The Arizona Border Study

An Extension of the
Arizona National Human Exposure Assessment Survey (NHEXAS)Study
Sponsored by the Environmental Health Workgroup of the Border XXI Program

Quality Systems and Implementation Plan for Human Exposure Assessment

The University of Arizona
Tucson, Arizona 85721

Cooperative Agreement CR 821560

Standard Operating Procedure **CDC-Compendium**

Title: Compendium of Method Summaries for Collection and Analysis of Metals, VOCs, and Pesticide Metabolites in Blood and Urine

Source: The University of Arizona

U.S. Environmental Protection Agency
Office of Research and Development
Human Exposure & Atmospheric Sciences Division
Human Exposure Research Branch

COMPENDIUM OF METHOD SUMMARIES FOR COLLECTION AND ANALYSIS OF METALS AND VOCs IN BLOOD AND URINE

TABLE OF CONTENTS

Protocol for Urine and Blood Specimen Collection and Shipping for Metals

Introduction

The proper collection, processing, storage and shipment of physiologic specimens from participants is critical to the success of the study. The following sections describe the procedures to be followed for all specimen collections. Please familiarize yourself with the study protocol and insure that you understand the concept of the study, the role of all the personnel involved, and your own role.

Note that subjects are not required to report for blood and urine collection in a fasting state although blood and urine collection should be accomplished early in the visit to avoid discomfort to the subject. Blood and urine collection must be completed and processed under carefully controlled conditions of good laboratory practice. Blood separation and processing must be accomplished promptly to avoid degradation of the specimen.

It is extremely important that all records associated with each subject be maintained in an organized and complete manner to ensure that all information is properly collected and accurate. Specimens should be labeled promptly and processed as a unit or "run" and precautions must be taken to avoid patient-specimen-label-record mix-ups. Careful planning and a well organized work area will keep such errors at a minimum. Some of the information required for the specimen label and shipping list will be collected at the time of specimen collection. Problems in blood and/or urine collection should be noted in the sample log and in the comments section of the shipping list.

URINE COLLECTION AND PROCESSING

Urine collection procedure

1. Materials needed per participant.

   - Urine collection cup (250 mL. plastic, sterile)
   - Preprinted label

2. Preparation of urine collection cup for participant.

   - Remove the collection cup with the cap in place from its plastic wrapping being careful not to dislodge the cap or touch the inside of the container or cap.
   - With the cap securely attached to the container affix the participant's preprinted label marked "URINE CONTAINER" to the urine specimen cup.

3. Instructions for urine collection.

   - The following instructions should be explained to the participant prior to urine collection:

     o Hands should be washed with soap and water.
     o The collection cup should not be opened until just before voiding.
     o The person should leave the cap turned up while voiding, then recap the filled container immediately.
     o It is most important that the inside of the container and the cap not be touched or come into contact with any parts of the body or clothing or external surfaces. Exposure to air should be minimized.
     o The participant should deliver the capped specimen immediately to the clinic personnel.

Urine processing procedure

1. Materials and equipment needed per participant.

   - 15 mL Conical-bottom plastic tube with ORANGE cap (containing Triton X-100 and sulfamic acid as preservative for URINE MERCURY)
   - 15 mL Conical-bottom plastic tube with BLUE cap for URINE METALS
   - Powder-free lab gloves
   - Safety glasses
   - Racks
   - Deionized water (for preparing lab blanks)
   - Preprinted labels
   - Freezer (-20°C)

2. Special safety precautions.
   UNIVERSAL PRECAUTIONS (See CDC Publications for HIV-Hepatitis)

3. Processing (urine specimen)

   - Wear powder-free lab gloves, safety glasses, and work under a laboratory hood, if available.
   - Using the preprinted labels provided for each participant, add the date collected and the initials of the person preparing the aliquots and label each of the plastic tubes as follows:

Priority	Size/Type Bottle	Label
1.	15 mL ORANGE capped plastic	"URINE MERCURY"
2.	15 mL BLUE capped plastic	"URINE METALS"

- Gently swirl the specimen in the capped collection container to resuspend any solids.
- The ORANGE capped tube for "URINE MERCURY" should be filled to the 10 mL mark. THIS TUBE MUST BE EXACT (+/- 1 mL) AS TO THE AMOUNT OF URINE ADDED TO ENSURE THE PROPER RATIO OF URINE TO PRESERVATIVE.
- The BLUE capped tube for "URINE METALS" should have urine poured to the 10 mL mark. Concentrated nitric acid will be added later (at CDC) as a preservative.
- Recap and tightly seal the ORANGE capped tube and the BLUE capped tube.
- Dissolve the preservative in the ORANGE capped tube for "URINE MERCURY" by gently mixing 5-10 times on a mixer or by gently inverting the tube by hand.

4. Preparation of laboratory blanks

   - Wearing protective clothing and working under a hood, prepare one laboratory blank for "URINE MERCURY" and "URINE METALS" each day on which urine specimens are collected.
   - Select one of the 15 mL ORANGE capped plastic tubes with preservative already added and slowly add deionized water to the 10 mL line. Mix well and add a label printed for "URINE MERCURY LAB BLANK" and add the date on which it was prepared.
   - Select one of the 15 mL BLUE capped plastic tubes and add deionized water to the 10 mL line (SEE BELOW FOR INSTRUCTIONS). Cap, label with the preprinted label "URINE METALS LAB BLANK" and invert the tube five times.

5. Urine storage and shipping
   - Urine specimens should be kept frozen (-20°C) until shipment.
   - For shipment instructions, see following section: "Frozen and refrigerated specimen packing and shipping"

WHOLE BLOOD COLLECTION AND PROCESSING

NOTE: **Universal Precautions** - procedures to prevent exposure to HIV; hepatitis; etc are ASSUMED during all collection and handling of biological specimens. ALL specimens should be considered POTENTIALLY INFECTIOUS (if needed, see CDC guidelines on universal precautions).

Whole blood collection procedure

1. Materials needed per participant.

   - Gauze sponges
   - Alcohol wipe
   - Bandaid
   - 3 mL purple top tube
   - 21g 3/4" butterfly assembly with multiple sample luer adapter, sterile
   - 23g 3/4" butterfly assembly with multiple sample luer adapter for children and difficult sticks.
   - 21g or 22g Vacutainer multiple sample needles
   - 10 cc plastic syringe for children
   - Preprinted labels
   - Tourniquet
   - Vacutainer holder and adapters for pediatric tubes
   - Refrigerator
   - White storage boxes

2. Venipuncture procedure.

   - Locate a suitable table and chair for blood collecting and lay out blood collection supplies.
   - Locate the puncture site. Hold with 2 fingers on one side of the "alcohol wipe" so that only the other side touches the puncture site. Wipe the area in a circular motion beginning with a narrow radius and moving outward so as not to cross over the area already cleaned. Repeat with a second alcohol wipe.
   - Locate vein and cleanse in manner previously described, then apply the tourniquet. If it is necessary to feel the vein again, do so; but after you feel it, cleanse with alcohol prep again, and dry with a sterile gauze square.
   - Fix the vein by pressing down on the vein about 1 inch below the proposed point of entry into the skin and pull the skin taut.
   - Approach the vein in the same direction the vein is running, holding the needle so that it is at an approximately 15^{0} angle with the examinee's arm.
   - Push the needle, with bevel facing up, firmly and deliberately into the vein. Activate the vacuum collection tube. If the needle is in the vein, blood will flow freely into the tube. If no blood enters the tube, probe for the vein until entry is indicated by blood flowing into the tube.

- For collection, loosen the tourniquet immediately after blood flow is established and release entirely as the last tube fills.
- If a syringe is required to obtain the blood, attach it to the appropriate size butterfly needle and withdraw 2-3 mLs blood. After withdrawing the needle from the arm, quickly change the needle on the syringe and transfer the blood from the syringe by puncturing the top of the tube with the new needle and allowing the vacuum to draw the blood into the tube. Mix well with the anticoagulant.
- When the needle is out of the arm, press gauze firmly on the puncture. Heavy pressure as the needle is being withdrawn should be avoided because it may cause the sharp point of the needle to cut the vein.
- Have the examinee raise his arm (not bend it) and continue to hold the gauze in place for several minutes. This will help prevent hematomas.
- Report to the physician any reaction experienced by the participant during the venipuncture procedure.
- Label all tubes with the preprinted labels provided, and use a ballpoint pen to add the date collected and your initials to the label. The tubes should be affixed with the label showing the participant's ID number (e.g.92-0024-0001-B1).
- Place a bandaid on the subject's arm.

Whole blood processing procedure

General processing instructions

1. Determine the times 'FEDERAL EXPRESS' packages are picked up in order to connect with the best flights to Atlanta, Georgia provided the specimens are to shipped there. If Federal Express is not used as the overnight carrier, then schedules for package pick-up should be established at the outset of the study with the carrier that is selected. If a contractor is used to coordinate collection and shipment of specimens, they will provide this information.

2. Inquire about regulations in your area concerning shipment of human blood, serum, and urine specimens with dry ice and the quantity of dry ice allowed per shipper. Also, make sure the specimens will be received at CDC within 24 hours if possible.

3. For specimens that should be shipped frozen: Schedule a delivery of dry ice from a local supplier for shipping specimens. A block should be sawed at the plant into 1" slabs. Then each of these should be sawed lengthwise. A 7"x10" slab would fit easily into the shipper without having to break the slab. (Large pieces are preferable to small chunks, since they do not volatilize as rapidly.)

5. For all shipments, do not pack shippers with frozen specimens and dry ice until just before shipment.

6. Telephone the laboratory at CDC the day the shipment is mailed at (404) 488-4305. Speak with Charles Dodson.

Specimen shipping list

1. For each shipment, fill out a blank Specimen Shipping List provided by CDC. If the number of specimens in a shipment is too large to fit on one page, please use the continuation sheets provided. Please give the following information on the blank shipping lists:

   a. Page number - e.g. 1 of 4.
   b. Shipment Number- number shipments sequentially starting with 1.
   c. Number of shippers- total number of shippers containing frozen, refrigerated or unrefrigerated serum and urine specimens which are being mailed in this shipment.
   d. Type of Specimens- whole blood, serum, or urine.
   e. Number of Specimens- number of each type of specimen shipped.
   f. Name, Title, Signature, and Phone Number of person sending shipment or initials as indicated on the continuation sheets.
   g. Date shipped.
   h. Specimen ID for each participant- (e.g. 92-0024-0001). For each participant, check (X) each individual specimen type/aliquot included in this shipment.
   i. Date Collected- e.g. 01-25-92.
   j. Comments- Specify any deviations from collection, storage, and shipment protocols, and date of occurrence.

   Photocopy 2 extra copies of the completed shipping list. As will be described again later, the original will be shipped with the specimens, a copy mailed to CDC under separate cover, and a copy retained for your records.

Frozen and refrigerated specimen packing and shipping

1. Materials needed per shipper

- 1 styrofoam shipper
- 10-12 lbs dry ice FOR FROZEN SPECIMENS ONLY
- Frozen ice packs FOR REFRIGERATED SPECIMENS ONLY
- Freezer boxes
- Safety glasses or eye shield
- Strapping tape
- Gloves for handling dry ice and frozen specimens
- Sheets of bubble-pack packing material
- 'FEDERAL EXPRESS' label, preaddressed by Centers for Disease Control personnel
- DRY ICE label FOR FROZEN SPECIMENS ONLY
- KEEP REFRIGERATED label FOR REFRIGERATED SPECIMENS ONLY
- HUMAN BLOOD-THIS SIDE UP label
- CDC 'Specimen Shipping List' (completed)
- Zip-lock bag
- Whole blood, serum and urine specimen

2. Packing procedure

- When packing the shippers, use gloves to handle the dry ice to avoid burning the hands. Glasses or an eye shield should also be worn if the dry ice cakes are to be broken into small pieces. Dry ice will be used to pack the urine samples. Frozen ice packs will be used to pack the blood tubes.
- Place the specimens from each participant in the specimen boxes provided (white cardboard freezer boxes). Place each of these boxes inside one of the zip-lock bags provided.
- Pack the boxes in the bottom of the shipper. If necessary, use sheets of bubble-pack packing material to ensure the specimens vertical position. Put one layer of bubble-pack on top.
- Fill the shipper with dry ice (probably will hold 10-12 lbs) for the urine samples or with several of the frozen ice packs for the blood samples.
- Place more bubble material to even the top and place the polyfoam lid on top of the shipper.
- Insert the completed 'Specimen Shipping List' in a 12"x12" Zip-lock bag and secure to the top of the polyfoam lid with filament tape. (Remember to photocopy 2 copies of the 'Specimen Shipping List'. Keep one copy for your records and mail the other copy in a separate envelope to the following address:

  Charles Dodson Mailstop F25
  Chamblee Bldg. 17, Loading Dock
  Centers for Disease Control
  1600 Clifton Road N.E.
  Atlanta, GA 30333

- Secure the outer cardboard lid on the shipper with filament tape.

3. Shipping procedure. SEE NOTE BELOW

- Cover or remove previous address labels on all shippers.
- Label each shipper with the following:

  Charles Dodson
  Chamblee Bldg. 17, Loading Dock
  Centers for Disease Control
  4770 Buford Highway
  Chamblee, GA 30341

- HUMAN BLOOD label
- DRY ICE label for shipper with the URINE specimens - fill in the spaces with the amount of dry ice
- REFRIGERATE-DO NOT FREEZE label for the BLOOD specimens
- GENERAL DIAGNOSTIC SPECIMENS - NON-INFECTIOUS label

If 'FEDERAL EXPRESS' is being used as the carrier, call their office at 1-800-238-5355 to arrange for pick-up. Telephone the laboratory at CDC the day the shipment is mailed at (404) 488-4305 and speak with Charles Dodson.

NOTE: Any correspondence sent through the postal service will only be delivered to the Clifton Road address and forwarded from there to the Chamblee facility. Federal Express delivers to the actual physical address zip-code, therefore use the Chamblee address for any shipments by FedEx.

Pesticide metabolites in human urine

1.0 Biomarkers Measured

Pesticide metabolites can be measured in 10 mL of human urine. Table 1 lists these analytes and their corresponding parents.

Table 1. Pesticide metabolites and their parent compounds

Metabolites	Parent Compound(s)
2-isopropoxyphenol (IPP)	propoxur
2,5-dichlorophenol (25DCP)	1,4-dichlorobenzene
2,4-dichlorophenol (24DCP)	1,3-dichlorobenzene, dichlofenthion, prothiofos, phosdiphen
carbofuranphenol [2,3-dihydro-2,2-dimethyl-7-hydroxybenzofuran] (CFP)	carbofuran, benfuracarb, carbosulfan, furathiocarb
2,4,6-trichlorophenol (246TCP)	1,3,5-trichlorobenzene, hexachlorobenzene, lindane
3,5,6-trichloro-2-pyridinol (TCPY)	chlorpyrifos, chlorpyrifos-methyl
4-nitrophenol (NP)	parathion, methyl parathion, nitrobenzene, EPN
2,4,5-trichlorophenol (245TCP)	1,2,4-trichlorobenzene, fenchlorphos, trichloronate
1-naphthol (1NAP)	naphthalene, carbaryl
2-naphthol (2NAP)	naphthalene
2,4-dichlorophenoxyacetic acid (24D)	2,4-D
pentachlorophenol (PCP)	pentachlorophenol

2.0 Background and overview

Exposure to hazardous chemicals, such as pesticides or industrial waste, in water, air, or waste dump sites, continues to be a concern for residents living near industrial or contaminated sites. Pesticide applicators and users are clearly at risk of excessive exposure.

These groups would include farmers, farm workers, road workers, exterminators, gardeners, greenhouse workers, fumigators, wood workers, and others making frequent use of pesticides.

Public health officials are frequently asked to assess community and individual risk of health problems from potential exposure to these pesticides. This task is difficult because there is little information to help make decisions about these possible health risks. Measurements of environmental contaminants in air, water, food or soil provide some estimation of potential exposure; however, these multiple sources of exposure make exposure measurements by environmental measurements difficult or impossible. A better measure of an individual's exposure to these contaminants is the direct determination of those chemicals or their metabolites in human tissues or fluids such as serum or urine, a technique sometimes called biological monitoring.

As part of the Priority Toxicant Reference Range Study, we developed a reliable method for the measurement of a diverse group of urinary analytes representing exposure to pesticides. The analytes, shown in Table 1, are common pesticides or their metabolites to which many "normal" people are exposed through their food, water, or air. We had previous experience in measuring urinary phenols in low parts-per-billion concentrations [1,2], and used that experience as basis for method development, but modified the procedure to better suit our present needs.

The method was used to measure these 12 analytes in the urine of 1000 U.S. residents. The data from this study provide a baseline of "normal" or reference values for use in judging the extent of exposure among other study populations. This information can be used in making assessments of health risk from exposure to these compounds. It may also be useful in determining the relative priority of future studies.

3.0 Sample Collection

3.1 Collection procedure

Urine samples are collected in standard urine collection cups and are transferred to solvent-rinsed 60-mL glass Wheaton vials, which are sealed with Telfon®-coated stoppers. A minimum of 25 mL is requested for analysis in order to provide two 10-mL aliquots for the metabolite measurements, and a 1-mL aliquot for creatinine measurement.

3.2 Short term storage

Samples should be frozen as soon as possible after collection. The frozen samples are transported to the laboratory in shipping containers with dry ice. Samples are stored at -40 °C until analysis.

4.0 Sample Analysis

4.1 Analytical method

The method utilizes capillary gas chromatography tandem mass spectrometry (GC/MS/MS) and an isotope dilution technique with carbon-13 labelled internal standards for all analytes. It involves enzyme hydrolysis of urine, solvent extraction using laboratory

robotics, formation of a chloropropyl derivative using phase-transfer catalysis technique, chromatographic cleanup, sample concentration, and finally analysis using GC/MS/MS.

4.2 Analytical sensitivity and specificity

The limit of detection for all analytes, except 246TCP, is 1 parts per billion (ppb). The limit of detection for 246TCP is 3 ppb.

This highly specific method incorporates the use of capillary gas chromatography combined with tandem mass spectrometric determination. In conventional mass spectrometry used by many laboratories, analytes are broken apart (fragmented) into pieces (ions) and the resulting ion fragment pattern that is detected by the mass spectrometer is characteristic of that analyte. Tandem mass spectrometry, also known as mass spectrometry-mass spectrometry (MS/MS), is a more specific technique in which the ions are originally formed in the mass spectrometer are chosen and selectively broken down into smaller fragments in a second mass spectrometer. The result of tandem mass spectrometry is a very specific analysis for each compound tested. The overall combination of capillary gas chromatography and tandem mass spectrometry provides highly specific determination to assure that the resulting analyses measure the analytes of interest.

4.3 Analytical accuracy and precision

There is no standard reference material containing these pesticide metabolites in urine. The accuracy of this method is based the recovery from urine spiked with the analytes of interest. These spiked urines are used as quality control materials and are analyzed with each run of samples to insure the accuracy of each determination.

The relative standard deviations for quality control pools, spiked with the analytes and analyzed over several months, were generally about 15%.

5.0 Quality Assurance/Quality Control

A critical part of all of our analytical programs is the extensive use of quality assurance and quality control procedures. Quality control procedures adhere to standards recommended in reference 3. The performance of the mass spectrometer is evaluated and optimized before every analysis. Analytical standards are analyzed with samples and quality control samples to assure that all systems were performing according to established criteria. Quality control urine pools were developed from bulk urine spiked with analytes. The urine was sterile-filtered, dispensed into 20 mL-solvent rinsed, sterile glass Wheaton vials. These quality control samples were stored at -40 °C until analysis. Quality control samples were analyzed with each sample run and individual quality control charts were maintained to assure that the analyses were within 99% quality control limits.

6.0 Banking of samples

Quality control samples spiked with these pesticide metabolites have been stored at -40 °C for extended periods of time. Analyses of these samples have shown that the analytes are stable for at least one year.

Urine samples should be stored at -40 °C for extended periods of time. Numerous or frequent freeze-thaw cycles may lead to loss of some analytes.

7.0 Interpretation of Biomarker Measurements

We recently completed measurements of the pesticide metabolites in Table 1 in 1000 persons selected from the Third National Health and Nutrition Examination Survey (NHANES III). Although this population may not be representative of the U.S. population, it provides a good approximation of concentrations of these analytes in the urine of persons living in the U.S. Preliminary results of the analyses of these 1000 persons are shown in Table 2.

Table 2. Preliminary results of analyses of pesticide metabolites in urine of 1000 persons living in the U.S.

ANALYTE	% DETECTABLE	MEDIAN	95% PERCENTILE
IPP	7%	ND	1.6 ppb
25DCP	97%	28 ppb	760 ppb
24DCP	61%	1.7 ppb	47 ppb
CFP	1.5%	ND	ND
246TCP	12%	ND	3.6 ppb
TCPY	70%	2.1 ppb	11 ppb
NP	34%	ND	3.0 ppb
245TCP	18%	ND	3.0 ppb
1NAP	91%	4.2 ppb	37 ppb
2NAP	75%	3.0 ppb	30 ppb
24D	9.6%	ND	1.8 ppb
PCP	57%	1.3 ppb	7.7 ppb

This data can be used as a basis for comparison for populations that have potential exposure to the parent pesticides (see Table 1). For example it would be expected that most people would have concentrations of IPP (from exposure to propoxur) of 1.6 ppb [the 95% percentile] or less. Most people would not have detectable concentrations of IPP, since only 7% of the 1000 we tested had detectable concentrations.

The half lives of the parent pesticides is relatively short, usually 24 hours or less, resulting in the relatively rapid elimination of the metabolites in urine. Detection of these metabolites indicates relatively recent exposure to the parent pesticide.

References

1. RH Hill Jr, T. To, JS Holler, DM Fast, SJ Smith, LL Needham, S Binder. Residues of chlorinated phenols and phenoxy acid herbicides in the urine of Arkansas children. Arch. Environ. Contam. Toxicol., 18, 469-474 (1989).

2. JS Holler, DM Fast, RH Hill Jr, FL Cardinali, GD Todd, JM McCraw, SL Bailey, LL Needham. Quantification of selected herbicides and chlorinated phenols in urine by using gas chromatography/mass spectrometry/mass spectrometry. J. Analyt. Toxicol., 13, 152-157 (1989).

3. Taylor, JK., "Quality Assurance of Chemical Measurements", Lewis Publishers, Chelsea, MI. 1987.

DIVISION OF ENVIRONMENTAL HEALTH LABORATORY SCIENCES
LABORATORY PROTOCOL

ANALYTE:	**Atrazine, Malathion, and 2,4-D Metabolites**
MATRIX:	**Urine**
METHOD:	**National Human Exposure Assessment Survey (NHEXAS) Organic Liquid Extraction, Liquid Chromatography, Tandem Mass Spectrometry**
METHOD CODE:	
BRANCH:	**Toxicology**

SUPERVISOR: Larry L. Needham _____ _____
supervisor's name | signature | date

PREPARED BY: William J. Driskell _____ _____
author's name | signature | date

Susan L. Head _____ _____
author's name | signature | date

ADOPTED: _____
date

UPDATED: _____
date

DIRECTOR'S SIGNATURE BLOCK:

REVIEWED: _____ _____
date

_____ _____
date

_____ _____
date

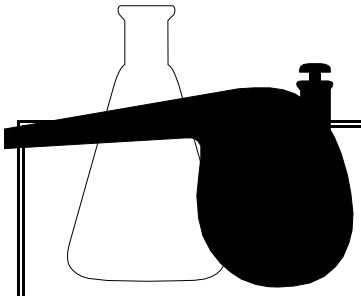
_____ _____
date

_____ _____
date

_____ _____
date

_____ _____
date

MODIFICATIONS/CHANGES: see Procedure Change Log - page I



CLIA Laboratory Account Number: _____

HCFA Analyte Code Number: _____

HCFA Method Code Number: _____

EHLS Method Code: _____

ANALYTE: **National Human Exposure Assessment Survey (NHEXAS) ATRAZINE, MALATHION, and 2,4-D METABOLITES**

MATRIX: **URINE**

as performed by:

Toxicology Branch
Division of Environmental Health Laboratory Sciences

contact:

Dr. Eric J. Sampson, Director
Division of Environmental Health Laboratory Sciences
(404) 488-4151

CDC
CENTERS FOR DISEASE CONTROL AND PREVENTION

PROCEDURE CHANGE LOG

PROCEDURE: **NATIONAL HUMAN EXPOSURE ASSESSMENT SURVEY (NHEXAS) ATRAZINE, MALATHION, and 2,4-D METABOLITES**

EHLS METHOD CODE: _____

DATE	MODIFICATIONS			REVIEWED	
	MADE	BY (Initials)	Date	BY (Initials)	Date

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This method is used for determining concentrations of select pesticide metabolites in the human population. Urine samples are prepared by extracting with an organic solvent and concentration of the solute. The concentrate is analyzed using liquid chromatography combined with tandem mass spectrometric (LC\MS\MS) determination.

The analytes (MS parent compound) are: Atrazine mercapturate (N-acetylcysteine adduct of atrazine), malathion dicarboxylic acid, and 2,4-dichlorophenoxyacetic acid.

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: Formal training and experience in mass spectrometry is necessary.

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 JDNHEX has been set up on the EHLS-PC Network using R:Base SYSTEM V, copyrighted by Microrim INC., 1986. This database is used for storage, retrieval, and analysis of data from the NHEXAS PROJECT. Statistical analysis of data, programming, and reporting is performed using Statistical Analysis System (SAS) software, copyrighted by SAS Institute INC., 1985. Extensive knowledge of the above two software systems is required to design, utilize, maintain, and analyze such a database as this. Additional knowledge of other software systems is encouraged for further data analysis, manipulation, and reporting.

b. Sample Information

In the preparation of each sample run, samples are selected and each sample id # is entered into the R:Base SAMPREP table. Certain fields in the R:Base sample information SAMPREP table have been assigned a default value. Once the first entry is made subsequent entries are not necessary for that particular field unless a different value must be assigned for that particular sample id #. Use of the these tools minimizes operator transcription errors and reduces the amount of keystrokes.

c. Data Maintenance

Integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data, and storage of data in multiple computer systems. Routine backup procedures include: 1) daily backup of hard disks onto magnetic tape and 2) daily backup of the networks R:Base database files onto 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. The specimen should be transferred to specimen vials within 4 hours of collection. A minimum of ten milliliters of urine is collected, and poured, not pipetted, into 60 mL solvent-rinsed glass Wheaton vials. Teflon coated stoppers are used to plug vial and the vial is sealed with an aluminum seal. The specimens are then labeled and frozen immediately to -20 EC, and stored on dry ice for shipping. All samples should be stored at -40EC until processed. Special care must be taken in packing to protect bottles from breakage during shipment. All samples should be stored at -70 EC until processed.

b. Sample Handling

Some problems with breakage due to freezing and subsequent thawing have occurred (see note 9). Samples are thawed, aliquoted, and the residual specimen is again stored at -70 EC 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. Reagents and Sources

REAGENT	MANUFACTURER
Methanol	Sigma Chemical Co.
Acetonitrile	Eastman-Kodak Co.
Acetic acid	Fisher Scientific Co.
Ethyl Ether	Fisher Scientific Co.
Methylene Chloride	Fisher Scientific Co.
Mercapturic acid of atrazine	University of California, Davis
Malathion Dicarboxylic Acid	U.S. Environmental Protection Agency

REAGENT	MANUFACTURER
2,4 Dichlorophenoxyacetic Acid	Sigma Chemical Co.
$^{13}C_6$ Atrazine Mercapturate	Cambridge Isotope Labs, Inc.
D_7 Malathion Dicarboxylic Acid	Cambridge Isotope Labs, Inc.
$^{13}C_6$ 2,4 Dichlorophenoxyacetic Acid	Cambridge Isotope Labs, Inc.

b. Reagent preparation

(1) Liquid chromatography mobile phases:
Phase A: 25:75 acetonitrile:water with 0.2% acetic acid
Combine 250 ml of methanol with 750 ml of HPLC grade water, add 2 ml of glacial acetic acid.
Phase B: 75:25 acetonitrile:water with 0.2% water
Combine 750 ml methanol with 250 ml of HPLC grade water, add 2 ml of glacial acetic acid.

c. Standard Preparation

Due to the toxic nature of the pesticide compounds, all are weighed out on a Sartorius Microbalance inside a plexiglass box that is equipped with an exhaust system.

(1) Stock Solutions of Pesticide metabolites (80 ng/µL):
Weigh 2.0 mg of the pesticide metabolite into a 25-mL volumetric flask. Dilute to volume with acetonitrile. Mix thoroughly. Repeat for each individual compound.

(2) Stock Solutions of Labeled Isotopes (20 ng/µL):
Weigh 0.5 mg of the labeled isotopes into a 25 ml volumetric flask. Dilute to volume with acetonitrile. Mix thoroughly. Repeat for each individual compound.

(3) Combined Labeled Isotope Solution (2.5 ng/µL):
Pipet 1.88 mL of each of the 3 labeled stock isotopes into a 15 -mL volumetric flask. Dilute to volume with acetonitrile. Mix thoroughly. Aliquot 300 uL volumes into 3 ml crimp-sealed vials, which are frozen until use.

(4) Combined Pesticide Metabolite Standard Solution (13 ng/µL):

Pipet 3 mL of each of the stock pesticide metabolites into a 25-mL volumetric flask.

d. Manual Equipment

(1) Vacuum Concentrator - one capable of holding 50-ml tubes and 15-ml tubes. [We use an AS290 Speedvac, Savant Instruments, Inc., Farmingdale, NY]
(2) Microbalance - one capable of weighing accurately and precisely [We use a Sartorius Ultramicro, Westbury, NY]

(3) Rotator - one capable of rotating 50-mL tubes [We use a Glas-Col, RD-230]

(4) Centrifuge - one capable of holding 50-mL tubes [We use an IEC Centra-7, International Equipment Co.]

e. Other Materials

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

(2) Graduated, conical bottom 15-mL tubes (Pyrex or Kimax)

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

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

(5) Pipetman (Gilson Co.)

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

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

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

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

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Calibration of the Mass Spec Systems

The Finnigan TSQ 7000 Tandem Mass Spectrometer requires extensive training and experience and must be calibrated by qualified personnel before analyses.

In this procedure the mass spectrometer is tuned and calibrated on clusters of 2-methoxyethanol. The procedure has three steps: Prepare for tuning and calibrating; cool down the APCI (atmospheric pressure chemical ionization) probe; introduce the tuning solution; autotune in the APCI mode. The tuning solution consists of 200 uL 2-methoxyethanol to which 10 uL of ammonium hydroxide is added. The mass spectrometer is set up in the automatic tuning mode for APCI. Turn off the vaporizer heater. Set the heated capillary to 80 C. Start the flow of sheath gas and set at 100 psi. Introduce the tuning solution to the APCI ion source by flushing about 100 uL with a syringe around the interior surface of the spray shield. Go to the Guide program called "APCI Autotuning". Go to "Setup" and then "Select". Type 3 for 2-methoxyethanol. Click on OK. Click on Both to tune both the first and third quads. The following 2-methoxyethanol cluster masses are used for tuning and mass calibration: 94.087; 170.139; 398.296;626.453. After tuning the mass spectrometer, a standard containing the pesticide analytes is run under the mass spectrometry conditions used for unknown samples. Signal to noise ratios for all the analytes are obtained and compared to signal to noise ratios obtained after previous tunings; signal to noise ratios that vary no more than 20% from the mean of historical values validate the current tune of the mass spectrometer.

b. Standard Calibration Curve

(1) A calibration curve is constructed using at least a five-point curve of relative response factor versus standard concentration. At least seven 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 these curves must in all cases be greater than 0.85 and in more than 95% of the cases greater than 0.95. Linearity of standard curves should extend over the entire standard range, at least two orders of magnitude. 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. Possible sources include incorrect ion ratios, standard concentration error, and transcription errors.

(5) The standard curve should be recalculated to incorporate the newest data points after every five analytical runs. Whenever new labeled compound spiking solutions are prepared, the standard curve must be re-evaluated.

(6) Currently, there are no available standard reference or proficiency testing materials for evaluation of method accuracy. Thus, the proficiency of the method is tested by a system developed by in-house statisticians which involves the semi-annual blind analysis of five (5) randomly selected QC materials. The accuracy is evaluated by analyzing spiked samples and evaluating the results of these analyses. Spiked samples are routinely incorporated as quality control materials in each run. Following any significant change in the procedure, this recovery must be re-established.

c. Calibration Verification

Linearity of calibration curves for each analyte extends over range encompassed in calibration materials used for calibration verification.

(1) Testing Frequency: After every substantive change in method or instrumentation such as cleaning a MS source or using a new lot of reagent or standard. Calibration verification should be performed a minimum of once per 6 months.

(2) Samples per Test: Three samples: one standard representing the low detection end of the method (CV1); one standard representing the mid-range (CV2); one standard representing the high detection end (CV3).

CV Sample	Database Code	Concentration (ppb)		
		Atrazine	Malathion	24D
Low end	CV1	2.5	2.5	2.5
Mid-range	CV2	5.0	5.0	5.0
High end	CV3	15.0	15.0	15.0

(3) Documentation: The preparation and analysis dates and analysis results of the calibration verification samples will be documented in a notebook kept by the database manager. Additionally, any remedial action taken to correct problems encountered during calibration verification will be recorded in the notebook.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Preparation for a Run

Twenty-five unknown samples (including one "blind" QC), two QC samples (a low and a high), and two randomly selected standards are selected for each analytical run (a total of 29 samples per run). These samples are removed from the freezer and thawed (see note 2). The samples are logged into the sample prep analyst procedure Notebook and logged into the R:Base database SAMPREP table in ascending order of position with the run. The following samples must always occupy the same position within any run.

Position 1:	Standard
Position 2:	Standard
Position 3:	Quality Control
Positions 4-28:	Unknowns
Position 29:	Quality Control

The following information is contained within the R:Base database SAMPREP table:

VARIABLE	COMMENTS AND/OR EXAMPLES
SampleID	NHEXAS sample # or XXXXXXX for non NHEXAS samples notebook XXXX-XXX, in the analyst procedure notebook positions 1-4 are equal to the notebook #, positions 6-7 are equal to the notebook page #, and position 8 is equal to the position of the sample within the run (as an alphanumeric character)
CDATE	sample prep date, MM/DD/YY
SampType	unknown (UNK), blank (BLK), standard (STD), quality control pool _ (QC_), calibration verification _ (CV_), proficiency testing _ (PT_), etc.
Matrix	solvent or urine
Std#	defines the standard concentration: if the sample is a standard (STDA, STDB, STDC, STDD, or STDE), for all non standard samples (STDZ)

CRunNo	sample prep date, YYMMDD
CAnalyst	sample prep analyst, 3 initials
InitVol	initial urine volume used, in mL
FinalVol	final sample volume, in uL
SampComm	sample comments
MethComm	method comments
ISCode	vial # of internal standard used in the sample prep run
AnalRun	analytical run #
DBMVer	database manager verification initials, 3 initials
VerDate	database manager verification date, MM/DD/YY

The information entered in the analyst procedure notebook and in the database must be checked for errors and all errors must be corrected. The information is rechecked for errors by the database manager. If no corrections are necessary, a verification date and initials are entered into the database for each of the samples within a given sample prep run #. Once a record has a verification initial and date assigned, the data can no longer be edited by anyone other than the database manager.

After input of sample data, the sample preparation analyst automatically generates a run sheet for the mass spectrometer analyst and labels for the autosampler vials from the R:Base database SAMPREP table.

b. Preparation of Standards

The standards are made using the same urine base pool that was used to make the quality control pools. The urine base pool was split into 5 aliquots. Each pool was spiked with the appropriate amount of combined metabolite stock standard solution to establish a STDA (1 ppb), STDB (2.5 ppb), STDC (5 ppb), STDD (15 ppb), and STDE (50 ppb).

c. Extraction Procedure and Liquid Chromatography Injection

Aliquot 10 ml of urine into a 50 ml round bottom screw cap centrifuge tubes that have been examined (see note 2). Add 100 uL of the combined labeled ISTD to urine and vortex briefly. Adjust the pH of the urine to 3.7 by adding 1 ml of glacial acetic acid. Add 15 ml of 1 part methylene chloride:4 parts ethyl ether to each sample. Place the tube on a rotator and mix for 5 minutes at 30 rpm. After mixing, the tube is centrifuge for 10 min at 2000 rpm. Remove the organic layer to a clean 15 mL conical bottom screw cap tube. Concentrate extractant to between 100 and 200 ul using the AS290 Speedvac set at 25EC. Transfer residual sample to a 250 ul liquid chromatography injection vial. The sample injection volume is 50 ul. Note:

Since there are two injections (one for negative ions and one for positive ions) for two LC runs from each vial, the sample volume used is 100 ul.

d. Liquid Chromatography Conditions

A linear gradient over 6 min with a 1 ml/min flow rate is used from 100% solution A (25:74.9:0.1 methanol:water:acetic acid) to 100% solution B (75:24.9:0.1 methanol:water:acetic acid); the last step is a constant 100% B for 4 min. To reequilibrate column change to 100% A over 1 min by linear gradient; flow 100% A for 6 min. The analytes elute between 3 and 6 min. The chromatography column is a Waters NovaPak 5 micron 2.2 mm diameter, 15 cm length.

e. Mass Spectrometry Conditions

The Finnigan TSQ-7000 was used in the positive ion APCI LC-MS/MS mode to analyze atrazine mercapturate and in the negative ion APCI LC-MS/MS to analyze 2,4-D and malathion mercapturate. Separate runs were made to analyze the positive and negative ions with two injections from each LC sample vial. The vaporizer was set at 450 C. The heated capillary at 150 C. The corona discharge at 5 microamps. The sheath gas was set at 90 psi. Argon was the collision gas and was set at 5 millitorr. In the positive ionization mode the mass spectrometer was set to scan between 3 and 6 min the following parent (par) daughter (dau) masses: Par 343.1 dau 214.1 (atrazine mercapturate); Par 346.1 dau 217.1 (atrazine mercapturate internal standard). In the negative ionization mode the mass spectrometer was set to scan consecutively between 3 and 6 min retention time the following parent daughter masses: Par 219.1 dau 161.1, par 221.1 dau 163.1 (2,4-D). Par 225.1 dau 167.1, par 227.1 dau 169.1 (2,4-D internal standard). Par 273.1 dau 141.1 (malathion diacid). Par 280.1 dau 147.1 (malathion diacid, internal standard). The electron multiplier is set at 2600 kV and the collision offset is set at 25 V.

f. Integrity Check of MS Peak Detection and Integration

A criterion of three times noise level is used to identify a valid peak. The integration software selects peaks that fit this criterion. The mass spec operator monitors the integration process and checks the automatic integration with the option of overriding the software decision.

g. Integrity Check of Importation of MS Data

The data is transferred from the R:Base database MSpec table to an ASCII file and analyzed using a SAS program. The data is evaluated as follows:

(1) All files have been imported for a given mass spec run #
(2) Each files has all of the 3 compounds associated with it

Any missing file or compound are identified and the MS data is reloaded into the R:Base database MSpec table

h. Preliminary Mass Spec Data Evaluation

(1) The data is transferred to an ASCII file and analyzed to eliminate all data which does not qualify as correct. A SAS program is run and the data is examined for the following status:

STATUS	VALUE	REASON
ConcSel	13	Ion Ratio of Ion 1 to Ion 2 is < ion ratio range permitted for that compound, indicating possible contamination with Ion 2
ConcSel	24	Ion Ratio of Ion 1 to Ion 2 is > ion ratio range permitted for that compound, indicating possible contamination with Ion 1
DBMAct	ND	Ion 1 and/or Ion 2 is not present
DBMAct	NA	Ion Ratio of Ion 3 to Ion 4 is < or > ion ratio range permitted for that compound, indicating the predominant ion of the internal standard ion is missing or possible contamination with the second ion of the internal standard OR Ion 3 and /or Ion 4 is not present.
DBMAct	INR	Interference (not resolvable) with Ion 1 and/or Ion 2 indicating possible contamination
DBMAct	ERR	Sample lost in clean-up (i.e., no sample for mass spec analysis)

All of the above conditions will require an examination of the MS chromatograms. Any discrepancy between the chromatogram and the data in the database must be resolved and all errors corrected.

It may require the data being transferred and the SAS program being run several times to resolve all discrepancies.

(2) Examine 'MethComm' (method comment) and 'SAMPCOMM' (sample comment) in the R:Base database SAMPREP table for indication of a sample preparation error or any abnormal observations found during the sample preparation which might affect the results.

(3) After all data as been verified, any result still qualifying to the conditions listed in step 1 must be flagged in the database. The columns - DBMAct, ConcSel, RptStat - in the R:Base database MSpec table are edited as follows:

COLUMN	VALUE	DBMAct	ConcSel	RptStat
IR12	Low		13	
IR12	High		24	
Ht1	. or 0	ND		
Ht2	. or 0	ND		
Ht3	. or 0	NA		NARPT
Ht4	. or 0	NA		NARPT
IR34	Low	NA		NARPT
IR34	High	NA		NARPT

where:

MNEMONIC	REASON
ND	Not acceptable
NA	Not acceptable
13	Concentration calculated on the ion ratio of Ions 1 to 3
24	Concentration calculated on the ion ratio of Ions 2 to 4
NARPT	Not Acceptable, repeat analysis necessary
INRRPT	Interference (not resolvable), not acceptable, repeat analysis necessary
ERRRPT	Sample lost in clean-up, repeat analysis necessary

I. MS Standard Curve Data Verification

(1) All standard data files are transferred to an ASCII file and analyzed using a SAS program which produces a plot of the calibration curve of the relative response between compound and labeled internal standard versus concentration. Examine the outliers to attempt to

ascertain causes. Any determined errors in the R:Base database MSpec table must be corrected and the data transferred again.

(2) Step 1 may have to be repeated to ensure that all errors have been corrected in the R:Base database MSpec table.

(3) Results determined to be a standard curve outlier are flagged by editing the column DBMAct in the R:Base database MSpec table to 'SCO' indicating that particular data point is a 'Standard Curve Outlier'.

(4) Steps 1 thru 3 may have to be repeated to ensure that all standard curve outliers have been either corrected or flagged in the R:Base database MSpec table.

(5) Once all outliers have been identified a SAS program is run which generates current slopes and intercepts for each compound. The results of the regression analysis are examined. Check the R^2 results to make sure they are all above 0.85 and generally above 0.95. Examine any results which do not qualify to determine underlying causes. The newest slopes and intercepts are examined against the slopes and intercepts currently in the R:Base database MSpec table. A significant difference between old and new slopes and intercepts must be examined for underlying causes.

j. Slope and Intercept Update

An automated command file updates the slopes and intercepts, for all the compounds in the data files in the R:Base database MSpec table.

k. Quality Control Material Data Verification

(1) The 'blind' quality control material sample is 'UN-BLINDED' (identified).

(2) All quality control material data files are transferred to an ASCII file and analyzed using a SAS program which produces a graphical plot of the quality control material using the non-updated quality control limits. Examine the plots for outliers of the 95% and 99% confidence limits. Review outliers data to attempt to ascertain causes and correct errors where possible. Any errors in the R:Base SPEC table are corrected and the data is reanalyzed in SAS. [Flag any result determined to be a quality control outlier by editing the column DBMAct in the R:Base database MSpec table to 'QCO' indicating the data point is a 'Quality Control Outlier'.]

(3) The now edited quality control material data files are transferred to an ASCII file. A SAS program is run which produces the numerical data (no plots), without outliers, for the updated quality control limits.

(4) The SAS program that produces the plots of the quality control material is edited to change the plot limits, means, 95 and 99% confidence limits to allow all values to be plotted including the quality control outliers. The SAS program is run and up-to-date quality control material plots are generated.

I. Calculations

(1) Data is transferred from R:BASE to an ASCII file, and calculations are made using a SAS program.

(2) For all analytes the calculations are made as described below (assuming the data passed all previous tests as acceptable).

When the ratio of Ion 1: Ion 2 falls within acceptable ion ratio limits, the relative response factor is calculated as:

$$R_{1234} = \frac{\text{Peak Height Ion 1} + \text{Peak Height Ion 2}}{\text{Peak Height Ion 3} + \text{Peak Height Ion 4}}$$

Where Ion 1 and Ion 2 are the primary analyte ions and where Ion 3 and Ion 4 are the internal standard ions.

When the ratio of Ion 1:Ion 2 falls outside of the acceptable ion ratio limits, alternate methods of calculation are used.
When the ratio of Ion 1:Ion2 is greater than the acceptable ion ratio limits, the relative response factor is calculated as:

$$R_{24} = \frac{\text{Peak Height Ion 2}}{\text{Peak Height Ion 4}}$$

When the ratio of Ion 1:Ion 2 is less than the acceptable ion ratio limits, the relative response factor is calculated as:

$$R_{13} = \frac{\text{Peak Height Ion 1}}{\text{Peak Height Ion 3}}$$

(3) The concentration of the analyte in the sample is calculated using the response factors R_{1234}, R_{13}, or R_{24} in the formula:

$$\text{Conc} = \frac{((R_{1234}, R_{13}, \text{or } R_{24}) - \text{intercept})}{\text{Slope}} \times \frac{10}{\text{Vol. of Sample Used}}$$

The intercept and slope were calculated from the standard curve. The volume of the sample was usually 10 mL. All calculations were automated using an ASCII file transferred from R:BASE and a SAS program.

m. Creatinine Corrected Concentration

Urinary creatinine concentrations, determined elsewhere, are loaded into the R:Base database MSpec table using an automated command file. Creatinine values exist only for the unknown material. Urinary analyte concentrations in µg/L (ng/mL) may be corrected using the following formula:

$$\text{Concentration (µg/g creatinine)} = \frac{\text{Concentration (µg/L)} \times 100}{\text{Creatinine (mg/dL)}}$$

n. Data Verification

The data verification step involves analyzing each of the previous data analysis results to eliminate all data which does not absolutely qualify as correct. The flag columns DBMAct, RptStat, or Report in the database MSpec table are edited as follows:

MNEMONIC			REASON
DBMAct	RptStat	Report	
		NO	Eliminates results not selected for final reporting for samples analyzed more than once
		YES	Selects results for final reporting for samples analyzed more than once
	QCRPT		Analytical run is “Out of Control”, repeat analysis necessary
NA	NARPT		Internal standard ions are not present or the ion ratio is outside

		the acceptable limits, repeat analysis necessary
DIL	DILRPT	Outside of standard curve range, repeat analysis necessary
INR	INRRPT	Interference not resolvable, repeat analysis necessary
ERR	ERRRPT	Sample lost in clean-up, repeat analysis necessary

o. Data Review

Reexamine unknown results for any concentrations which appear to exceed the 95th percentile of the reference population. Also, review results which are above the detection limits for compounds which are often or usually non-detectable. Make any necessary changes.

p. Reporting Results

Using SAS, a report of final results is generated. Concentrations, typically rounded to two significant figures are reported in µg/L and µg/g creatinine.

9. REPORTABLE RANGE OF RESULTS

The linear range of the standard calibration curves determine the highest analytical value of an analyte that is 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 1 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 1 ppb.

c. Accuracy: The accuracy of this method is reflected by spiking samples with known concentrations of analytes and evaluating the results in terms of deviations from expected values.

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 one or two parent ions at specific masses; 3) that it has one or 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 material used for each unknown run was urine spiked with known amounts of the pesticide metabolites.

b. Collection: Two quality control pools were made and are used in each run of unknown samples. The urine for each pool was collected from volunteers.

c. Spiking of Urine: The urine was split into three equal amounts and individually analyzed to determine the baseline values for the pesticide analytes. After establishing this, each pool was spiked with the appropriate amount of the three pesticides to establish a high (15 ppb), low (5 ppb), and blank pool which was left unspiked.

d. Filtration and Dispensing: Each pool was then sterile filtered and dispensed into 20-mL solvent rinsed sterile vials. The vials where plugged, sealed and labeled appropriately. Samples were then frozen at -40 EC until needed.

e. Use of Quality Control Samples: Before beginning runs of unknown samples, each QC pool was characterized. This involved making consecutive runs of QC samples over a period of two weeks. Samples were analyzed and control limits were determined. Subsequent unknown runs contained a high QC, a low QC, and a randomly selected blind QC (either high of low).

f. Data from Quality Control Pools: All data are recorded and stored in an extensive database created for this project.

11. LIMITATION OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The specificity of LC-MS/MS, the use of stable isotope internal standards, and the monitoring of isotope ratios in analytes that contain chlorine atoms cause the effect of interfering substances to be small.

12. REFERENCE RANGES (NORMAL VALUES)

Reference values for concentrations in human urine are presently unavailable.

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

Data is checked to verify there were no sample preparation problems in the run. If the sample is out of established criteria, the sample is repeated for new value.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

These analyses are not applicable to the usual clinical situation and critical call values are not applicable.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Sample extracts are stored in autosampler vials in a -20EC freezer until needed for the mass spectrometer.

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

There are no alternate methods for performing this analysis.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable.

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

Standard record keeping systems (i.e. data files, notebooks) should be employed to keep track of all specimens. All data should be kept a minimum of 3 years. All login sheets are kept with the data coordinator and the project coordinator. Additional copies of unique samples maybe kept by the sample preparation personnel

REFERENCES

There are no references available for this method. Publications describing this method in a general manner are in preparation.

NOTES

(1) OPH_2O is organically pure water made by purifying, in a Barnstead Organic pure system tank, deionized water run through a Mill-Q filtering system.

(2) Special care should be taken to avoid loss of sample when thawing since cracks may occur in the sample containers. It is recommended to put samples in beakers and thaw in refrigerator over night so samples thaw slowly. This practice will prevent loss of sample and injury to laboratory personnel.

(3) All tubes are solvent rinsed with hexane and acetone before being used.

Dioxin and Persistent Organic Pollutants (POPs) Method Summary

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), non-ortho substituted or coplanar polychlorinated biphenyls (cPCBs), other polychlorinated biphenyls (PCBs), persistent chlorinated pesticides and selected pesticide metabolites are measured in serum by high-resolution gas chromatography/isotope-dilution high-resolution mass spectrometry (HRGC/IDMS-HRMS). Serum samples are spiked with $^{13}C_{12}$-labeled internal standards and the analytes of interest are isolated using a C_{18} solid phase extraction (SPE) procedure followed by a multi-column automated cleanup and enrichment procedure using a Fluid Management Systems Power-Prep/6. The analytes are chromatographed on a DB-5ms capillary column (30m x 0.25 mm x 0.25 μm film thickness) using a Hewlett-Packard 6890 gas chromatograph and selected analytes quantified by ID-HRMS using selected ion monitoring (SIM) at 10,000 resolving power using a Thermo Finnigan MAT95 XP mass spectrometer in the EI mode. The concentration of each analyte is calculated from an individual standard linear calibration. Each analytical run is blinded to the analyst and consists of nine unknown serum samples, a method blank, and two quality control samples. After all data are reviewed using comprehensive quality assurance and quality control (QA/QC) procedures, the analytical results are reported on both a whole-weight and lipid-adjusted basis. Serum total lipids are calculated using an enzymatic "summation" method. International toxicity equivalents (I-TEQs) are also reported for PCDDs, PCDFs, cPCBs and other "dioxin-like" PCBs, based on the WHO-TEQ system. Detection limits, on a whole-weight and lipid-adjusted basis, are reported for each sample, corrected for sample weight and analyte recovery. All human serum specimens are handled using *Universal Precautions*. The references for these analytical techniques and QA/QC procedures are listed below.

REFERENCES

Cleanup and Mass Spectrometry

1. Patterson D.G. Jr., Holler J.S., Lapeza C.R., *et al.* High-Resolution Gas Chromatographic/High-Resolution Mass Spectroscopic Analysis of Human Adipose Tissue for 2,3,7,8-TCDD. *Anal. Chem*. 58: 705-713 (1986).

2. Lapeza C.R. Jr., Patterson D.G. Jr., Liddle J.A.. An Automated Apparatus for the Extraction and Enrichment of 2,3,7,8-TCDD in Human Adipose. *Anal. Chem. 58:* 713-716 (1986)..

3. Patterson D.G. Jr., Holler J.S., Belser W.T., Boozer E.L., Lapeza C.R. Jr., Needham L.L. Determination of 2,3,7,8-TCDD in Human Adipose Tissue on Whole Weight and Lipid Bases. *Chemosphere*16: 935-936 (1987).

4. Patterson D.G. Jr., Hampton L., Lapeza C.R. Jr., *et al.* High-Resolution Gas Chromatographic/High-Resolution Mass Spectrometric Analysis of Human Serum on a Whole-Weight and Lipid Basis for 2,3,7,8-TCDD. *Anal. Chem*. 59: 2000-2005 (1987).

5. Patterson D.G. Jr., Alexander L.R., Turner W.E., Isaacs S.G., and Needham L.L. (1990). The Development and Application of a High Resolution Mass Spectrometry Method for Measuring Polychlorinated Dibenzo-p-dioxins and Dibenzofurans in Serum. Chapter 9 In: Instrumentation for Trace Organic Monitoring. Clement R.E., Sui K.M., and Hill H.H. Jr., eds, Lewis Publishers.

6. Patterson D.G. Jr., Isaacs S.G., Alexander L.R., Turner W.E., Hampton L., Bernert J.T., Needham L.L. (1990). Determination of Specific Polychlorinated Dibenzo-p-dioxins and Dibenzofurans in Blood and Adipose Tissue by Isotope-Dilution High Resolution Mass Spectrometry, Method 5 in "Environmental Carcinogens - Methods of Analysis and Exposure Measurement. Volume 11 - Polychlorinated Dibenzo-p-dioxins, Dibenzofurans, and Biphenyls," C. Rappe and H.R. Buser, Eds., WHO, International Association for Research on Cancer, Lyon, France.

7. Turner W., DiPietro E., Cash T.P., McClure P.C., Patterson, D.G Jr., and Shirkhan H. An Improved SPE Extraction and Automated Sample Cleanup Method for Serum PCDDs, PCDFs, and Coplanar PCBs. *Organohalogen Compounds* 19: 31-35 (1994).

8. Turner W., DiPietro E., Lapeza C., Green V., Gill J., Patterson, D.G. , Jr. A Fast Universal Automated Cleanup System for the Isotope-Dilution High-Resolution Mass Spectrometric Analysis of PCDDs, PCDFs, Coplanar PCBs, PCB Congeners, and Persistent Pesticides from the Same Serum Sample. *Organohalogen Compounds* 31: 26-31 (1997).

9. Barr J.B., Maggio V.L., Barr D.B., Turner W.E., Sjodin A., Sandau C.D., Pirkle J.L., Needham L.L., and Patterson D.G. Jr. New High-Resolution Mass Spectrometric Approach for the Measurement of Polychlorinated Biphenyls and Organochlorine Pesticides in Human Serum. J. Chromatography B, 794: 137-148 (2003).

Quality Control and Limit of Detection

1. Taylor J.K. Quality Assurance of Chemical Measurements. *Anal. Chem*. 53: 1588A-1592A, 1596A (1981)..

2. Keith H.K., Crummett W., Deegan J. Jr., *et al.* Principles of Environmental Analysis. *Anal. Chem*. 55: 2210-2218 (1983).

3. Keith L.H . Report Results Right, Part I. *Chemtech* June: 352-356 (1991).

4. Keith L.H . Report Results Right, Part II. *Chemtech* August: 486-489 (1991).

Total Lipid Measurement

1. Akins J.R., Waldrep K., and Bernert J.T. Jr. The Estimation of Total Serum Lipids by a Completely Enzymatic 'Summation' Method. *Clin. Chim. Acta.* 184: 219-226 (1989).

Toxic Equivalency Factors (TEFs).

1. Van den Berg M, Birnbaum L, Bosveld ATC *et al*. Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, and PCDFs for Humans and Wildlife. *Environmental Health Perspectives* 106: 775-792 (1998).

Universal Precautions

1. Universal Precautions. Recommendations for Prevention of HIV Transmission in Health-Care Settings. *MMWR* (Aug 21, 1987) Vol 36 / No 2S: 2S-18S.

Metals

1.0 Biomarkers measured

The following elements can be measured as biological markers of exposure to inorganics in either whole blood or urine.

Metal	Matrix	Analytical Method[1]	Reference
Lead	blood	GFAAS	Miller et al. **Analyst**, 112, 1701-4 (1987)
Mercury	blood	CVAAS	Greenwood et al. **J. Analyt. Tox.** , 1, pp. 265-9 (1977)
Cadmium	blood	GFAAS	Stoeppler and Brandt, **Fresenius Z. Anal. Chem.** 300, 372-80 (1980).
Arsenic	urine	GFAAS	Paschal, Kimberly, and Bailey, **ACA**, 181, 179-86 (1986).
Beryllium	urine	GFAAS	Paschal and Bailey, **At. Spectrosc.** 7, 1-3 (1986).
Cadmium	urine	GFAAS	Pruszkowska et al. **Clin. Chem.** 19, 477-80 (1983)
Chromium	urine	GFAAS	Paschal and Bailey, **At. Spectrosc.** 12, 151-4 (1991).
Lead	urine	GFAAS	Paschal and Kimberly, **At. Spectrosc.** 6, 134-6 (1985).
Mercury	urine	CVAAS	Littlejohn et al., **Clin. Chem.** 22, 1719-23 (1976).
Nickel	urine	GFAAS	Paschal and Bailey, **Sci. Tot. Env.** 89, 305-310 (1989).
Thallium	urine	GFAAS	Paschal and Bailey, **J. Analyt. Tox.** 10, 252-254 (1986).
Vanadium	urine	GFAAS	Paschal and Bailey, **At. Spectrosc.** 11, 65-69 (1990).

[1] Method Abbreviations- GFAAS- graphite furnace AAS; CVAAS- cold vapor AAS; ICP-MS - inductively coupled plasma mass spectrometry; ICAP-OES - inductively coupled argon plasma optical emission spectroscopy

2.0 Background and overview

Exposure to many inorganic toxicants can be determined by measurement of these toxicants in appropriately chosen biological fluids. A classic example is the determination of lead in whole blood, used to determine the "at risk" status of children and adults exposed to lead. EHLS has historically provided support for a wide variety of "field" studies, using well-established biomarkers to document exposure to inorganic toxicants. Among the areas of expertise of this group are modern graphite furnace AAS (GFAAS), cold vapor AAS (CVAAS), inductively coupled argon plasma optical emission spectroscopy (ICAP-OES), and inductively coupled plasma mass spectrometry (ICP-MS).

The following laboratory procedures have been found to significantly enhance quality measurements.

1) use of standards from or traceable to the National Institute of Standards and Technology (NIST);
2) use of the "best available" quality control materials in each analytical run;
3) use of methods and sample collection protocols that have proven to be rugged and reliable;
4) maintenance of a high degree of training of all analysts;
5) use of the appropriate analytical technology for measurement- not necessarily the most "high tech" approach;
6) participation (frequently as a reference laboratory) in a number of external quality control/proficiency testing programs including those from NY State, the State of Pennsylvania, the College of American Pathologists (CAP), the Center of Toxicology at Quebec, Canada (CTQ), and the CDC/Wisconsin PT program.

Most of our analytical methods are "traditional" in approach, typically one element is determined in the appropriate analytical specimen- for example, blood lead - determined by graphite furnace AAS. We have had extensive experience in developing, adapting, and troubleshooting methods of this type. Recently, we have begun to explore the usefulness of multielement techniques for either "screening" specimens for high concentrations of toxic elements, or as a truly quantitative multielement technique. Both ICAP-OES and ICP-MS lend themselves to this approach.

We have developed a "screening" method for toxic elements in urine using ICAP-OES (1), and are in the process of developing a method for ICP-MS (2). The ICP-MS methods are based on either dilution of urine (1+4) with HNO3 containing one or more internal standards; the blood analytical method is based on digestion of whole blood via microwave oven with ultrapure HNO3, followed by dilution with ultrapure water. The final dilution for blood treated this way is typically (1+19).

This general approach for blood analysis has been primarily applied to determination of lead in blood by isotope dilution mass spectrometry (3), a method we use to establish accurate target values for whole blood pools used in a national standardization program (the Blood Lead Laboratory Reference System- BLLRS). To date, we have applied the "multielement" approach in the ICAP-OES determination of multiple toxic elements in drinking water and serum (4), and to establish reference values for elements in urine (5). The

ICP-MS determination of multiple toxic elements in blood and urine, prepared as described above, has been applied to an investigation in Texas (6).

3.0 Sample Collection

Success in biological monitoring depends on a carefully developed and field tested specimen collection and shipping protocol. An extract of this protocol is attached, describing the details of collection and shipping of specimens to the laboratory. This protocol has been field tested and improved for five years, and has served as the basis of training from our staff to personnel tasked with specimen collection in a number of studies sponsored by CDC.

4.0 Sample Analysis

4.1 Method description

The analytical methods used vary from Zeeman effect background correction graphite furnace AAS to inductively coupled plasma mass spectrometry. Literature references for these methods are presented in the Introduction to this document. In many cases, the biological fluid is diluted with an appropriate matrix modifier, and vaporized thermally in a graphite furnace by electrical resistive heating. The resulting ground state neutrally charged atoms are then measured by absorbance of resonance radiation from a "line" source - either a hollow cathode lamp (HCL) or electrodeless discharge lamp (EDL). The methods based on inductively coupled argon plasma (ICP) spectroscopy use the ICP as an emission source (ICAP-OES) or radiation or as a source of positively charged ions (M+) which are subsequently measured by a quadrupole mass spectrometer (ICP-MS).

Quantification is accomplished by measurement of standards (external calibration) carried through the same analytical processes as specimens. In some cases, the same matrix (e.g. blood or urine) is spiked with the element(s) of interest and the resulting spiked material is used for calibration (matrix matched calibration).

4.2 Analytical sensitivity and specificity

Detection limits for the analytes listed are presented in Table 1. These data were calculated by estimation of the standard deviation of a "blank" or a low-concentration processed specimen, usually with ten replicate measurements. The detection limit is the concentration equal to three SD, as recommended by IUPAC.

4.3 Analytical accuracy and precision

Accuracy of these methods has been evaluated using standard reference materials (SRMs) when available. The referenced publications for each method document the accuracy, which in general is about 10% absolute. Precision of the methods is similarly referenced; generally in the 5-15% RSD range.

5.0 Quality assurance and quality control

Our system of quality control for the different methods described here has been detailed in a number of reports and publications. QC methods adhere to standard procedures described in reference 10. The basic approach is the analysis of materials (blood or urine) with known target values in duplicate with each analytical run (blanks, standards, and unknown specimens). The means and ranges of duplicate measurements are plotted on a Shewhart chart and the degree of statistical control of the measurement system is evaluated using standard statistical control criteria (9,10).

Table 1- Detection Limits for Metals

Metal	Matrix	Detection limit (µg/L or PPB)
Arsenic	urine	6
Beryllium	urine	0.6
Cadmium	urine	0.2
Cadmium	blood	0.3
Chromium	urine	0.4
Mercury	urine	0.2
Mercury	blood	0.2
Nickel	urine	1.0
Lead	urine	2.0
Lead	blood	10 (equals 1.0 µg/dL)
Thallium	urine	0.4
Vanadium	urine	1.0

6.0 Banking of samples

Recommended conditions for the storage of collected biological specimens are:

1) for whole blood: 1.5 mg/mL EDTA; -20 °C - stability at least 24 months;

2) for urine (all but mercury)- 1% v/v ultrapure nitric acid; -20 ^{o}C- stability at least 36 months;
3) for urine mercury - 20 mg sulfamic acid; 10 mg (ca. 10 uL) Triton-X-100/10 mL; -20 ^{o}C- stability at least 12 months.

These preservatives are incorporated into the collection and shipping protocol. Stability data are based on published data (D.H. Cox, **J. Analyt Tox.**, 13, pp. 367-70, 1989), and unpublished data from our laboratory (D.C. Paschal, unpublished investigation).

7.0 Interpretation of biomarker measurement

Reference intervals for concentrations of elements in "normal" or non-exposed persons is important for appropriate interpretation of biomarker levels. We have used reference ranges from human studies involving our laboratory and other studies in the literature. In general, the 95% upper limit of concentrations from "normal" subjects may be used as the upper limit of "normal" values for an element in a specific tissue. This limit should be carefully distinguished from the "lowest adverse effect" level, if known. For example, WHO published the 95% upper limit from a large population based study for blood mercury as 30 μg/L total (inorganic and methyl) mercury in whole blood (7). The "lowest adverse effect" level for total blood mercury is reported to be 200 μg/L (8). These two levels are almost one order of magnitude apart- clearly illustrating the difference in these two "limits". A table of reference intervals from selected literature is provided in Tables 2 and 3.

Table 2. Reference Ranges for Metals in Urine

Element	Method	Limit of Detection	Reference Range	Literature Reference
Ag (Sliver)	ICP-MS	0.4	9 µg/dL	1
Al (Aluminum)	ICP-MS	18.5	100 µg/dL	1
As (Arsenic)	GFAA	6	100 µg/L	1
B (Boron)	ICP-MS	180	1000 µg/dL	1
Ba (Barium)	ICP-MS	0.15	80-400 µg/L	2,3
Be (Beryllium)	ICP-MS	0.5	<20 µg/L	4
Bi (Bismuth)	ICP-MS	0.80	<20 µg/L	4
Cd (Cadmium)	GFAA	0.1	<5 µg/L	5
Ce (Cerium)	ICP-MS	0.02	-	-
Co (Cobalt)	ICP-MS	0.30	100-750 µg/L	1
Cs (Cesium)	ICP-MS	0.20	-	-
Ga (Gallium)	ICP-MS	0.05	-	-
Ge (Germanium)	ICP-MS	1.35	1400 µg/dL	1
In (Indium)	ICP-MS	0.05	-	-
Ir (Iridium)	ICP-MS	0.10	-	-
Li (Lithium)	ICP-MS	0.15	-	-
Mn (Manganese)	ICP-MS	0.75	<3 µg/L	1
Mo (Molybdenum)	ICP-MS	0.15	150 µg/dL	1
Pb (Lead)	ICP-MS	0.60	<50 µg/L	6
Pt (Platinum)	ICP-MS	0.25	-	-
Rb (Rubidium)	ICP-MS	1.55	1900 µg/dL	1
Sb (Antimony)	ICP-MS	0.45	6.2 µg/L	1
Se (Selenium)	ICP-MS	59.5	0-150 µg/L	8,9
Sn (Tin)	ICP-MS	2.05	20 µg/d L	1

Element	Method	Limit of Detection	Reference Range	Literature Reference
Sr (Strontium)	ICP-MS	0.05	390 µg/dL	7
Ta (Tantalum)	ICP-MS	0.15	5-250 µg/L	1
Th (Thorium)	ICP-MS	0.05	-	-
U (Uranium)	ICP-MS	0.05	0.05-0.5 µg/dL	1
V (Vanadium)	ICP-MS	0.65	15 µg/dL	1
W (Tungsten)	ICP-MS	0.20	-	-
Y (Yttrium)	ICP-MS	0.02	-	-
Zn (Zinc)	ICP-MS	25.1	500 µg/dL	1
Zr (Zirconium)	ICP-MS	0.05	150 µg/dL	1

Table 3. Metals in Blood - Reference Ranges

Element	Method	Limit of Detection	Reference Range	Literature Reference
Al (Aluminum)	ICP-MS	72.5	6240 µg/L	1
B (Boron)	ICP-MS	81.0	-	-
Ba (Barium)	ICP-MS	11.2	80-400 µg/L	1
Bi (Bismuth)	ICP-MS	1.80	<12 µg/L	1
Ce (Cerium)	ICP-MS	2.16	-	-
Co (Cobalt)	ICP-MS	2.88	0.5-238 µg/L	1
Cs (Cesium)	ICP-MS	2.88	-	-
Ga (Gallium)	ICP-MS	2.70	-	-
Ge (Germanium)	ICP-MS	3.06	-	-
Hg (Mercury)	CVAA	0.2	<30 µg/L	5
In (Indium)	ICP-MS	2.70	-	-
Ir (Iridium)	ICP-MS	1.62	-	-
Li (Lithium)	ICP-MS	3.96	17 µg/L	1
Pb (Lead)	GFAA	4.0	<100 µg/L	6
Pt (Platinum)	ICP-MS	2.16	-	-
Mo (Molybdenum)	ICP-MS	4.86	5-157.3 µg/L	1
Rb (Rubidium)	ICP-MS	156	2800 µg/L	1
Sb (Antimony)	ICP-MS	3.78	3 µg/L	1
Sn (Tin)	ICP-MS	7.38	7-11 µg/L	7
Sr (Strontium)	ICP-MS	4.50	<3.6 µg/L	1
Ta (Tantalum)	ICP-MS	1.80	16 µg/L	1
W (Tungsten)	ICP-MS	2.70	-	-
Y (Yttrium)	ICP-MS	173	-	-
Zr (Zirconium)	ICP-MS	3.42	10-20 µg/L	1

[1] Analytical methods: GFAA = graphite furnace atomic absorption spectroscopy; ICP-MS = inductively coupled plasma - mass spectroscopy; CVAA = cold vapor atomic absorption spectroscopy.

[2] All elements are reported in concentration units of µg/L or ppb. Det. Limit = Detection Limit; Ref. Range = Reference Range. Reference ranges for some elements are presented as the estimated total excretion in µg per day (µg/d).

1. B.L. Carson et al., **Toxicology and Biological Monitoring of Metals** in Humans, Lewis Publishers, Inc. 1986.
2. W. Gooddy, E.I. Hamilton, T.R. Williams (1975) **Brain 98**:65.
3. Y. Mauras, P. Allain (1979) **Anal. Chim. Acta 110**:271.
4. Norbert W. Tietz, **Textbook of Clinical Chemistry** W.B. Saunders (1986).
5. WHO, Special Publication 647 (1980), Geneva, Switzerland.
6. CDC, internal report.
7. Eric J. Underwood, **Trace Elements in Human and Animal Nutrition**, 4th ed., 1977.
8. R.C. Dickson, R.H. Tomlinson (1967) **Clin. Chim. Acta 16**:311.
9. B. Welz, M. Melcher, G. Schlemmer (1983) **Fresenius Z. Anal. Chem. 316**:271.

References:

1) Kimberly and Paschal, **ACA**, 174, 203-10 (1985).
2) Paschal, Caldwell, and Ting, manuscript in preparation.
3) Paschal, Caldwell, and Ting, manuscript in preparation.
4) DiPietro et al., **Sci. Tot. Env.**, 74, 249-62 (1988).
5) Paschal- private communications to Fisher Chemical Co.
6) Paschal et al. "Multi-Element Screening of Blood and Urine"- Supplemental Report August 28, 1992.
7) Goldwater et al. (cited in Mercury in the Environment, CRC Press Boca Raton FL 1977)
8) Sherlock et al. **Arch. Env. Health** 37, 271-8 (1982).
9) Hainline et al., "Selected Methods for the Small Clinical Chemistry Laboratory", AACC, Washington, D.C. 1982.
10) Taylor, JK., "Quality Assurance of Chemical Measurements", Lewis Publishers, Chelsea, MI, 1987.

Volatile Organic Compounds (VOCs)

1.0 Biomarkers measured

The following VOCs can be measured in 10 mL of human blood:

1,1,1-Trichloroethane
1,1,2,2-Tetrachloroethane
1,1,2-Trichloroethane
1,1-Dichloroethane
1,1-Dichloroethene
1,2-Dichlorobenzene
1,2-Dichloroethane
1,2-Dichloropropane
1,3-Dichlorobenzene
1,4-Dichlorobenzene
2-Butanone
Acetone
Benzene
Bromodichloromethane
Bromoform
Carbon Tetrachloride
Chlorobenzene
Chloroform
cis-1,2-Dichloroethene
Dibromochloromethane
Dibromomethane
Ethylbenzene
Hexachloroethane
m-/p-Xylene
Methylene chloride
o-Xylene
Styrene
Tetrachloroethene
Toluene
trans-1,2-Dichloroethene
Trichloroethene

2.0 Background and overview

In our society, individuals are exposed to volatile organic compounds (VOCs) from many sources. VOC exposure occurs during many normal everyday activities which involve most if not all of the citizens of developed countries. These activities include drycleaning of clothes, house painting (especially indoor), furniture refinishing, gasoline dispensing, automobile transportation (especially stop and go), showering, and tobacco smoking. In addition, many individuals are exposed at their workplaces, including chemical manufacturing industries, oil refineries, offices in close proximity to copiers or other printing machines, drycleaning establishments, oil fields, as well as many other industries.

VOCs have been linked to neurological, hematologic, and hepatic health effects [1,2]. The combination of ubiquitous exposure and possible serious health effects makes VOCs a major public health concern.

VOCs can be measured in environmental media (air, water, soil, food) and biological specimens. A discussion of the usefulness of toxicant measurements in environmental media and the usefulness of toxicant measurements in biological specimens was given earlier in: Rationale for the use of biomarkers in exposure assessment. For the purposes of this discussion, breath will be included as a biological specimen.

Attempts to determine internal dose levels of VOCs have concentrated on measuring the native compounds in blood, the native compounds in exhaled breath or metabolites of these compounds in urine. Since breath is considered to be the major pathway for removing VOCs from the body, numerous attempts have been made to relate breath levels to levels of VOCs in environmental media [3-6]. Approaches which have focused on measurement of metabolites in urine have run into difficulty with specificity since many metabolites of VOCs are also metabolites of other compounds [7]. Directly measuring VOCs in blood estimates the concentrations of these compounds that are circulating through the body and contacting the potential target site(s) of effect.

Early studies of VOCs in blood concentrated on only one or a few particular analytes [8-11], whereas, in more recent studies, the number of VOCs under investigation has expanded [12-17]. Generally, the methods used in these studies have had detection limits for VOCs in blood in the high parts per trillion (ppt) to high parts per billion (ppb) range. These detection limits are sufficient to measure VOC levels in persons with occupational or high non-occupational exposure, but are not sufficient to examine the exposures which occur commonly in individuals who encounter VOCs in their homes, automobiles, and in workplaces not normally associated with chemical exposure. To assess these exposures, a detection limit in the low ppt range is needed. Previously published methods have also 1) required large blood samples (50 mL or more), and 2) not adequately addressed sample contamination, especially from sample collection materials.

3.0 Sample collection

3.1 Collection procedure

Vacutainer tubes obtained from commercial sources contain VOC contamination which can greatly interfere with the ability to obtain analytical results which estimate the extent of exposure. We therefore modify commercially available blood collection tubes so that they no longer contain measurable levels of most VOCs. The vacuum on individual vacutainers is released and the glass tubes and rubber stoppers are both heated for 2 weeks at 70°C under vacuum. The vacutainers are reassembled, the vacuum restored and the vacutainers sterilized using a Cobalt-60 radiation source. This process produces a sampling container with substantially less contamination.

Isopropanol used to disinfect the venipuncture site has appeared as a contaminant in measuring VOCs. This contaminant is effectively eliminated by swabbing the venipuncture site with a dry gauze bandage after wiping with isopropanol and allowing the site to dry for 5 - 10 seconds.

Whole blood samples are collected by venipuncture into grey top vacutainers which have been prepared as described above. Within 15 minutes of collection, whole blood samples are placed on wet ice or stored at refrigerator temperatures. The anticoagulant used in the prepared tubes is a mixture of sodium oxalate and sodium fluoride. This anticoagulant is chiefly intended to stop metabolism so that VOC levels do not change appreciably during storage. This mixture's ability to prevent clotting of blood is not as great as many other anticoagulants. Thus, once samples have been collected, they must be mixed thoroughly to allow the complete distribution of the anticoagulant. If a blood mixer is available, samples should be placed on this mixer for at least 3 minutes. If a mixer is not available, the blood

tube should be rocked back and forth by hand at least 30 times to completely mix the anticoagulant into the blood sample.

3.2 Short term storage

Since VOCs are highly volatile, care must be taken to insure that samples are kept at refrigerator temperatures during storage and shipment. All samples should be placed on wet ice or into a refrigerator within 30 minutes of sample collection. In addition, samples should be shipped with enough wet ice or equivalent cooling material to insure that the samples will remain cool throughout the shipment process. Samples should not be frozen or stored at freezer temperatures at any time during sample collection and shipment.

4.0 Sample analysis

4.1 Analytical method

The method is a purge and trap gas chromatographic method using high resolution isotope dilution mass spectrometric detection in the full scan mode. Stable isotopically labelled analogs of the compounds of interest are added to 10 mL of blood and this entire sample is injected into a specially designed sparging vessel which is already attached to the purging apparatus via air-tight seals. Prepurified helium gas is bubbled through the blood which is heated to approximately 35°C. This process removes volatile compounds from the sample into the gas stream. The purged volatile compounds pass into and are captured by a Tenax trap.

Once the 15 minute purge cycle is complete, the Tenax trap is purged with dry helium gas for 6 minutes to remove absorbed water. The trap is then heated to 180°C for 4 minutes to desorb all volatile compounds. As the compounds are desorbed, they are trapped at the gas chromatograph injection port by a liquid nitrogen trap at -150°C. Following this period, the site is ballistically heated to 200°C injecting the compounds onto the DB-624 capillary column which is interfaced to the mass spectrometer. The mass spectrometer is operated in the full scan mode (40 - 200 amu) with one scan collected per second. Quantitation is accomplished from specific ion responses relative to those of the corresponding isotopically labelled analogs. Correction is made for the o-xylene contribution to the styrene signal. Final concentrations are calculated based on six-point calibration curves.

4.2 Analytical sensitivity and specificity

Detection limits for these analytes are given in Table I. These values were determined from the plot of the standard deviation of calculated concentration of standards versus concentration [18]. The y-intercept of the least squares fit of this line equals s_0, with $3s_0$ being the calculated detection limit [18]. The detection limits are generally in the low ppt range except for analytes in which the range of standards had to be adjusted because blood levels were much higher (e.g., acetone).

High resolution (3000 resolving power) eliminates most interferences that can have substantial effects on results. By going to high resolution measurements, the mass window

can be reduced to 0.03 mass units. The high selectivity of this small mass window combined with the GC separation provides excellent analytic specificity.

Additional steps are also critical in promoting analytical specificity by removing extraneous compounds from the sample analysis system. Interferences which have their source in the measurement apparatus itself are examined by measuring instrument blanks. For this purpose, a pure water sample remains attached to the measuring apparatus and is examined regularly to check for operational levels of instrument blanks.

Glassware used for standards is treated to remove possible interferences and contamination. All glassware is heated in a vacuum oven at 150°C for at least 8 hours to remove adsorbed volatiles. The vacuum oven used contains an independent vacuum source since cross-contamination from other laboratory operations has been determined to be a major source of contamination of laboratory glassware. This glassware is cooled to room temperature before removal from the oven and sealed to diminish exposure to volatile compounds which are present in laboratory air.

The water used for dilution of standards and as water blanks is an extremely critical potential source of interference. No commercial filtering or purification system was found which could consistently yield water with acceptably low levels of VOCs (< 20 ppt for most analytes). An acceptable source of water was discovered at a non-commercial site and all further studies make use of this source. Under some circumstances even this source of water failed to yield acceptable levels of volatile organic compounds. In this case, the water is further purified by helium refluxing. To prevent further contamination from the laboratory air, water samples are sealed in glass ampules. In all cases, typical blank water levels are below the detection limits given above.

4.3 Analytical accuracy and precision

Since volatile organic compounds are not stable for extended periods in blood, no standard reference material is available. The accuracy basis for this method is established by determining the recovery of spiked blood samples. In addition, new quality control pools are overlapped with existing pools to insure a verified stable accuracy base is maintained over time.

For spiked samples, relative standard deviations are in most cases less than 30%. As expected, most of the exceptions were found in the low spike samples. These standard deviation results are actually higher than would be encountered in typical blood determinations since they include variation in the blood both before and after spiking. Multiple measurements on spiked QC materials show somewhat lower standard deviation results, averaging 19.4% for all analytes combined.

5.0 Quality assurance/quality control

Quality assurance and quality control procedures follow standard practices [18]. Daily experimental checks are made on the stability of the analytical system and standards and quality control materials which are added to each day's run sequence. A 'pure-water' blank sample is run at the beginning of each day to check for the presence of contamination in the purge and trap system or the labelled analog solution. In addition, determination of label ion

counts for this material is used to check daily method sensitivity. Relative retention times are examined for each analyte to insure the choice of the correct chromatographic peak.

Serum quality control materials were developed from bulk serum spiked with the analytes being examined in this study. This material was sealed in ampules and frozen at -60°C to insure long term stability. Quality control materials were developed at two different analyte concentration levels. These samples have proven to be stable over at least two years. Individual quality control charts are created for each analyte examined and quality control limits are used to insure analytical stability. 99% confidence limits are used for analytical control limits.

Table I. Detection limits for VOCs in whole blood

VOC	Detection limit (ppb)
1,1,1-trichloroethane	0.045
1,1,2,2-tetrachloroethane	0.010
1,1,2-trichloroethane	0.018
1,1-dichloroethane	0.007
1,1-dichloroethene	0.010
1,2-dichlorobenzene	0.048
1,2-dichloroethane	0.016
1,2-dichloropropane	0.012
1,3-dichlorobenzene	0.041
1,4-dichlorobenzene	0.042
2-butanone	0.38
acetone	200
benzene	0.026
bromodichloromethane	0.005
bromoform	0.020
carbon tetrachloride	0.011
chlorobenzene	0.010
chloroform	0.015
cis-1,2-dichloroethene	0.032
dibromochloromethane	0.009
dibromomethane	0.032
ethylbenzene	0.025
hexachloroethane	0.035
m-/p-xylene	0.034
methylene chloride	0.081

VOC	Detection limit (ppb)
o-xylene	0.024
styrene	0.022
tetrachloroethene	0.022
toluene	0.059
trans-1,2-dichloroethene	0.012
trichloroethene	0.007

6.0 Banking of samples

Repeat measurements of samples stored at 4°C indicate that whole blood VOC samples may be banked for at least 7 weeks. Since these are whole blood samples, longer storage results in samples which are harder to manipulate and produce additional analytical problems. Thus, even though analytical results may not change over this time, samples may be less amenable to analysis. Preliminary results suggest that the 7 week storage time may not be appropriate for acetone and 2-butanone. These compounds occur naturally in the body, and metabolism may alter their concentration with storage.

Whole blood samples for VOC measurement should be stored at 4°C. This prevents blood cell rupture which would occur at lower temperatures. In addition, freezing of blood can lead to breakage of vacutainers and loss of sample in some cases. Since VOCs are lost whenever the containers in which they are contained are opened, it is not appropriate to transfer the blood samples to another container which would be more resistant to breaking.

7.0 Interpretation of biomarker measurement

Reference ranges for these VOCs have been measured in a sample of 700 to 1000 persons selected from the Third National Health and Nutrition Examination Survey (NHANES III). The sample is not representative of the U.S. population but it is designed to examine the influence of age, sex, race/ethnicity, urban/rural status and region of the country on VOC levels. At this writing, the analytical results are complete and the analysis examining the relationship of demographic variables to blood VOC levels is in progress.

Detectable analyte levels of 12 VOCs have been found in most if not all samples from this reference population. For these 12 VOCs, preliminary results (N approximately 400) are given in Table II for the 5th percentile, mean, median, and 95th percentile.

Table II. Preliminary results: Volatile Organic Compounds levels in a reference population (ppb)

Analyte	N	5th percentile	Mean	Median	95th percentile
1,1,1-trichloroethane	363	ND	0.29	0.13	0.71
1,4-dichlorobenzene	395	0.071	2.40	0.34	14.4
2-butanone	426	2.37	7.72	5.63	18.5
acetone	415	629	2960	1690	8520
benzene	398	0.030	0.14	0.063	0.53
chloroform	421	ND	0.046	0.022	0.099
ethylbenzene	397	ND	0.11	0.061	0.27
m-/p-xylene	426	0.071	0.42	0.19	0.83
o-xylene	422	0.038	0.14	0.10	0.29
styrene	425	ND	0.079	0.041	0.19
tetrachloroethene	426	ND	0.19	0.058	0.60
toluene	405	0.13	0.52	0.29	1.54

Some VOCs are found in less than half of the samples examined in the reference population. These compounds and the percent of samples found above the detection limit are bromodichloromethane, 22%; dibromochloromethane, 22%; and trichloroethene, 20%.

Sixteen of the analytes determined by our method have been found above the detection limits in less than 5% of the normal human blood samples investigated to date. The compounds are 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, 1,1-dichloroethane, 1,1-dichloroethene, 1,2-dichlorobenzene, 1,2-dichloroethane, 1,2-dichloropropane, 1,3-dichlorobenzene, bromoform, carbon tetrachloride, chloro-benzene, cis-1,2-dichloroethene, dibromomethane, hexachloroethane, methylene chloride, and trans-1,2-dichloroethene.

Measurements carried out in our laboratory and previous reports by others [19-23] agree that the elimination of VOCs is multi-phasic with at least three components. The half lives for these components are short compared to many other organic compounds. Preliminary estimates of the three half-lives are within the range 3 - 10 min, 20 - 100 min, and 150 - 1000 min. Preliminary results suggest, at least for some VOCs, that the final long half-life component only accounts for approximately 5-10% of the total elimination following acute exposure, thus these VOCs appear to be largely eliminated from blood within a few hours after exposure has ceased. It is not clear at this time to what extent bioaccumulation occurs in persons with chronic exposure to "low-level" VOC exposures.

Comparison of blood levels of VOCs with environmental measurement is in its initial stages. Our laboratory in collaboration with the Environmental Protection Agency has made

measurements of blood levels resulting from exposure to low levels of some VOCs and compared them with chamber air levels [19]. The results of these measurements are summarized in Table III. This table gives the analyte being examined, the exposure conditions, the air concentrations during exposure, and the blood levels immediately after exposure has ended.

Table III. Volatile Organic Compounds Levels in Blood After Acute Air Exposure.

Analyte	Conditions	Air concentration (mg/m^3)	Peak blood concentration (ppb)
1,1,1-trichloroethane	2-hr exercise	2.92	7.65
1,1,1-trichloroethane	10-hr at rest	1.30	7.38
1,1,1-trichloroethane	2-hr at rest	0.95	5.68
ethylbenzene	10-hr at rest	3.30	14.4
ethylbenzene	2-hr exercise	1.30	7.82
ethylbenzene	4-hr at rest	0.825	2.16
ethylbenzene	4-hr at rest	0.412	1.18
ethylbenzene	2-hr at rest	0.400	1.84
methylene chloride	10-hr at rest	3.05	8.16
methylene chloride	2-hr exercise	3.04	14.5
methylene chloride	2-hr at rest	1.05	5.34
o-xylene	10-hr at rest	2.80	8.22
o-xylene	2-hr exercise	1.00	5.23
o-xylene	2-hr at rest	0.35	1.57
p-xylene	10-hr at rest	10.50	83.4
p-xylene	4-hr at rest	8.250	17.5
p-xylene	2-hr exercise	4.54	36.8

Analyte	Conditions	Air concentration (mg/m^3)	Peak blood concentration (ppb)
p-xylene	4-hr at rest	4.125	10.2
p-xylene	2-hr at rest	1.55	10.8
toluene	10-hr at rest	2.50	10.8
toluene	2-hr exercise	2.08	13.9
toluene	2-hr at rest	0.90	3.70

References

1. Hayden, J.W.; Comstock, E.G.; Comstock, B.S. Clin. Toxicol. **1976**, 9, 169-184.
2. Ramsey, J.D.; Flanagan, R.J. J. Chromatogr. **1982**, 240, 423-444.
3. Droz, P.O.; Krebs, Y.; Nicole, C.; Guillemin, M. Am. Ind. Hyg. Assoc. J. **1988**, 49, 319-324.
4. Berlin, M. Am. J. Ind. Med. **1985**, 7, 365-73.
5. Wallace, L.A.; Pellizzari, E.D.; Hartwell, T.D.; Sparacino, C.; Whitmore, R.; Sheldon, L.; Zelon, H.; Perritt, R. Environ. Res. **1987**, 43, 290-307.
6. Brugnone, F.; Perbellini, L.; Faccini, G.B.; Pasini, F.; Maranelli, G.; Romeo, L.; Gobbi, M.; Zedde, A. Int. Arch. Occup. Environ. Health **1989**, 61, 303-311.
7. Carlsson, A. Scand. J. Work Environ. Health **1982**, 8, 43-55.
8. Snyder, C.A.; Laskin, S.; Goldstein, B. Am. Ind. Hyg. Assoc. J. **1975**, 36, 833-836.
9. Dowty, B.; Carlisle, D.; Laseter, J.L.; Storer, J. Science **1975**, 187, 75-77.
10. Dowty, B.J.; Laseter, J.L.; Storer, J. Pediatr. Res. **1976**, 10, 696-701.
11. Peoples, A.J.; Pfaffenberger, C.D.; Shafik, T.M.; Enos, H.F. Bull. Environ. Contam. Toxicol. **1979**, 23, 244-249.
12. Pfaffenberger, C.D; Peoples, A.J.; Enos, H.F. Int. J. Environ. Anal. Chem. **1980**, 8, 55-65.
13. Barkley, J.; Bunch, J.; Bursey, J.T.; Castillo, N.; Cooper, S.D.; Davis, J.M.; Erickson, M.D.; Harris, B.S.; Kilpatrick, M.; Michael, L.C.; Parks, S.P.; Pellizzari, E.D.; Ray, M.; Smith, D.; Tomer, K.B.; Wagner, R.; Zweidinger, R.A. Biomed. Mass Spectrom. **1980**, 7, 139-47.
14. DeLeon, I.R.; Antoine, S.R. Clin. Ecol. **1985**, 2, 108-109.
15. Kroneld, R. Bull. Environ. Contam. Toxicol. **1985**, 34, 486-496.
16. Antoine, S.R.; DeLeon, I.R.; O'Dell-Smith, R.M. Bull. Environ. Contam. Toxicol. **1986**, 36, 364-371.

17. Cramer, P.H.; Boggess, K.E.; Hosenfeld, J.M.; Remmers, J.C.; Breen, J.J.; Robinson, P.E.; Stroup, C. Bull. Environ. Contam. Toxicol. **1988**, 40, 612-618.
18. Taylor, J.K. Quality Assurance of Chemical Measurements; Lewis Publishers, Inc.: Chelsea, MI, 1987.
19. Raymer, J.H.; Thomas, K.W.; Pellizzari, E.D.; Cooper, S.D.; Smith, D.J.; Kizakevich, P.; Pugh, D.. Preliminary Draft. RTI/4948-31A-02FR. **1992.**
20. Fernandez, J.; Guberan, E.; Caperos, J. Am. Ind. Hyg. Assoc. J. **1976**, 37, 143-150.
21. Monster, A.C.; Boersma, G.; Steenweg, H. Int. Arch. Occup. Environ. Health **1979**, 42, 303-309.
22. Nise, G.; Attewell, R.; Skerfving, S.; Orbek, P. Brit. J. Ind. Med. **1989**, 46, 407-411.
23. Sabourin, P.J.; Medinsky, M.A.; Bechtold, W.E.; Henderson, R.F. Adv. Mod. Environ. Toxicol. **1989**, 16, 153-176.