Laboratory Procedure Manual

Analyte:	**Desethyldesisopropylatrazine (DCZ), Desisopropylatrazine (SIS), Desethylatrazine (DTZ), Atrazine (AAZ), Desisopropilatrazine mercapturate (SISM), Atrazine mercapturate (ATZ)**
Matrix:	**Urine**
Method:	**Atrazine-related Metabolites in Urine**
Method No:	**6107.03**
Revised:	**08/24/2013**

As performed by:

Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

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Important Information for Users

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

File Name	Variable Name	SAS Label
UAM_E	URXAAZ	Atrazine
	URXATZ	Atrazine mercapturate (ug/L)
	URXDCZ	Diaminochloroatrazine(ug/L)
	URXDTZ	Desethyl atrazine(ug/L)
	URXSIS	Desisopropyl atrazine(ug/L)
	URXSISM	Desisopropyl atrazine mercapturate(ug/L)

Clinical Relevance and Summary of Test Principle

Atrazine (6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine; CAS Number: 1912-24-9; AAZ) is a triazine herbicide used to kill weeds, primarily on crops such as sugarcane, corn, pineapples, sorghum, and macadamia nuts. It has also been used on evergreen tree farms, evergreen forest re-growth, highway and railroad rights-of-way, and turf application. Atrazine was the most used active ingredient in agriculture in the United States for nearly two decades, until it was replaced by glyphosate (EPA 2012).

Atrazine enters into the environment, particularly soil and water. Its primary degradation can occur via soil bacteria and abiotic processes with an environmental half-life of a few weeks to several months (ATSDR 2003, Mandelbaum *et al*., 1993). Atrazine and its degradation products tend to migrate out of the soil into water systems including surface runoff to streams, river, and lakes, or deep ground water systems and aquifers (ATSDR 2003) creating a potential for human exposure

The atrazine metabolism may differ based on the exposure scenario (Barr et al. 2007). Occupational exposures may result in increased exposure to atrazine itself while environmental exposures are likely dominated by the dealkylated environmental degradates which are presumed to be biologically active if they retain the chlorine atom. Thus, measurement of multiple potential metabolites is preferable to best assess exposures to atrazine and its related degradates.

The method uses solid phase extraction coupled with high performance liquid chromatography-tandem mass spectrometry (SPE-HPLC-MS/MS) for quantifying atrazine and several of its metabolites and/or hydrolysis products (Kuklenyik et al. 2012). The method relies on automated off-line SPE to pre-concentrate the target compounds while reducing or eliminating most of the urine matrix potential interferences to increase sensitivity and specificity (Kuklenyik et al. 2012). Off-line SPE is coupled with on-line two-dimensional (2D) HPLC combining cation exchange (SCX) and reversed phase (RP) chromatography in one integrated HPLC system followed by isotope dilution tandem mass spectrometry for the quantitative determination of the target analytes.

1. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some reagents used in this procedure are toxic. Avoid inhalation of or dermal exposure to these reagents. Consult the chemical hygiene plan specific for the pesticide laboratory if any questions about special precautions arise.

b. Radioactive Hazards

None

c. Microbiological Hazards

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 recommended for laboratorians who are exposed to human fluids and tissues. Observe universal precautions. Laboratory

personnel handling human fluids and tissues are also required to take the "Bloodborne Pathogens Training" course offered at CDC to insure proper compliance with CDC safe work place requirements.

d. Mechanical Hazards

Follow standard safety practice while performing this procedure to minimize the risk for mechanical hazard. Avoid any direct contact with the electronic components of the mass spectrometer unless all power to the instrument has been shut off. Generally, only qualified technicians perform electronic maintenance and repair of the mass spectrometer. Contact with the heated surfaces of the mass spectrometer should be avoided; also, care must be taken to avoid puncture wounds from the corona discharge needle when removing the APCI interface.

e. Protective Equipment

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

f. Training

Anyone performing this procedure should be trained and experienced in the use of an automated solid phase extraction and HPLC systems, and a triple-quadrupole mass spectrometer. Although formal training is not necessary, personnel are appropriately trained by an experienced operator of the instrument. All instrument operators must also read all operation manuals.

g. Personal Hygiene

Use caution when handling any biological specimen. Be sure to 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 or equivalent storage space (follow CDC's guidelines entitled Hazardous Chemical Waste Management). Use caution when handling containers, glassware, etc., that come in direct contact with the specimens. Decontaminate sample preparation surfaces with 10% bleach. Wash the glassware or dispose of it in an appropriately labeled autoclave pan. To insure proper compliance with CDC requirements, laboratory personnel are required to take annual hazardous waste disposal training courses.

2. Computerization; Data-System Management

a. Software and Knowledge Requirements

A database named Starlims was developed on the CDC internal network. This database is used to store, retrieve, and analyze data generated at the Contemporary Pesticides Laboratory. The Statistical Analysis System (SAS)® software package is used to maintain the data-management structure.

b. Sample Information

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

c. Data Maintanance

After uploading 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.

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

a. Sample Handling

Use standard urine collection cups to collect urine specimens from participants. Samples should be refrigerated as soon as possible. Preferably at least 10 mL of urine should be transferred into polypropylene vials or Qorpak® vials with screw-cap tops. Samples are subject to labeling, and are stored at or below -20˚C. Dry ice should be used for shipping. Carefully pack vials to avoid breakage during shipment. Store all samples at or below -20˚C until analysis.

b. Sample Rejection

Reject specimens with insufficient volume if they cannot be reliably processed.

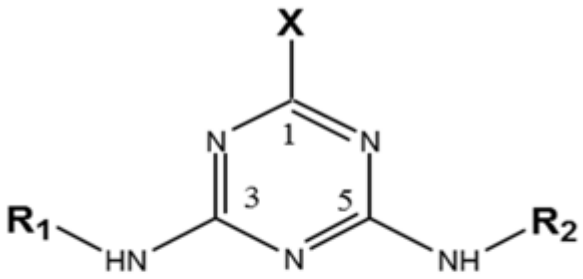
4. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

5. Preparation of Reagents, Calibration Standards, Controls, and All Other Materials; Equipment and Instrumentation

a. Reagents and Sources

Analytes nomenclature and structures



	X	R1	R2
AAZ	Cl	Et	i-Pr
DTZ	Cl	i-Pr	H
SIS	Cl	Et	H
DCZ	Cl	H	H
ATZ	Mer	Et	i-Pr
SISM	Mer	Et	H

Table 1: Calibration Standards, Reagents and their Manufacturers

Solvents and other reagents of similar specifications but from other sources may also be used.

Reagents	Manufacturers
Atrazine (10mg) **AAZ**	AccuStandard (New Haven, CT, USA)
Atrazine D5 (100ppm) **AAZ-L**	EQ Laboratories (Augsberg, Germany)
Atrazine Mercapturate (100ppm) **ATZ**	Cambridge Isotope laboratory (Andover, MA, USA)
Atrazine Mercapturate (100ppm) (Ring 13C3) **ATZ-L**	Cambridge Isotope laboratory (Andover, MA, USA)
Atrazine-desethyl (10mg) **DTZ**	AccuStandard (New Haven, CT, USA)
Atrazine-desethyl D6 (100ppm) **DTZ-L**	EQ Laboratories (Augsberg, Germany)
Atrazine-desethyl-desisopropyl (10mg) **DCZ**	AccuStandard (New Haven, CT, USA)
Atrazine-desethyl-desisopropyl (Ring-13C3) **DCZ-L**	Cambridge Isotope laboratory (Andover, MA, USA)
Atrazine-desisopropyl (10mg) **SIS**	AccuStandard (New Haven, CT, USA)
Atrazine-desisopropyl D5 (100ppm) **SIS-L**	EQ Laboratories (Augsberg, Germany)
Atrazine-desisopropyl Mercapturate **SISM**	Cambridge Isotope laboratory (Andover, MA, USA)
Atrazine-desisopropyl Mercapturate (Ring-13C3) **SISM-L**	Cambridge Isotope laboratory (Andover, MA, USA)
Methanol	Fisher Scientific
Ammonium hydroxide	Fisher Scientific
Formic Acid, MS grade 98% (1000mL)	Sigma-Aldrich Corp.
Deionized water	NANOpure Dlamond Barnstead
Ammonium Acetate (500g)	Sigma-Aldrich Corp.
Argon Gas (99.99%)	Airgas, Inc.

b. Reagent Preparation

Prepare as necessary.

1) Liquid Chromatography Mobile Phases

Mobile Phase A (pump 1 & 2) = ~0.5% formic acid in deionized water (v/v). This solution is prepared by adding 10 mL of formic acid into 2.0L of deionized water and mixing well.

Mobile Phase B (pump 1 & 2) = ~20mM ammonium acetate in deionized water. This solution is prepared by dissolving 3.08g of ammonium acetate in 2.0L of deionized water and mixing well.

Mobile Phase D (pump 2) = ~0.1% ammonium hydroxide in methanol (v/v). This solution is prepared by adding 1 mL of ammonium hydroxide into 1.0L of methanol and mixing well.

2) Sample Clean-up Reagents

2%formic acid in water (v/v) aqueous solution: The preparation is done by adding 20 mL of formic acid into 980 mL of water (store refrigerated).

1%formic acid in water (v/v) aqueous solution: The preparation is done by adding 10 mL of formic acid into 990 mL of water (store refrigerated).

~2% ammonium hydroxide in methanol (v/v): The preparation is done by adding 2 mL of ammonium hydroxide into 100 mL of methanol (prepare fresh).

c. Standards Preparation

1) Stock Solutions of Analytes

Prepare the individual stock solutions of commercially available neat standards by dissolving them in acetonitrile. Store the stock solutions in freezer safe vials below 0˚C.

2) Stock Solutions of Labeled Isotope Standards

Prepare the individual stock solutions of commercially available neat standards by dissolving them in acetonitrile. Store the stock solutions in freezer safe vials below 0˚C.

3) Labeled Isotope (ISTD) Spiking Solution

Prepare the spiking solution of isotope-labeled standards in 1:1 methanol: water.

4) Native Spiking Standard Solutions

Prepare ten working standard solutions of all target analytes in 1:1 methanol: water from the individual stock solutions. The final concentrations from a 20-µL spike would cover a range of 0.05 ng/mL to 50 ng/mL.

5) Calibration Verification Materials

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

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. Equipment/Supplies

1) Sartorius Ultramicro® Microbalance (Westbury, NY)
2) Electronic 6-channel pipette (Rainin Instrument Company, Oakland, CA)
3) 450 µL pipette tips (TOMTEC, Hamden, CT)
4) EDP2® pipettes (Rainin Instrument Company, Oakland, CA)
5) Presterilized filter pipette tips (Rainin Instrument Company, Oakland, CA)
6) Vortex Genie® vortex mixer (Scientific Industries Inc., Springfield, MA)
7) 96-well plates (2 ml square well, Varian, Lake Forest, CA)
8) 96-well format cartridges 60 mg Strata-X-C polymeric SCX (Phenomenex, Torrance, CA)
9) Luna 5u SCX 100A Guard Column 30 x 4.6 mm (Phenomenex, Torrance, CA)
10) Gemini 5u C6-Phenyl 100x4.6 mm Column(Phenomenex, Torrance, CA)
11) Luna 5 µm SCX 100 Å 50 x 4.6 mm Column (Phenomenex, Torrance, CA)
12) TOMTEC Quadra3 automated SPE station (Hamden, CT)
13) Turbovap 96 concentration workstation (Caliper Life Sciences, Hopkinton, MA)
14) NANOpure Infinity ultrapure water system
15) Qorpak bottles (Lab Depot, Inc., Cumming, GA)

e.Instrumentation

The 2D HPLC system is constructed in-house by use of Agilent 1200 modules (Agilent Technologies, Waldbronn, Germany), specifically two quaternary pumps (Pump I and II) with built in degassers, one autosampler, three external ten-port switching valves (Valve I, II, and III), and one column compartment with a six-port valve (Valve D) (Kuklenyik et al. 2012). A high-pressure mixing T is also incorporated between Valves II and III to allow for in-line mixing with an aqueous 0.5% formic acid flow provided by Agilent 1100 isocratic pump, Pump T (Agilent Technologies, Waldbronn, Germany); Pump T is turned on/off from the autosampler by contact closure signals. The solvent gradients, switching valves movements, and contact closures are controlled by the

Chemstation B.04.02 SP1 software (Agilent Technologies). Sample separation is performed using this 2D HPLC system and three HPLC columns (Phenomenex, Torrance, CA): Luna SCX (30 mm × 4.6 mm, 5 µm p.s.), Gemini C6-Phenyl RP (100 mm × 4.6 mm, 5µm p.s.), and Luna SCX (100 mm × 4.6 mm, 5µm p.s.).

Mass spectrometry analysis is performed on a TSQ Vantage mass spectrometer (ThermoFisher, San Jose, CA) equipped with an atmospheric pressure chemical ionization (APCI) interface to generate gas phase ions of the target analytes. The mass spectrometer is programmed and controlled using Xcalibur software (ThermoFisher, San Jose, CA). The APCI is set in the positive ion mode with the multiple reaction monitoring (MRM) setup.

The HPLC and MS/MS systems are operated from separate computers, and they communicated only through a contact closure circuit.

6. Calibration and Calibration Verification Procedures

a. Calibration Plot

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

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

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

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

b. Calibration Verification

1) Calibration verification is not required by the manufacturer(s) of the HPLS-MS/MS system. However, it should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.
2) All calibration verification runs and results shall be appropriately documented.
3) According to the updated CLIA regulations from 2003 (http://www.cms.hhs.gov/CLIA/downloads/6065bk.pdf), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months.
4) 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 a minimum of once approximately every 6 months. Because no formal PT testing program exists for the target analytes of this method, an in-house program is used. This in-house program currently includes pools prepared in-house but could include independently prepared materials whose preparation was contracted out to an external laboratory. Where applicable, NIST matrix-based certified reference materials may be included as PT materials. 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 selected PT materials will be analyzed in the same manner as unknown samples. These PT materials will be selected from among four different concentration ranges spanning the linear range of the method. The concentration range for each sample will be blinded to all analysts. Following analysis, the results will be forwarded to the PT administrator for evaluation. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the PT administrator. The PT administrator will notify the laboratory chief or his/her designee of the PT results (i.e. pass/fail). If a PT challenge is failed, a second attempt to demonstrate proficiency by analyzing a second set of PT samples is undertaken. If the second attempt fails, laboratory operations will cease until an appropriate corrective action is taken. After correction action is taken, laboratory operations can resume. All proficiency results shall be appropriately documented.

7. Operating Procedures; Calculations; Interpretation of Results

a. Analytical Runs

A typical analytical run consists of 36 unknown/study urine samples (total of 72 in one 96-well plate), four quality control (QC) samples (two low-concentration QC [QCL] and two high-concentration QC [QCH], so a total of 8 QCs in one 96-well plate), a blank (total of 2 in one 96-well plate), and nine calibration standard samples. Remove unknown samples and QC materials from storage freezer and allow them to come to room temperature prior to preparation.

b. Sample Preparation

In a 96-well plate (2 ml square well, Varian, Lake Forest, CA), 1 mL aliquots of urine and QCs are spiked with 20 μL of internal standard solution. Reagent blanks and calibration standards are prepared by adding 20 μL of internal/calibration solutions to each well. Then the 96-well plate is placed on a TOMTEC Quadra3 automated SPE station (Hamden, CT). Study samples, QCs, reagent blanks, and calibration standards are processed by use of the same automated procedure. A 96-well format cartridge bed (60 mg Strata XC polymeric SCX packing with 1.5 mL liquid space, Phenomenex, Torrance, CA) is conditioned with 2×430 μL of HPLC-grade methanol and 2×430 μL of 2% formic acid in water. Several of the target analytes are acid-

labile. Therefore, to minimize their exposure to the required acidic media for the optimal performance of the method, 2×430 μL of 2% formic acid in water is added to each sample well and pipette-mixed twice right before loading the urine onto the SPE cartridge. After sample loading (6×310 μL), the cartridges are washed with 2×430 μL of 2% formic acid in water. The target analytes are eluted with 3×400 μL of freshly prepared ~2% NH_4OH in MeOH. The eluted samples in the second half of the 96-well plate are transferred to a clean 96-well plate and all the SPE extracts (two partially occupied 96-well plates) are evaporated to ~20 µL under a stream of dry nitrogen (UHP grade) at 50 ºC in a Turbovap 96 concentration workstation (Caliper Life Sciences, Hopkinton, MA). The evaporated SPE extracts are reconstituted only right before the injection into the HPLC system by use of the Agilent 1200 HPLC autosampler (Agilent Technologies, Wilmington, DE), programmed to add 80 µL 1% formic acid in water to each SPE extract. During the sample analysis, the autosampler is kept at 4ºC by using an Agilent 1200 thermostat module.

c. Liquid Chromatography Conditions

The HPLC separation uses a stepwise-gradient elution as shown in Table 2 and Table 3 to maximize the resolution of target compounds against their matrix interferences.

Table 2: HPLC Separation – Pump I

Time (min)	Flow Rate (mL/min)	%A	%B	%C
0.00	0.200	30.0	30.0	40.0
12.00	0.200	30.0	30.0	40.0
13.00	1.000	0.0	0.0	100.0
15.00	1.000	0.0	0.0	100.0
15.50	0.200	95.0	0.0	5.0
20.00	0.200	95.0	0.0	5.0

Table 3: HPLC Separation – Pump II

Time (min)	Flow Rate (mL/min)	%A	%B	%C	%D
0.00	0.500	95.0	0.0	5.0	0.0
1.00	0.500	80.0	0.0	20.0	0.0
1.01	1.000	70.0	10.0	20.0	0.0
4.00	1.000	70.0	10.0	20.0	0.0
5.00	1.000	40.0	30.0	30.0	0.0
7.00	1.000	40.0	30.0	30.0	0.0
7.25	1.000	20.0	30.0	50.0	0.0
13.00	1.000	20.0	30.0	50.0	0.0
14.00	1.000	0.0	20.0	80.0	0.0
16.00	1.000	0.0	20.0	80.0	0.0
16.50	1.000	0.0	0.0	0.0	100.0
18.00	1.000	0.0	0.0	0.0	100.0
18.50	1.000	95.0	0.0	5.0	0.0
20.00	1.000	95.0	0.0	5.0	0.0

1) **Sequence Setup**

Open Chemstation; Instrument 1 (Online)

- To create a sequence: Choose **Sequence > Sequence Table**
- Manually define the following necessary parameters;
  Vial Position→ Enter each individual sample vial position
  Method Name → Enter the selected method
  Injection per Location → Enter 1
  Injection Volume → Enter 10 µL
- To Save Sequence: Choose **Sequence > Save Sequence,** then identify sequence name.

2) **Run the sequence**

- To open a sequence: Click **Sequence > Load Sequence**
- Choose specific sequence and then click **OK**
- Then click **Sequence > Sequence Table**
- On the bottom of the **Sequence Table**, Click **Run Sequence**
- Click the "**OK**" button when ready to proceed

d. Mass Spectrometer Conditions

Configuration parameters for this instrument are found in Table 4. The optimized precursor/product ion pairs, as well as the collision off-set energy and the retention time for the target compounds, are summarized in Table 5.

Table 4: MS Configuration

MS Parameter	Setting
Ionization Type	APCI
Ion Polarity Mode	positive ion
Discharge Current	4.0 µA
Vaporizer Temperature	400˚C
Collision Gas Pressure	1.5 torr
Sheath Gas Pressure	25
Auxiliary gas	5
Capillary Temperature	260˚C

Table 5: Select HPLC-Mass Spectrometry Parameters

Analyte	Precursor ion; $(M+H)^+$ (m/z)	Product ion (Confirmation ion) (m/z)	Retention time (min)
DCZ	146	79 (104)	4.3
SIS	174	68 (104)	8.6
DTZ	188	104 (146)	10.2
AAZ	216	174 (104)	15.1
SISM	301	172 (102)	12.3
ATZ	343	214 (172)	15.3
DCZ-L	149	113	4.3
SIS-L	179	101	8.6
DTZ-L	194	104	10.1
AAZ-L	221	179	15.1
SISM-L	305	172	12.3
ATZ-L	346	217	15.2

1) Sequence Setup

Open Xcalibur; Xcalibur allows importing data created by the Starlims database

- To import a sequence: Choose **File > Import Sequence**
- Use the Browse button to select the file for import.
- Select all sequence columns to be included in the sequence file.
- Click on **OK** to import the sequence: Xcalibur displays the imported file in Sequence Setup
- Manually define the following parameters;
  File name→ Enter each individual sample file name
  Path → Enter the directory path where the sample's raw file will be stored;
  Inst. Meth → Enter the directory of the instrument method file
  Comments (If needed)
- To Save File: Choose **File > Save As**

2) Run the sequence

- Click on the Run Sequence Icon on the tool bar.
- Check to make sure that the selected rows are displayed in the Run Sequence dialogue Box.
- Click Instrument Setting to "Standby" after the sequence has been run at the bottom of the Run Sequence dialogue Box.
- Click the "OK" button when ready to proceed.

e. Processing data

To process a batch of samples:

- Save all selected raw files and sequence file onto a removable drive.
- Transfer all data onto desktop PC used for data processing
- Specific sequence associated with target raw files has to be modified by identifying **"Level" "Path" and "Processing Method"** prior data processing
- Open **Quan® browser** and select the modified sequence
- Click **Process** button

f. Quantification

- After processing the sample batch, visually evaluate for correct peak detection and baseline selection in the Quan® browser. If needed, correct integrations and/or peak selection manually
- To save the settings in a Quan Browser file *(*.XQN)*. Choose **File>Save As**
- Export data files to EXCEL® using the long report format
- To export data files to EXCEL® → **File > Export Excel > Long Report**
- Quan Browser allows the operator to view quantitative results, to evaluate standard curve, QCs and unknowns samples, to integrate chromatogram peaks manually, and to analyze detailed quantification information.

g. Rearrangement of Data Files

Data are automatically rearranged into a multiple worksheet (Excel format)® that is compatible with the Division's database, Starlims, using an Excel® macro.

h. Transfer of Data

Data are transferred using CDC approved McAfee Encrypted USB removable drive.

i. Importation of Data into Database

Select "Import new data" option in database. A password is required to import the data.

j. Statistical Analysis and Interpretation of Data

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

k. Routine and Periodic Maintenance of Key Components

The instrumentation used is serviced according to the manufacturer's guidance included in the instrument manuals or based on the recommendation of experienced analysts/operators after following appropriate procedures to determine that the instrument performs adequately for the intended purposes of the method.

Mass Spectrometer

Preventive maintenance (PM) is required every 6 months and it is performed by a Thermo Service Engineer. Instrument inspections, testing, cleaning and part replacements are done according to the manufacturer guidelines. Open vacuum manifold, cleaning of the Q0, Q1, Q2 and Q3 quadrupoles should only be done by a Thermo Service Engineer. All maintenance records are kept electronically in the instrument computer.

In general, the maintenance procedures of calibration and cleaning API stack (ion sweep cone, tube lens, skimmer and Q00) are performed by the operator if there is a decrease in the system performance (sensitivity or S/N ratio) without any other apparent technical reasons.

Clean corona needle with sand paper followed by water and methanol, as needed.

Clean the ion transfer tube as needed. Use metal needle to check for internal blockage; if internal blockage exists, discard ion transfer tube and use a new one. If not, clean ion transfer tube by sonicating in 30% HNO_3 in water (v/v) for about 30 minutes, then in methanol and in acetone (for about 15 minutes in each solvent).

The frequency of cleaning the components of the mass spectrometer depends on the type and number of samples analyzed and solvents used.

Agilent 1200 HPLC-Systems

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

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

Check the solvent tubing and connections for leaks.
Back-flush or change analytical column as necessary (when peak tailing or high pressure is observed).
Replace HPLC filter as needed.
Check needle position into vials & sample wells.
Ensure that the solvent reservoir contains sufficient running solvent for all samples.

8. Reportable Range of Results

The linear range of the standard calibration curve and the method LOD determine the highest and lowest analytical values of an analyte that are reportable. The reportable range must be within the range of the calibration curves.

a. Linear Limits

Analytical standard curves are linear for all analytes through the range of concentrations evaluated. The high linearity limit is determined by the highest standard analyzed. The low end of the linear range is limited by the method LOD. Concentrations below the method LOD are reported as non-detectable. Samples with concentrations exceeding the highest calibration standard are re-extracted using less urine. The lowest calibration point should be at or below the LOD and the highest calibration point has to be above the expected range of results for most samples. The linear range for all target analytes is from LOD to 50 ng/mL.

b. Analytical Sensitivity

The LODs are presented in Table 6.

Table 6: Method Detection Limits

Analyte	LOD (ng/ml)
DCZ	0.50
SIS	0.25
DTZ	0.25
AAZ	0.50
SISM	0.10
ATZ	0.50

c. Accuracy

The accuracy of this method is defined as the degree of agreement between the mean of measured concentrations of samples and their nominal values (measured/expected). Accuracy is shown in Table 7.

Table 7: Accuracy of the Method

Analyte	%Accuracy (measured/expected)	
	Low Level	High Level
DCZ	96	101
SIS	97	99
DTZ	95	97
AAZ	94	100
SISM	87	99
ATZ	99	99

d. Precision

The precision is determined by calculating the coefficient of variation (CV) of repeat measurements (n=40) of quality control materials at two concentrations and includes all sources of variability (Table 8).

Table 8: Precision of the Method

Analyte	%CV	
	QCL	QCH
DCZ	4	3
SIS	4	4
DTZ	8	7
AAZ	4	3
SISM	2	4
ATZ	4	3

e. Analytical Specificity

This is a highly selective method that requires the following of each analyte detected: 1) that it elutes at a specific retention time, 2) that it has one precursor ion at a specific mass, 3) that it has two specific product ions formed from each precursor ion at specific masses, and 4) that the ion ratios of the two product ions are within a predetermined range.

9. Quality Control (QC) Procedures

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

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

ii. The area counts of IS for each analyte should meet minimum requirements. Low IS area count could indicate strong ion suppression from sample matrix, or simply missing of IS (i.e., error in spiking). Depending on the findings, either re-extract the original sample or dilute the sample first and re-extract it.

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

iv. The ratio of the calculated concentration of quantitation ion (Q1) and the calculated concentration of confirmation ion (Q2) for a given analyte should follow the same ratio (Q1/Q2) for the standards and QCs for the same analyte.

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

vi. If a given analyte concentration in a study sample is above the highest calibration standard, the sample needs to be re-analyzed with a smaller urine volume.

b. Analytical batch quality control procedures

a. QC Materials

Urine pools enriched with known amounts of pesticides residues as the control material should be analyzed in each analytical run. QC materials for each analytical run are aliquoted at the same time when samples are prepared.

b. Collection of Urine for QC Pools

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

c. Filtration and Dispensing

The urine pool is pressure filtered with a 0.45-μm filter capsule. Mix with 1:1 water and homogenize well ~24 hrs.

d. Urine Enrichment

Take two equal pools (~500 ml) from the base pool and reserve the rest for later use at or below -10°C. Enrich one of the pools with an appropriate amount of the stock solution of each pesticide residue to yield an approximate concentration of 5.0 ng/mL (QCL) and enrich the other pool to yield an approximate concentration of 10 ng/mL (QCH). Mix the individual pools thoroughly (~24hrs), aliquot in to 2.2 mL portions and store at or below -20°C until use.

e. Characterization of QC Materials

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

f. Use of QC Samples

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

g. Final Evaluation of Quality Control Results

The results of the QC materials analyzed with the study samples are evaluated according to standard Westgard multi-rule criteria (Caudill et al. 2008) to determine a run either in-control or out-of-control. No data from runs considered out-of-control will be reported.

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

For 1 QC result per pool

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

For 2 or more QC results per pool

1) If both QC run means are within $2S_m$ limits and individual results are within $2S_i$ limits, then accept the run.
2) If 1 of the 2 QC run means is outside a $2S_m$ limit - reject run if:
   Extreme Outlier – Run mean is beyond the characterization mean $\pm$ $4S_m$
   1 3S Rule – Run mean is outside a $3S_m$ limit
   2 2S Rule – Both run means are outside the same $2S_m$ limit
   10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

3) If one of the 4 QC individual results is outside a $2S_i$ limit - reject run if:
   R 4S Rule – Within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit). Since runs have multiple measurements per pool for 2 pools, the R 4S rule is applied within runs only.

Abbreviations:

S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).

S_m = Standard deviation of the run means (the limits are shown on the chart).
S_w = Within-run standard deviation (the limits are not shown on the chart).

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

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

11. Limitations of Method; Interfering Substances and Conditions

This method is an isotope-dilution mass spectrometry method, which is widely regarded as the definitive method for the measurement of organic toxicants in human specimens. By using tandem mass spectrometry, a large number of analytical interferences are eliminated. However, because of the urinary matrix used in this procedure, occasional interfering of unknown substances has been encountered. Interferences with the internal standards result in rejection of that analysis. If a repeat analysis still results in an interference with the internal standard, do not report the results for that analyte.

12. Reference Ranges (Normal Values)

Reference range values are not available

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

Insufficient data exist to correlate urinary concentrations of this pesticide or its metabolites with serious health effects in humans. Therefore, critical call values have not been established.

14. Specimen Storage and Handling during Testing

All samples and standards must remain frozen prior to use. Stability studies suggest that the analytes are stable up to four days at room temperature after preparation (at neutral pH but not at acidic pH) (Kuklenyik et al. 2012).

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

The method is designed to run on an LC-MS/MS instrument and is not transferable to other instrumentation. If the system fails during the injection, samples have to be re-prepared. If the system fails before reconstitution with 1% formic acid solution, the urine extracts can be stored up to four days at -70 °C.

16. Test-Result Reporting System; 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.

17. 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 CLIA-specimens is only permitted to certified laboratories.

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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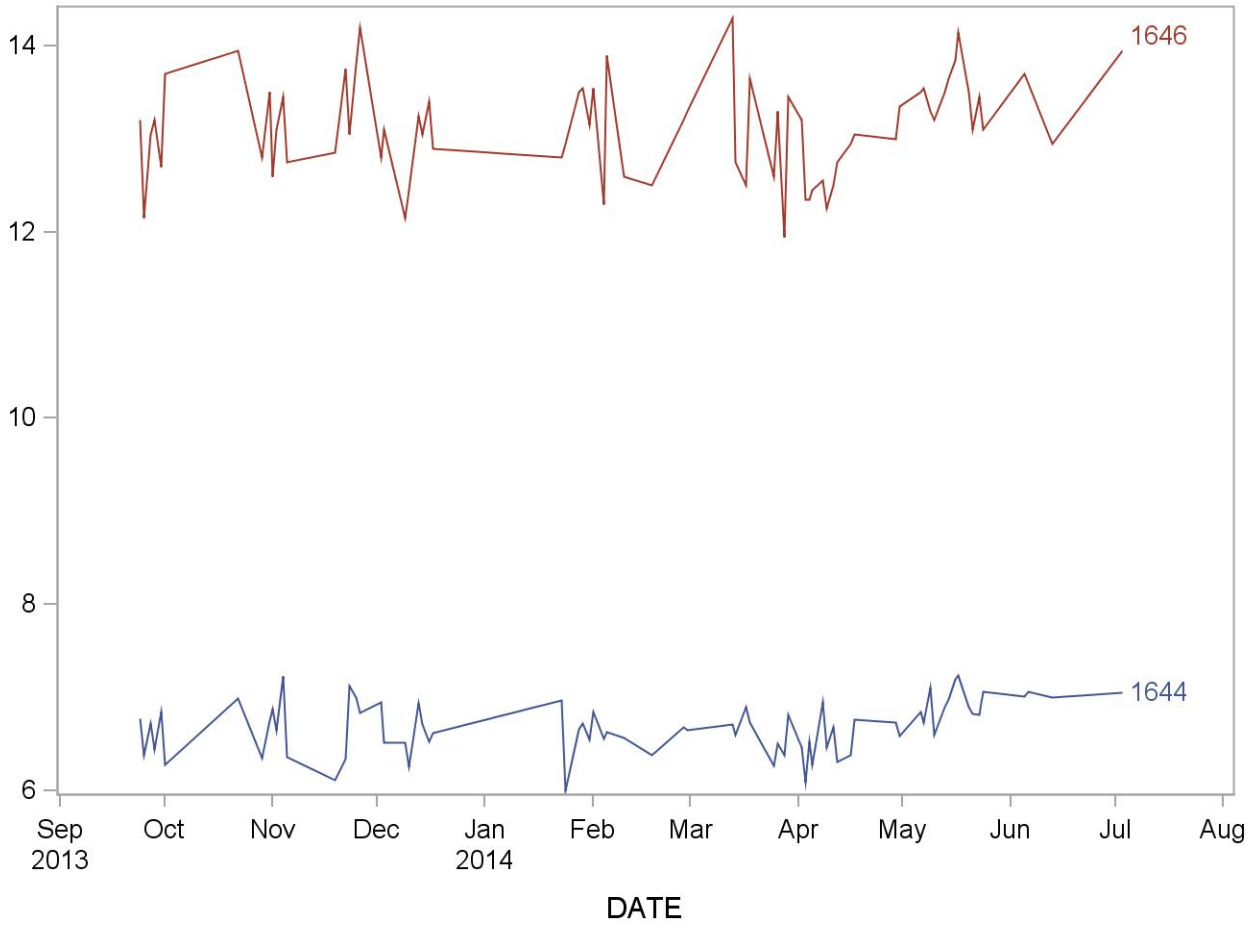
18. Summary Statistics and QC Graphs

See following pages

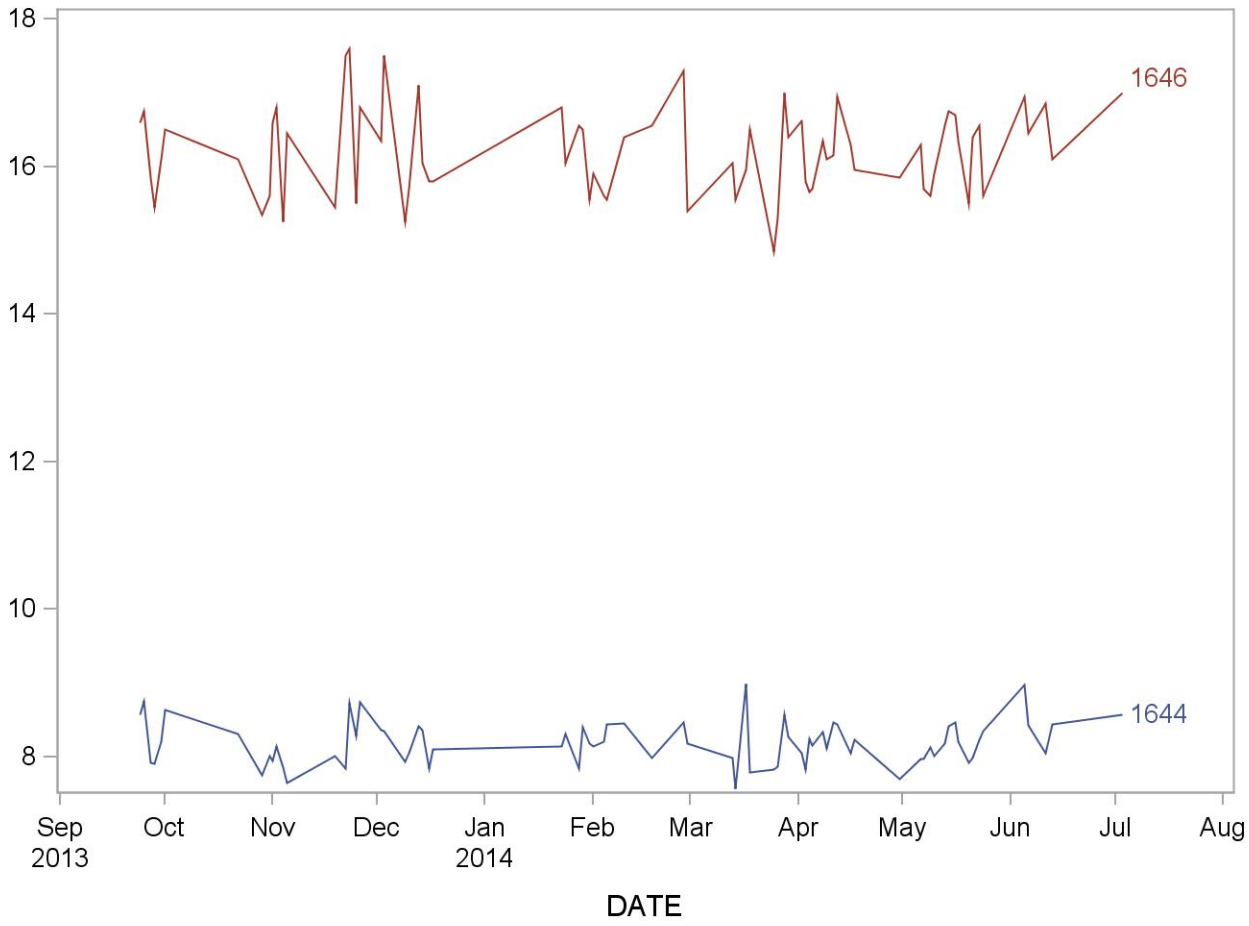
2007-2008 Summary Statistics and QC Chart for Atrazine in urine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1646	63	24SEP13	03JUL14	13.16111	0.54532	4.1
1644	63	24SEP13	03JUL14	6.70857	0.26252	3.9
1646	12	25SEP13	09MAY14	12.96250	0.39550	3.1
1644	12	25SEP13	09MAY14	6.53458	0.35026	5.4



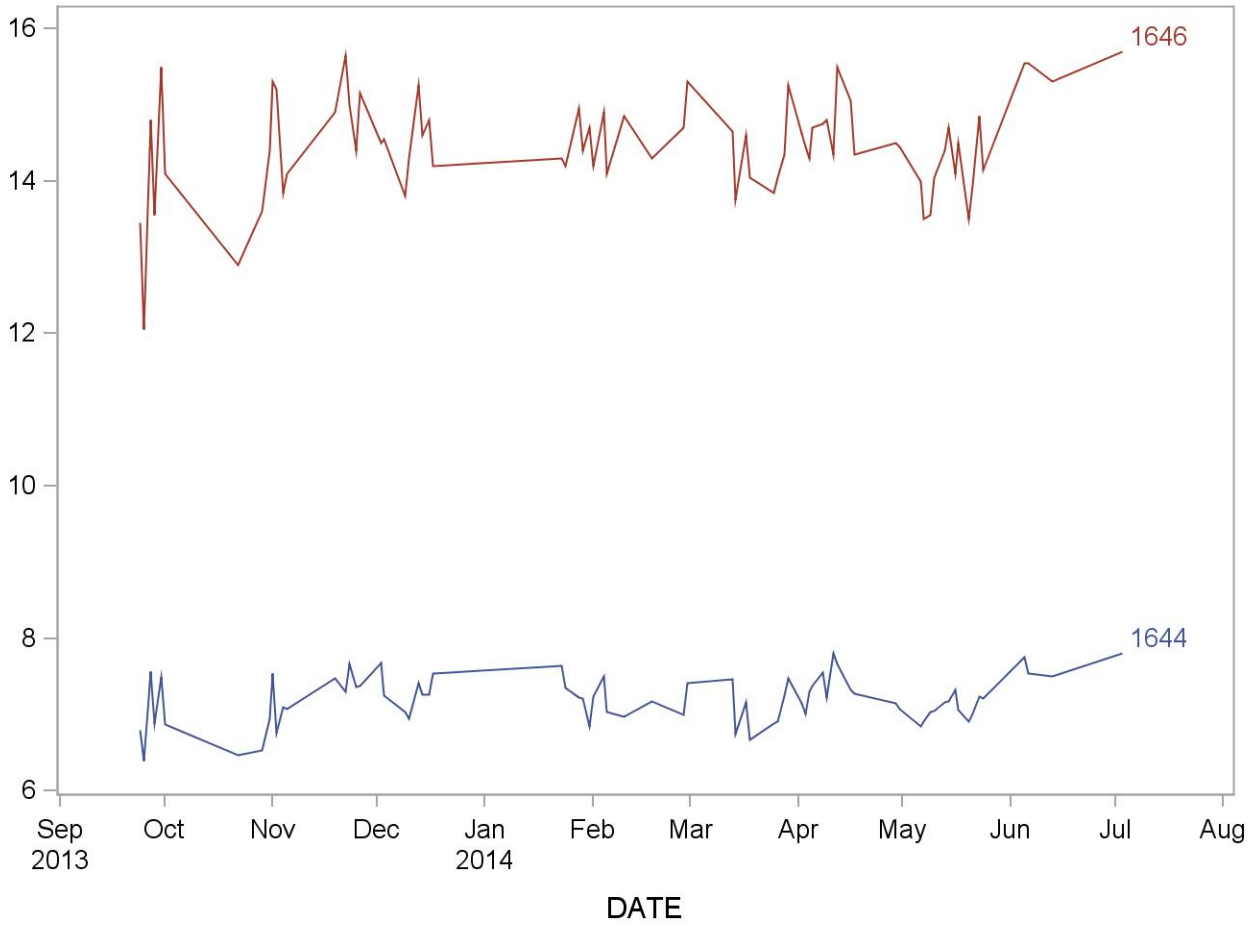
2007-2008 Summary Statistics and QC Chart for Atrazine mercapturate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1646	63	24SEP13	03JUL14	16.24762	0.58007	3.6
1644	63	24SEP13	03JUL14	8.19754	0.30428	3.7
1646	12	25SEP13	09MAY14	15.93750	0.66610	4.2
1644	12	25SEP13	09MAY14	8.17208	0.27041	3.3



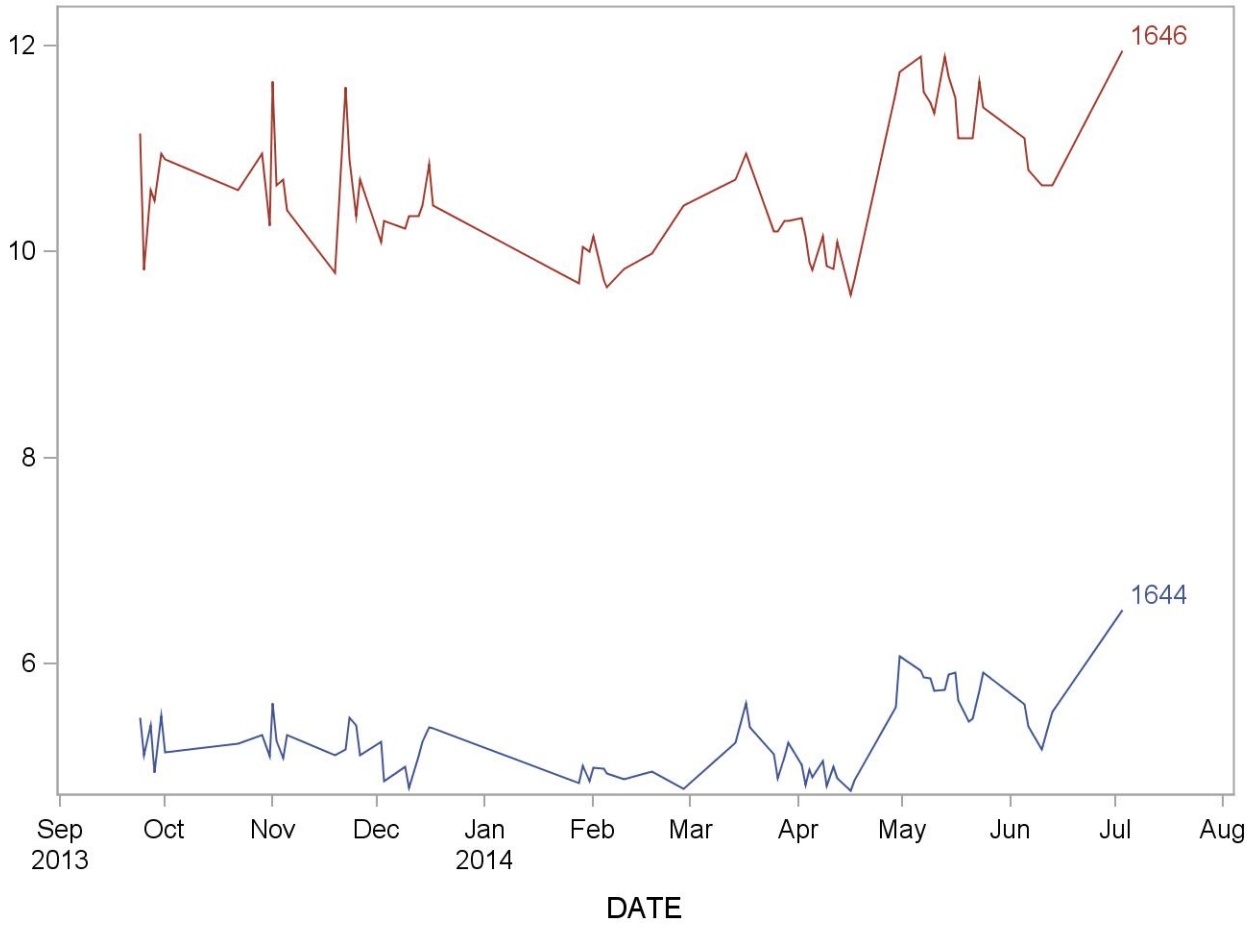
2007-2008 Summary Statistics and QC Chart for Diaminochloroatrazine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1646	63	24SEP13	03JUL14	14.52063	0.56495	3.9
1644	63	24SEP13	03JUL14	7.20119	0.29065	4.0
1646	12	25SEP13	09MAY14	14.19583	0.97897	6.9
1644	12	25SEP13	09MAY14	7.17042	0.41729	5.8



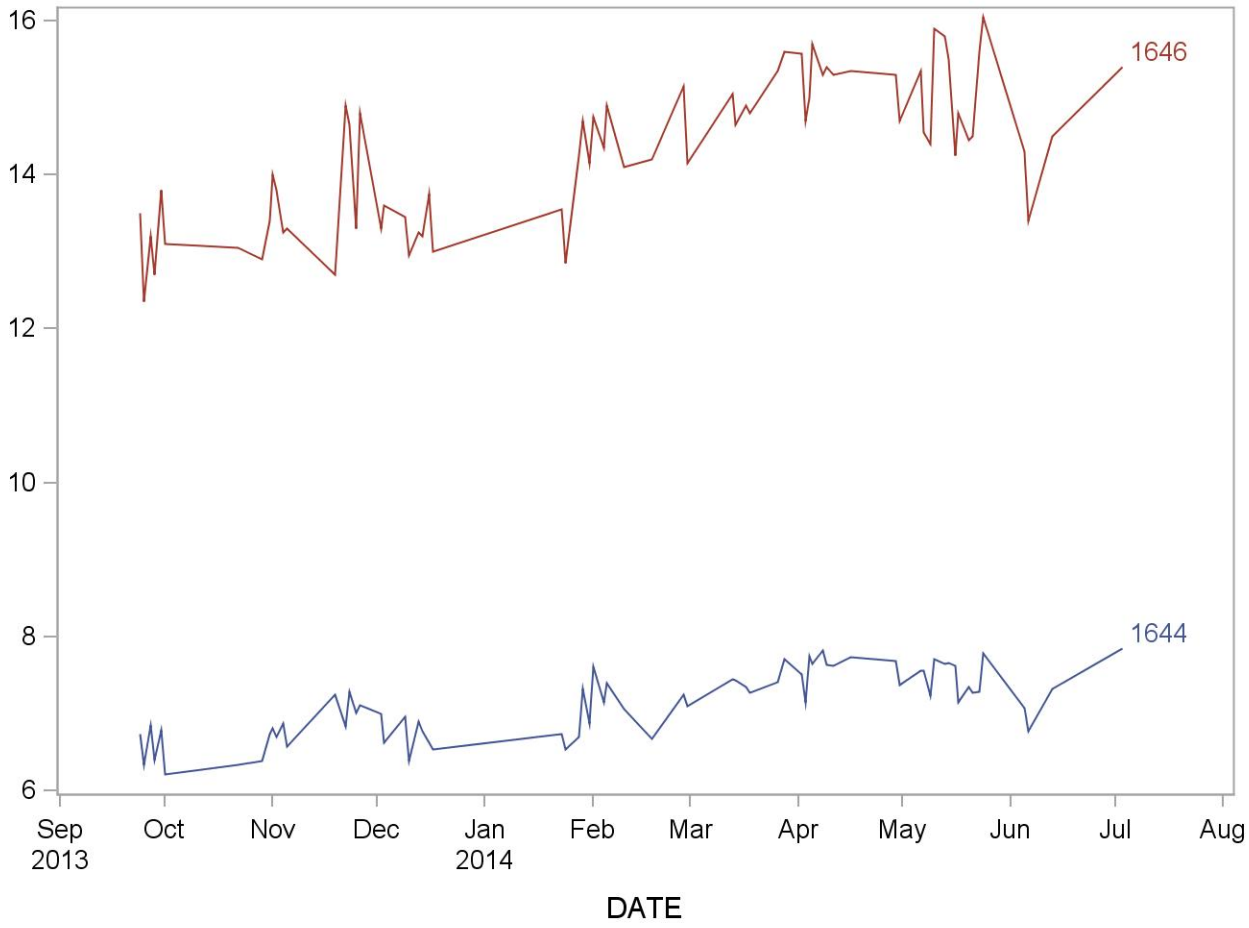
2007-2008 Summary Statistics and QC Chart for Desethylatrazine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1646	63	24SEP13	03JUL14	10.64460	0.63456	6.0
1644	63	24SEP13	03JUL14	5.27476	0.37479	7.1
1646	9	25SEP13	09MAY14	10.43389	0.70681	6.8
1644	9	25SEP13	09MAY14	5.26278	0.36695	7.0



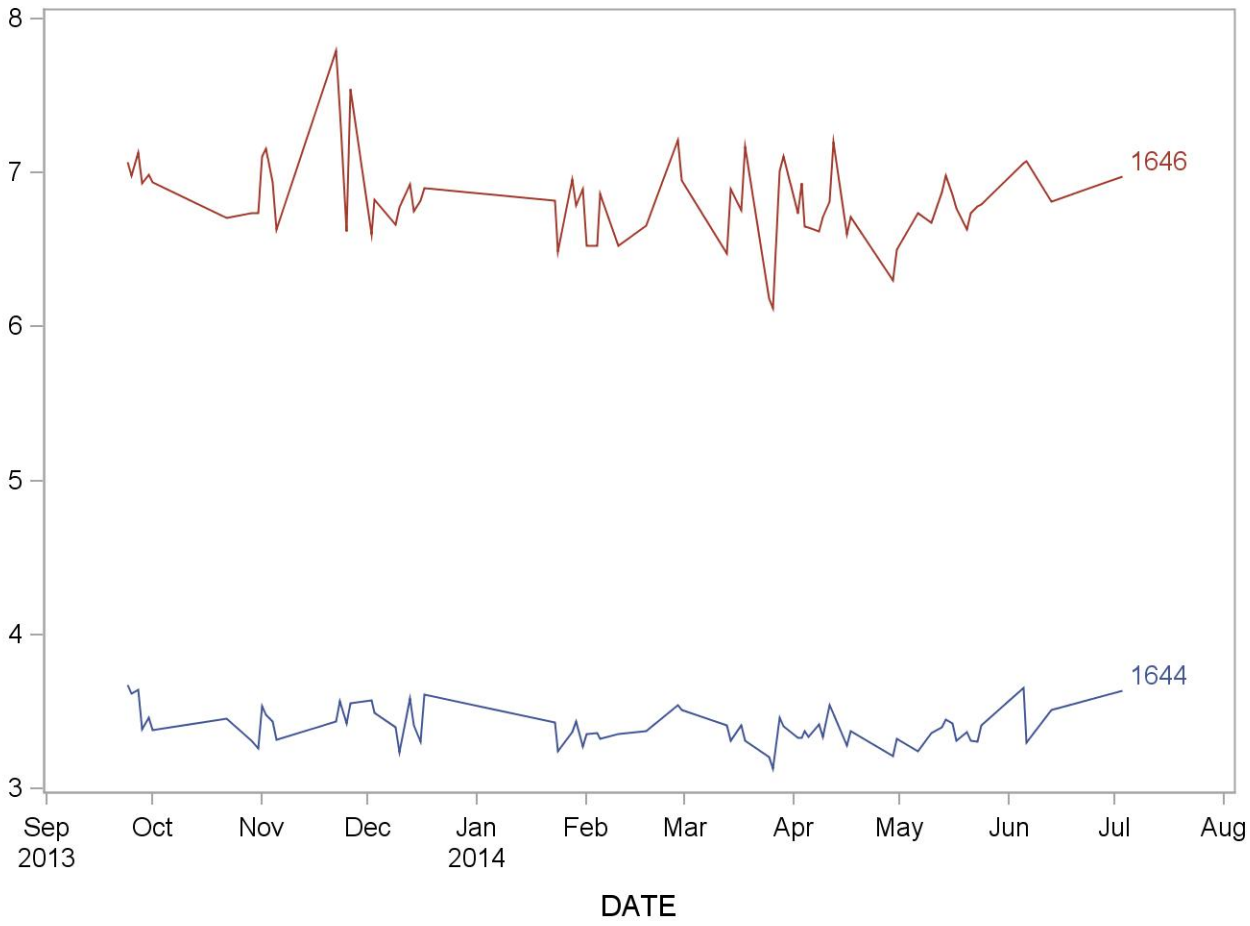
2007-2008 Summary Statistics and QC Chart for Desisopropyl atrazine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1646	63	24SEP13	03JUL14	14.44206	0.90830	6.3
1644	63	24SEP13	03JUL14	7.16429	0.44059	6.1
1646	8	25SEP13	09MAY14	13.43125	0.84892	6.3
1644	8	25SEP13	09MAY14	6.89438	0.45339	6.6



2007-2008 Summary Statistics and QC Chart for Desisopropyl atrazine mercapturate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
1646	63	24SEP13	03JUL14	6.83214	0.26924	3.9
1644	63	24SEP13	03JUL14	3.40643	0.11724	3.4
1646	9	25SEP13	17APR14	6.79944	0.31591	4.6
1644	9	25SEP13	17APR14	3.39889	0.13160	3.9



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