Laboratory Procedure Manual

Analyte: **Dialkyl Phosphate Metabolites of Organophosphorus Pesticides**

Matrix: **Urine**

Method: **Gas Chromatography-Tandem Mass Spectrometry and Isotope Dilution Quantification**

Method No.:

Revised:

as performed by: *Toxicology Branch*
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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
L26OPD_C	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. Summary of Test Principle and Clinical Relevance

    Urinary dialkylphosphate (DAP) metabolites have been used to estimate human exposure to organophosphorus pesticides. A method for quantifying the six DAP urinary metabolites of at least 28 organophosphorus pesticides using lyophilization and chemical derivatization followed by analysis with isotope-dilution gas chromatography–tandem mass spectrometry (GC–MS/MS) was developed. Urine samples were spiked with stable isotope analogues of the DAPs and the water was removed from the samples using a lyophilizer. The dried residue was dissolved in acetonitrile and diethyl ether, and the DAPs were chemically derivatized to their respective chloropropyl phosphate esters. The chloropropyl phosphate esters were concentrated and analyzed with GC–MS/MS. The limits of detection of the method were in the low mg/l (parts per billion) to mid pg/ml range (parts per trillion), with coefficients of variation of 7–14%. The use of stable isotope analogues as internal standards for each of these metabolites allows for sample-specific adjustment for recovery and thus permits a high degree of accuracy and precision. Use of this method with approximately 1100 urine samples collected from pregnant women and children indicate that the low limits of detection allow this method to be used in general population studies (J Exp Anal Environ Epidem. 2004:14; 249–259.)

    Pesticides are used extensively in both agricultural and residential applications worldwide. These current-use pesticides include organophosphorus (OP), carbamates, triazines, chloroacetanilides, and synthetic pyrethroids. Among these, OP pesticides are the most widely used in agriculture and pest-control applications that contribute about 8% to the total amount of pesticides used in the United States (EPA, 1999). Because of the widespread use of OP pesticides, exposure of the general population to these chemicals is almost impossible to avoid. To properly evaluate risks associated with these exposures and to document exposure trends over time, viable techniques for evaluating exposure are required. One technique that has been widely accepted and used in the scientific community is the measurement of biomarkers of exposure (Needham et al., 1995; Anwar, 1997; Azaroff, 1999; Wessels et al., 2003). However, to produce meaningful data, we must have analytical methods that are accurate, precise, and are adequately sensitive to detect biomarker concentrations resulting form incidental human exposures. Since the 1970s, urinary dialkylphosphate (DAP) metabolites of OP pesticides have been quantified in human urine as a dosimeter for exposure and bodily adsorption of OP pesticides (Bradway and Shafik, 1977; Reid and Watts, 1981; Bardarov and Mitewa, 1989; Fenske and Leffingwell, 1989; Weisskopf and Seiber, 1989; Drevenkar et al., 1991; Aprea et al., 1994, 1996a, b, 1999, 2000; Davies and Peterson, 1997; Loewenherz et al., 1997; Moate et al., 1999; Simcox et al., 1999. Fenske et al., 2000; Hardt and Angerer, 2000; Heudorf and Angerer, 2001; Lu et al., 2001; Ogobline et al., 2001a, b; Bravo et al., 2002; CDC, 2002; Cocker et al., 2002; Garfitt et al., 2002a, b). The six common DAP metabolites measured are dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), dimethyldithio-phosphate (DMDTP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP). Table 1 shows a list of organophosphorus pesticides approved by the U.S.

    Environmental Protection Agency (US EPA) has approved these pesticides for use and their corresponding phosphorous-containing urinary metabolites. DAP metabolites do not retain the specific chemical structure of the parent pesticides; therefore, individual pesticides from these metabolites are impossible to identify. However, because these metabolites are common to the majority of OP pesticides (Table1) and laboratory methods are not available for pesticide-specific metabolites for many of these pesticides, the nonspecific DAPs provide valuable information about cumulative exposure to the OP class.

Table 1. A total of 28 EPA-registered organophosphorus pesticides and their potential dialkyl phosphate metabolites

Pesticide	DMP	DMTP	DMDTP	DEP	DETP	DEDTP
Azinphos methyl								
Chlorethoxyphos							
Chlorpyrifos							
Chlorpyrifos methyl							
Coumaphos							
Dichlorvos (DDVP)						
Diazinon							
Dicrotophos						
Dimethoate								
Disulfoton								
Ethion								
Fenitrothion						
Fenthion						
Isazaphos-methyl						
Malathion							
Methidathion							
Methyl parathion						
Naled						
Oxydemeton-methyl						
Parathion							
Phorate								
Phosmet							
Pirimiphos-methyl						
Sulfotepp							
Temephos						
Terbufos								
Tetrachlorviphos						
Trichlorfon						

DMP¼ dimethylphosphate; DEP¼ diethylphosphate; DMTP¼ dimethylthiophosphate; DMDTP¼ dimethyldithiophosphate; DETP¼diethylthiophosphate; DEDTP¼diethyldithiophosphate.

2. Safety Precautions

    A. Reagent Toxicity or Carcinogenicity. Some of the reagents used are toxic. Special care should be taken to avoid inhalation or dermal exposure to the reagents necessary to carry out the procedure.

    B. Radioactive Hazards. None

    C. Mechanical Hazards. There is only minimal mechanical hazard when performing this procedure using standard safety practices. Laboratory workers should avoid any direct contact with the electronics of the mass spectrometer unless all power to the instrument is off. Generally, electronic maintenance and repair should only be performed by qualified technicians.

    D. Protective Equipment. Standard personal protective equipment must be used when performing this procedure. This apparatus includes lab coat, safety glasses, appropriate, durable gloves. A chemical fume hood must be used for this procedure.

    E. Training. Training and experience in the use of a triple quadrupole mass spectrometer must be obtained by anyone using this procedure. Formal training is not necessary; however, personnel should be trained appropriately by an experienced operator of the instrument and are required to read the operation manuals.

    F. Personal Hygiene. Care should be taken in handling any biological specimen. Routine use of gloves and proper hand washing must be practiced.

    G. Disposal of Wastes. Solvents and reagents must always be put to waste in an appropriate container clearly marked for waste products and temporarily stored in a flame resistant cabinet (follow CDC guidelines for Hazardous Chemical Waste Management). Containers, glassware, etc., that come in direct contact with the specimens must be autoclaved or decontaminated with 10% bleach. The glassware must be washed and recycled or disposed of in an appropriate manner.

3. Computerization; Data System Management

    A. Software and Knowledge Requirements. A database has been developed on the EHLS-PC Network using Microsoft ACCESS. This database is used for storage, retrieval, and analysis of data from the pesticide residue analyses. Statistical analyses of data are performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC) or Microsoft EXCEL. Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

    B. Sample Information. Information pertaining to particular specimens is transferred electronically into the database or manually entered. Data that are manually entered include the sample identification number, the notebook number associated with the sample preparation, the sample type, standard number, and any other information not associated with the mass spectral analysis. The analytical information obtained from the sample is electronically transferred via a PC-based instrument interface. The data are then transferred electronically into the database.

    C. Data Maintenance. All sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The database is routinely (at least once weekly) backed up onto a computer hard drive.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

    A. Sample Collection. Urine specimens are collected from subjects in standard urine collection cups. Samples should be refrigerated as soon as possible and transferred to specimen vials within 4 hours of collection. A minimum of 10 ml of urine is collected, and poured into sterile 30 mL Qorpak vials with screw cap tops. The specimens are then labeled, frozen immediately to $-20°C$, and stored on dry ice for shipping. Special care must be taken in packing to protect vials from breaking during shipment. All samples should be stored at $-20°C$ until analysis.

B. Sample Handling. Samples are thawed, aliquoted, and the residual specimen is again stored at –70°C until needed.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

   Not applicable for this procedure.

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

   A. Materials

      (1) All solvents used were analytical grade with purity greater than 98%.

      (2) 1-Chloro-3-iodopropane (CIP)was obtained from Aldrich Chemical Co. (Milwaukee, WI).

      (3) Seven sets of calibration spiking standards in acetonitrile with analyte concentrations ranging from 0.0145 to 1.7 ng/ml were prepared under contract by Battelle Memorial Institute (Bel Air, MD).

      (4) Native standards of DMP and DEP (98% purity) were purchased from AccuStandard Inc (New Haven, CT) and ChemServices (West Chester, PA), respectively. Although the manufacturer did not determine the purity of DMP, Battelle determined it to be 33% pure by using nuclear magnetic resonance analysis. The impurities primarily consisted of the O-monoalkyl-substituted phosphate and inorganic phosphate with none of the other five metabolites.

      (5) DETP (98%) and DEDTP (97%) were both purchased from Aldrich Chemical Co. (Milwaukee, WI).

      (6) Native standards of DMTP (98%) and DMDTP (98%) were purchased from AppliChem (Darm-stadt, Germany). No further purifications were performed for the native analytes, but the purities were used to calculate the final concentration of the native analytes.

      (7) Isotopically labeled internal standards (ISTD) of DMP (dimethyl-d6), DEP (diethyl-d10), DMTP (dimethyl-d6), DMDTP (di-methyl-d6), DETP (diethyl-d10), DEDTP (diethyl-13C4) were custom synthesized by Cambridge Isotope Laboratories (Andover, MA) with isotopic purities greater than 99%.

      (8) Gases used by the instrumentation had a minimum purity of 99.999% and were purchased from Holox (Atlanta, GA).

      (9) Synthetic urine was prepared according to the procedure of Gustafsson and Uzqueda (Gustafsson and Uzqueda, 1978) with bioanalytical Grade I water prepared in-house using a Solutions 2000 water purification system (Solution Consultants, Jasper, GA).

   B. Standard Preparation

      (1) Isotopically Labeled Internal Standard Solution (ISTD): An isotopically labeled ISTD was prepared by weighing approximately 0.5 mg of each isotopically labeled analyte into a 100-ml volumetric flask and dissolving with acetonitrile (HPLC grade, Tedia, Fairfield, OH). The 5-ng/ml solution was divided into 1-ml aliquots and stored at 20°C until used. This solution was used as an ISTD spiked in all unknown samples, quality control (QC) materials, and calibration standards.

      (2) Calibration Standards: Calibration standards were prepared daily by spiking 2.0 ml of "blank" urine with 10 ml of the ISTD solution and with an amount of the appropriate calibration spiking standard solution to produce urinary concentrations ranging from 0.09 to 50 mg/l. The urine samples were prepared for analysis according to the procedure described below.

C. QC Materials

Urine was collected from multiple (430) donors, combined, diluted with water (1:1 v/v) to reduce endogenous levels of the analytes of interest, and mixed overnight at 20°C. After pressure-filtering with a 0.2-mm filter capsule, the urine was divided into three pools. The first pool (QC low pool) was spiked with the native standard stock solution to yield an approximate concentration of 8 mg/l for DEP, DMTP, DMDTP, and DETP; 4 mg/l for DMP; and 0.6 mg/l for DEDTP. The second pool (QC high pool) was spiked with the native standard stock solution to yield an approximate concentration of 20 mg/l for DEP, DMTP, DMDTP, and DETP; 10 mg/l for DMP; and 1.3 mg/l for DEDTP. The third pool was not spiked. After being screened for possible endogenous analytes, it was used, along with synthetic urine, as matrix material for calibration standards and blanks. All QC pools were characterized to determine the mean and the 99th and 95th control limits by a consecutive analysis of at least 20 samples from each QC pool. After establishing the control limits of the pools, individual QC samples contained within each analytical run were evaluated for validity using Westgard multirules (Westgard, 2002).

D. Sample Preparation

All urine, reagents, and standards were brought to room temperature. A 2-ml aliquot of urine was pipetted into a 15-ml tube and spiked with 10 ml of the ISTD solution to give a urinary ISTD concentration of 25 mg/l for each analyte. After the sample was mixed, it was placed in a commercial lyophilizer system (Labconco, Kansas City, MO). The lyophilizer was operated overnight in the program mode without further manual manipulation. For a run of 50 samples, the samples were initially frozen for 4 h at –34°C and atmospheric pressure. After the samples were sufficiently frozen, the vacuum was set to 25.5 mT and the samples remained at –34°C for an additional 4 hours. The samples were then taken to –20°C for 2 h, 0°C for 1 h, and finally 20°C for 1 h. The following day after completion of the lyophilization process, 2 ml of acetonitrile and 2 ml of ethyl ether were added to the residue in each sample tube, then vortex-mixed for about 1 min. The supernatant was then poured into a different 15-ml centrifuge tube to separate it from the undissolved residue. The extraction tubes with the undissolved residue were rinsed with another 1 ml of acetonitrile, vortex-mixed, and combined with the supernatant. The samples were concentrated to approximately 1 ml in about 10 min using a Turbovap LV (Zymark, Hopkinton, MA) at 30°C and 10 psi of nitrogen. The concentrated samples were poured into a 15-ml test tube, which contained a few grains of potassium carbonate. The CIP (50 ml) was added to the samples and mixed. The samples were then placed in a dry bath set at 60°C for 3 h. The supernatant was transferred to a clean tube and evaporated to dryness. The samples were reconstituted using 75 ml of toluene, transferred to autosampler vials, capped, and stored under refrigeration until analyzed.

E. Calibration Verification standards: To ensure the continued accuracy of our method, we independently contracted out the purchase and preparation of standards for this method, in addition to the standards prepared in house. These standards are run periodically to ensure accuracy, linearity, and acceptable calibration. In addition, these standards are analyzed after substantive changes in the analytical system.

F. Equipment/Supplies

(1) TurboVap LV Evaporator,(Zymark Corporation, Framingham, MA)

(2) Microbalance (Sartorius Ultramicro, Westbury, NY)

(3) Isotemp 145D Heating block (Fisher Scientific)

(4) Conical bottom 15-mL screw capped tubes (Pyrex or Kimax, Scientific Services, CDC).

(5) Graduated, conical bottom 15-mL tubes (Pyrex or Kimax, Scientific Services, CDC).

(6) Phenolic screw caps with Teflon seals for both sizes of tubes (Corning, Scientific Services, CDC).

(7) EDP2 Pipettes (Rainin Instrument Co., Woburn, MA).

(8) Vortex Genie (Scientific Industries Inc., Springfield, MA).

(9) Qorpak bottles (Lab Depot, Inc., Cummings, GA).

(10) Micro autosampler vials with aluminum seals (Alltech, Milwaukee, WI)

(11) Lyophilizer (Lab Conco,Kansas City, MO).

G. Instrumentation and Instrumental Analysis

(1) GC Conditions Samples (1 ml) were injected into the gas chromatograph (TraceGC, ThermoQuest, San Jose, CA) by splitless injection using an autosampler (CTC A200s, Carrboro, NC) with an injection purge delay of 60 s. The GC was coupled to a triple quadrupole mass spectrometer (FinniganTSQ-7000, ThermoFinnigan, San Jose, CA). A 30-m J & W (Folsom, CA) DB5MS ([5% phenyl]-methyl polysiloxane, 0.25 mmfilm thickness, 0.25 mm i.d.) capillary column was used for separation of the chloropropyl phosphate esters. A guard column (deactivated fused silica column, Restek, Bellefonto, PA) was used to help extend the useful life span of the analytical column. The temperatures of the injector and transfer line were 250°C. The column temperature was initially 80°C for 2 min and was then heated linearly to 250°C at 17°C/min. The final temperature of 250°C was held for 2 min.

(2) Mass Spectrometric Conditions. The chloropropyl phosphate esters were analyzed using multiple reaction monitoring (MRM). All of the precursor ions were the pseudomolecular ($[M_bH]_b$) ions produced by chemical ionization in the positive ion mode. Methane was used as a reagent gas with a pressure of 1500 mT and argon as a collision-induced dissociation gas with a pressure of 2 mT. A full autotune of the mass spectrometer was performed before analysis of every set of samples. MS conditions were as follows: source temperature was 150°C, electron energy was 200 eV, and the potential for the continuous dynode electron multiplier varied depending upon multiplier lifetime. Table 1 summarizes the characteristic precursor/product ion combinations and collision offsets used in measuring each analyte and ISTD with a width mass window of 0.4 amu and a scan rate of 0.03 s. The product ions for Cl precursor ions were selected to maximize specificity, sensitivity, and linear dynamic range. The product ions for Cl precursor ions were used only for confirmation purposes and added to the selectivity of the analysis.

Table 2. Multiple reaction monitoring analysis of dialkylphosphate metabolites of organophosphorus pesticides.

Metabolite I Quantification Precursor Ion	CO (eV)	^{35}Cl Quantification Pair Product Ion		^{37}Cl Confirmation Precursor Ion	Confirmation Pair Product Ion	Possible Fragment (MH-R)+ for the Quantification Ion
DMP	12	203	127	205	127	$(MH-C_3H_5\ Cl)^+$
DMP (dimethyl-d_6)	12	209	133	211	133	$(MH-C_3H_5\ Cl)^+$
DEP	13	231	127	233	127	$(MH-C_5H_9\ Cl)^+$
DEP (diethyl-d_{10})	13	241	133	243	133	$(MH-C_5H_5D_4Cl)^+$
DMTP	13	219	143	221	143	$(MH-C_3H_5\ Cl)^+$
DMTP (dimethyl-d^6)	13	225	149	227	149	$(MH-C_3H_5\ Cl)^+$
DMDTP	10	235	125	237	125	$(MH-C_3H_7ClS)^+$
DMDTP (dimethyl-d_6)	10	241	131	243	131	$(MH-C_3H_7ClS)^+$
DETP	12	247	191	249	193	$(MH-C_4H_8)^+$
DETP (diethyl-d_{10})	12	257	193	259	195	$(MH-C_4H_8)^+$
DEDTP	12	263	153	265	153	$(MH-C_4H_7ClS)^+$
DEDTP (diethyl-$^{13}C_4$)	12	267	157	269	157	$(MH-^{12}C_4H_7ClS)^+$

CO¼ collision offset; eV¼ electron volts.

(3) Data Processing and Analysis Peaks were automatically integrated using the Xcalibur[s] software (version 1.3) provided with the MS. The background signal was subtracted, and all data were smoothed (3-point smooth). The analyst checked and corrected any discrepancies in peak selection yielding an accurate integration. Peak areas and other pertinent data were exported into a Microsoft EXCEL[s] file and loaded into a Microsoft ACCESS[s] database for permanent storage. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

(4) Quantification Calibration plots were constructed for each analytical run, with seven analyte concentrations (urinary concentrations ranging from 0.09 to 1.7 mg/l) plotted against the response factors. Response factors were calculated as the area of the native analyte ion divided by the area of the labeled analyte ion. Calibration standard concentrations encompassed the entire linear range of the analysis. The lowest standard concentrations were at or below the limits of detection (LOD) to ensure linearity and accuracy at the low concentration end. A linear regression analysis of the calibration plot provided a slope and intercept from which unknown sample concentrations could be determined. Except for DMP, the y-intercepts were statistically indistinguishable from zero.

H. Method Validation

(1) Daily Operating Protocol. A typical sample batch included one reagent blank, 36 unknown samples, one low QC, one high QC, and seven standards. Before daily instrumental analysis, a known standard was analyzed to confirm acceptable chromatographic resolution and mass spectral sensitivity. We required that the reagent blank be free of analytes and QC samples pass the Westgard multirules before we approved as valid a batch of sample data.

(2) Limits of Detection. The LOD was calculated for each analyte as $3s_0$, where s_0 is the standard deviation at zero concentration. S_0 was estimated as the y-intercept of a linear regression analysis of a plot of the standard deviation (in units of concentration) versus the concentrations of the four lowest standards (Taylor, 1987). The calculated LODs were verified by the analysis of similar concentrations of analytes spiked into urine.

(3) Absolute Analyte Recoveries. The recovery of the method was determined at two concentrations, 10 and 50 mg/l, by spiking six ''blank'' urine samples (2.0 ml) with the appropriate native standard spiking solution and lyophilizing according to the method. Six additional ''blank'' urine samples (unspiked) were lyophilized concurrently. After the drying step, all the extracts were spiked with a known amount of the ISTD solution to correct for instrument variation. This resulted in a more accurate recovery calculation. The samples that were not spiked before preparation were then spiked with the appropriate native standard spiking solution to serve as control samples representative of 100% recovery. Samples were derivatized, evaporated, and reconstituted with toluene. Then, the samples were analyzed. The recovery was calculated by a comparison of the ratio of the native standard and ISTD areas in the recovery samples to those in the control samples.

(4) Relative Recoveries. The relative recoveries of the method were determined by spiking ''blank'' urine samples, at different concentrations and calculating the concentrations using this method. The relative recovery was determined at each of the concentration levels by comparing the measured concentrations with the expected, or spiked, concentrations. A ratio of 1.00 indicated 100% relative recovery.

(5) Accuracy. The absolute accuracy of this method is difficult to determine because no reference materials were available for evaluation. However, we linked the results of this method to our previous method by analyzing, in parallel, a series of QC materials and several unknown samples representing a wide concentration range using both methods.

(6) Selectivity. No objective way exists to determine the degree of selectivity of an analytical method. However, as a matter of consensus, chromatographic separation coupled with MS/ MS is considered the most selective analytical technique available (Persson and Vessman McDowall, 1998). The use of a chlorinated derivatizing agent allowed us to analyze ^{35}Cl and ^{37}Cl fragments

for greater selectivity. The use of the isotope-dilution technique, which provides a chromato-graphic reference for peak selection, adds another degree of selectivity to the method. Apparent interferences in the form of extra chromatographic peaks or peak shoulders were rarely encountered. Unknown interferences typically resulted in an increased background signal.

(7) Precision. The method precision was determined by calculating the coefficient of variation (CV) of repeat measurements of the QC materials at two concentrations (about 8 and 20 mg/l). At least 63 repeat measurements over a 2-month period were used in the calculations.

(8) Human Studies. Urine samples were collected as part of a study conducted by Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) at the University of California at Berkeley (Eskenazi et al., 1999). This study is multifaceted, with one portion focusing on pesticide exposures in Latina women and their children in the Salinas Valley in southern California. Upon collection, samples were frozen within 4 h and were stored at 20ºC before analysis. All protocols were reviewed and approved by a human subjects review committee and complied with all institutional guidelines for the protection of human subjects. Approximately 1100 urine samples from adults and children were analyzed using this method to validate the speed and ruggedness of the analysis. About 5% of the samples tested were QC materials inserted blindly among the study samples. These QC materials were thawed in the field, aliquoted into regular unknown sample vials, and then shipped with the samples on dry ice to CDC.

7. Calibration and Calibration Verification Procedures

A. Mass Spectrometer

The mass spectrometer is calibrated and tuned according to the instructions in the operator's manual. The program can be modified and executed to determine the optimum for each parameter. After the instrument is calibrated with unit resolution and maximum sensitivity, the instrument is prepared for analysis of the pesticide metabolites as described in the Procedure Operating Instructions.

B. Calibration Curve

(1) A seven-point calibration curve is constructed by performing a linear reqression analysis of relative response factor (i.e., area native/area label) versus standard concentration. A full calibration curve is run for each analytical run.

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

(3) The slope and intercept of this curve is determined by linear least squares fit using SAS software.

(4) R-squared values for the curve must be greater than 0.97. 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 it is, the source of this bias should be identified.

(5) Alternatively, a continuing calibration curve can be constructed. The standard curve should be calculated using a minimum of 5 points at each concentration and should be periodically to incorporate the newest data points. Whenever a new combined labeled isotope solution is prepared, the standard curve must be re-established.

C. Calibration Verification (CV)

(1) Calibration verification materials are analyzed, using the same procedure used with the unknown samples, after any substantive change in the method or instrumentation to verify the continuation of integrity of the calibration curve slope, linearity, and dynamic range. For example, CV materials should be analyzed before samples are analyzed if the instrument has been used in another method, after a new column is installed, after preventative maintenance is performed, or after the source of the mass spectrometer is cleaned.

(2) Calibration verification should be performed a minimum of once every 6 months while the method is in use.

(3) Three CV materials (described in standard preparation section) are analyzed per calibration verification run. The slope, intercept, and linearity of a regression analysis of the CV materials should not differ significantly from that of the calibration curve.

(4) If there is a significant difference, analyses using this method should be halted until corrective actions are taken and CV materials are consistent with the calibration curve.

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

8.   Procedure Operating Instructions; Calculations; Interpretation of Results

A.   Analytical Runs

Unknown urine samples (usually 20), a blank urine, 2 QC samples, and 2 blank urines used for standards are allowed to thaw and reach room temperature.

B.   Procedure

Two mL of urine are aliquoted into 20-mL flat-bottomed vials. Twenty-five µL of combined labeled ISTD are added to the urine and briefly vortexed. The samples are lyophilized overnight using the following derivatization procedure:

The urine residue is reconstituted in 1 mL of acetonitrile. 50 µL of chloroiodopropane are added and mixed. The samples are allowed to sit at room temperature for 1 hour. The vials are mixed and the supernatants are transferred to clean tubes containing a few granules of potassium carbonate. The samples are incubated at 80°C for 2 hours. The reaction mixture is evaporated to 100 µL in a TurboVap at 50°C with 15 psi head pressure. The concentrated samples are then transferred to labeled micro autosampler vials. The vials are stored at –20°C until analysis.

C.   Analysis

(1) Preliminary system setup and performance check

(a) Install a clean CI ion volume.

(b) Open reagent gas valve. Check the methane peaks in the 10-50 m/z range. The intensity should be greater than 50×106 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) In the log book, periodically record the manifold and CI source pressures.

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

(2) To check the performance of the system, a mass spectrometer check standard is run first under the same conditions as samples. The S/N and area counts for standard and labeled standard are assessed. If the minimum S/N and area counts are met, the GC 3400-TSQ 7000 system is ready to start a run.

(3) Final setup and operation

(a) In ANALYSIS view, the filename should correspond to the date and sample number in the format YYMMDD##. The 'samp' field has to be the sample notebook number and the 'oper' field should correspond to the operator initials. Before pressing START or LSTART (for a list) to run OPNRBCNM, make sure the acquisition time (SCN/TIM) is XX min. Press LSTART to start the analysis.

(b) In the GC system, place the sample vials in the autosampler. Be sure that the needle rinse vial contains a sufficient quantity of hexane to rinse the needle after each injection. Check the needle plunger operation by injecting from a blank solvent vial.

(4) System shutdown

      (a)   To turn off the mass spectrometer, turn off ionization gas, filament, CID gas and multiplier.

      (b)   Type .CANCEL to cancel the ICL procedure (as many times as needed). Type .Q3MS, then .SW 28 (to collect Q3 mass spectra). Finally, type .Q1MS, and next .SW 28 to collect Q1 mass spectra and scan a window of width 5 about m/z = 28.

(5)   Processing of data

      (a)   Quantification: All raw data files are quantified using the XCalibur quantitation software of the TSQ7000. Automatic processing is performed after each analysis. After the sample batch is processed, peaks can be manually evaluated for correct peak detection and baseline selection in the QUAN Browser. Data files are exported to EXCEL using the long export format.

      (b)   Transfer of data: The file can then be transferred to a floppy disk or transferred via electronic mail.

(6)   Importation of data into database

The file is read into the ACCESS database using specially constructed import coding. To import the data, a password is required.

(7)   Statistical analysis and interpretation of data

Data can be exported from the database to an EXCEL file and imported into SAS. SAS programs for standard curve generation, QC analysis, blank analysis, limit of detection determination, unknown calculations, data distribution, etc. have been created and may be executed in SAS when this information is needed.

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. However, urine samples with analytical data values exceeding the highest reportable limit may be diluted and reanalyzed so that the result will be in the reportable range.

A.   Linearity Limits: Analytical standards were linear for all analytes through the range of concentrations evaluated. The linear range for all analytes was LOD to 50 ppb. Urine samples, whose concentrations exceed these ranges, must be re-sampled and re-analyzed using a smaller aliquot.

B.   Analytical Sensitivity: The detection limits for all analytes was calculated as $3S_0$, where S_0 is the standard deviation at zero concentration, and is determined by linear regression analysis of the absolute standard deviation vs. concentration. The detection limits vary based upon the current operating precision and the cleanliness of the analytical system.

C.   Accuracy: The accuracy of this method was determined by enriching urine samples with known concentrations of the pesticide residues and comparing the calculated and expected concentrations. The accuracy was consistent across the entire linear range. The accuracy can be expressed as the slope of a linear regression analysis of the expected value versus the calculated value. A slope of 1.0 indicates the results are identical. Another way of expressing a method's accuracy is as a percentage of the expected value.

D.   Precision: The precision of this method is reflected in the variance of quality control samples over time. The major contributor to the overall CV is the variation between runs. Variation and the total coefficient of variation (CV) were determined from multiple analyses of quality control materials throughout the study. The total CVs of the method vary from study to study and are dependent upon instrument, operators, and sample preparation analyst.

E.   Analytical Specificity: This is a highly specific method that requires 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) the ion ratios of the two product ions be within a predetermined range.

10. Quality Control (QC) Procedures

    A.   Quality Control Material. The control materials used for each unknown run were urine pools enriched with known amounts of pesticide residues.

    B.   Collection of Urine for QC Pools. Two quality control pools were prepared and are used in each run of unknown samples. The urine for each pool was collected from volunteers and was screened to ensure that the endogenous levels of pesticide residues were low or non-detectable. The urine samples were combined and homogenized to form a base pool.

    C.   Urine Enrichment. The base pool was split equally into three smaller urine pools. One pool was reserved for blank and standard analyses (see sample preparation section). Another of the pools was enriched with an appropriate amount of the stock solution of each pesticide residue to yield an approximate concentration of 8 µg/L (low pool). The final pool was enriched with an appropriate amount of each pesticide stock solution to yield an approximate concentration of 20 µg/L (high pool).

    D.   Filtration and Dispensing. Each pool was clean filtered to 0.2 µ. The urine was dispensed in 12-mL aliquots into 25-mL sterile screw-capped vials. The vials labeled appropriately and the QC materials were then frozen at –20°C until needed.

    E.   Characterization of QC Materials. The QC pools (including the unspiked pool) were characterized by 20 consecutive runs of each QC material. Using the data from these runs, the mean and upper and lower 99th and 95th confidence intervals were established. The confidence intervals were determined and adjusted according to the number of each QC material analyzed in each run.

    F.   Use of Quality Control Samples. During each analytical run, blank urine and two QC materials are analyzed. The QC materials can be any combination of the high and low pools.

    G.   Final Evaluation of Quality Control Results. QC material is evaluated using standard Westgard criteria.

    The quality control rules are as follows:

    (1)   If both QC results are within the 2s limits, then accept the run.

    (2)   If one of two QC results is outside the 2s limits, then apply the rules below and reject the run if any condition is met.

        (a)   13s – Either QC is outside of a 3s limit.

        (b)   22s – Both QCs are outside of 2s limit on the same side of the mean.

        (c)   R4s sequential – Both QCs are outside of 2s limit on opposite sides of the mean.

        (d)   10x sequential – The previous 9 QC results (for the previous 9 runs) were on the same side of the mean.

    No data from runs considered out-of-control will be reported. Repeat out-of-control runs if residual sample is available.

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

    If the calibration or QC systems fail, all operations are suspended until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for example failure of the mass spectrometer or a pipetting error, the problem is immediately corrected. Otherwise, fresh reagents are prepared and the mass spectrometer system is cleaned. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure) are re-analyzed. After calibration or quality control has been re-established, analytical runs may be resumed.

12. Limitations of Method; Interfering Substances and Conditions

    This method is an isotope dilution mass spectrometry method, widely regarded as the definitive method for

the measurement of organic toxicants in human body fluids. Using low resolution tandem mass spectrometry eliminates 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 the results for that analyte are not reportable.

13. Reference Ranges (Normal Values)

Reference values) were determined from a small, nonrepresentative subset of NHANES III (1988–1994) and were determined from 703 individuals (adults and children) participating in NHANES 1999. These studies were performed to provide reference values in the human population to determine length or severity of an exposure incidence.

14. Critical Call Results (Panic Values)

It is unlikely that any result would be a "critical call", which would only be observed in poisonings.

15. Specimen Storage and Handling During Testing

Urine samples may be stored overnight in refrigeration to expedite thawing prior to aliquoting the sample. The urine extracts are stored in autosampler vials in a $-20°C$ freezer after analysis. Stability studies suggest that the extracts remain stable at room temperature for up to 5 days.

16. Alternate Methods for Performing Test or 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, sample vials may be placed in a refrigerator for storage. The extract samples can be stored for as long as three weeks. If long-term interruption is anticipated, store samples between $-80°C$ and $-20°C$.

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

Once the validity of the data has been established by the QC/QA system outlined above and has been verified by a DLS statistician, one hardcopy and one electronic copy of the data will be generated. These data, a cover letter, and a table of method specifications and reference range values will be routed through the appropriate channels for approval (i.e., supervisor, branch chief, division director). Once approved at the division level, they will be sent to the contact person who requested the analyses.

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

Standard record keeping systems (i.e. notebooks, sample logs, data files, creatinine logs, demographic logs) should be employed to keep track of all specimens. Specimens will only be transferred or referred to CLIA-certified laboratories.

19. Summary Statistics and Graphs

   A.   Dimethylphosphate
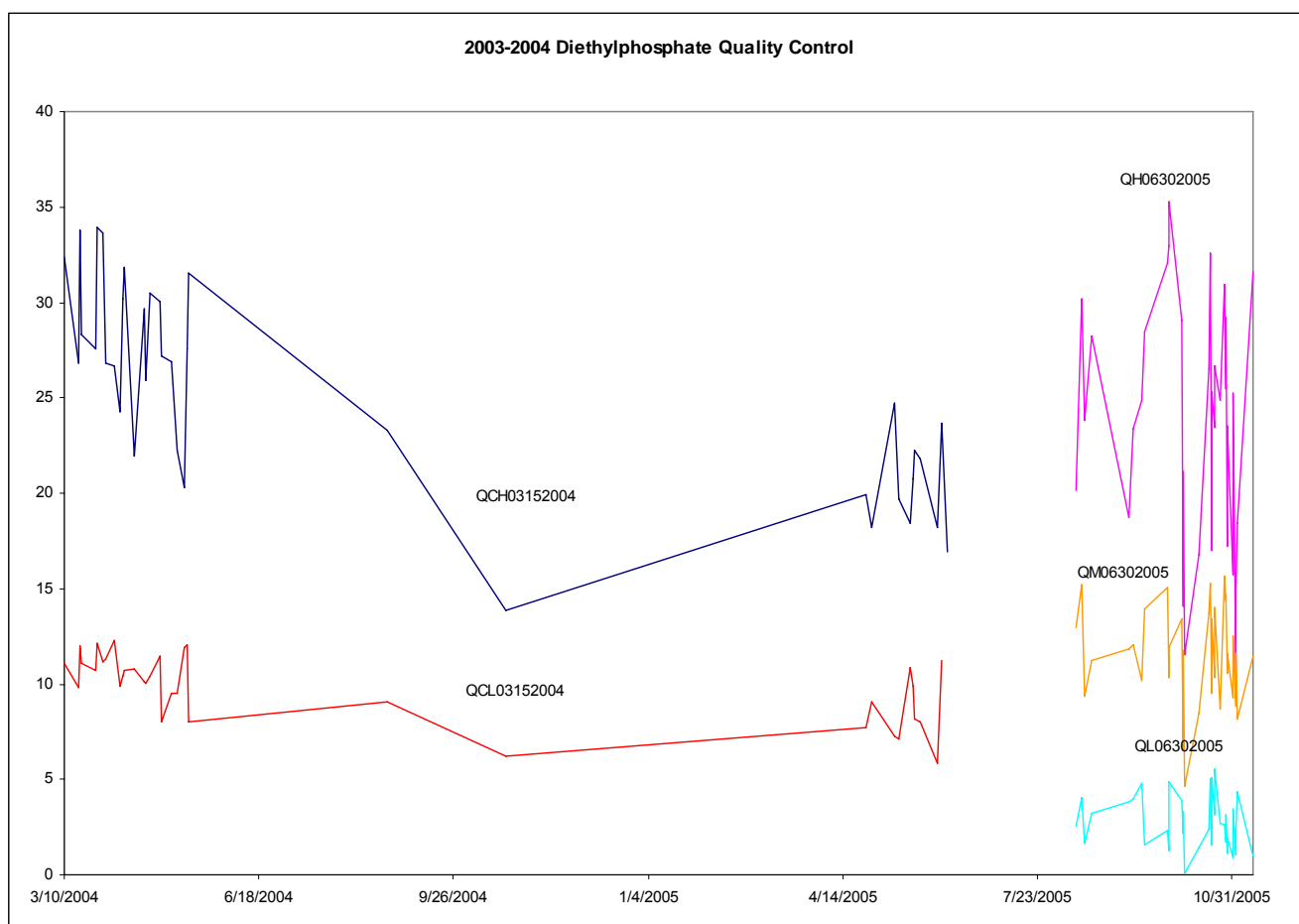
Summary Statistics for Dimethylphosphate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL03152004	36	3/10/2004	6/7/2005	5.647	1.461	25.9
QCH03152004	36	3/10/2004	6/7/2005	11.290	2.028	18.0
QL06302005	36	8/12/2005	11/11/2005	1.277	0.600	47.0
QM06302005	36	8/12/2005	11/11/2005	7.500	1.096	14.6
QH06302005	36	8/12/2005	11/11/2005	14.929	2.624	17.6

2003-2004 Dimethylphosphate Quality Control
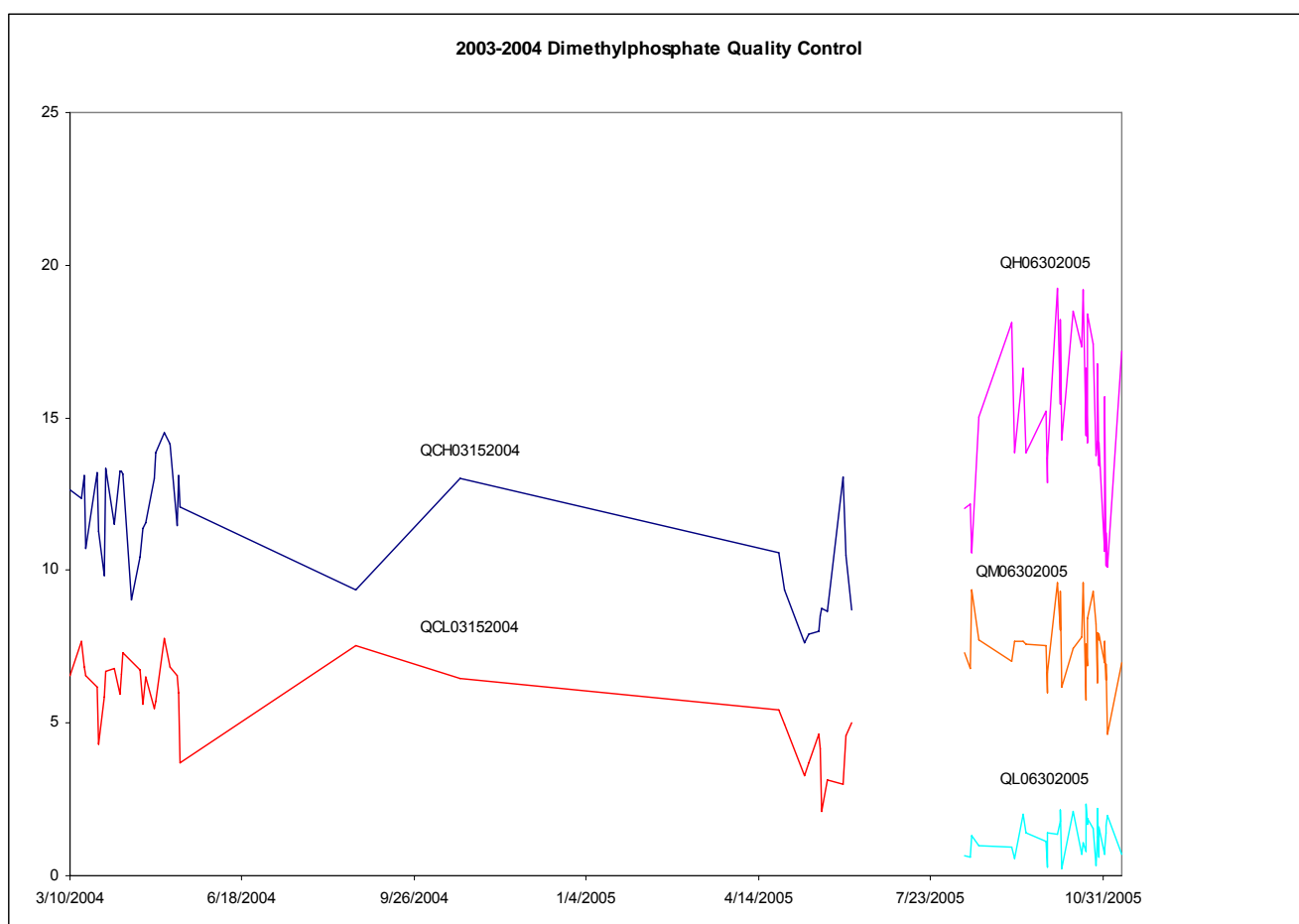
B.   Diethylphosphate

Summary Statistics for Diethylphosphate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL03152004	35	3/10/2004	6/4/2005	9.854	1.741	17.7
QCH03152004	36	3/10/2004	6/7/2005	25.325	5.287	20.9
QL06302005	36	8/12/2005	11/11/2005	2.846	1.422	50.0
QM06302005	36	8/12/2005	11/11/2005	11.573	2.602	22.5
QH06302005	36	8/12/2005	11/11/2005	23.817	6.394	26.8

2003-2004 Diethylphosphate Quality Control

C. Dimethylthiophosphate

Summary Statistics for Dimethylthiophosphate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL03152004	36	3/10/2004	6/7/2005	14.439	1.833	12.7
QCH03152004	36	3/10/2004	6/7/2005	32.073	3.415	10.6
QL06302005	36	8/12/2005	11/11/2005	1.459	0.125	8.6
QM06302005	36	8/12/2005	11/11/2005	7.884	0.459	5.8
QH06302005	36	8/12/2005	11/11/2005	16.526	0.939	5.7

2003-2004 Dimethylthiophosphate Quality Control
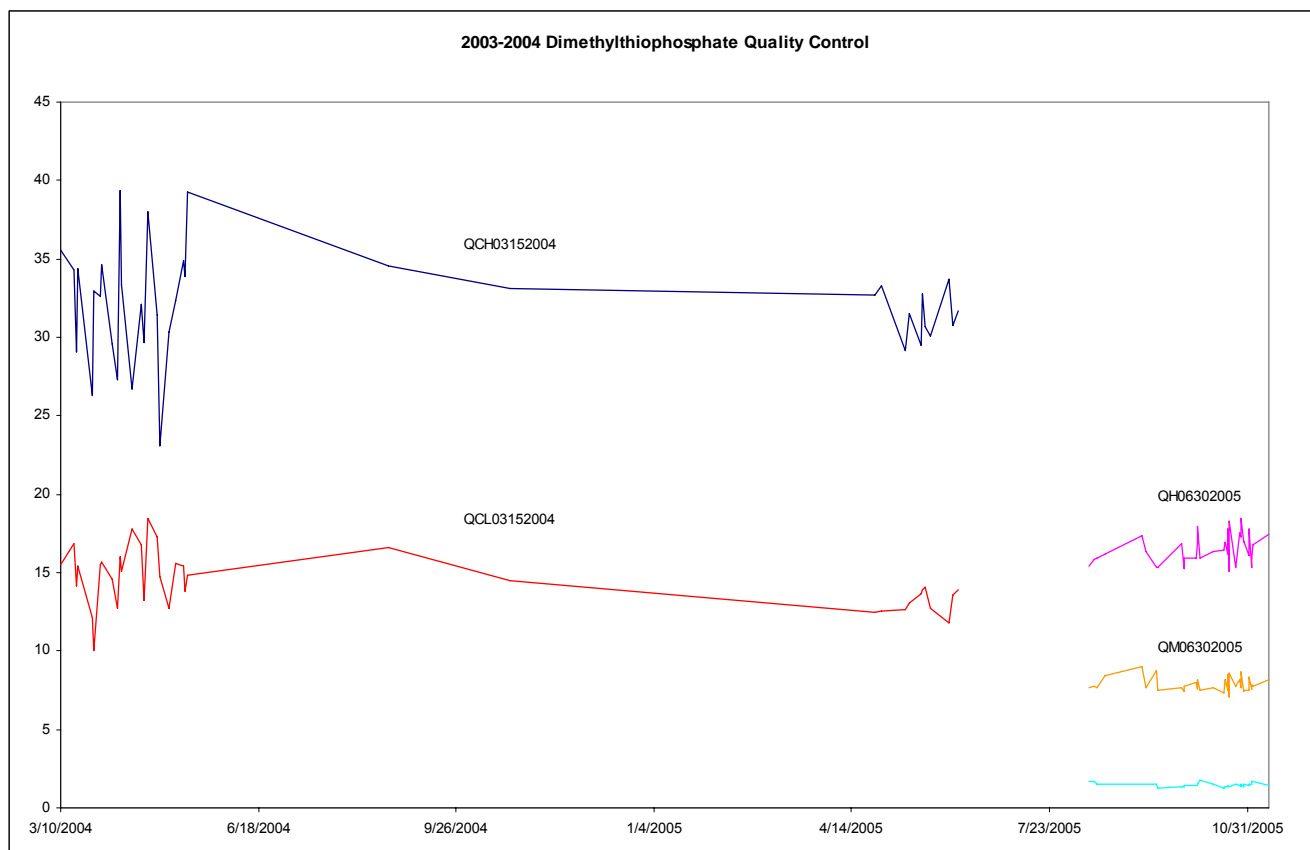
D.   Diethylthiophosphate

Summary Statistics for Diethylthiophosphate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL03152004	34	3/10/2004	6/7/2005	8.787	1.058	12
QCH03152004	36	3/10/2004	6/7/2005	20.682	3.062	14.8
QL06302005	36	8/12/2005	11/11/2005	1.386	0.168	12.1
QM06302005	36	8/12/2005	11/11/2005	7.979	0.458	5.7
QH06302005	36	8/12/2005	11/11/2005	16.792	0.932	5.5

2003-2004 Diethylthiophosphate Quality Control
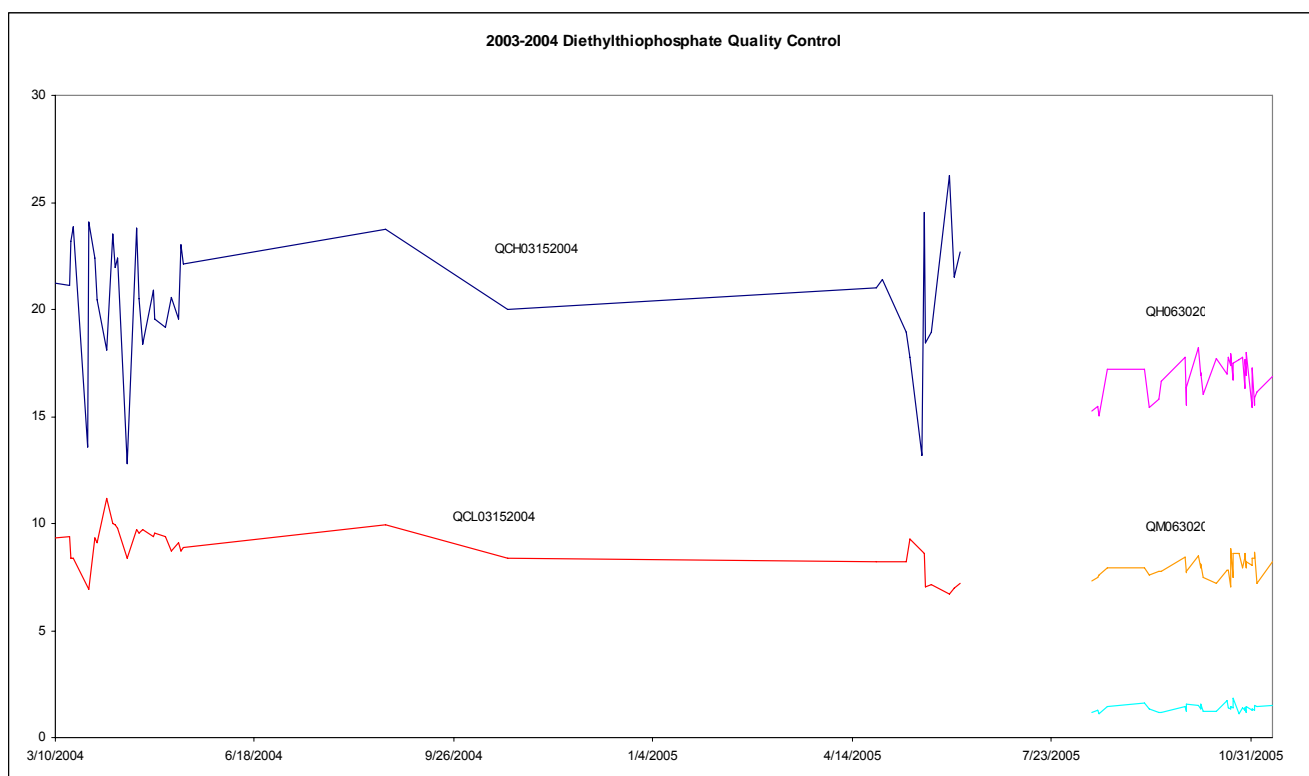
E.   Dimethyldithiophosphate

Summary Statistics for Dimethyldithiophosphate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL03152004	35	3/17/2004	6/7/2005	5.291	1.132	21.4
QCH03152004	35	3/17/2004	6/7/2005	11.990	3.581	29.9
QL06302005	36	8/12/2005	11/11/2005	1.319	0.293	22.2
QM06302005	36	8/12/2005	11/11/2005	6.765	0.613	9.1
QH06302005	36	8/12/2005	11/11/2005	12.266	1.880	15.3

2003-2004 Dimethyldithiophosphate Quality Control
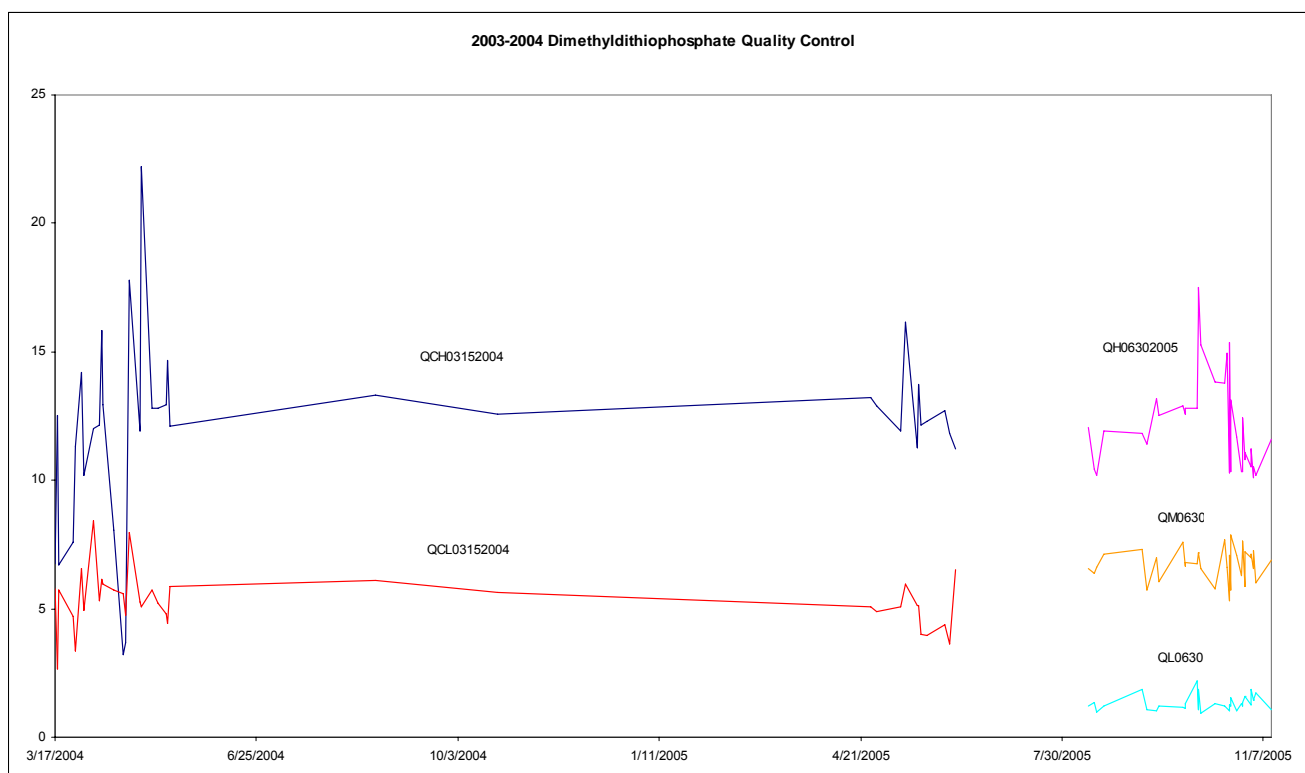
F.   Diethyldithiophosphate

Summary Statistics for Diethyldithiophosphate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QCL03152004	36	3/10/2004	6/7/2005	4.986	0.672	13.5
QCH03152004	36	3/10/2004	6/7/2005	11.497	1.133	9.9
QL06302005	36	8/12/2005	11/11/2005	1.762	0.300	17.0
QM06302005	36	8/12/2005	11/11/2005	8.832	0.833	9.4
QH06302005	36	8/12/2005	11/11/2005	15.396	2.261	14.7

2003-2004 Diethyldithiophosphate Quality Control

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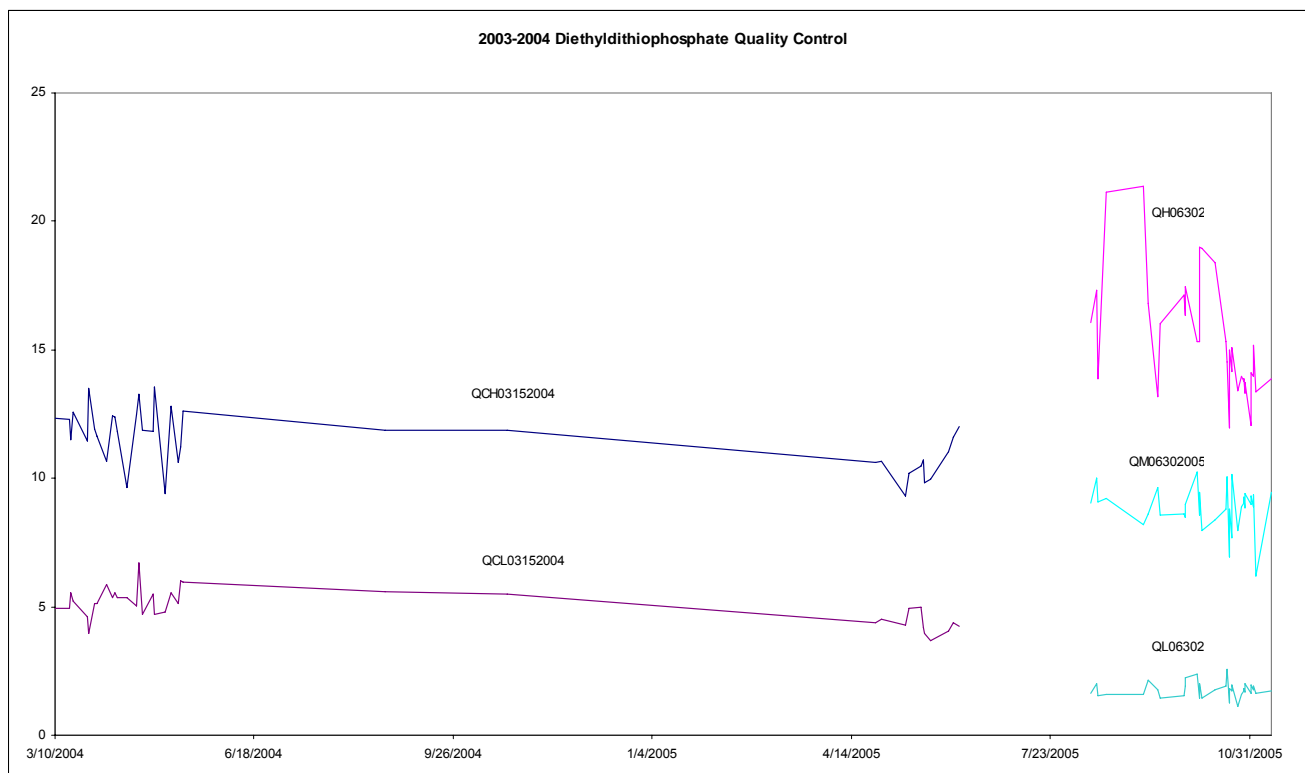
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