Laboratory Procedure Manual

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

Matrix: **Urine**

Method: **Isotope Dilution Gas Chromatography/High Resolution Mass Spectrometry (GC/HRMS)**

Method No: **6703.02**

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as performed by:

Organic Analytical Toxicology Branch
Division of Laboratory Sciences
National Center for Environmental Health

contact:

Andreas Sjodin, Ph.D.
PAH Biomarker Laboratory
Phone: 770-488-4711
Fax: 770-488-0142
Email: ASjodin@cdc.gov

Dr. James Pirkle, Director
Division of Laboratory Sciences

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.

PAHs in Urine
NHANES 2007-2008

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
PAH_E	URXPO1	1-napthol (ng/L)
	URXPO2	2-napthol
	URXPO3	3-fluorene
	URXPO4	2-fluorene
	URXPO5	3-phenanthrene
	URXPO6	1-phenanthrene
	URXPO7	2-phenanthrene
	URXP10	1-pyrene
	URXP17	9-fluorene

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. Common routes of occupational exposure may include work involving diesel fuels and coal tars such as paving and roofing. Possible environmental exposures include smoking, diet, smog and forest fires. Threshold levels for carcinogenicity have not been determined for most PAHs. Application of this method to analyze samples obtained from participants in the National Health and Nutrition Examination Survey (NHANES) will help determining the reference range of these chemicals in general U.S. population, aged 6 years and higher.

b. Test Principle

The specific analytes measured in this method are monohydroxy-PAH (OH-PAH). The procedure involves enzymatic hydrolysis of urine, extraction, derivatization and analysis using capillary gas chromatography combined with high resolution mass spectrometry (GC-HRMS). This method uses isotope dilution with carbon-13 labeled internal standards. Ions from each analyte and each carbon-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. The analytes measured in this procedure are shown in Table 1. By evaluating these analytes in urine, a measurement of the body burden from PAH exposure is obtained.

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

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

2. Safety Precautions

a. Reagent toxicity or carcinogenicity

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

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.

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 high resolution mass spectrometer is necessary. Users are required to read the operation manuals and must demonstrate safe techniques in performing the method. Anyone involved in sample preparation must be trained in 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. The 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.

> ***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 Thermo Finnigan GC/HRMS system controlled by Xcalibur™ Software 1.3. Analyte peaks are integrated by Quan Browser under Xcalibur™. Results are exported from Quan Browser result files to Microsoft Excel files that are subsequently used for calculations. Final results are stored in Excel 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 prior to being entered into the MS Excel for transcription errors and overall validity. The database is routinely backed up locally through the standard practices of the NCEH 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 have 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 must be refrigerated as soon as possible, and must be transferred to specimen vials within 24 hours of collection. A minimum of 5 milliliters of urine is collected and poured into sterile vials with screw-cap tops. The specimens are then labeled, frozen immediately to -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 at -70 ºC until analysis.

c. Sample handling

Specimen handling conditions are outlined in the Division of Laboratory Science (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 must be transported and stored at frozen (< -10 ºC). Once received, they can be frozen at -70 ± 10 °C until time for analysis. Portions of the sample that remain after analytical aliquots are withdrawn must be refrozen at -70 ± 10 °C. Samples are not compromised by repeated freeze and thaw cycles.

d. Sample quantity

The minimum amount of specimen required for analysis is 0.5 mL, with the optimal amount being 2.0 mL.

e. Unacceptable specimens

Specimens must be frozen at a minimum of -20 ± 5 °C when delivered to the lab. The minimum volume required is 0.5 mL. If either of these criteria is violated, then specimen must 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 by contact with dust, dirt, etc. 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*
organic de-ionized water (D.I. H_2O)	Prepared in house, CDC (Aqua Solutions, Inc.)
β-glucuronidase/arylsulfatase, H-1, powder enzyme	Sigma Chemical, St. Louis, MO
pentane, hexane, acetonitrile, toluene, and methanol (ABSOLV grade)	Tedia Company, Fairfield, OH
glacial acetic acid	Sigma Chemical, St. Louis, MO Fisher Scientific, Pittsburgh, PA
Silver nitrate	Fisher Scientific, Pittsburgh, PA Sigma Chemical, St. Louis, MO
sodium acetate	Sigma Chemical, St. Louis, MO
N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)	Sigma Chemical, St. Louis, MO
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-FLU	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 (10 mg enzyme/mL buffer)

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) 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 repeator 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

All standard preparations are based on gravimetric determination, not by volume. Therefore, the final actual concentrations have minor deviation from the target concentrations, e.g. target concentration for an individual stock solution is 100 ng/uL; the actual concentration is 98.7 ng/uL. Actual calculated concentrations based on weight are used in all data calculation and processing.

All OH-PAH are light sensitive and precautions to minimize exposure to light must be taken, such as use of UV-filtered yellow light in lab areas where samples are handled.

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

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 OH-PAH 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 10 native OH-PAH mix (W.S.A)

Combine 400 μL from each of the individual native OH-PAH standard stock solutions in a silanized screw cap amber vial to generate the working standard solution (W.S.A, target concentration at 4 ng/μL for each native OH-PAH 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 stored them in a refrigerator until needed.

3) Working Standard Solution of 10 C13-labeled OH-PAH mix (W.S.I)

Combine individual ^{13}C-labeled OH-PAH standard stock solutions (90 ng/μL) in a silanized screw cap amber vial to generate the working internal standard solution (W.S.I, target concentration at 6 ng/μL for each of the 10 ^{13}C-labeled OH-PAHs, except for C13-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 stored them in a refrigerator until needed.

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

External calibration standards (E.C.S.) are prepared as presented in Table 3 below. 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 high concentrations. Target concentrations for ^{13}C-labled internal standards are 100 pg/μL (400 pg/μL for C13-labeled 1- and 2-NAP) in all calibration standards.

In addition, due to high concentrations for 1- and 2-NAP often found in specimens, two additional calibration standards were prepared. The two calibration standards are at levels of 2000 and 4000 pg/μL (8000 and 16,000 pg/μL for 1- and 2-NAP), and were 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	5	4	20
2	2	10	8	40
3	5	25	20	100
4	10	50	40	200
5	50	250	200	1,000
6	100	500	400	2,000
7	500	2500	2,000	10,000
8	1000	5,000	4,000	20,000

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 target concentration of each ^{13}C-labeled OH-PAH 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.

6) Mass Spectrometric Check Solution

This solution is used daily to monitor the operating performance of the GC column and the mass spectrometer. A solution of 20 fg/μL of a tetra-chlorinated dioxin is used as the check solution. For a 1 μL injection, a minimum signal-to-noise of 4 on the m/z 321.894 peak must be obtained.

d. Preparation of Control Materials

1) Quality Control (QC) materials

Prepare quality control materials by spiking a known amount of native OH-PAH mixture (in acentonitrile) into 2000 mL of an anonymous filtered urine pool (500 pg/mL urine). Homogenize the QC solutions overnight for equilibration. On the next day aliquot the QC solutions into 16 x 100 mm test tubes (2 mL in each tube) and store them at -70 ± 10 °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 ± 5 °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), the PT samples were then randomized by an external PT administrator, labeled by external lab technicians, and store them at -70 ± 10 °C until use.

e. Other equipment, materials, and supplies

Materials / supplies and sources used during the development, validation, and application of this method are listed below. Materials / supplies procured from other sources must meet or exceed these specifications.

- Gilson 215 liquid handler (Gilson Inc., Middleton, WI)
- 818 Automix mixer (Gilson Inc., Middleton, WI)
- Water bath/sonicator (Branson Ultrasonics, Danbury, CT)
- TurboVap LV evaporator (Caliper LifeSciences, Hopkinton, MA)
- 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)
- Pasteur pipettes and bulbs (VWR).
- Maxi-mix Vortex mixer (Barnstead International)
- Allegra-6 centrifuge (Beckman-Coulter)
- Amber screw top vials with various volume (Supelco, Inc., Bellefonte, PA)
- Clear autosampler vials, 0.5 mL (SunSri)

f. Instrumentation

The analyses are performed on a ThermoFinnigan MAT-95XL high resolution mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electron impact

ionization and interfaced to an Agilent Technologies 6890 gas chromatograph (GC) system (Agilent Technologies, Palo Alto, CA)

1) Gas chromatograph configuration

Chromatographic separation is performed on an Agilent 6890 gas chromatograph fitted with a DB-5 30-m fused silica capillary column. The column ID is 0.25-mm and the film thickness is 0.25-micron (J&W, # 22-5022 or equivalent). The temperature program lasts a total of 28 minutes. (See Table 4 for GC configuration and Table 5 for the GC temperature program.)

Table 4. GC configuration

GC Parameter	Setting
Carrier gas	Helium
Constant flow rate	1 mL/minute
GC purge flow rate	70 mL/minute
GC saver time	5 minutes
GC save flow rate	15 mL/minute
Injection mode	Splitless
Injector purge delay	2 minutes
Injector temperature	270 ºC

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

Time (min.)	Ramp (C/min)	Temperature (ºC)
0	0	95
2	15	95
6	5	155
20	40	225
22.4	0	320
27.4	-	320

2) High Resolution Mass spectrometer (HRMS) configuration

Thermo Finnigan MAT 95XL HRMS configuration is presented in Table 6. The mass spectrometer is operated under Multiple Ion Detection (MID) mode. The masses used to quantify analytes are presented in Table 7.

Table 6. Thermo Finnigan MAT 95XL HRMS configuration

HRMS Parameter	Setting
Scan mode	Multiple ion detection
Ionization type	Electron impact
Ion polarity mode	Positive
Electron energy	45 eV
Resolution	10,000
Ion source	250 $^{\circ}$C
Conversion dynode voltage	Positive
Electron multiplier voltage	1.45 – 2.25 kV (or 10^6 gain)

Table 7. Analyte masses

Analyte	Molecular Ion [$M^{\cdot +}$]
1-NAP, 2-NAP	216.0970
2-FLU, 3-FLU, 9-FLU	254.1127
1-PHE, 2-PHE, 3-PHE, 4-PHE	266.1127
1-PYR	290.1127
$^{13}C_6$ 1-NAP, $^{13}C_6$ 1-NAP	222.1172
$^{13}C_6$ 2-FLU, $^{13}C_6$ 3-FLU, $^{13}C_6$ 9-FLU	260.1328
$^{13}C_4$ 4-PHE, $^{13}C_4$ 1-PHE	270.1263
$^{13}C_6$ 3-PHE, $^{13}C_6$ 2-PHE	272.1328
$^{13}C_6$ 1-PYR	272.1328

7. Calibration and Calibration Verification

a. Calibration of Mass Spectrometer

Calibrate and tune the Finnigan MAT 95XL mass spectrometer using FC43 (perfluorotributylamine) according to the instructions in the operator's manual located next to the instrument. After calibrated with 10,000 resolution and maximum

sensitivity, the instrument is prepared for the analysis of OH-PAHs as described in Section 8.

b. Creation of calibration curve

1) Calculation data

A linear log-log calibration curve, using eight ECS with concentration ranging from 1 to 1000 pg/μL, is generated using the log ratio of the peak area of the analyte to the labeled internal standard against the log ratio of the native analyte concentrations to those of the labeled internal standards. The concentrations in ECS correspond to 5 - 5,000 pg/mL (20-20,000 pg/mL) levels in 2 mL urine. For urine samples with concentrations higher than the calibration curve, the highest two standards (2000 & 4000 pg/ μL, 8,000 and 16,000 pg/μL for 1-&2-NAP) are prepared to extend the calibration curve and to accurately quantify those samples.

2) Evaluation of curve statistics

The R-squared value of the curve must be equal to or greater than 0.990. Linearity of the standard curve must extend over the entire standard range.

3) Use of the calibration curve

The lowest point on the calibration curve is the lowest reportable level and the highest point is above the expected range of results. The remainders of the points are distributed between these two extremes, with the majority of points in the concentration range where most unknowns fall.

c. Calibration verification

In order to verify that this calibration of this test system is accurate and stable throughout reportable range, a full calibration curve is run monthly. Calculated concentration must be within 10% deviation from expected concentration.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of two blanks, two QCs, and 16 unknown urine samples.

a. Sample Preparation

All samples are prepared in a laboratory with non-UV yellow fluorescent lights.

1) Hydrolysis

Allow urine samples and QCs to thaw and reach room temperature. Aliquot 2 mL of urine sample (or 2 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) 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 ± 2 °C oven and incubate the sample overnight (~17-18 hours).

2) Automated Liquid-Liquid Extraction

Add D.I. water (2 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 (~20-40 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 until a total of 10mL of organic phase is 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 TurboVap LV evaporator. Under a stream of N_2 and in a 40 °C water bath, evaporate the pentane fraction (~10-15 minutes). Transfer the remaining extract volume (~2mL) to a second TurboVap LV evaporator warmed with an 80 °C water bath. Evaporate extracts to ~10μL within 20-30 minutes. Spike each sample tube with 20 μL toluene and 5 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 ± 5 °C for 30 minutes. The samples are then ready for analysis on the mass spectrometer.

b. Instrument and software setup for the GC/HRMS

1) Preliminary MAT 95 system setup and performance check

Turn on the MAT 95; inject 2 μL FC-43 into the reference inlet. In the TUNE window, adjust resolution between 9900 and 10100. Perform a GC/HRMS analysis of the mass spectrometer check solution (2, 3, 7, 8-TCDD, 10 fg/μL, 1 μL injection) and verify chromatographic resolution and peak intensity. In the lab note book, record the signal to noise of the check compound.

2) Final setup and operation

a) *Create the run sequence*

In the Xcalibur Sequence Setup window, create a sequence for the run using the template. Make sure that the appropriate number of samples is loaded, the appropriate filenames are assigned, and the appropriate sample positions on the autosampler tray are included in the run sequence. Make sure that the correct process method and instrument method are selected. The latter defines GC, MID, Autosampler, and ICL methods. The methods used are listed in Table 8.

Table 8. GC/HRMS methods

Program	Method
Instrument	NHANES.met
GC	NHANES.mcr
MID	NHANES.mid
Autosampler	pah_1ul.mcs
ICL	Sleep90.icl 7
Process	NHANES.pmd

Filenames conform to the following format: PMYYNNN, KMYYNNN or JMYYNNN where P = PAH samples run on the MAT 95 instrument named as CASSI, K = PAH samples run on the MAT 95 instrument named as MIKE, J = PAH samples run on the MAT 95 instrument named as ICE; M = month (A = January, B = February, C = March, etc.); YY = year (04 = year 2004), and NNN = run number for the month. For example, the filename PB01012 corresponds to PAH-February-2001-sample #12 run on MAT 95 – CASSI, and KD02267 would correspond to PAH-April-2002-sample #267 run on MAT 95 – MIKE.

b) *Start the sequence*

Click **Run Sequence** in the main menu under Action. Verify the sequence set up and make sure the whole sequence instead of one single sample is selected to be run. Click **OK;** the system will immediately start by turning green on the first sample to run.

3) System standby

To place the MAT 95 in standby mode, enter the command **.bye** at the ICL prompt. This command turns off the accelerator, multiplier and dynode voltage, and vents the reference compound (FC-43). To reactivate the MAT 95, enter **.run 0.50** at the command prompt. Add the reference compound and tune.

c. Processing of data

After the run sequence finishes, process all raw data files in the whole sequence using the "NHANES.pmd" process method after which the analyte peaks in data files are automatically integrated using the process method. Visually review and manually correct if needed the integration of each peak in the QuanBrowser window of the Xcalibur software. Save the reviewed result file, export it as a MS Excel file, and save the Excel file on the Q:\ share 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 RBase database located on the Q:\ share drive.

d. Replacement and Periodic Maintenance of Key Components

1) MAT 95 XL Mass Spectrometer

Check cooling water level and temperature monthly
Clean the ion volume or replace it monthly
Clean the ion source or replace it annually
Replace the calibration gas septum monthly
Trained Thermo Finnigan technicians perform all other maintenance based on an annual schedule, or as needed

2) Agilent 6890 GC

Change the injection port liner and septum daily
Clean the injection port, clean or replace the gold seal monthly
Cut the GC column for ~10" monthly or as needed
Replace the GC column at 1000 analyses or sooner
Replace Helium tank when the pressure is below 500 psi.

Note: Accelerate the above maintenance schedules if necessary.

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 and re-analyzed so that the result is in the reportable range.

a. Linearity Limits

Analytical standards were linear for all analytes through the range of concentrations evaluated. The linear range for all analytes except 1-NAP and 2-NAP is 5 pg/mL to 40,000 pg/mL. Calibration curves for 1-NAP and 2-NAP are extended to 160,000 ng/L, because their high concentrations detected from unknown samples. Therefore, the linear range for 1-NAP and 2-NAP were 20 pg/mL to 160,000 pg/mL.

Urine samples whose concentrations exceed these ranges must be diluted and re-analyzed using a smaller aliquot.

b. Limit of Detection

The limit of detection (LOD) for this method is defined as the greater LOD calculated by two methods: (i) in direct relation to method blanks prepared in parallel with the unknown samples, as 3 times the standard deviation of the method blanks, and (ii) according to the instrumental detection limit defined as the lowest point in the calibration curve (1 pg/uL, or 5 pg/mL in 2-mL urine samples) verified to give a signal with the S/N equal to or greater than 5. 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 9 (updated after NHANES 2005-06).

Table 9. Limits of detection (LOD)

Analyte	LOD (pg/mL)
1-NAP	48
2-NAP	13
9-FLU	5
3-FLU	5
2-FLU	5
4-PHE	5
3-PHE	5
1-PHE	5
2-PHE	5
1-PYR	5

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 the 318 QC samples over the analysis of NHANES 2005-06 are listed in Table 10 below. These QC samples were prepared over 6 months by two different analysts using two automated liquid handlers and two GC/HRMS instruments.

Table 10. Mean, standard deviation, and CV for QC samples. The parameters are QC pool specific.

Analyte	Mean (pg/mL)	Stdev (pg/mL)	CV (%)
1-NAP	519	33	6.4%
2-NAP	791	28	3.5%
9-FLU	539	12	2.1%
3-FLU	433	10	2.3%

2-FLU	507	12	2.4%
4-PHE	508	16	3.1%
3-PHE	501	13	2.6%
1-PHE	339	10	2.8%
2-PHE	493	12	2.5%
1-PYR	485	17	3.6%

d. Analytical specificity

The HRMS system provides excellent analytical specificity. The analyte peaks are located in well defined regions of the chromatogram with no visible interferences and low background. In addition, the retention time for the analytes relative to the isotope internal standards give additional confirmation of the presence of analytes in the sample.

e. Accuracy

Presently no established standard reference material (SRM) exists for OH-PAHs in human urine. Therefore, we cannot evaluate the accuracy of this method by analyzing an SRM and compare to its certified concentrations. Nonetheless, the usage of isotopically labeled internal standards can adjust for any deviates occurred on the OH-PAH analytes during sample preparation and instrumental analysis, and thus provide assurance on the accuracy of this method.

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 QCs are prepared and analyzed with 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) <u>Establishing QC limits</u>

Quality control limits are established by characterizing assay precision with repetitive analyses of the QC pool. Different variables are included in the analysis (e.g. different analysts and instruments) to capture realistic assay variation over time. The mean, standard deviation, 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 in NHANES 2005-06 are listed in Table 11. The characterization statistics are pool specific.

Table 11. Sample QC Characterization Statistics. The data are QC pool specific.

Analyte	Mean - 3σ (pptr)	Mean - 2σ (pptr)	Mean (pptr)	Mean + 2σ (pptr)	Mean + 3σ (pptr)
1-NAP	420	453	519	585	618
2-NAP	707	735	791	847	875
9-FLU	503	515	539	563	575
3-FLU	403	413	433	453	463
2-FLU	471	483	507	531	543
4-PHE	460	476	508	540	556
3-PHE	462	475	501	527	540
1-PHE	309	319	339	359	369
2-PHE	457	469	493	517	529
1-PYR	434	451	485	519	536

2) Quality Control evaluation

After the completion of a run, the quality control limits are consulted 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:

If both of the QCs are within the 2σ limits, then accept the run.

If one of two QC results is outside the 2σ limits, then apply the rules below and reject the run if any condition is met.

Extreme outliner: the result is outside the characterization mean by more than 4σ limit.
$1_{3\sigma}$ – Average of both QCs is outside of a 3σ limit.
$2_{2\sigma}$ –QC results from two consecutive runs are outside of 2σ limit on the same side of the mean.
$R_{4\sigma}$ sequential –QC results from two consecutive runs are outside of 2σ limit on opposite sides of the mean.
10_x sequential – QC results from ten consecutive runs are on the same side of the mean.

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

There are no established PT materials; currently we are the only laboratory running this essay. Therefore, the proficiency testing (PT) scheme for this method is administered by an in-house PT coordinator. Because no standard reference materials exist for urinary analysis of hydroxy-PAH levels, PT samples are prepared in-house by spiking a known amount of standard into a well characterized urine pool, and blink-coded by in-house PT coordinator.

2) Frequency of PT

Five samples of unknown PT concentrations are analyzed 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.

3) Documentation of PT

Analytical 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. 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, reviewed 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 high resolution mass spectrometry, most interference is eliminated. Due to the matrix used in this procedure, occasional unknown interfering substances have been encountered. If chromatographic interference with the internal standards occurs, reject

that analysis. If repeat analysis still results in an interference with the internal standard, the results for that analyte are not reportable.

13. Reference Ranges (Normal Values)

Population-based reference ranges were determined from a subset of National Health and Nutritional Survey (NHANES) 2003-2004 urine samples. The reference values are presented in Table 12.

Table 12. Reference values from NHANES 2003-2004

Analyte	Reference range concentrations								
	Fresh weight (ng/L)					Creatinine adjusted (ng/g crea)			
	50th	75th	90th	95th		50th	75th	90th	95th
1-NAP	2260	7660	18500	26100		2100	6560	15100	21800
2-NAP	2960	7500	17300	25800		2560	6340	14100	19900
2-FLU	280	679	1850	2670		221	495	1510	2070
3-FLU	103	302	1090	1740		86.6	256	856	1330
9-FLU	269	541	929	1390		233	412	729	1100
1-PHE	166	287	464	625		141	222	352	487
2-PHE	62.2	117	206	291		52.3	85.8	150	212
3-PHE	118	219	424	647		99.5	172	321	497
4-PHE	25.9	53.7	96.8	152					
1-PYR	91.3	189	389	569		79.9	149	279	424

14. Critical Call Results (“Panic Values”)

It is unlikely that any result would be a "critical call", which would only be observed in acute poisonings. There are no established “critical call” values. Application of this method to NHANES studies will assist in determining levels of OH-PAH normally found in healthy US populations. Test results in this laboratory are reported in support of epidemiological studies, not clinical assessments. Data will help determine critical exposures.

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 in a -70 ^{o}C freezer after analysis. Current studies indicate (CDC data) that the extracts are stable for three weeks.

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

Alternate validated methods have not been evaluated for measuring OH-PAH in urine. If the analytical system fails, then samples must be refrigerated (at 4 ± 3 ^{o}C) until the analytical system is restored to functionality. If long-term interruption (greater that 4 weeks) is anticipated, then store urine specimens at -70 ± 10 ^{o}C.

The method is designed to run on a GC/HRMS instrument, and is not generally transferable to other instrumentation. If the system fails, then samples must be refrigerated (at 4 ± 3 ^{o}C) until the analytical system is restored to functionality. If long-term interruption (greater that 4 weeks) is anticipated, then store urine specimens at -70 ± 10 ^{o}C. Sample extracts in GC vials may be refrigerated for as long as three weeks. If long-term interruption is anticipated, store sample extracts at -70 ± 10 ^{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) and adjusted based on creatinine excretion (µg/g creatinine).

Once the validity of the data is established by the QC/QA system outlined above, these results are verified by a DLS statistician, and the data reported in both hard copy and electronic copy. This data, a cover letter, and a table of method specifications and reference range values will be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director) as outlined in the DLS policy and procedure manual. After approval at the division level, the report will be sent to the contact person who requested the analyses.

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 ± 10 °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.

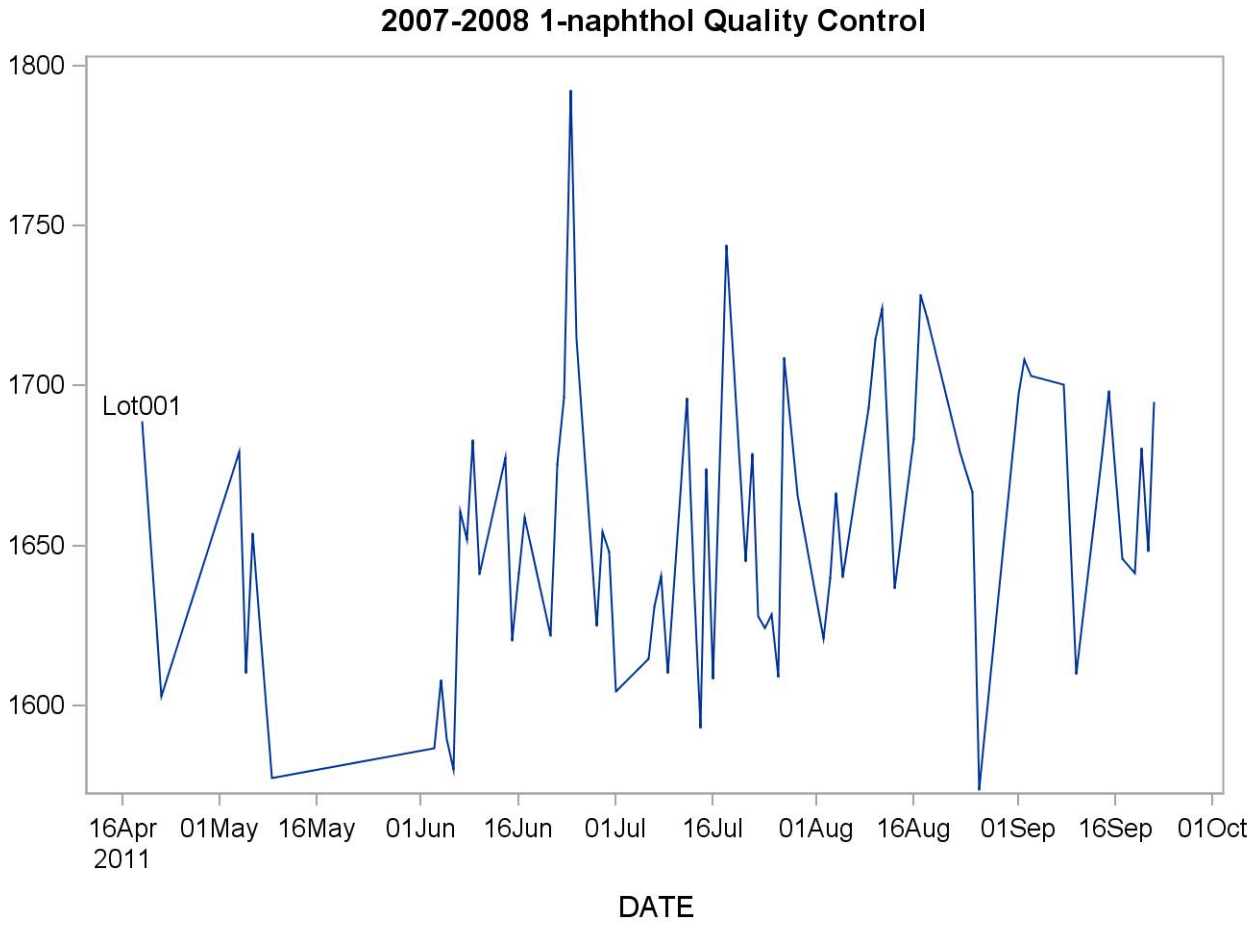
Standard record keeping (e.g., database, notebooks, and data files) is used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Transfer is carried out through the DLS Samples Logistic Group. Specimens may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

19. Summary Statistics and QC Graphs

See following pages.

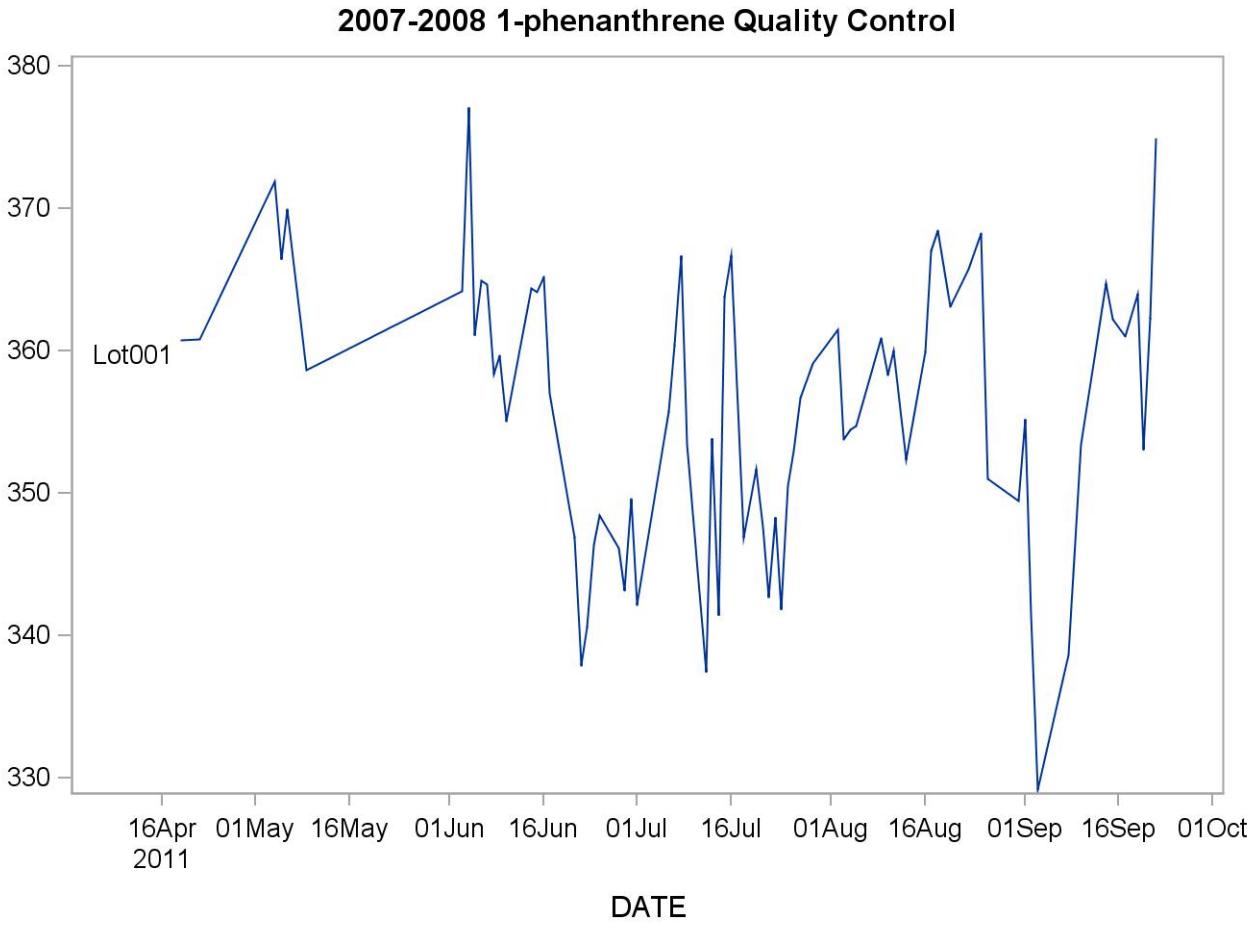
Summary Statistics for 1-naphthol

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	102	19APR11	22SEP11	1657.04	51.43	3.1



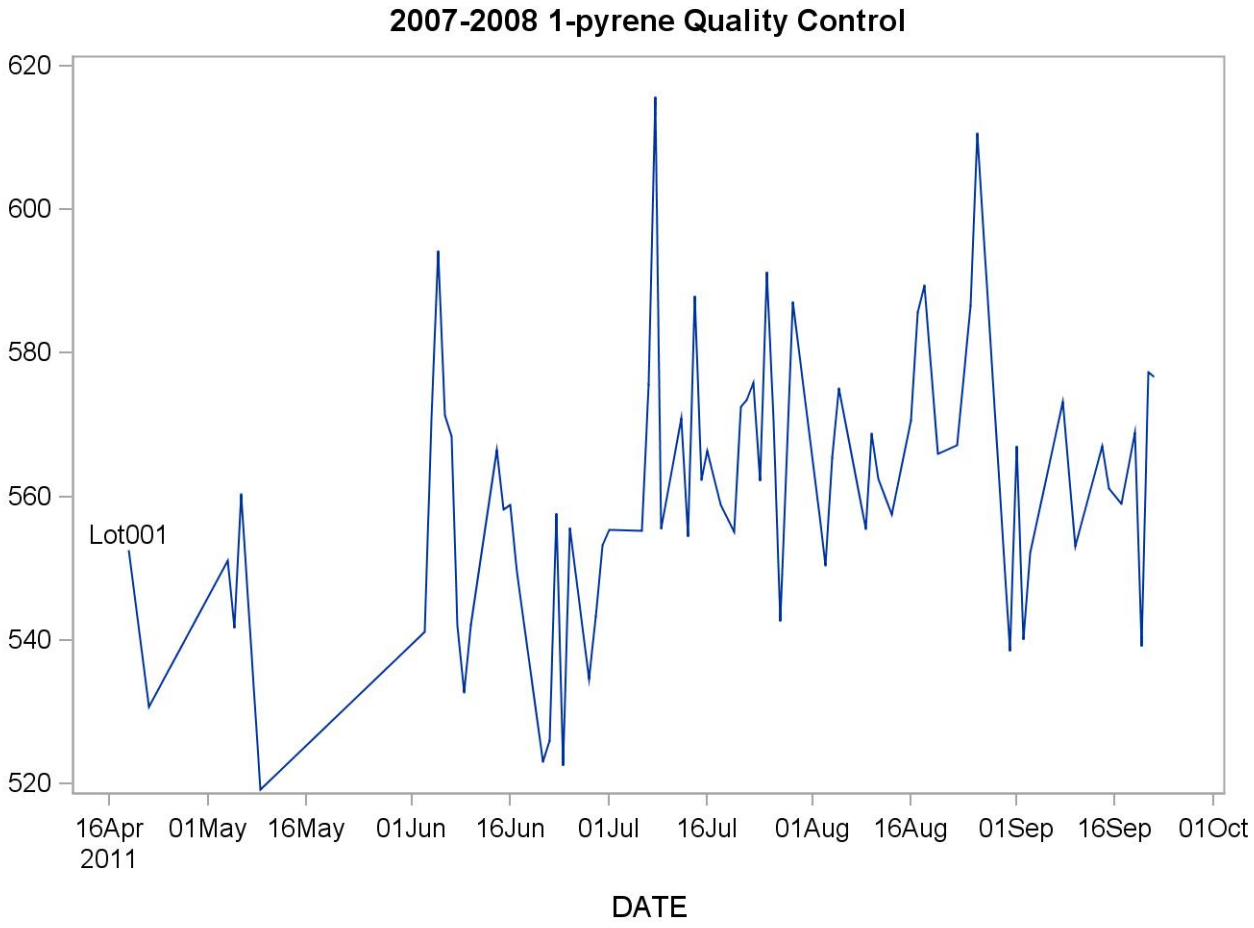
Summary Statistics for 1-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	107	19APR11	22SEP11	356.33	11.72	3.3



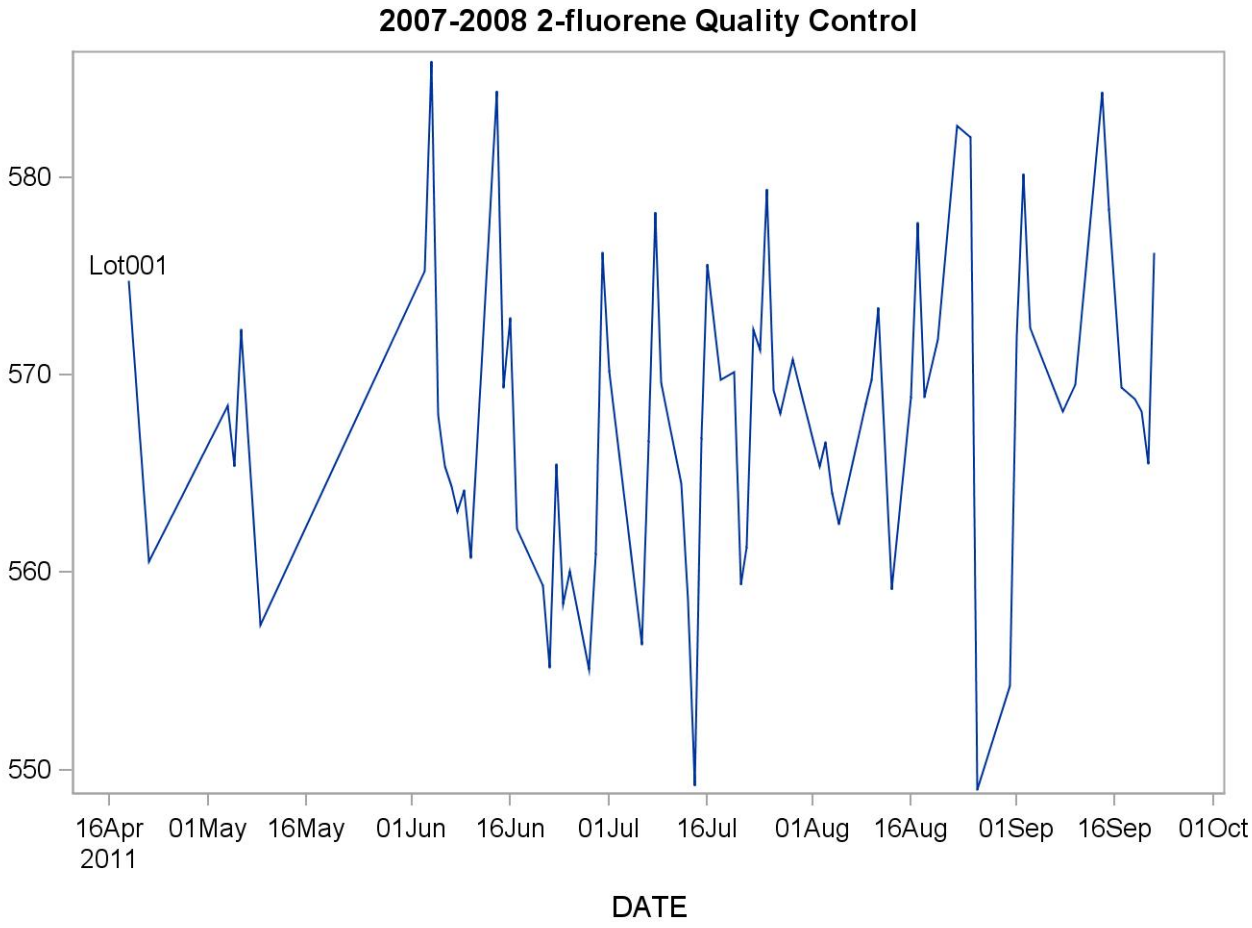
Summary Statistics for 1-pyrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	106	19APR11	22SEP11	561.19	21.20	3.8



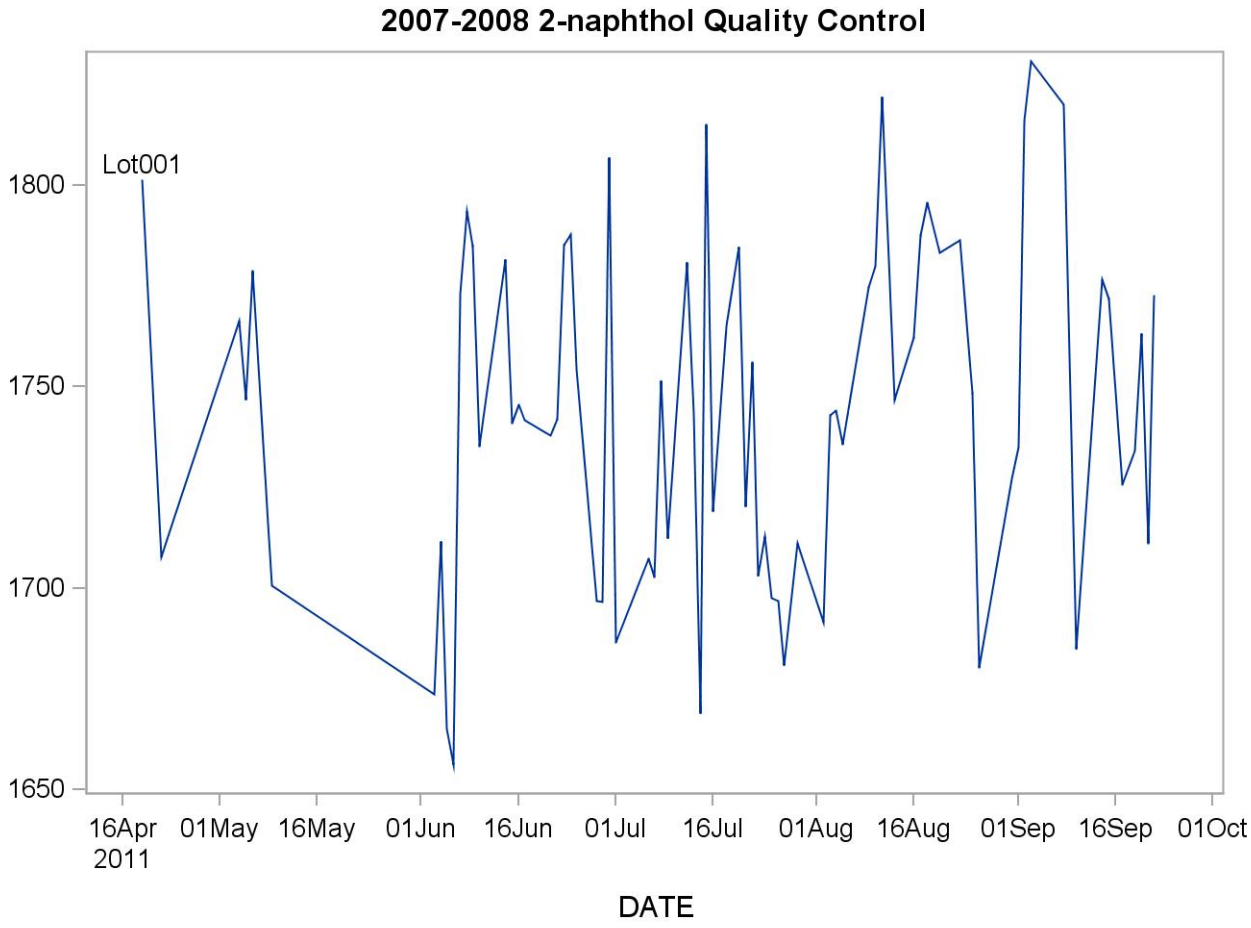
Summary Statistics for 2-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	106	19APR11	22SEP11	567.74	9.33	1.6



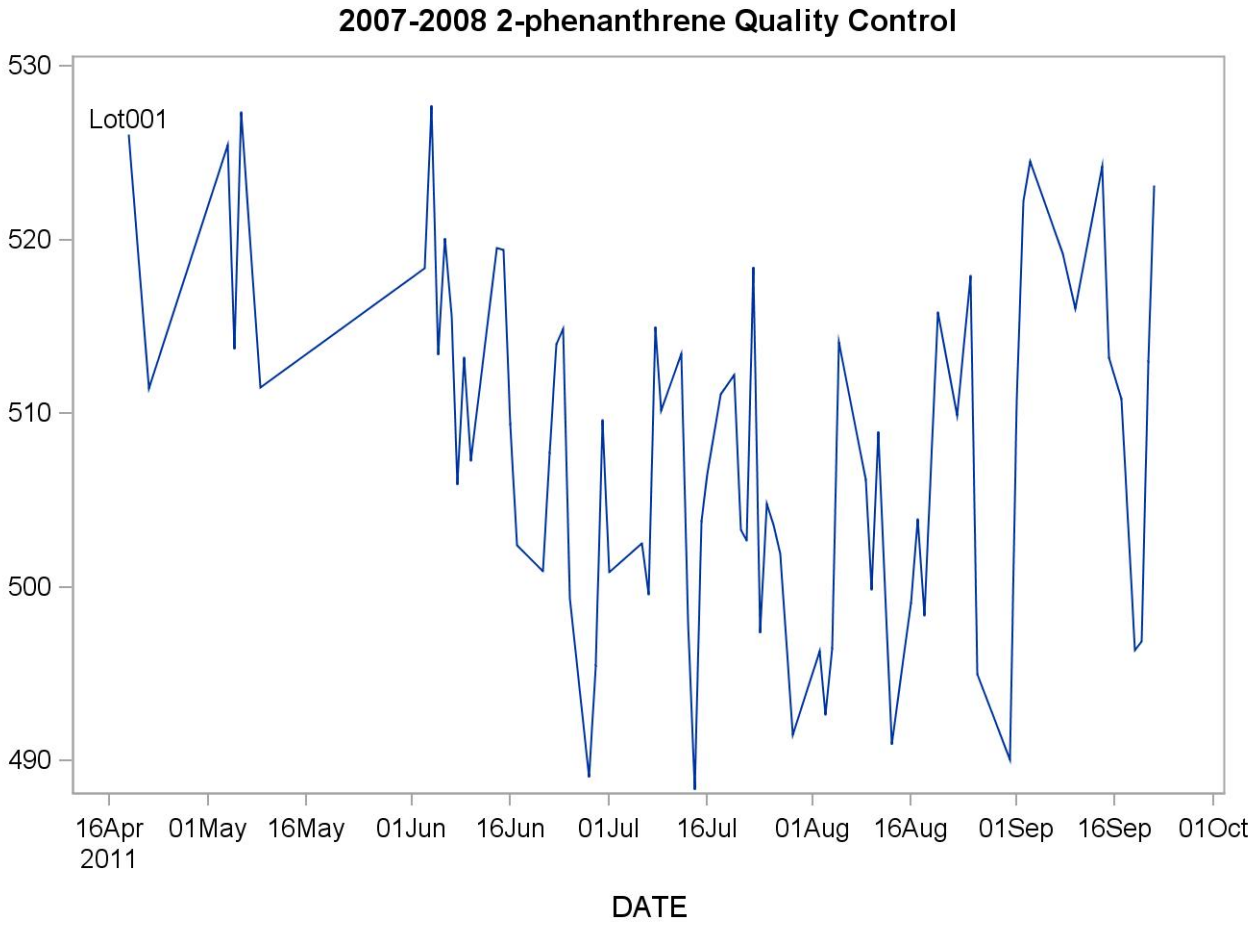
Summary Statistics for 2-naphthol

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	104	19APR11	22SEP11	1744.88	52.46	3.0



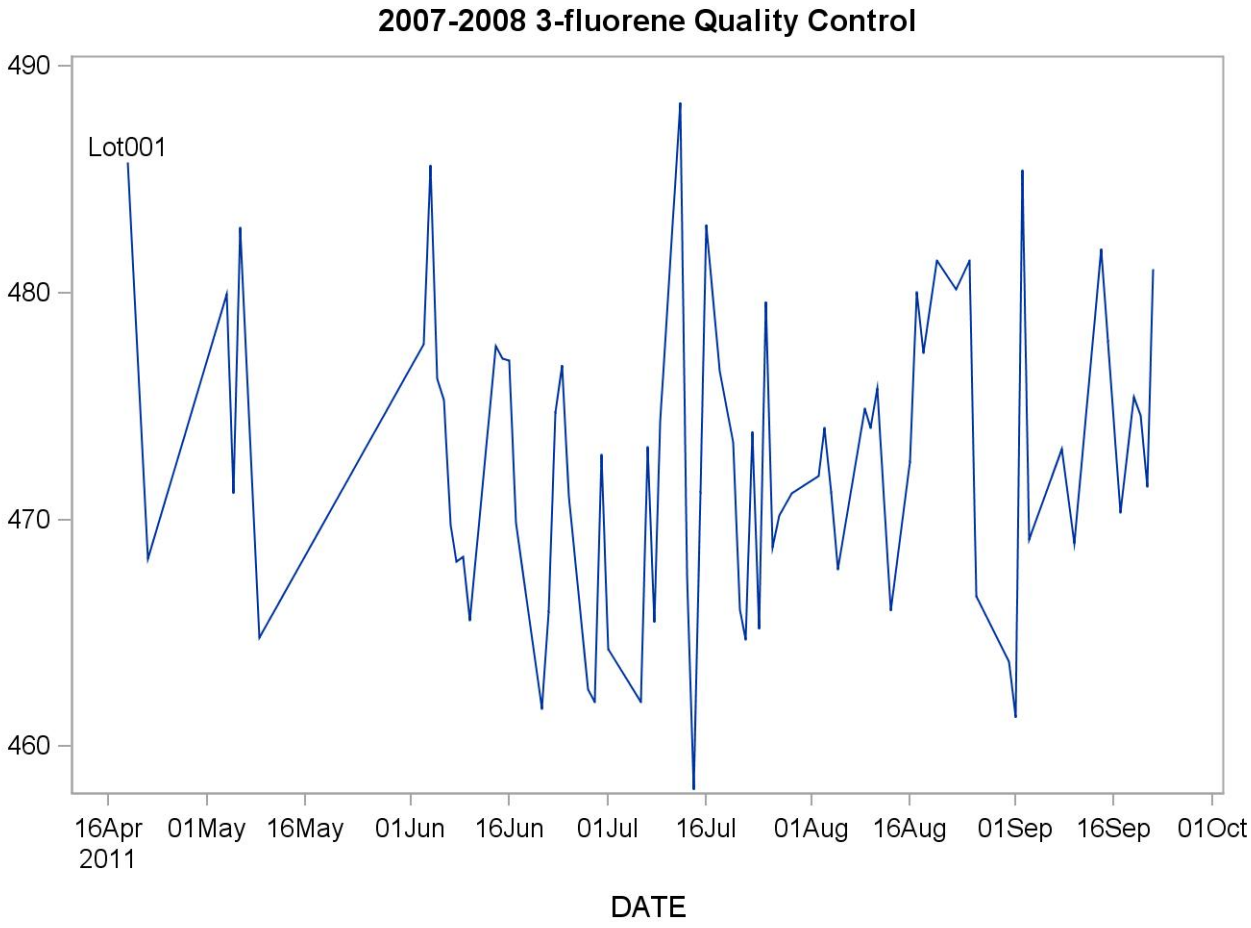
Summary Statistics for 2-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	106	19APR11	22SEP11	507.80	11.38	2.2



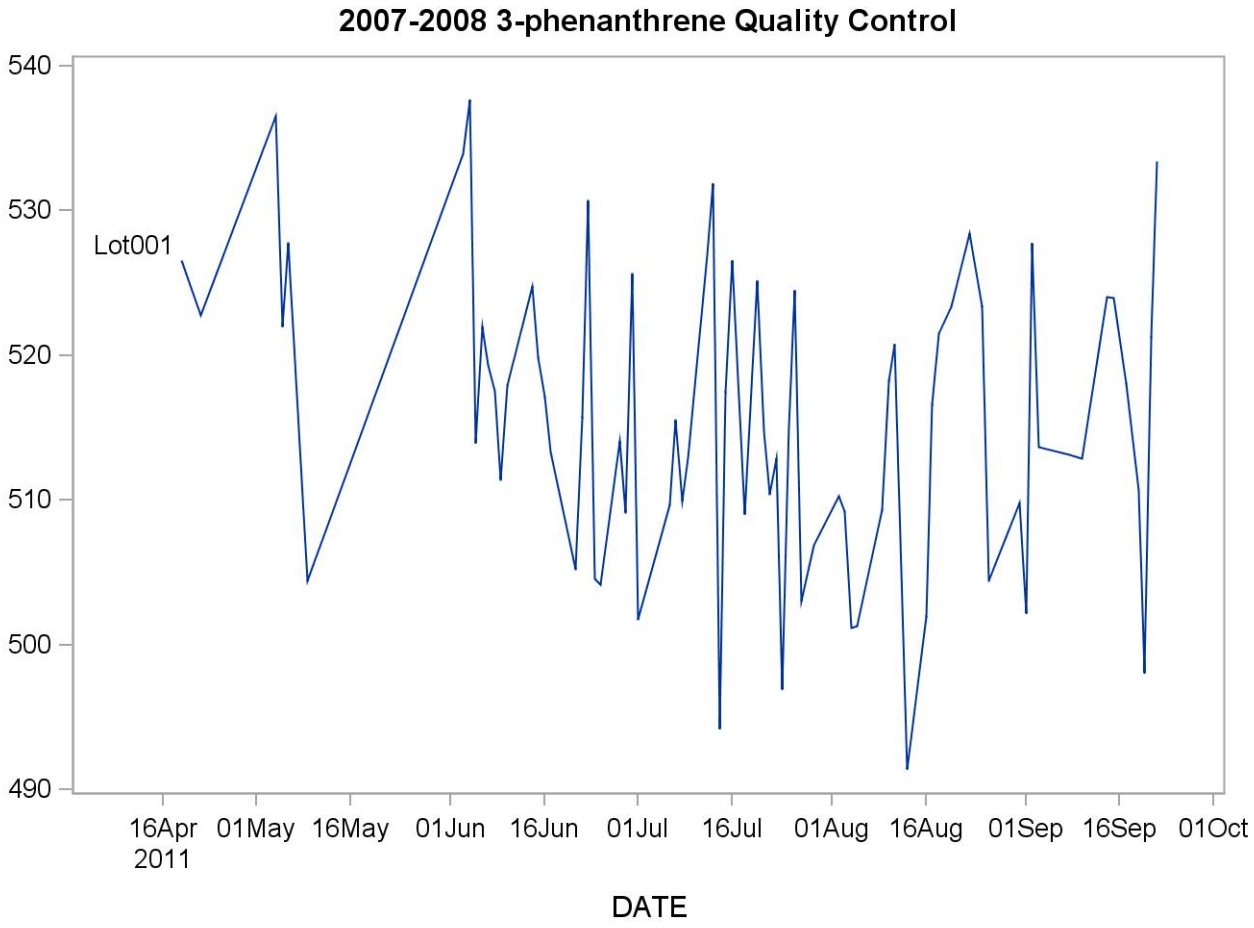
Summary Statistics for 3-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	106	19APR11	22SEP11	472.50	8.51	1.8



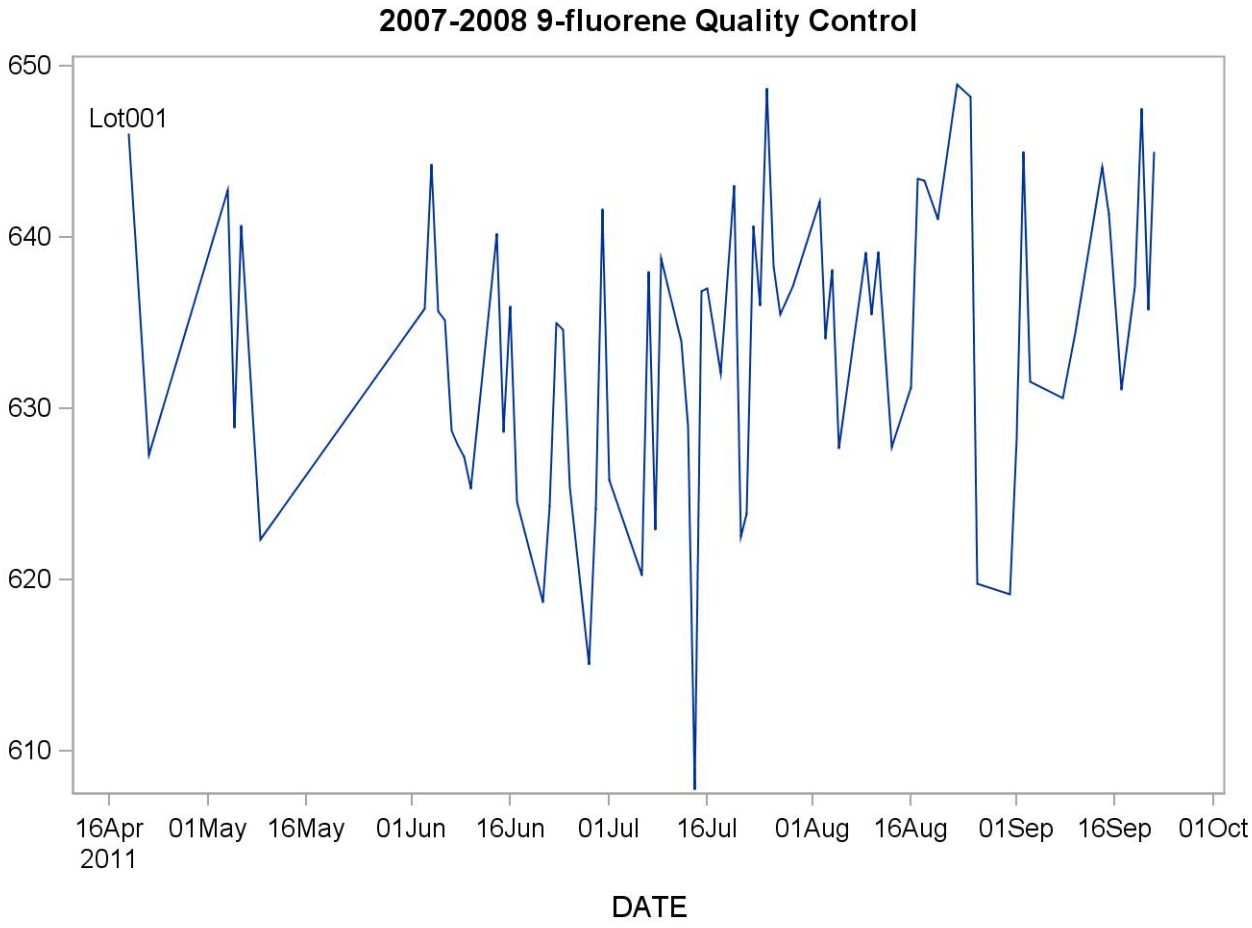
Summary Statistics for 3-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	107	19APR11	22SEP11	515.40	12.14	2.4



Summary Statistics for 9-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
Lot001	107	19APR11	22SEP11	633.52	10.40	1.6



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