Laboratory Procedure Manual

Analyte:	Phenolic Metabolites of Pesticides
Matrix:	*Urine*
Method:	GC-MS/MS
Method No.:	
Revised:	
as performed by:	*Toxicology Branch* *Division of Laboratory Sciences* *National Center for Environmental Health, CDC*
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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.

Public Release Data Set Information

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

File Name	Variable Name	SAS Label (and SI units)
L26UPP_C	URXCBF	Carbofuranphenol (ug/L)
	URXPCP	Pentachlorophenol (ug/L)
	URXPPX	2-isopropoxyphenol (ug/L)
	URXUCR	Creatinine, urine (mg/mL)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A method to measure 12 urinary phenolic metabolites of pesticides or related chemicals has developed. The target chemicals for our method are 2-isopropoxyphenol; 2,4-dichlorophenol; 2,5-dichlorophenol; carbofuranphenol; 2,4,5-trichlorophenol; 2,4,6-trichlorophenol; 3,5,6-trichloro-2-pyridinol; para-nitrophenol, ortho-phenylphenol, pentachlorophenol, 1-naphthol and 2-naphthol. The sample preparation involves enzyme hydrolysis, isolation of the target chemicals using solid phase extraction cartridges, a phase-transfer catalyzed derivatization, cleanup using sorbent-immobilized liquid /liquid extraction cartridges, and concentration of the sample. Derivatized samples are analyzed by capillary gas chromatography-tandem mass spectroscopy using isotope dilution calibration for quantification. The limits of detection are in the mid ng/L range and the average coefficient of variation was below 15% for most of the analytes. Using our method, we measured concentrations of the target chemicals in urine samples from the general population.

Numerous analytical methodologies for biological monitoring of urinary phenolic chemicals in occupationally exposed populations have been published (Hill et al., 1995; Shealy et al., 1997). The limits of detection (LODs) of these methods range from 1 µg/L to 60 µg/L. However, biomonitoring of the general population typically requires LODs of 1 µg/L or less. In previous work, we reported the simultaneous measurement of 12 urinary phenols with average LODs about 1 µg/L in a 10-mL urine sample. These methods have essentially eight major steps for sample preparation: (1) the addition of internal standards (Hill et al., 1995); the enzyme hydrolysis of urine; liquid-liquid extraction; back extraction into a basic solution; the formation of chloropropyl derivatives through the use of a phase-transfer catalysis reaction; 6) liquid-liquid extraction of the reaction mixture; silica column cleanup; and sample concentration. We analyzed concentrated derivatized samples by using gas chromatography coupled with tandem mass spectroscopy (GC-MS/MS). Although these methods are highly selective and relatively sensitive, they are still labor-intensive and require a great deal of time. Also, the properties of the method, such as sensitivity, precision, and extraction recoveries, needed further improvement.

The existing methods were significantly refined to produce a more streamlined method that was less labor-intensive and had a higher throughput, better precision and extraction recoveries, and lower LODs. These analytes are potential metabolites of 30 environmental chemicals including several contemporary pesticides. The refined method involves (Shealy et al., 1997) the addition of internal standards; (Hill et al., 1995) the enzyme hydrolysis of urine; extraction of the metabolites by solid phase extraction (SPE) using OASIS ® or STRATA ® cartridges; back extraction into a basic solution; the formation of chloropropyl derivatives through the use of a phase-transfer catalysis reaction; (6) cleanup of the derivatized sample mixture using sorbent-immobilized liquid/liquid extraction cartridge ChemElut ®; and concentration of the sample. Concentrated derivatized samples are analyzed by GC-MS/MS. In addition to improvements in recovery, precision, and sensitivity, a reduced volume of urine is used, and the

total analytical time (including sample preparation and analysis) is dramatically reduced.

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. NoneC. Microbiological Hazards. Although urine is generally regarded as less infectious than serum, the possibility of being exposed to various microbiological hazards exists. Appropriate measures should be taken to avoid any direct 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.

D.Mechanical Hazards. There is only minimal mechanical hazard when performing this procedure using standard safety practices. Laboratorians 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.

E Protective Equipment. Standard safety apparatus should be used when performing this procedure. This apparatus includes lab coat, safety glasses, durable gloves, and a chemical fume hood.

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 any biological specimen. Routine use of gloves and proper hand washing should be practiced.

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 in a flame resistant cabinet. Containers, glassware, etc., that come in direct contact with the specimens should be autoclaved or decontaminated with 10% bleach. The glassware should be washed and recycled or disposed of in an appropriate manner.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. Software and Knowledge Requirements. A database named NPP2 has been developed on the EHLS-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 20 milliliters of urine is collected, and poured into sterile 30mL 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 breakage 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. (Note 6)

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)Ethyl ether, butyl chloride (BuCl), methanol (MeOH), hexane, acetonitrile and toluene were purchased from Tedia Company INC. (Fairfield, Ohio).
(3)Acetic acid, sulfuric acid, sodium acetate, sodium hydroxide, sodium sulfate, tetrabutylammonium hydrogen sulfate ($TBAHSO_4$), and hydrochloric acid (HCl) were obtained from J. T. Baker Co. (Phillipsburg, N.J.).
(4)1-chloro-3-iodopropane and β-glucuronidase from *Helix pomatia* (G 0751, EC 3.2.1.31, type H-1) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo.).
(5) OASIS® HLB and Strata® X solid phase extraction cartridges were obtained from Waters Corporation, Milford, MA and Phenomenex, Torrance, Calif., respectively.
(6) ChemElut ® sorbent-immobilized liquid/liquid extraction cartridges were purchased from Sample Varian Preparation Products, Walnut Creek, Calif.
(7)The following native standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo.): 2-isopropoxyphenol (IPP; 97%), 2, 5-dichlorophenol (25DCP; 98+%), 2, 4-dichlorophenol (24DCP; 99%), 2, 4, 5-trichlorophenol (245TCP; 99+%), 4-nitrophenol (PNP 99+%), o-phenyl phenol (OPP; 99+%), and pentachlorophenol (PCP; 98%).
(8) 2, 4, 6-trichlorophenol (246TCP; 98%) was obtained from Eastman Kodak Co. (Rochester, N.Y.)
(9)3, 5, 6 trichloropyridinol (TCPY; 99%) was purchased from Dow Chemical Co. (Midland, Mich.).
(10) 1-naphthol (1N; 99%) and 2-naphthol (2N; 99%) were obtained from Janssen Chimica (Geel, Belgium).
(11)Carbofuranphenol (CFP; 99.5%) was purchased from Chem Service (West Chester, Penn.).
(12)All labeled standards were custom synthesized by Cambridge Isotope Laboratories (Andover, Mass.),
(13) $^{13}C_6$ 1-naphthol, which was synthesized in house at the Centers for Disease Control and Prevention (CDC). All labeled standards had chemical and isotopic purities of 99+%.
(14)Gases used by the instrumentation had a minimum purity of 99.999% and were purchased from Holox (Atlanta, Ga.).

B. Reagent preparation

All reagents were prepared according to Hill et al. [7] using bioanalytical grade I water. An acetate buffer solution was prepared by combining 3.4 g sodium acetate, 1.1 ml acetic acid, and 700 mL of bioanalytical water. The buffer-enzyme solution was prepared by dissolving 0.358 g β-glucuronidase (338,000 units/g) in 100 mL 0.1 M acetate buffer (pH 5).

C. Standard Preparation

(1) Isotopically Labeled Internal Standard
An isotopically labeled internal standard (ISTD) solution was

prepared by weighing approximately 0.5 mg of each of the 9 isotopically labeled analytes into a 2.5-mL volumetric flask and dissolving with acetonitrile to yield a 200-ng/µL solution. The individual stock solutions were stored at –20 °C until used. A multiple analyte ISTD solution was prepared by adding 250 µL of each of nine internal standard stock solutions into a 50 mL volumetric flask and diluting the solution with acetonitrile to make a final concentration of 1 ng/µL. This solution was used as an ISTD spiked in all unknown samples, quality control (QC) materials, and calibration standards. For the three target analytes without analogous labeled internal standards (2 IPP, CFP, and 2 N), the labeled compounds for 25 DCP and 246 TCP were used as ISTDs.

(2) Native Standards and Calibration Plots

A native standard stock solution was prepared by weighing approximately 5 mg of the native standard into a 25-mLvolumetric flask and dissolving with acetonitrile to yield a 200 ng/µL solution. Standard mixture solutions of the 12 target analytes were prepared by spiking 0.62, 1.25, 2.5, 5.0, 12.5, 25, 50, 125, and 250µL aliquots of each individual stock standard into 10 mL volumetric flask and dissolving with acetonitrile. The individual stock solutions and the standard mixture solutions were stored at –20 °C until used. Calibration standards were prepared daily by spiking 2 mL of "blank" urine with 40 µL of the appropriate concentration of standard mixture solutions prepared in acetonitrile. The calibration standards were prepared according to the sample preparation procedure described below.

D. Quality Control Materials

Urine was collected from multiple (> 30) 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-µm 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 10 µg/L for all of the metabolites. The second pool (QC high pool) was spiked with the native standard stock solution to yield an approximate concentration of 20 µg/L for all of the metabolites. The third pool was not spiked. After being screened for possible endogenous analytes, it was used as matrix material for calibration standards and blanks. All QC pools were characterized to determine the mean and $99^{th}$and 95^{th} control limits by a consecutive analysis of at least 20 samples from each QC pool. After establishing the control limits of the pools, both QC high and low samples contained within each analytical run were evaluated for validity using the Westgard multirules [8] shown below:

(1)If both QC results were within the 95% confidence limits, then the run was accepted as valid.

(2)If one of two QC results was outside the 95% confidence limits, then the following rules were evaluated. It the QC failed one of these additional rules, the run was considered invalid and the entire analysis was repeated.

(a)1_{3s} – Either QC is outside of a 99% confidence limit.

(b)2_{2s} – Both QCs are outside of 95% confidence limits on the same side of the mean

(c)R_{4s} sequential – Both QCs are outside of 95% confidence limits on opposite sides of the mean

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

The mean values and limits of each QC pool remained constant throughout each study; however, they were reestablished after each study to ensure the most accurate limits were used.

E. Sample Preparation

All urine, reagents, and calibration standards were brought to room temperature. A 2 mL aliquot of the appropriate sample was pipetted into a 15 mL tube with screw cap and spiked with 50 μL of the combined internal standard spiking solution, using an automatic Gilson 215 liquid handler (Gilson, Middleton, Wisc.), to give an approximate 12.5 μg/L concentration of the internal standard in the urine. To hydrolyze possible glucuronide or sulfate-conjugated metabolites, β-glucoronidase in 0.1 M acetate buffer (pH 4.5) was used. Samples were incubated for 17 hours at 37 °C. The urine hydrolysates were extracted using SPE cartridges. First, samples were acidified with 250 µL of 2 M H_2SO_4 and mixed. Generally, 3 cc Oasis ® cartridges were used for SPE and were preconditioned with 1 mL of a 20 % ethyl ether/BuCL solution followed by 1 mL MeOH, and 1mL of 0.05N HCl solution. Samples were applied to the cartridges and the cartridges were washed with a 5% MeOH solution. Samples were eluted with 4 mL ethyl ether: BuCl (1:4) into conical centrifuge tubes. 1 mL 3N NaOH was added to each extract and vortex was mixed to extract the analytes from the organic phase back into the aqueous phase. The organic layer was discarded and the aqueous layer was transferred into a round bottom 15 mL centrifuge tube. The chloropropyl ether derivatives of the target analytes were made by adding 0.5 mL 0.4 M $TBAHSO_4$ and 0.5 mL 1-chloro-3-iodopropane:BuCl (1:5) and incubating in a 60 °C dry bath for 1 hour. The target analyte derivatives were separated from the reaction mixtures using 3 cc ChemElut ® sorbent-immobilized liquid/liquid extraction cartridges. The reaction mixtures were applied to the cartridges and allowed to sit for about 5 min. The analytes were eluted from the cartridges with 8 mL (2 x 4mL) of hexane and collected in a conical 15 mL centrifuge tube. The samples were evaporated to dryness using a Turbovap LV Evaporator (Zymark, Hopkinton, Mass.) at 30 °C and 10 psi of nitrogen for approximately 30 minutes. Samples were reconstituted with 75 μL of toluene and transferred to autosampler vials, capped, and stored at – 20 °C until analyzed.

F. Quantification

Calibration plots were constructed for each analytical run with seven

analyte concentrations, ranging between 0.20 to 125 ng/mL, which were plotted against 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 LODs 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. All contributions from the labeled ion to the native ion channels and vice versa were accounted for in the final calculations.

G. Method Validation

(1) Daily Operating Protocol

A typical sample batch included 1 blank urine sample, 36 unknown samples, 1 low QC, 1 high QC, and 7 standards. Before daily instrumental analysis, a known standard was analyzed to confirm acceptable chromatographic resolution and mass spectral sensitivity. At the end of the run, we required that the data of blank and QC samples met clear specifications before we considered an unknown batch of sample data valid.

(2)Limits of Detection

The LODs for each analyte were calculated 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. The calculated LOD was verified as a reasonable estimate by injecting concentrations of the analytes at the LOD.

(3)Extraction Recoveries

The extraction recoveries of the method were determined at three concentrations (6, 25 and 100 μg/L) that spanned the calibration range and where at least one sample was near the expected range of unknown samples. The recoveries were measured by spiking six “blank” urine samples (2 mL) with the appropriate native standard spiking solution and preparing the samples according to the method. Six additional “blank” urine samples (unspiked) were prepared concurrently. After the SPE step, all extracts were spiked with 50 µL ISTD to correct for instrument variation, which 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. The sample preparation after the extraction step was completed according to the method and 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 Recovery

Relative recovery is defined as the ability of the method to quantify the spiked value, regardless of analyte losses through the sample preparation procedure. The relative recovery of the method was evaluated by spiking “blank” urine samples at different concentrations spanning the range of

expected unknown sample concentrations, processing through the method, and calculating the resulting concentration as if the sample had an unknown concentration. A linear regression analysis was performed on a plot of the measured concentration versus the expected concentration. The slope of the resulting line was evaluated. A slope of 1 would indicate 100% relative recovery.

(5)Precision

The method precision was determined by calculating the coefficient of variation (CV) of repeat measurements of the QC materials at two concentrations (about 10 μg/L and 20 μg/L). At least 42 repeat measurements over a 2-month period were used in the calculations. These measurements were made in consecutive runs representing data from a single human study described below.

(6) Human Studies

Urine samples were collected as part of the National Health and Nutrition Examination Survey conducted by the National Center for Health Statistics at CDC. Upon collection, samples were frozen within 4 hours and were stored at -20 °C until 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 2000 urine samples from adults and children were analyzed using this method to validate the speed and ruggedness of the analysis.

H. Manual Equipment

(1) Water bath - Equate, Curtin Matheson Scientific
(2) Solid phase extraction vacuum manifold - Supelco, Inc., Bellefonte, Pennsylvania
(3) Concentrator - AS290 Speedway, Savant Instruments, Inc., Farmingdale, NY or Turbovap® LV Evaporator, Zymark Corporation, Framingham, MA
(4) Microbalance - Sartorial Ultra micro, Westbury, NY
(5) Rotator - Glass-Col, RD-230
(6) Centrifuge - IEC Centra-7, International Equipment Co.

I. Other Materials

(1) Round bottom 50 mL screw capped tubes (Kim ax, Scientific Services, CDC).
(2) Conical bottom 15 mL screw capped tubes (Pyrex or Kim ax, Scientific Services, CDC).
(3) Graduated, conical bottom 15 mL tubes (Pyrex or Kim ax, Scientific Services, CDC).

(4) Phenolic screw caps with Teflon seals for both sizes of tubes (Corning, Scientific Services, CDC).
(5) EDP2 Pipettes (Rainin Instrument Co., Woburn, MA).
(6) Pipetman (Gilson Co.).
(7) Vortex Genie (Scientific Industries Inc., Springfield, MA).
(8) Silica solid phase extraction columns (Lab Depot, Inc., Cummings, GA).
(9) Micro autosampler vials with aluminum seals (Caltech, Milwaukee, WI)

J. Instrumentation

The analyses are performed on a Finnigan TSQ-7000 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a chemical ionization interface and interfaced to Varian 3400 gas chromatograph (GC) system (Varian, Palo Alto, CA).

(1) Mass spectrometer configuration

(2) Mass spectrometer instrument control (ICL) programs

This ICL procedure initiates a multiple reaction monitoring (MRM) experiment. It sets the instrument to centroid acquisition mode and the ion polarity to positive ions. It scans the precursor to product transformations of the masses. In addition, it sets the collision energy to -10 V and turns on the continuous dynode and sets the electron multiplier voltage (EMULT). While the procedure runs the TSQ-7000, the GC system operates using the temperature program. After the analytes elute from the GC, the ICL procedure turns off the electron current and sets the multiplier to 600V.

* Indicates ^{37}Cl isotope of chloropropyl derivative. Used only for confirmation of analyte.

(3) GC configuration and temperature program

Chromatographic separation is performed on a Varian 3400 gas chromatograph fitted with a model 1075 injector and a J&W DB-5, 30 m, fused silica capillary column. The column ID is 0.25 mm and the film thickness is 0.25 micron (J&W, # 22-5032 or equivalent). A two meter length of deactivated fused silica column (0.25mm ID) is attached to the

front of the analytical column using a glass, pre-fitted union (Caltech, #20416 or equivalent). (This 2 meter retention gap traps non-volatile residues that would normally accumulate on the first few inches of the analytical column. See Routine and Periodic Maintenance of Key Components.) Two μL of extract are injected using a CTC A200S autosampler fitted with a Hamilton 725N 10 μL syringe.

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

(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 minimum of five repeat determinations are performed for each point on the standard curve.

(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 0.90. 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) 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 (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; one standard representing the high detection end of the method; one standard representing the low detection end of the method; one standard representing the mid-range) are analyzed per calibration verification runs. 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.
documented.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Instrumental Analysis

(1) GC Conditions

Samples (1 µL) were injected into the gas chromatograph (TraceGC, ThermoQuest, San Jose, Calif.) by splitless injection using an autosampler (CTC A200s, Carrboro, N.C.) with an injection purge delay of 60 s. The GC was coupled to a triple quadrupole mass spectrometer (FinniganTSQ-7000, ThermoFinnigan, San Jose, Calif.). A 30-m J & W (Folsom, Calif.) DB-5MS ([5% phenyl]-methyl polysiloxane, 0.25 µm film thickness, 0.25 mm id) capillary column was used for separation of the chloropropyl ethers of the target analytes. A guard column (deactivated fused silica column, Restek, Bellefonto, Penn.) was used to help extend the useful life span of the analytical column. The temperature of the injector was 250 °C and transfer line was 260 °C. The column temperature was initially 80 °C for 2 min and was then heated linearly using two ranges: to 160 °C at 10 °C/min and then t o 260 °C at 4°C/min. The final temperature of 260 °C was held for 2 min.

(2) Mass Spectrometric Conditions

The chloropropyl ethers of the target analytes were analyzed using multiple reaction monitoring (MRM) except for pentachlorophenol. All of the precursor ions were the pseudomolecular ([M+H]+) 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. Pentachlorophenol was determined by using negative chemical ionization in selected ion mode (SIM). A full auto-tune 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. The product ions for ^{35}Cl precursor ions were selected to maximize specificity, sensitivity, and linear dynamic range. The product ions for ^{37}Cl precursor ions were used only for confirmation purposes and added to the selectivity of the analysis.

Peaks were automatically integrated using the Xcalibur® software (version 1. 3) (ThermoFinnigan, San Jose, Calif.). 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® file and loaded into a Microsoft Access® database for permanent storage. All statistical analyses were performed using SAS software (SAS Institute Inc., Cary, N.C.).

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 0.5 ppb to 50 ppb. Urine samples, whose concentrations exceed these ranges, must be resampled and reanalyzed 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.

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.

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 parent ions at specific masses; 3) that it has two specific daughter ions formed from each of the two parent ions at specific masses; and 4) the ion ratios of the two daughter 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 nondetectable. 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, one 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. An analytical run is considered "out-of-control" if the mean QC value or QC range values (for multiple QCs) are outside the 99% confidence intervals. If two consecutive mean QC values or QC range values are outside the 95% confidence intervals, the second of those runs is considered "out-of-control". Any data generated from a run that is not in control are not reported. If more than 8 consecutive QCs are on the same side of the mean of the characterized QC material, all operations will be suspended until it is determined whether a bias is present in the method. This is a preventative measure only; the run is not considered "out-of-control".

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 spectrometrsystem 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. 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 an EHLS 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.

13. 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.

14. 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. Due to 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.

15. REFERENCE RANGES (NORMAL VALUES)

Reference values were determined in the Priority Toxicant Reference Range Study. This study was performed to provide reference values in the human population to determine length or severity of an exposure incidence.

16. CRITICAL CALL RESULTS ("PANIC VALUES")

These measurements require significant time for completion. It is unlikely that any result would be a "critical call", which would only be observed in poisonings.

17. 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 five days.

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

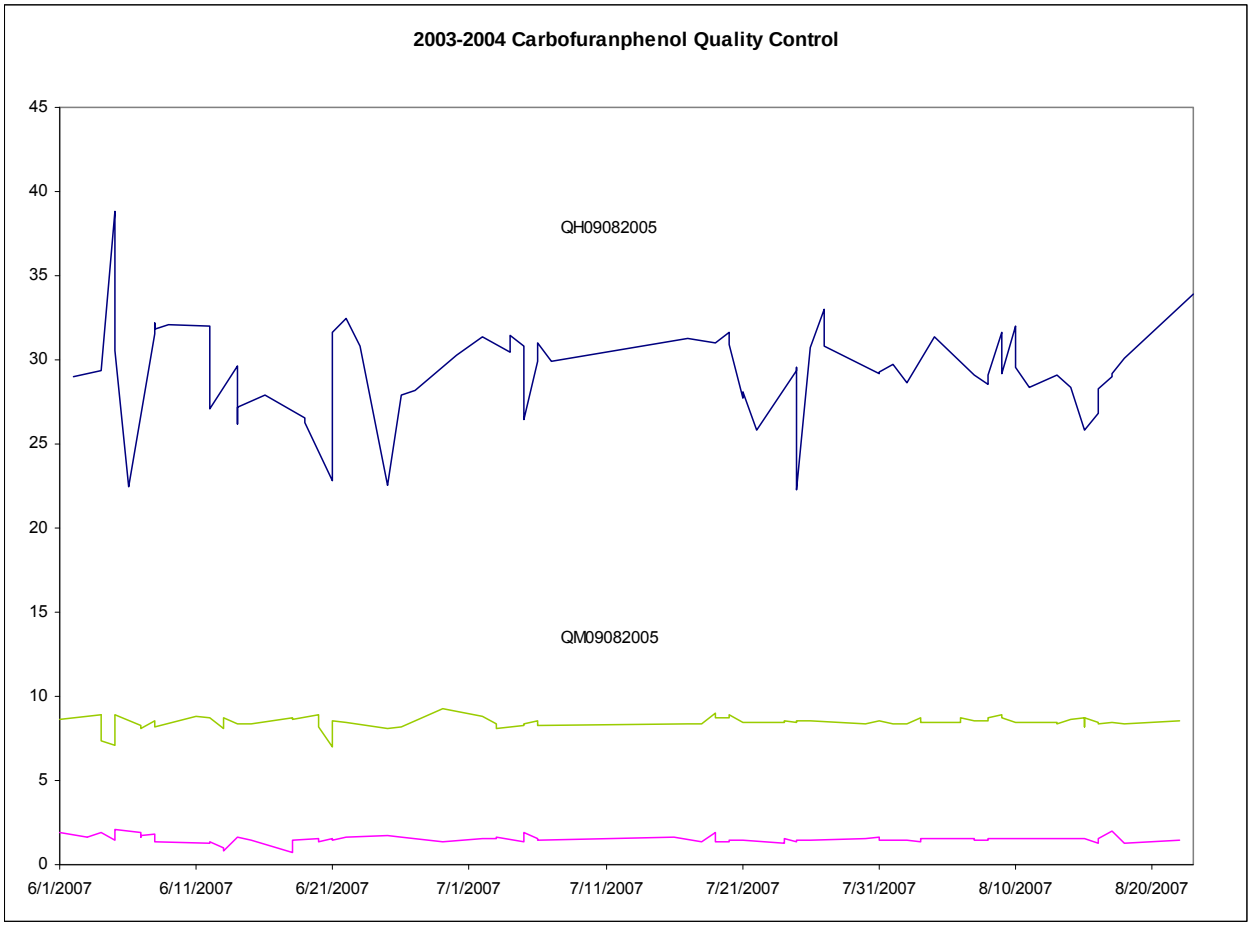
Standard record keeping systems (i.e., notebooks, sample logs, data files, creatinine logs, demographic logs) should be employed to keep track of all specimens. Specimens will only be transferred or refered to CLIA certified laboratories.

19. SUMMARY STATISTICS AND GRAPHS

Summary Statistics for Carbofuranphenol by Lot

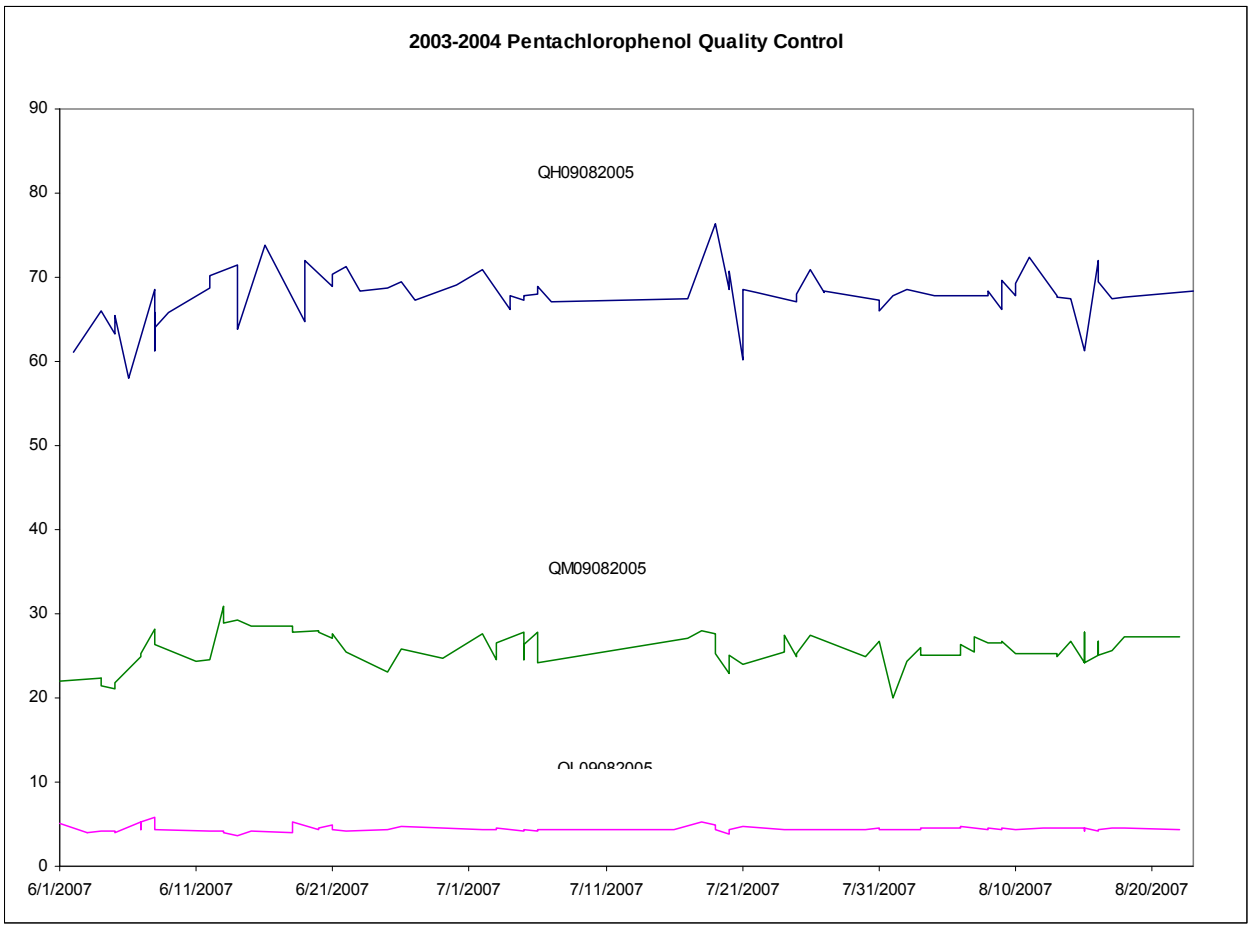
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL09082005	76	6/1/2007	8/22/2007	1.519	0.225	14.8

QM09082005	76	6/1/2007	8/22/2007	8.460	0.361	4.3
QH09082005	74	6/2/2007	8/23/2007	29.333	2.66	9.1



Summary Statistics for Pentachlorophenol by Lot

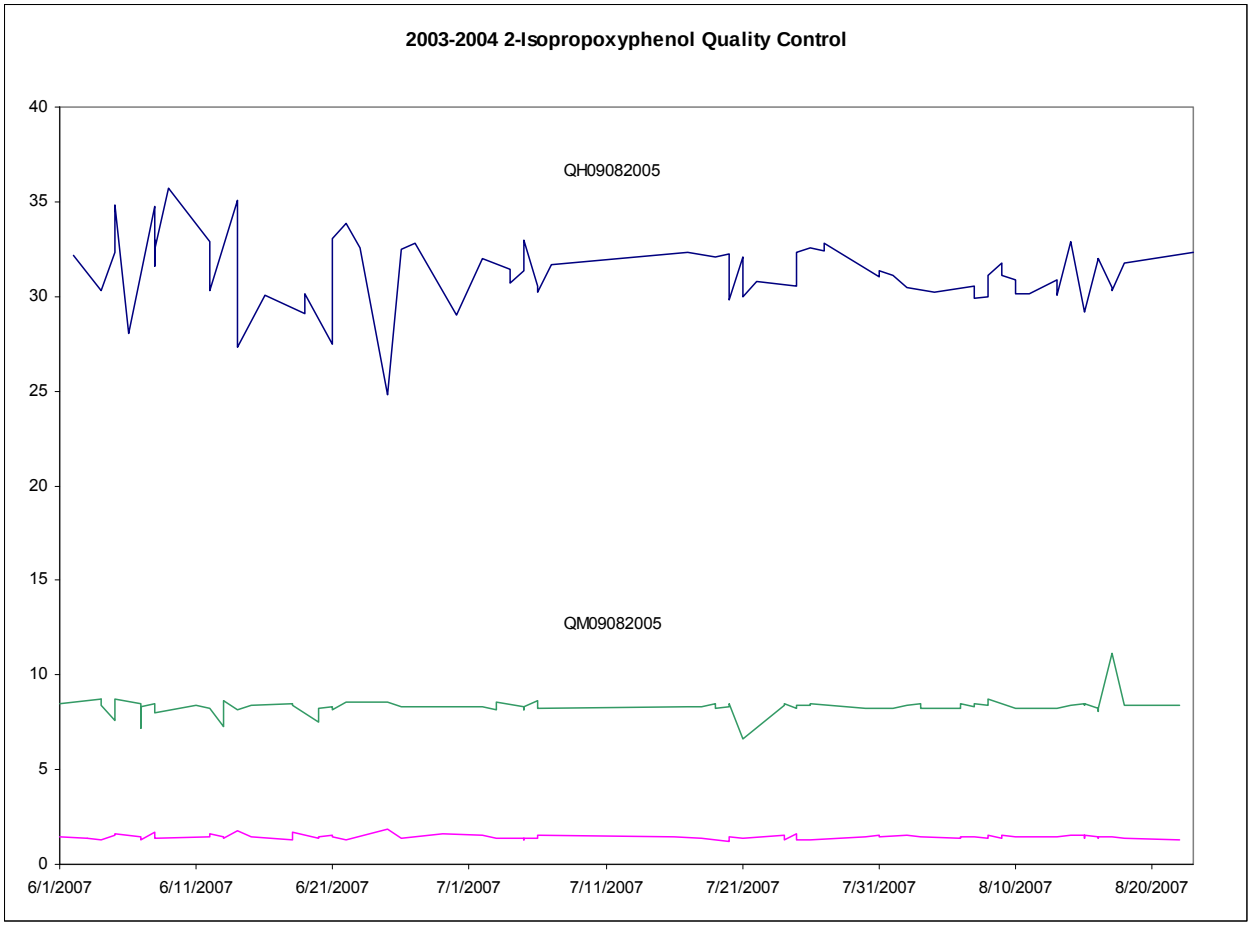
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QL09082005	76	6/1/2007	8/22/2007	4.438	0.342	7.7
QM09082005	75	6/1/2007	8/22/2007	25.848	1.995	7.7
QH09082005	74	6/2/2007	8/23/2007	67.806	2.962	4.4



Summary Statistics for 2-Isopropoxyphenol by Lot

Lot	N	Start Date	End Date	Mean	Standard	Coefficient of

					Deviation	Variation
QL09082005	76	6/1/2007	8/22/2007	1.445	0.113	7.8
QM09082005	76	6/1/2007	8/22/2007	8.371	0.568	6.8
QH09082005	74	6/2/2007	8/23/2007	31.333	1.775	5.7



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