Laboratory Procedure Manual

Analytes: **Methamidophos (MMP), o-methoate (Omet), Dimethoate (Dmet), Ethylenethiourea (ETU) and Propylenethiourea (PTU)**

Matrix: Urine

Method: High-performance Liquid Chromatography- Tandem Mass Spectrometry (HPLC-MS/MS)

as performed by: *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.

Acephate (AP), Methamidophos (MMP), Methoates and Thioureas Pesticides in Urine NHANES 2003-2004

Public Release Data Set Information for

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

Data file name	Variable name	SAS Label (and SI units)
L26UPP_C	URXAPE	acephate (ug/L)
	URXETU	ethylenethiourea (ng/mL)
	URXMMI	methamidophos (ng/mL)
	URXMTO	dimethoate (ng/mL)
	URXOMO	o-methoate (ng/mL)
	URXPTU	propylenethiourea (ng/mL)
	URXUCR	Creatinine, urine (mg/mL)

; ;

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The general sample preparation included lyophilization of the urine samples followed by extraction with dichloromethane. The analytical separation was performed by high-performance liquid chromatography (HPLC), and detection by a triple quadrupole mass spectrometer with and APCI source in positive ion mode using multiple reaction monitoring (MRM) and tandem mass spectrometry (MS/MS) analysis. Two different Thermo-Finnigan triple quadrupole mass spectrometers, a TSQ 7000 and a TSQ Quantum Ultra, were used.

Acephate (AP), methamidophos (MMP), o-methoate (Omet) and dimethoate (Dmet) are organophosphorus pesticides (OPs) pesticides. OP pesticides are the most commonly used insecticides in agriculture and the domestic field.[1, 2]

The most widely used fungicides in agriculture are the alkali and metal salts of the alkelenebis-(dithiocarbamate) acids. Ethylenethiourea (ETU) represents the main degradation product of the ethylene bisdithiocarbamates and propylenethiourea (PTU) is the main degradation product of the propylene bisdithiocarbamates[3]. 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.

2. SAFETY PRECAUTIONS

A. Reagent Toxicity or Carcinogenicity: The reagents used can be both toxic and carcinogenic. Special care should be taken to avoid inhalation or dermal exposure to the acids and solvents necessary to carry out the procedure.

B. Radioactive Hazards: None

C. Microbiological Hazards: The possibility of being exposed to various microbiological hazards exists since human urine is the matrix in which the pesticide metabolites are found. Measures should be taken to avoid any direct contact with the specimen. A Hepatitis B vaccination series is usually recommended for health care and laboratory workers who are exposed to human fluids and tissues.

D. Mechanical Hazards: There is minimal hazard when carrying out this procedure.

E. Protective Equipment: Lab coat, safety glasses, durable gloves, fume hood, face mask (optional).

F. Training: Training and experience in the use of a triple quadrupole mass spectrometer should 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.

G. Personal Hygiene: Care should be taken in handling urine samples. Use gloves and wash hands thoroughly after sample handling.

H. Disposal of Wastes: Solvents and reagents should always be put to waste in an appropriate container clearly marked for waste products and temporarily stored under a fume hood. Containers, glassware, etc., that come in direct contact with the specimens should be autoclaved and disposed of in a routine manner. Urine may also be decontaminated with 50% bleach.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. Software and Knowledge Requirements. A database named NPP2 has been developed on the DLS-PC Network using R: Base 4.5+ (Microrim Inc., Redmond, WA). 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). 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 from a UNIX-based system to a PC via an ethernet connection. 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 and onto a network magnetic tape.

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 milliliters of urine is collected, and poured into sterile 14 mL vials with snap cap tops. The specimens are then labeled, stored immediately at: -30 °C, and shipped on dry ice. Special care must be taken in packing to protect vials from breakage during shipment. All samples should be stored at -30 °C until analysis.

B. Sample Handling. Some problems with breakage due to freezing and subsequent thawing have occurred. 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. Chemicals

(1) All solvents used were of analytical grade.

(2) Acetonitrile was purchased from Tedia (Fairfield, OH).

(3) Deionized water was organically and biologically purified with a NANOpure Infinity ultrapure from Barnstead International (Dubuque, IA).

(4) Nitrogen was purchased from Airgas Inc. (Radnor, PA).

(5) The native standards of Acephate (O,S-Dimethyl acetylphosphoramidothioate), Methamidophos (O,S-Dimethyl phosphoramidothioate), Omethoate (O,O-Dimethyl S-methylcarbamoylmethyl phosphorothioate), Dimethoate (O,O-Dimethyl S-methylcarbamoylmethyl phosphorodithioate), Ethylenethiourea (Imidazolidine-2-thione), and Propylenethiourea (Tetrahydropyrimidine-2(1H)-thione) were all purchased from Chemservice (West Chester, PA).

(6) The labeled standards Acephate-d_6 (AP-label), Ethylene thiourea-ethylene-d_4 (ETU-label), and Methamidophos-d_6 (MMP-label) were custom synthesized by Cambridge Isotope Laboratories (Andover, MA).

(7) Methanol was purchased from Tedia (Fairfield, OH, USA). .

(8) Glacial Acetic acid was purchased from J.T. Baker (Phillipsburg, NJ).

(9) Dichloromethane was purchased from Tedia (Fairfield, OH). .

(10) Formic acid was purchased from Fisher Scientific (Pittsburgh, PA),

B. QC Materials

Urine samples were collected from multiple (> 30) donors, combined together, diluted with water (1:1 v/v) to reduce endogenous levels of the analytes of interest, and mixed overnight at 20°C. The urine pool was pressure filtered with a 0.2-µm filter capsule and divided into four pools. The first pool (QCL), the second pool (QCM) and the third pool (QCH) were spiked with the native standard stock solution to yield concentrations of 0.5 µg/mL, 5.0 µg/mL and 15 µg/mL, respectively. The fourth pool was not spiked. After being screened for possible endogenous analytes, the fourth pool was used as matrix material for calibration standards and blanks.

C. Standard and Internal Standard Preparation

Individual stock solutions of the native acephate (AP), methamidophos (MMP), dimethoate (Dmet), o-methoate (Omet), ethylenethiourea (ETU) and propylenethiourea (PTU) were prepared by weighing out 1 mg of each analyte and dissolving in 100 ml of acetonitrile. Stock solutions were stored at −70°C. Ten working standard solutions, each a mixture of an equal concentration of all the analytes, covering a range of 0.01 µg/mL to 8.0µg/mL, were prepared by diluting with acetonitrile appropriate volumes of the individual stock solutions in 100-ml volumetric flasks. The working standard solutions were stored at −20°C. Ten calibration standards were made by adding the working stock solutions to blank urine covering a range from 0.125 to 100 ng/mL. The calibration standards were made freshly before each analytical run.

The labeled internal standard stock solutions of AP-label, ETU-label, and MMP-label were prepared by weighing approximately 1.0 mg of each isotopically labeled analyte into a 100-mL volumetric flask and dissolving with acetonitrile. These were stored −70°C. An internal standard working solution mixture of all the labeled analytes was prepared at 1.0 µg/ml in acetonitrile and stored at −20°C.

D. Equipment

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

(2) Rotator - Glas-Col, RD-230

(3) TurboVap LV evaporator (Zymark, Farmingham, MA)

(4) Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany)
(a) binary pump
(b) degasser
(c) thermostattable column compartment

(5) TSQ Quantum high resolution triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA).

(6) Finnigan TSQ 7000 triple quadrupole mass spectrometer (ThermoQuest, San Jose, CA)
(a) Atmospheric pressure chemical ionization (APCI) application),

(7) SPE cartridge (3 capacity, Waters Corporation, Milford, MA)

(8) 3mlL polyethylene cartridges with 20 µm pore frits (Varian, Walnut Creek, CA);

(9) Polycap 75 TC filter device (0.6/.45 µm pore size, Whatman, NJ)

(10) Lyophilizer (Labconco, Kansas City, MO)

(11) Surveyor HPLC system (ThermoFinnigan, San Jose, CA)

E. Other Materials

(1) Conical bottom 15-mL screw capped tubes (Pyrex or Kimax)

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

(3) Micro/pettor and glass tips (SMI Liquid Handling Products)

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

(5) Round bottom 50-mL screw capped tubes (Pyrex or Kimax)

(6) Micro autosampler vials with resealing septa (Fisher Scientific, Norcross, GA)

(7) Compressed nitrogen, helium, and argon, liquid nitrogen (Holox Ltd, Atlanta, GA)

(8) Prodigy column (5µ, phenyl-3, 100 x 4.6 mm) from Phenomenex (Torrance, CA).

(9) Zorbax SB-C3, narrow-bore 4.6 x 150-mm, 5.0-µm (Agilent Tchnologies, Waldbronn, Germany)

(10) Large green cap glass vials (23 x 85 mm, BGC vials (Kimble Inc.)

(11) Small green cap glass vials (15 x 45 mm, SGC vials) (Kimble Inc.)

(12) 15 mL disposable conical centrifuge tubes (C tubes) (Kimble Inc.)

F. Instrumentation

Analytes were separated on a Prodigy column (5µ, phenyl-3, 100 x 4.6 mm). The column was connected to an Agilent 1100 high performance liquid chromatography (HPLC) system, consisting of an autosampler, a binary pump, a degasser, and a thermostattable column compartment. The HPLC system was interfaced to a Finnigan TSQ 7000 triple quadrupole mass spectrometer, with an atmospheric pressure chemical ionization (APCI) source.

Current throughput of the method is 36 unknown samples (48 samples including calibration standards, blanks, and QC materials) per day per laboratory analyst, with the analyst performing the entire procedure. Sample preparation requires approximately 4 hours of analyst time. Mass spectral analysis time is 12 min per sample; thus samples are being analyzed on the instrument for about 11 hours for one run. Final processing of the data requires about 2 hours of analyst time. Factoring in a 20% downtime, the predicted yearly throughput of this method is approximately 8,000 samples per laboratory analyst, which is suitable for large-scale studies.

This method provides a simple, fast, and specific tool for the analysis of several herbicides and some metabolites thereof. Sample clean-up requires only a basic SPE method and requires no pretreatment of samples (e.g., buffering, deconjugation, derivatization, or centrifugation). Inclusion of stable isotope analogues for some of the analytes and use of MS/MS analysis provides specificity and accuracy in their detection and quantification.

G. Method Validation.

In order to improve selectivity of the analysis, the most abundant product ion as a quantification ion and the least abundant as a confirmation ion were used. All transitions were based on the $[M+H]^+$ precursor ions, except for the MMP confirmation ion on the TSQ 7000, which was based on a cluster ion with methanol $[M+H–32]^+$. The liquid chromatography was optimized to achieve the best separation and retention for all analytes; it was performed under gradient conditions. The total run time was nine minutes. The divert valve of the HPLC system, which directs the solvent flow away from the mass spectrometer's ion source, was programmed to stay open for two minutes after injection to help reduce the chemical background and keep the ion source clean.

A summary of the method specifications is shown in Tables 2 and 3

(1) Stability and adsorption of the analytes

The stability of the analytes in stock and working solutions at different temperatures were determined by monitoring changes in concentrations in samples stored at different temperatures in relation to a sample stored at -70°C [4]. The storage temperatures were −70°C, −20°C, 4°C, room temperature and 37°C. Samples were analyzed after 100 days of storage at each temperature. If any standard degraded over 10% during the storage time, it was considered unstable at that temperature. In general, standards were less stable when stored at room temperature or 37°C.

To evaluate possible adsorption of the analytes onto glassware used in the method, 10 mL of a sample containing 50 ng/mL of each of the analytes dissolved in acetonitrile was placed in a silanized flask. Three different types of glassware used for sample storage or used during sample preparation were tested: large green cap glass vials (23 x 85 mm, BGC vials), small green cap glass vials (15 x 45 mm, SGC vials) and 15 ml disposable conical centrifuge tubes (C tubes). The sample in the silanized flask was considered the control at zero time adsorption. An aliquot of 1 mL (for each of the three types of glassware) was passed through 10 times. The test was done in triplicate. The samples were analyzed to discover any changes in concentration.

For the analysis of the samples from the stability test and the adsorption test, a 25 µl volume of the sample was combined with 25 µl of internal standard. The mixture was taken to dryness in a Turbo Vap LV (Zymark, Hopkinton, MA) at 40°C and 10 psi of nitrogen. The residues were reconstituted with 50 µl of methanol and transferred to auto-injection vials.

Overall, the data suggested that the analytes are stable at −20ºC and 4ºC, and are not adsorbed on any of the glassware (Table 3). However, long term (100 days) storage at room temperature or 37°C showed gradual

deterioration for the OP pesticide metabolites (AP, MMP, Omet and Dmet), but not for the fungicide metabolites.

(3) Limits of Detection.

The limits of detection (LOD) were calculated for each analyte as three times the standard deviation of the noise at zero concentration ($3s_0$), where s_0 were estimated as the y-intercept of a linear regression analysis of a plot of the standard deviation of the five lowest standards versus the expected concentration from 6 runs.[5]

The LODs of the method on the TSQ 7000 (Table 1) ranged from 0.06 ng/mL to 0.3 ng/mL. For ETU and PTU, the LOD was 0.1 ng/mL; for AP, it was 0.3 ng/mL; for MMP, it was 0.16 ng/mL; and for Omet and Dmet, it was lower than 0.06 ng/mL. The LODs for most of the analytes (except for ETU) improve significantly when the TSQ-Quantum was substituted for the TSQ 7000. The LODs ranged from 0.001 ng/mL to 0.1 ng/ml (Table 2). The LOD calculations were based on six runs of calibration curves.

Contributing to the better performance of the TSQ-Quantum are new design features that have improved the mass-resolution among other things. The LODs for both instruments are lower in magnitude than the LODs previously published for these analytes[6, 7]. The high sensitivity of this method makes it suitable for the measurement of internal doses resulting from incidental, low-level exposures such as those commonly occurring with environmental exposures.

(4) Extraction Efficiency.

The extraction recovery of the method was determined at two concentrations, 10 and 50 ng/mL, by spiking six blank urine samples with the appropriate standard concentration and processing according to the method. Four additional blank urine samples (unspiked) were processed concurrently. Before the evaporation steps, all of the samples were spiked with a known amount of labeled internal standard to correct for instrument variation. The samples that were not spiked before preparation were then spiked with the appropriate native standard to serve as control samples representative of 100% recovery. After evaporating and reconstituting, the samples were analyzed. The recovery was calculated by comparing the responses of the blank urine samples spiked before lyophilization to the responses of the blank urine samples spiked after lyophilization

The method's recovery efficiency ranged between 52% and 63%. Some analytes showed a small difference in recovery efficiency between the higher and the lower concentration. Using the isotope dilution technique, the individual recovery of each analyte in a sample is automatically

corrected so variable extraction recoveries do not negatively affect the accuracy of the data obtained.

(5) Precision

The precision of the method was determined by calculating the relative standard deviation (RSD) of repeat measurements of the QC materials at three different concentrations (0.5 µg/mL, 5 µg/mL, and 15 µg/mL. At least 30 repeat measurements of QC materials were used to determine the method RSDs for each analyte.

The method precision of each analyte, expressed as the RSD of repeated analyses of the QC materials, is also shown in Table 1 and Table 2. The QC values were calculated as an average of six runs with five at each level in each run. In most instances, the RSDs were less than 18%.

(6) Matrix Effects.

The matrix effects were evaluated by spiking three urine sample aliquots with different concentrations (1ng/mL, 8 ng/mL and 20 ng/mL), from each of seven different donors. Five replicates were analyzed from each urine sample aliquot. The replicates were divided in five analytical runs so that only one replicate from each aliquot was analyzed in each analytical run. A calibration plot with eight calibration standards was prepared and analyzed with each run. The urine samples were prepared for analysis according to the procedure already described above. An aliquot of each urine matrix was screened for possible endogenous analytes.

Individual variation in pH and concentrations of salts and biomolecules in urine might affect the sensitivity of the method. Urine collected from different donors was spiked with three concentrations of the analytes. The variation of matrix effect on each concentration for each analyte was calculated, and the data are shown in Table 4. The variation of matrix effect ranged between 1 to 10% suggesting that individual differences in matrix composition did not significantly affect the performance of the method.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Mass Spectrometer

The TSQ7000 mass spectrometer is calibrated and tuned using 2-methoxyethanol-ammonia clusters according to the instructions in the operator's manual. The "OPT" ICL 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

The calibration plots were linear throughout the entire standard range A best-fit line of the data showed good correlation coefficients. Errors about the slope were less than 1%.

For all analytes a slope average of a linear regression analysis of ten calibration standards of six runs of calibration curves were calculated on the two instruments. The R^2 values were greater than 0.990 for all analytes. In addition, the method's accuracy was indistinguishable from 99.8 % to 100%. The calculation was based on a slope average of linear regression analyses of plots of calculated concentrations of spiked samples versus the expected concentration of the same samples from six runs per instrument.

(1) 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.

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

(3) R-squared values for the curve must be greater than 0.95. 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.

(4) The standard curve should be recalculated periodically to incorporate the newest data points. Whenever a new combined labelled isotope solution is prepared, the standard curve must be re-established.

C. Calibration Verification

(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, QC 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.

Calibration plots were generated by plotting the area-of-the-analyte/area-of-the internal standard against the calibrant concentration. A best-fit line was generated that provided a slope and intercept from which unknown analyte concentrations could be calculated. A full calibrant set was analyzed with each analytic run.

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

(3) Three Quality Control (QC) Materials. The low-level QC pool (QCL) was spiked at 0.5 ng/mL urine for all analytes, the medium-level QC pool (QCM) was spiked at 5 ng/mL, and the high-level QC pool (QCH) was spiked at 15 ng/mL. The slope, intercept, and linearity of a regression analysis of the QC 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 QC 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. Sample Preparation

Urine samples were frozen within 1 hour of collection and stored at -20 °C before analysis. For each analytical run, calibration samples, two fortified urine samples (one high and one low dose), one blank urine sample, and one solvent blank were prepared, extracted, and analyzed in parallel with the unknown samples.

B. Sample Cleanup

A 2-ml urine sample was pipetted into a 15-ml vial and spiked with 25 µL of the labeled internal standard working solution to give a urinary concentration of 12.5 ng/mL. The urine samples were vortex mixed and placed into a -70°C freezer in custom-made Teflon racks for at least 4 hours. After the samples were frozen, they were placed in a lyophilizer. The lyophilizer was operated overnight in the automatic program mode without further manual manipulation. The program was set with the vacuum at 25.5 mT and the temperature at -34°C for 6 hours following by 2 hours at −20°C, 2 hours at 0°C, 3 hours at 20°C

and, finally, 1 hour at 22°C. The following day, after completion of the lyophilization process, dichloromethane (3 mL was added to the residue in each sample tube in order to extract the analytes. The tubes were vortex mixed for about 2 minutes. The samples were loaded onto 3mL polyethylene cartridges with 20 µm pore frits; the breakthrough was collected into 15-mL centrifuge tubes. The extraction vials were rinsed with an additional 1mL of dichloromethane, vortex mixed and applied to the cartridges to combine with the previous breakthrough.

The samples were concentrated to dryness using a Turbovap LV at 40°C and 10 psi of nitrogen. Methanol (0.5 mL) was added to each tube, followed by vortex mixing to rinse the tube. The samples were concentrated to dryness again. The residues were reconstituted with 50 µLof methanol and transferred to auto-injection vials.

C. Instrumentation

Analytes were separated on a Prodigy column (5µ, phenyl-3, 100 x 4.6 mm). The column was connected to an Agilent 1100 high performance liquid chromatography (HPLC) system, consisting of an autosampler, a binary pump, a degasser, and a thermostattable column compartment. The HPLC system was interfaced to a Finnigan TSQ 7000 triple quadrupole mass spectrometer, with an atmospheric pressure chemical ionization (APCI) source.

D. *HPLC and Mass Spectrometry Conditions*.

Chromatographic separation was performed using a Surveyor HPLC system composed of an autosampler and HPLC pump. The column used was a Zorbax SB-C3, narrow-bore 4.6 x 150-mm, 5.0-µm. The analytes were separated with gradient elution by using 0.1% formic acid in aqueous solution (solvent A) and 0.1% formic acid in methanol (solvent B). The initial mobile phase composition was 95% solvent A and 5% solvent B. The mobile phase was changed linearly over the next 5 minutes until the mobile phase composition was 40% solvent A and 60% solvent B. Over the next minute, the mobile phase composition was changed to 20% solvent A and 80% solvent B then changed to 5% solvent A and 95% solvent B over the next minute. Finally, the mobile phase composition was allowed to return to the initial conditions and allowed to equilibrate for 2 minutes prior to the next injection. The total HPLC run time including equilibration was 9 min. The flow rate was 1000 µL/min and the injection volume was 5 µL. The divert valve was programmed to go to waste for the first 2 minutes and the two last minutes of the run. The Surveyor HPLC pump pressure was 400 bar.

For the MS/MS analysis two different instruments were used: a TSQ 7000 triple quadrupole mass spectrometer and a TSQ Quantum high resolution triple quadrupole mass spectrometer. The instruments were operated with an APCI source in positive ion mode with multiple reaction monitoring (MRM). For the TSQ 7000 the heated capillary and vaporizer temperatures were 250°C and 450°C, respectively. The corona current was set at 4 KV and the sheath gas pressure was 60 psi. Collision-induced dissociation (CID) was performed using argon at 2.0 mT. The electron multiplier was set at 1400 V. The instrument parameters for the TSQ Quantum were as follows: vaporizer temperature 450°C, sheath gas pressure 45, auxiliary gas pressure 5 (arbitrary units), capillary temperature 350°C and collision gas 1.5 mTorr.

F. Processing of data

(1) Quantification

Just before each analytical run, calibration standards were prepared by diluting the working standard stock solutions in blank urine. The concentrations of the ten calibration standards ranged from 0.125 to 100 ng/mL for each of the analytes. To each run was added the ten calibration samples, three quality control samples (QCL, QCM, and QCH), and one blank urine sample; these were extracted and analyzed in parallel with the unknown samples. The area of the analyte divided by the area of the internal standard and plotted against the concentration of the sample to derive a calibration plot. The best fit line of a linear regression analysis of the plot was used to derive an equation from which unknown sample concentrations could be calculated.

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.

10. QUALITY CONTROL (QC) PROCEDURES

All QC pools were characterized before use to determine the mean and 99th and 95th control limits by consecutively analyzing at least 30 samples from each QC pool. QC samples were analyzed in runs with 5 replicates in 6 runs over 3 days. After establishing the control limits of the pools, individual QC samples contained within each analytical run were evaluated for validity using Westgard multirules.[8]

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. Three 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 nondetectable. The urine samples were combined and homogenized to form a base pool.

C. Urine Enrichment. Pooled, filtered urine was used to prepare QC pools at three concentration levels by fortifying with the appropriate native standard and mixing well. The low-level QC pool (QCL) was spiked at 0.5 ng/mL, urine for all analytes, the medium-level QC pool (QCM) was spiked at 5 ng/mL, and the high-level QC pool (QCH) was spiked at 15 ng/mL. All QC materials were stored at -40°C until analyzed.

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. QC materials were characterized by 6 replicates on each of 5 days, thus in total 30 replicates, to determine the mean and relative standard deviation (RSD). The QC characterization results were used to determine the within and between day variation, as well as the accuracy of the method. The accuracy was defined as the mean of estimated value divided by the theoretical value.

F. Use of Quality Control Samples. For a given analytic run of 36 unknown samples, three QC materials were prepared along with the urine blank sample and the full set of calibration samples. .

G. Out of Control Runs. The run was considered "out-of-control" if the calculated values of the QC samples violated the Westgard multirules[8].

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the calibration or QC systems, all operations are suspended until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for instance, 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 reanalyzed. After calibration or quality control has been reestablished, analytical runs may be resumed.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The measurement of ETU, PTU, AP, MMP, Omet and Dmet in urine is challenging because of their chemical and physical properties. These polar analytes are extremely difficult to extract from a polar urine matrix. A variety of methods reported in the literature have noted these difficulties; most methods use LLE requiring large volumes of organic solvents such as chloroform and dichloromethane[,9,6,7]. The sample preparation is simple, efficient and reproducible. The lyopholization step has proven to be critical in enhancing recovery efficiency for these highly water-soluble compounds. Even though the lyophilization process takes approximately 14 hours for 50 samples, it can be performed overnight and is an automated process. Lyophilization of the urine sample yields the driest possible sample; so that a small volume of organic solvent is required to extract these analytes. In our laboratory, the lyophilization technique has been applied with success for the measurement of other compounds including dialkyl phosphate metabolites of organophosphorus pesticides[10] .

13. REFERENCE RANGES (NORMAL VALUES)

Reference range values for most of these analytes do not exist. However, in the time that atrazine mercapturate has been measured

by this lab, only a few samples have had detectable amounts, resulting from incidental exposures.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

There are no panic values for these analytes.

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.

The stability of the analytes and the possible adsorption of the analytes on the specific glassware used for storage or during sample preparation were investigated. Overall, the data suggested that the analytes are stable at −20ºC and 4ºC, and are not adsorbed on any of the glassware (table 3). However, long term (100 days) storage at room temperature or 37°C showed gradual deterioration for the OP pesticide metabolites (AP, MMP, Omet and Dmet), but not for the fungicide metabolites.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If testing cannot be performed, the specimens are stored at -70°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. This 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.

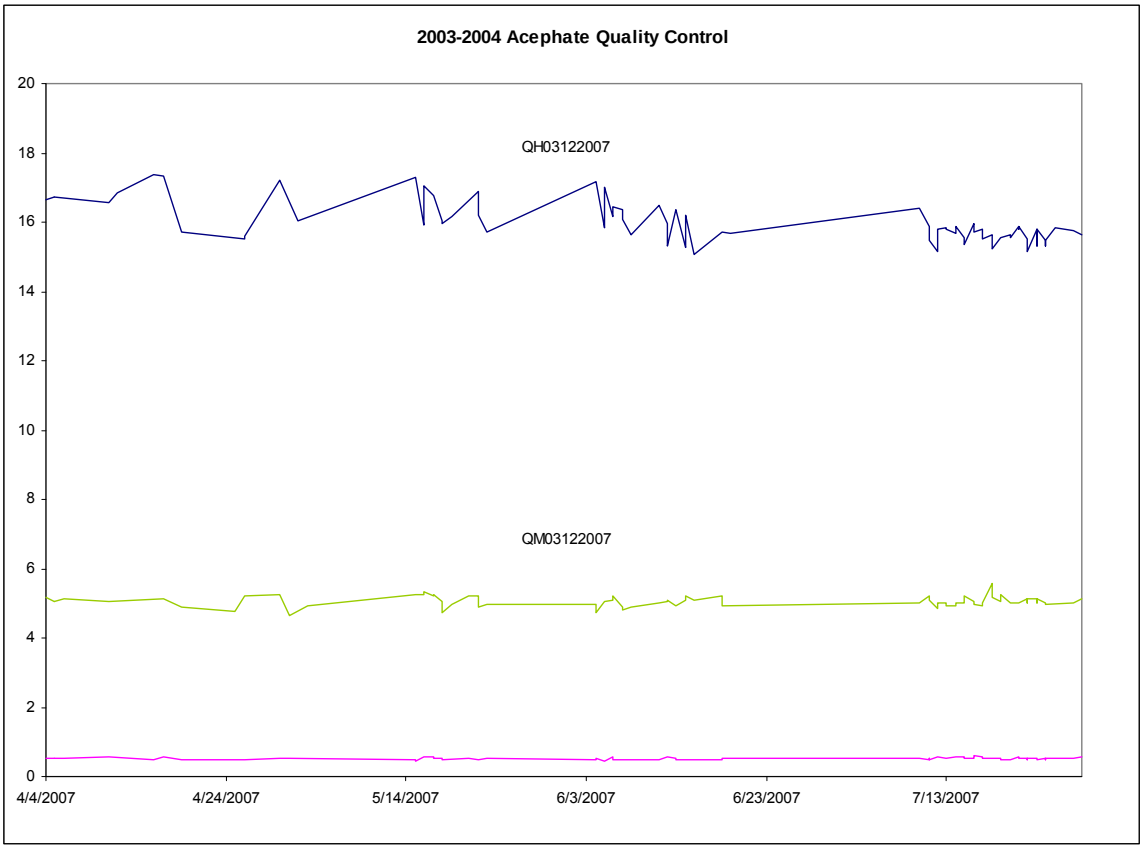
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

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

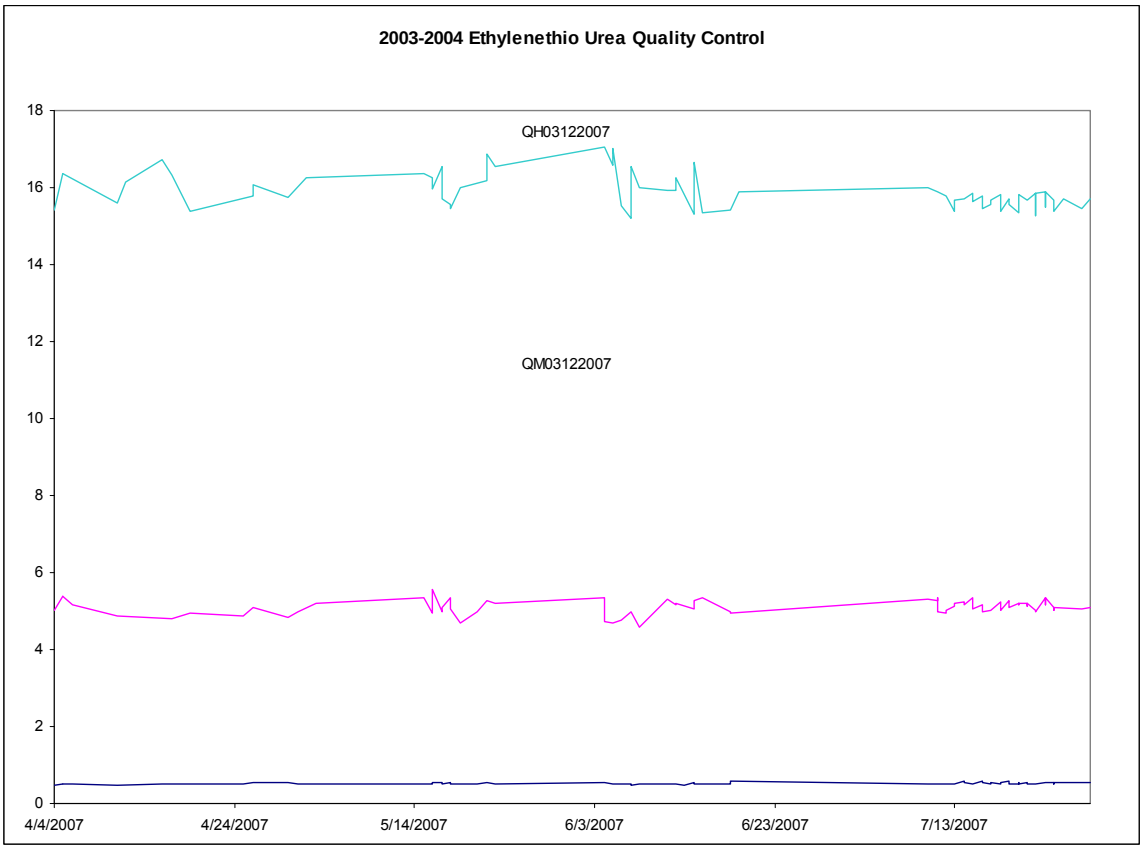
Summary Statistics for Acephate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL03122007	78	4/4/2007	7/28/2007	0.5187	0.0293	5.6
QM03122007	78	4/4/2007	7/28/2007	5.0522	0.1499	3.0
QH03122007	78	4/4/2007	7/28/2007	15.9813	0.5715	3.6



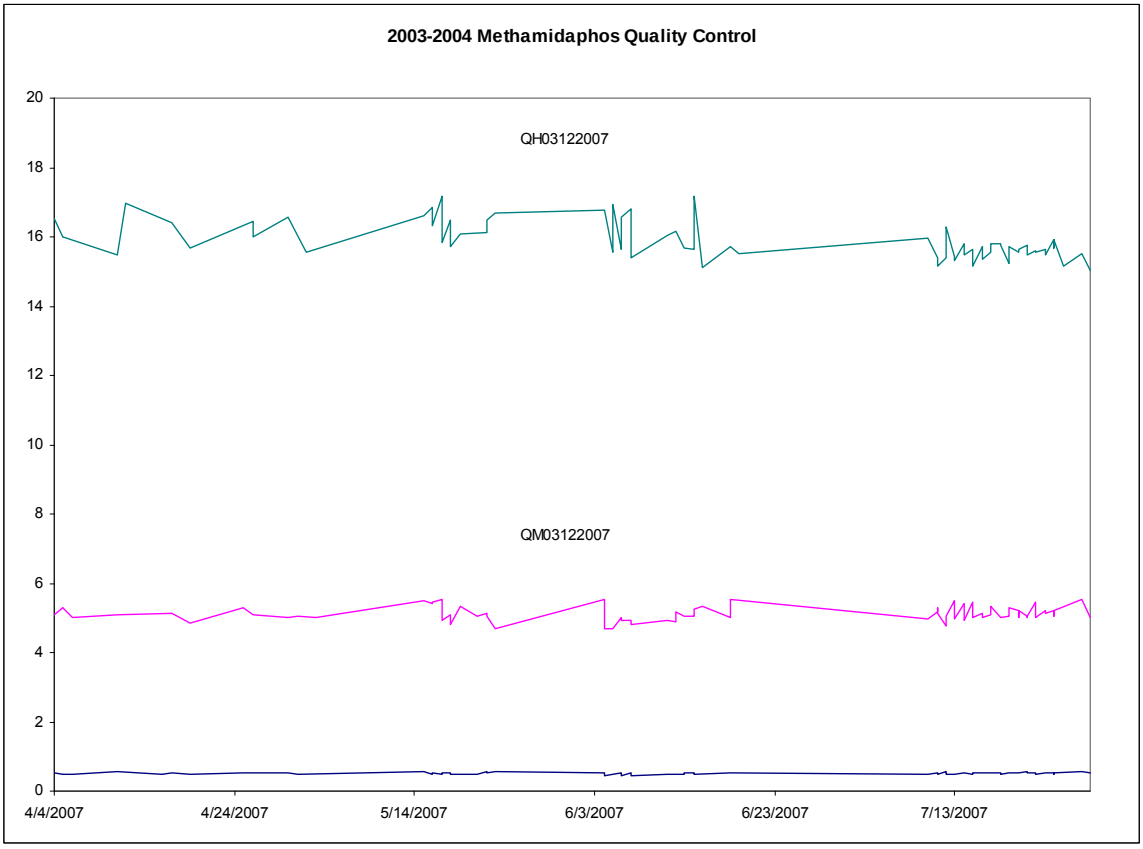
Summary Statistics for Ethylenethio Urea by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL03122007	76	4/4/2007	7/28/2007	0.5246	0.0217	4.1
QM03122007	75	4/4/2007	7/28/2007	5.0997	0.1841	3.6
QH03122007	72	4/4/2007	7/28/2007	15.8759	0.4363	2.7



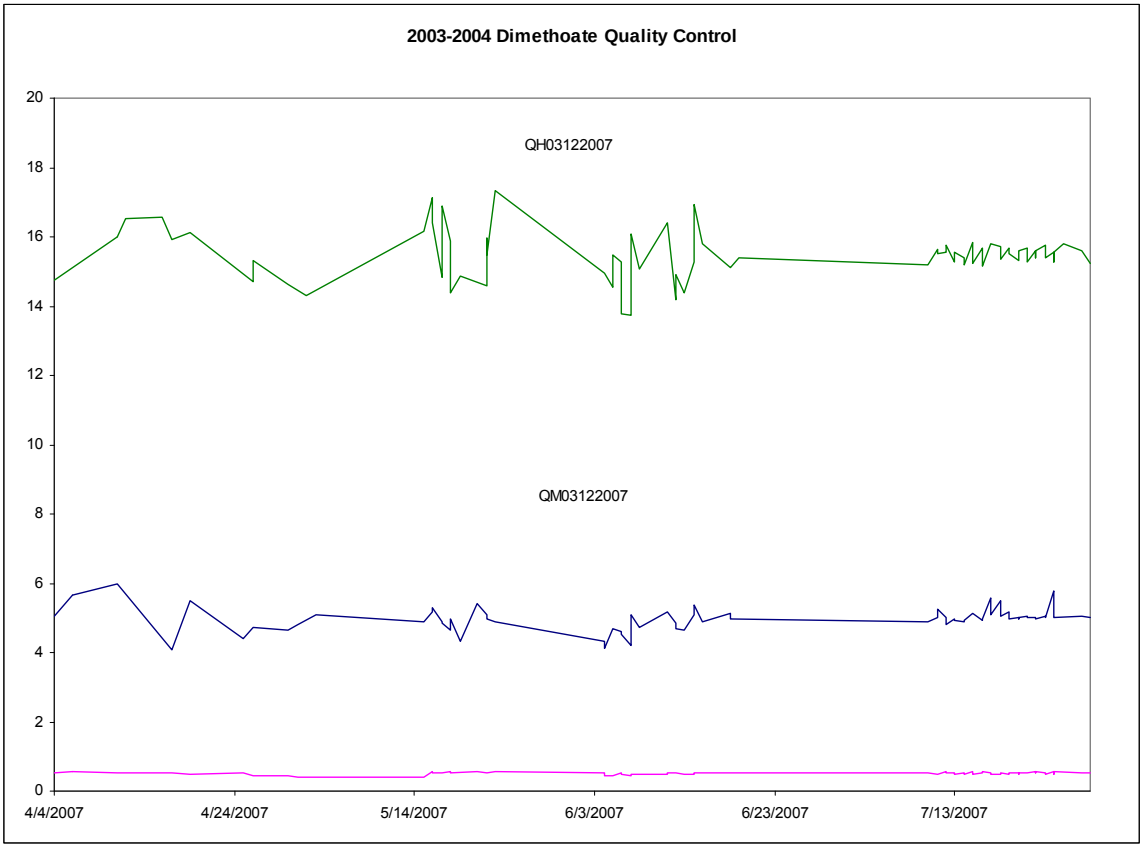
Summary Statistics for Methamidaphos by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL03122007	77	4/4/2007	7/28/2007	0.5111	0.0246	4.8
QM03122007	77	4/4/2007	7/28/2007	5.1206	0.2115	4.1
QH03122007	76	4/4/2007	7/28/2007	15.8757	0.5406	3.4



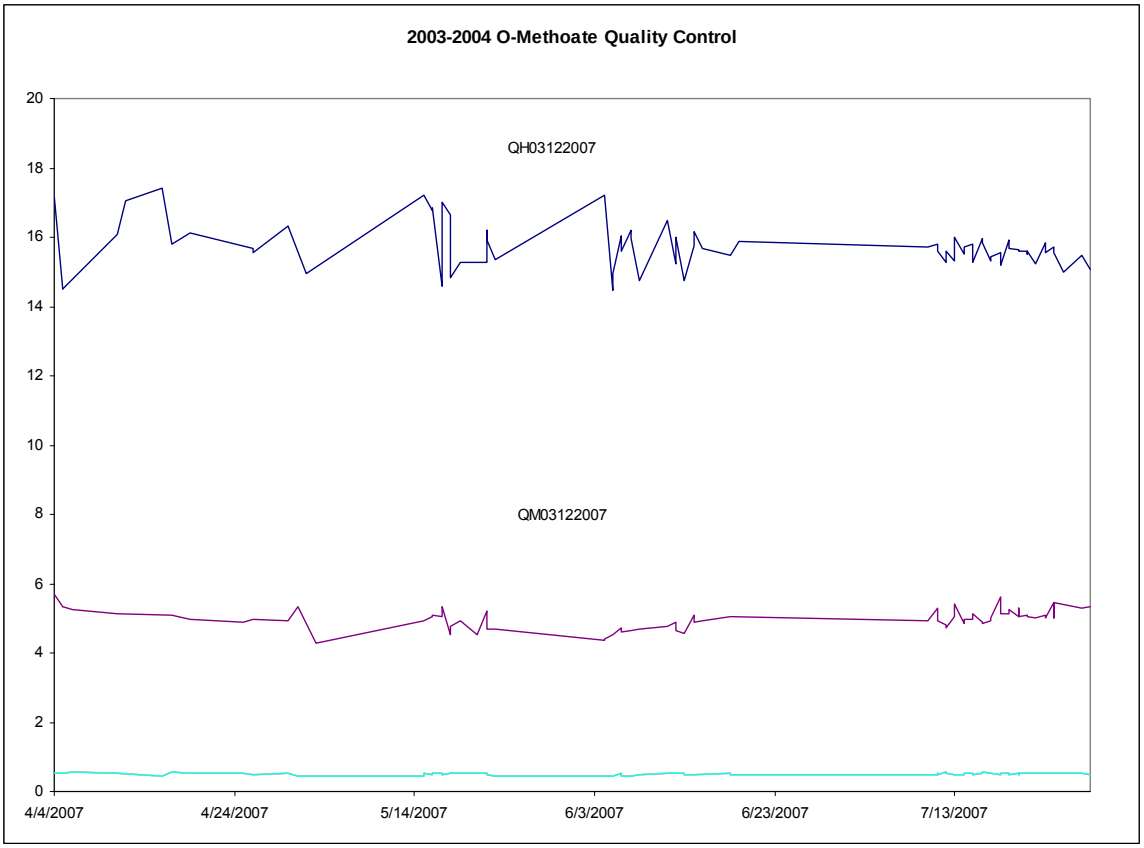
Summary Statistics for Dimethoate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL03122007	77	4/4/2007	7/28/2007	0.5106	0.0351	6.9
QM03122007	77	4/4/2007	7/28/2007	4.9818	0.3397	6.8
QH03122007	77	4/4/2007	7/28/2007	15.4787	0.6771	4.4



Summary Statistics for O-Methoate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL03122007	78	4/4/2007	7/28/2007	0.5076	0.0286	5.6
QM03122007	78	4/4/2007	7/28/2007	4.9838	0.2791	5.6
QH03122007	78	4/4/2007	7/28/2007	15.7062	0.639	4.1



Summary Statistics for Propylenethio Urea by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL03122007	77	4/4/2007	7/28/2007	0.5453	0.0294	5.4
QM03122007	78	4/4/2007	7/28/2007	5.1125	0.2889	5.7
QH03122007	78	4/4/2007	7/28/2007	15.8199	0.4942	3.1

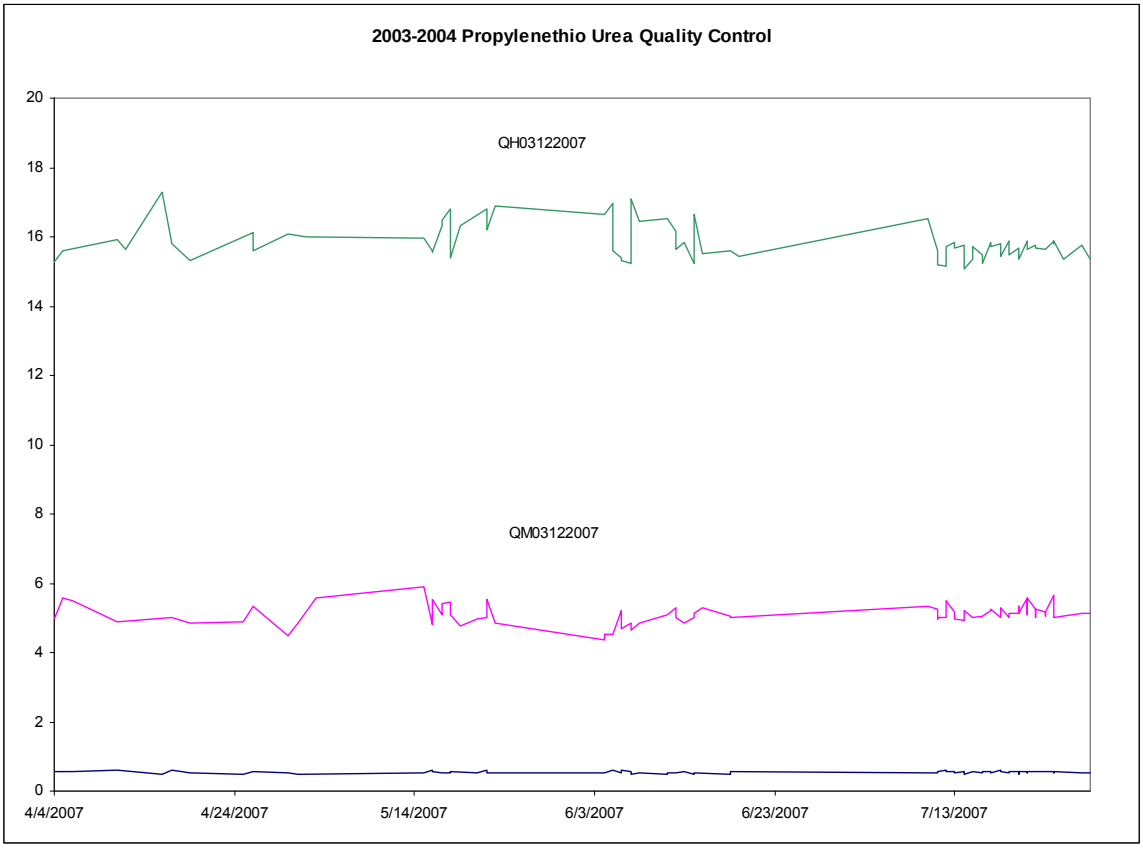


Table 1. Summary of method specifications on TSQ 7000.

Analyte	LOD ng/ml (ppb)	Standard Curve R^2	Accuracy (%)	QC values			RSD		
				QCL	QCM	QCH	QCL	QCM	QCH
ETU	0.106	0.9994	100	0.54	5.11	15.90	10.39	6.53	6.99
PTU	0.119	0.9990	100	0.55	5.08	15.39	12.37	6.51	9.11
AP	0.282	0.9993	100	0.52	5.07	17.10	15.32	7.25	4.62
MMP	0.639	0.9949	100	0.46	4.97	14.74	9.39	9.69	11.84
Omet	0.053	0.9987	100	0.53	5.31	15.49	11.94	17.76	14.42
Dmet	0.030	0.9980	99.9	0.55	5.03	14.87	14.66	17.24	10.16

LOD: Calculated as $3S_0$. Standard deviation at zero concentration (S_0) was estimated as the y-intercept of a plot of the standard deviation of the five lowest calibration standards from 6 runs versus the expected concentration.
Standard Curve: Slope average of a linear regression analysis of ten calibration standard from six runs.
Accuracy: Expressed as the percentage of the expected concentration that was quantified.
QC (Quality Control) values: Average of QCL (low), QCM (medium) and QCH (high) from 6 runs. Blank urine pools were spiked with the native standard stock solution to yield a concentration of 0.5 µg/ml (QCL), 5.0 µg/ml (QCM) and 15.0 µg/ml (QCH).
RSD: Relative standard deviation of the QC values from 6 runs.

Table 2. Summary of method specifications on TSQ-Quantum.

Analyte	LOD ng/ml (ppb)	Standard Curve R^2	Accuracy (%)	QC values			RSD		
				QCL	QCM	QCH	QCL	QCM	QCH
ETU	0.160	0.9999	100	0.51	5.36	13.37	7.86	4.90	3.47
PTU	0.0045	0.9996	99.9	0.48	5.05	14.75	6.20	5.21	4.87
AP	0.0234	0.9994	99.9	0.51	5.01	15.12	8.20	6.54	3.83
MMP	0.0015	0.9995	99.9	0.50	5.26	15.26	4.49	4.72	2.51
Omet	0.0258	0.9929	99.8	0.48	4.94	14.87	13.54	6.28	4.82
Dmet	0.0048	0.9987	99.9	0.49	5.16	15.16	7.45	9.04	6.54

LOD: Calculated as $3S_0$. Standard deviation at zero concentration (S_0) was estimated as the y-intercept of a plot of the standard deviation of the five lowest calibration standards from six runs versus the expected concentration.
Standard Curve: Slope average of a linear regression analysis of ten calibration standard from six runs.
Accuracy: Expressed as the percentage of the expected concentration that was quantified.
QC (Quality Control) values: Average of QCL (low), QCM (medium) and QCH (high) from 6 runs. Blank urine pools were spiked with the native standard stock solution to yield a concentration of 0.5 µg/ml (QCL), 5.0 µg/ml (QCM) and 15.0 µg/ml (QCH).
RSD: Relative standard deviation of the QC values from 6 runs.

Table 3. Stability and adsorption of the analytes

Analytes	Stability (%) -100 days incubation				Variation on Adsorption (%)		
	−20ºC	4ºC	RT *	37ºC	BGC vials	SGC vials	C tubes
ETU	100	100	100	100	1.2	0.8	0.9
PTU	100	100	100	100	0	1.5	0.3
AP	100	100	97	81	0	1.3	0.2
MMP	100	100	89	82	1.5	0.3	1.1
Omet	100	100	61	48	0.7	0.1	1.8
Dmet	100	100	68	49	1.4	1.2	1.6

*RT- room temperature.
At −70ºC was considered 100% stability.
Percentage of variation on adsorption was calculated as:
{100-[(0 time adsorption/10 times adsorptions) x 100]}

Table 4. Matrix effects

Analyte	Spiked matrix			Variation of matrix effect (%)		
	1ng/ml	8 ng/ml	20 ng/ml	1 ng/ml	8 ng/ml	20 ng/ml
ETU	1.11 ± 0.14	8.44 ± 0.40	20.73 ± 0.63	9.1	5.3	3.5
PTU	1.10 ± 0.15	8.37 ± 0.37	20.53 ± 0.49	9.1	4.4	2.6
AP	1.07 ± 0.07	8.10 ± 0.30	20.17 ± 0.54	6.5	1.2	0.8
MMP	1.01 ± 0.05	8.19 ± 0.34	20.25 ± 0.61	1.0	2.3	1.2
Omet	1.07 ± 0.12	8.33 ± 0.36	20.41 ± 0.49	6.5	4.0	1.1
Dmet	1.06 ± 0.08	8.31 ± 0.47	20.22 ± 0.72	5.7	3.6	1.1

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