

Laboratory of Organic Analytical Chemistry
Wadsworth Center
New York State Department of Health

STANDARD OPERATING PROCEDURE

Analyte: Monohydroxy-Polycyclic Aromatic Hydrocarbons (OH-PAHs)

Matrix: Urine

Method: Isotope Dilution Gas Chromatography/High-Resolution Mass Spectrometry *

**This Method was Modified Based on CDC Method No. 09-0D*

Revised: July 27, 2006

As performed by:
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1. Clinical Relevance and Summary of Test Principle

This method is used to assess human exposure to selected polycyclic aromatic hydrocarbons (PAHs), a class of potential human carcinogens (1, 2), by determining the concentrations of their mono-hydroxy metabolites in urine. Common routes of occupational exposure to PAHs may include work involving diesel fuels and coal tars through paving and roofing as well as possible environmental exposures including smoking, diet, smog, and forest fires. Threshold levels for carcinogenicity have not been determined for most PAHs.

The specific analytes measured in this method are monohydroxy-polycyclic aromatic hydrocarbons (OH-PAHs). The procedure involves enzymatic hydrolysis of urine (to hydrolyze PAH conjugates), liquid-liquid extraction, derivatization, and analysis using capillary gas chromatography coupled to high-resolution mass spectrometry (GC/HRMS). This method uses isotope dilution with carbon C-13-labeled internal standards. Ions from each analyte and each C-13-labeled internal standard are monitored, and the abundances of each ion are measured. The ratios of these ions are used as criteria for evaluating the data. (See **Table 1** for the analytes measured in this procedure.) By evaluating the concentrations of these analytes in urine, laboratorians can assess human exposure to the particular PAH analyzed.

Table 1. Analytes Measured and Their Abbreviations

	Parent PAH	Metabolite/Analyte	Abbreviation
1	Naphthalene	1-hydroxynaphthalene	1-nap
2	Naphthalene	2-hydroxynaphthalene	2-nap
3	Fluorene	2-hydroxyfluorene	2-fluo
4	Fluorene	3-hydroxyfluorene	3-fluo
5	Fluorene	9-hydroxyfluorene	9-fluo
6	Phenanthrene	1-hydroxyphenanthrene	1-phen
7	Phenanthrene	2-hydroxyphenanthrene	2-phen
8	Phenanthrene	3-hydroxyphenanthrene	3-phen
9	Phenanthrene	4-hydroxyphenanthrene	4-phen
10	Phenanthrene	9-hydroxyphenanthrene	9-phen
11	Fluoranthene	3-hydroxyfluoranthene	3-flran
12	Pyrene	1-hydroxypyrene	1-pyr
13	Benzo(c)phenanthrene	1-hydroxybenzo(c)phenanthrene	1-bcp
14	Benzo(c)phenanthrene	2-hydroxybenzo(c)phenanthrene	2-bcp
15	Benzo(c)phenanthrene	3-hydroxybenzo(c)phenanthrene	3-bcp
16	Chrysene	1-hydroxychrysene	1-chry
17	Chrysene	2-hydroxychrysene	2-chry
18	Chrysene	3-hydroxychrysene	3-chry
19	Chrysene	4-hydroxychrysene	4-chry
20	Chrysene	6-hydroxychrysene	6-chry
21	Benzo(a)anthracene	1-hydroxybenzo(a)anthracene	1-baa
22	Benzo(a)anthracene	3-hydroxybenzo(a)anthracene	3-baa
23	Benzo(a)anthracene	9-hydroxybenzo(a)anthracene	9-baa
24	Benzo(a)pyrene	3-hydroxybenzo(a)pyrene	3-bap

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents necessary to perform this procedure are toxic. Exercise caution to avoid inhalation of or dermal exposure to these reagents by wearing a lab coat and gloves.

b. Radioactive Hazards

There are no radioactive hazards associated with this procedure.

c. Microbiological Hazards

Although urine is generally regarded as less infectious than serum, the possibility of a laboratorian being exposed to various microbiological hazards exists. Take appropriate measures to avoid any direct contact with the specimen (see Section 2.e.). A Hepatitis B vaccination series and a baseline test for health care and laboratory workers who may be exposed to human fluids and tissues are recommended. Observe universal precautions.

d. Mechanical Hazards

Following standard safety practices while performing this procedure minimizes the risk for mechanical hazards. Avoid any direct contact with the electronic components of the mass spectrometer unless all power to the instrument has been shut off. Only qualified technicians should perform electronic maintenance and repair.

e. Protective Equipment

Use standard personal protective equipment when performing this procedure. This includes wearing a lab coat, safety glasses, durable gloves (nitrile medical examination gloves or equivalent gloves), and appropriate footwear. Process specimens and prepare samples using a chemical fume hood.

f. Training

Anyone performing this procedure must be trained and experienced in the use of a high-resolution mass spectrometer. Formal training by an experienced operator of the instrument is necessary. Personnel must also read the operation manuals from the manufacturer. Anyone involved in sample preparation must be trained in liquid-liquid extraction and have basic chemistry laboratory skills.

g. Personal Hygiene

Follow universal precautions. Exercise special care when handling any biological specimen. Use personal protective equipment and wash hands properly after completing lab work.

h. Disposal of Wastes

According to Wadsworth Hazardous Chemical Waste Management, collect solvents and reagents in appropriate containers clearly marked for waste products, and temporarily store them in a flame-resistant cabinet. Autoclave or decontaminate with 10 percent bleach containers, glassware, etc., that come in direct contact with the specimens. Wash, recycle, or dispose of the glassware in an appropriate manner according to the Wadsworth Center Safety Guidelines. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis according to Wadsworth Center Safety Guidelines for disposal of hazardous waste.

3. Computerization, Data-System Management**a. Software and Knowledge Requirements**

Raw data are processed for qualitative and quantitative analysis using QualBrowser[®], QuanBrowser[®]/QuanDesk[®] (Thermo Electron Corporation), respectively. Perform statistical data analyses using Statistical Analysis System (SAS)[®] software (SAS Institute, Cary, NC), or Excel[®] (Microsoft). Knowledge and experience with these software packages (or their equivalent) are required to use and maintain the data-management structure.

b. Sample Information

Electronically transfer or manually enter information pertaining to particular specimens into the database. Data that are manually entered should include the sample identification number, sample type, standard number, and other information not associated with the mass spectral analysis.

c. Data Maintenance

After inputting sample and analytical data into the database, backup the database onto a computer hard drive and/or writable compact disk (CD-R) weekly. Backup raw data onto the CD-RW, and/or DVD-RW monthly.

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

a. Sample Collection

Use standard urine collection cups to collect urine specimens from donors. Refrigerate samples as soon as possible and transfer them within 4 hours of collection. Collect a minimum of 20 ml of urine and pour it into sterile, 30-ml Qorpak vials with screw-cap tops. Label specimens, immediately freeze them to -20°C , and store them in dry ice for shipping. Carefully pack vials to avoid breakage during shipment. If long-term storage is anticipated, then store all samples at -80°C .

b. Sample Handling

Samples and residual specimens are stored at -80°C until needed to be thawed, aliquoted, and analyzed.

c. Criteria for Specimen Rejection

Specimens must be frozen when delivered to the lab. The minimum volume required is 1.0 ml. If either of these criteria is violated or contamination due to improper collection procedures or collection devices is suspected, the specimen should be rejected.

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

a. Reagents and Sources (see Table 2)

Table 2. Reagents and the Respective Manufacturers

Reagent	Manufactures*
1-nap; 2-nap, 2-fluo, 3-fluo, 9- fluo, 9-phen, 1-pyr	Sigma Chemicals, St Louis, MO
1-phen, 2-phen, 4-phen	RCT, Laramie, WY
3-phen, 3-flran, 1-bcp, 2-bcp, 3-bcp, 1-chry, 2-chry, 3-chry, 4-chry, 1-baa, 3-baa, 3-bap	Midwest Research Institute, National Cancer Institute, Kansas City, MO
6-chry, PCB-61	AccuStandard,
¹³ C ₆ 1-nap	CDC, synthesized in house, Atlanta, GA
¹³ C ₆ 1-baa, ¹³ C ₆ 3-bcp, ¹³ C ₆ 3-chry, ¹³ C ₆ 3- flran, ¹³ C ₆ 1-pyr	Midwest Research Institute, NCI Kansas City, MO
¹³ C ₆ 3-phen, ¹³ C ₆ 6-chry	Cambridge Isotope Laboratories, Andover, MA
β-Glucuronidase / arylsulfatase (Type H-3, 98,000 Unit/ml, from Helix pomatia)	Sigma Chemicals, St Louis, MO
N-methyl-N-(trimethylsilyl)- trifluoroacetamide (MSTFA)	Sigma Chemicals, St Louis, MO
Toluene (Nanograde)	Mallinckrodt Baker, Inc., Paris, KY
Pentane (HPLC grad)	J.T. Baker, Philipsburg, NJ
Organic Pure Water (o.p. H ₂ O)	Prepared in house, (Milli Q water)
n-Dedocane	ChemService, West Chester, PA 19381
Buffer Solution, (pH 5.00)	Anachemia, Rouses Point, NY
Argon	BOC Group Inc., Murray Hill, NJ

* Use an equivalent manufacturer when the manufacture noted is not available.

All OH-PAH compounds are stored at -70°C and protected with Argon.

(1). Reagent Preparation

- i. **MSTFA.** Open the sealed vial containing N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) from the vendor and place the MSTFA solution in a clean GC sample vial. Displace the air over the MSTFA with a gentle stream of argon. Immediately screw the cap on the vial. Store the MSTFA in the vial at 4°C could last more than 1 month.
- ii. **Enzymatic-buffer** Combine 250mL of buffer solution (pH=5.0, Anachemia) and 0.6mL of β -glucuronidase / sulfatase enzyme (derived from Helix Pomatia, 98900units/ml, Sigma) in a sterilized 250ml Wheaton bottle to generate Enzymatic-buffer (β -glucuronidase / sulfatase enzyme 300 units/ml). Store at 4°C could last more than 1 month.

(2) Standards Preparation

Due to the potential carcinogenicity of these analytes, weigh each analyte on a microbalance inside a ventilated plexiglass box. Because all OH-PAHs are light-sensitive, take precautions to minimize their exposure to light.

- i. **Stock solutions of Individual Analytes (100ng/ μ L).** Weigh approximately 1mg of neat OH-PAH in a screw-cap vial. Add exactly 1 mL of acetonitrile and allow the OH-PAH material to dissolve by gently swirling it or placing it in an ultrasonic bath. Calculate and perform the dilution to 100 ng/ μ L for a final volume of ~10 mL of acetonitrile. Ampouelize the individual standard solution in clean amber ampoules, use argon to displace any air in the ampoule and seal it. Stored at -80°C (for up to 1.5 years).
- ii. **Working Standard Solution of 23 OH-PAH Mix.** Combine 500 μ L of each OH-PAH standard stock solutions (100 ng/ μ L) and 1mL acetonitrile in a 20mL scintillant tube to generate a mixture of 23 OH-PAHs working standard solution (each OH-PAH concentration is 4 ng/ μ L). Solutions of other concentrations may also be prepared, if needed. Use argon to displace any air in the vials. Then cap the vials tightly, place the vials in a sealed double layer plastic bag filled with argon, and store them at -80 °C until needed (for up to 6 months).
- iii. **Calibration Curve Standards.** Concentrations for the six points that establish the calibration curve are 10, 50, 100, 500, 2000, 5000 and 10000 ng/L. These

standards are made from the dilution of 23 OH-PAH working standard solution [see Section 6.c.(2)].

- iv. **Working Solution of C-13-labeled OH-PAH.** For each C-13-labeled OH-PAH, make 100 ng/ μ L of the stock solution, combine and dilute aliquots of the individual C-13-labeled OH-PAH solutions with acetonitrile to produce the working solution with 100 pg/ μ L of each C-13-labeled OH-PAH. Use argon to displace any air in the vials. Then cap the vials and store them at -80 °C until needed (for up to 12 months).
- v. **Mass Spectrometric Check Solution.** Use this solution at least weekly to monitor the operating performance of the GC column and the mass spectrometer. Use a solution of 10 fg/ μ L of a tetra-chlorinated dioxin (2378 **TCDD**) as the check solution. For a 1- μ L injection, a minimum signal-to-noise ratio (using 4 standard deviations of baseline noise) of 4 is required. Use the ion intensity of mass 321.894 for the signal-to-noise ratio measurement.

b. Manual Equipment (indicated manufacture or its equivalent)

- (1) Water bath/sonicator (Cole Parmer, Vernon Hills, IL).
- (2) Incubator (Fisher Scientific, Pittsburgh, PA).
- (3) Oven for derivatization (Cole Parmer, Vernon Hills, IL).
- (4) Microbalance (Sartorius Ultramicro, Westbury, NY).
- (5) TurboVap® LV evaporator (Zymark Corp., Hopkinton, MA).
- (6) Centrifuge 5804 (Eppendorf Corp., Westbury, NY)
- (7) Repeater Plus (Eppendorf Corp., Westbury, NY)
- (8) Vortex Genie vortex mixer (Scientific Industries Inc., Springfield, MA).

c. Other Materials (indicated manufacture or its equivalent)

- (1) Miscellaneous glassware (Pyrex or Kimax, Scientific Services).
- (2) 1.5ml amber autosampler vials (National Scientific, Inc.).
- (3) 1.1ml 12x32mm, Pulled PT Vial (Sun Sri, Rockwood, TN)
- (4) 4-mL, amber screw-top vials (Supelco, Inc.).
- (5) 10-ml screw-top culture tubes

- (6) 15-mL conical centrifuge tubes (Kimble, Vineland, NJ).
- (7) 2-micron filter (Nalgene, Rochester, NY)
- (8) Pipetman 10 μ L, 25 μ L, 100 μ L, 250 μ L, 1mL, 5mL (Gilson Co., Middletown, WI)

d. Instrumentation (indicated manufacture or its equivalent)

Perform the analyses on a Finnigan MAT -95XL high-resolution mass spectrometer that is equipped with an electron impact ionization source and is interfaced to a Thermo Finnigan Trace Ultra 2000 gas chromatograph system (Thermo Finnigan, San Jose, CA). A PAL injection system (CTC Analytics, Switzerland) handles the GC injections automatically. All instruments are connected and controlled by a Dell[®] computer with Microsoft[®] Windows XP[®] operating system

(1) High Resolution Mass Spectrometer (HRMS) Configuration (see Table 4)**Table 4. Thermo Finnigan MAT 95 XL HRMS Configuration**

MS Parameter	Setting
Scan mode	Multiple ion detection
Ionization type	Electron impact
Ion polarity mode	Positive
Electron energy	40 eV
Resolution	10,000
Ion source	260 °C
Transfer line	290 °C
Conversion dynode voltage	Positive
Continuous dynode electron multiplier voltage	1.45 -2.25 kV (106 gain)

(2) Masses and retention times of targeted OH-PAHs on MAT 95 XP HRMS are shown in the **Table 5**.

Table 5. Masses and Retention Time of Targeted OH-PAHs

Analyte-O-Si (CH ₃) ₃	Mole. Mass	Ion Mass	R.T. min	I-Std.
1-nap	216.0970	201.0735	8.44	
¹³ C ₆ 1-nap	222.1172	207.0937		x
2-nap	216.0970	201.0735	8.70	
9-fluo	254.1127	239.0892	11.13	
¹³ C ₆ 9-fluo	260.1328	245.1093		
3-fluo	254.1127	239.0892	12.65	
¹³ C ₆ 3-fluo	260.1328	245.1093		
2-fluo	254.1128	239.0892	12.85	
¹³ C ₆ 2-fluo	260.1328	245.1093		
4-phen	266.1127	251.0890	13.73	
9-phen	266.1127	251.0890	14.10	
3-phen	266.1127	251.0890	14.37	
¹³ C ₆ 3-phen	272.1328	257.1093		x
1-phen	266.1127	251.0890	14.43	
2-phen	266.1127	251.0890	14.73	
¹³ C ₆ 2-phen	272.1328	257.1093		
1-bcp	316.1283	301.1048	16.79	
3-flran	290.1127	275.0892	17.03	
¹³ C ₆ 3-flran	296.1328	281.1093		x
1-pyr	290.1127	275.0892	17.40	
¹³ C ₆ 1-pyr	296.1328	281.1093		x
2-bcp	316.1283	301.1048	19.07	
1-baa	316.1283	301.1048	19.13	
¹³ C ₆ 1-baa	322.1485	307.1250		x
4-chry	316.1283	301.1048	19.22	
6-chry	316.1283	301.1048	19.30	
¹³ C ₆ 6-chry	322.1485	307.125		x
3-bcp	316.1283	301.1048	19.71	
¹³ C ₆ 3-bcp	322.1485	307.1250		x
3-chry	316.1283	301.1048	20.03	
¹³ C ₆ 3-chry	322.1485	307.1250		
1-chry	316.1283	301.1048	20.10	
3-/9-baa	316.1283	301.1048	20.35	
2-chry	316.1283	301.1048	20.43	
3-bap	340.1282	325.1042	23.63	
¹³ C ₆ 3-bap	346.1484	331.1249		
7-bap	340.1282	325.1042		

(3) Gas Chromatography (GC) Configuration and Temperature Program

Chromatographic separation is performed on a Finnigan Thermo Trace GC Ultra 2000 gas chromatograph fitted with a J&W DB-5MS, 25-m, fused silica capillary column. The column ID is 0.25 mm and the film thickness is 0.25 micron (J&W, # 22-5022 or its equivalent). The GC purge flow is 10 mL/min, the saver time is 2 min, and the saver flow is 20 mL/min. The temperature program lasts a total of 33.3 min. (See **Table 6** for GC Configuration and **Table 7** for the GC Temperature Program.)

Table 6. GC Configuration

GC Parameter	Setting
Carrier gas	Helium
Constant flow rate	1.0 ml/minute
Injection mode	Splitless
Injector purge delay	2.0 minutes
Injector temperature	270 °C
Mode	Constant pressure

Table 7. GC Oven Temperature Program for Hydroxy PAHs

Time (minutes)	Temperature (°C)
0	100
2	100
6	160
19.5	295
25.5	295
26	300
35	300

6. Calibration and Calibration-Verification Procedures

a. Mass Spectrometer

Calibrate and tune the Thermo Finnigan MAT 95 XP mass spectrometer using FC43 (perfluorotributylamine) according to the instructions in the operator's manual. After calibrating the instrument with 10,000 resolution and maximum sensitivity, prepare the instrument to analyze the PAH metabolites (see Section 8).

b. Calibration Curve

- (a). Construct a 6-point calibration curve by performing a weighted regression (i.e., weighted to 1/concentration) analysis of relative response factor (i.e., area native/area internal label) versus standard concentration. Perform a minimum of seven repeat determinations for each point on the standard curve.
- (b). The lowest point (10 ng/L) on the calibration curve is near the measurable detection limits; the highest point (10,000 ng/L) is above the expected range of normal results.
- (c). Measure and calculate blank levels; subtract the blank amounts from the calibration points.
- (d). Determine the slope and intercept of the calibration curve by linear least squares fit with weighted 1/concentration using QuanBrowser[®], QuanDesk[®], or SAS[®] software.
- (e). R-squared values for the curve must be greater than 0.90. Linearity of standard curves should extend over the entire standard range. Intercepts (calculated from the least squares fit of the data) should not be significantly different from 0; if they are, then identify the source of bias, make necessary corrections, and run again.
- (f). Periodically recalculate the standard curve to incorporate the newest data points. Reestablish the standard curve when new working standard solutions or new isotope solutions are prepared.

c. Calibration Verification

- (a). Analyze the calibration-verification materials after any substantive change in the method or instrumentation occurs to verify the integrity of the calibration curve, slope, linearity, and dynamic range (e.g., the instrument has used in another method; installed a new column; performed preventative maintenance; or cleaned the ion source of the mass spectrometer).
- (b). Perform calibration verification at least once every 6 months while the method is in use.
- (c). If there is a significant difference from the established calibration-verification pool limits, stop doing analyses using this method until you can correct the problem and ensure that calibration-verification materials are consistent with the calibration curve.
- (d). Document all calibration-verification runs and results.

d. Proficiency Testing (PT)

Because no standard reference materials are available for urinary analysis of hydroxy-PAH levels within an analytical study, duplicate measurements of hydroxy-PAH levels were performed on a series of selected individual samples at designated intervals during sample analysis. The analytical reproducibility of a set of individual samples where levels of duplicate samples were determined after every calculated sample increment in the study was considered an acceptable measure of the proficiency of the method. The calculated increment is a value that ranges from $N/0.05N$ to $N/0.02N$ (N = number of samples in the study). PT criteria are under evaluation.

7. Operating Procedures; Calculations; Interpretation of Results

a. Sample Preparation

Allow unknown urine samples, QC samples, and a blank (2-mL o.p. water) to thaw slowly in a refrigerator overnight to avoid cracking the glass containers.

b. Hydrolysis

In this stage, carefully pre-washed culture tubs with screw-cap tops, caps with PTFE liner, glass pipets, and conical centrifugal tubes are used. Aliquot 2 mL of

urine into 10mL culture tube. Add 3mL enzymatic-buffer (pH=5.0, with β -glucuronidase / sulfatase enzyme 300 units/ml, derived from *Helix Pomatia*), and 10 μ L of combined C-13-labeled standards (100 pg/ μ L of each labeled compound). Prepare QC samples, the same way. Aliquots of calibration standards and QCs are usually made earlier in batches and stored in a freezer. Place the caps loosely on the tubes and use a vortex mixer to mix the resulting solution well. Incubate the samples at 37 °C over night. Remove samples from the incubator and cool down them for a brief period. Urine samples can be hydrolyzed and then place them in a refrigerator for extraction in the following morning. (Note: This is an acceptable stopping point if you immediately refrigerate the hydrolysates.) For the blank, and the calibration standard the solid phase extraction should be done same day of the preparation.

c. Extraction

Add 5mL pentane to hydrolyzed samples tube. Screw on cap, mix and vent pressure twice. Screw on cap tightly and mix samples by shaking rocker for 5 min. Evenly place the culture tubes on rocker of Eppendorf centrifuge and centrifuge for 15min at 3000rpm, check the separation and set another 15min run. Upper layer solvent transferred to a cleaned conical centrifugal tubes using glass pipet. Due to the extracts are directly concentrated without further dry-up in next stage, the transferring the solvent needs special care, no aqueous is allowed to touch. Extraction repeated with another 5mL of pentane. Add 5 μ L dodecane (keeper) to each tube prior to further concentrate.

d. Concentration

Concentrate the extracts using a TurboVap® (Caliper Life Sciences), set at 40 °C and with 5 psi of nitrogen, the final volume is about 10 μ L. The evaporation takes approximately 10 minutes (Do not let the nitrogen stop until remove the tubes from TurboVap®). Reconstitute the residual with 20 μ L high purity toluene. Add 10 μ L PCB-61 (50pg/ μ L) as the external recovery standard (ERS), use vortex mixer 15 seconds and transfer solution into a cleaned pulled 1.1 ml GC vial use 100 μ L pipettor.

e. Derivatization Procedure

The derivatization step converts the OH functional group to an O-trimethylsilyl group. This step produces better limits of detection (LOD) and chromatography. To derivatize the analytes after evaporation, add 20 μL of MSTFA in each vial and then displace the air in the vial with a gentle stream of argon. Quickly screw the vial. Place the vials in an incubator set at 60 $^{\circ}\text{C}$ for 1 hour. Cover the samples with aluminum foil. The derivatized samples are then ready for analysis on the mass spectrometer. The samples can also be stored in a refrigerator (-4°C) for up to 1 week.

f. Analysis

Preliminary MAT 95 XL System Setup and Performance Check

- (a) In the TUNE window, adjust the resolution between 9950 and 10050 using 2 μL of FC43.
- (b) Run a GC/HRMS analysis of 10 fg tetra-chlorinated dioxin, check the solution, and verify chromatographic resolution and peak intensity.
- (c) In the logbook, record the signal-to-noise ratio of the check compound.

g. Processing of Data

(1) Quantification

All raw data files are quantified using the quantitation software of the MAT 95 XP in the QUAN window. This allows manual peak selection and baseline determination for each ion. The entire quantification procedure is accomplished by using the user buttons defined in the QUAN application.

(2) Transfer of Data

Use the USB jump drive or CD-R/RW copy the data files to other computer.

(3) Backup Data

Backup data to CD-R or other net hard drive weekly.

(4) Statistical Analysis and Interpretation of Data

Export information in the cleanup and mass spectrometry database tables from the database to a fixed text (ASCII) file, and then import the file into SAS[®]. Use SAS[®] programs for standard curve generation, QC analysis, blank analysis, limit-of-detection determination, unknown calculations, data distribution, etc. as needed.

h. Replacement and Periodic Maintenance of Key Components

(1) Routine Maintenance

(a) MAT 95 XL Mass Spectrometer

- (i) Check cooling water level and temperature monthly.
- (ii) Clean the ion volume or replace it monthly.
- (iii) Clean the ion source or replace it annually.
- (iv) Replace the calibration gas septum every two weeks.
- (v) Trained Thermo Finnigan technicians will perform all other maintenance based on an annual schedule or as requested.

(b) Trace Ultra 2000 GC gas chromatograph

- (i) Change the injection port liner and septum daily.
- (ii) Clean the injection port, clean or replace the gold seal monthly. (iii) Replace the GC column at 1000 analyses or sooner.
- (iv) Replace Helium tank when the pressure is lower than 500 psi.

(Note: The above maintenance schedules may be accelerated if necessary.)

(2) Performance Maintenance

Perform maintenance procedures if you detect a decrease in system performance (i.e., sensitivity and/or S/N ratio) without other apparent technical reasons.

8. Reportable Range of Results

The linear range of the standard calibration curves determines the highest and lowest analytical values of an analyte that are reportable. The calibration verification of the method encompasses this reportable range. However, you can dilute and analyze a urine sample with an analytical data value exceeding the highest reportable limit so that the result will be within 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 10 *ng/L* to 5,000 *ng/L*. Urine samples whose concentrations exceed these ranges must be analyzed again with a diluted solution or using a smaller aliquot.

b. Analytical Sensitivity

The method detection limit (MDL) is defined as 3 times the standard deviation (SD) at zero concentration (3SD). It is determined by weighted regression (1/concentration) analysis of the absolute SD versus the concentration. For analytes with detectable blank signals, the MDL is calculated by adding the average blank level to 3SD (see Table 8 for the MDL determined for the analytes). Generally, MDL range from 2ng/L to 10 ng/L, depending upon blank levels.

Table 8. Method Detection Limits (MDLs)

Analyte	MDL (ng/L)
1-nap	5.7
2-nap	4.7
2-fluo	4.4
3-fluo	7.0
9-fluo	2.0
1-phen	8.0
2-phen	4.4
3-phen	1.2
4-phen	1.3
9-phen	1.9
3-flran	5.8
1-pyr	4.9
1-bcp	4.5
2-bcp	2.2
3-bcp	6.9
1-chry	4.8
2-chry	3.8
3-chry	1.3
4-chry	9.5
6-chry	5.3
1-baa	8.6
3-baa	8.7
3-bap	72.1

c. Accuracy

Enriching urine samples with known concentrations of the OH-PAH and comparing the calculated and expected concentrations determine the accuracy of the method.

The accuracy can be expressed as the slope and the intercept of a weighted regression (1/concentration) analysis of the expected values versus the calculated values. A slope of 1.0 and an intercept of 0 indicate that the results are identical. The method accuracy is shown in **Table 9**.

Table 9. Accuracy of the Method

Analyte	Slope	Intercept	R-Squared	Weighting index
1-nap	0.0015489	0.0142736	0.9982	1/ Conc.
2-nap	0.0016431	0.00441331	0.9982	1/ Conc.
2-fluo	0.0029785	0.00425533	0.9968	1/ Conc.
3-fluo	0.0019075	0.0050077	0.9975	1/ Conc.
9-fluo	0.0014534	0.000940282	0.9971	1/ Conc.
1-phen	0.0029675	0.00299927	0.9969	1/ Conc.
2-phen	0.0026923	0.005438	0.9979	1/ Conc.
3-phen	0.0028350	0.00563754	0.9959	1/ Conc.
4-phen	0.0029070	0.00333788	0.9972	1/ Conc.
9-phen	0.0021514	0.00336125	0.9983	1/ Conc.
3-fran	0.0015743	0.00224427	0.9975	1/ Conc.
1-pyr	0.0025646	0.00356015	0.9958	1/ Conc.
1-bcp	0.0013202	0.00498609	0.9939	1/ Conc.
2-bcp	0.0016979	0.00632489	0.9973	1/ Conc.
3-bcp	0.0019940	0.00548789	0.9961	1/ Conc.
1-chry	0.0034276	0.0211208	0.9962	1/ Conc.
2-chry	0.0017045	0.00689675	0.9962	1/ Conc.
3-chry	0.0028450	0.0210635	0.9977	1/ Conc.
4-chry	0.0035895	0.00712345	0.9987	1/ Conc.
6-chry	0.0021580	0.00605751	0.9950	1/ Conc.
1-baa	0.0020282	0.00339295	0.9917	1/ Conc.
3-/9-baa	0.0007313	0.00185813	0.9968	1/ Conc.
3-bap	0.0009369	-0.00932439	0.9940	1/ Conc.

d. Precision

The precision of this method is reflected in the variance of QC samples over time. The major contributor to the overall coefficient of variation (CV) is the variation between runs. (See **Table 10** and **Table 12** for CV values.)

9. Quality Control (QC) Procedures**a. QC Material**

Use urine pools enriched with known amounts of OH-PAH as the control materials for each analytical run.

b. Urine Enrichment

Split the urine pool into four smaller urine pools. Reserve the first pool for blank check. Enrich the second pool with the 23 OH-PAH working solution to yield a low pool with an added concentration of 100 ng/L. Enrich the third pool with the 23 OH-PAH working solution to yield a middle pool with an added concentration of 300 ng/L. Enrich the fourth pool with the 23 OH- PAH working solution to yield a High pool with an added concentration of 900 ng/L.

c. Dispensing

Dispense 2-mL aliquots of urine into screw-capped vials. Mark the vials appropriately with identification stickers and store the QC materials at -70 °C until time of analysis. Buffer and internal standards could be added at the day of analysis when unknown urines are prepared.

d. Characterization of QC Materials

Characterize the QC pools (including the unspiked pool) by 20 consecutive runs of each QC material. Use the data from these runs to establish the mean and upper- and lower- 99th and -95th confidence intervals. Determine the confidence intervals and adjust them according to the number of each QC material analyzed in each run.

e. Use of QC Samples

During each analytical run, analyze at least one QC material from each pool.

f. Final Evaluation of QC Results

After the completion of a run, consult the QC limits to determine if the run is in control. **The following QC rules apply to the average of the beginning and ending analyses of each of the bench QC pools:**

- (1) If both the low and the high QC results are within the 2s limits, then accept the run.
- (2) If one of two QC results is outside the 2s limits, then apply the rules below and reject the run if any condition is met.

(Note: each one is given a code [0- 4] to be entered in the database.)

Code

- 0** -Extreme outlier: the result is outside the characterization mean by more than 4SD.
- 1 -1_{3s}** -The average of both low QC results QB the average of both high QC results is outside a 3s limit.
- 2 -2_{2s}** -The average of both low QC results AND the average of both high QC results is outside a 2s limit on the same side of the mean.
- 3- R_{4s} 5sequential**- The average of both low QC results AND the average of both high QC results is outside a 2s limit on opposite sides of the mean.
- 4 -10_x sequential** -The previous nine average QC results (for the previous nine runs) were on the same side of the mean for either the low QB high QC results.

If the run is declared out of control, the analysis results for all patient samples analyzed during that run are invalid for reporting. If more than ten consecutive QCs are on the same side of the mean of the characterized QC material, suspend all operations until you determine whether a bias is present in the method. (See **Tables 10- 12** for the characterization of both QC pools.)

Table 10. Mean Value, SD, and CV of Low QC Pool

(Replicates =20)

Analyte	Mean (ng/L)	SD	CV
1-nap	231	30	25
2-nap	590	66	50
2-fluo	166	29	24
3-fluo	114	17	13
9-fluo	108	19	15
1-phen	168	25	19
2-phen	146	12	8
3-phen	171	24	17
4-phen	166	29	24
9-phen	100	12	9
3-flran	116	8	5
1-pyr	135	7	4
1-bcp	66	14	12
2-bcp	95	10	8
3-bcp	93	6	4
1-chry	88	9	6
2-chry	98	13	9
3-chry	98	11	7
4-chry	82	9	7
6-chry	90	17	11
1-baa	90	10	7
3-baa	104	16	11
3-bap	137	20	5

Table 11. Mean Value, SD, and CV of Middle QC Pool

(Replicates =20)

Analyte	Mean (ng/L)	SD (%)	CV
1-nap	369	41	34
2-nap	781	59	44
2-fluo	357	72	59
3-fluo	317	71	54
9-fluo	326	80	72
1-phen	429	94	76
2-phen	361	63	47
3-phen	291	16	11
4-phen	357	73	59
9-phen	214	45	33
3-flran	238	13	9
1-pyr	248	12	10
1-bcp	173	24	18
2-bcp	222	17	10
3-bcp	222	13	9
1-chry	215	16	12
2-chry	246	23	20
3-chry	234	17	15
4-chry	195	21	17
6-chry	221	26	19
1-baa	219	17	15
3-baa	248	25	19
3-bap	288	51	36

Table 12. Mean Value, SD, and CV of High QC Pool

(Replicates =20)

Analyte	Mean (ng/L)	SD	CV
1-nap	930	82	60
2-nap	1189	127	93
2-fluo	806	90	73
3-fluo	766	97	71
9-fluo	656	97	71
1-phen	868	94	73
2-phen	813	63	42
3-phen	862	47	35
4-phen	801	81	67
9-phen	656	97	67
3-flran	752	55	43
1-pyr	789	55	45
1-bcp	628	82	64
2-bcp	790	49	37
3-bcp	765	31	22
1-chry	759	70	54
2-chry	859	88	72
3-chry	812	78	55
4-chry	698	82	64
6-chry	813	107	84
1-baa	762	57	45
3-baa	861	105	84
3-bap	1148	161	116

10. Recovery of Method

The recoveries of OH-PAHs use this method are calculated based on the amount of spiked isotope labeled internal standards vs measured amount with the external recovery standard correction. The recovery values reflect the performance of sample preparation.

Table 13 Internal Standard Acceptable Recovery Ranges

Spike	Recovery Range, %
1-OH-Naphthol C13	35-95
3-OH-Phenanthrene C13	35-95
3-OH-Fluoranthene C13	35-95
1-OH-Pyrene C13	50-120
1-OH-Benzo(a)anthracene C13	50-120
6-OH-Chrysene C13	50-120
3-OH-Benzo(c)phenanthrene C13	50-120

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

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until you identify the source or cause of failure. If you can easily identify the source of failure (e.g., failure of the mass spectrometer or an error in pipeting), then correct the problem. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, reanalyze several QC materials (in the case of QC failure) or calibration-verification materials (in the case of calibration failure). After reestablishing calibration or QC, resume analytical runs.

12. Limitations of Method; Interfering Substances and Conditions

This method is an isotope-dilution mass-spectrometry method, which is widely regarded as the definitive method for the measurement of organic toxicants in human body fluids. By using high-resolution mass spectrometry, it eliminates most interferants. Occasional unknown interferants have been encountered because of the matrix used in this procedure. Chromatographic interferences with the internal standards will result in the rejection of analysis. If repeat analysis results in an interference with the internal standard, do not report the results for that analyte.

13. Reference Ranges (Normal Values)

The reference ranges for OH-PAH in the U.S. population are based on the NHANES 2005 study. (See **Table 14** for reference range values from NHANES 2005.)

Table 14. Reference Range of OH-PAHs in Urine

Analyte	Without Creatine Correction			Creatine Corrected		
	Mean 95% C.I. (ng/L)	Low 50 th (ng/L)	High 95 th (ng/L)	Mean 95% C.I. (ng/L)	Low 50 th (ng/L)	High 95 th (ng/L)
1-nap	2050	1490	26600	1910	1370	20800
2-nap	2470	1920	29700	2310	1670	19200
2-fluo	441	422	9470	408	382	6860
3-fluo	170	86.3	4680	157	79.9	4060
9-fluo	219	185	1300	205	181	1120
1-phen	154	105	1390	142	102	1270
2-phen	98.3	79.7	937	90.3	67.8	748
3-phen	127	113	721	116	101	574
4-phen	41.9	46.0	339	39.2	56.5	411
9-phen	33.9	36.0	396	31.7	36.3	290
3-flran	13.4	17.5	147	12.3	14.7	127
1-pyr	79.8	76.9	926	74.2	63.1	607
1-bcp	*	< LOD	54.0	*	< LOD	70.8
2-bcp	*	< LOD	28.1	*	< LOD	30.9
3-bcp	*	< LOD	16.0	*	< LOD	16.7
1-chry	*	< LOD	138	*	< LOD	104
2-chry	*	< LOD	45.0	*	< LOD	35.0
3-chry	*	< LOD	46.0	*	< LOD	62.5
4-chry	*	< LOD	< LOD	*	< LOD	< LOD
6-chry	*	< LOD	10.1	*	< LOD	76.4
1-baa	*	< LOD	30	*	< LOD	32.4
3-/9-baa	*	< LOD	31.0	*	< LOD	35.5
3-bap	*	< LOD	251	*	< LOD	248

< LOD means less than the limit of detection

* Not calculated. Portion of results below limit of

From CDC *Third Report on Human Exposure to Environmental Chemicals*

14. Critical-Call Results ("Panic" Values)

There are no established critical-call values for this method. It is unlikely that any result would be a critical-call, which would only occur with poisonings.

15. Specimen Storage and Handling During Testing

Refrigerate urine samples overnight at -4 °C to expedite thawing prior to aliquoting the samples. Store the urine extracts in autosampler vials at -70 °C after analysis. Current studies indicate (CDC data) that the extracts are stable for up to 3 weeks.

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

The method is designed to run on a GC/HRMS instrument and is not generally transferable to other instrumentation.

There are no acceptable alternative methods for analysis or backup systems. If the analytical system fails, store specimens in a refrigerator overnight. If long-term interruption is anticipated, store at -80 °C.

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

Use standard record-keeping systems (i.e., notebooks, sample logs, data files, creatinine logs, demographic logs) to keep track of all specimens.

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