Laboratory Procedure Manual

Analyte: **Dialkylphosphate metabolites: dimethylphosphate, diethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, diethylthiophosphate, and diethyldithiophosphate.**

Matrix: **Urine**

Method: **Organophosphorus Pesticides Screen in Urine**

Method No: **6104.03**
Revised: **02/14/2011**

as performed by:

Organic Analytical Toxicology Branch
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Important Information for Users

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

Important Information for Users

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

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table

: **Data File Name**	**Variable Name**	**SAS Label**
OPD_E	URXOP1	Dimethylphosphate (ug/L)
	URXOP2	Diethylphosphate (ug/L)
	URXOP3	Dimethylthiophosphate(ug/L)
	URXOP4	Diethylthiophosphate ug/L)
	URXOP5	Dimethyldithiophosphate (ug/L)
	URXOP6	Diethyldithiophosphate (ug/L)
	URXUCR	Creatinine, urine (mg/mL)

1. Clinical Relevance and Summary of Test Principle

Organophosphate (OP) pesticides represent about 8% of the total pesticides used in the United States and about half of the total insecticides used worldwide. More than 75 million pounds are applied annually, with about 15% being used in nonagricultural applications. OP pesticides include malathion, diazinon, chlorpyrifos, Guthion® (azinphosmethyl), malathion, parathion, and many others. All OP pesticides have a common mode of toxicity because they are competitive inhibitors of acetylcholinesterase, the enzyme responsible for deacetylation of the neurotransmitter acetylcholine. Unfortunately, the toxic effects of OP pesticides are not unique to insects; high doses can similarly affect wildlife and people. Because of their potential to adversely affect especially vulnerable populations, such as children, and because of their common mode of toxicity, OP pesticides were among the first of the US Environmental Protection Agency (EPA)-registered pesticides whose food tolerances were reassessed by EPA.

There are 39 organophosphates pesticides registered for use in the United States by the EPA. Of these, about 75% are O,O-dimethyl or O,O-diethyl substituted that are metabolized to six common dialkylphosphate (DAP) metabolites: dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP). DAP metabolites do not retain any of the structure unique to the pesticides from which they were derived, so it is impossible to identify individual pesticides from these metabolites. However, because these metabolites are common to the majority of OP pesticides, they can provide invaluable information about cumulative exposure to the OP class.

The test principle utilizes gas chromatography-tandem mass spectrometry (GC-MS/MS) to quantify six DAP metabolites of organophosphate pesticides in urine. The procedure involves lyophilization of the urine, derivatization of the DAP metabolites using chloroiodopropane, and quantification of the resulting chloropropyl phosphate esters using GC-MS/MS. This procedure uses isotope dilution with deuterium- or ^{13}C-labeled internal standards for all analytes. During analysis, the protonated analytes dissociate into charged fragments (ions) that are specific to each analyte. Several ions from each analyte and labeled internal standard are monitored, and the abundances of each ion are measured. The ratios of these ions are used as criteria for evaluating the data. By evaluating the concentrations of DAP metabolites in urine, a combined measurement of the body burden of common organophosphate pesticides (cumulative exposure) is obtained.

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents 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 hoods for this procedure.

f. Training

Anyone performing this procedure should be trained and experienced in the use of a triple-quadrupole mass spectrometer. Although formal training is not necessary, personnel are appropriately trained by an experienced operator of the instrument. Untrained personnel must work under the supervision of a trained person. All personnel who operate the instrument must also read all operation manuals.

g. Personal Hygiene

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

h. Disposal of Wastes

Always dispose of solvents and reagents in an appropriate container clearly marked for waste products, and temporarily store them according to CDC's guidelines for 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. 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 onto a computer hard drive to avoid loss of data.

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

a. Sample Collection

Use standard urine collection cups to collect urine specimens from participants. Refrigerate samples as soon as possible and transfer them to specimen vials preferably within 4 hours of collection. Aliquot and label the specimens in adequate vials, freeze them at or below -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.

b. Sample Handling

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

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

Not applicable for this procedure.

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

a. Reagents and Sources

Table 1
Reagents and their Manufactures

Reagents	Manufacturer
Potassium carbonate	Fisher Scientific Co.
1-Chloro-3-iodopropane	Aldrich Chemical Co
Acetonitrile, Toluene, Ethyl Ether	Burdick and Jackson Co.
Dimethylphosphate	Acros Organics Co.
D_6-Dimethylphosphate	Cambridge Isotope Laboratories (custom synthesis)
Dimethylthiophosphate	Cambridge Isotope Laboratories (custom synthesis)
D_6-Dimethylthiophosphate	Cambridge Isotope Laboratories (custom synthesis)
Dimethyldithiophosphate	Los Alamos Co.
D_6-Dimethyldithiophosphate	Cambridge Isotope Laboratories (custom synthesis)
Diethylphosphate	ChemService Co (Sigma-Aldrich)
D_{10}-Diethylphosphate	Cambridge Isotope Laboratories (custom synthesis)
Diethylthiophosphate	Aldrich Chemical Co.
D_{10}-Diethylthiophosphate	Cambridge Isotope Laboratories (custom synthesis)
Diethyldithiophosphate	Aldrich Chemical Co.
$^{13}C_4$-Diethyldithiophosphate	Aldrich Chemical Co.

b. Standards Preparation

1) Stock Solutions of Analytes

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 an accuracy of 3 decimal places. Place into 10 mL volumetric flasks. Add a few milliliters of acetonitrile to the flask and the swirl the flask. Once the analytes are dissolved, dilute the contents of the flask to volume with acetonitrile and mix. Divide stock solutions into 5-mL aliquots and calculate the final concentration. Label each one with the exact concentration and store them at -10°C or below.

2) Stock Solutions of Labeled Isotopes

Individually weigh approximately 2.0 – 5.0 mg of each labeled isotopes in 10-mL volumetric flasks. Add a few milliliters of acetonitrile to the flask and then swirl the

flask. Once the analytes are dissolved, dilute contents of the flask to 10 mL volume with acetonitrile and mix. Divide stock solutions into 5-mL aliquots and calculate the final concentration. Label each one with the exact concentration and store them at -10°C or below. The mixed labeled isotopes solution should contain an equal concentration of each labeled analyte. Calculate the final concentration. Transfer stock solutions to 5-mL ampoules; flame seal, and label each one with the exact concentration and store at -10°C or below.

3) Working Solution of Mixed Labeled Isotopes (ISTD)

Prepare the mixed labeled isotope working solution diluting the contents of one ampoule (5 mL) into acetonitrile such that 125 µL of labeled isotope in 1.0 mL of urine results in the concentration between 15 ppb to 30 ppb. Store the working mixed labeled isotopes at -10°C or below.

Add 125µL of the mixed labeled working solution to a 1 mL urine sample via the Gilson Liquid Handler.

4) Working Standard Solutions

Make nine solutions of varying concentrations ranging from 4 ng/mL to 1000 ng/mL from the stock analyte solutions and dilute with acetonitrile. Calculate concentrations so that a 125-µL aliquot of working standard, via Gilson Liquid Handler, gives the desired standard concentrations of 0.1 ppb, 0.5 ppb, 1 ppb, 2.5 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb, and 125 ppb.

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

5) Mass Spectrometric Check Solutions

These are standard solutions of sufficient concentration (0.1 ng/mL and 125 ng/mL) to produce a reasonable analytical signal. The concentration of check solutions are the same throughout the study. Use these standards daily to evaluate the operating condition of the GC and mass spectrometer.

6) 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.

7) 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.

c. Equipment/Supplies

1) TurboVap® LV evaporator (Zymark Corporation, Framingham, MA).
2) Sartorius Ultramicro® Microbalance (Westbury, NY).
3) Isotemp® 145D heating block (Fisher Scientific).
4) 15-mL, conical-bottom screw-capped tubes (Pyrex or Kimax, Scientific Services, CDC).
5) 15-mL, graduated, conical-bottom tubes (Pyrex or Kimax, Scientific Services, CDC).
6) EDP2® pipettes (Rainin Instrument Co. Woburn, MA).
7) Vortex Genie® vortex mixer (Scientific Industries Inc., Springfield, MA).
8) Micro® autosampler GC vials with aluminum seals (Alltech, Milwaukee, WI)
9) Gilson Liquid Handler
10) Genesis Freeze Dryers.

d. Instrumentation

Perform the analyses on a Finnigan TSQ-7000® triple-quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) that is equipped with a chemical ionization interface and is interfaced to a Trace (GC) system (ThermoFinnigan, San Jose, CA).

1) Mass-Spectrometer (MS) Configuration

Table 2

MS Configuration

MS Parameter	Setting
Scan mode	Product
Ionization	CI
Ion polarity mode	Positive ion
Electron energy	200 V
Ionization gas pressure (methane)	1500 mTorr
CID gas (Ar)	2 mTorr
Collision energy (offset)	Varies for each analyte
Conversion dynode voltage (CDYN)	-15 kV
Continuos dynode électron multiplier voltage (EMULT)	Varies depending upon multiplier lifetime

2) Mass-Spectrometer Instrument-Control (ICL) Programs (Xcalibur)

This ICL procedure initiates a multiple-reaction monitoring (MRM) experiment. It sets the instrument to centroid acquisition mode and the polarity to positive ions. It scans the precursor to product ion transformations of the masses in Table 3

below. In addition, it sets the collision energy for each analyte, turns on the continuous dynode, and sets the electron multiplier voltage (EMULT). This program also initiates another ICL procedure (OP2), which continues the MRM experiment until it is complete. While OPNRBCNM runs the TSQ-7000®, the GC system operates using the temperature program described in Table 5. After the analytes elute from the GC, the ICL procedure turns off the electron current and multiplier.

Table 3

Analyte Masses

Analyte	Collision Offset (V)	Precursor Ion	Product Ion
DMP	-12	203	127
DMP*	-12	205	127
d_6-DMP	-12	209	133
d_6-DMP*	-12	211	133
DEP	-13	231	127
DEP*	-13	233	127
d_{10}-DEP	-13	241	133
d_{10}-DEP*	-13	243	133
DMTP	-13	219	143
DMTP*	-13	221	143
d_6-DMTP	-13	225	149
d_6-DMTP*	-13	227	149
DMDTP	-10	235	125
DMDTP*	-10	237	125
d_6-DMDTP	-10	241	131
d_6-DMDTP*	-10	243	131
DETP	-12	247	191
DETP*	-12	249	193
d_{10} DETP	-12	257	193
d_{10} DETP*	-12	259	195
DEDTP	-12	263	153
DEDTP*	-12	265	153
$^{13}C_4$ DEDTP	-12	267	157
$^{13}C_4$ DEDTP*	-12	269	157

*Indicates ^{37}Cl isotopes of chloropropyl derivative. Used only to confirm analyte

3) GC Configuration and Temperature Program

Perform chromatographic separation on a Trace gas chromatograph fitted with a factor (Four) Agilent 30-m column. The column ID is 0.25 mm and the film

thickness is 0.25 micron (#CP8944 or equivalent). Inject 1 µL of extract using a CTC Analytics-GCPAL (Leap Technologies) autosampler fitted with a Hamilton 701-10 µL syringe.

Table 4
GC Configuration

GC Parameter	Setting
Carrier gas	He
Constant pressure	14.5 psi He
Injection mode	Splitless
Injector purge delay	60 s
Injector temperature	250°C
Transfer line	270°C

Table 5

Oven Temperature Program

Time (min)	Temperature
2	80°C
9.2	235°C
0.7	270°C

7. Calibration and Calibration-Verification Procedures

a. Mass Spectrometer

Calibrate and tune the TSQ7000® mass spectrometer using FC-43 gas according to the instructions in the operator's OPT ICL program to determine the optimum for each parameter. After calibrating the instrument with unit resolution and maximum sensitivity, prepare the instrument to analyze the pesticide metabolites as described in the "Operating Procedures" sections.

b. Calibration Plot

1) Construct a 9-point calibration plot by performing a linear regression analysis of relative response factor (i.e., area native/area label) versus standard concentration.

2) The lowest point on the calibration curve should be at or below the measurable detection limits and the highest point is above the expected

range of results.

3) Determine the slope and intercept of this curve is by linear least squares fit using SAS® software.

4) R-squared 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.

c. Calibration Verification

1) Calibration verification is not required by the manufacturer. However, it should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.

2) Calibration verification must be performed at least once every 6 months.

3) All calibration verification runs and results shall be appropriately documented.

4) According to the updated CLIA regulations from 2003 (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.

d. Proficiency Testing

Proficiency testing should be performed a minimum of once every 6 months. We are participating in the ongoing German External Quality Assessment Scheme (G-EQUAS) organized and managed by the Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (Erlangen, Germany). A minimum of once per year since 2010, we analyze two reference urine samples fortified with the six DAPs metabolites. The program, evaluation, and certification are based on the guidelines of the German Federal Medical Council (http://www.g-equas.de/). In addition to the G-EQUAS PT program, an in-house program including pools prepared in-house is available. Five randomly selected PT materials are analyzed in the same manner as unknown samples. These PT materials are selected from among three different concentration ranges spanning the linear range of the method. 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 notifies the laboratory of its PT status (i.e. pass/fail).

8. Operating Procedures; Calculations; Interpretation of Results

a. Analytical Runs

Each analytical run consists of unknown urine samples (usually 36), 2 QCL, 2 QCM a blank and nine standards in acetonitrile (125 ppb, 50 ppb, 25 ppb, 10 ppb, 5 ppb, 2.5 ppb, 1 ppb, 0.5 ppb, and 0,1ppb). Remove these samples from -70˚C freezer and allow them to come to room temperature.

b. Lyophilization Procedure

Pipette 1 mL of urine into 20 mL green-capped vials. Spike calibration standard tubes with 125 µL of standards spiking solutions and all tubes with 125 µL of combined labeled isotope solution (ISTD) using an automatic Gilson 215 liquid handler. Vortex and place QCs, blank and unknown samples in the corresponding freezing tray and freeze at -70^{0}C for a minimum of three hours; once frozen, transfer to one of the freeze driers and follow the appropriate operating procedure:

On the Genesis freeze drier: Run the instrument for about 20 minutes by pressing the "FREEZE" button (till the shelf temperature is below -300 ºC). Place the frozen samples on the shelf, select "Program n°" and press "START" button to run overnight. Samples will run approximately 14 hours on this mode. The freezer, condenser and vacuum parameter set point are -34°C, 40, and 500 mTorr respectively. The drying phase mode is specifying below.

Table 6
Drying Phase Program

STEP	Temperature (°C)	Time (min)	Vacuum (mTorr)	R/H (Run or Hold)
1	-34	360	200	H
2	-20	15	200	R
3	-20	120	200	H
4	0	20	200	R
5	0	120	200	H
6	+20	20	200	R
7	+20	180	200	H
8	+24	20	200	H

c. Extraction Procedure

After blank QC and unknown samples are dried, extract with 2-mL of Acetonitrile, shake for at least 10 minutes using the Gilson Liquid Handler shaker and pour

the extract into a 15-mL centrifuge tube. Extract a second time with 1-mL of Acetonitrile and 1-mL of Ethyl Ether, shake for at least 10 minutes and add extract to the first. Then extract sample with 2-mL of Ethyl Ether, shake for at least 10 minutes and add extract to the previous 2 extracts. Shake extracts; place them in the TurboVap® at 30°C, 10 psi of nitrogen head pressure and blow down until 1-mL of solvent remains.

d. Derivatization Procedure

Add 50 µL of chloroiodopropane and approximately 10 mg of potassium carbonate into above extracts and into the standard curve samples and then vortex each tube. Place all tubes in Isotemp® heating block at 60°C for 3 hours. Place all tubes in the centrifuge for 10 minutes at 3200 rpm or more; then, pour off the supernatant into new set of 15-mL centrifuge tubes. Evaporate the extracts to dryness using a Turbovap LV Evaporator at 30 ˚C, 10 psi of nitrogen for approximately 30 minutes. Add 150 µL of toluene and vortex. Blow down to dryness (30 ˚C, 10 psi of nitrogen). Reconstitute samples with 70 µL of toluene, vortex thoroughly, transfer to autosampler vials, cap and inject to mass spectrometer or store at -10 ˚C or below until analyzed.

e. Analysis

1) Preliminary System Setup and Performance Check

(a) Install a clean chemical ionization (CI) ion volume.

(b) Open the CI gas valve. Check the calibration gas peaks including m/z of 131, 219, 414, 502 and 652. The intensity should be greater than 50 x 10^6 counts with the electron multiplier (EMULT) set at 800V. If the counts are low, be sure that the ion volume is well seated in the source.

(c) Print out both Q1 and Q3 tune views and place in the log book, along with the standard check.

(d) Check the GC helium gas pressure. It should be between 13-15 psi. Check the helium pressure at the tank.

(e) To check the performance of the system, first run the mass-spectrometer check standards under the same conditions as you run the samples. Assess the signal to noise ratio (S/N) and area counts are met, the TSQ 7000® system is ready to start a run.

2) Final Setup and Operation

(a) In “Sequence Setup” view (TSQ-7000)®, import the sequence created from the database. The database creates each run sheet with random positions for the QCs. The filename should correspond in order: chemical class O for

organophosphates), 1st letter of study name, instrument number, year, month and date (*e.g*. OF2041105). Save the sequence with the same prefix as the file name with XX at the end.

(b) In the GC system, place the sample vials in the autosampler. Be sure that the needle-rinse vial contains a sufficient quantity of toluene to rinse the needle after each injection. Check the needle plunger operation by injecting from a blank solvent vial. Run a solvent injection before, in the middle before and after QCs and at the end of a run sequence.

3) System Shutdown

The instrument shutdowns automatically or:

(a) To turn off the mass spectrometer, turn off ionization gas, collision-induced dissociation (CID) gas and multiplier.

(b) Type .alloff and "enter".

f. Processing of Data

1) Quantification

To process a batch of samples, select the rows to be processed from the current sequence then click on the left-most column of the first sample and drag 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. After Clicking on "OK", Xcalibur initiates batch reprocessing of the select samples.

After processing the sample batch, manually evaluate for correct peak detection and baseline selection in the Quan® browser.

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

2) Rearrangement of Data Files to data-compatible EXCEL file

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 for analyst evaluation of quantification and confirmation ions.

3) Transfer of Data

Transfer the file to a CDC approved transfer data system.

4) Importation of Data into Database

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

5) Statistical analysis and interpretation of data

The Branch's Statisticians are responsible for the statistical analysis and interpretation of data.

g. Replacement and Periodic Maintenance of Key Components

1) Routine Maintenance

a) TSQ7000® Mass Spectrometer

- Instrument undergoes routine maintenance by Thermo service technicians on a bi-annual basis.
- All records for this maintenance are kept in a bench side notebook.
- Perform the following maintenance as suggested:
  Replace the ion volume before each run.
  Clean the source lenses once per month.

b) TRACE® GC

- System undergoes routine maintenance by service technicians during the same time period that the mass spectrometer undergoes maintenance.
- All records for this maintenance are kept in a bench side notebook.
- Perform the following maintenance as suggested:
  Change the injection port liner once per each run.
  Change the retention gap on the front of the GC column for each analytical run.

2) Periodic Maintenance

In general, these maintenance procedures are performed by instrument operator if there is a decrease in the system performance (sensitivity or S/N ratio) without any other apparent technical reasons.

a) TSQ 7000® Mass Spectrometer

Maintenance of the L1x lenses and the analyzer assembly requires that you vent the system. After venting, the system will usually require about 24 hours to re-establish high vacuum.

L11, L12, and L13 Lenses

These lenses get less dirty than the ion source and they are cleaning during the routine maintenance by Thermo service technicians on a bi-annual basis.

They are cleaned with slurry of aluminum oxide in water. Follow the instructions in the ThermoFinnigan TSQ/SSQ 7000® Hardware Operator's and Service Manual: Rev. A (chapter 6).

b) The Analyzer Assembly

The mass analyzers (Q1, Q2, and Q3) and their associated lenses get dirty at a rate significantly slower than the electron impact (El) or CI source assembly. The analyzer assembly is cleaned during a scheduled preventative maintenance (PM) by Thermo service technicians in order to achieve adequate instrument performance. Again, follow the notes in the ThermoFinnigan TSQ/SSQ 7000® Hardware Operator's and Service Manual: Rev. A (chapter 6).

9. Reportable Range of Results

The linear range of the standard calibration curve determines the highest and lowest analytical values of an analyte that are reportable. However, you can dilute and re-analyze urine samples whose analytical data value exceeds the highest reportable limit so that the result will be in the reportable range.

a. Linear Limits

Analytical standard curves are linear for all analytes through the range of concentrations evaluated. The linear range for all analytes is the concentration of the lowest standard to 125 ppb.

b. Analytical Sensitivity

The detection limits for all analytes are calculated as $3S_0$, where S_0 is the estimated standard deviation (SD) at zero concentration. The range of detection limits are presented in Table 7.

Table 7

Analyte Detection Limits

Analyte	$3S_0$
DMP	0.47
DMTP	0.55
DMDTP	0.51
DEP	0.37
DETP	0.56
DEDTP	0.39

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

c. Accuracy

The accuracy of this method is calculated as a percentage of an expected value. This value is determined by enriching urine samples with known concentrations of the pesticide residues and comparing the calculated and expected concentrations. The accuracy is consistent across the entire linear range. Values for accuracy for the metabolites in this method are listed in Table 8.

Table 8

Accuracy of the Method

Analyte	Slope (Calculated vs. Expected)
DMP	0.992
DMTP	0.995
DMDTP	0.990
DEP	0.994
DETP	0.997
DEDTP	0.999

d. Precision

The precision of this method is reflected in the variance of QC samples over time. The major contributor to the variance is the variation between runs. The coefficients of variation were determined from multiple analyses of quality control materials throughout the study (Table 9, N= 83 runs).

Table 9
Precision of the Method

Analyte (ppb)	CV
DMP-QCL	0.150
DMP-QCM	0.139
DMTP-QCL	0.184
DMTP-QCM	0.162
DMDTP-QCL	0.160
DMDTP-QCM	0.138
DEP-QCL	0.135
DEP-QCM	0.164
DETP-QCL	0.158
DETP-QCM	0.144
DEDTP-QCL	0.211
DEDTP-QCM	0.143

Note: QC limits and means can vary over time as additional studies are completed and their QC data are added to the characterization.

e. Analytical Specificity

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

10. Quality Control (QC) Procedures

a. QC Materials

Quality Control materials for each unknown run are made from human urine pools which are enriched with known amounts of pesticide residues.

b. Collection of Urine for QC Pools

Prepare and use three QC pools in each run of unknown samples. Collect the urine form participants for each pool. 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.

c. Urine Enrichment

Split the base pool into three smaller urine pools. Enrich one of the pools with an appropriate amount of the stock solution of each pesticide to yield an approximate concentration range of 3-10 ng/mL (QCL). Enrich the other pool to yield an approximate concentration range of 12-20 ng/mL (QCM). Use the other pool as

the matrix blank (QCB). Allow the pools to stir over night in a refrigerator to ensure a homogeneous mixture. Store at -10^{0}C or below.

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

d. Filtration and Dispensing

Clean filter the base pool through 0.2µm filter capsule. Store this urine in 1-L glass bottles in a refrigerator until enrichment.

e. Characterization of QC Materials

Characterize the QC pools by analyzing at least 30 runs of each QC material. Use the data from these runs to establish the mean and upper-and lower- 99th and 95th control limits. Determine the control limits and adjust them according to the number of each QC material analyzed in each run.

f. Use of QC Samples

During each analytical run, analyze four QC materials: two QC low and two QC medium.

g. Final Evaluation of Quality Control Results

Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control [6]. Repeat out-of-control runs if residual sample is available. No data from runs considered out-of-control will be reported.

When using 2 QC pool levels (1QCL and 1 QCM) per run, the rules are:

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
   3. 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
   4. 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.

When using 2 QCs per QC pool levels (2QCL and 2 QCM) per run, the rules are:

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).

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

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until you identify the source or cause of failure is identified and corrected. If the source of failure is with 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 tandem mass spectrometry, you can eliminate most analytical interferences. Because of the matrix used in this procedure, occasional interfering, unknown substances have been encountered. Interferences with the internal standards result in rejection of that analysis. If repeat analysis still results in an interference with the internal standard do not report the results for that analyte.

13. Reference Ranges

The results from the National Health and Nutrition Examination Survey (NHANES) 1999-2004 can be used as the reference ranges among the general US population.

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

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

15. Specimen Storage and Handling during Testing

All samples must be stored frozen prior to use.
However, urine samples can be refrigerating overnight to expedite thawing prior to the analysis. Store the urine extracts in autosampler vials in a -10˚C or below in the freezer after analysis. Stability studies suggest that the extracts remain stable at

room temperature for up to five days.

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

The method is designed to run on a GC/MS/MS instrument and is not generally transferable to other instrumentation. If the system has failed, store sample vials in a refrigerator. You can store the extract samples for as long as 3 weeks. For long-term interruption, store samples between -80˚C and -10˚C.

17. 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 release of 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.

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

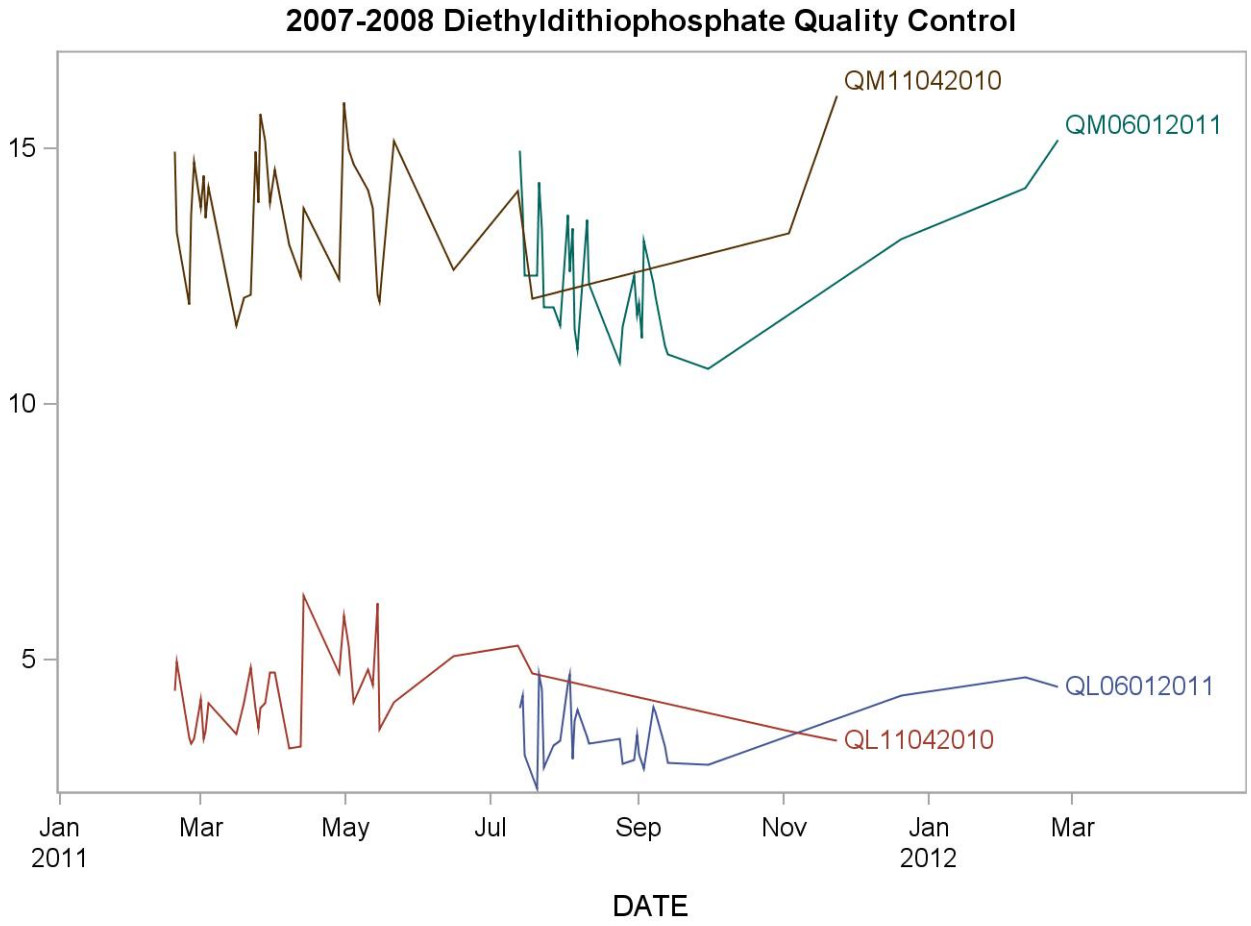
Use standard record-keeping systems (i.e., notebooks, sample logs, data files, creatinine logs, demographic logs) to keep track of all specimens. Transfer of refer to CLIA-specimens only certified laboratories. Any transfer of study samples is handled through the DLS special studies coordinator.

19. Summary Statistics and QC Graphs

See next pages.

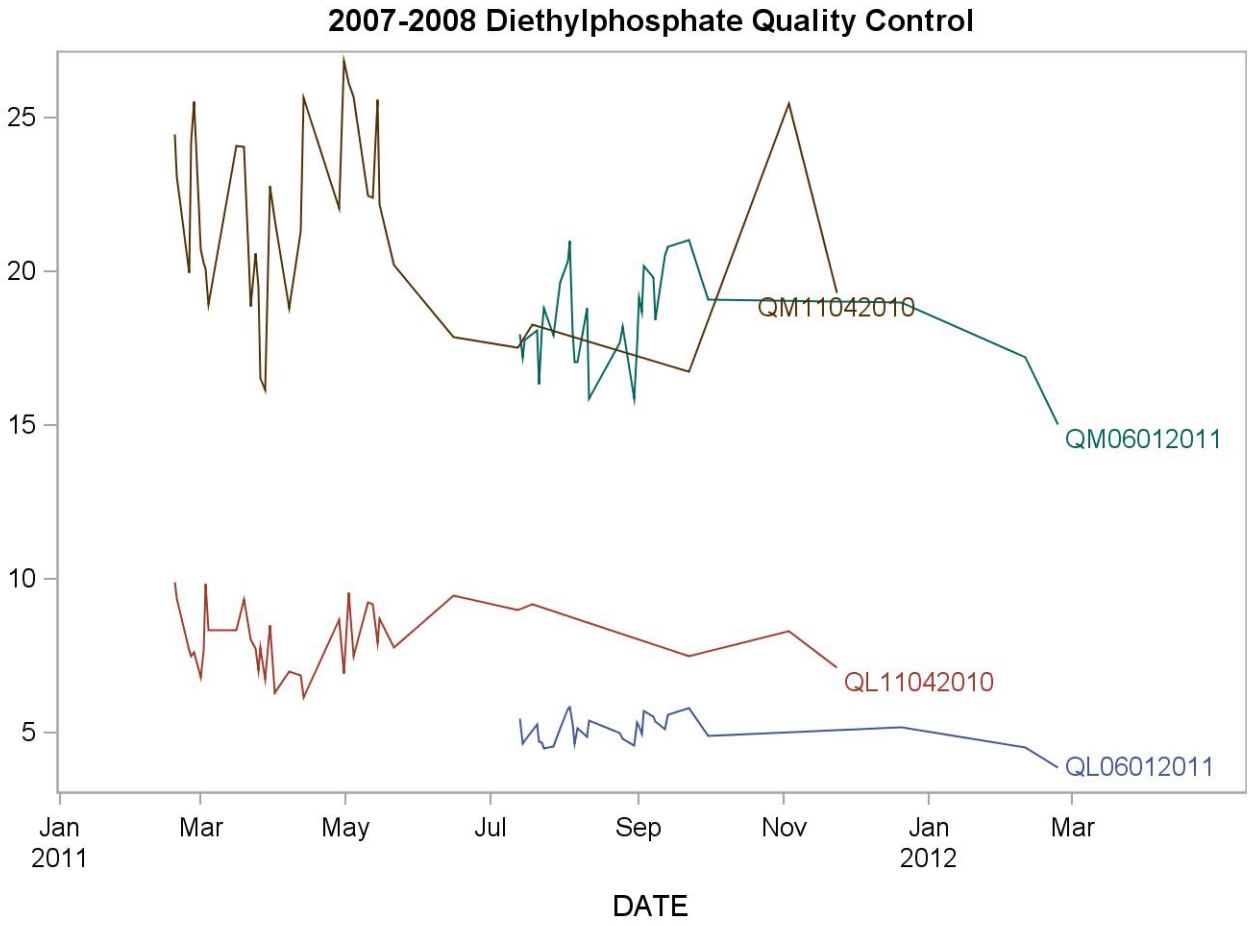
Summary Statistics for Diethyldithiophosphate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL11042010	36	18FEB11	23NOV11	4.318	0.799	18.5
QM11042010	36	18FEB11	23NOV11	13.768	1.233	9.0
QL06012011	41	13JUL11	24FEB12	3.643	0.631	17.3
QM06012011	41	13JUL11	24FEB12	12.457	1.183	9.5



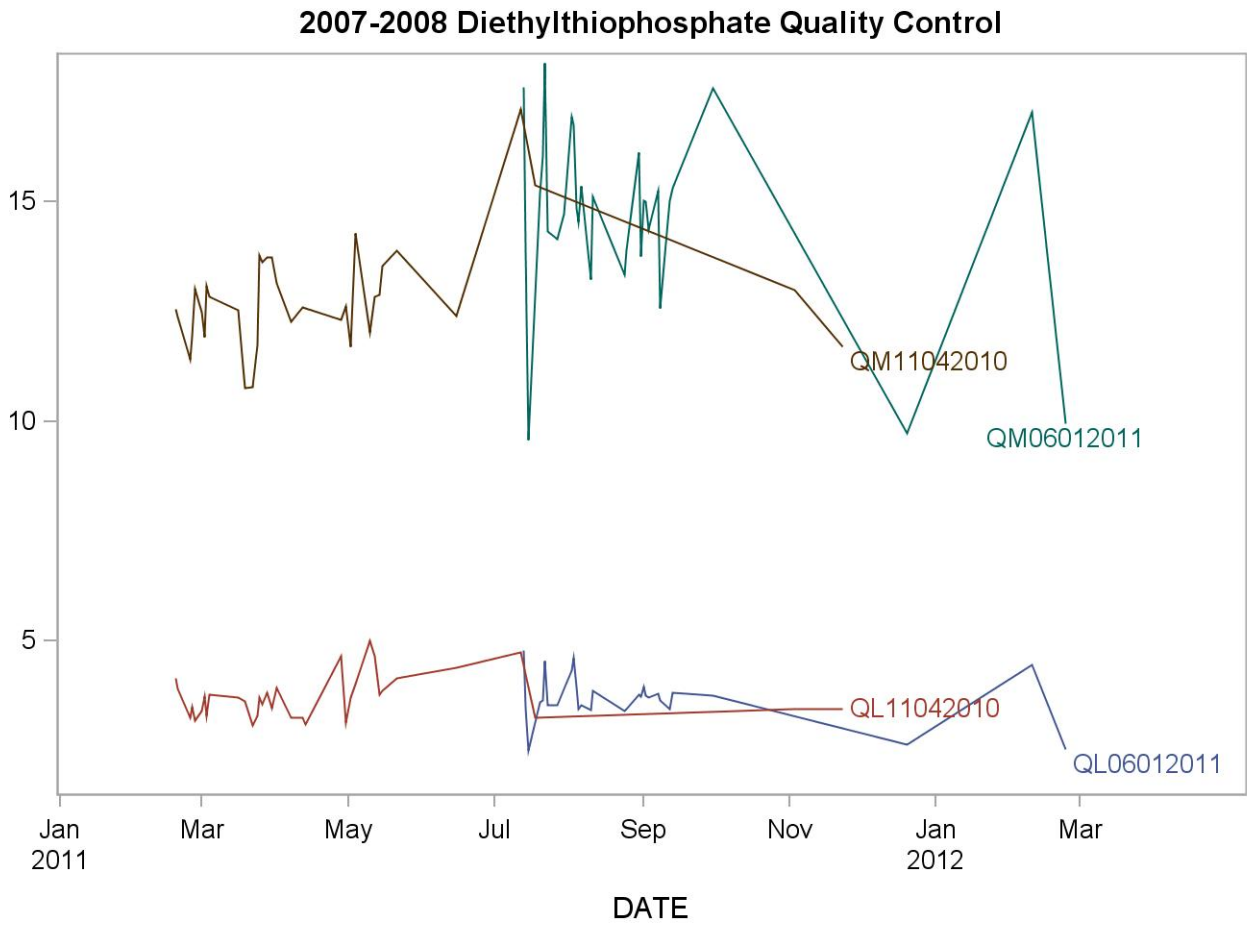
Summary Statistics for Diethylphosphate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL11042010	37	18FEB11	23NOV11	8.089	1.029	12.7
QM11042010	37	18FEB11	23NOV11	21.611	3.011	13.9
QL06012011	42	13JUL11	24FEB12	5.066	0.427	8.4
QM06012011	42	13JUL11	24FEB12	18.355	1.524	8.3



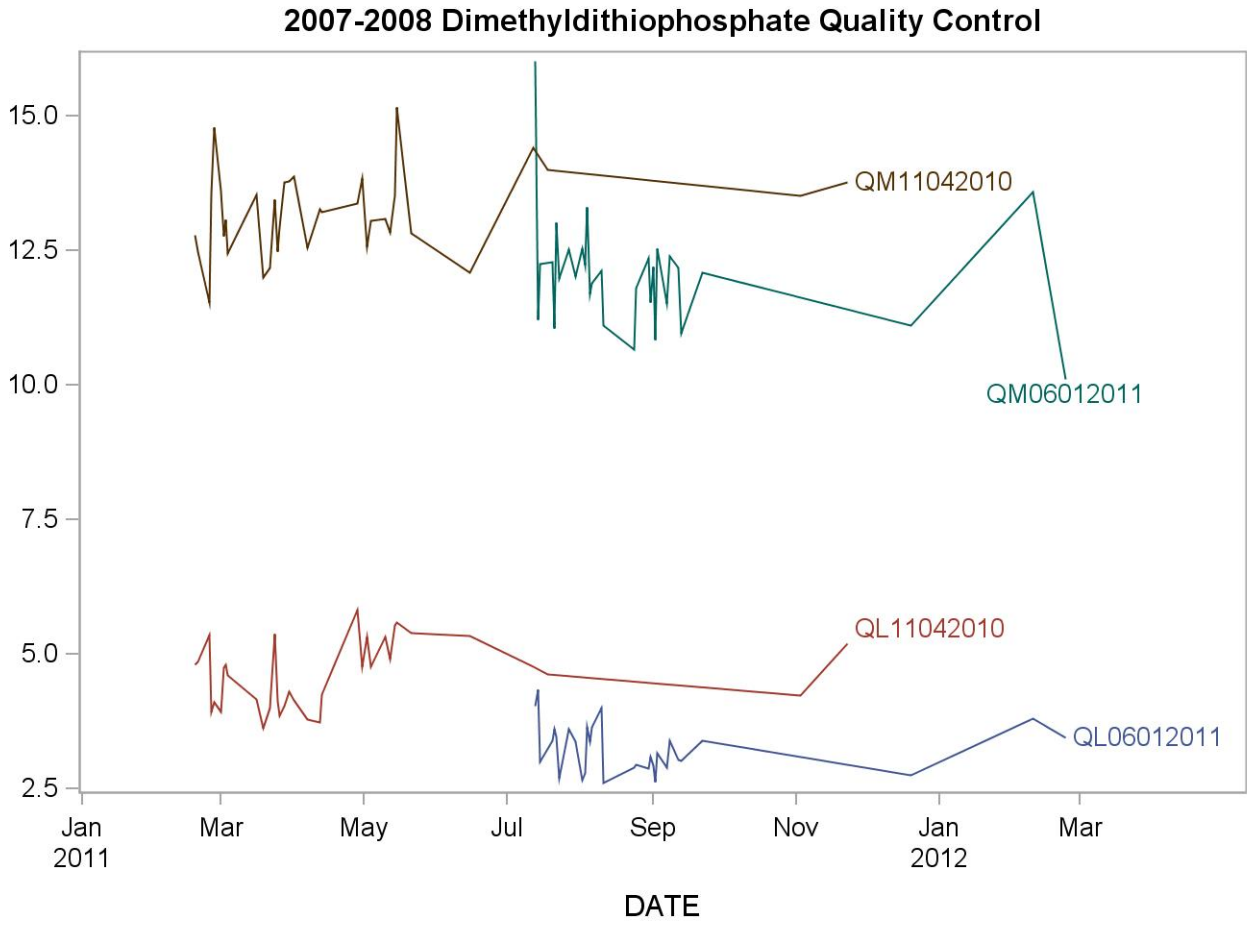
Summary Statistics for Diethylthiophosphate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL11042010	36	18FEB11	23NOV11	3.747	0.506	13.5
QM11042010	36	18FEB11	23NOV11	12.778	1.214	9.5
QL06012011	41	13JUL11	24FEB12	3.753	0.518	13.8
QM06012011	41	13JUL11	24FEB12	14.882	2.041	13.7



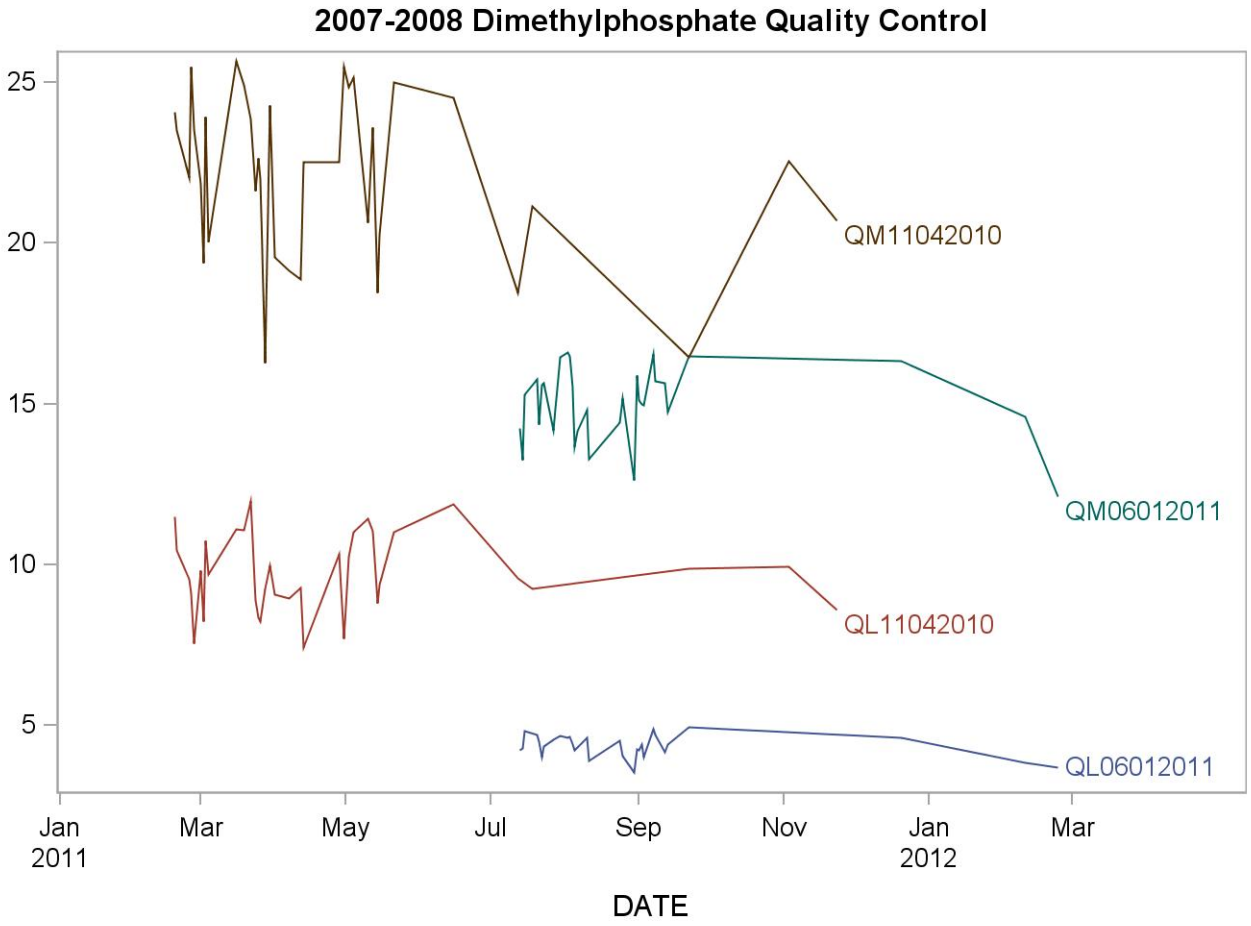
Summary Statistics for Dimethyldithiophosphate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL11042010	36	18FEB11	23NOV11	4.637	0.614	13.2
QM11042010	36	18FEB11	23NOV11	13.163	0.796	6.0
QL06012011	42	13JUL11	24FEB12	3.239	0.426	13.2
QM06012011	42	13JUL11	24FEB12	12.163	1.091	9.0



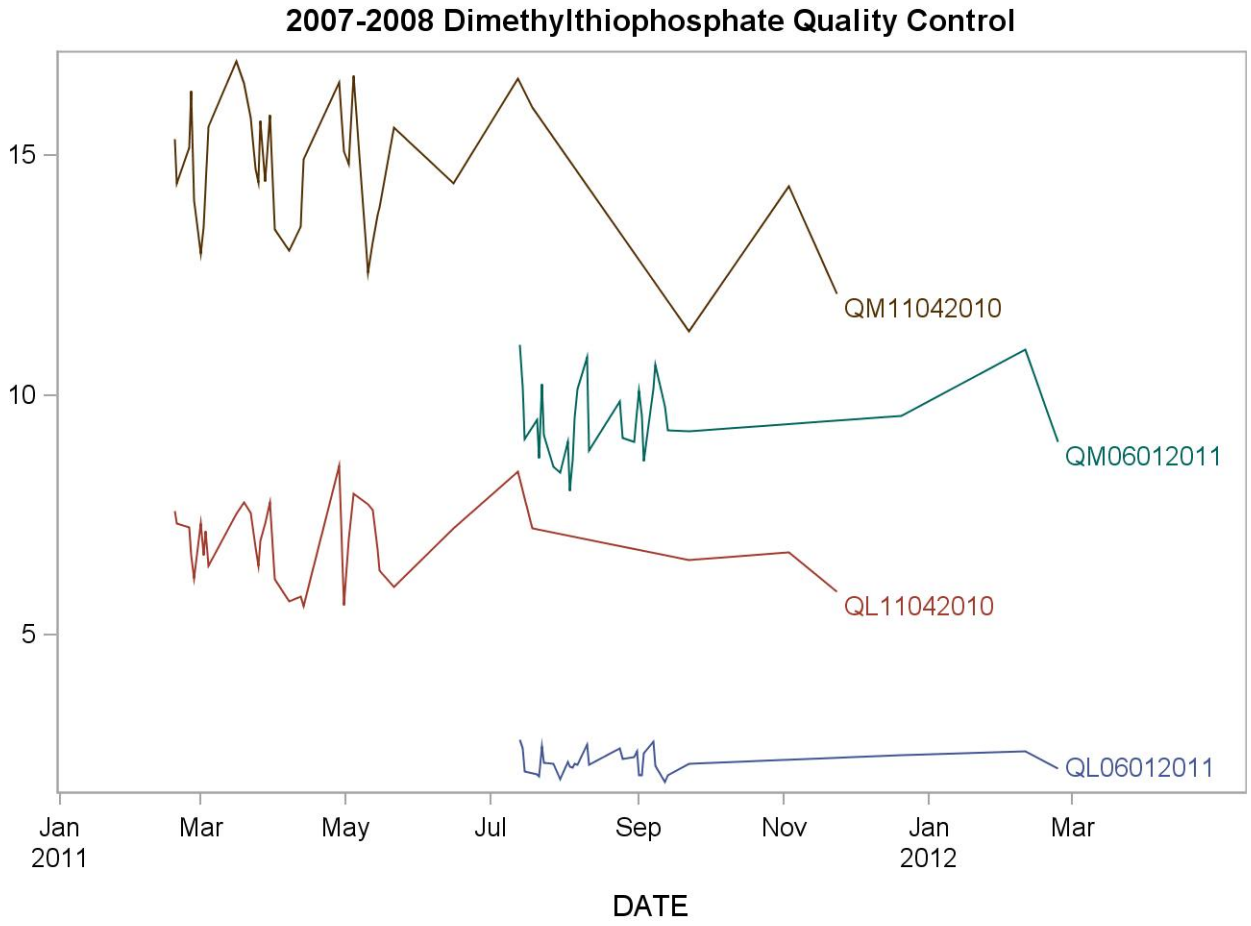
Summary Statistics for Dimethylphosphate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL11042010	37	18FEB11	23NOV11	9.761	1.218	12.5
QM11042010	37	18FEB11	23NOV11	22.129	2.572	11.6
QL06012011	42	13JUL11	24FEB12	4.370	0.334	7.7
QM06012011	42	13JUL11	24FEB12	15.019	1.090	7.3



Summary Statistics for Dimethylthiophosphate

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL11042010	37	18FEB11	23NOV11	6.972	0.788	11.3
QM11042010	37	18FEB11	23NOV11	14.686	1.367	9.3
QL06012011	42	13JUL11	24FEB12	2.355	0.240	10.2
QM06012011	42	13JUL11	24FEB12	9.629	0.779	8.1



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