Laboratory Procedure Manual

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

Matrix: **Urine**

Method: **Isotope Dilution Gas Chromatography/Tandem Mass Spectrometry (GC-MS/MS)**

Method No: **6703.04**

Revised: **12/03/2013**

as performed by:

Organic Analytical Toxicology Branch
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Important Information for Users

The Centers for Disease Control and Prevention (CDC) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

This document details the Lab Protocol for NHANES 2005–2006 data.

A tabular list of the released analytes follows:

Data File Name	Variable Name	SAS Label
PAHS_G	URXPO1	1-napthol(ng/L)
	URXPO2	2-napthol(ng/L)
	URXPO3	3-fluorene(ng/L)
	URXPO4	2-fluorene(ng/L)
	URXPO5	3-phenanthrene(ng/L)
	URXPO6	1-phenanthrene(ng/L)
	URXPO7	2-phenanthrene(ng/L)
	URXP10	1-pyrene(ng/L)
	URXP17	9-fluorene(ng/L)
	URXP19	4-phenanthrene (ng/L)

1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous environmental contaminants formed during incomplete combustion processes. Many of them have been identified as suspected human carcinogens (1), but threshold levels for carcinogenicity have not been determined for most PAHs. Occupational exposure may occur through work involving diesel fuels and coal tars such as paving and roofing. Possible environmental exposures include smoking, diet, smog and forest fires (2, 3). Because of potential widespread human exposure and potential risk to health, biomonitoring of PAHs is relevant for environmental public health. Application of this biomonitoring method to analyze samples obtained from participants in the National Health and Nutrition Examination Survey (NHANES) will help determine reference ranges for these chemicals in the general U.S. population, aged 6 years and older.

b. Test Principle

The specific analytes measured in this method are monohydroxylated metabolites of PAHs (OH-PAHs). This procedure involves enzymatic hydrolysis of glucuronidated/sulfated OH-PAH metabolites in urine, extraction, derivatization and analysis using isotope dilution capillary gas chromatography tandem mass spectrometry (GC-MS/MS) (4, 5). Ion transitions specific to each analyte and carbon-13 labeled internal standards are monitored, and the abundances of each ion are measured. The analytes measured in this procedure are shown in Table 1.

Table 1. Analytes measured their parent compounds, and their abbreviations.

No.	Metabolite/Analyte	Parent PAH	Abbreviation
1	1-hydroxynaphthalene	Naphthalene	1-NAP
2	2-hydroxynaphthalene	Naphthalene	2-NAP
3	9-hydroxyfluorene	Fluorene	9-FLU
4	2-hydroxyfluorene	Fluorene	2-FLU
5	3-hydroxyfluorene	Fluorene	3-FLU
6	1-hydroxyphenanthrene	Phenanthrene	1-PHE
7	2-hydroxyphenanthrene	Phenanthrene	2-PHE
8	3-hydroxyphenanthrene	Phenanthrene	3-PHE
9	4-hydroxyphenanthrene	Phenanthrene	4-PHE
10	1-hydroxypyrene	Pyrene	1-PYR

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Some of the reagents needed to perform this procedure are toxic. Special care must be taken to avoid inhalation or dermal exposure to these reagents.

β-Glucuronidase is a known sensitizer. Prolonged or repeated exposure to the sensitizer may cause allergic reactions in certain sensitive individuals.

Note: Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure can be found at http://www.msdsxchange.com/english/index.cfm. Laboratory personnel are advised to review the MSDS before using chemicals.

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 being exposed to various microbiological hazards exists. Appropriate measures must be taken to avoid any direct contact with the specimen (See Section 2.e.). CDC recommends a Hepatitis B vaccination series and a baseline test for health care and laboratory workers who are exposed to human fluids and tissues. Observe Universal Precautions. Also, laboratory personnel handling human fluids and tissues are required to take the “Blood borne Pathogens Training” course offered at CDC to insure proper compliance with CDC safe work place requirements.

d. Mechanical Hazards

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratory analysts must read and follow the manufacturer’s information regarding safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of the mass spectrometer unless all power to the instrument is off. Generally, mechanical and electronic maintenance and repair must only be performed by qualified technicians. The autosampler and the mass spectrometer contain a number of areas which are hot enough to cause burns. Precautions must be used when working in these areas.

e. Protective Equipment

Standard safety precautions must be followed when performing this procedure, including the use of a lab coat/disposable gown, safety glasses, appropriate gloves, and chemical fume hood. Refer to the laboratory Chemical Hygiene Plan and CDC

Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

f. Training

Formal training in the use of a GC/MS-MS is necessary. Users are required to read the operation manuals and must demonstrate safe techniques in performing the method. Laboratorians involved in sample preparation must be trained for all sample preparation equipment, chemical handling, and have basic chemistry laboratory skills.

g. Personal Hygiene

Follow Universal Precautions. Care must be taken when handling chemicals or any biological specimen. Routine use of gloves and proper hand washing must be practiced. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences safety policies and procedures for details related to specific activities, reagents, or agents.

h. Disposal of Wastes

Waste materials must be disposed of in compliance with laboratory, federal, state, and local regulations. Solvents and reagents must always be disposed of in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood. All disposable items that come in direct contact with the biological specimens are to be placed in a biohazard autoclave bag that must be kept in appropriate containers until sealed and autoclaved. Unshielded needles, pipette tips and disposable syringes must be placed immediately into a sharps container and autoclaved when this container becomes full. Wipe down all surfaces with a freshly prepared bleach solution (a 10% dilution of commercial sodium hypochlorite (bleach) or equivalent) when work is finished. Any non-disposable glassware or equipment that comes in contact with biological samples must be washed with bleach solution before reuse or disposal. Any other non-disposable glassware must be washed and recycled or disposed in an appropriate manner. To insure proper compliance with CDC requirements, laboratory personnel are required to take annual hazardous waste disposal training courses.

***Observe Universal Precautions**. Dispose of all biological samples and diluted specimens in a brown glass bottle; disinfect the bio-hazardous material with bleach (10% in final volume), and dispose according to CDC/DLS guidelines for disposal of hazardous waste. Dispose all used disposable laboratory supplies (tubes, pipette tips, etc.) in an autoclave bag at the end of the analysis according to CDC/DLS guidelines for disposal of hazardous waste.*

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

This method has been validated using the Agilent GC/QQQ 7000 GC-MS/MS system controlled by Agilent MassHunter Workstation™ software. Analyte chromatographic peaks are integrated by Quantitative Analysis under MassHunter™. Results are exported from MassHunter result files to Microsoft Excel files that are subsequently used for calculations. Final results are processed using SAS and stored in both Excel and SAS format. Knowledge of and experience with these software packages (or their equivalent) is required to utilize and maintain the data management structure.

b. Sample Information

Information pertaining to particular specimens is entered into the database either manually or electronically transferred. The result file is transferred electronically into the database. No personal identifiers are used, and all samples are referenced to a blind coded sample identifier.

c. Data Maintenance

All sample and analytical data are checked in MS Excel and SAS for overall validity. The database is routinely backed up locally through the standard practices of the CDC network. The local area network manager must be contacted for emergency assistance.

d. Information Security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the individual laboratories, buildings, and site. Confidentiality of results is protected by referencing results to blind coded sample IDs (no names or personal identifiers are used).

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

a. Special Instructions

No special instructions such as fasting or special diets are required.

b. Sample Collection

Urine specimens are collected from subjects in standard urine collection cups. Samples should be refrigerated as soon as possible, and preferably transferred to specimen vials within 24 hours of collection. If at all possible, a minimum of 5 milliliters of urine is collected and poured into sterile polypropylene or glass vials with screw-cap tops. The specimens should be labeled, frozen at or below -20 ºC, and stored on dry ice for shipping. Special care must be taken in packing to protect vials from breakage during shipment. All samples in long-term storage must be kept frozen, preferably at -70 ºC, until analysis.

c. Sample Handling

Specimen handling conditions are outlined in the Division of Laboratory Sciences (DLS) protocol for urine collection and handling (copies available in branch, laboratory and special activities specimen handling offices). Collection, transport, and special requirements are discussed in the division protocol. In general, urine specimens should be transported and stored frozen. Once received, they should be frozen, preferably at -70 °C, until time for analysis. Portions of the sample that remain after analytical aliquots are withdrawn must be refrozen as soon as possible after analysis.

d. Sample Quantity

The regular sample size for analysis is 1.0 mL, and the minimum amount of specimen required for analysis is 20 μL.

e. Unacceptable Specimens

Specimens must be frozen when delivered to the lab. The minimum volume required is 0.2 mL. If either of these criteria is violated, then specimen would be rejected. Specimens are also rejected if suspected of contamination due to improper collection procedures or devices. Specimen characteristics that may compromise test results include contamination of urine from improper handling. Samples with visible microbiological growth (e.g. mold, bacteria) must also be rejected. In all cases, request a second urine specimen if available. A description of reasons for each rejected sample must be recorded on the sample transfer sheet, such as low sample volume, leaking or damaged container.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

6. Preparation of Reagents, Calibration Materials, Control Materials, and all Other Materials; Equipment and Instrumentation

a. Reagents and Sources

See Table 2.

Table 2. Reagents and the respective manufacturers

Reagent	Manufacturers*
de-ionized water (D.I. H_2O)	Prepared in house, CDC (Aqua Solutions, Inc.)
β-glucuronidase/arylsulfatase (H-1, powder enzyme), glacial acetic acid, sodium acetate, N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)	Sigma Chemical, St. Louis, MO
pentane, hexane, acetonitrile, toluene, and methanol (ABSOLV grade)	Tedia Company, Fairfield, OH
argon, nitrogen	Air Products and Chemicals, Allentown, PA
$^{13}C_6$ 3-PHE, $^{13}C_{12}$ -PCB105, $^{13}C_6$ 1-NAP, $^{13}C_6$ 2-NAP, $^{13}C_6$ 9-FLUO	Cambridge Isotope Laboratories, Andover, MA
$^{13}C_6$ 3-FLU, $^{13}C_6$ 1-PYR	ChemSyn, Lenexa, KS
$^{13}C_6$ 3-FLU, $^{13}C_6$ 9-FLU, $^{13}C_6$ 2-PHE	Los Alamos National Laboratory, Los Alamos, NM
1-NAP, 2-NAP, 2-FLU, 3-FLU, 9-FLU, 1-PYR	Sigma-Aldrich Chemicals, St. Louis, MO
1-PHE, 2-PHE, 3-PHE, 4-PHE	Promochem, Wesel, Germany

* Equivalent products from other manufacturers may be used.

b. Preparation of Reagents

1) Sodium Acetate Buffer Solution (1 M, pH 5.5)

Place 41 g sodium acetate powder in a 500-mL vitro bottle and add approximately 300 mL de-ionized water (D.I. H_2O). Stir on a stir plate until sodium acetate is completely dissolved. Fill flask to the 500 mL line with D.I. H_2O. Adjust the pH to 5.5 with glacial acetic acid.

2) β-glucuronidase/arylsulfatase Enzyme/Buffer solution

Weigh 0.5 g of β-glucuronidase/arylsulfatase, H-1, powder enzyme into a 60-mL ASE glass vial. Add 50 mL of the pre-prepared sodium acetate buffer and cap the vial. Place vial on a rotating mixer at 40 rpm until the enzyme is completely dissolved.

3) Ascorbic Acid Solution

Weigh 1.25g of L-ascorbic acid into a 20mL borosilicate glass vial. Add 5.0mL of the deionized water and cap the vial. Place vial on a rotating mixer at 40 rpm until the solute is completely dissolved.

4) N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)

Open the sealed vial containing MSTFA from the vendor and place the MSTFA solution in an amber screw-cap vial (2-mL). Displace the air over the MSTFA with a gentle stream of argon. The MSTFA can be stored in the amber screw-cap vial for up to 1 month. To add MSTFA to multiple samples, use an Eppendorf repeater pipette with a 100-μL pipette tip, set the pipette volume at 10 μL, and then withdraw 100 μL of MSTFA. Discard the first two aliquots and the last aliquot of MSTFA (10 μL per aliquot), aliquot 10 μL into each of the sample vials.

c. **Preparation of Calibration Materials**

1) Stock Solutions of Individual Analytes (target concentration: 400 ng/μL for 1- and 2-NAP, 100 ng/μL for remaining analytes)

Approximately 5-10 mg of neat standard is weighed into a silanized screw cap amber vial. Add 3 mL of acetonitrile into the vial and record the exact weight of the solvent. Allow the standards to dissolve by gentle swirling or placing in an ultrasonic bath. Dilute the individual standards using toluene to 100 ng/μL (400 ng/μL for 1- and 2-NAP) with a final volume of 3.0 mL. All solutions are stored in a refrigerator with an inert argon atmosphere in the vials.

2) Working Standard Solution of Native Standard Mix (W.S.A)

Combine 400 μL from each of the individual native standard stock solutions in a silicanized screw cap amber vial to generate the working standard solution (W.S.A, target concentration: 4 ng/μL for each native compound concentration, except for 1- and 2-NAP at 16 ng/μL). Homogenize the mixture by gentle swirling and vortexing. Solutions of other concentrations may also be prepared, if needed. Displace air in the vial with argon, cap the vials, and store them in a refrigerator until needed.

3) Working Standard Solution of ^{13}C-labeled Standard Mix (W.S.I)

Combine individual ^{13}C-labeled standard stock solutions (90 ng/μL) in a silicanized screw cap amber vial to generate the working internal standard solution (W.S.I, target concentration: 6 ng/μL for each of the ^{13}C-labeled compounds, except for ^{13}C-labeled 1- and 2-NAP at 24 ng/μL). Homogenize the mixture by gentle swirling and vortexing. Displace air in the vial with argon, cap the vials, and store them in a refrigerator until needed.

4) External Calibration Standards (E.C.S)

A typical set of external calibration standards (E.C.S.) are prepared as presented in Table 3 below. The preparation of standards is done by gravimetric determination. Therefore, the exact concentration for each standard will slightly deviate from the target concentration listed in Table 3. Concentrations for 1- and 2-NAP in all standards are 4 times higher than the rest of the native compounds because these two compounds are present in urine samples at higher concentrations. Target concentrations for ^{13}C-labeled internal standards are 100 pg/μL (400 pg/μL for ^{13}C-labeled 1- and 2-NAP) in all calibration standards.

In addition, due to higher concentrations of 1- and 2-NAP often found in specimens, two additional calibration standards are prepared for 1-NAP and 2-NAP. The two calibration standards are at levels of 8000 and 16,000 pg/μL and only contain 1- and 2-NAP. These two calibration standards are used to evaluate and extend the linear range of the instrument calibration curves.

Table 3. Preparation of external calibration standards (ECS)

Standard No.	Analytes, excluding 1- & 2-NAP		1- and 2- NAP	
	Target concentration (pg/μL)	Equivalent concentration in urine (pg/mL)	Target concentration (pg/μL)	Equivalent concentration in urine (pg/mL)
1	1	10	4	40
2	2	20	8	80
3	5	50	20	200
4	10	100	40	400
5	50	500	200	2,000
6	100	1000	400	4,000
7	500	5000	2,000	20,000
8	1000	10,000	4,000	40,000
9	n/a	n/a	8,000	80,000

10	n/a	n/a	16,000	160,000

5) Internal Qualification Standards (I.Q.S)

Weigh in 4.167 mL of W.S.I into a 1-L silanized volumetric flask. Dilute the solution with acetonitrile to the 1-L line to get the internal qualification standard (I.Q.S). The concentration of each ^{13}C-labeled IS 25 pg/μL. Aliquot 1.9 mL of I.Q.S. into amber 2-mL standard vials, cap and seal the vials with argon. Store all I.Q.S. vials in the fridge until use.

d. Preparation of Control Materials

1) Quality Control (QC) Materials

Prepare quality control materials by spiking a known amount of native standard mixture (in acetonitrile) into 2000 mL of an anonymous filtered urine pool (300 pg/mL for QCL, 800 pg/mL for QCH). Homogenize the QC solutions overnight for equilibration. On the next day aliquot the QC solutions into 16 x 100 mm test tubes and store them at -70 °C until use.

2) Recovery Spiking Solution (R.S.S)

The recovery standard ($^{13}C_{12}$ PCB105) was purchased as a solution (40 μg/mL, in nonane). Dilute the standard solution using toluene to 100 pg/μL. This will be used as the recovery spiking solution (R.S.S.). Aliquot 1.7 mL of E.R.S. into amber 2-mL standard vials, cap and seal the vials with argon. Store all E.R.S. vials at 4 °C until use.

3) Proficiency Test Material (PT)

Prepare quality control materials by spiking a known amount of W.S.A (at a different level as the QC) into 100 mL of an anonymous urine pool (filtered) to achieve the target concentration. Prepare four urine pools at levels within the linear range of the method. After spiking the urine pool with a known amount of W.S.A, homogenize the PT solutions overnight for equilibration. On the next day aliquot the PT solutions into 16 x 100 mm test tubes (2 mL in each tube). PT samples are then randomized by an external PT administrator, labeled by external lab technicians, and stored at -70 °C until use.

e. Other Equipment, Materials, and Supplies

Materials / supplies and sources, or their equivalent, used during the development, validation, and application of this method are listed below.

- Gilson 215 liquid handler (Gilson Inc., Middleton, WI)

- 818 Automix mixer (Gilson Inc., Middleton, WI)
- Water bath/sonicator (Branson Ultrasonics, Danbury, CT)
- RapidVap evaporator (Labconco, Kansas City, MO)
- Incubator ovens (Fisher Scientific)
- pH meter (Thermo)
- Microbalance (Mettler-Toledo)
- Stirring/heating plates (Corning)
- Miscellaneous glassware (Pyrex, Kimax, Wheaton or Corning)
- Eppendorf Repeater Plus Pipette (Brinkmann Instruments Inc., Westbury, NY).
- Rainin Electronic Pipettes (Rainin, California)
- Maxi-mix Vortex mixer (Barnstead International)
- Allegra-6 centrifuge (Beckman-Coulter)
- Amber screw top vials with various volume (Supelco, Inc., Bellefonte, PA)
- Amber autosampler vials, 350 μL (National Scientific)

f. Instrumentation

The analyses are performed on an Agilent 7000A tandem mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an electron ionization ion source and interfaced to an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA).

1) Gas Chromatograph Configuration

Chromatographic separation is performed on an Agilent 7890A GC fitted with a ZB-5MS (Phenomenex or equivalent) 30-m fused silica capillary column. The column ID is 0.25-mm and the film thickness is 0.25-micron. The temperature program runs a total of 22 minutes. (See Table 4 for GC configuration and Table 5 for a typical GC temperature program.)

Table 4. GC configuration

GC Parameter	Setting
Carrier gas	Helium
Constant flow rate	0.9 mL/min

GC septum purge flow rate	3 mL/min
GC gas saver after	3 min
GC gas saver flow rate	20 mL/min
Injection mode	Pulsed Splitless
Injection pulse pressure	25 psi until 0.4 min
Injector temperature	270 ºC

Table 5. Typical GC temperature program. Slight modifications might be required to accommodate separation variations on commercial GC columns.

Time (min.)	Ramp (C/min)	Temperature (ºC)
0	0	95
1.00	15	195
7.67	2	206
13.2	0	206
16.2	40	320
19.0	0	320
22.0	-	320

2) Tandem Mass Spectrometer (MS/MS) Configuration

The Agilent 7000A MS/MS configuration is presented in Table 6. The mass spectrometer is operated under multiple reaction monitoring (MRM) mode. The ion transitions used to quantify analytes are presented in Table 7.

Table 6. Thermo TSQ Quantum MS/MS configuration

MS/MS Parameter	Setting
Scan mode	Multiple reaction monitoring
Ionization type	Electron ionization

MS/MS Parameter	Setting
Ion polarity mode	Positive
Electron energy	-70 eV
Emission current	35 μA
Ion source temperature	270 °C
Electron multiplier voltage	operated at GAIN + 100

Table 7. Analyte MS/MS Ion Transitions.

Analyte	Ion transition	CE (eV)
1-NAP	216.1 → 185.1	27
2-NAP		27
$^{13}C_6$ 1-NAP	222.1 → 191.2	27
$^{13}C_6$ 2-NAP		27
9-FLU	254.1 → 165.0	23
3-FLU		20
2-FLU		20
$^{13}C_6$ 9-FLU	260.1 → 170.9	23
$^{13}C_6$ 3-FLU		30
$^{13}C_6$ 2-FLU		30
4-PHE	234.8 → 220.0	30
$^{13}C_4$ 4-PHE	270.1 → 238.8	30
3-PHE	266.1 → 235.2	27
2-PHE		27
1-PHE		27
$^{13}C_6$ 3-PHE	272.1 → 241.3	27
$^{13}C_6$ 2-PHE		27
$^{13}C_4$ 1-PHE	270.1 → 239.3	27
1-PYR	290.1 → 258.9	30
$^{13}C_6$ 1-PYR	296.1 → 265.2	30

7. Calibration and Calibration Verification

a. Tuning and Calibration of Mass Spectrometer

The Agilent 7000A GC-MS/MS should be tuned before each analytical run. Under the Instrument menu in the MassHunter Workstation Software Data Acquisition window, select “MS Tune” to open the tune window. Select the “Autotune” tab and then the “Autotune” tab again in the lower view. Select “EI high sensitivity autotune” from the options and “Print Autotune report” if desired, then click the “Autotune” button. Once the autotune is complete and saved, review the results either by printout (if that option was selected) or by the PDF that was auto-generated by the software. The abundance of m/z 69 should be >1,000,000, and the isotope ratio of m/z 70 to m/z 69 should be roughly 1%. The relative abundance of m/z 502 to m/z 69 should be >2.5%, and the isotope ratio of m/z 503 to m/z 502 should be roughly 10%. The repeller voltage should be <16 V. The EMV (Gain) should be < 2000 V and gain factor >200. If these values are not met, proceed to clean the ion source or perform other maintenance as needed.

b. Creation of Calibration Curve

1) Calibration data

A full calibration curve is analyzed with every analytical run. A linear log-log calibration curve, using eight ECS with concentrations ranging from 1 to 1000 pg/μL (4 to 4,000 pg/μL for 1- and 2-NAP), is generated using the log ratio of the analyte peak area to the labeled internal standard peak area against the log ratio of the native analyte concentrations to those of the labeled internal standards. The concentrations in ECS correspond to 10 - 10,000 pg/mL urine for all analytes except for 1- and 2-NAP, and 40-40,000 pg/mL urine for 1-NAP and 2-NAP. For urine samples with concentrations higher than the calibration curve, the highest two standards for 1- & 2-NAP are prepared to extend the calibration curve and to accurately quantify those samples up to 160,000 pg/mL urine.

2) Evaluation of Curve Statistics

The R-squared value of the curve must be equal to or greater than 0.98. Linearity of the standard curve must extend over the entire calibration range.

3) Use of the Calibration Curve

The lowest point on the calibration curve is the lowest reportable level and the highest point is the highest reportable level. The remainders of the points are distributed between these two extremes, with the majority of points in the concentration range where most unknowns fall. When sample results are over the highest reportable level, the samples are repeated with up to 50 fold dilution.

c. Calibration Verification

1) Calibration verification is not required by the manufacturer. However, it should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.

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

3) According to the updated Clinical Laboratory Improvement Amendments (CLIA) regulations from 2003 the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months.

4) All of the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of eight calibration standards, two blanks, two QCLs, two QCHs and 34 unknown urine samples.

a. Sample Preparation

1) Enzyme Hydrolysis

Allow urine samples and QCs to thaw and reach room temperature. Aliquot 1 mL of urine (or 1 mL of D.I. H_2O as blank) into a 16x100 mm test tube. Add 1 mL of 1 M sodium acetate butter (pH = 5.5) containing β-glucuronidase/ arylsulfatase enzyme from *Helix pomatia* (10 mg enzyme/1 mL buffer) into the test tube. Place the uncapped sample tubes on the Gilson 215 liquid handler (Gilson Inc., Middleton, WI) and initiate the automated spiking procedure. All samples are spiked with 40 µL of I.Q.S on the Gilson 215 liquid handler. Cap the test tubes and invert gently several times to mix well. Place the samples in a 37 °C oven and incubate the sample overnight (~17-18 hours).

2) Automated Liquid-Liquid Extraction

Add D.I. water (3 mL) to all samples prior to using the Gilson 215 liquid handler (Gilson Inc., Middleton, WI) fitted with an 818 AutoMix for automation. Place the uncapped sample tubes in the Gilson 215 and initiate the extraction procedure. The automated procedure will add 20% toluene/80% pentane (5 mL) to each sample. Manually cap the tubes then place back on the AutoMix to mix for 5 minutes (20 rpm). Centrifuge samples at 2800 rpm on an AllegraTM 6 centrifuge (Beckman Coulter Inc., Fullerton, CA) until clear separation can be seen between layers (20-30 minutes). Uncap the tubes and return them to the Gilson 215 to resume automation. The liquid handler probe will transfer the

organic phase to clean 16x100 test tubes. Repeat extraction a second time which gives a total of approximately 9mL of organic phase collected in each test tube. The urine sample test tubes are no longer needed and may be discarded.

3) Evaporation

Spike the extract with 10 μL dodecane and place in a RapidVap evaporator. The evaporation is a two-stage process. First, the pentane fraction is evaporated at 45 °C, with 40% rotation speed, and 400-450 mbar vacuum (~10 minutes). Then, the sample tube is transferred to a second RapidVap, and the toluene fraction is evaporated to a final volume of ~10uL at 80 °C, with 50% rotation speed, and 200-230 mbar vacuum (20-25 minutes). Spike each sample tube with 20 μL R.S.S., vortex for a few seconds, and then transfer the contents to an amber autosampler vial.

4) Derivatization

Add 10 μL of MSTFA into the GC vial and then displace the air in the vial with a gentle stream of argon. Quickly screw a cap onto the vial. Place the vials in an incubator or oven set at 60 °C for 30 minutes. The samples are then ready for analysis on the mass spectrometer.

b. Instrument and software setup for the GC-MS/MS

1) Preliminary MS/MS System Setup and Performance Check

The MS/MS tune must be verified in the MassHunter Workstation Software Data Acquisition window either by a new Autotune procedure (described above in 7a) or by performing a Check Tune. Under the Instrument menu, select “MS Tune” to open the tune window. Select the “Autotune” tab and then the “Check Tune” tab in the lower view. Select “Print Check Tune report” if desired, then click the “Check Tune” button. The results of the check tune must all pass as “OK” and the abundance of PFTBA (69.0) should be >1000000. If these results are not met, the instrument is not ready for operation and maintenance (cleaning, check for air leaks, etc) needs to be performed.

2) Final Setup and Operation

a) *Create the run sequence*

The sequence may be created in two ways: in the MassHunter software or in Microsoft Excel. The choice is left to the discretion of the analyst.

In the MassHunter Workstation Software Data Acquisition window, select “Edit sequence” under the Sequence menu. Make sure that the appropriate number of samples filenames, sample IDs, and sample positions are

included in the run sequence. Include two solvent blanks after upper level calibration standards. Make sure that each sequence row is labeled for "Type" (Cal, Sample, etc.) and that all "Cal" standards have corresponding "Level." Ensure that the correct instrument method (NHANES_UU_20130611.M) is selected. The latter defines GC, MS, and autosampler methods.

If creating a sequence in a Microsoft Excel spreadsheet, make sure there are the appropriate number of rows with correct filenames, sample IDs, vial positions, sample types, and calibration levels (if applicable). The information can then be copied and pasted from Excel into the appropriate columns within the MassHunter sequence table.

Filenames conform to the following format: AGMYYNNNN or ARMYYNNNN where AG = samples run on the MS/MS instrument named as AGIL, AR = samples run on the MS/MS instrument named as ARCHIE; M = month (A = January, B = February, C = March, etc.); YY = year (13 = year 2013), and NNNN = run number for the month. For example, the filename AGB130012 corresponds to the 12th sample run in February 2013 on MS/MS instrument AGIL.

b) *Start the sequence*

In the MassHunter Workstation Software Data Acquisition window, select "Run sequence" under the Sequence menu. In the pop-up window that appears, select "Disable Barcode for this sequence" and fill out the fields for Operator Name and Data File Directory. Click **RunSequence**.

c. Processing of Data

Data is processed using the MassHunter QQQ Quantitative Analysis software. In the software, select raw data files and batch them by analytical run (e.g. ABC-011). Select and apply the quantitation method with the most recent calibration information (2013-06_OH-ME_ECS71-78.quantmethod.XML) to the batch. The batch is then analyzed and automatically integrated. Visually review, and manually correct as needed, the integration of each peak. Save the batch and re-analyze to account for updates to the calibration curve and calculated concentrations in samples due to manual integrations. Export the results table as an MS Excel file, and place a copy of this Excel file on the CDC shared network drive. Perform all further calculations such as standard curve generation, QC analysis, blank analysis, limit-of-detection determination, unknown sample calculations, data distribution, etc. in MS Excel and in SAS. Import final results and all supporting information into a SAS dataset and save it on the share drive.

d. Replacement and Periodic Maintenance of Key Components

1) Agilent 7000A GC/MS/MS Mass Spectrometer

- Clean the ion source every 4-6 weeks or sooner as needed
- Replace the ion source filaments every 4 months or sooner as needed
- Replenish the calibration gas every 12 months or sooner as needed
- Trained Agilent technicians perform all other maintenance based on an annual schedule, or as needed

2) Agilent 7890A GC

- Clean the injection port and change the injection port liner and septum biweekly or sooner as needed
- Cut the GC column as needed
- Replace the GC column after 2000 injections or sooner as needed
- Replace Helium tank when the pressure is below 500 psi

9. Reportable Range of Results

The linear range of the standard calibration curves determines the highest and lowest analytical values of an analyte that are reportable. The calibration verification of the method encompasses this reportable range. However, urine samples with analytical data values exceeding the highest reportable limit may be re-extracted using a smaller volume (e.g., 0.2 mL or 5 fold dilution) and re-analyzed so that the result is in the reportable range. For samples with extremely high values, samples can be diluted up to 50 folds (0.02 mL sample size).

a. Linearity Limits

Analytical standards are linear for all analytes through the range of concentrations evaluated. The linear range is 40 pg/mL to 160,000 pg/mL urine for 1-NAP and 2-NAP and 10 pg/mL to 20,000 pg/mL urine for the remaining analytes. Urine samples whose OH-PAH concentrations exceed these ranges must be diluted and re-analyzed using a smaller aliquot (first use 0.2mL, and then 0.02 mL if needed).

b. Limit of Detection

The limit of detection (LOD) for each analyte is defined as the higher LOD calculated by two methods, 1) In relation to method blanks: as three times the standard deviation of the method blanks run over a 6-month period of time; 2) In relation to instruments detection limit: defined as the lowest point on the calibration curve verified to give a signal with the signal-noise-ratio (S/N) equal to or greater than 10. The detection limits determined for each analyte are evaluated periodically (e.g. every 6 months or after an NHANES cycle) based on observed blank levels over the period. Typical LODs are presented in Table 8 (updated after NHANES 2011-12).

Table 8. Limits of detection (LOD)

Analyte	LOD (pg/mL)
1-NAP	48
2-NAP	40
9-FLU	10
2-FLU	10
3-FLU	10
1-PHE	10
2-PHE	10
3-PHE	10
4-PHE	10
1-PYR	10

c. Precision

The precision of the method is reflected in the variance of quality control samples analyzed over time. The mean and coefficients of variation (CV) of 318 QC samples are listed in Table 9. These QC samples were prepared over 6 months by two different analysts using two automated liquid handlers and two GC/MS/MS instruments.

Table 9. Mean, standard deviation, and CV for QC samples. The parameters are QC pool specific.

Analyte	Low QC (n=30)				High QC (n=30)			
	Mean (pg/mL)	Between day SD (pg/mL)	Within day SD (pg/mL)	CV	Mean (pg/mL)	Between day SD (pg/mL)	Within day SD (pg/mL)	CV
1-NAP	812	55	27	6.3%	1233	111	59	8.3%
2-NAP	1807	112	65	5.6%	2273	153	102	5.9%
9-FLU	310	23	9	7.3%	628	48	24	7.1%
3-FLU	268	22	9	7.9%	689	56	28	7.6%
2-FLU	465	28	12	5.6%	789	85	31	10%
4-PHE	317	18	9	5.2%	773	42	31	4.7%
3-PHE	273	17	9	5.8%	704	40	31	4.8%
1-PHE	274	17	10	5.7%	691	45	31	5.6%
2-PHE	210	29	8	13%	596	81	25	13%
1-PYR	348	26	17	6.5%	906	71	58	6.4%

d. Analytical Specificity

The use of a triple-quadrupole MS allows a means of monitoring ion/mass transitions specific to each analyte. The analyte peaks are located in well-defined regions of the chromatogram with no visible interferences and low background. The retention time for the analytes relative to the isotope internal standards give additional confirmation of the presence of analytes in the sample. The relative retention time (RRT) ratio of a native analyte against its labeled internal standard cannot deviate more than 0.15% (OH-PAHs) from that in calibration standards.

e. Accuracy

The accuracy of this method was evaluated by analyzing two NIST Standard Reference Materials (SRMs) and compared to its certified concentrations for the 10 OH-PAHs (Table 10).

Table 10. Measured concentrations using CDC method in comparison to the certified concentrations in two NIST SRMs

Analyte	SRM 3672 Smoker urine			SRM 3673 Non-smoker urine		
	This method	NIST Certified*	accuracy	This method	NIST Certified*	accuracy
1-NAP	33868	34,400	98%	197277	211,000	93%
2-NAP	8768	8,730	100%	1342	1,345	100%
9-FLU	357	337	106%	109	110	99%
3-FLU	404	428	94%	35	39	90%
2-FLU	823	870	95%	94	107	88%
4-PHE	36	49	74%	8	10	77%
3-PHE	97	125	78%	20	28	70%
1-PHE	141	136	103%	50	49	102%
2-PHE	89	84	106%	23	25	94%
1-PYR	201	173	116%	32	30	106%

* Certified concentrations on OH-PAHs were obtained from the SRMs' draft Certificate of Analysis (COAs, internal communication with Dr. Michele Schantz, NIST). Mass fraction concentrations (μg/kg) were converted to urinary concentration (pg/mL) using a urine density value of 1.019 g/mL, as specified on the COAs.

The accuracy of the method was further assessed in a 6-point matrix standard addition experiment. We spiked a urine pool with 10, 20, 50, 100, 500 and 1000 pg/mL of standards (four times higher spike for 1- and 2-NAP). The un-spiked and spiked urine pools were analyzed, each in six replicates. A linear regression analysis was carried out by plotting the measured concentrations against spiked concentrations to evaluate correlations and determine concentrations of analyte in the non-spiked urine sample. As shown in Table 11, the matrix-spiked samples gave good linearity for all compounds with correlation coefficients ranging 0.92−1.00. The intercept from the linear regression reflected 80−109% of the measured concentrations in the un-spiked urine pool and the differences were not statistically significant (alpha = 0.05), demonstrating a non-biased and accurate method.

Table 11. Matrix-spiked standard addition experiment parameters

Analyte	Slope	Intercept (pg/mL)	r^2	unspiked Conc (pg/mL)
1-NAP	1.00	868	1.00	901
2-NAP	0.98	1208	1.00	1200
9-FLU	1.01	247	1.00	248
3-FLU	1.02	257	1.00	255
2-FLU	1.02	408	1.00	406
4-PHE	0.87	10	1.00	11
3-PHE	0.92	124	1.00	122
1-PHE	0.98	78	1.00	82
2-PHE	0.97	54	1.00	56
1-PYR	1.02	109	0.99	108

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

Quality assessment procedures follow standard practices (6). Daily experimental checks are made on the stability of the analytical system. Blanks and standards, as well as QC materials, are added to each day's run sequence. The blank and standard are analyzed at the beginning of each run to check the system for possible contamination or in the spiking solutions and/or reagents. Two QCLs and two QCHs are prepared and analyzed at the beginning and the end of each run; their concentrations are compared with acceptance criteria to assure the proper operation of the analysis. Relative retention times are examined for the internal standard to ensure the choice of the correct chromatographic peak.

b. Quality Control Procedures

1) Individual Sample Quality Checks

Each individual sample will be subjected to a number of quality checks: a) auto integrations must be reviewed and integrated manually if needed; b) the calculated recovery for the spiked C^{13}-labeled internal standards must be above 15% and lower than 150%; c) the relative retention time of each analyte in relation to its respective internal standard must be within ±0.15% (for OH-PAHs) of its established value.

2) Establishing QC limits

Quality control limits are established by characterizing assay precision with repetitive analyses of the QC pools. Different variables are included in the analysis (e.g. multiple analysts and instruments) to capture realistic assay variation over time. The mean, standard deviation (within day and between days), coefficient of variation, and confidence limits are calculated from this QC characterization data set. Individual quality control charts for the characterization runs are created, examined, and quality control limits are used to verify assay precision and accuracy on a daily basis. QC characterization statistics for OH-PAH analytes are listed in Table 9. The characterization statistics are pool specific.

3) Quality Control Evaluation

After the completion of a run, the quality control limits are evaluated to determine if the run is "in control." The quality control rules apply to the average of the beginning and ending analyses of each of the QC pools. The quality control results are evaluated according to Westgard rules (6).

Two QC pools *per run* with two or more QC results *per pool*

A) If both QC run means are within $2S_m$ limits and individual results are within $2S_i$ limits, then accept the run.

B) If 1 of the 2 QC run means is outside a $2S_m$ limit - reject run if:

a) Extreme Outlier – Run mean is beyond the characterization mean +/- $4S_m$
b) 3S Rule - Run mean is outside a $3S_m$ limit
c) 2S Rule - Both run means are outside the same $2S_m$ limit
d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

C) If one of the 4 QC individual results is outside a $2S_i$ limit - reject run if:

a) Extreme Outlier – One individual result is beyond the characterization mean +/- $4S_i$
b) R 4S Rule – Within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit). Note: Since runs have multiple results per pool for 2 pools, the R 4S rule is applied within runs only.

Abbreviations:

S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).
S_m = Standard deviation of the run means (the limits are shown on the chart).
S_w = Within-run standard deviation (the limits are not shown on the chart).

If the QC result for an analyte is declared "out of control", the results of that analyte for all patient samples analyzed during that run are invalid for reporting.

c. Proficiency Testing (PT)

1) Scope of PT

The proficiency testing (PT) scheme for this method is administered by an in-house PT coordinator. PT samples (4 different levels) were prepared in-house by spiking a known amount of standard into a well characterized urine pool and blind-coded by an in-house PT coordinator.

In addition, since 2013, we participate in the German External Quality Assessment Scheme (G-EQUAS) for Analyses in Biological Materials, which covers 1-NAP, 2-NAP and 1-PYR. G-EQUAS is organized and managed by the Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (Erlangen, Germany). The program, evaluation, and certification are based on the guidelines of the German Federal Medical Council.

2) Frequency of PT

For the in-house PT scheme, five samples of unknown concentrations are analyzed once or twice a year using the same method described for unknown samples. The PT administrator will randomly select five of the PT materials for analysis. A passing score is obtained if at least four of the five samples fall within the prescribed limits established beforehand.

For G-EQUAS, two materials of unknown concentrations are sent from G-EQUAS twice a year. The samples are analyzed using the same method described for unknown samples and the results are reported to G-EQUAS for evaluation and certification.

3) Documentation of PT

For the in-house PT scheme, PT results are reviewed by the analyst and laboratory supervisor, and then submitted to the in-house PT Coordinator. The PT results are evaluated by the PT Coordinator; the analysis passes proficiency testing if at least four of the five sample results deviate $\leq$ 20% from the known value.

For G-EQUAS, PT results are reviewed by the analyst and laboratory supervisor. The results and QC report are approved by a DLS statistician, Branch Chief, and DLS Director. The final results are submitted on G-EQUAS website.

All proficiency results shall be appropriately documented. If the assay fails proficiency testing then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error.

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 the source or cause of failure is identified and corrected. If the source of failure is easily identifiable, for instance, failure of the mass spectrometer or a pipetting error, the problem is immediately corrected. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration standards (in the case of calibration failure). After re-establishing calibration or quality control, resume analytical runs. Document the QC failures, review the cases with supervisor to determine source(s) of problem, and take measures to prevent re-occurrence of the same problem.

12. Limitations of Method, Interfering Substances and Conditions

This method is an isotope dilution mass spectrometry method, widely regarded as the definitive method for the measurement of organic toxicants in human body fluids. By using tandem mass spectrometry, most interferences are eliminated. Due to the matrix used in this procedure, occasional unknown interfering substances have been encountered. If chromatographic interference with the native analyte or internal standard occurs, reject that analysis. If repeat analysis still results in an interference that cannot be separated chromatographically, the results for that analyte are not reportable.

13. Reference Ranges (Normal Values)

The reference range values for the OH-PAH metabolites, established based on the National Health and Nutrition Examination Survey (NHANES), can be found at http://www.cdc.gov/exposurereport.

14. Critical Call Results ("Panic Values")

Insufficient data exist to correlate urinary OH-PAH concentrations with serious health effects in humans. Therefore, no established "critical call" values exist. Test results in this laboratory are reported in support of epidemiological studies, not for clinical assessments.

15. Specimen Storage and Handling During Testing

Urine specimens may reach and maintain ambient temperature during analysis. The urine extracts are stored in GC vials at -70 ^{o}C after analysis. Current studies indicate (CDC data) that the extracts are stable for at least three weeks.

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

Alternate validated methods have not been evaluated for measuring these PAH metabolites in urine. If the analytical system fails, urine extracts can be refrigerated until the analytical system is restored to functionality. If long-term interruption (greater than 4 weeks) is anticipated, then store urine specimens at -70 ^{o}C.

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

Study subject data is reported in both concentration units (ng/L, pg/mL, parts per trillion, or ppt) and adjusted based on creatinine excretion (ng/g creatinine).

a. The data from each analytical run are initially processed and reviewed by the laboratory supervisor or Quality Control officer using MS Excel with build-in macros to check sample Quality Control parameters, including recovery, relative retention time, blank levels, calibration curve, etc. The supervisor provides feedback to the analyst and/or his/her designee and requests confirmation of the data as needed.
b. The Quality Control officer reviews each analytical run and identifies the quality control samples within each analytical run and determines whether the analytical run is performed under acceptable control conditions.
c. One of the Division statisticians reviews and approves the quality control charts pertinent to the results being reported.
d. If the quality control data are acceptable, the laboratory supervisor or his/her designee generates a memorandum to the Branch Chief, and a letter reporting

the analytical results to the person(s) who requested the analyses to be signed by the Division Director.

e. The data are sent (generally electronically by e-mail) to the person(s) that made the initial request.
f. All data (chromatograms, etc.) are stored in electronic format.

Final hard copies of correspondence are maintained in the office of the Branch Chief and/or his/her designee and with the quality control officer.

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

If greater than 1 mL of sample remains following successful completion of analysis, this material must be returned to storage at -70 °C in case reanalysis is required. These samples shall be retained until valid results have been obtained and reported and sufficient time has passed for review of the results.

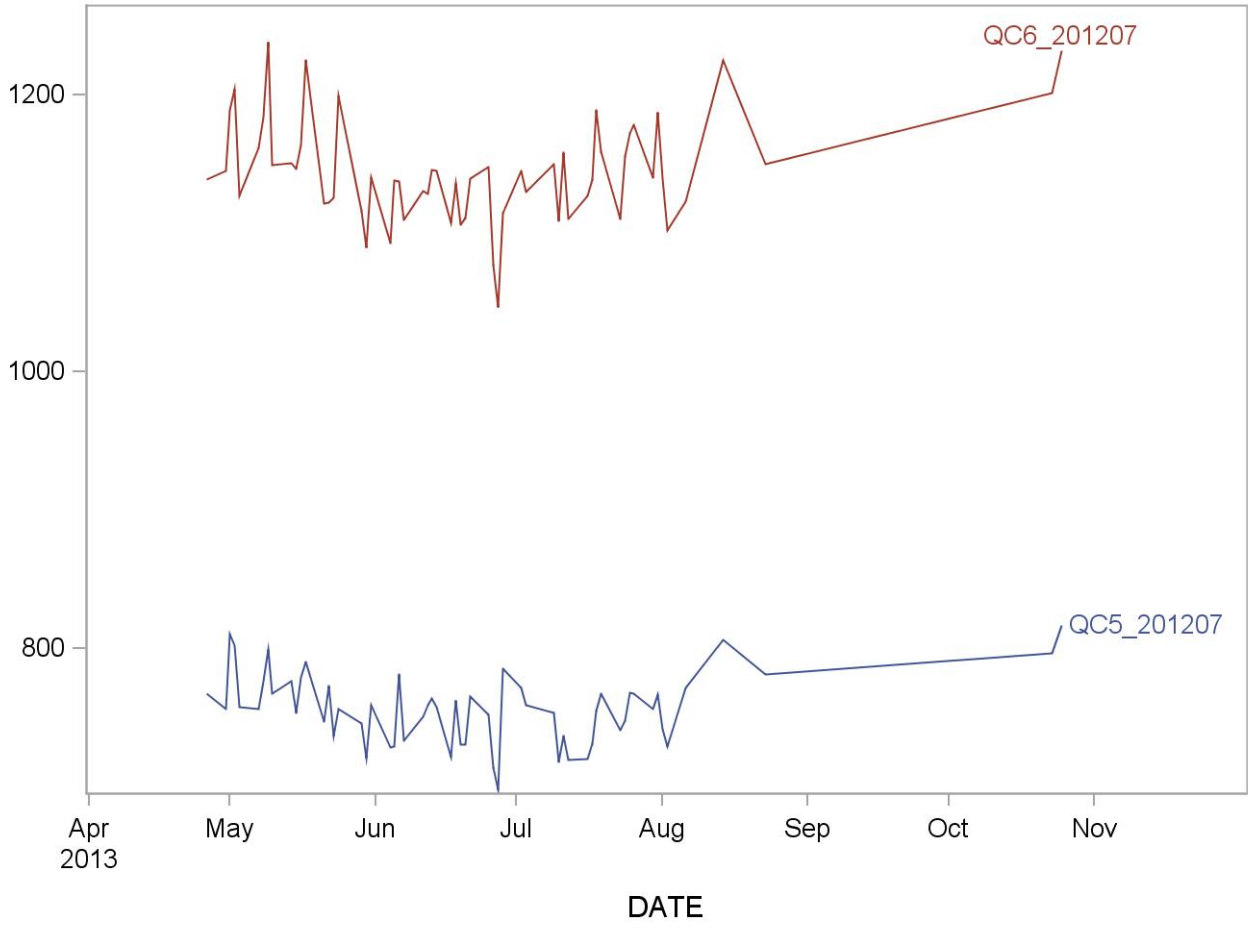
Standard record keeping (e.g., database, notebooks, and data files) is used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Transfer is carried out through the DLS Samples Logistic Group. Specimens may be stored at CDC specimen handling and storage facility (CASPIR).

19. Summary Statistics and QC Graphs

See following pages

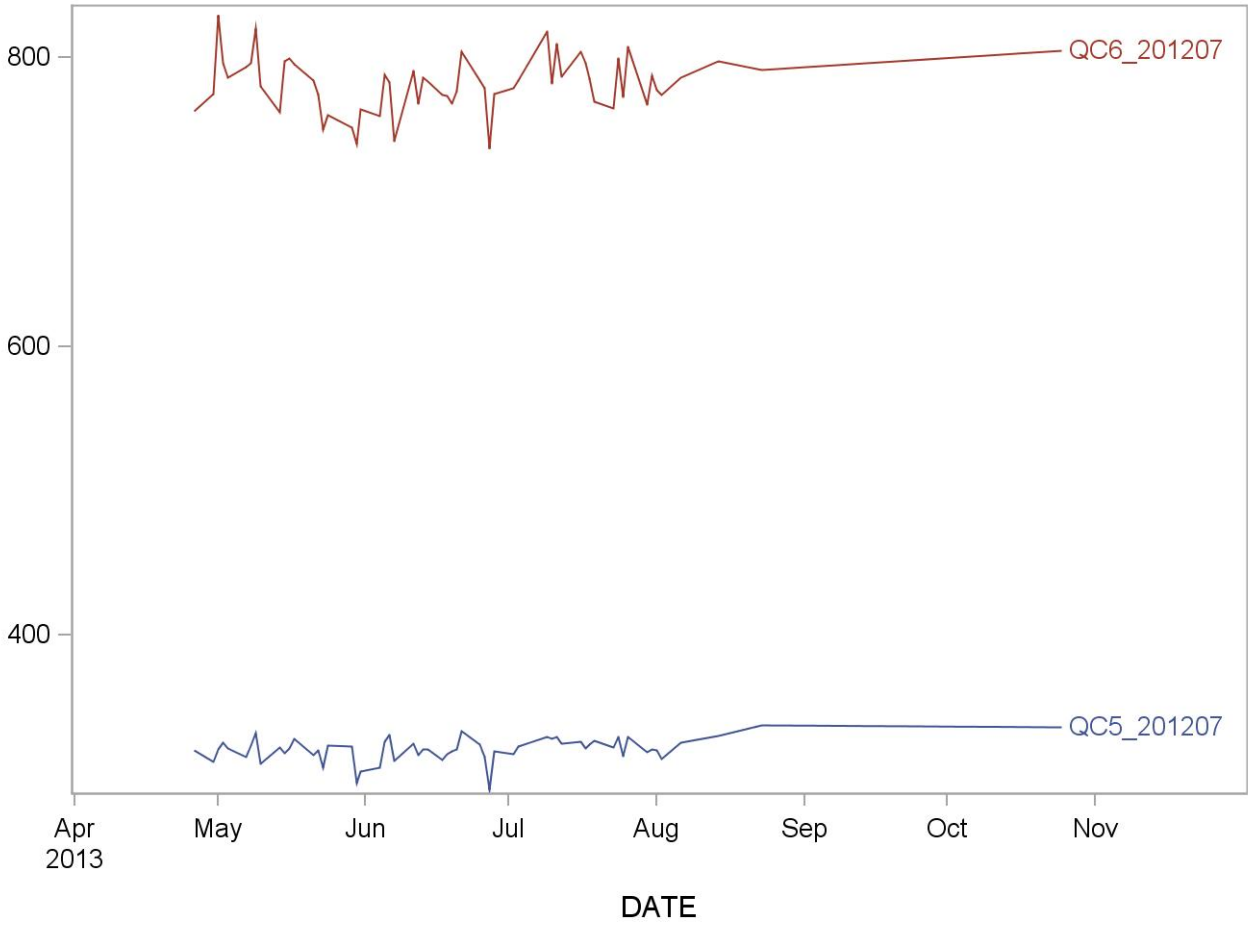
2011-2012 Summary Statistics and QC Chart for 1-naphthol

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	94	26APR13	25OCT13	1141.60	38.27	3.4
QC5_201207	94	26APR13	25OCT13	754.38	27.23	3.6



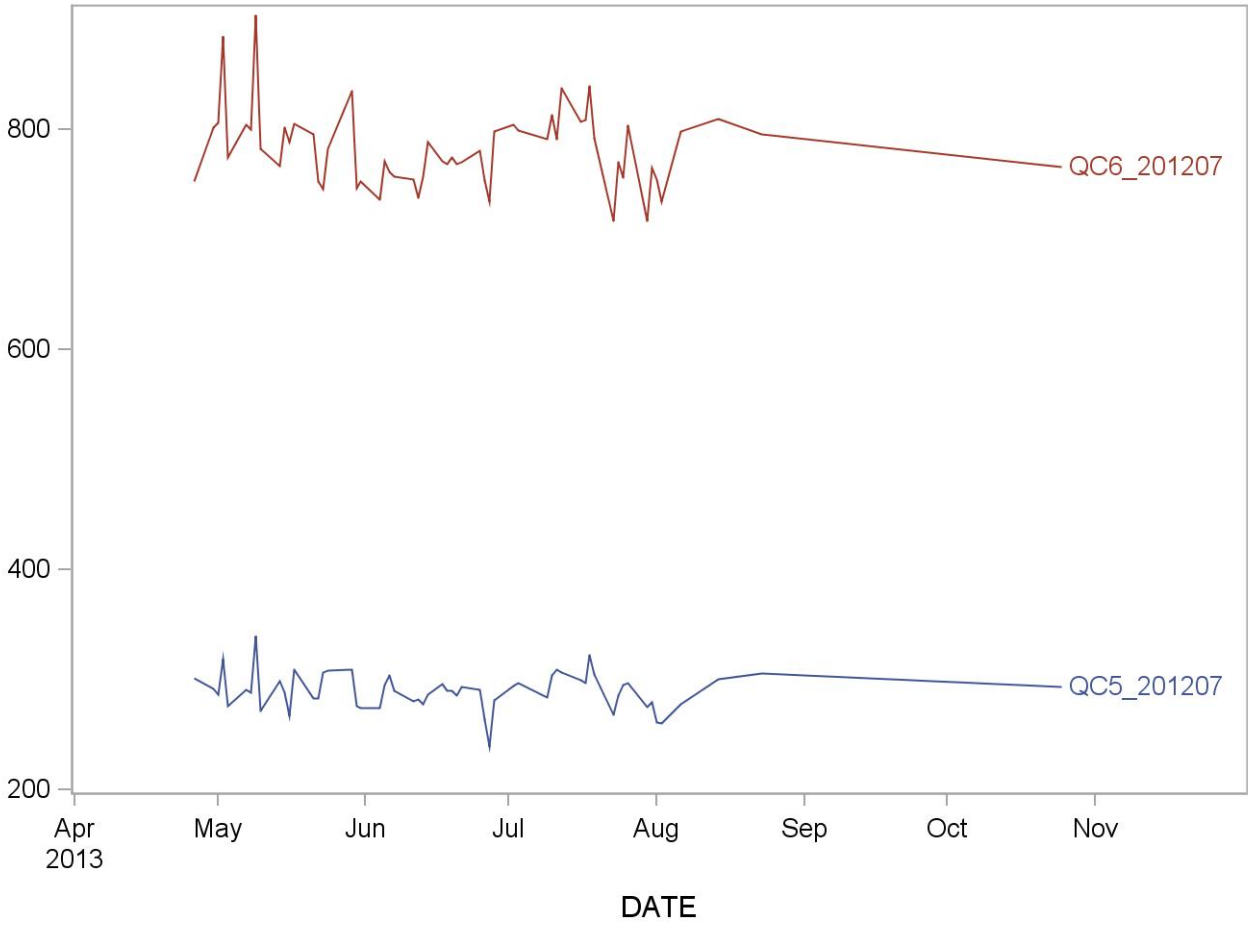
2011-2012 Summary Statistics and QC Chart for 1-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	781.88	21.58	2.8
QC5_201207	93	26APR13	25OCT13	321.26	9.18	2.9



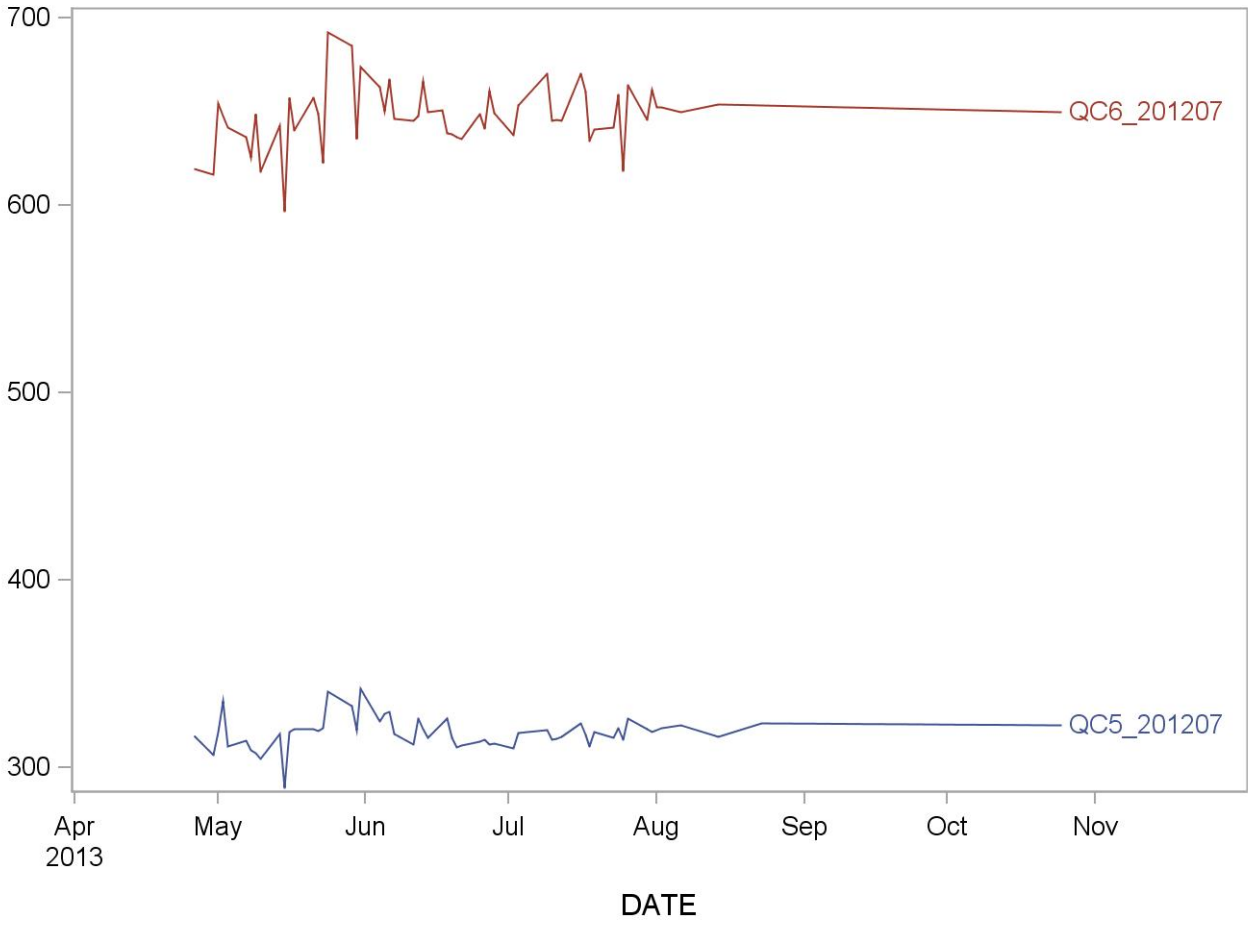
2011-2012 Summary Statistics and QC Chart for 1-pyrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	782.13	39.42	5.0
QC5_201207	93	26APR13	25OCT13	290.39	17.09	5.9



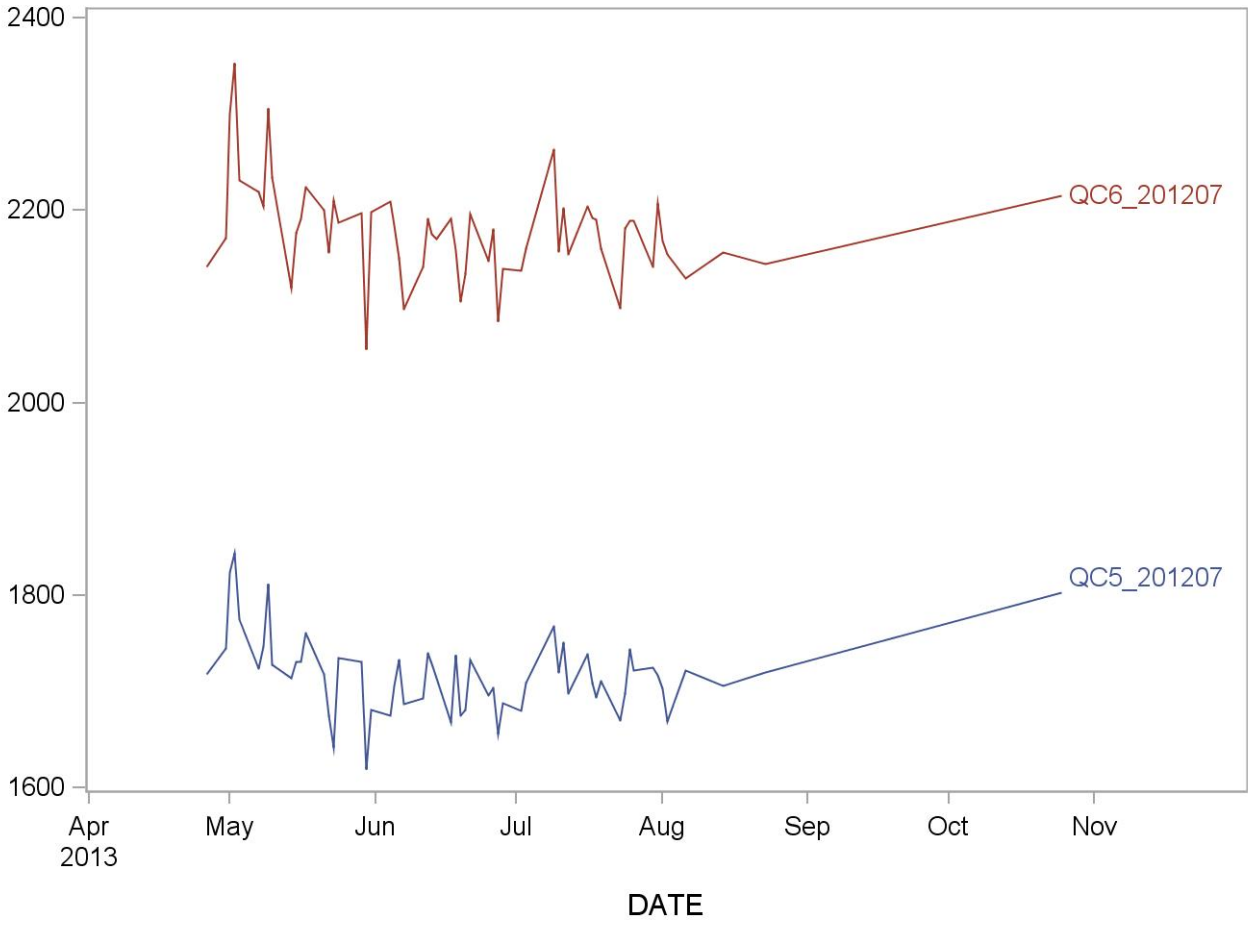
2011-2012 Summary Statistics and QC Chart for 2-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	646.83	21.47	3.3
QC5_201207	93	26APR13	25OCT13	317.88	9.94	3.1



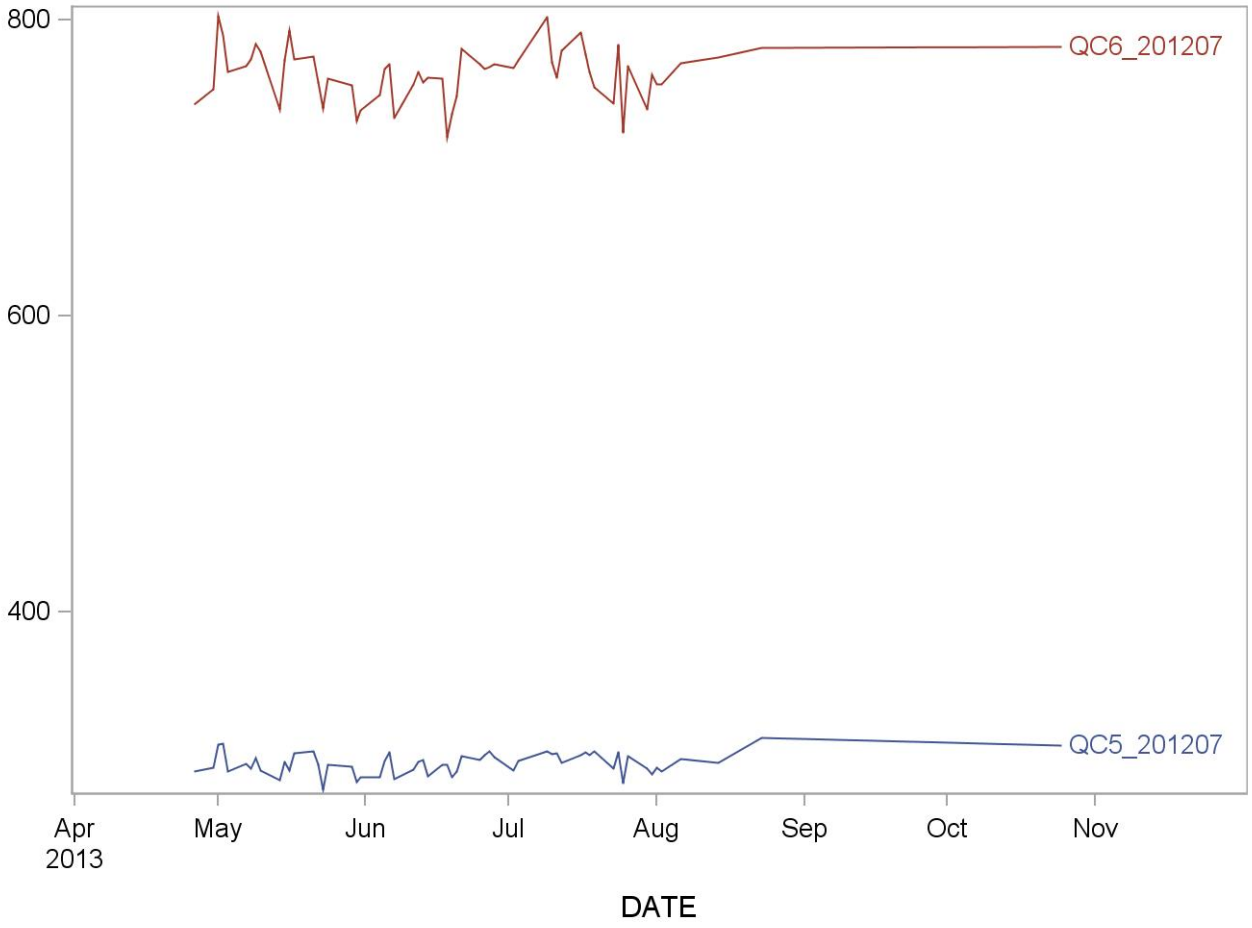
2011-2012 Summary Statistics and QC Chart for 2-naphthol

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	2176.54	53.49	2.5
QC5_201207	93	26APR13	25OCT13	1716.19	41.45	2.4



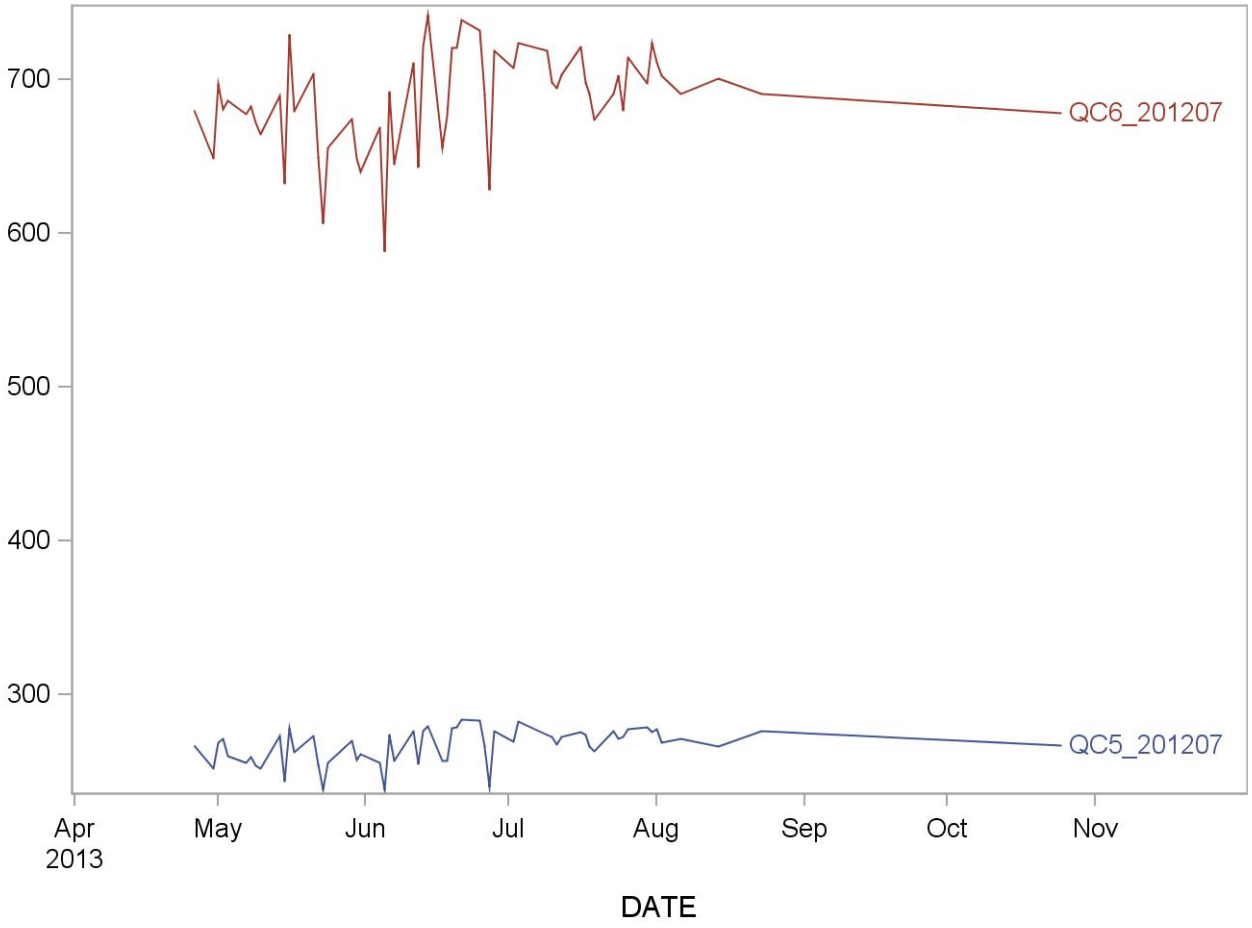
2011-2012 Summary Statistics and QC Chart for 2-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	763.70	20.87	2.7
QC5_201207	93	26APR13	25OCT13	298.19	9.03	3.0



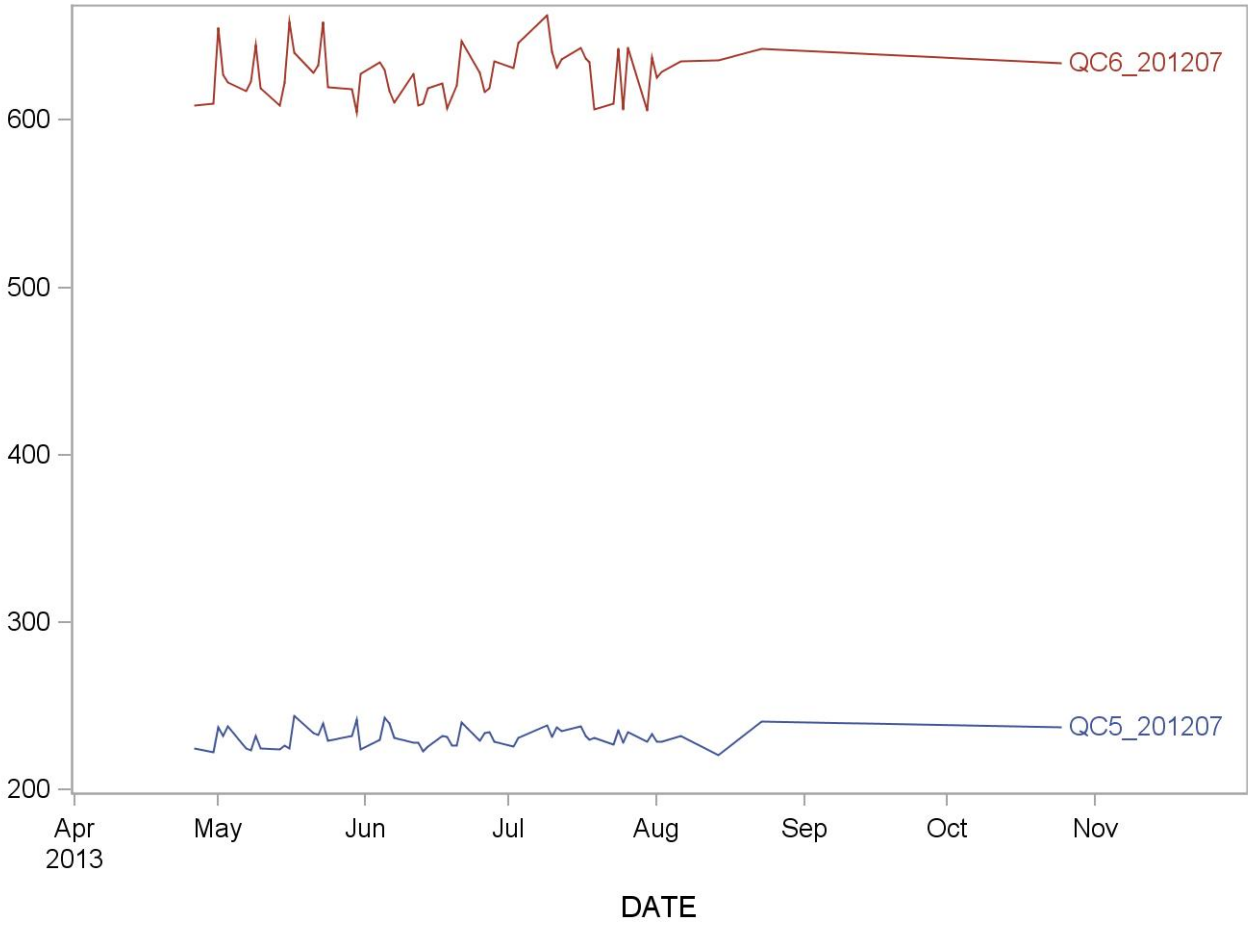
2011-2012 Summary Statistics and QC Chart for 3-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	687.53	38.53	5.6
QC5_201207	93	26APR13	25OCT13	267.14	14.01	5.2



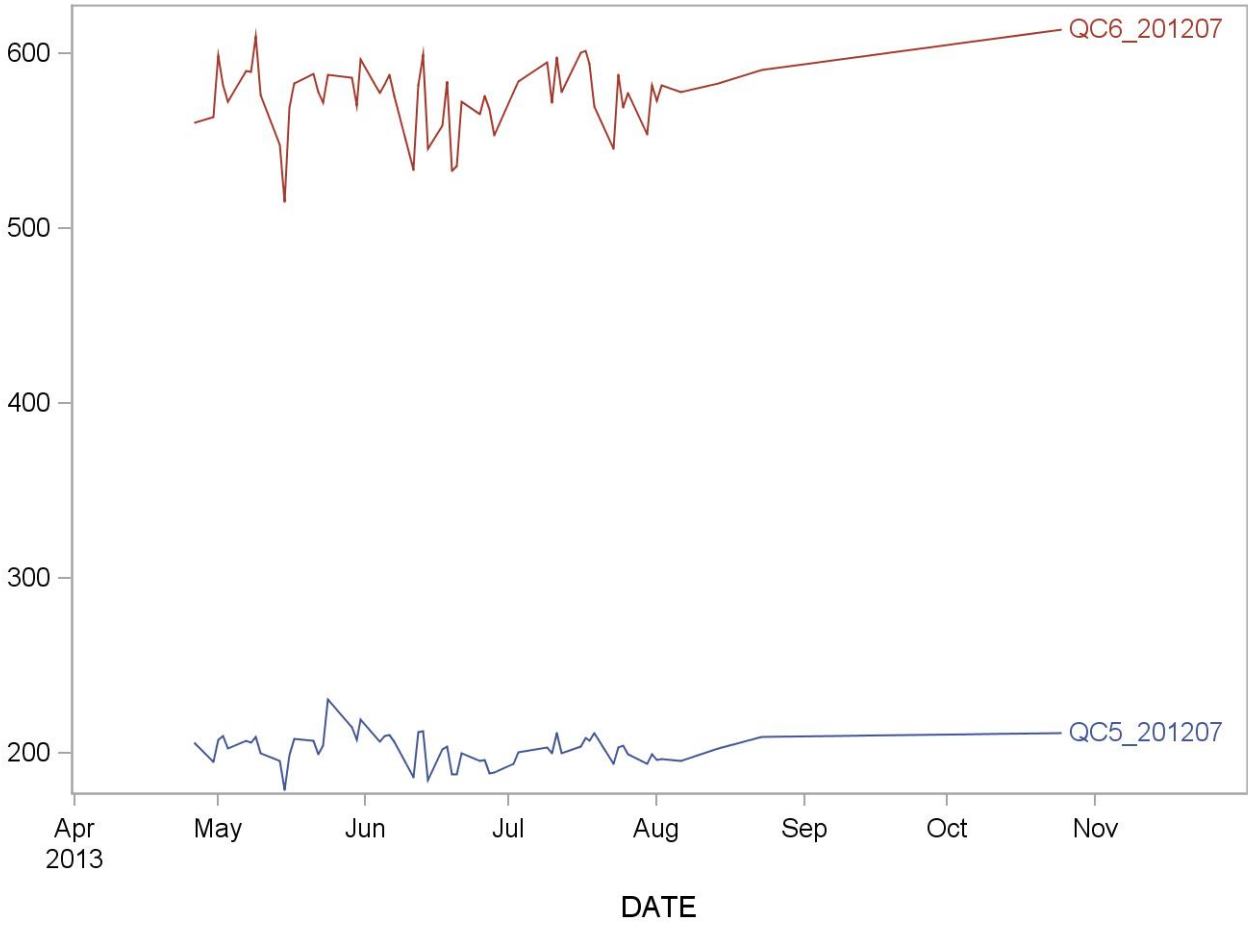
2011-2012 Summary Statistics and QC Chart for 3-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	627.17	18.39	2.9
QC5_201207	93	26APR13	25OCT13	231.29	6.54	2.8



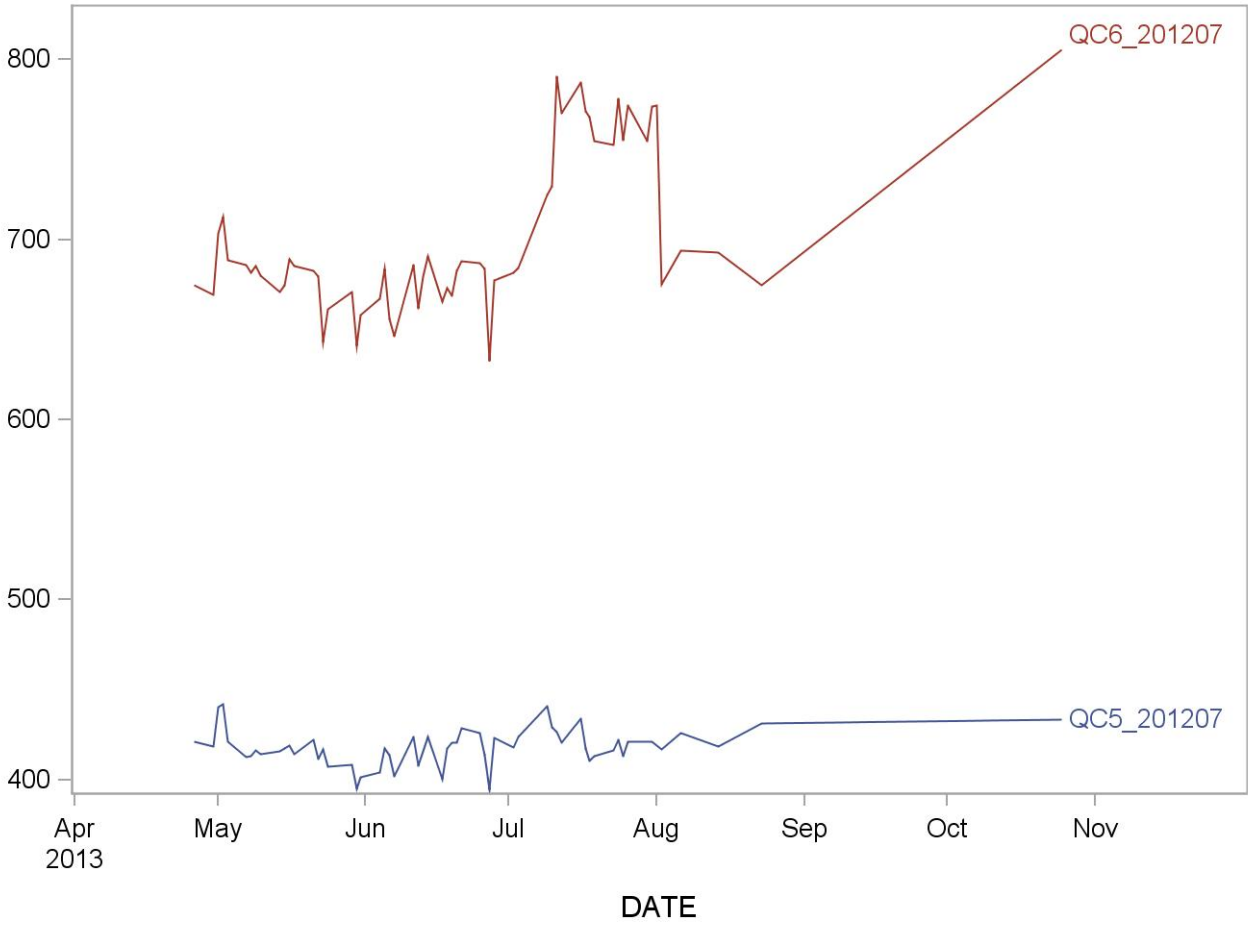
2011-2012 Summary Statistics and QC Chart for 4-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	575.06	22.67	3.9
QC5_201207	93	26APR13	25OCT13	201.66	9.77	4.8



2011-2012 Summary Statistics and QC Chart for 9-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207	93	26APR13	25OCT13	703.54	46.21	6.6
QC5_201207	93	26APR13	25OCT13	418.23	12.11	2.9



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Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

Appendix A – Ruggedness Testing for Analytical Method

Procedure

Ruggedness testing was conducted to evaluate 5 parameters in this method: enzyme amount, buffer strength, buffer pH value, de-conjugation time and de-conjugation temperature. For each parameter, 3 or 5 levels were tested, including 1 or 2 lower level(s), 1 or 2 higher level(s), and the method level. An anonymous urine pool was used in the ruggedness experiments. Samples were run in triplicates to ensure the precision of analytical results. Reported below are results on the major detectable OH-PAH analytes.

Results in Tables

Enzyme amount (mg/sample)		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		Average concentration (pg/mL)									
Lower level1	5mg	32664	38389	5682	1767	3062	390	1902	1701	1387	1288
Lower level2	9mg	33012	37555	5616	1763	3064	398	1864	1683	1371	1267
Method	10mg	33089	37914	5629	1759	3088	483	1868	1697	1373	1271
Higher level1	11mg	33915	39543	5641	1767	3062	489	1883	1703	1396	1291
Higher level2	20mg	33499	38383	5653	1785	3058	485	1891	1688	1385	1275
		Standard deviation									
Lower level1	5mg	20	432	120	5	18	10	24	12	5	15
Lower level2	9mg	97	249	256	34	24	7	9	15	3	11
Method	10mg	647	181	137	15	39	1	17	3	8	11
Higher level1	11mg	1118	281	99	26	31	13	32	21	9	17
Higher level2	20mg	312	312	90	13	21	3	10	10	4	27

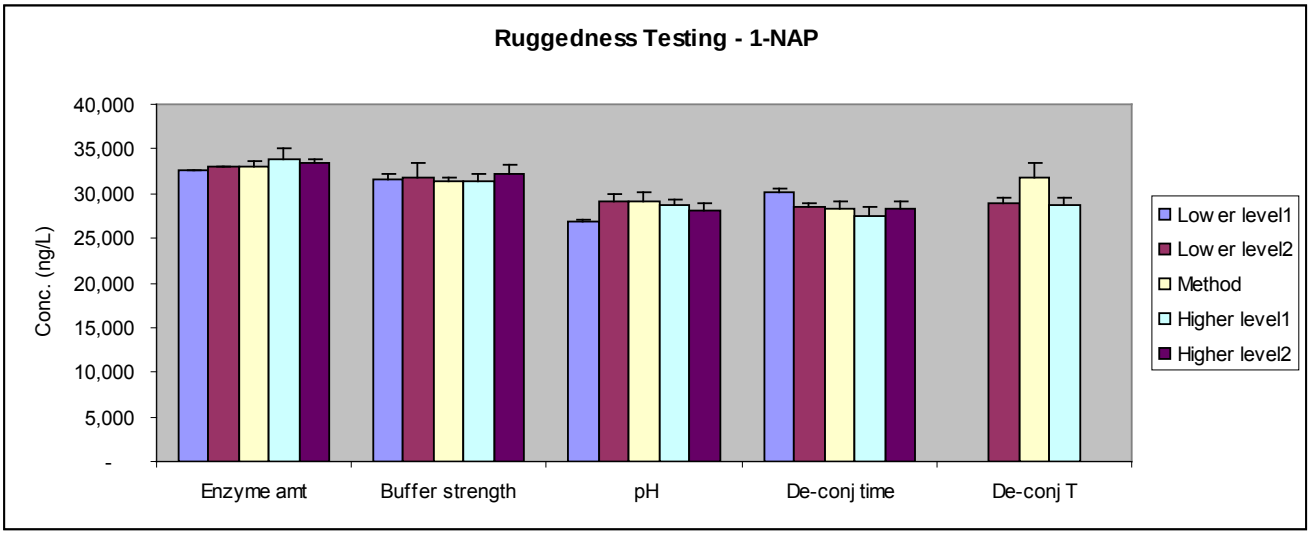
Buffer strength (M)		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		average concentration (pg/mL)									
Lower level1	0.5M	31599	35592	5706	1732	3087	492	1851	1735	1363	1221
Lower level2	0.9M	31708	36069	5684	1746	3028	452	1860	1711	1369	1231
Method	1M	31289	35629	5764	1753	3070	445	1856	1693	1368	1229
Higher level1	1.1M	31310	35516	5646	1753	3040	373	1860	1707	1350	1246
Higher level2	1.5M	32175	36013	5672	1751	3065	374	1843	1747	1358	1228
		Standard deviation									
Lower level1	0.5M	514	533	77	27	43	45	27	38	10	18
Lower level2	0.9M	1699	1429	145	20	17	31	25	65	23	23
Method	1M	537	580	79	22	47	16	47	32	18	24
Higher level1	1.1M	806	871	136	32	44	12	36	9	17	6
Higher level2	1.5M	1098	349	102	18	81	19	32	41	25	16

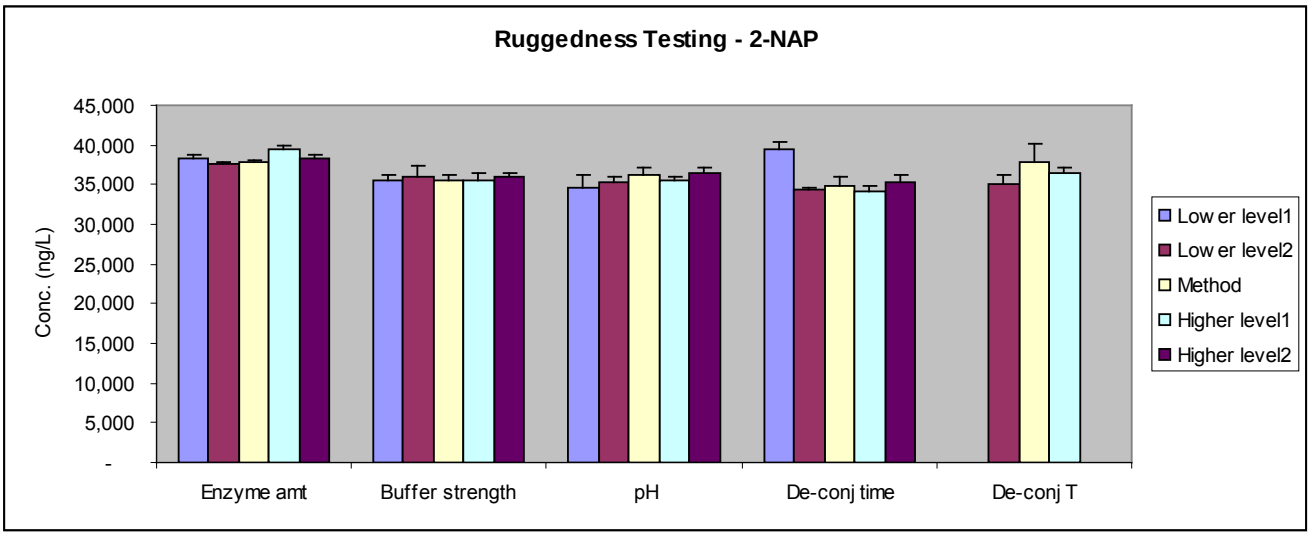
Buffer pH		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		Average concentration (pg/mL)									
Lower level1	pH4.5	26966	34660	5554	1774	2943	442	1878	1699	1374	1188
Lower level2	pH5.3	29123	35246	5757	1746	3061	401	1837	1671	1364	1290
Method	pH5.5	29221	36267	5773	1750	3101	467	1875	1699	1382	1263
Higher level1	pH5.7	28717	35474	5682	1729	3019	442	1856	1630	1363	1205
Higher level2	pH6.5	28169	36460	5863	1788	3112	440	1889	1632	1378	1243
		Standard deviation									
Lower level1	pH4.5	182	1675	77	118	8	20	10	22	15	29
Lower level2	pH5.3	920	718	42	21	26	50	37	17	11	23
Method	pH5.5	907	833	146	11	50	3	16	33	24	36
Higher level1	pH5.7	547	510	203	41	75	9	45	30	31	12
Higher level2	pH6.5	761	729	15	68	75	28	48	53	7	27

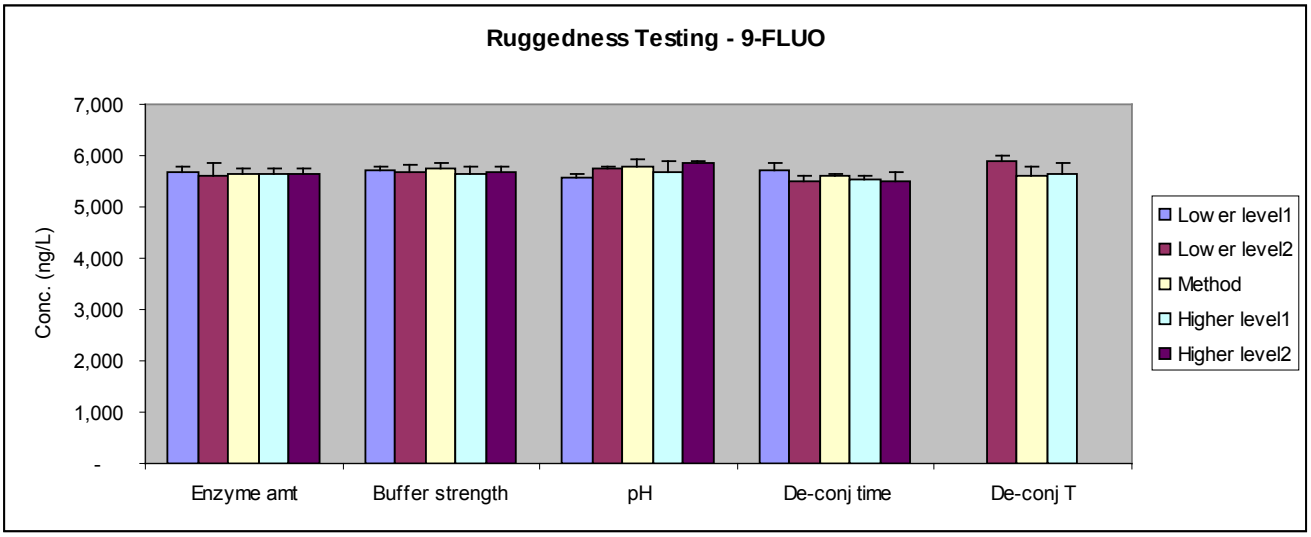
De-conjugation time (hours)		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		Average concentration (pg/mL)									
Lower level1	4 hr	30121	39503	5721	1766	3111	478	1846	1690	1377	1246
Lower level2	17 hr	28590	34326	5516	1740	2982	429	1741	1631	1257	1226
Method	18 hr	28293	34936	5614	1721	2954	406	1756	1604	1268	1211
Higher level1	19 hr	27462	34265	5521	1710	2947	411	1724	1591	1263	1201
Higher level2	24 hr	28283	35314	5502	1729	2976	331	1769	1629	1288	1214
		Standard deviation									
Lower level1	4 hr	484	809	146	7	19	3	19	19	15	13
Lower level2	17 hr	261	330	74	19	36	6	21	21	22	36
Method	18 hr	921	1158	26	3	11	7	18	12	10	2
Higher level1	19 hr	1150	580	94	40	88	18	23	21	39	30
Higher level2	24 hr	861	983	162	11	28	4	40	26	10	12

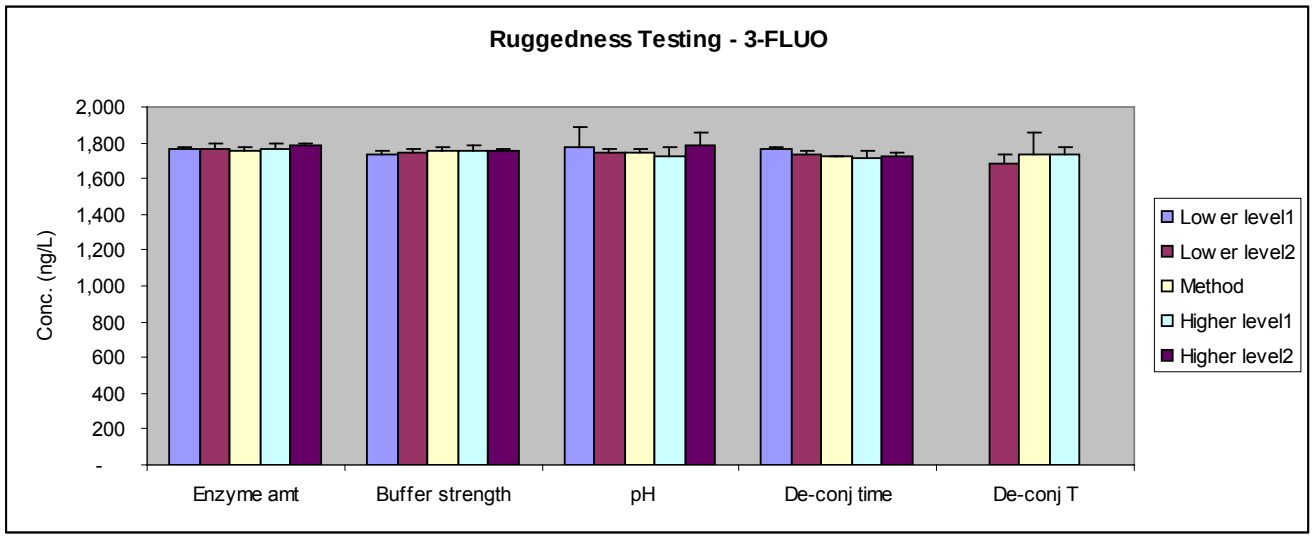
De-conjugation temperature (ºC)		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		Average concentration (pg/mL)									
Lower level	32ºC	28842	35031	5882	1681	3065	495	1752	1640	1355	1223
Method	37ºC	31709	37941	5601	1738	2985	416	1727	1568	1273	1196
Higher level	42ºC	28793	36394	5647	1730	2992	448	1813	1604	1341	1139
		Standard deviation									
Lower level	32ºC	694	1131	122	48	55	14	22	10	19	27
Method	37ºC	1771	2142	201	120	214	32	93	106	88	65
Higher level	42ºC	654	733	202	44	71	16	45	41	29	28

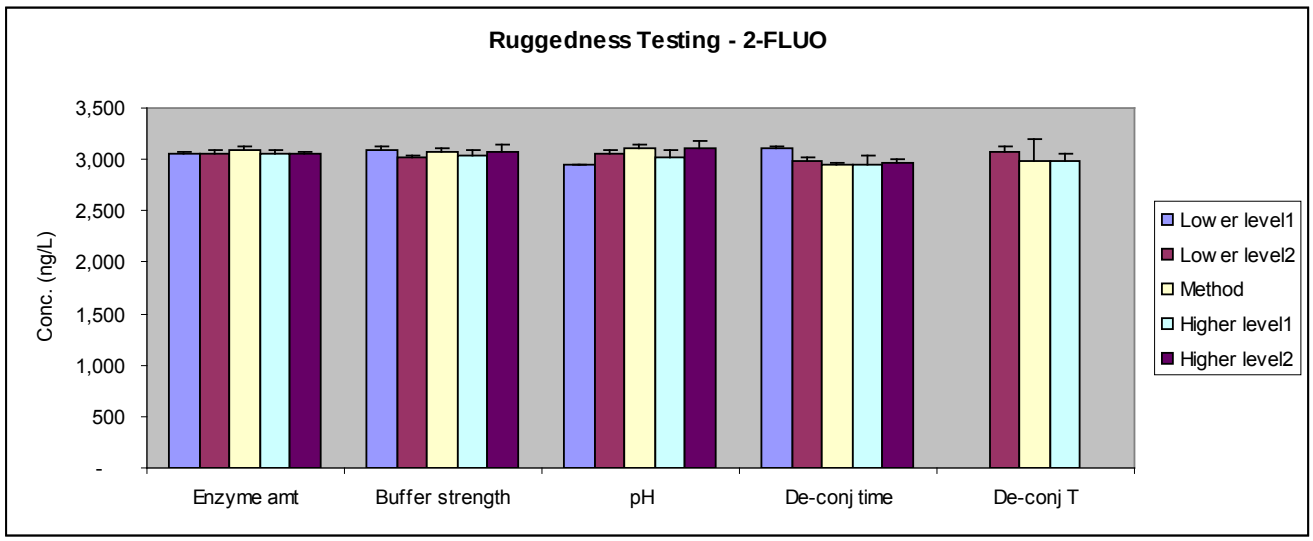
Results in Graphs

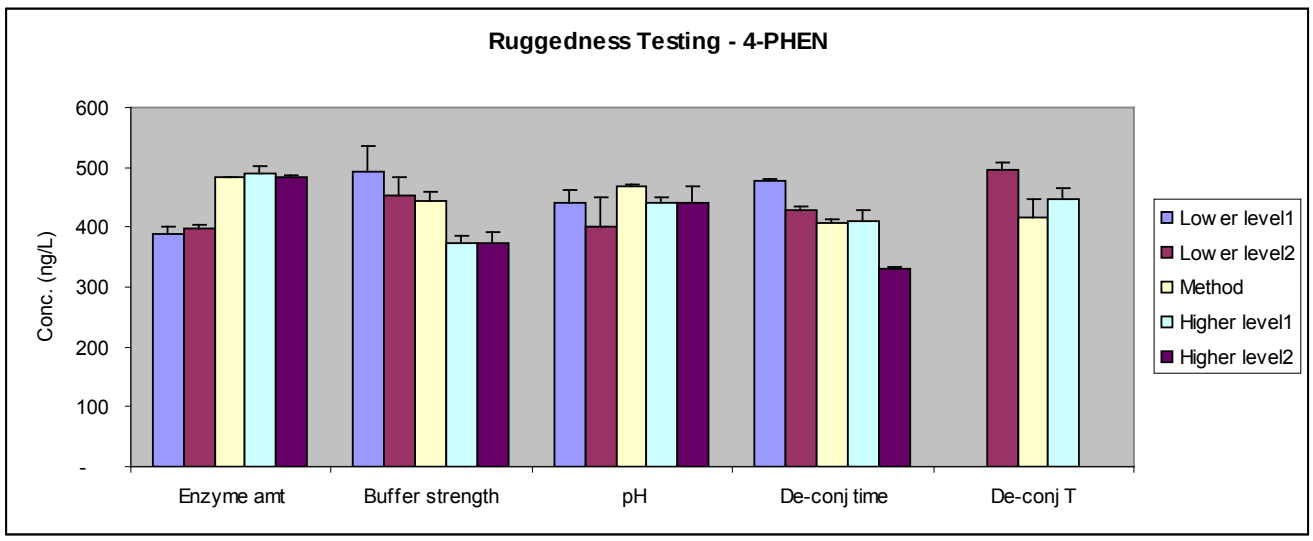


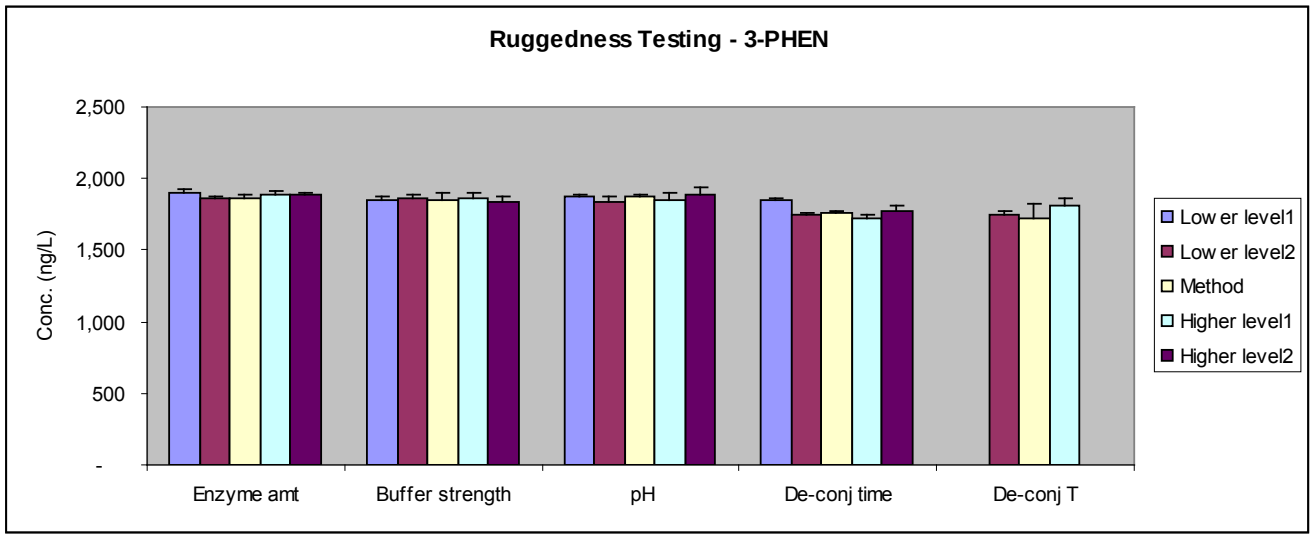


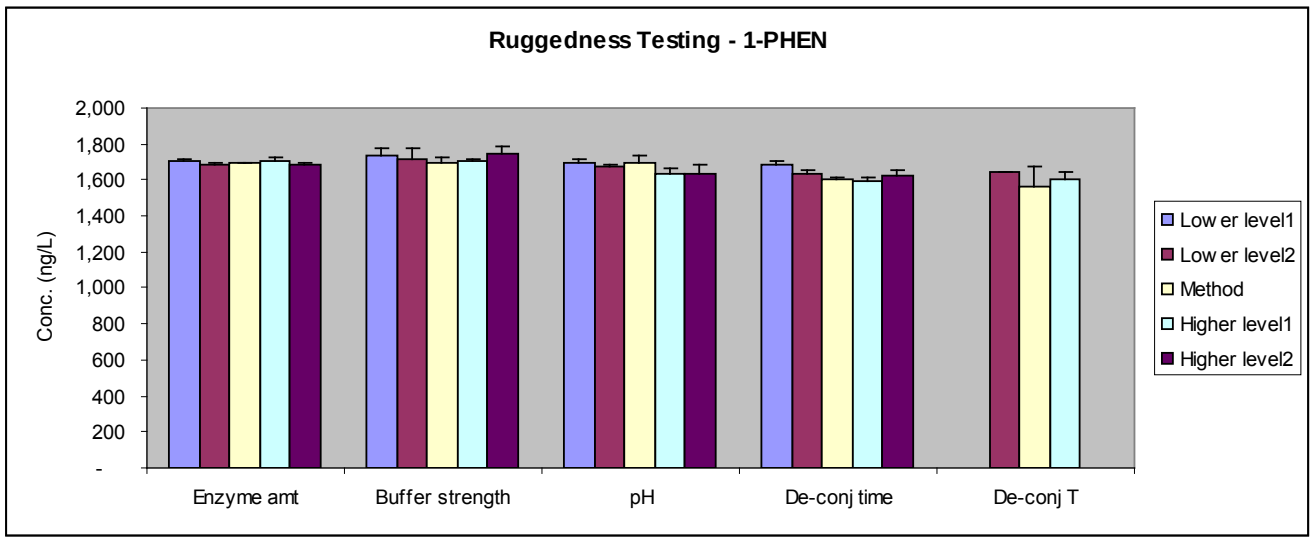


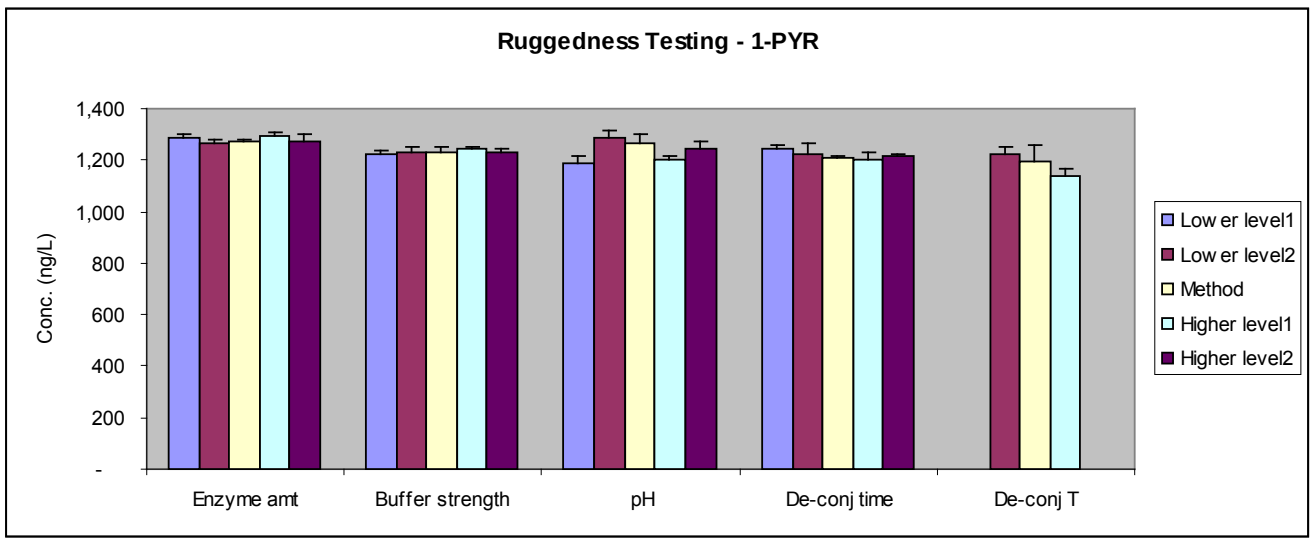

Ruggedness Testing - 3-FLUO
Conc. (ng/L)
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
-
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Low er level1
Low er level2
Method
Higher level1
Higher level2


Ruggedness Testing - 2-FLUO
Conc. (ng/L)
3,500
3,000
2,500
2,000
1,500
1,000
500
-
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Low er level1
Low er level2
Method
Higher level1
Higher level2


Ruggedness Testing - 4-PHEN
Conc. (ng/L)
600
500
400
300
200
100
-
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Low er level1
Low er level2
Method
Higher level1
Higher level2


Ruggedness Testing - 3-PHEN
Conc. (ng/L)
2,500
2,000
1,500
1,000
500
-
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Low er level1
Low er level2
Method
Higher level1
Higher level2


Ruggedness Testing - 1-PHEN
Conc. (ng/L)
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
-
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Low er level1
Low er level2
Method
Higher level1
Higher level2


Ruggedness Testing - 1-PYR
Conc. (ng/L)
1,400
1,200
1,000
800
600
400
200
-
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Low er level1
Low er level2
Method
Higher level1
Higher level2

Appendix B – Quick Method Guide

This quick method guide was compiled for new lab analysts to get familiar with the method and for using in the lab for quick reference.

1. The current OH-PAH Method (as of September 2013)

   The current method is a two-day method. Urine samples are aliquoted & spiked on day one, deconjugated overnight, then extracted, evaporated, and derivatized on day two.

2. Day One:
   a. Print runsheet(s) of day's run(s).
   b. Pull samples out to thaw. Also thaw 2 QCL and 2 QCH per sample set.
   c. Make sure that enough buffer/enzyme solution is prepared for the day's samples. If not, prepare additional solution.
   d. Hand label culture tubes (2 sets, size 16x100) & conical tip centrifuge tubes (15mL) with run ID #s. *(Note: QCs are stored in culture tubes.)*
   e. Gently invert the urine specimen vial to ensure homogeneity. Aliquot 1mL urine into test tubes. For blanks, use 1mL DI-water.
   f. To all samples, add 1mL buffer/enzyme solution (recommend using Eppendorf repeater pipette with 10mL tip) and 5uL ascorbic acid solution.
   g. Load samples onto TRACY Gilson 215 Liquid Handler.
      i. Transfer contents of one vial of IQS into clean amber V-vial & cap with new septum. Weigh capped V-vial with IQS for "before" weight. Note on runsheet.
      ii. Purge transfer lines.
      iii. Use TRACY to spike 40uL of IQS to all samples.
      iv. Weigh capped V-vial with IQS for "after" weight.
      v. Return any remaining IQS back to its GC vial. Write date on vial cap. Return to refrigerator.
   h. Cap all samples with solid black phenolic caps. Gently invert each sample twice to mix the buffer and urine.
   i. Incubate samples in 37°C oven overnight (~17-18) hours.
   j. Record buffer solution # & date, enzyme # & date, IQS #, and any other comments/observations on the runsheet.

3. Day Two:
   a. Remove samples from incubation oven. Note time on runsheet.
   b. Uncap tubes. Add DI-water to all samples (3mL, or enough to bring total volume in test tube up to 5mL).

c. Gilson 215 Liquid Handler
   i. Place samples on Gilson 215 Liquid Handler automix. Lock lid. Power off the automix, and then turn back on. Rotate automix until red "NOT READY' light goes away.
   ii. Load pre-labeled conical tip tubes & cover with aluminum foil.
   iii. Fill solvent bottles with fresh 80/20 pentane/toluene.
   iv. Check hexane level. Replace if necessary.
   v. Check waste level. Replace if necessary.
   vi. Open Trilution LH software. Log on as Administrator, no password is required. The interface will open & default to the Liquid Handling menu.
   vii. Click once on the Application bar to open the Application window.
   viii. In the left pane, click the [+] next to Applications. Our method is **OHPAH Full Method**. Double-click to select it.
   ix. The top pane will load all the methods that make up the application. The lower pane will show the Gilson 215 bed layout.
   x. The default settings are for a batch of 20 samples, as shown in the *'#Range of Samples'* & '*Number of Samples'* columns. If running more than 20 samples, you will need to change those entries.
   xi. Return to the desktop & open Home818.exe. A small popup will appear. If not already preset, enter Unit ID = 20 & the appropriate tilt (OMAR = 6 clicks to the right; SIM = 15 clicks to the right). Click Send Calibration.
   xii. Return to Application window.
   xiii. Make sure all samples are loaded, the automix is properly locked, all solution bottles are sufficiently filled, and all empty glass tubes are loaded in the correct position.
   xiv. Click **RUN** button in bottom left of window. A popup will ask to refresh the run name. Click **YES**. The application will run.
   xv. At first prompt, take samples out of automix & cap with solid caps. Return to automix. Power off the automix, and then turn back on. Rotate automix until red "NOT READY' light goes away. Click **OK** on prompt.
   xvi. After 5 minute mix, another prompt will appear. Move samples from automix to the centrifuge. Centrifuge until 2 layers are clearly separated. Usually, this needs two (2) 15-20 minute periods in the centrifuge.
   xvii. Load samples back to automix rack UNCAPPED. Return rack to automix. Power off the automix, and then turn back on. Rotate automix until red "NOT READY' light goes away. Click **OK** on prompt.
   xviii. Turn on both RapidVaps to preheat.
   xix. Repeat steps xv-xvii for second solvent extraction. Application will be finished after this step.

d. Evaporate extracts in the RapidVaps.

i. Spike 10uL dodecane into each sample, using Eppendorf repeater pipette & 0.1 mL tip.
ii. Load samples into preheated 45*C RapidVap and evaporate under 400-450mbar vacuum at 40% rotation speed until ~1-2 mL remains. This step takes ~10 minutes.
    1. Note time on runsheet.
    2. Print labels for your GC vials.
        a. On SIM's computer, open Brady LabelMark software. Choose Part Family – Standard, Printer Type – TLS2200/TLS PC Link, Label Part – PTL-72-461. Click OK.
        b. Under the Tools menu, choose 'Create Template.' Type your desired template info on the label. At the end of each line of text, click the lock icon in the lower left corner. When done creating template, click the save icon in the lower left corner. Give template a name or overwrite an existing template. Minimize the template.
        c. Under the File menu, choose 'Open Template' and choose the template you just created.
        d. Above the labels are a row of buttons. Click the 'SN' button. Enter the first sample number (1) and your last sample number (most likely 40) then click 'Generate.' When you've created all the labels you need for that set of samples, click the printer icon to print.
    3. Label your GC vials.
iii. Move extracts to 80*C RapidVap with 50% rotation speed and 200-230mbar vacuum and evaporate until ~10uL remains.
    1. This step will take roughly 25-30 minutes.
    2. Use this time wisely: fill out runsheet, prep next set of samples, label tomorrow's glassware, start dishwasher, clean up, etc.

e. Using Eppendorf repeater, reconstitute samples by spiking 20uL Recovery Standard (RS) into each tube. Vortex tubes for roughly 5 seconds.
f. Transfer extracts to labeled GC vials using electronic Rainin pipette.
g. Using Eppendorf repeater, spike 10uL MSTFA into all vials.
h. Displace air in MSTFA vial and GC vials using a gentle stream of Argon. Cap vials.
i. Derivatize extracts in 60*C oven for 30 minutes. Use this time to fill out any missing information on the runsheet.
j. When the extracts are ready, give them to the analyst. *Make sure your runsheet is complete before you give the samples to the analyst!*

Appendix C – Matrix and non-matrix calibration curve comparison

Four different types of calibration curves were prepared. They are solvent based (current in use), water-spiked, synthetic urine spiked, and urine-spiked calibration curves. The urine-spiked curve is referred to matrix calibration curve, and the remaining three types of curves are non-matrix curves. An experiment was set up to compare the slopes from these four types of calibration curves, according to the DLS Policy and Procedure Manual (Version February 27, 2012), Section 10.5.

Slope

	1-nap	2-nap	9-fluo	3-fluo	2-fluo	4-phen	3-phen	1-phen	2-phen	1-pyr
Solvent	0.96	0.89	0.99	1.10	1.15	2.00	1.36	0.97	0.94	1.36
Water	0.94	0.89	0.97	1.08	1.14	2.01	1.36	0.96	0.94	1.31
Syn-urine	0.96	0.89	0.97	1.09	1.14	2.00	1.36	0.96	0.94	1.31
Urine	0.93	0.89	0.97	1.08	1.14	1.93	1.36	0.96	0.93	1.31

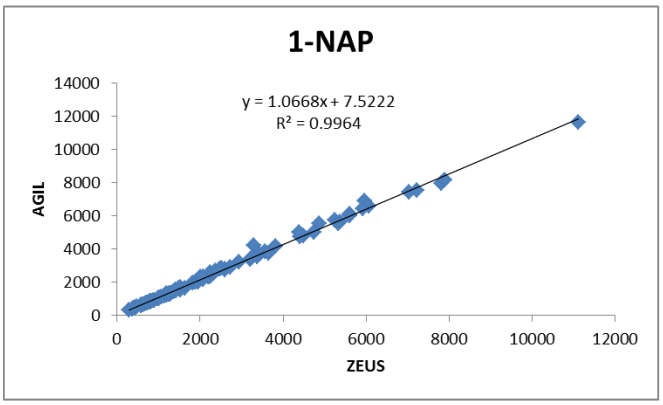
% difference compared to urine-spiked curve

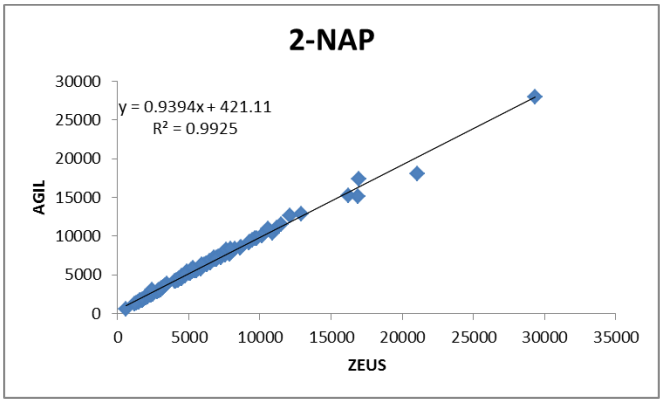
Solvent	3.5%	0.5%	1.4%	1.5%	1.5%	3.7%	-0.3%	0.6%	0.4%	3.5%
Water	1.4%	0.6%	-0.4%	-0.1%	0.4%	4.2%	-0.1%	-0.7%	0.2%	-0.6%
Syn-urine	3.2%	0.7%	-0.1%	0.2%	0.2%	3.7%	0.3%	-0.9%	0.4%	-0.7%
Urine	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

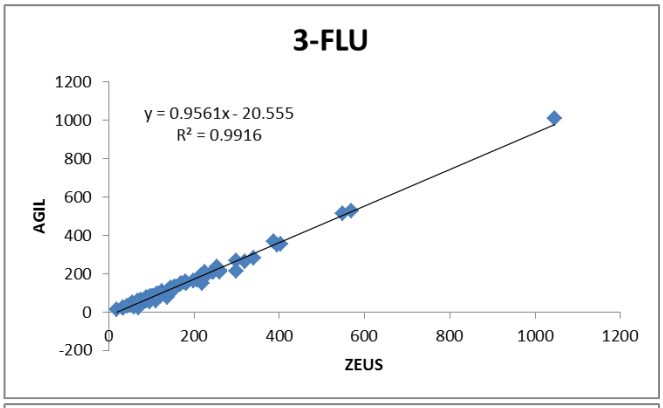
Appendix D – Method Comparison Graphs

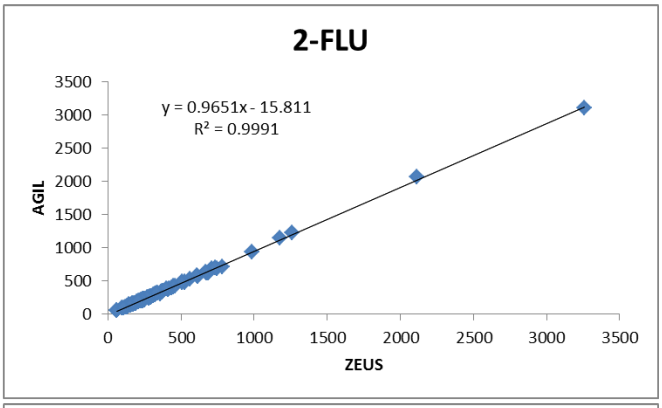
Method comparison was performed by running 100 clinical samples using the previous method by Thermo Quantum GC/MS/MS (Method #6703.03) and the updated method (Method #6703.04) by Agilent GC/QQQ 7000 GC/MS/MS. The results are evaluated by both the linear regression plots between the Thermo ("ZEUS") and Agilent ("AGIL") results, as well as Bland-Altman plots by plotting the differences between the results from the two methods against the mean of the two results.

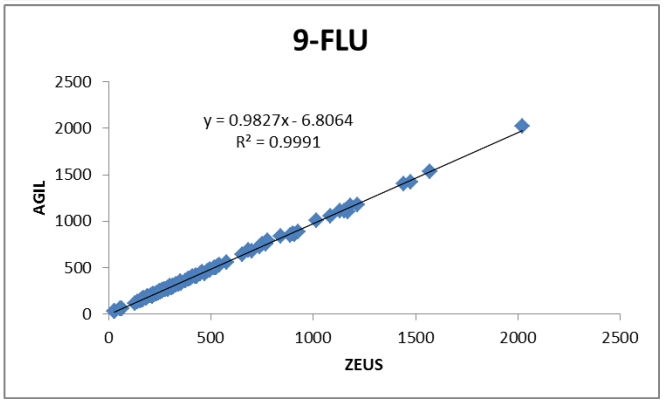
Linear regression plots: New method "AGIL" vs. old method "ZEUS"

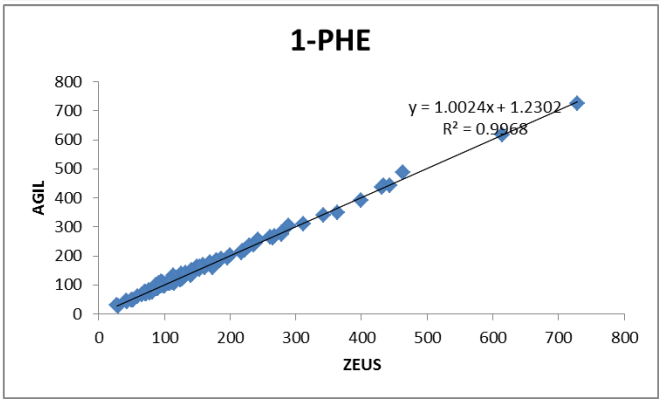


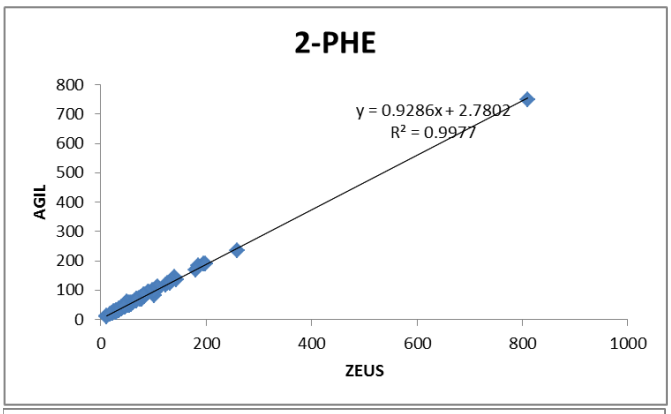


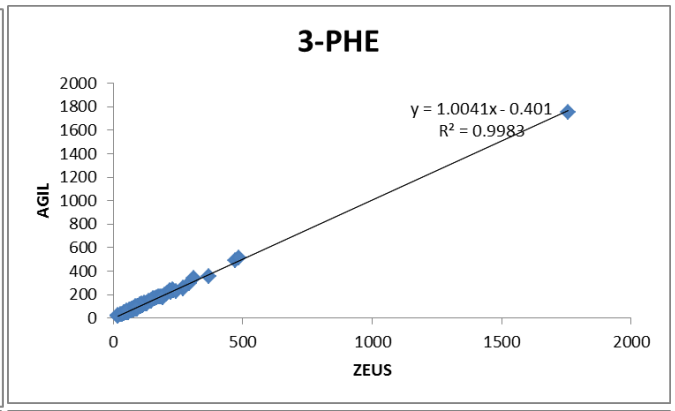


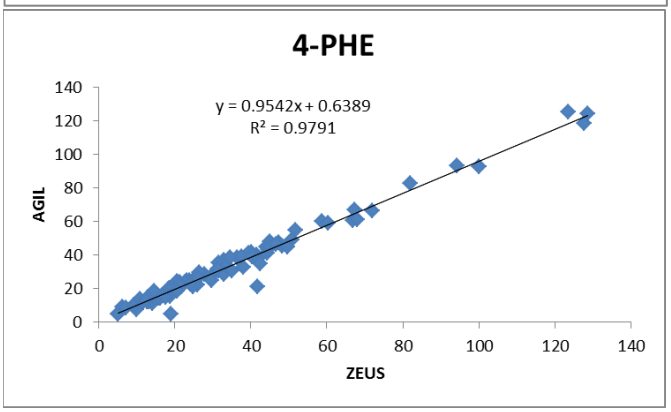


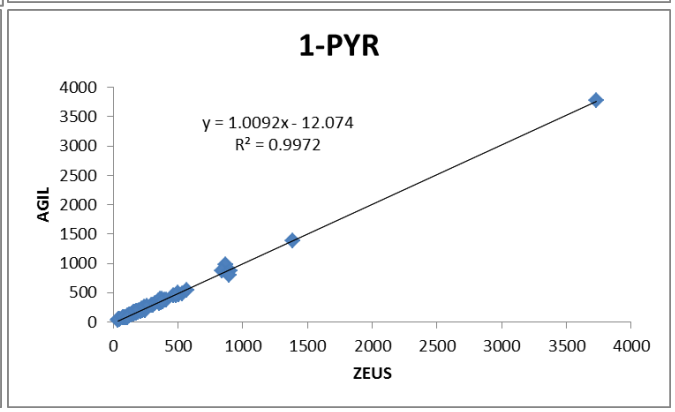




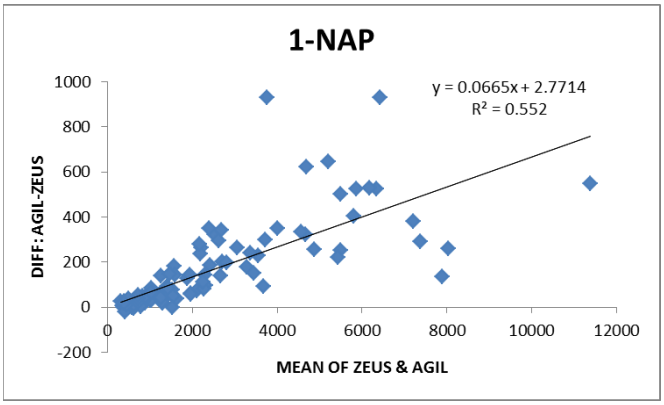


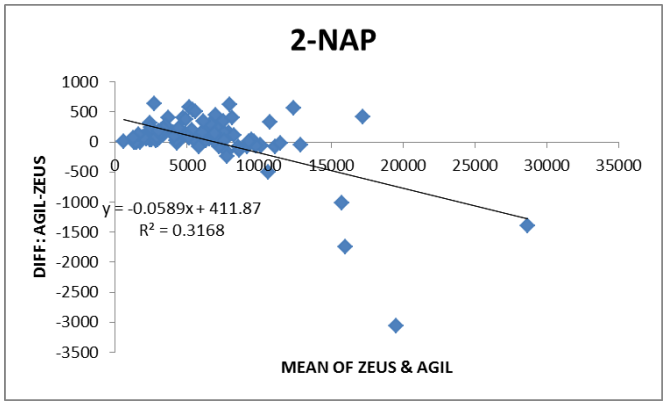


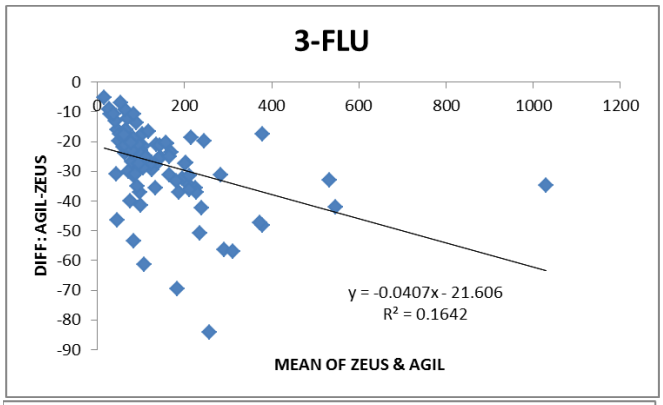


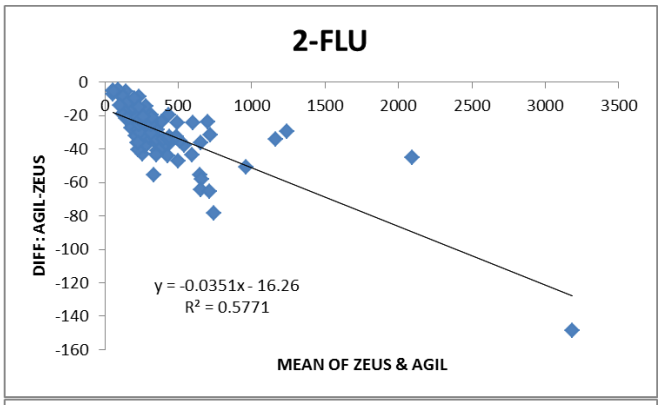


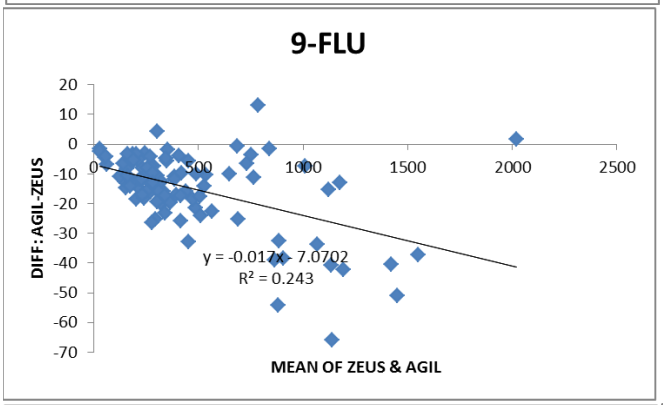
Bland-Altman plots: Difference (AGIL-ZEUS) vs. Mean of AGIL and ZEUS

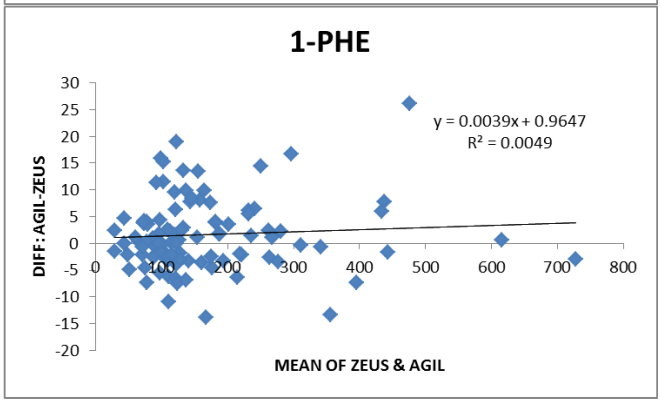


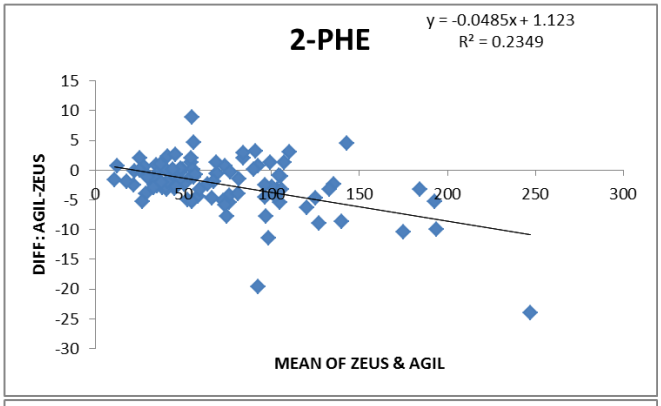


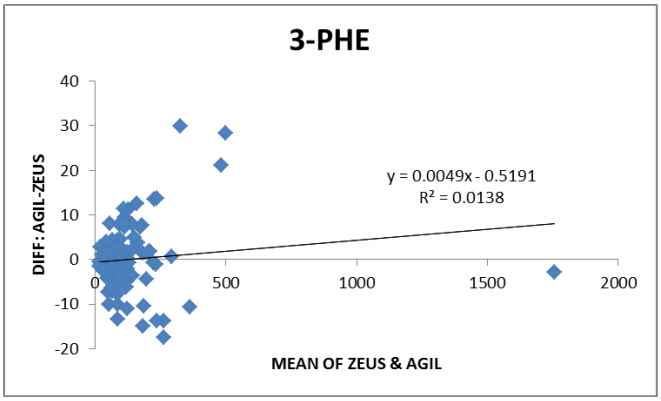

3-FLU
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.0407x - 21.606
R² = 0.1642

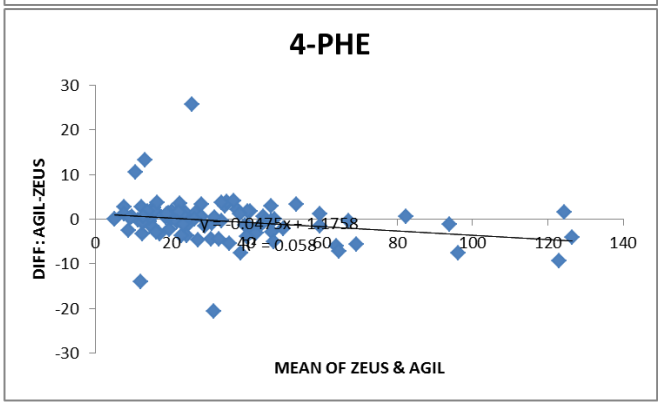

2-FLU
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.0351x - 16.26
R² = 0.5771

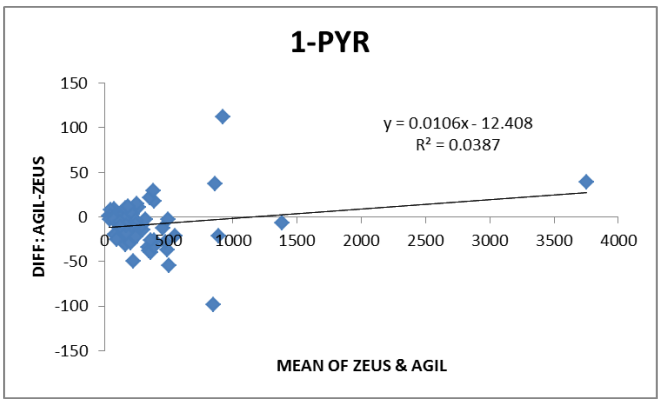

9-FLU
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.017x - 7.0702
R² = 0.243


1-PHE
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = 0.0039x + 0.9647
R² = 0.0049


2-PHE
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.0485x + 1.123
R² = 0.2349


3-PHE
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = 0.0049x - 0.5191
R² = 0.0138


4-PHE
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.0475x + 1.1758
R² = 0.058


1-PYR
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = 0.0106x - 12.408
R² = 0.0387