FP	10.5	13.7	-2.1	5.8	9.7	0.6
CCC	5.4	9.7	-1.1	5.7	11.5	-10.2
OXY2	11.7	10.6	-5.3	9.4	7.5	-10.6
MAL	10.8	12.9	3.2	3.8	4.5	-8.9
PAR	11.7	13.8	-5.3	7.4	5.6	-8.0
CPM	7.4	11.7	-5.3	10.1	10.1	-5.7
TCC	9.4	10.4	-5.2	5.4	5.4	-9.2
CBC3	10.5	10.5	0.0	8.0	9.9	-3.1

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 analyte concentrations exceeding the highest reportable limit may be diluted, re-extracted, and reanalyzed so that the measured concentration 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. A minimum of eight standards is used for the calibration curve which is obtained by plotting the area ratios ([analyte peak area]/ [internal standard peak area]) of freshly analyzed standards versus the concentrations of the standards. The slopes and intercept are calculated by linear regression analysis and the 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 should be above the expected range of results for most samples. The linear range for all compounds is from the LODs to 50 ppb.

10. Quality Control (QC) Procedures

a. Analytical batch quality control procedures

QC Materials

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 pesticides 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.

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. Considering 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. Considering 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.

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.

Use of QC Materials

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

Final Evaluation of Quality Control Results

QC materials are evaluated using the Westgard multi-rule criterion that has been modified for use within the Division (Caudill et al. 2008) as described below.

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 result 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

1. The concentration measured in the qualification ion transition should be within 25% of the concentration measured in the confirmation ion transition. If the concentrations are not within these criteria, 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 11.
2. The relative retention time (RRT) of the native to label must fall within 0.95 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. No result should be reported outside the RRT criteria.

Table 11. Minimum Concentration of Qualifying Ion for Confirmation Comparison

Analyte	Minimum Concentration (ng/mL)
OXY2	0.4
MAL	1.5
PAR	1.5
24D	0.4
CPM	1.0
OPM	1.0
4FP	0.4
TCC	2.0
CCC	2.0
CBC3	2.0

Analytical columns exhibit minor variations from column to column and from one manufactured batch to another. It is therefore expected that upon replacement of a new column, or as the analytical column ages, the retention times of analytes will shift. If the shift is such that the analyte peaks are outside of the method-defined analyte windows, such a column would be deemed unfit for the method. If the shift is such that the peaks are skewed onto one side of the window or partially cut off, then the retention time for the affected analytes will be adjusted. This will center the peaks in the acquisition window to allow for reliable data collection and integration. Such a

method change will be documented in the Acquisition Method Change Log located on the instrument computer.

3. 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 ratio. 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.
4. The minimum signal-to-noise ratio for all native quantification ion peaks will be 3:1. These criteria are applied using the processing software but needs to be confirmed by the analyst.
5. The minimum signal-to-noise ratio for all labeled ion peaks will be 10:1 except for the labeled TCC and CCC peaks which will be 3:1. As with the native ratios, the analyst must visually confirm the acceptable criteria.
6. 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.
7. 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.
8. 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.
9. 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, samples must 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. All CLIA methods conducted in the Branch follow the Division-wide guidelines for Quality Improvements, Corrective Actions and Preventive Actions (QI/CAPA) Application described in STARLIMS document SG-017.

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 quantification of organic toxicants in human body fluids. By using tandem mass spectrometry, most analytical interferences can be eliminated. Because of the matrix used in this procedure, occasional interferences of 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) reported at the National Report on Human Exposure to Environmental Chemicals website (http://www.cdc.gov/exposurereport) are used as reference ranges to describe exposure to these pesticides among the general U.S. population (CDC 2022).

14. Critical-Call Results ("Panic Values")

Report test results in this laboratory support of epidemiological studies, rather than clinical assessments.

15. Specimen Storage and Handling during Testing

On the day of sample analysis, samples are retrieved from the freezer and kept on the bench until fully thawed before aliquoting. After analysis, sample plates are stored at -20 °C should a reanalysis be required.

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. Critical calls do not apply to this program.

1. The data from analytical runs of unknowns are reviewed by the laboratory supervisor. The supervisor provides feedback to the laboratory statistician and program lead and requests confirmation of the data as needed.
2. Laboratory statistician generates the quality control charts and pertinent documents to be reviewed by lab chief, branch chief and for the final review of the Division statistician who approves the quality control charts so that the results can be reported.
3. If the quality control data are acceptable, the laboratory supervisor or his/her designee generates a letter reporting the analytical results to the person(s) who requested the analyses to be signed by the CLIA Laboratory Director.
4. The data are sent (generally electronically by e-mail) to the person(s) that made the initial request.
5. All data (chromatograms, etc.) are stored in electronic format.

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. Robustness Test

The robustness of an analytical method is an estimation of its capability to remain unaffected by small changes in method variables. It provides a qualitative estimation of reliability when analyses are performed in standard conditions. The robustness test focused on steps where analyst errors are most likely to occur, specifically, in the aliquoting of various reagents: The variations chosen for the method are outlined below. Two samples were run in duplicate, and the results averaged out. Measured differences between duplicates were acceptable.
Enzyme concentration at 80%
Enzyme concentration at 120%.
Enzyme volume at 80%
Enzyme volume at 120%.
Enzyme pH at 4
Enzyme pH at 6
Quenching solution pH at 75%
Quenching solution pH at 125%

Sample 1 (Concentrations in ng/mL)

Analyte Code	Method Settings	Quench 12.5% Acetic	Quench 7.5% Acetic	120% Enzyme conc	80% Enzyme conc	60 µL Enzyme	40 µL Enzyme	Enzyme at pH 6	Enzyme at pH 4
24D	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
4FP	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
CBC3	1.5	1.4	1.4	1.5	1.6	1.6	1.6	1.5	1.6
CCC	1.8	1.9	2.0	1.9	2.0	2.0	1.9	1.9	1.9
CPM	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.5
MAL	1.1	1.1	1.0	0.9	0.9	0.9	0.9	0.9	0.9
OPM	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
OXY2	0.8	0.8	0.8	0.7	0.8	0.8	0.8	0.7	0.8
PAR	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
TCC	1.7	1.9	1.8	1.8	1.8	1.8	1.8	1.8	1.9

Sample 2 (Concentrations in ng/mL)

Analyte Code	Method Settings	Quench 12.5% Acetic	Quench 7.5% Acetic	120% Enzyme conc	80% Enzyme conc	60 µL Enzyme	40 µL Enzyme	Enzyme at pH 6	Enzyme at pH 4
24D	3.8	3.8	4.1	3.9	3.9	3.9	3.9	3.8	4.0
4FP	3.6	3.7	3.7	3.7	3.8	3.7	3.7	3.8	3.9
CBC3	10.5	10.0	10.0	11.1	10.5	10.4	11.2	10.8	10.5
CCC	8.2	8.2	8.3	8.6	8.1	8.2	8.0	8.4	8.4
CPM	9.1	9.0	9.4	9.2	9.1	9.2	8.8	8.7	8.9
MAL	6.5	6.4	6.7	6.4	6.5	6.4	6.4	6.3	6.6
OPM	3.7	3.8	3.9	3.8	3.9	3.9	3.9	3.9	4.0
OXY2	5.3	5.4	5.7	5.4	5.5	5.5	5.4	5.4	5.6
PAR	5.6	5.6	5.9	5.6	5.7	5.6	5.6	5.5	5.6
TCC	8.0	8.1	8.0	8.0	8.0	7.9	8.0	8.2	8.3

20. 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 place frozen samples in the 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. QC limits do not vary within studies.

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.

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.

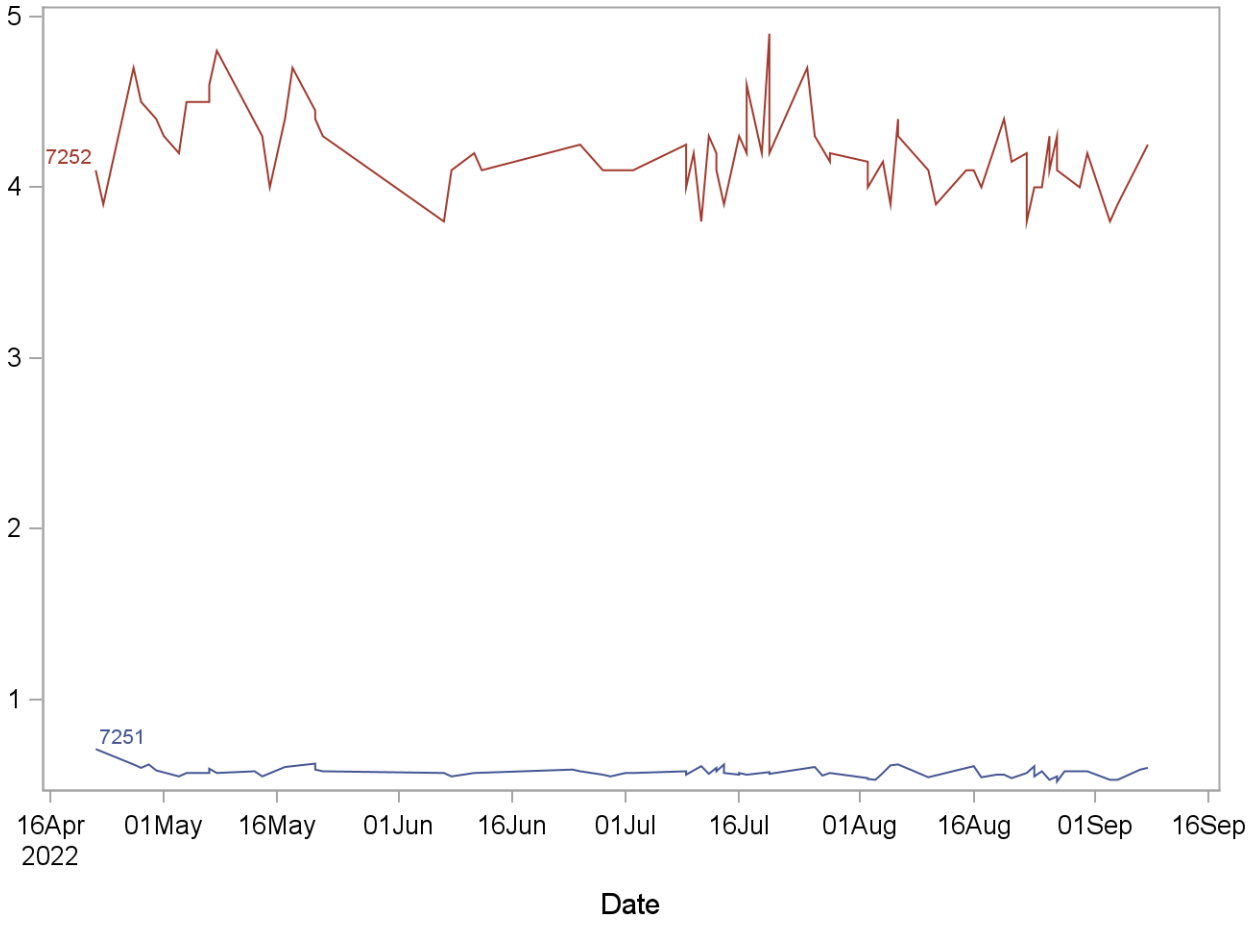
7. The frequency of cleaning the components of the mass spectrometer depends on the types and amounts of samples and solvents that are introduced into the instrument.

21. Summary Statistics and QC Graphs

Please see following pages

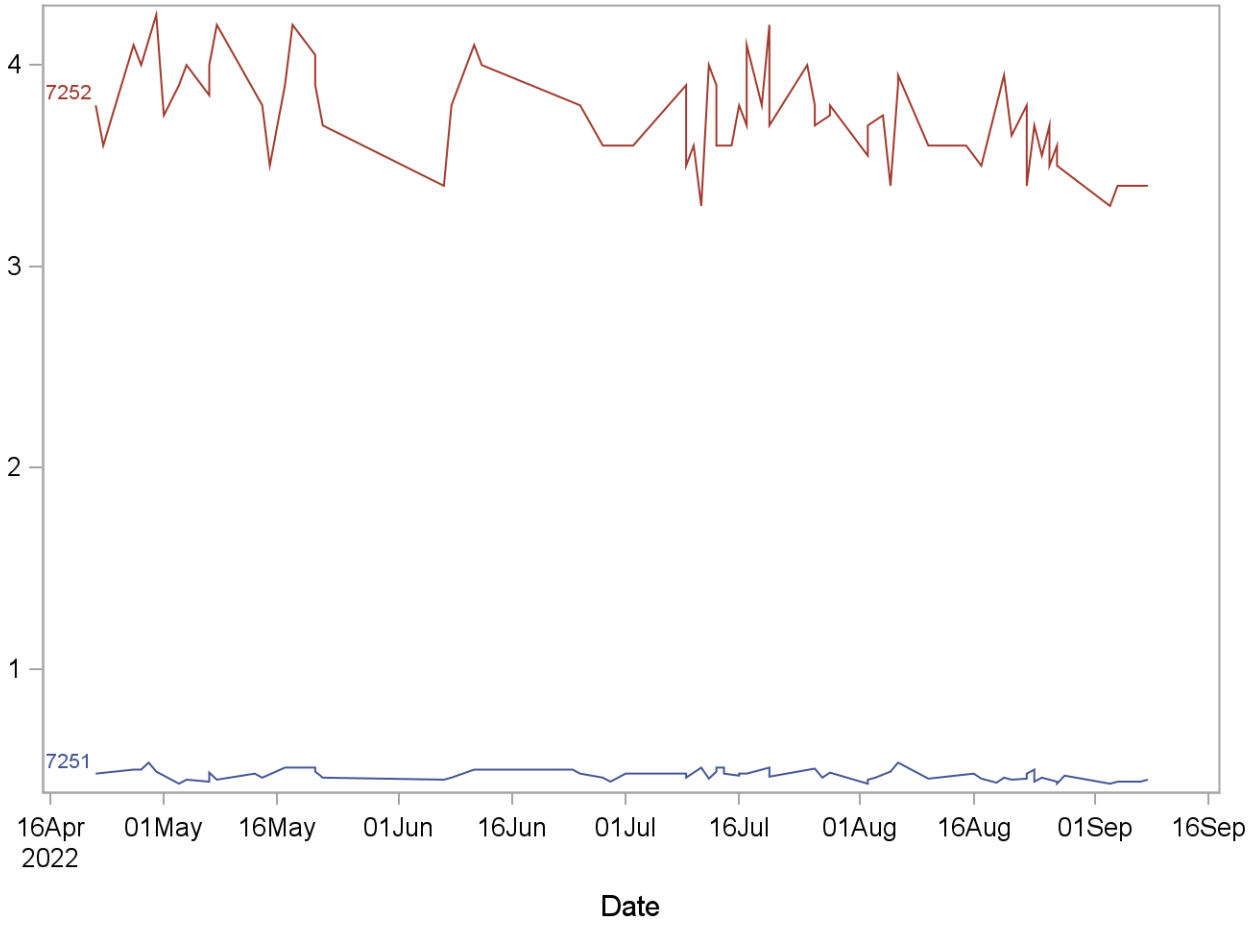
2015-2016 Summary Statistics and QC Chart
URX24D (2,4-D (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	67	22APR22	08SEP22	0.57515	0.03069	5.3
7252	71	22APR22	08SEP22	4.21408	0.24041	5.7



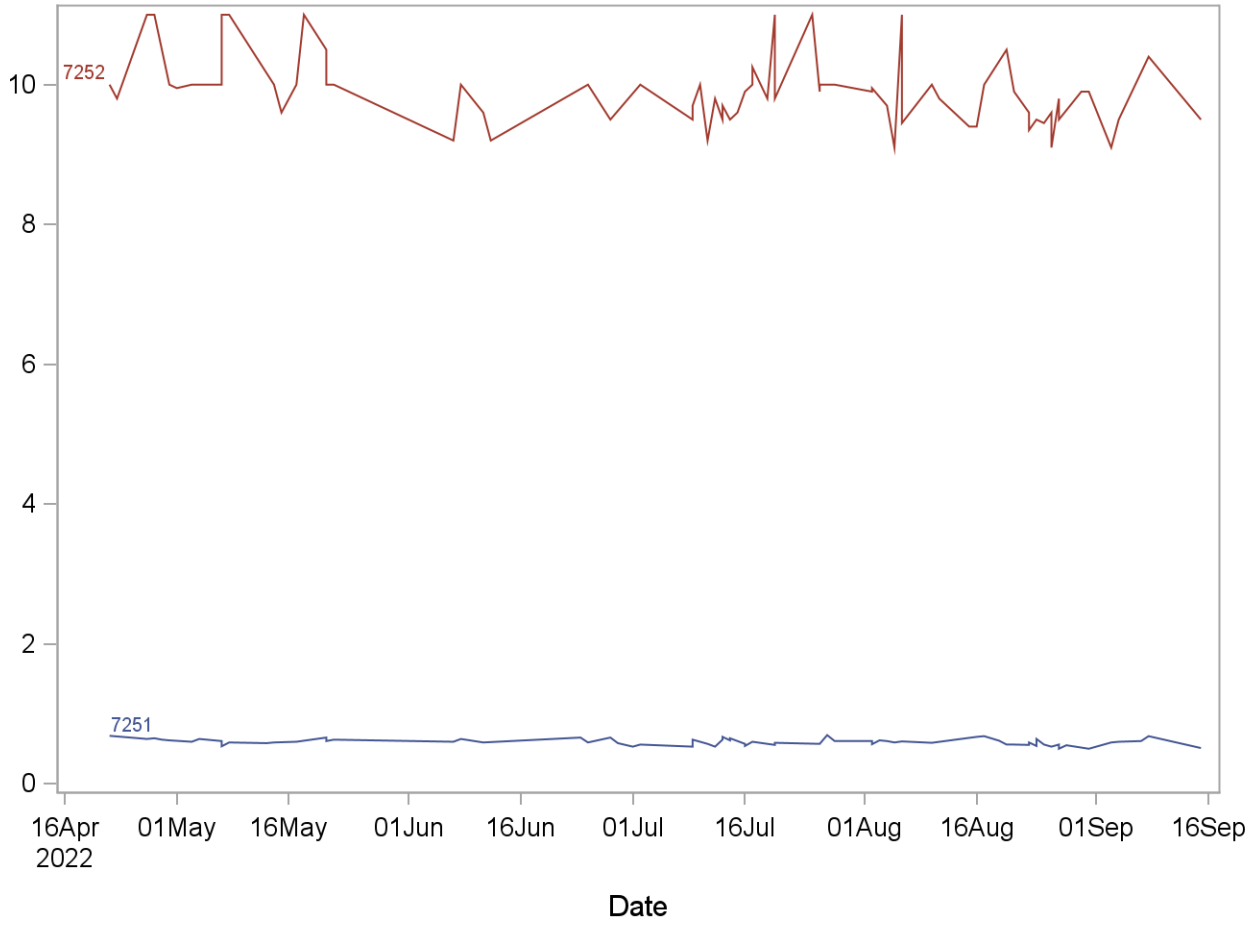
2015-2016 Summary Statistics and QC Chart
URX4FP (4-fluoro-3-phenoxy-benzoic acid (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	66	22APR22	08SEP22	0.47167	0.02614	5.5
7252	69	22APR22	08SEP22	3.74928	0.23208	6.2



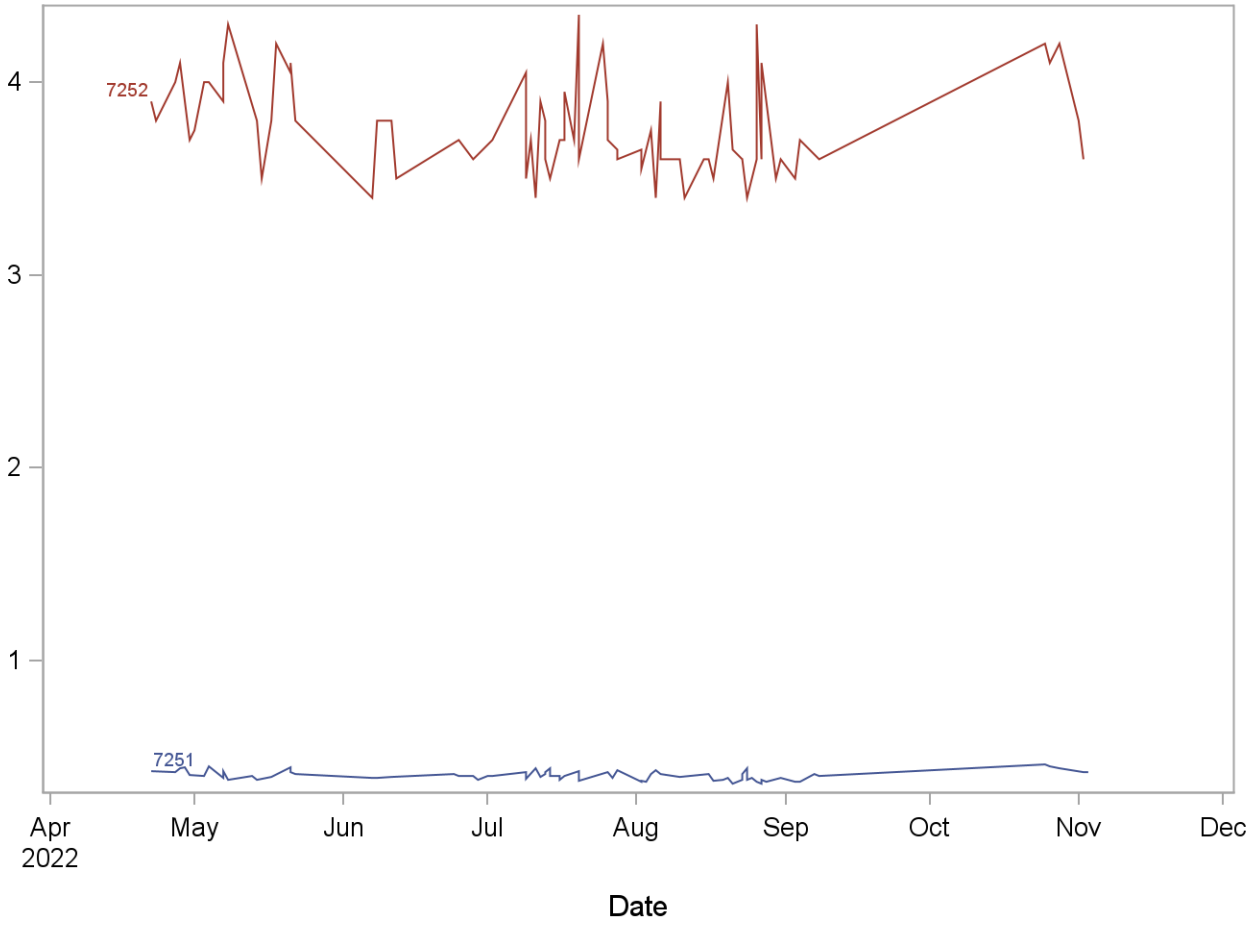
2015-2016 Summary Statistics and QC Chart
URXCPM (3,5,6-trichloropyridinol (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	68	22APR22	15SEP22	0.59735	0.04664	7.8
7252	72	22APR22	15SEP22	9.90000	0.49611	5.0



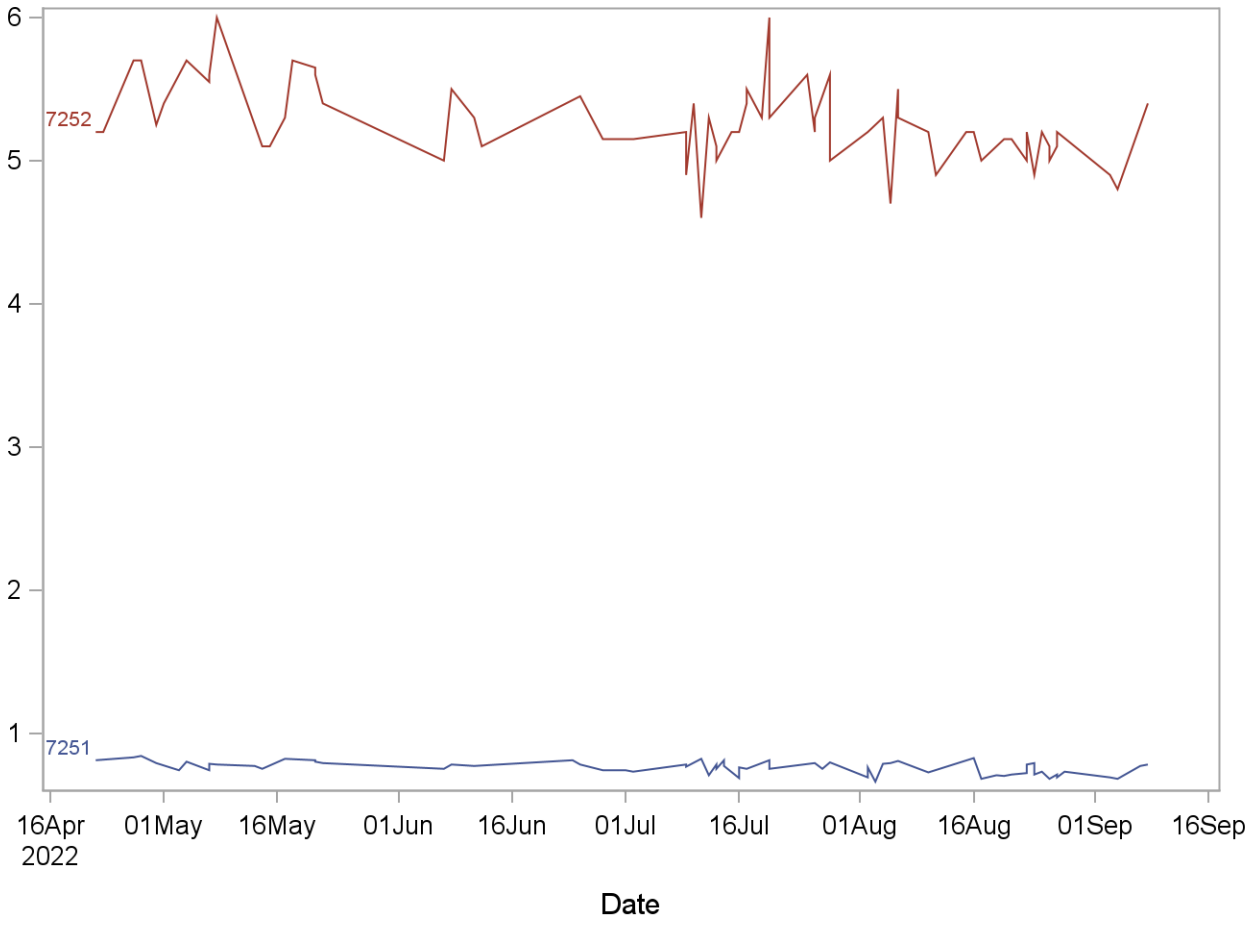
2015-2016 Summary Statistics and QC Chart
URXOPM (3-phenoxybenzoic acid (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	72	22APR22	03NOV22	0.40292	0.02433	6.0
7252	76	22APR22	02NOV22	3.76447	0.24601	6.5



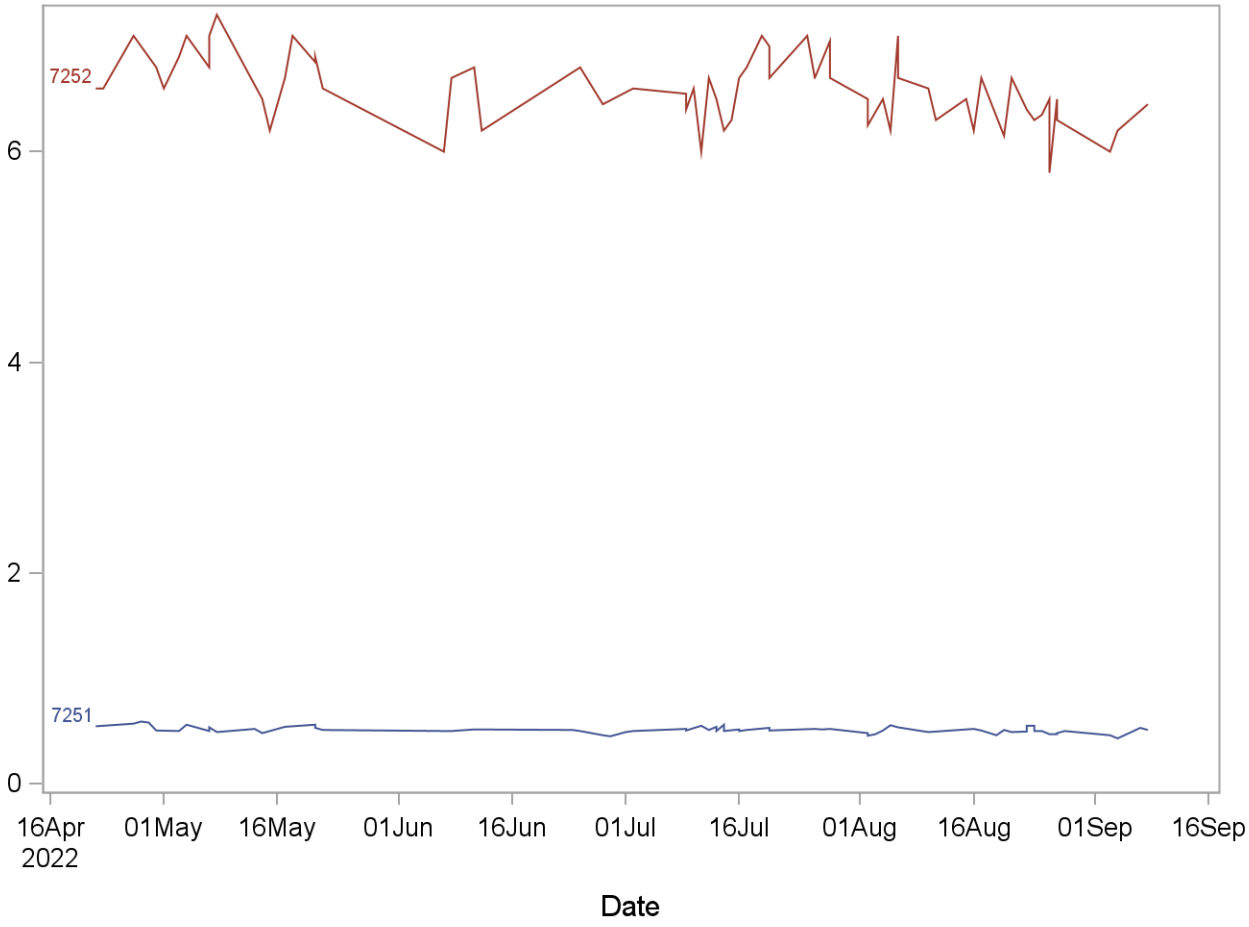
2015-2016 Summary Statistics and QC Chart
URXOXY (Oxypyrimidine (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	66	22APR22	08SEP22	0.75871	0.04444	5.9
7252	69	22APR22	08SEP22	5.26667	0.27556	5.2



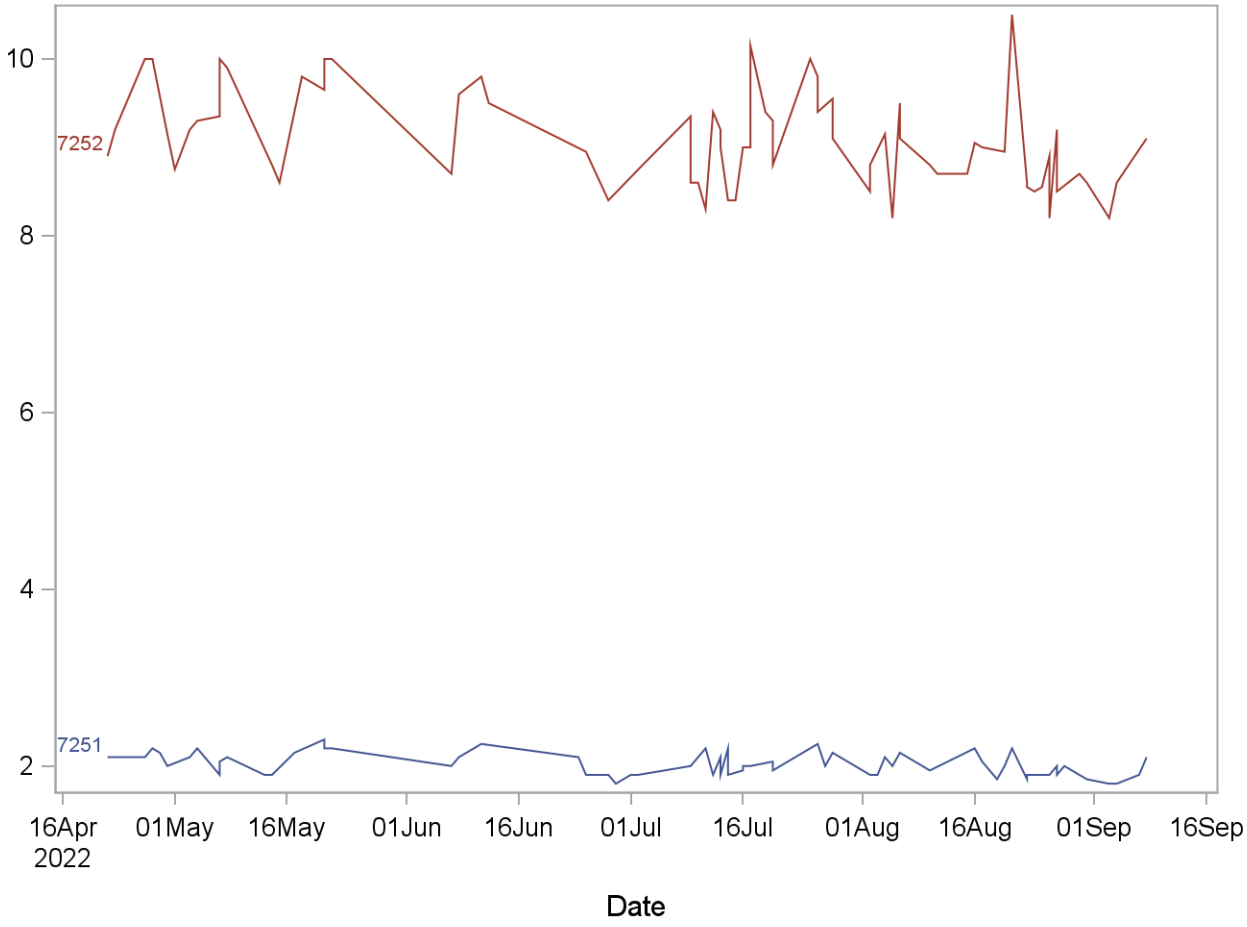
2015-2016 Summary Statistics and QC Chart
URXPAR (para-Nitrophenol (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	66	22APR22	08SEP22	0.51000	0.03197	6.3
7252	69	22APR22	08SEP22	6.59420	0.32148	4.9



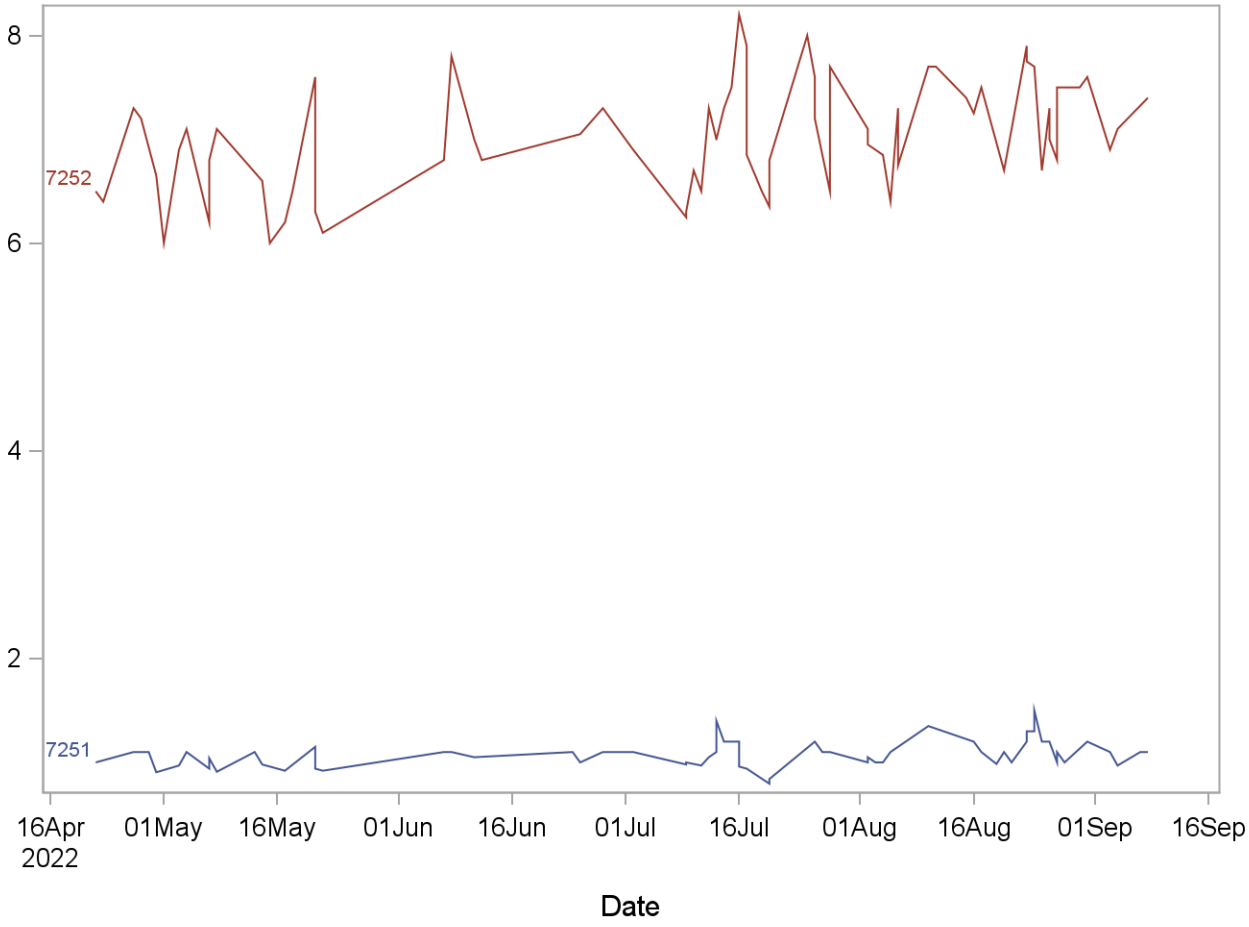
2015-2016 Summary Statistics and QC Chart
URXTCC (Dichlorovnl-dimeth prop carboacid (ug/L))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	67	22APR22	08SEP22	2.01269	0.12949	6.4
7252	71	22APR22	08SEP22	9.09296	0.54578	6.0



2015-2016 Summary Statistics and QC Chart
URXMAL (Malathion Diacid)

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
7251	67	22APR22	08SEP22	1.07858	0.12592	11.7
7252	71	22APR22	08SEP22	7.01972	0.52437	7.5



References

Atwood, D., & Paisley-Jones, C. (2017). Pesticides Industry Sales and Usage, 2008-2012 Market Estimates. In: U.S. Environmental Protection Agency Washington, DC 20460.

Caudill SP, Schleicher RL, Pirkle JL, Multi-rule quality control for the age-related eye disease study. Statist. Med. (2008) 27(20):4094-4106.

Centers for Disease Control and Prevention. National Report on Human Exposure to Environmental Chemicals (September 2022). Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. https://www.cdc.gov/exposurereport/

Davis MD, Wade EL, Restrepo PR, Roman-Esteva W, Bravo R, Kuklenyik P, Calafat AM. (2013) Semi-automated solid phase extraction method for the mass spectrometric quantification of 12 specific metabolites of organophosphorus pesticides, synthetic pyrethroids, and select herbicides in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci*, *929*, 18-26. https://doi.org/10.1016/j.jchromb.2013.04.005

Taylor, J.K. (1987). Quality Assurance of Chemical Measirements. Lewis Publishers, Chelsea, MI.