Laboratory Procedure Manual

Analyte: **Specific Organophosphorous 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: 6103.03

Revised: 05/1/2013

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:

File Name	Variable Name	SAS Label (and SI units)
UPHOPM_E	URXCB3	c-diBrvinyl-diMecyclopropane (µg/L)
	URXCPM	3,5,6-trichloropyridinol (µg/L)
	URXOXY	Oxypyrimidine (ug/L)
	URXMAL	Malathion Diacid (µg/L)
	URXPAR	Paranitrophenol (µg/L)
	URXOPM	3-phenoxybenzoic acid (µg/L)
	URXTCC	Trans-3-(2,2-d carboxylic acid(µg/L)
	URX4FP	4 fluoro-3-phenoxybenzoic acid(µg/L)
	URX24D	2,4, D (µg/L)
	URX25T	2,4,5, T (µg/L)
	URXUCR	Creatinine, urine (mg/mL)

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 four organophosphorous insecticide metabolites (i.e., 2-isopropyl-6-methyl-4-pyrimidiol; 2-[(dimethoxyphosphorothioyl) sulfanyl] succinic acid; 3, 5, 6-tricholor-2-pyridinol; 4-nitrophenol), four synthetic pyrethroid metabolites (3-phenoxybenzoic acid; 4-fluoro-3-phenoxybenzoic acid; trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid; trans-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid)) and two herbicides (2,4-dichlorophenoxyacetic acid; 2,4,5-trichlorophenoxyacetic acid)**.**

b. Test Principle

The approach followed is a modification of previous methodology (Beeson et al. 1999; Olsson et al. 2004). 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 (Davis et al. 2013). 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	OXY	Diazinon	O
2-[(dimethoxyphosphorothioyl) sulfanyl] succinic acid	MDA	MAL	Malathion	O
para-Nitrophenol	PNP	PAR	Methyl parathion, Parathion	O
3,5,6-tricholor-2-pyridinol	TCPY	CPM	Chlorphrifos, Chlorpyrifos-methyl	O
2,4-dicholorphenoxyacetic acid	2,4-D	24D	2,4-D	H
2,4,5-tricholorphenoxyacetic acid	2,4,5-T	25T	2,4,5-T	H
3-phenoxybenzoic acid	3-PBA	OPM	Permethrin, Cypermethrin, Cyfluthrin, others	P
4-fluoro-3-phenoxy-benzoic acid	4F-3PBA	4FP	Cyfluthrin	P
trans-dichlorovinyl-dimethylcyclopropane carboxylic acid	*trans*-DCCA	TCC	Permethrin, Cypermethrin	P
cis-dibromovinyl-dimethylcyclopropane carboxylic acid	*cis*-DBCA	CB3	Deltamethrin	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

The PSTARS database is a Microsoft Access database used to store, retrieve, and analyze data for this method along with other methods in the Contemporary

Pesticides Laboratory. The database is stored on the CDC network and is backed up regularly. Statistical data analyses are performed with Statistical Analysis System (SAS) ® software package.

b. Sample Information

Electronically transfer or manually enter into the database information pertaining to particular specimens. If you manually enter data, include the sample-identification (ID) number, the sample type, the standard number, and any other information not associated with the mass-spectral analysis. Electronically transfer the analytical information obtained from the sample to the database via a PC-based instrument interface. Then transfer the data electronically into the database.

c. Data Maintanance

After inputting all sample and analytical data into the database, check for transcription errors and overall validity. The database is on the CDC network which is backed up regularly.

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 4 hours of collection. If possible, collect at least 10-mL of urine and pour it into preferably sterile vials (30-mL Qorpak ® vials with screw-caps tops are suggested). Label the specimens, freeze them at ≤-20 ^{o}C, and ship them on dry ice. Carefully pack vials to avoid breaks during shipment. Store all samples at ≤-20 ^{o}C until analysis.

b. Sample Rejection

Reject specimens with volumes less than 0.5 mL because they cannot be reliably processed.

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

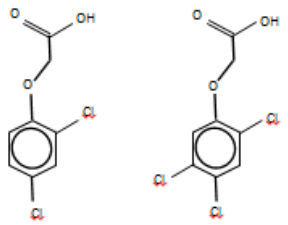
Not applicable for this procedure.

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

a. Reagents and Sources

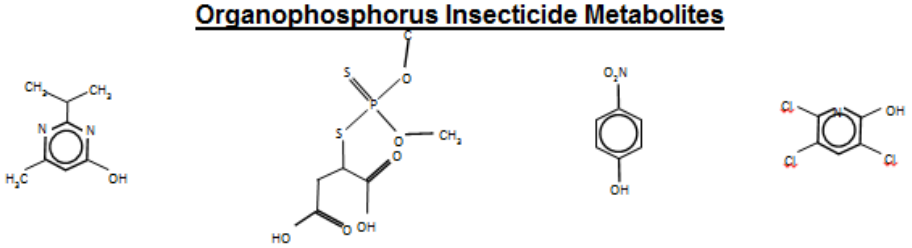
Table1. Compound Structures

Herbicides



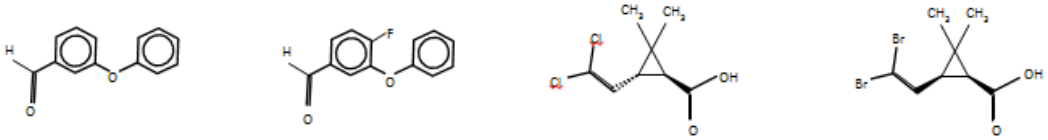
2,4-D 2,4,5-T

Organophosphorus Insecticide Metabolites



IMPY MDA PNP TCPY

Pyrethroid Insecticide Metabolites



3-PBA 4-F-3-PBA *trans*-DCCA *cis*-DBCA

Table 2a. Reagents and their Suggested Manufactures

Reagents	Suggested Manufacturers
Acetonitrile	Burdick and Jackson Inc.
Acetone	Tedia Company Inc.
Methanol	Fisher Scientific Inc.
HPLC Grade Water	JT Baker
Deionized water	NANOpure Infinity ultrapure water system
Glacial Acetic Acid	Sigma-Aldrich Co.
Sodium Acetate	Anachemia Chemicals NY
β-glucuronidase type H-1 from *Helix pomatia*	Sigma-Aldrich Co.

Table 2b. Analytical Standards and their Sources

Compound	Source
Oxypyrimidine-methyl-4,5,6	Cambridge Isotope Labs
$^{13}C_{4-}$Oxypyrimidine-methyl-4,5,6	Cambridge Isotope Labs
2-[(dimethoxyphosphorothioyl) sulfanyl] succinic acid	EQ Laboratories
d_{7-}Malathion dicarboxylic Acid	California Dept of Health Services
para-Nitrophenol	Cambridge Isotope Labs
$^{13}C_6$-*para*-Nitrophenol	Cambridge Isotope Labs
3,5,6-trichloro-2-pyridinol	Chem Services
$^{13}C_5$-^{15}N-3,5,6-tricholor-2-pyridinol	Cambridge Isotope Labs
2,4 Dichlorophenoxyacetic acid	Cambridge Isotope Labs
$^{13}C_{6-}$2,4-Dichlorophenoxyacetic	Cambridge Isotope Labs
2,4,5-trichlorophenoxyacetic acid	Cambridge Isotope Labs
$^{13}C_6$-2,4,5-trichlorophenoxyacetic acid	Cambridge Isotope Labs
3-phenoxybenzoic acid	Cambridge Isotope Labs
$^{13}C_6$-3-phenoxybenzoic acid	Cambridge Isotope Labs
4-fluoro-3-phenoxy benzoic acid	Cambridge Isotope Labs
$^{13}C_{6-}$4-fluoro-3-phenoxy benzoic acid	Cambridge Isotope Labs
trans-dichloro-dimethyl-cyclopropane carboxylic acid	Erlangen University
$^{13}C_4$-*trans*-dichlorovinyl-dimehtylcyclopropane carboxylic acid	Los Alamos National Lab
cis-dibromovinyl-dimethylcyclopropane carboxylic acid	EQ Laboratories
$^{13}C_6$-*cis*-dibromovinyl-dimethylcyclopropane carboxylic acid	Los Alamos National La
3-chloro-2-phenoxybenzoic acid	Aldrich Chemicals

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. Mix thoroughly. This solution can be stored at room temperature.

c. Analytical Standards 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 caps of solutions should never be labeled.

2) Labeled Spiking Solution

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

Table 3a. Labeled Spiking Solution Concentrations

Compound	Conc. (ng/mL)
$^{13}C_4$ IMPY	120
$^{13}C_6$ PNP	120
$^{13}C_6$ 2,4-D	120
$^{13}C_6$ 2,4,5-T	120
$^{13}C_5$ TCPY	240
d_7 MDA	480
$^{13}C_6$ 3-PBA	120
$^{13}C_6$ 4F-3-PBA	120
$^{13}C_4$ trans-DCCA	480
$^{13}C_4$ cis-DBCA	240

3) Calibration Standard Solutions

The concentrations of the individual compounds in the calibration solutions are given in Table 3b. 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. Typically 2 mL or 5 mL is used as the final volume. For sample analysis, 50µL of each solution is aliquotted into separate aliquots of diluted blank urine.

Table 3b. Calibration Standard Solution Concentrations

Compound	CS0	CS1	CS2	CS3	CS4	CS5	CS6	CS7	CS8	CS9	CS10	CS11
3-PBA	0.6	0.8	1.6	3	6	16	30	60	120	240	500	1000
4F-3-PBA	0.6	0.8	1.6	3	6	16	30	60	120	240	500	1000
2,4-D	0.6	0.8	1.6	3	6	16	30	60	120	240	500	1000
2,4,5-T	0.6	0.8	1.6	3	6	16	30	60	120	240	500	1000
IMPY	2	4	8	14	20	30	44	60	120	240	500	1000
MDA	2	4	8	14	20	30	44	60	120	240	500	1000
PNP	2	4	8	14	20	30	44	60	120	240	500	1000
TCPY	2	4	8	14	20	30	44	60	120	240	500	1000
trans-DCCA	8	12	16	20	24	30	60	120	160	240	500	1000
cis-DBCA	8	12	16	20	24	30	60	120	160	240	500	1000

Values given are concentrations in ng/mL.

4) 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, a 10µL aliquot of high level solution is diluted to 20mL in acetonitrile by weight to produce the spiking solution. The concentration of the current spiking solution is 1.4ng/mL. From the spiking solution, 10µL is added to every extract after concentration on the Turbo-Vap.

5) 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.

6) Proficiency-Testing Materials

Proficiency testing materials are matrix-based samples (typically spiked samples) with a known or characterized concentration. These samples may be spiked or have endogenous levels of the target analytes.

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 Micrewtube *(Simport, Beloeil, Canada)*
22) Screw caps for micrewtubes 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 compatable, 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) Agilent 1100 Series LC system *(Agilent Technologies, Santa Clara, CA)* including:
   a. Degasser
   b. Binary or Quaternary LC Pump
   c. AutoSampler
   d. Heated column Compartment

2) ThermoFisher TSQ Quantum Ultra 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 0.99. 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.

5) Due to 96-well plate technology, one calibration curve is prepared for two analytical runs. This calibration curve may be injected with each of the two runs. If the instrumental conditions have not changed since the injection of the first calibration curve, and the instrument sensitivity can be demonstrated to be similar, the same injection of the calibration curve used to quantitate the first run can be used for quantification of the second 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. 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 36 unknown samples, a solvent blank, a urine blank, 12 calibration samples, two low-concentration QC samples and two high-concentration QC samples. Due to the use of the 96-well plate technology, two analytical runs are prepared on one plate and share the calibration curve. The samples should be allowed to thaw and reach room temperature before aliquotting for analysis.

b. Sample Preparation

1. Sample Aliquotting and Incubation

1) Because the actual plate cannot be labeled, a plate diagram will be generated in Microsoft Excel detailing the contents of all positions on plate including if a well is not being used. This document is then kept with the run sheet as raw data.
2) Before aliquotting samples, the pipette used will be checked for proper calibration by pipetting 1 mL of water into a vial (after noting its tare weight) and recording the weight on the run sheet. If the result deviates by more than 0.02 grams from 1.0 grams, the pipette should not be used and should be noted for repair.
3) Before adding samples, 50µL of labeled spiking solution is added to every well and 50µL 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. This allows the addition of the sample to also mix the sample and standard solution.
4) Each thawed, unknown sample and QC sample is first vortexed, and then immediately, 1 mL of urine is aliquotted into the appropriate well on a 96-well collection plate. For the calibration samples, 1 mL of diluted blank urine is aliquotted into each designated well. One more aliquot of the blank urine is designated as the blank urine sample. The solvent blank sample is 1 mL of deionized water.
5) If a well is not to be used for extraction, aliquot 1 mL of deionized water into the position and treat as a sample through the SPE procedure. This reduces the variability of the applied vacuum.
6) Add 750 µLof enzyme solution to every well.
7) The samples are placed in an incubator at 37°C for at least 6 hours, but typically overnight.
8) 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 3 SPE system. 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. Below is a description of the procedure. Lines given in bold type are directions for analyst intervention. Lines given in italic type are performed automatically by the Quadra 3 system. 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 of the same volume.

b. The procedure will run as follows:

1. Initial shuttle layout
    a. Position 1: New tips on tip jig
    b. Position 2: Acetone
    c. Position 3: Conditioning Solution
    d. Position 4: Used tip jig
    e. Position 5: Sample Plate
    f. Position 6: OASIS HLB plate with waste reservoir below.
2. **Apply a small amount of vacuum to the SPE plate and begin the automated program**
3. *Load tips from Position 1*
4. *Mix 250µL of Acetone - 2 repetitions*
5. *250µL of Acetone is dispensed on Position 6 - 2 repetitions*
6. **Program will pause to allow the acetone to elute. Ensure that all the acetone has eluted before moving to the next step.**
7. *Acetone is blown out of tips at Position 2.*
8. *250µL of Conditioning Solution is dispensed on Position 6 – 2 repetitions.*
9. **Program will pause to allow elution of Condition Solution. This will require increasing the vacuum from the previous setting.**
10. *Conditioning Solution is blown out of tips at Position 3.*
11. *The samples are mixed by aspirating and dispensing 200µL of sample at Position 5.*
12. *350µL of sample is dispensed on Position 6 – 5 repetitions.*
13. **Program will pause after dispensing each increment.**
14. **It is recommended to dispense three increments at once and then add the final two increments as there is space in the well.**
15. **The vacuum will have to be increased to get all the samples to elute.**
16. *The tips are shucked to position 4 and discarded.*
17. *The program pauses for 10 minutes.*
18. **The vacuum should be turned off.**
19. **During the pause, the waste reservoir below the SPE plate should be emptied.**
20. **Position 3 should be changed to Wash Solution.**
21. **New tips should be placed in Position 1.**
22. **After the 10 minutes, apply a small amount of vacuum**
23. *The tips are loaded from Position 1.*
24. *The tips are wet with 250µL of Wash Solution*
25. *190µL of Wash Solution is dispensed on Position 6 – 2 repetitions*
26. *The tips are shucked to Position 4.*

27. *The program pauses to allow all the wash solution to elute.*
28. **After the solution has eluted, the vacuum should be turned up to high and left on.**
29. **The program will them pause for 20 minutes.**
30. **After the pause, the vacuum should be turned off and the waste reservoir replaced with an empty sample collection plate.**
31. **New tips should be placed in Position 1.**
32. **A small amount of vacuum should be applied.**
33. *The tips will be loaded from Position 1.*
34. *The tips will be wet with acetone from Position 2*
35. *325µL of Acetone will be dispensed on Position 6 – 2 times.*
36. *The tips are shucked to Position 4.*

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. If this does not help, the top frit of the well can be scored with a pick type instrument. If the well is still blocked, the top frit can be broken but left in place.

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 .

b. 10µL of injection standard spiking solution is added to each extract and the sample plate is placed on the Quadra 3 in position 2.

c. The analyst will prepare an LC vial plate by placing salinized vial inserts into a specially made collection plate.

d. The reconstitution program runs as follows:

37. Shuttle layout
    a. Position 1: New tips on tip jig
    b. Position 2: Sample Plate
    c. Position 3: not used
    d. Position 4: Used tip jig
    e. Position 5: LC vial plate
    f. Position 6: not used
38. *Load tips from Position 1*
39. *Mix 200µL of Reconstitution solution - 2 repetitions*
40. *110µL of Reconstitution Solution is dispensed on Position 2*
41. *Mix 110µL of solution in Sample Plate 10 times*
42. *Dispense 110µL of solution from Sample plate to LC vial plate*

43. Shuck tips to position 4

d. Due to the small diameter of the vial inserts, the analyst may choose to run the last step of the procedure manually to ensure each pipet tip is lined up correctly to transfer the extract.

e. The inserts are transferred to labeled LC vials using the plate diagram as reference and capped.

f. Samples are stored in a freezer if not analyzed immediately.

5. Data Considerations

a. A run sheet is generated from the PSTARS database that is used to track the samples through the analytical process.

b. Once a run of samples has been prepared, the analyst must log into PSTARS and specify that run as being cleaned up along with any comments about the prep. A sequence file for the mass spectrometer cannot be generated until the run has been cleaned up.

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

1) Instrumental conditions for liquid chromatography are specified in the Xcalibur instrument method file.
2) Pre-column setup: 2µm stainless steel frit, 0.5µm stainless steel frit, Betasil C-18 10x 4mm guard column.
3) HPLC Column : Betasil C-18, 2.1 x 100-mm, 3.0 µm particle size
4) Mobile Phase A: 95:5 0.1% Acetic Acid in HPLC-Grade Water: Methanol
5) Mobile Phase B: Acetonitrile
6) Gradient:
    a. Initial – 98% A, 2% B, hold for 2 minutes
    b. 4.0 minutes – 80% A, 20% B, hold for 2 minutes
    c. 7.0 minutes – 60% A, 40% B, hold for 1 minute
    d. 13.0 minutes – 40% A, 60% B, hold for 1 minute
    e. 14.5 minutes – 0% A, 100% B, hold for 2.5 minutes
    f. 17.01 minutes – 98% A, 2% B, hold for 3 minutes
7) Flow Rate: 0.5mL/min
8) Injection Volume: 30µL
9) Column Temperature: 40°C
10) Prior to running samples, place the vials in the sample tray of the autosampler according the position in the Xcalibur sequence file.
11) Ensure the solvent vial in tray position 99 is full with acetonitrile.
12) Ensure the wash vial in tray position 100 is full with mobile phase A.
13) Ensure that the solvent reservoirs contain sufficient solvent for the run.

d. 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. Table 4 list suggested tuning parameters that are optimized for this method.

Table 4.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 mT

3) 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.
4) The method is run in Selected Reaction Monitoring Mode.
5) The parent and product m/z's for each compound 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 5. For TCPY and DCCA, a higher mass resolution represented by a peak width of 0.2 is used only 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: 0.04 seconds.
8) The data mode: Centroid.
9) Divert valve timing
   a. Inject to waste 0.00 – 4.49 min
   b. Load to detector 4.50 – 14.99 min
   c. Inject to waste 15.00 – 20.00 min

Table 5. Compound Specific Mass Spectrometry Conditions

Analyte	Ion Polarity Mode	Segment	Precursor (m/z)	Product (m/z)	Peak Width of Q1	Collision Energy (V)
IMPY-Q	Positive	1	153	84	0.7	26
IMPY-C			153	70		25
IMPY-L			157	88		26
MDA-Q	Negative	2	273	141	0.7	11
MDA-C			273	157		23
MDA-L			280	147		23
PNP-Q	Negative	2	138	108	0.7	21
PNP-C			138	92		28
PNP-L			144	114		21
2,4-D-Q	Negative	3	219	160	0.7	15
2,4-D-C			221	163		16
2,4-D-L			225	167		31
TCPY-Q	Negative	3	195.91	196	0.2	7
TCPY-C			197.91	198		7
TCPY-L			202	202	0.7	7
2,4,5-T-Q	Negative	3	253	195	0.7	21
2,4,5-T-C			255	197		21
2,4,5-T-L			261	203		21
*Trans*_DCCA-Q	Negative	3	206.97	207	0.2	5
*Trans*_DCCA-C			208.97	209		5
*Trans*_DCCA-L			210	210	0.7	6
4F-3-PBA-Q	Negative	3	231	93	0.7	42
4F-3-PBA-C			231	187		19
4F-3-PBA-L			237	99		42
3-PBA-Q	Negative	3	213	93	0.7	42
3-PBA-C			213	169		17
3-PBA-L			219	99		42
DBCA-Q	Negative	3	294.9	79	0.7	31
DBCA-C			296.9	79		31
DBCA-L			303.8	79		31
3C-2PBA	Negative	3	247	93	0.7	21

Q = Quantification ion
C = Confirmation ion
L = Labeled compound ion

e. 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 HPLC system is also switched on with the initial conditions of the solvent gradient flowing and the column compartment heated coming to temperature.
4) Once the system has equilibrated, an instrument check sample is run to establish the instrument is working correctly with good sensitivity. 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.

5) A solvent blank is run after the instrument check samples.
6) The analytical run is then set up and run with the Xcalibur software.
7) A solvent blank is run after the highest calibration point to demonstrate no carry over. The analyst may choose to run additional solvent blanks throughout the run to keep the system clean for the entire run keeping in mind the entire analytical run needs to be completed in a 24 hour time period.
8) After the analytical run, a column wash procedure is run with a solvent blank to clean the HPLC column.
    a. Mobile Phase A= 95:5 0.1% Acetic Acid in HPLC-Grade Water: Methanol
    b. Mobile Phase B= 100% Acetonitrile
    c. Flow Rate 0.500 mL/min
    d. Divert valve set to Inject to waste
    e. Initial – 65% A, 35% B, hold for 1 minute
    f. 5.0 minutes – 95% A, 5% B, hold for 5 minutes
    g. 15 minutes – 0% A, 100% B, hold for 45 minutes
9) After the column wash, a probe wash procedure is run with a solvent blank to clean the HESI probe. This procedure is run with the analytical column taken out of the line.
    a. Mobile Phase C= 100% Methanol
    b. Mobile Phase D= 100% Acetone
    c. Flow Rate 2.00 mL/min
    d. Divert valve set to load to detector
    e. Initial – 100% C, 0% D, hold for 15 minutes
    f. 16.5 minutes – 0% C, 100% D, hold for 13.5 minutes
    g. 37.00 minutes – 100% C, 0% D
10) At the end of routine analysis, the system is placed in Stand-by mode until the next session. 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 PSTARS 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.
    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 check samples at the beginning of the sequence, a solvent blank after the calibration curve, a column wash procedure at the end of the run and a probe wash procedure after the column wash. 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 flash 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 PSTARS 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.
    1. All compounds in this method except cis-DCCA have their own stable isotope labeled analog for quantitation.
    2. For the quantitation of cis-DCCA, use the labeled trans-DCCA.
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 PSTARS 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. The following suggested maintenance schedule has been demonstrated to keep the systems is good working order during high throughput analysis times.

- **Daily**
  a. Recap all vials on the auto-sampler tray immediately following previous day's run.
  b. Look for bacterial growth in the mobile phase "A" bottle. If present, dispose of mobile phase to proper waste bottle and prepare fresh mobile phase. Wash mobile phase bottle with isopropanol (IPA) and water. Put away for later use and use a new mobile phase bottle for the freshly prepared mobile phase.
  c. Change HPLC pre-column filter.
  d. Flush the HPLC lines without column attached:
     1. Open the valve (black knob on HPLC) so that the solvents go to the waste line
     2. Flush with 100% of mobile phase B @ 5 mL/min (look for pressure ~ 20 bar)
     3. If pressure is stable at ~20bar, flush with 50% of mobile phase A and 50% mobile phase B @ 5 mL/min (usually between ~30 –40 bar)
     4. Flush with 100% of mobile phase A @ 5mL/min (look for pressure usually between ~40 -50 bar)
  e. If HPLC lines have very high pressure before the column and will not stabilize when you perform the above test, it is most likely the needle seat getting backed up with salts and proteins. Make sure that the needle is in the up position, remove the needle seat and place in the correct injector to the HPLC line. Back-flush with 100% acetonitrile for ~ 1 hour. If pressure is really high, you can also sonicate for ~30 minutes in organic solvent. Then, reconfigure and test pressure again. If still too high change the needle seat.
  f. Attach column and flush with mobile phases at 0.5 mL/min for ~30 minutes @ initial method parameters for %A and %B.
  g. Inject the daily standard check 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)

h. If problems are found with the first injection, inject the standard again. If problem still exist, do not continue until appropriate action is taken.
i. Change the guard column and re-inject the daily check standard.
j. If tailing is still present, back-flush the column with 0.3 mL/min or higher of 100% ACN overnight. Make sure to change back column configuration the next day before the next run.

- **Weekly**
  a. Replace ion transfer tube while instrument is in "stand-by"
     1. Do not cool the ion transfer tube
  b. Clean the sweep cone, if needed, with aluminum oxide powder, water, and methanol.
  c. Clean ion transfer tubes once several have accumulated, use a metal needle to look for internal blockage. If an internal blockage is present discard the tube. If no internal blockage is present, sonicate for 30-45 minutes in 15 - 20% nitric acid in water, then 100% methanol for 5-10 minutes, and finally 5 minutes in 100% acetone
  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.
- **Monthly**
  a. Wash mobile phase A bottle with isopropanol (IPA)
  b. Turn off electronics and then turn off the vacuum.
  c. Remove probe, probe mount housing, and API stack.
  d. Remove ion transfer tube if still inserted when removed
  e. Clean API stack- remove tube lens and skimmer plate
     4. Wipe both with methanol and a cotton-tip or tissue; paying special attention the orifice.
     5. If burn spots are still present, clean the skimmer plate with aluminum oxide slurried water using a cotton-tip. This should only be done as a last resort since the oxide will scratch the plate.
     6. Sonicate both tube lens and skimmer plate in methanol for ~10 minutes.
     7. Dry both the tube lens and skimmer plate with nitrogen gas by using another MS that is ON but not running anything.

a. Unscrew the blue sheath gas line
b. Turning sheath gas up to 80 on the tune file
c. Gas will come out from the line.
d. Blow all over the tube lens and skimmer plate until both are completely dry.

f. Clean probe mount (housing) with water, methanol, and acetone (in that order). Pay special attention to drain tube connector. If it is very dirty, the metal elbow can be removed by screws and sonicated in water, methanol, and acetone. Do not use abrasives.
g. Assemble the API stack and install on the MS.
h. Turn the vacuum and electronics on.
i. Wait at least 2-4 hours to run anything on instrument**

- **Quarterly**

j. Flush IPA through the HPLC lines, A & B, (without column attached) for ~ 30 minutes each at 5 mL/min if pressure allows.
k. Change the PTFE filter attached to the valve.
l. Equilibrate lines with 100% mobile phase A at 1-5 mL/min and then with 100% mobile phase B at 1-5mL/min.

2. Breaking vacuum and turning system off.
    a. Turn the capillary temperature down to room temperature (30° C) and then put the instrument in Stand-By mode.
    b. After cooling the capillary, turn the electronics switch off.
    c. After 20-30 seconds, turn the hardware off.
    d. Limit the amount of time that the MS is OFF and the heater cage assembly is removed.
3. Turning system back on.
    a. Un-plug one of the fore pump plugs on side of instrument.
    b. Turn on the hardware.
    c. Make sure there is not a constant hissing noise indicating the API Stack was not installed correctly.
    d. Re-plug the fore pump on the side of the instrument.
    e. Install a clean ion transfer and the sweep cone.
    f. Place a GC septa over the hole so that the vacuum will return in ~ 2 hours,
    g. Do not turn on the electronics for 45 minutes.
    h. Install the source housing, including probe.
    i. Turn on the electronics.

9. Reportable Range of Results

The linear range of the standard calibration curves determines the highest and lowest analytical values of an analyte that are reportable. The calibration verification of the method encompasses this reportable range. Samples with results that exceed the

highest reportable limit will be re-extracted and analyzed with a smaller volume to bring the result within the reportable range.

a. Linear Limits

Analytical standards are linear for all compounds through the range of concentrations evaluated. The linear range for all compounds is from the LODs to 50 ppb. Resample urine samples whose concentrations exceed these ranges, and re-analyze them using a smaller volume.

b. Analytical Sensitivity

The detection limits for each compound can be set by the concentration of the lowest detected calibration standard or by calculating as $3S_0$ where S_0 is the standard deviation of blank samples at near-zero concentrations (Taylor 1987). The concentration in the S0 standard is assumed to be below the LOD; therefore, in Table 8, the concentration of S1 is given as the detected lowest standard. This is compared to the $3S_0$ concentration for a recent study. The reported LOD will be the higher of the two values and will be determined for each project.

Table 8. Analyte Detection Limits

Compound	(ng/mL)
IMPY	0.1
MDA	0.5
PNP	0.1
TCPY	0.1
2,4-D	0.4
2,4,5-T	0.1
3-PBA	0.1
4f-3-PBA	0.1
***trans*-DCCA**	0.6
***cis*-DBCA**	0.5

c. Accuracy

The accuracy of this method was determined by enriching urine samples with known concentrations of the pesticides residues and comparing the calculated and expected concentrations. The accuracy was consistent across the entire linear range. The accuracy can be expressed as the slope of a linear regression analysis of the expected value versus the calculated value. A slope of 1.0 indicates that the results are identical. Another way of expressing a method's accuracy is as a percentage of the expected value. Data given in Table 9 are taken from the most recent study using the average calculated concentration from 28 determinations.

Table 9. Accuracy of the Method

Compound	Slope (Calculated vs. Actual)
IMPY	1.01

MDA	0.994
PNP	1.00
TCPY	1.03
2,4-D	1.04
2,4,5-T	1.05
3-PBA	1.03
4F-3-PBA	1.03
trans-DCCA	1.00
cis-DBCA	1.01

d. Precision

The precision of this method is reflected in the variance of (QC) samples over time. The major contributor to the overall calibration verification is the variation between runs. Variation and the total coefficient of variation were determined from multiple analyses of quality control materials throughout a set time period. The total calibration verifications of the method vary from study to study and are dependent upon instrument, operators, and sample preparation analyst. The calibration verifications shown in Table 10 are representative of the most recent study.

Table 10. Precision of the Method

	QC High			QC Low		
Compound	Average (ng/mL)	Standard Deviation	%RSD	Average (ng/mL)	Standard Deviation	%RSD
IMPY	3.74	0.17	4.4	0.987	0.055	5.6
MDA	6.18	0.90	15	1.63	0.275	17
PNP	2.80	0.19	6.7	0.413	0.045	11
TCPY	12.0	0.59	4.9	1.88	0.198	11
2,4-D	3.90	0.25	6.3	2.33	0.148	6.4
2,4,5-T	2.31	0.24	11	0.254	0.049	19
3-PBA	3.19	0.23	7.2	0.578	0.082	14
4F-3-PBA	3.22	0.17	5.4	0.355	0.034	9.7
trans-DCCA	8.11	0.83	10	3.29	0.528	16
cis-DBCA	5.06	0.86	17	3.24	0.417	13

e. Analytical Specificity

This is a highly selective method that requires the following of each compound detected:

a. A specific retention time determined by the labeled analog of the compound.
b. Two precursor ions at specific m/z's.
c. Two specific product ions formed from the two precursor ions at specific m/z's.
d. The ion ratios of the two product ions are within a predetermined range.

10. Quality Control (QC) Procedures

a. **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

1) The ratio of the quantification ion and confirmation ion must fall within the ranges presented in Table 11. If the ratio falls outside this range, the integration of each ion should be checked. If the ratio is still out, the extract can be re-injected with a different gradient to attempt to separate out interfering compounds. If the ratio is out, the result must be reported as "Interfering Compound Present."
   a. For non-halogenated compounds, the ion ratio is highly dependent on the mass spectrometer tune. If the ion ratio begins to consistently fall out of the ranges given, the average ion ratio of the calibration standards plus or minus 30% can be used as an alternate criteria for these compounds.
2) The relative retention time (RRT) of the native to label must fall within the ranges presented in Table 11. 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.

Table 11. Compound Specific QC Criteria

Analyte	Ion Ratio Range	RRT Range
IMPY *	1.17-2.18	0.95-1.05
MDA *	1.94-3.60	0.95-1.05
PNP *	6.09-11.3	0.95-1.05
TCPY	0.73-1.36	0.95-1.05
2,4-D	1.14-2.11	0.95-1.05
2,4,5-T	0.71-1.31	0.95-1.05
3-PBA *	0.70-1.30	0.95-1.05
4F-3-PBA *	0.48-0.90	0.95-1.05
trans-DCCA	0.88-1.64	0.95-1.05
cis-DBCA	0.74-1.38	0.95-1.05

*- denotes a non-halogenated compound

3) The minimum signal-to-noise ratio for all native quantification ion peaks will be 3:1. This is an examination done by the analyst during the initial data processing. The analyst can choose to use the software generated ratio, but needs to visually confirm the ratio since software algorithms do not always accurately calculate the ratio.
4) The minimum signal-to-noise ratio for all labeled ion peaks will be 10:1 except for the labeled trans-DCCA peak which will be 3:1. As with the native ratios, the analyst must visually confirm the acceptable criteria.
5) The minimum area count for all native quantification peaks will be 800. If area counts fall below this criterion, the mass spectrometer should be inspected and possibly cleaned, and the extract can be re-injected to give appropriate area counts.
6) All results found in trans-DCCA and cis-DCCA must have a corresponding result for 3-PBA. If this does not occur, the mass spectrometer should be inspected and possibly cleaned, and the extract can be re-injected.
7) 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.
8) The injection standard (3C-2-PBA) is used to evaluate the instrument function during the run on a sample basis. It is used primarily 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 analyst can conclude that the injection of the extract was compromised and re-inject.

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

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until you identify the source or cause of failure is identified and corrected. If the source of failure is failure of the mass spectrometer, a pipetting error or other instrumental problems, correct the problem immediately. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration-verification samples (in the case of calibration failure). After re-establishing calibration or QC, resume analytical runs.

12. Limitations of Method; Interfering Substances and Conditions

This method is an isotope-dilution mass spectrometry method, which is widely regarded as the definitive method for the measurement of organic toxicants in human 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) 1999-2004 are used as the reference range to describe levels of exposure to these pesticides among the general US population (CDC 2009).

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. The Quality Control officer reviews each analytical run and identifies the quality control samples within each analytical run and determines whether the analytical run is performed under acceptable control conditions.
c. One of the Division statisticians reviews and approves the quality control charts pertinent to the results being reported.
d. If the quality control data are acceptable, the laboratory supervisor or his/her designee generates a memorandum to the Branch Chief, and a letter reporting the analytical results to the person(s) who requested the analyses to be signed by the Division Director.
e. The data are sent (generally electronically by e-mail) to the person(s) that made the initial request.
f. All data (chromatograms, etc.) are stored in electronic format.
g. 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

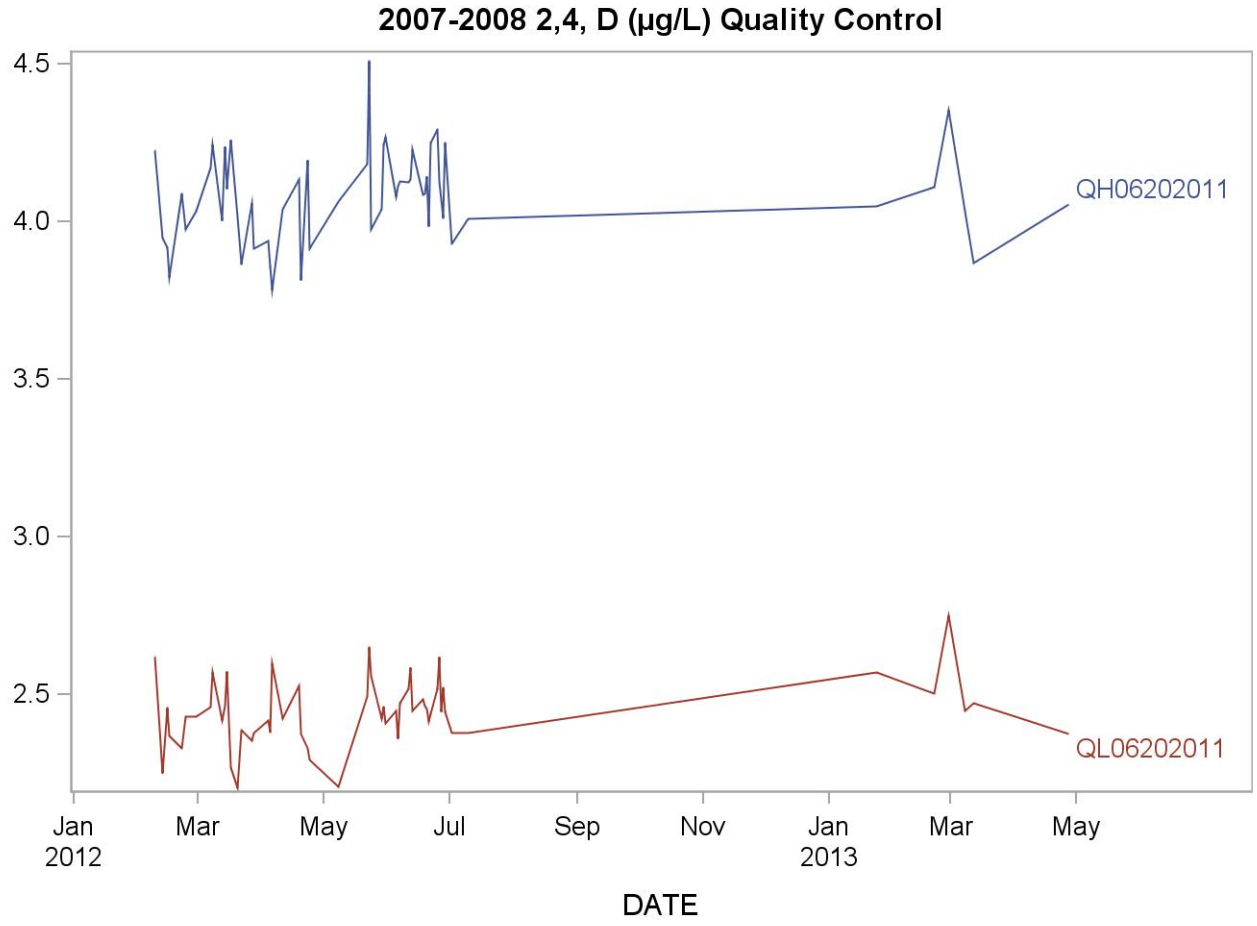
Use standard record-keeping systems (i.e., notebooks, sample logs, data files, creatinine logs, demographic logs) to keep track of all specimens. Transfer of refer to CLIA-specimens only certified laboratories. Any transfer of study samples is handled through the DLS special studies coordinator.

19. Summary Statistics and QC Graphs

See next page(s).

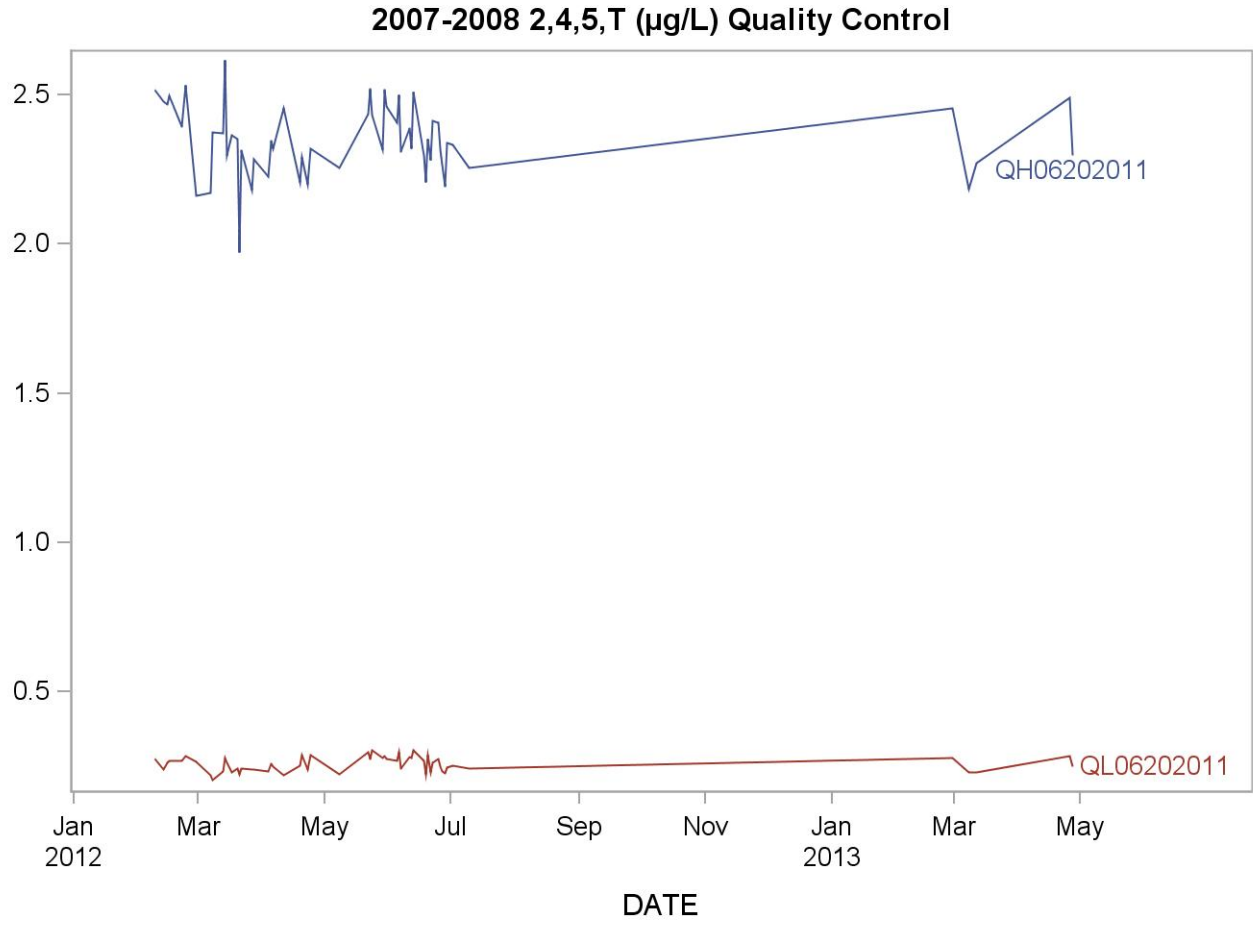
Summary Statistics for 2,4, D (µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	77	09FEB12	27APR13	4.092	0.167	4.1
QL06202011	77	09FEB12	27APR13	2.454	0.117	4.8



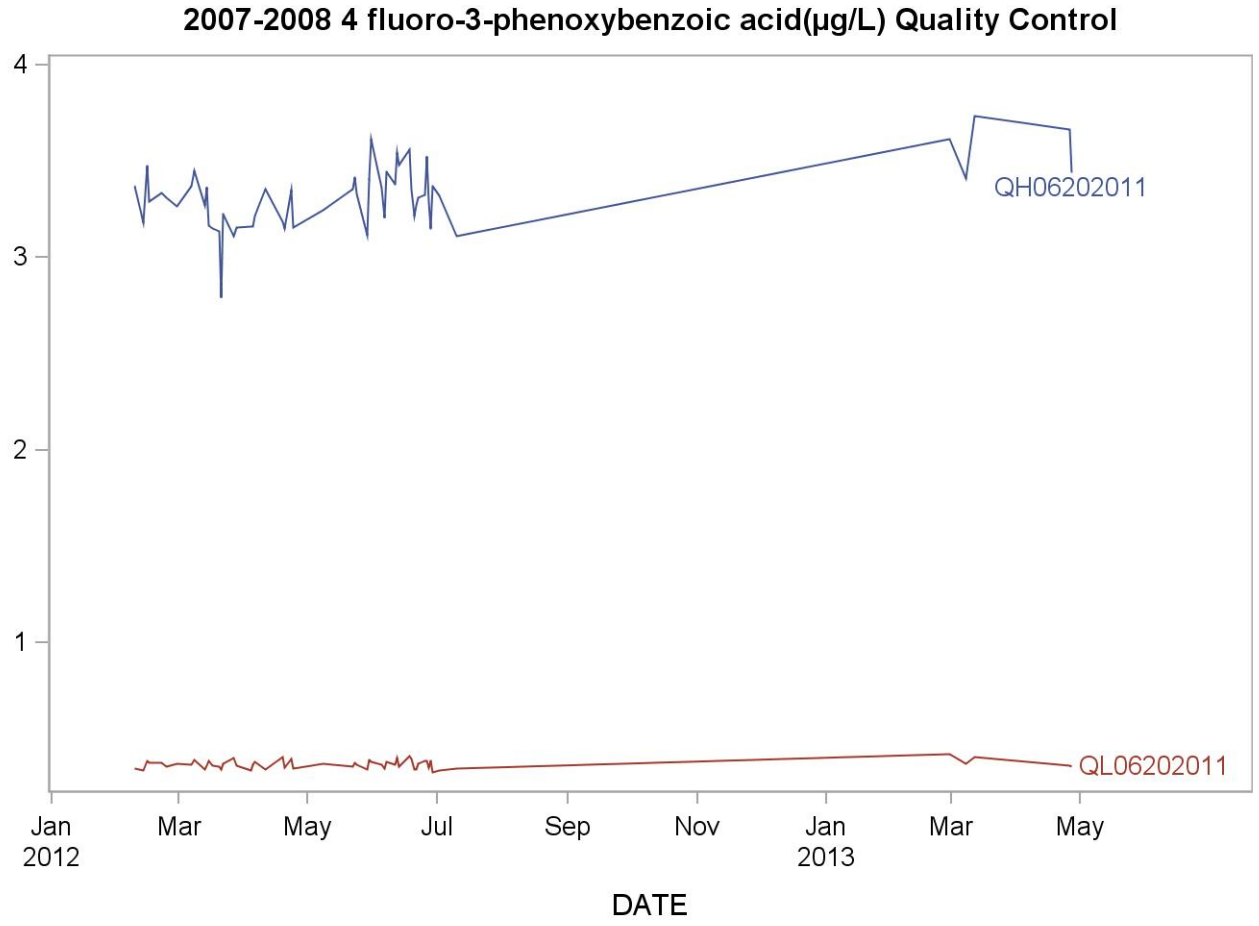
Summary Statistics for 2,4,5,T (µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	76	09FEB12	27APR13	2.354	0.124	5.3
QL06202011	76	09FEB12	27APR13	0.259	0.025	9.8



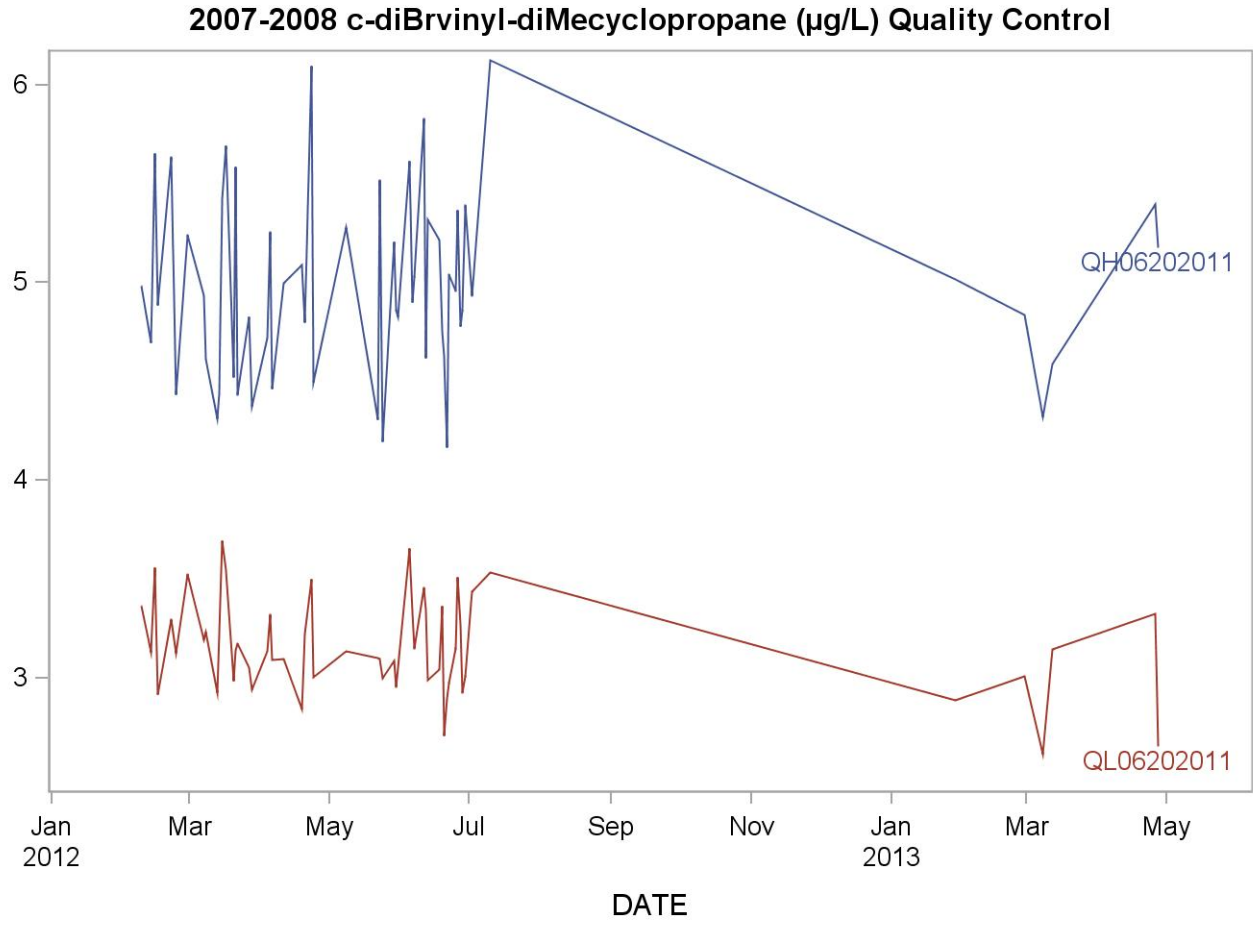
Summary Statistics for 4 fluoro-3-phenoxybenzoic acid(µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	76	09FEB12	27APR13	3.323	0.161	4.8
QL06202011	76	09FEB12	27APR13	0.368	0.025	6.7



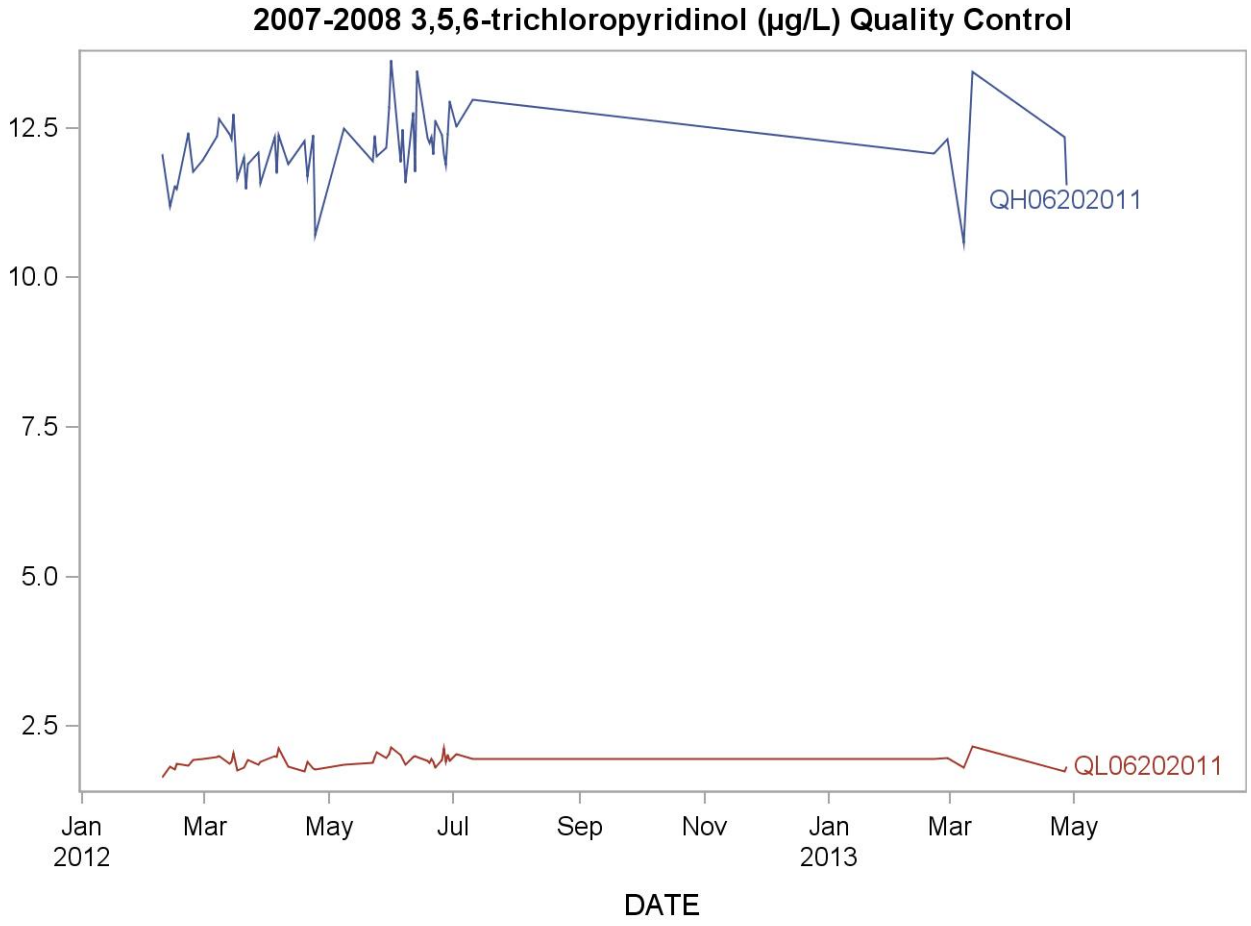
Summary Statistics for c-diBrvinyl-diMecyclopropane (µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	76	09FEB12	27APR13	5.018	0.539	10.7
QL06202011	76	09FEB12	27APR13	3.169	0.286	9.0



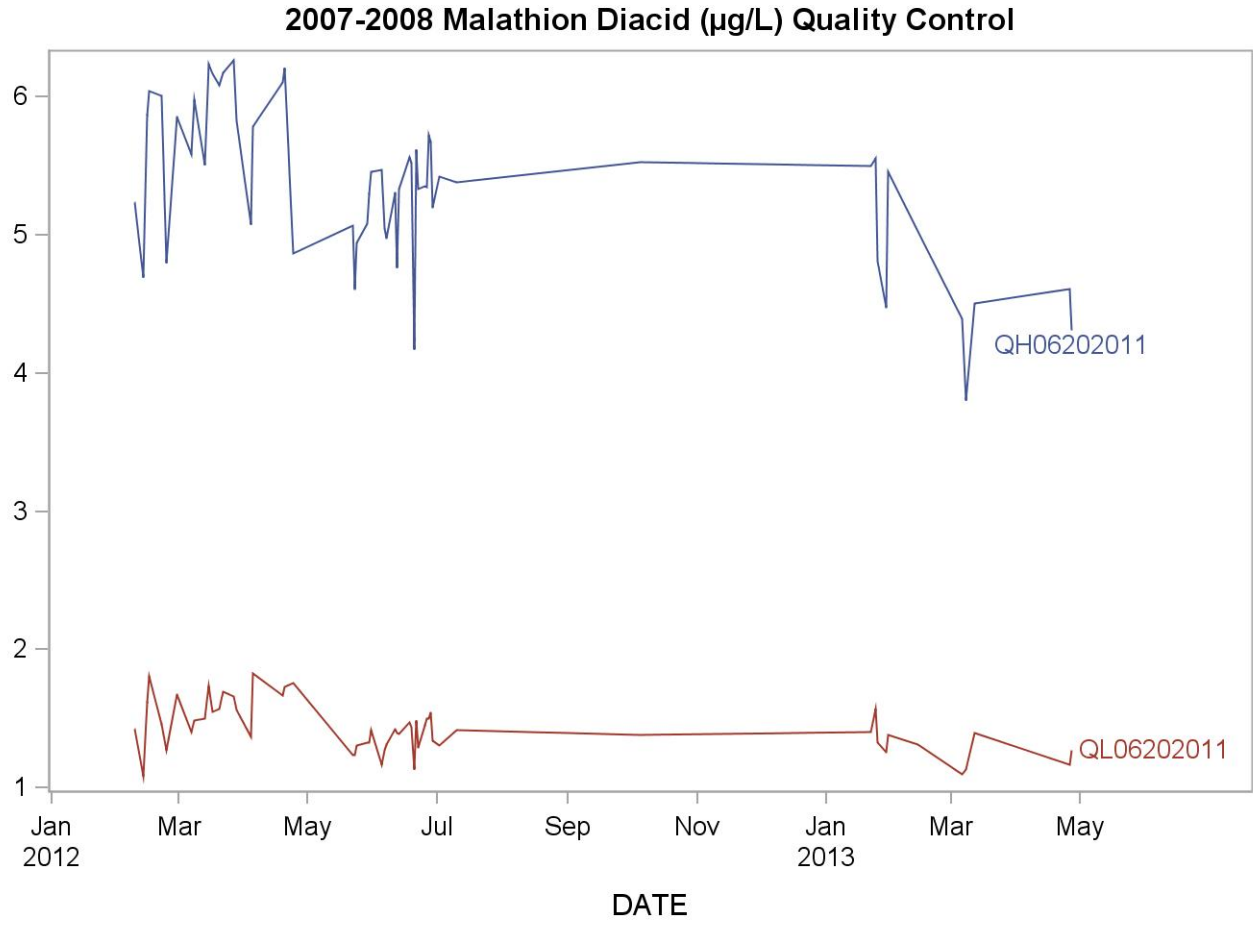
Summary Statistics for 3,5,6-trichloropyridinol (µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	77	09FEB12	27APR13	12.182	0.583	4.8
QL06202011	77	09FEB12	27APR13	1.916	0.123	6.4



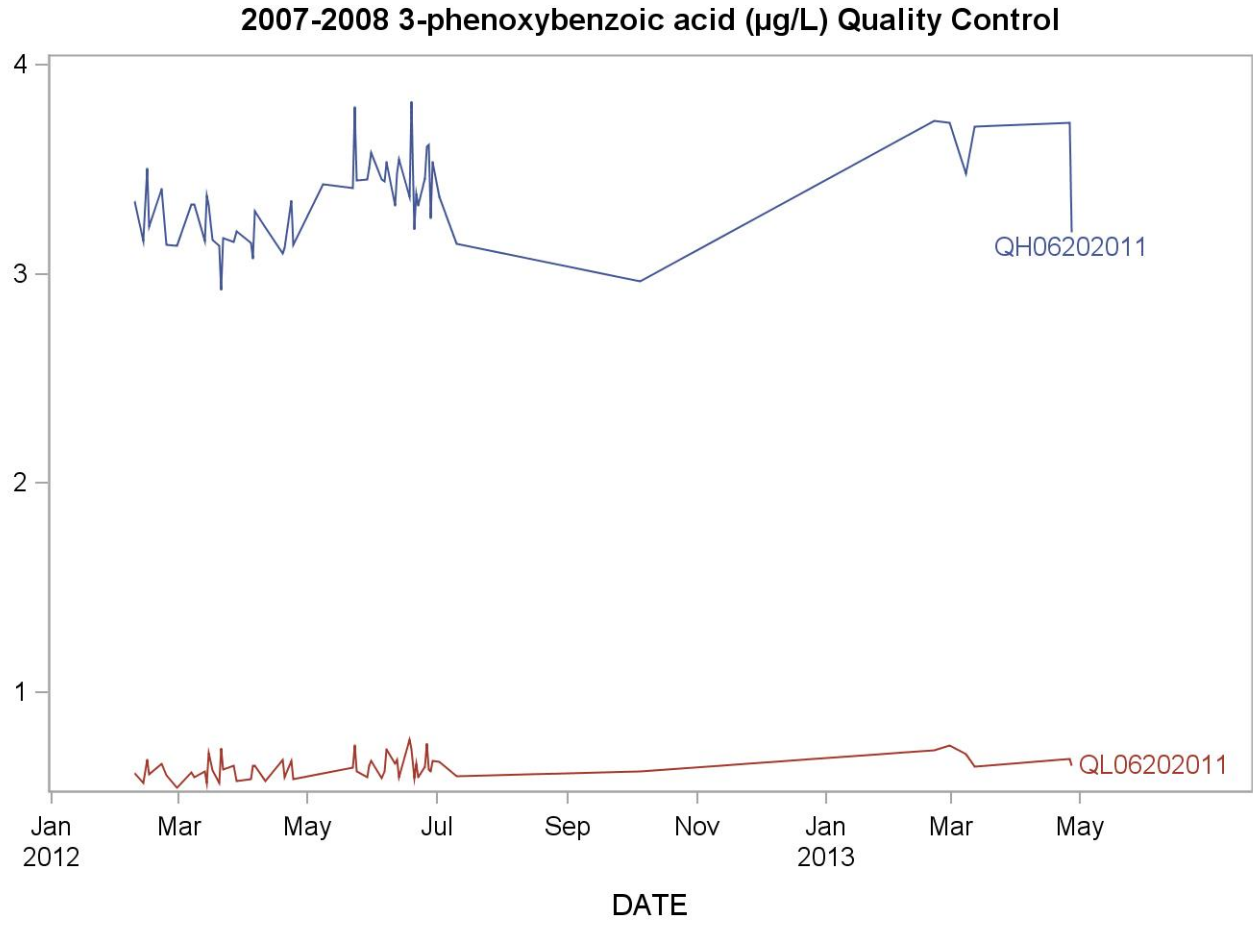
Summary Statistics for Malathion Diacid (µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	76	09FEB12	27APR13	5.333	0.562	10.5
QL06202011	76	09FEB12	27APR13	1.422	0.181	12.7



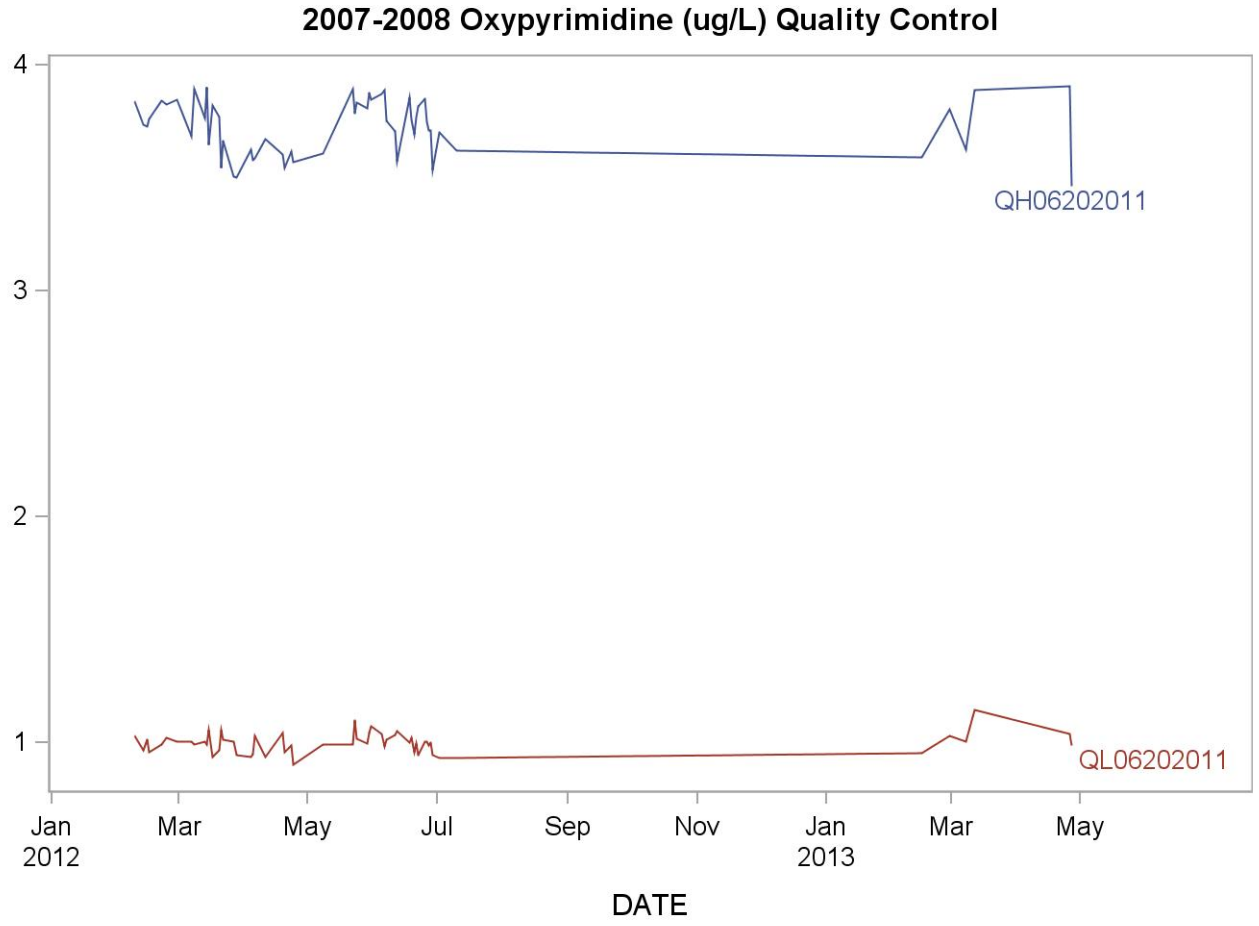
Summary Statistics for 3-phenoxybenzoic acid (µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	79	09FEB12	27APR13	3.363	0.224	6.7
QL06202011	79	09FEB12	27APR13	0.647	0.061	9.5



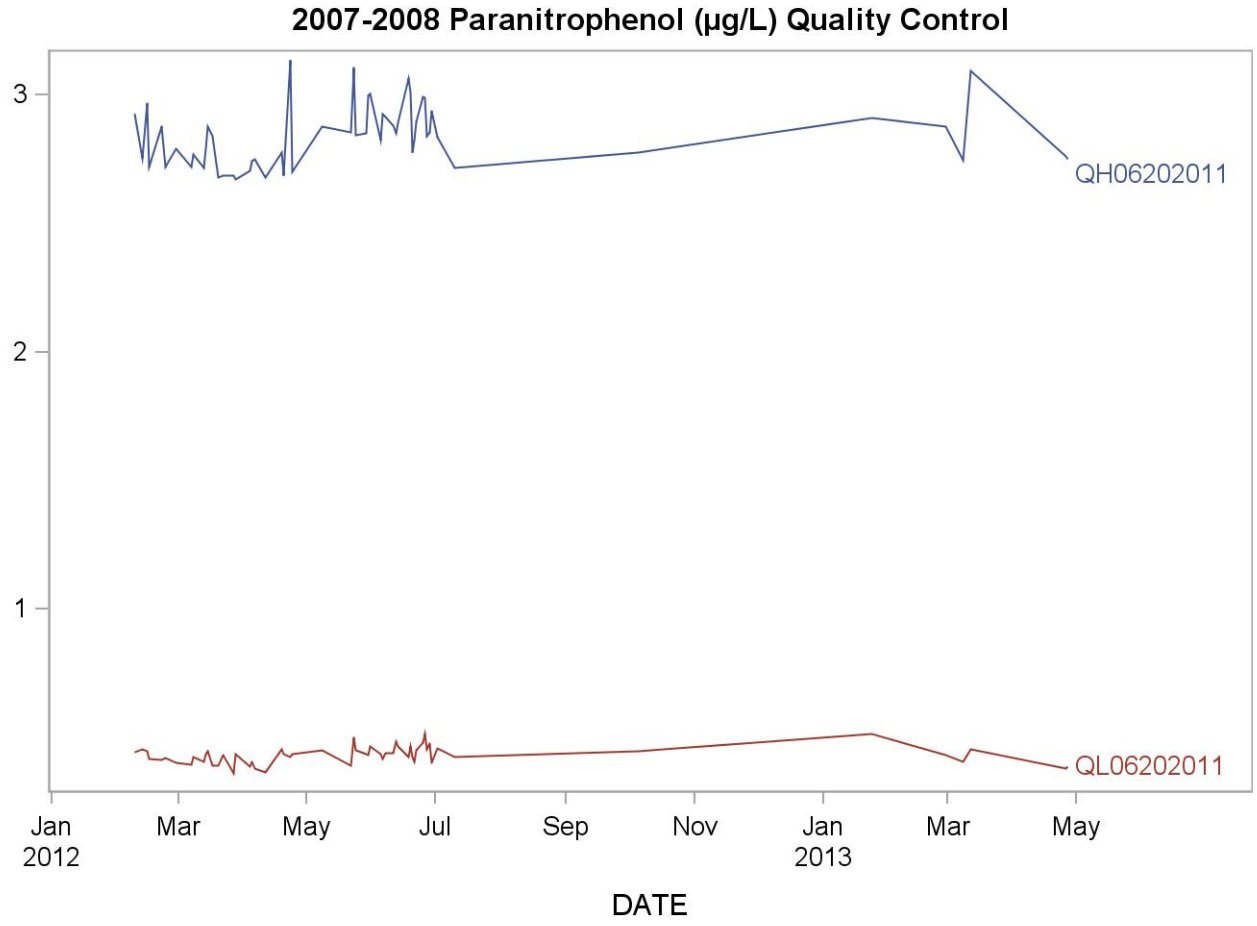
Summary Statistics for Oxypyrimidine (ug/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	76	09FEB12	27APR13	3.735	0.128	3.4
QL06202011	76	09FEB12	27APR13	0.998	0.052	5.3



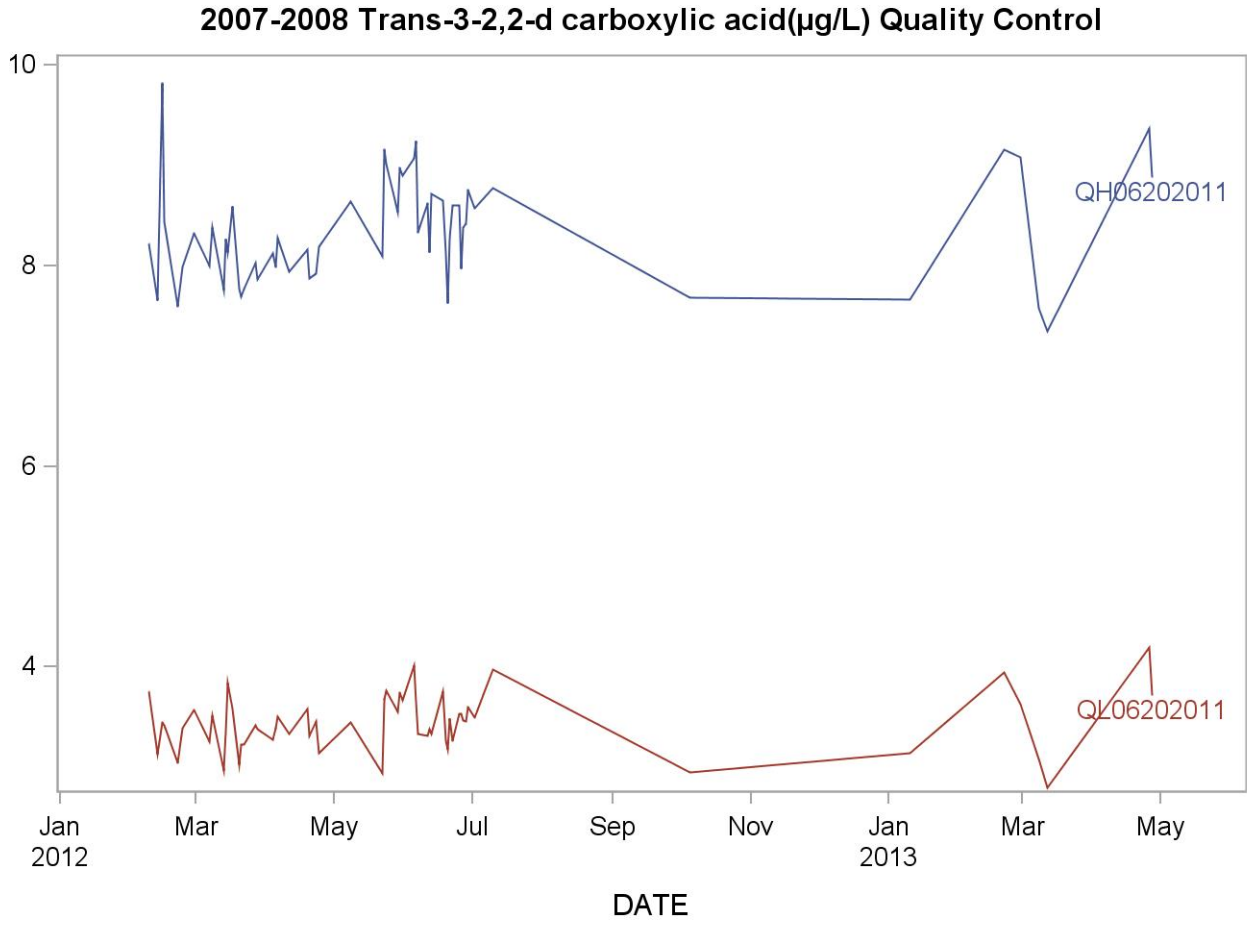
Summary Statistics for Paranitrophenol (µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	77	09FEB12	27APR13	2.854	0.130	4.5
QL06202011	77	09FEB12	27APR13	0.434	0.038	8.8



Summary Statistics for Trans-3-2,2-d carboxylic acid(µg/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06202011	79	09FEB12	27APR13	8.354	0.570	6.8
QL06202011	79	09FEB12	27APR13	3.426	0.292	8.5



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

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