



Laboratory Procedure Manual

Analyte: **Acephate, Methamidophos, Omethoate, Dimethoate, Ethylenethiourea and Propylenethiourea**

Matrix: **Urine**

Method: **Carbamates and Organophosphorus Pesticides in Urine**

Method No: **6106.03**

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As performed by:

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Important Information for Users

NCEH, 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)
CARB_E	URXAPE	Acephate (ug/L)
	URXETU	Ethylenethio urea (ug/L)
	URXOMO	Methamidaphos (ug/L)
	URXMMI	Dimethoate (ug/L)
	URXMTO	O-methoate (ug/L)
	URXPTU	Propylenethio urea (ug/L)

1. Clinical Relevance and Summary of Test Principle

Acephate (AP), methamidophos (MMP), omethoate (Omet), and dimethoate (Dmet) are organophosphorus pesticides (OPs). Organophosphates are the most commonly used insecticides in agriculture and the domestic field (1, 2). Extensive and widespread use makes it virtually impossible for a person to avoid exposure to these compounds completely. One property of OPs that has led to their widespread use is they are less persistent in the environment than other classes of insecticides. It has been known that the mode of toxicity of this group is through inhibition of acetylcholinesterase, the enzyme responsible for catalyzing the breakdown of the neurotransmitter acetylcholine (3, 4). The inhibition of the enzyme causes the accumulation of acetylcholine which leads to symptoms related to the autonomous nervous system (abdominal cramps, nausea, diarrhea, salivation) and the central nervous system (dizziness, tremor, anxiety, confusion) (5, 6).

The most widely used fungicides in agriculture are the alkali and metal salts of the alkylenebis-(dithiocarbamate) acids. The alkylenebis-(dithiocarbamates) can be divided into three sub-groups, namely, dimethyl dithiocarbamates (ferbam, thiram, ziram), ethylene bisdithiocarbamates (EBDCs) (mancozeb, maneb, nabam, zineb), and propylene bisdithiocarbamates (propineb). Ethylenethiourea (ETU) represents the main degradation product of the ethylene bisdithiocarbamates and propylenethiourea (PTU) is the main degradation product of the propylene bisdithiocarbamates (7). They are foliar applied compounds that control many fungal diseases including early and late blights, leaf spots, rust mildew and scabs in various field crops such as fruits, nuts, cucurbits, vegetables, grapes and ornamentals. The occurrence of ETU and PTU as contaminants, environmental decomposition products (8, 9, 10), and urinary metabolites (11, 12, 13) is a cause for concern due to their mutagenic, teratogenic, carcinogenic, and goitrogenic properties, as described in experimental models (14, 15).

This method is used for determining concentrations of specific organophosphorus and carbamate metabolites, in particular, AP, MMP, Omet, Dmet, ETU and PTU from a urine matrix. The general sample preparation includes lyophilization of the urine followed by extraction with dichloromethane using a 96-well plate automated sample handler. The analytical separation is performed by high-performance liquid chromatography and detection by a triple quadrupole mass spectrometer with APCI source in positive ion mode. Isotopically labeled internal standards were used for three of the analytes (16).

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents that are necessary to perform this procedure are toxic. Avoid inhalation of or dermal exposure to these reagents. Consult the Pesticides Laboratory's chemical hygiene plan if any questions about special precautions arise.

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

To follow standard safety practice while performing this procedure minimizes 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. Only qualified technicians perform electronic maintenance and repair.

e. Protective Equipment

Use standard personal protective equipment when performing this procedure. Always wear safety glasses. Wear a lab coat and appropriate, durable gloves when they are required. 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. All personnel who will be operating the instrument also must read the operation manuals.

g. Personal Hygiene

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

h. Disposal of Waste

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). Use caution when handling containers, glassware, etc., that come in direct contact with the specimens. Decontaminate sample preparation surfaces with 1-10% bleach. Wash the glassware or dispose it in an appropriately labeled autoclave pan.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

A database named CCEHIP_PSTARS was developed on the CDC/NCEH (SQL server 2005). The Front-End is Microsoft Access on a DLS-PC network (share drive) named PSTARS.adp. This database is used to store, retrieve, and analyze data from the pesticide-residue analyses. Statistical Analysis System (SAS) ® software packages (or their equivalent) are used to perform statistical data analysis and are required to use and maintain the data-management structure.

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 notebook number associated with the sample preparation, 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 Maintenance

After inputting all sample and analytical data into the database, check them for transcription errors and overall validity. Back up the database at least once weekly onto a computer hard drive.

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

a. Sample Handling

Use standard urine collection cups to collect urine specimens from participants. Refrigerate samples as soon as possible and transfer them to specimen vials within 4 hours of collection. Collect a minimum of 10-ml of urine and pour it into sterile, 30-ml Qorpak ® vials with screw-caps tops. Label the specimens, immediately freeze them to -20°C, and store them in dry ice for shipping. Carefully pack vials to avoid breakage during shipment. Store all samples at -20°C or below until analysis.

**Carbamates and Organophosphorus Pesticides in Urine
NHANES 2007-2008**

b. Sample Rejection

Reject specimens with volumes less than 0.1 ml because they cannot be reliably processed.

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

Not applicable for this procedure.

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

a. Reagents and Sources

**Table 1.
Reagents and their Manufacturers**

Reagents	Manufactures
Acephate(O,S-Dimethyl acetylphosphoramidothioate)	Chemservice (West Chester, PA, USA)
Methamidophos (O,S-Dimethyl phosphoramidothioate)	Chemservice (West Chester, PA, USA)
Omethoate (O,O-Dimethyl S-methylcarbamoylmethyl phosphorothioate)	Chemservice (West Chester, PA, USA)
Dimethoate (O,O-Dimethyl S-methylcarbamoylmethyl phosphorodithioate)	Chemservice (West Chester, PA, USA)
Ethylenethiourea (Imidazolidine-2-thione)	Chemservice (West Chester, PA, USA)
Propylenethiourea (Tetrahydropyrimidine-2(1H)-thione)	Chemservice (West Chester, PA, USA)
Acephate-d ₆	Cambridge Isotope Laboratories (custom synthesis)
Ethylene thiourea –ethylene-d ₄	Cambridge Isotope Laboratories (custom synthesis)
Methamidophos-d ₆	Cambridge Isotope Laboratories (custom synthesis)
Dichloromethane	Tedia Company Inc.
Acetonitrile	Tedia Company Inc.
Methanol	Tedia Company Inc.
Formic Acid	Fisher Scientific
Deionized water	NANOpure Infinity ultrapure water system

b. Reagent Preparation

Liquid chromatography mobile phases: Mobile Phase A= 0.1% Formic Acid in aqueous solution. For example: pipette 1 ml of Formic Acid in 999 ml in deionized water and mix. Mobile Phase B= 0.1% Formic Acid in Methanol. For example: pipette 1 ml of Formic Acid in 999 ml of Methanol and mix.

c. Standard Preparation

1) Stock Solutions of Analytes (approximately 200 µg/ml)

Concentration of the stock solution for each analyte should be approximately 200 - 500 µg/mL. Individually weigh approximately 3.0 - 5.0 mg of each analyte to 10 mL volumetric flasks. Add a few milliliters of acetonitrile to the flasks and then swirl the flasks. Once the analytes are dissolved, adjust the final volumes to 10 mL with acetonitrile and mix. Calculate the final concentration and label each one with the exact concentration. Store them at -10°C or below.

2) Stock Solutions of Labeled Isotopes (approximately 200–500 µg/ml)

Individually weigh approximately 2.0 – 5.0 mg of the labeled isotopes to 10 mL volumetric flasks. Add a few milliliters of acetonitrile to the flasks and then swirl the flasks. Once the analytes are dissolved, adjust the volumes to 10 mL with acetonitrile and mix. Calculate the final concentrations, label and then store at -10°C or below.

3) Working Labeled Isotopes Solution (ISTD)

The working solution for the ISTD (200ml) contains an equal concentration of each labeled analyte. Prepare the labeled isotope working solution mixture such that a 10 µL aliquot in 0.8 mL of urine results in the concentration approximately 15 ng/mL. Transfer working labeled solution to 10 mL vials, label and then store at -10°C or below.

4) Working Standard Solutions

An intermediate working standard solution (25 ml) containing an equal concentration (8.0 µg/ml) of each analyte was prepared by combining the aliquots of each primary stock solution and diluting with acetonitrile. By serial dilutions of this intermediate standard solution, we prepared nine additional working standard solutions for a total of 10 standard solutions (0.01, 0.02, 0.04, 0.08, 0.2, 0.4, 0.8, 2.0, 4.0 and 8.0 µg/ml). Transfer working standard solutions to 10 ml vials, label and then store at -10°C or below. 10µl aliquot of working standards in 0.8 ml of urine gives the desired standard concentrations ranging from 0.125 ppb to 100 ppb (0.125, 0.25, 0.50, 1.0, 2.5, 5.0, 10, 25, 50 and 100 ppb).

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

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

5) Calibration-Verification Standards

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 Standards

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.

**Carbamates and Organophosphorus Pesticides in Urine
NHANES 2007-2008**

d. Equipment/Supplies

- 1) TurboVap® 96 concentration workstation (Zymark Corporation, Framingham, MA).
- 2) TOMTEC Quadra 3 (Hamden, CT).
- 3) Sartorius Ultramicro® Microbalance (Westbury, NY).
- 4) VirTis 96-well freeze drying system (i.e., aluminum 96-well plate and 2ml glass vials) (VirTis, Gardiner, NY).
- 5) EDP2® pipettes (Rainin Instrument Co. Woburn, MA)
- 6) Electronic 6-channel pipette (Rainin Instrument Co. Oakland, CA)
- 7) Presterilized filter pipette tips (Rainin)
- 8) 450 µL pipette tips (TOMTEC, Hamden, CT).
- 9) Flat-bed large scale mixer (VWR, Suwanee, GA).
- 10) Vortex Genie® vortex mixer (Scientific Industries Inc., Springfield, MA).
- 11) Qorpak bottles (Lab Depot, Inc., Cumming, GA).
- 12) VirTis Genesis Lyophilizer (Gardiner, NY)
- 13) Captiva 96-well filtration plates (Varian, Inc., Palo Alto, CA).
- 14) Square 96-well collection plate, 2ml (Varian, Ince. Palo Alto, CA).
- 15) Micro auto sampler vials with insert and screw caps (Kimble Glass Inc).
- 16) Compressed nitrogen, helium, and argon, liquid nitrogen (Holox Ltd.)
- 17) Zorbax SB-C3 analytical column (4.6 x 150 mm, 5.0 µm) (Agilent Technologies, Santa Clara, CA).

e. Instrumentation

The ThermoFisher TSQ Quantum Ultra, a high resolution triple-quadrupole mass spectrometer (ThermoFisher, San Jose, CA) is equipped with a syringe pump, a divert/inject valve, an atmospheric pressure ionization (API) source, and the Xcalibur data system. Also, the TSQ quantum is connected to the ThermoFisher Surveyor LC System.

1) Mass-Spectrometer TSQ Quantum Ultra

- The instrument operates with an APCI source in positive ion mode with selective reaction monitoring (SRM)

**Carbamates and Organophosphorus Pesticides in Urine
NHANES 2007-2008**

- The tuning with the target analytes optimizes several mass spectrometer parameters such as discharge current, APCI vaporize temperature, sheath gas pressure, auxiliary gas pressure, and capillary temperature. Table 2 shows the optimum setting after the mass spectrometer was tune with the target compound (ETU, PTU, AP, MMP, Omet and Dmet) with the same solvents and flow rate used for the LC method.
- 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 3.

Note: The mass spectrometer parameters such as discharge current, APCI vaporize temperature, sheath gas pressure, auxiliary gas pressure, and capillary temperature can vary between mass spectrometers and needs to be optimized individually.

2) Surveyor LC System

- Chromatographic separation is performed using a Thermo Electron Finnegan Surveyor LC System composed of an autosampler and HPLC pump. The surveyor MS pump pressure is 400 bar.

Table 2
MS Configuration

MS Parameter	Setting
Ionization type	APCI
Ion polarity	Positive ion
Heated capillary	270 °C
Vaporizer temperature	400 °C
Sheath gas pressure	35
Auxiliary gas pressure	5
Discharge current	4.0

Table 3
Analyte Masses

Analyte	Precursor	Product	Collision Energy (V)	Retention Time* (min)
ETU - Q	103	44	- 18	2.96
ETU - C	103	86	- 18	2.97
d ₄ -ETU	107	48	- 18	2.95
MMP - Q	142	94	- 13	3.36
MMP - C	142	125	- 14	3.35
d ₆ - MMP	148	97	- 20	2.31
PTU - Q	117	58	- 15	3.76
PTU - C	117	60	- 27	3.75

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

AP - Q	184	143	- 10	4.53
AP - C	184	49	- 27	4.51
d ₆ - AP	190	148	- 10	4.48
Omet - Q	214	155	- 18	5.71
Omet - C	214	125	- 23	5.72
Dmet - Q	230	171	- 14	7.20
Dmet - C	230	125	- 20	7.20

"Q" Quantification ion

"C" Confirmation ion

Note: * Retention Time can shift within an acceptable range.

7. Calibration and Calibration-Verification Procedures

a. Calibration Plot

- 1) Construct an 8-point calibration plot by performing a linear regression analysis response factor (i.e., area native/area label) versus standard concentration.
- 2) The lowest point on the calibration curve is at or below the measurable detection limits and highest point is above the expected range of results.
- 3) Determination of the slope and intercept of this curve is done by linear least squares fit using SAS® software.
- 4) R² values for the curve must be greater than 0.990. 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, identify the source of this bias.
- 5) Periodically recalculate the standard curve to incorporate the newest data points. Whenever you prepare a new, combined, labeled-isotope solution, re-establish the standard curve.

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.

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

- 3) All calibration verification runs and results shall be appropriately documented.
- 4) According to the updated CLIA regulations from 2003 (<http://www.cms.hhs.gov/CLIA/downloads/6065bk.pdf>), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months.
- 5) All 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 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 may also 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. 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 an auditor (e.g, branch statistician) 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 auditor. The auditor will notify our 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 correction action is taken, laboratory operations can resume.

8. Operating Procedures; Calculations; Interpretation of Results

a. Analytical Runs

Allow the following to thaw and reach room temperature: unknown urine samples (usually 72), six QC samples (2 of each QC level), and approximately 10 ml blank urine used for 1 blank and standard curve.

b. Sample Preparation

VirTis 96-well freeze drying system consisting of an aluminum 96-well plate and 2-ml glass vials is used for the initial sample preparation. Each analytical run includes a blank, standard calibration curve, two each from three levels of QCs and unknowns. An 800 μ l urine sample volume is used for the standard curve, QCs, and unknowns. All samples are spiked with 10 μ l of the labeled internal standard working solution. Then the wells are covered using a cover plate and mix using a flat-bed, large-scale mixer in the pulse mode. To prevent any possible splashes, the mixer's speed has to be carefully adjusted. The samples are frozen for at least 3 hours at -70°C . The frozen samples are then placed in a lyophilizer that operates overnight in the automatic program mode.

The following day, after completion of the lyophilization process, the TOMTEC Quadra 3 is set up with a clean 2 ml square collection plate and a Captiva 96-well filtration plate (both placed on the vacuum box), 450 μ L pipette tips, dichloromethane (placed into a solvent reservoir) and the lyophilized sample plate. 900 μ L of dichloromethane is added to the residue in each sample vial and mixed thoroughly on flat-bed large scale mixer for approximately 10 minutes. The samples are aspirated from the vials and dispensed onto the Captiva 96-well filtration plate and collected on the 2 ml square 96-well collection plate. The sample vials are rinsed again with an additional 900 μ L of dichloromethane (using clean pipette tips), mixed well, aspirated, and dispensed onto the filtration plate to combine with the previous breakthrough. Vacuum is applied to the filtration and collection plates to collect all breakthroughs. The samples are concentrated to dryness using a Turbovap[®] 96 concentration workstation at 40°C and $30\text{-}70\text{ ft}^3/\text{hr}$ flow of nitrogen. After dryness, 0.3 ml of acetonitrile is added to each well, followed by vortexing to rinse the wells. The samples are concentrated to dryness again. The residues are reconstituted with 100 μ l of methanol and transferred to auto-injection vials.

c. Lyophilization (Freeze Drying) Procedure

Let the shelf temperature of the lyophilizer reach -34°C or below by pressing the "FREEZE" button once (this will take about 30 minutes). Then press the "FREEZE" button again, take the samples from the freezer at -70°C and place immediately in the lyophilizer. Select "Program 1" and press "START" button to run the program (make sure the "Vacuum Release" valve is closed). The lyophilizer operates overnight in the automatic program mode without further manipulation. The program named "Program 1" is set with the vacuum at 25.5 mTorr and the temperature at -34°C for 6 hours followed by at -20°C for 2 hours, at 0°C for 2 hours and at 20°C for 3 hours respectively and finally, at 22°C for 1 hour. The following day, after completion of the lyophilization process, press "STOP" button to stop the program & then the "Vacuum Release" button to release the vacuum in order to take the samples out.

d. Liquid Chromatography Conditions

The HPLC separation is a gradient separation, using 0.1% Formic Acid in aqueous solution (Solvent A) and 0.1% Formic Acid in methanol (Solvent B), as follows: t=0 min – 95% solvent A and 5% solvent B, t=5 min – 40% solvent A and 60% solvent B, t=5.01 min – 20% solvent A and 80% solvent B, t=6 min – 5% solvent A and 95% solvent B, t=7min – 95% solvent A and 5% solvent B, and 2 min for equilibration at 95% solvent A and 5% solvent B before the next injection. The column used is a Zorbax SB-C3, narrow-bore 4.6 x 150-mm, 5.0 µm. The analytes elute between 2.5 and 6.5 minutes. The run time for each injection is 9 minutes. The flow rate is 1000 µl/min and the injection volume is 10 µl. The divert valve is programmed to go to waste for the first 2 minutes and the last minute of the run.

e. Mass Spectrometry Conditions

1) Sequence Setup

- Open Xcalibur Software - Xcalibur allows importing data that has been created by Microsoft Excel.
- To import a sequence: Choose **File > Import Sequence** to open the Import Sequence dialog box.
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- To import a sequence: Choose **File > Import Sequence** to open the Import Sequence dialog box.
- Use the Browse button to select the file for import. In the select Columns to Import group box, select the 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:
 - Path → Enter the directory path where the sample's raw file will be stored;
 - Inst. Meth → Enter the path and filename of the instrument method file; and
 - Proc Method → Enter the path and filename of the processing method file.
- For Save File: Choose **File > Save As**.

2) Autosampler

- On the Surveyor Autosampler, place the vials into the sample tray (A, B, C, D and E) according the position on the sequence file. Each tray contains 40 vial positions arranged in two rows of 20. The first vial position is A01 and the last position is E40.

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

- Ensure that the solvent reservoir contains sufficient running solvent for all the samples and that the solvent rinse reservoir contains enough of the rinse solvent (5% methanol in water) for a complete rinse.

3) Gases

- Ensure that there is enough N₂ gas (utilized as the sheath and auxiliary gases) and argon (for the collision induced dissociation gas – CID).

4) Run the sequence

- In Tune Master, click on the **On/Standby** button on the Control/Scan Mode toolbar to turn on the mass spectrometer.
- On the Control/Scan Mode toolbar, click on the **AS/LC Direct Control** Button to display the Inlet Direct Control view.
- Set up the Survey MS pump to deliver a solution of 95% of 0.1% Formic Acid in Water and 5% of 0.1% Formic Acid in Methanol.
- Let the instrument and pump system to be on conditioning for about 30 minutes before start running samples (make sure the divert valve is set to “waste”).
- Always run a standard check (test sample) before running real samples.
- Highlight the samples you want to run. Click on the left-most column of the first sample and drag down to the last sample on the sequence.
- Choose **Actions > Run Sequence** or click on the Run Sequence toolbar button. The Run Sequence dialog box is then displayed.
- On the Sequence dialog box on the After Sequence Set System select “STANDBY” to put the instrument in Standby mode and turn off the pump after the last sample has been analyzed.
- Click on **OK** to start analyzing the run.

f. Processing data

To process a batch of samples:

- Select the rows to be processed from the current sequence → Click on the left-most column of the first sample and drag down to the last sample on the sequence.
- Choose **Actions > Batch Reprocess** or click on the Batch Reprocess toolbar Button to display the Batch Reprocess Setup Dialog box.
- Select the Quan check box and select the Peak Detection & Integration and Quantification boxes.
- Click on OK. Xcalibur initiates batch reprocessing of the selected samples.

g. Quantification

- After processing the sample batch, manually evaluate for correct peak detection and baseline selection in the Quan® browser.
- Export data files to EXCEL® using the long report format.

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

- Quan Browser allows: to view quantitative results, to evaluate standard curve, QCs and unknown samples, to integrate chromatogram peaks manually, and to analyze detailed quantification information.
- To start Quan browser: Click on the **Quan Browser** icon on the Home Page.
- Quan Browser displays an Open dialog to select an existing file (.SLD).
- Xcalibur displays the View Sample Types dialog box that offers the following viewing options to choose: Show standard and QC sample types and Show All Samples types.
- Click on OK to start the session.
- Save the settings in a Quan Browser file (.XQN). Choose **File>Save As**.
- Export data files to EXCEL → **File > Export Excel > Long Report**.

h. Rearrangement of Data Files

Data are automatically rearranged into a single worksheet (Excel format)[®] that is compatible with our existing database using an Excel[®] macro. This macro also allows the analyst to evaluate quantification and confirmation ions.

i. Transfer of Data

Transfer the file to an external drive.

j. Importation of Data into Database

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

k. Statistical analysis and interpretation of data

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

I. Maintenance of Key Components

1) Routine Maintenance

a) Mass Spectrometer

- The instrument needs to be calibrated for better applications. Preventive maintenance (PM) is required every 6 months and it is performed by Thermo Service Engineer. Instrument inspections, testing, cleaning and part replacements are done according to the manufacture guidelines. Open vacuum manifold, cleaning of the Q0, Q1, Q2 and Q3 quadrupoles should only be done by a Thermo Service Engineer.
- In general, the maintenance procedures of calibration and cleaning API stack (ion sweep cone, tube lens, skimmer and Q00) are performed by

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

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, discard it and use a new one. If not, clean it by sonicating in 5% HNO₃ in water, then in water and in methanol respectively (for about 5 minutes in each solvent).
- The frequency of cleaning the components of the mass spectrometer depends on the types and amounts of samples and solvents that are introduced into the instrument.

b) Surveyor Autosampler

The autosampler requires only a few simple maintenance procedures to keep it in optimum working condition.

- Check the solvent tubing and connections for leaks
- Back-flush or change analytical column as necessary (when tailing or high pressure is observed).
- Replace HPLC filter as needed (when high pressure is observed).
- Check needle position into vials.
- Ensure that the solvent reservoir contains sufficient running solvent for all samples and that the solvent rinse reservoir contains enough of the rinse solvent (5% methanol/ 95%water) for a complete rinse.

2) Tune and Calibration

a) Calibration

- Install the ESI Probe
- Place the LC/MS System in Standby
- Use the following procedure to set up the syringe pump for introducing tuning and calibration solution into ESI source:
 - Install a sample transfer line between the LC unions on the syringe adapter.
 - Load a clean, 500 µL Unimetrics ® syringe with 420 µL of the polytyrosine-1, 3, 6 tuning and calibration solution.
 - Insert the tip of the syringe needle into the end of the Teflon® tube on the syringe adapter assembly.
 - Place the syringe into the syringe holders of the syringe pump and push the handle down until it just contacts the syringe plunger.

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

- Set up the mass spectrometer for tuning and calibration as follows:
 - In Tune Master, click on the On/Standby button on the Control/Scan Mode toolbar to turn on the mass spectrometer.
 - The Tune Master is automatically placed in the ESI source mode.
 - Click on the Compound Optimization Workspace button on the Control/Scan Mode toolbar to display the Compound Optimization work space.
 - Set the values for several of the compound dependent devices: Spray voltage 4000, Sheath Gas Pressure 5, Aux Valve Flow 5, Capillary Temperature 270, Tube Lens Offset 90.
 - Choose Setup>Syringe Pump & Sample Loop to set up the Syringe pump: Flow rate (5 μ L/min), Syringe Type (Unimetrics or Hamilton) and Syringe Size (500 μ L). After specify the syringe type and syringe volume (500), Tune Master automatically sets the proper syringe ID value.
 - Click on Apply to apply these settings and to start the syringe Pump.
- Establish a stable ion beam with the ESI source.
 - Set the scan parameters in preparation for observing the intensity and stability of the ion beam: in Tune Master, on Control/Scan Mode toolbar, Click on the Instrument Method Development Workspace button to display the Instrument Method Development workspace.
 - Select: Scan Type \rightarrow Full Scan; Scan Mode \rightarrow Q1MS; Scan Range/Entry Mode \rightarrow Center Mass; Center Mass list box \rightarrow enter 508.208; Scan Width spin box \rightarrow enter 10.000; Scan Time spin box \rightarrow enter 0.20; Peak Width/Q1 box \rightarrow enter 0.70. Confirm AutoSim box is not selected, Micro Scans is set to 1 and Accurate Mass Mode is off. Click Apply.
 - On the Control/Scan Mode toolbar: display TIC button to begin an ion current trace; ensure Profile/Centroid button in the profile state; place the mass spectrometer in the positive ion polarity mode.
 - Click on the Optimize Compound Dependent Devices button to Display the Optimize Compound Dependent Devices view. Ensure that the compound dependent devices are set to the established values.
 - Determine if the ion beam is stable.
- Verify operation in the ESI/MS Mode.
 - Select: Scan Type \rightarrow Full Scan; Scan Mode \rightarrow Q1MS; Scan Range/Entry Mode \rightarrow FM/LM; First Mass list box \rightarrow enter 150.000; Last Mass list box \rightarrow 1050.000; Scan Time spin box \rightarrow enter 1.20; Peak Width/Q1 box \rightarrow enter 0.70; Data Processing spin box \rightarrow

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

- select average option button and select 10 on the box. Confirm AutoSim box is not selected, Micro Scans is set to 1 and Accurate Mass Mode is off.
 - Click Apply.
 - Click on the Creep toolbar button to toggle to Normalize toolbar button to normalize the spectrum.
 - In the Spectrum view, observe the mass spectrum of the singly charged ions of the tuning calibration solution. The ions are as follows: torsion monomer → m/z 182.082; polytyrosine trimer → m/z 508.208; polytyrosine hexamer → m/z 997.398.
 - Set the scan parameters for verifying the operation of the mass spectrometer in Q3.
- Tuning and calibration automatically in the ESI/MS/MS positive mode.
 - In Tune Master, click on the System Tune and Calibration workspace.
 - In the System Tune and Calibration view in the top left corner of the workspace, select polytyrosine- 1, 3, 6 from the Compound list box (this automatically selects the 3 positively charged polytyrosine ions).
 - Select Auto Tune-Calibration to specify a full tune and calibration.
 - Select both to tune and calibrate in both the first and third quadruples.
 - Click on Start to start the automatic tuning and calibration procedure.
 - Click on Accept to accept the results of the tuning and calibration procedure.
 - Click on Save Calib. As to open the Save Calibration File dialog box.
 - Save the Tune Method file.
 - Click on the Standby button on the Control/Scan Mode toolbar to turn off the mass spectrometer.

b) Tuning with the target analytes in APCI/MS/MS

- Install the APCI Probe
- Place the LC/MS System in **Standby**
- Click on the Optimize Compound Dependent Devices button on the Control/Scan Mode toolbar to display the Optimize Compound Dependent Devices view.
- Set the typical values used for APCI :
 - Discharge Current → 4
 - APCI Vaporizer Temperature → 500
 - Sheath Gas Pressure → 30

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

- Aux Valve Flow → 0
- Capillary Temperature → 350
- Tube Lens Offset → 160
- Source CID → 0
- Collision Pressure → 1.5
- Collision Energy → -38
- Quad MS/MS Bias → -3.0
- Set up the syringe pump for introduce the target solution in the APCI source:
 - Load a clean, 500 μ L Unimetrics® syringe with 420 μ L of a ETU, PTU, AP, MMP, Omet and Dmet solution around 20 ng/ml.
 - Insert the tip of the syringe needle into the end of the Teflon® tube on the syringe adapter assembly.
 - Place the syringe into the syringe holders of the syringe pump and push the handle down until it just contacts the syringe plunger.
 - Choose Setup>Syringe Pump & Sample Loop to set up the Syringe pump: Flow rate (5 μ L/min), Syringe Type(Unimetrics or Hamilton) and Syringe Size (500 μ L). After specify the syringe type and syringe volume (500), Tune Master automatically sets the proper syringe ID value.
 - Click on Apply to apply these settings and to start the syringe pump.
- Start the solvent flow:
 - On the Control/Scan Mode toolbar, click on the AS/LC Direct Control Button to display the Inlet Direct Control view.
 - Set up the Survey MS pump to deliver a solution of 50% of 0.1% Formic Acid in Water and 50% of 0.1% Formic Acid in Methanol.
 - In the Flow Rate text Box, enter 1000 to set a flow rate of 1000 μ L/min
 - In the Direct Control Panel box, click on the start button to start the Survey MS pump.
- Set up the mass spectrometer to automatically optimize with the target Compounds:
 - In Tune Master, on the Control/Scan Mode toolbar, click on the Compound Optimization Workspace button to display the Compound optimization workspace.
 - Select SRM in the Optimization Modes
 - Select Standard in the Optimization Options.

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

- In the Optimization table, enter the parent Mass and product Mass (Table 3).
- In the Inlet Types group box, select Syringe Pump Infusion.
- Click on Start to start the automatic tuning procedure.
- Click on Accept to accept the results of the compound Optimization.
- Save the Tune Method file: click on Save Tune As to open The Save As dialog box → enter file name → click on Save.

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.

a. Linear Limits

Analytical standard were linear for all analytes through the range of concentrations evaluated. The linear range for all analytes is from the lowest standard to 50 ppb.

b. Analytical Sensitivity

The detection limits for all analytes were calculated as $3S_0$, where S_0 is the standard deviation (SD) at zero concentration and is determined by linear regression analysis of the absolute standard deviation (SD) versus concentration. The detection limits vary based upon the current operating precision and the cleanliness of the analytical system. The detection limits are presented in Table 4.

Table 4
Analyte Detection Limits

Analyte	LOD ng/ml
ETU	0.21
PTU	0.36
AP	0.27
MMP	0.41
Omet	0.10
Dmet	0.10

Note: Method specifications, including LOD and Calibration Verification, are calculated for each individual study; therefore they may vary slightly from study to study.

**Carbamates and Organophosphorus Pesticides in Urine
NHANES 2007-2008**

c. Accuracy

The accuracy of this method was determined as the degree of agreement between the mean of measured concentrations of samples and their nominal values. The accuracy was consistent across the entire linear range. The accuracy in this method is expressed as the slope of a linear regression analysis of the measured values versus the nominal values. A slope of 1.0 is considered 100 % accuracy. Accuracy is shown in Table 5.

Table 5
Accuracy of the Method

Analyte	Accuracy (%)
ETU	99.9
PTU	100.0
AP	99.9
MMP	100.0
Omet	99.9
Dmet	100.0

d. Precision

The precision of this method is determined by calculating the relative standard deviation (RSD) of repeated measurements of quality control materials at three concentrations: (QCL: 1.0 ng/ml; QCM: 5 ng/ml; and QCH: 15 ng/ml). Table 6 shows the precision of the method of the most recent study.

Table 6
Precision of the Method

Analyte	Total RSD (%)		
	QCL	QCM	QCH
ETU	13.1	12.3	8.9
PTU	9.0	12.2	4.9
AP	9.3	8.6	10.8
MMP	11.4	9.7	10.6
Omet	7.1	8.1	10.5
Dmet	11.2	7.9	9.4

RSD= Relative Standard Deviation of the QC Values

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

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 two specific product ions (Quantification ion and Confirmation ion) for each specific mass.

10. Quality Control (QC) Procedures

a. QC Materials

Urine pools enriched with known amounts of pesticides residues as the control material should be used for each unknown run.

b. Collection of Urine for QC Pools

Collect the urine samples from multiple (>30) donors, and screen the urine to ensure that the endogenous levels of pesticide residues are low or non-detectable. Combine and homogenize the urines samples to form a base pool. The protocol for anonymous collection of urine was reviewed and approved by CDC's Institutional Review Board (IRB).

c. Filtration and Dispensing

The urine pool is pressure filtered with a 0.45- μ m filter capsule.

d. Urine Enrichment

Split the base pool equally into four smaller urine pools. Reserve one pool for blank and standard analyses (see "Sample Preparation" section). Enrich one of the pools with an appropriate amount of the stock solution of each pesticide residue to yield an approximate concentration of 1.0 μ g/L (QCL). Enrich the other pool to yield an approximate concentration of 5 μ g/L (QCM) Enrich the final pool with an appropriate amount of each pesticide stock solution to yield an approximate concentration of 15 μ g/L (QCH).

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

e. Characterization of QC Materials

Characterize the QC pools (including the unspiked pool) by 20 consecutive runs (1 analytical run per day for 20 days) of each QC material. Use the data from these runs to establish the mean and upper-and lower- 99th and 95th confidence intervals.

f. Use of QC Samples

Carbamates and Organophosphorus Pesticides in Urine NHANES 2007-2008

During each analytical run, analyze one blank urine and three QC materials: QCL, QCM, QCH.

g. Final Evaluation of Quality Control Results

Evaluate QC material are evaluated according to standard Westgard multirule criteria (www.westgard.com). Repeat out-of-control runs if residual sample is available. No data from runs considered out-of-control will be reported.

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

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

12. Limitations of Method; Interfering Substances and Conditions

This method is an isotope-dilution mass spectrometry method, which is widely regarded as the definitive method for the measurement of organic toxicants in human 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. Critical-Call Results (“Panic Values”)

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

14. Specimen Storage and Handling during Testing

All samples must be remaining under freezing temperature prior to use. However, urine samples can be refrigerating overnight to expedite thawing prior analyzes. Store the urine extracts in autosampler vials in a -10°C or below in freezer after analysis.

15. 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 vials at -70°C.

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

Once the validity of the data has been established by the QC/Quality Assurance system outlined above and has been verified by a DLS QAO, generate one hard copy and one electronic copy of the data. Route these data, a cover letter, and a table of method specifications and reference range values through the appropriate channels for approval (i.e., supervisor, branch chief, division director). Once division personnel have approved, the information, send it to the contact person who requested the analyses.

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

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 only certified laboratories. Any transfer of study samples is handled through the DLS special studies coordinator.

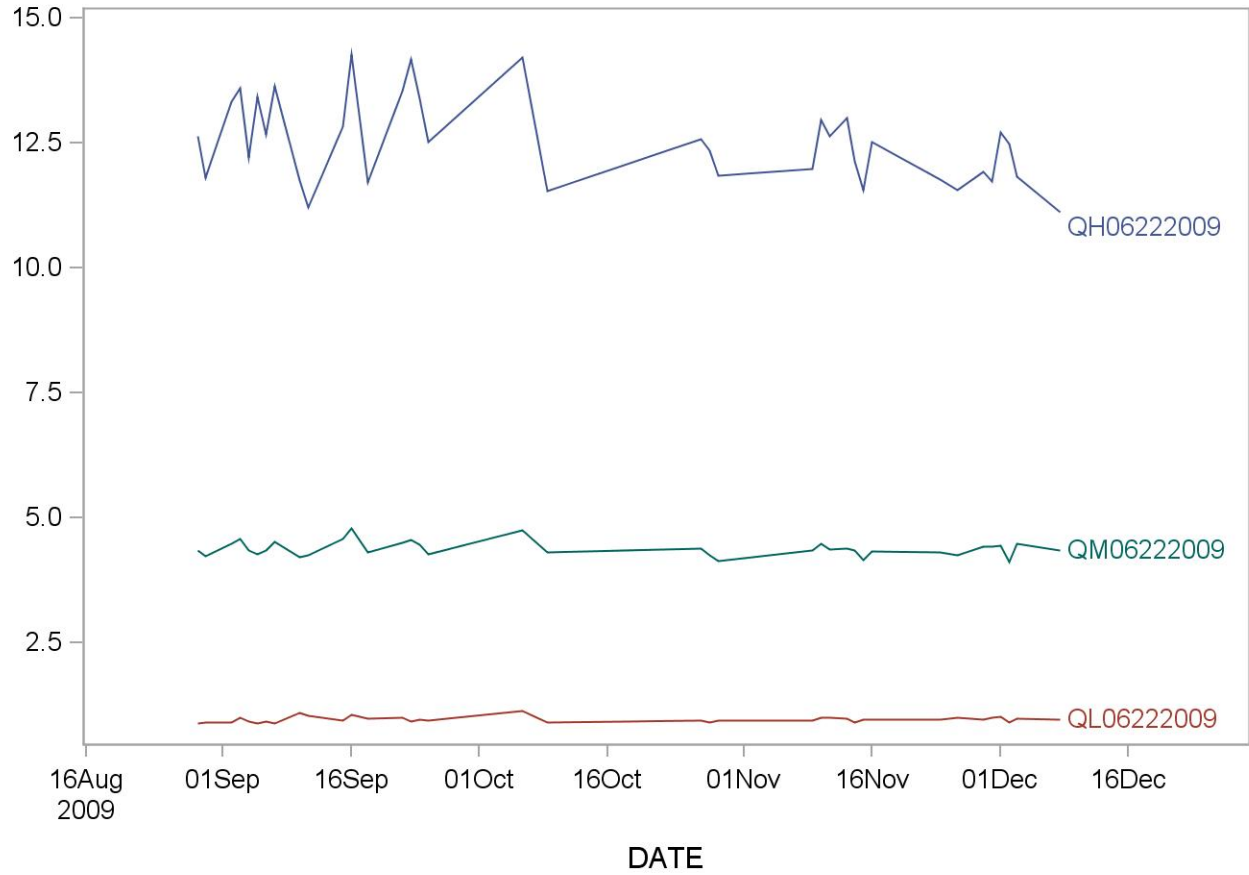
18. Summary Statistics and QC Graphs

See following pages.

Summary Statistics for Acephate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06222009	38	29AUG09	08DEC09	12.498	0.850	6.8
QL06222009	38	29AUG09	08DEC09	0.961	0.057	6.0
QM06222009	38	29AUG09	08DEC09	4.381	0.151	3.5

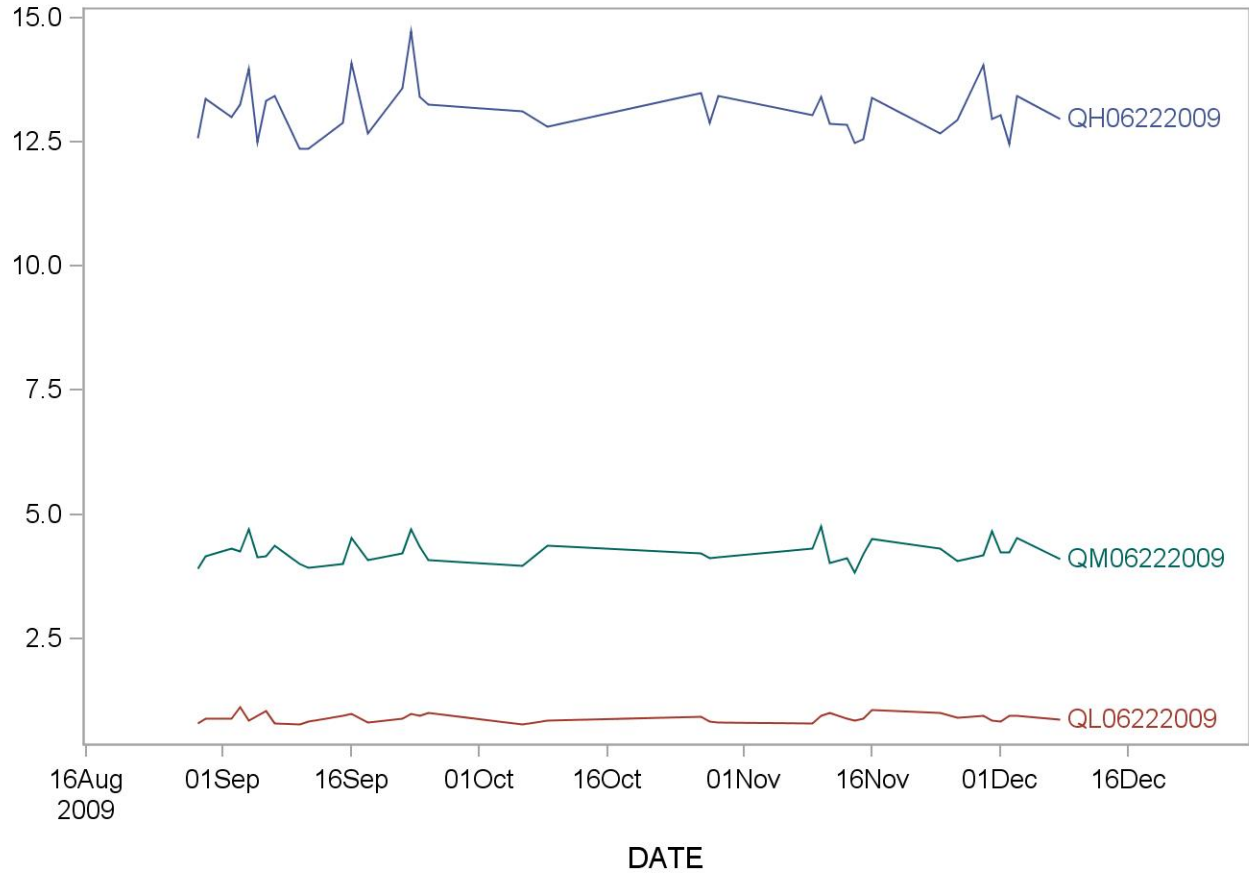
2007-2008 Acephate Quality Control



Summary Statistics for Ethylenethio urea

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06222009	38	29AUG09	08DEC09	13.136	0.522	4.0
QL06222009	38	29AUG09	08DEC09	0.905	0.086	9.5
QM06222009	38	29AUG09	08DEC09	4.245	0.266	6.3

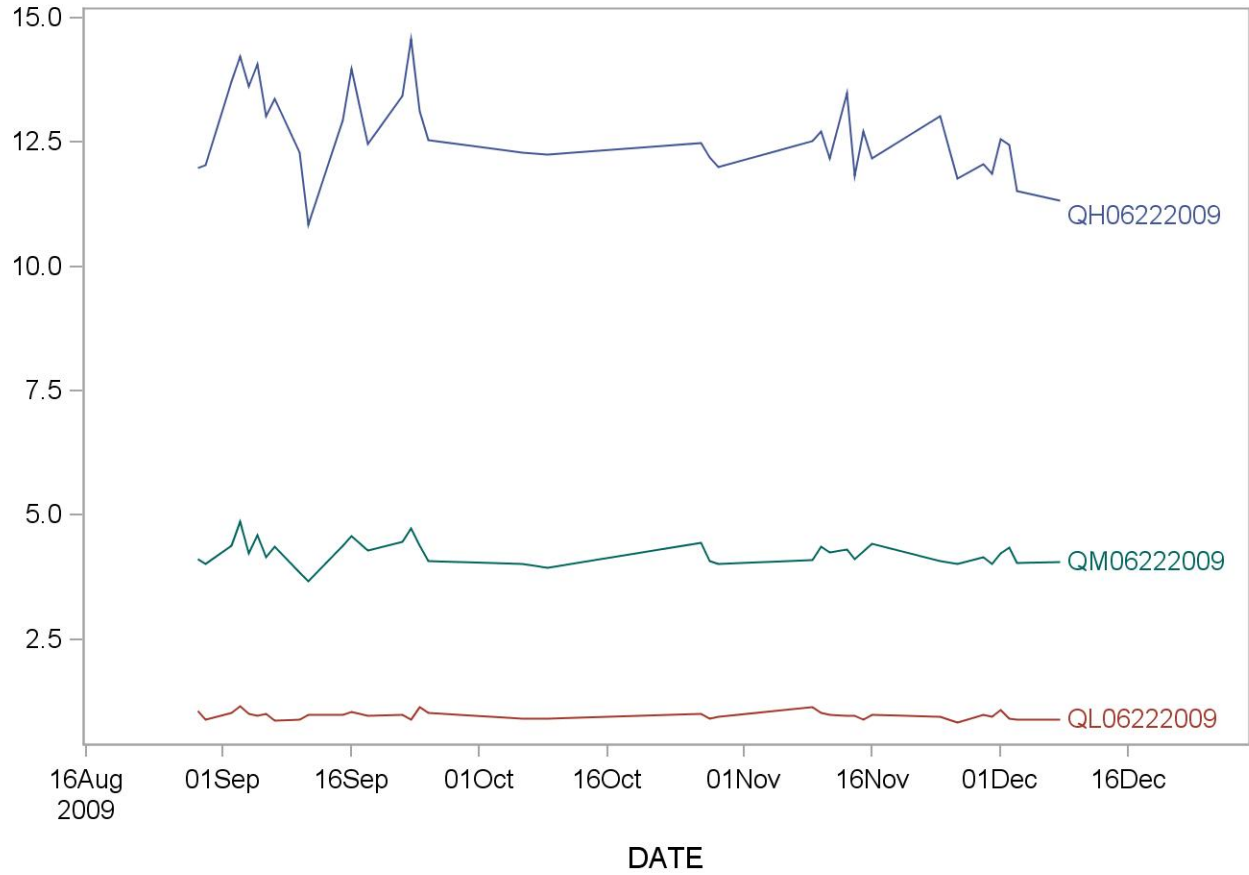
2007-2008 Ethylenethio urea Quality Control



Summary Statistics for Methamidaphos

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06222009	38	29AUG09	08DEC09	12.610	0.855	6.8
QL06222009	38	29AUG09	08DEC09	0.967	0.078	8.0
QM06222009	38	29AUG09	08DEC09	4.224	0.243	5.7

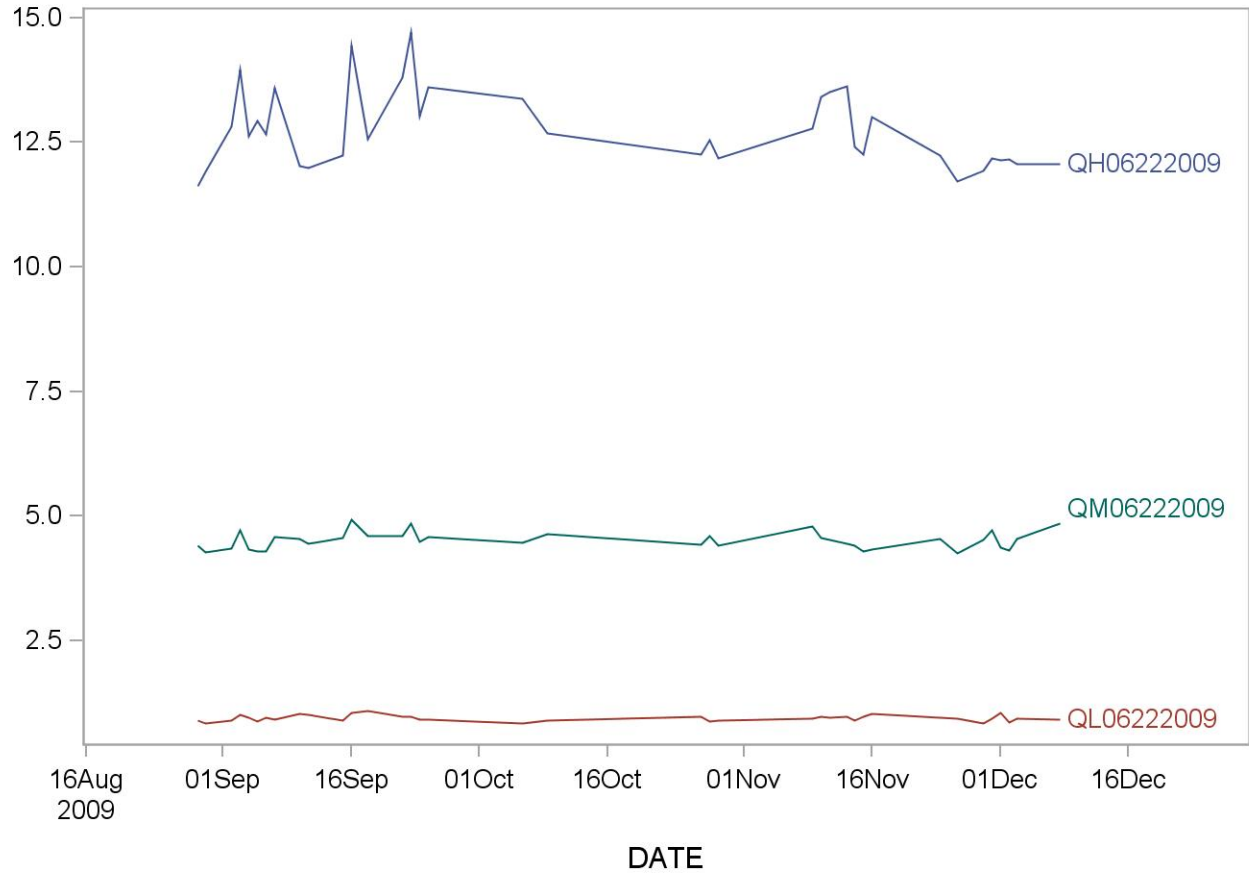
2007-2008 Methamidaphos Quality Control



Summary Statistics for Dimethoate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06222009	38	29AUG09	08DEC09	12.716	0.769	6.0
QL06222009	38	29AUG09	08DEC09	0.937	0.061	6.5
QM06222009	38	29AUG09	08DEC09	4.508	0.174	3.9

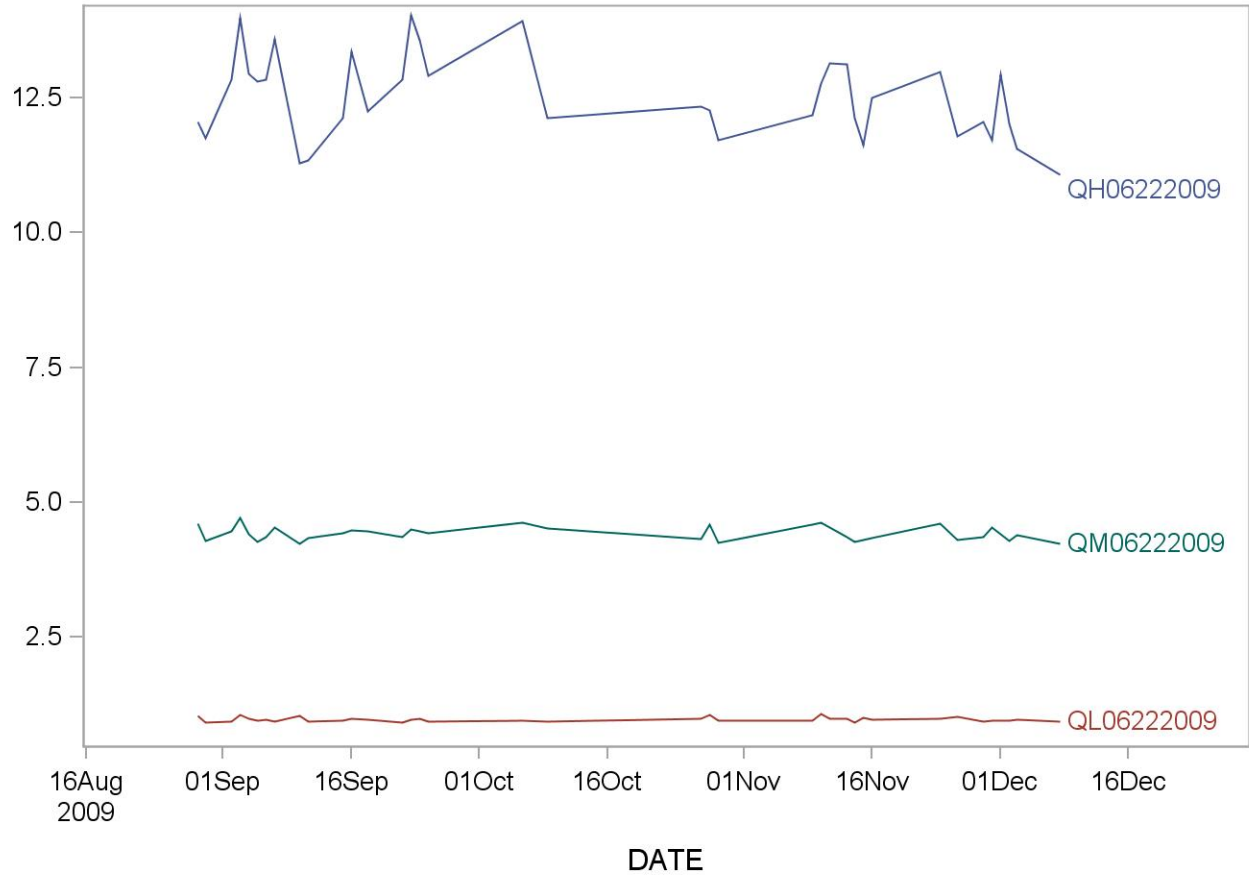
2007-2008 Dimethoate Quality Control



Summary Statistics for O-methoate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06222009	38	29AUG09	08DEC09	12.478	0.783	6.3
QL06222009	38	29AUG09	08DEC09	0.964	0.042	4.3
QM06222009	38	29AUG09	08DEC09	4.418	0.127	2.9

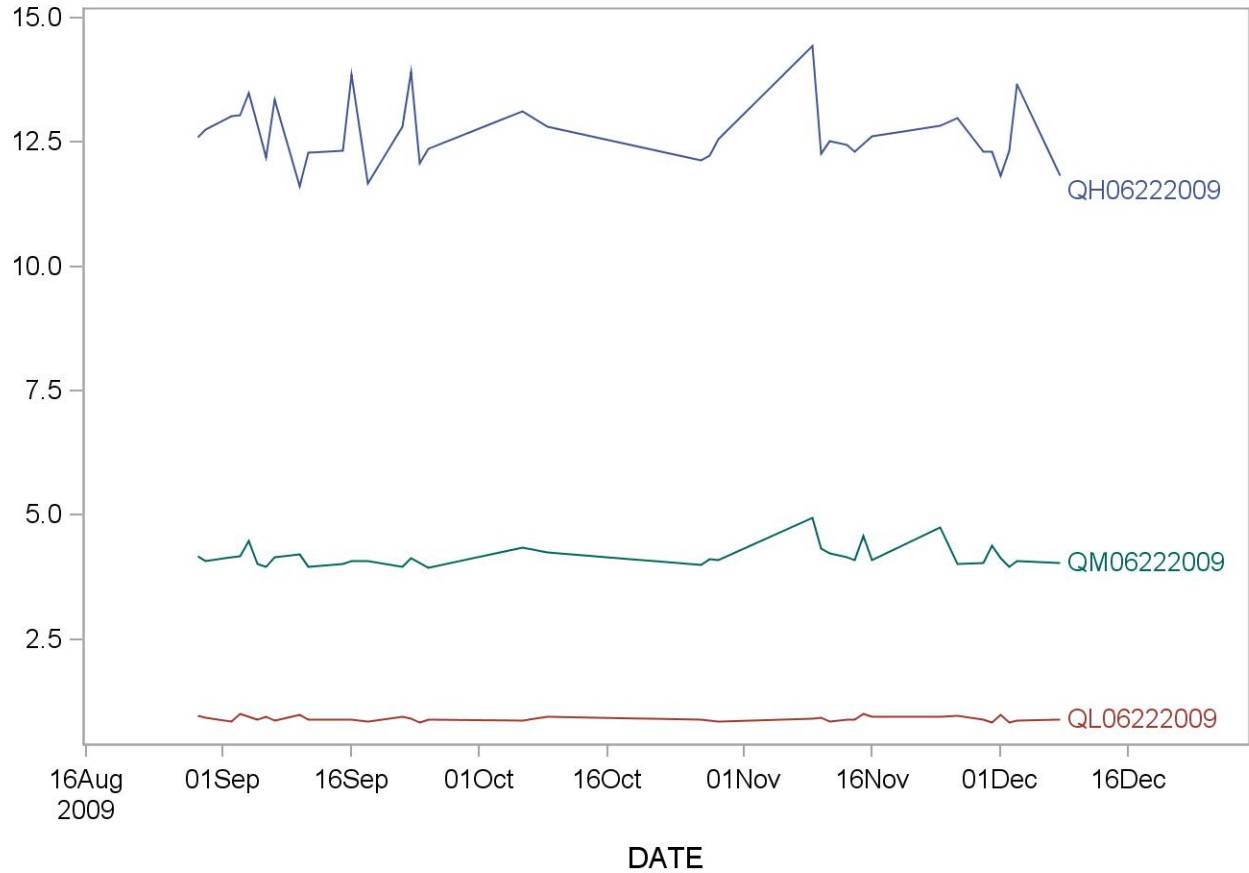
2007-2008 O-methoate Quality Control



Summary Statistics for Propylenethio urea

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QH06222009	38	29AUG09	08DEC09	12.684	0.653	5.2
QL06222009	38	29AUG09	08DEC09	0.902	0.048	5.4
QM06222009	38	29AUG09	08DEC09	4.166	0.220	5.3

2007-2008 Propylenethio urea Quality Control



**Carbamates and Organophosphorus Pesticides in Urine
NHANES 2007-2008**

*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.*

**Carbamates and Organophosphorus Pesticides in Urine
NHANES 2007-2008**

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**Carbamates and Organophosphorus Pesticides in Urine
NHANES 2007-2008**

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