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The association of urinary polycyclic aromatic hydrocarbon biomarkers and cardiovascular disease in the US population

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ABSTRACT

Background: Polycyclic aromatic hydrocarbons (PAHs) are potent atmospheric pollutants produced by incomplete combustion of organic materials. Pre-clinical and occupational studies have reported a positive association of PAHs with oxidative stress, inflammation and subsequent development of atherosclerosis, a major underlying risk factor for cardiovascular disease (CVD). The aim of the current study is to estimate the association between levels of PAH biomarkers and CVD in a national representative sample of United States (US) adults.
Methods: We examined adult participants (≥20 years of age) from the merged US National Health and Nutrition Examination Survey 2001–2010. Logistic regression models were used to estimate the associations of each urinary PAH biomarker and CVD. Post-exploratory structural equation modeling was then used to address the interdependent response variables (angina, heart attack, stroke and coronary heart disease) as well as the interdependencies of PAH biomarkers.
Results: PAH biomarkers were positively associated with cardiovascular disease in multiple logistic regression models, although some associations were not statistically robust. Using structural equation modeling, latent PAH exposure variable was positively associated with latent CVD level variable in the multivariable adjusted model ($\beta = 0.12$; 95% CI: 0.03, 0.20).
Conclusion: A modest association between levels of PAH biomarkers and CVD was detected in US adults. Further prospective studies with adequate sample size are needed to replicate or refute our findings.

1. Introduction

Despite advances in prevention, diagnosis and treatment, cardiovascular disease (CVD) remains the number one cause of mortality in United States (U.S.) adults (Kochanek et al., 2014). Cardiovascular disease refers to numerous conditions, many of which are related to atherosclerosis which develops when plaque builds up in arterial walls, narrowing the arteries, and decreasing or sometimes completely blocking tissue blood flow (Goff et al., 2014). Such mechanisms can manifest themselves eventually with adverse clinical outcomes such as coronary heart disease, angina pectoris, heart attack, and stroke.

Epidemiological evidence suggests that exposure to particulate matter present in ambient air may pose an increased CVD risk in humans (Bhatnagar, 2006). Polycyclic aromatic hydrocarbons (PAHs) are potent atmospheric pollutants that occur in oil, coal, and tar deposits, and are produced as byproducts of smoking, indoors and outdoors fuel burning and food grilling (Liu et al., 2008; Ramesh et al., 2004; Simko, 2005). Pre-clinical studies have reported a positive association between exposure to PAHs, oxidative stress and atherosclerosis (Jeng et al., 2011; Curfs et al., 2005). Epidemiological studies have found a positive association between biomarkers of PAH exposure and serum inflammatory markers of CVD (Alshaarawy et al., 2013). In addition, a positive association between occupational exposure to PAHs and CVD morbidity and mortality has been reported (Brucker et al., 2014; Burstyn et al., 2005). However it is not clear if exposure to PAHs is associated with cardiovascular disease in the general population.

With advantages of recent nationally representative sample surveys and standardized data collection approaches with relatively large samples, we examined the association of PAH exposure and CVD in the United States (US) general population. In addition, we used Structural Equation Modeling approach trying to account for the interdependencies that must be confronted when different PAH biomarkers are studied all at once. We also are accounting for the shared pathogenesis of the response variables, namely the CVD events (angina, heart attack, stroke and coronary heart disease).

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2. Methods

2.1. Study population

The National Health and Nutrition Examination Surveys (NHANES) consist of a series of surveys designed by the National Center for Health Statistics to continuously monitor the health status of the U.S. civilian non-institutionalized population (United States Centers for Disease Control and Prevention. National Center for Health Statistics (NCHS), 2010). The NHANES survey includes a stratified multistage probability sample. Selection is based on counties, blocks, households and individuals within households, and included oversampling of non-Hispanic Blacks and Mexican Americans in order to provide stable estimates of these groups. Data are collected for a two-year survey cycle.

In the current study we merged 2001–02, 2003–04, 2005–06, 2007–08 and 2009–10 data cycles, where eight analytes of PAHs have been consistently measured. There were 27,584 participants who were ≥20 years of age. Urinary PAH biomarkers were only measured in a subsample NHANES ($n = 7848$). Participants with missing information on serum cotinine and other covariates were not included in the final model. This resulted in 7301 participants included in the final analyses.

2.2. Outcome: Cardiovascular disease

A standardized medical condition questionnaire was administered during the personal interview on a broad range of health conditions including coronary heart disease, angina pectoris, heart attack and stroke. Participants were asked "has a doctor or other health professional ever told you that you have: coronary heart disease (CHD)/angina pectoris/heart attack/stroke?" (These were 4 separate questions with the same wording style). A participant was considered a CVD case if she/he answered "yes" to any of the previous questions (0: no event occurrence/1: occurrence of at least one of the 4 clinical events).

2.3. Main exposure: Urinary levels of PAH biomarkers

Detailed urine specimen collection and processing instructions are discussed in the NHANES Laboratory/Medical Technologists Procedures Manual (United States Center for Disease Control and Prevention. National Center for Health Statistics (NCHS), 2006). Specific analytes measured in NHANES are monohydroxy-PAH (OH-PAH). The procedure involves enzymatic hydrolysis of urine, with extraction, derivatization and analysis using capillary gas chromatography combined with high resolution mass spectrometry (GCHRMS). 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 abundance of each ion is measured. The ratios of these ions are used as criteria for evaluating the data. By evaluating these analytes in urine, a measurement of the body burden from PAH exposure is obtained (A detailed description is available online at http://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/l31pah_c_met.pdf).

The limit of detection (LOD) was defined as the higher 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 (0.5 pg/uL, or 5 pg/mL in 2-mL urine samples) verified to give a signal with the S/N equal to or >3. The limit of detection for each biomarker and each data cycle can be found in the corresponding NHANES PAH lab files provided as supplementary materials. <1% of the study population has PAH levels below lower detection limit.

Checks were made on the stability of the analytical system. Blanks and standards, as well as quality control materials, were added to each day's run sequence. The blank and standard were 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 were prepared and analyzed at the beginning and the end of each run; their concentrations were compared with acceptance criteria to assure the proper operation of the analysis. Relative retention times were examined for the internal standard to ensure the choice of the correct chromatographic peak. More details on quality control procedures can be found in the online supplemental materials.

Seven urinary low molecular weight PAH analytes (1-hydroxynapthol, 2-hydroxynapthol, 2-hydroxyfluorene, 3-hydroxyfluorene, 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene) and one urinary high molecular weight analyte (1-hydroxypyrene) were consistently available in NHANES 2001–10. Few other biomarkers are not included in the current study because they were not available in all years. All analytes were measured in the same unit; ng/L. Urinary analytes of PAHs were corrected for creatinine concentration to reduce their variability by differences in urinary outputs. Urinary levels of OH-PAH (ng/L) were divided by urinary creatinine level (mg/dL) multiplied by 0.01, i.e., [(ng/L) ÷ (mg/dL * 0.01)] and expressed as nanogram per gram of creatinine (ng/g creatinine).

2.4. Covariates

In NHANES, information on age, gender, ethnic self-identification (ESI), alcohol drinking, and poverty-income ratio (PIR) were obtained during a standardized questionnaire during a home interview. Information on anthropometric, physical and laboratory components were obtained during the medical center examination. Body mass index (kg/m^2) was calculated as weight in kilograms divided by height in meters squared. Serum total cholesterol (mg/dL) was measured enzymatically. Serum cotinine (ng/mL) was measured by an isotope dilution-high performance liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry.

2.5. Statistical analysis

Exploratory data analysis techniques were used to assess the univariate distribution of the study variables. Urinary PAH biomarker levels were log-transformed as a result of their skewed distribution. We ran logistic regression models to calculate the odds ratio ([OR] and 95% confidence interval [CI]) of self-reported CVD, for each urinary OH-PAH, controlling for age (years) and sex. Final models were additionally adjusted for ESI (non-Hispanic White, non-Hispanic Black, Hispanics, and all others), education (less than high school, high school, and above high school), PIR, past-year alcohol drinking, BMI (normal, overweight, obese), total cholesterol (mg/dL), and serum cotinine (ng/mL).

Structural Equation Modeling (SEM) was then used to construct a series of models to estimate the association of PAHs and CVD level. Buncher et al. discussed the application of SEM in Environmental Epidemiology early in 1991 (Buncher et al., 1991). Structural equation modeling is a group of statistical methods that can model relationships between one or more independent variables and one or more dependent variables (Ullman, 2006). In the current analysis, PAH exposure is a latent construct that is not directly measured but rather assessed indirectly by PAH biomarkers. Instead of simply combining PAH biomarkers by taking the sum and assigning equal weight to each biomarker, they are employed as indicators of a latent construct and hence allows for estimation and removal of the measurement error (Ullman, 2006). Limitations of including multiple indicators of the same exposure in regression models (such as collinearity) are accounted for in the SEM approach (Gefen et al., 2000; Suhr, 2006). Similarly a latent construct for CVD level was created via self-reported physician diagnosis of coronary heart disease, angina, heart attack and/or stroke. For both constructs, we defined factor loadings of 0.4 or greater as strongly correlated indicators with the latent construct. This part of the model that relates the measured variables to the corresponding latent construct is the measurement part of the model. The hypothesized association between

the two constructs is considered the structural part of the model, regressing the latent CVD level construct as the response variable on PAHs construct. In the initial models, we used maximum likelihood estimation with robust standard errors (MLR). Because MLR does not provide fit indices, in our post-exploratory step we used a robust weighted least squares estimator (WLSMV). We determined goodness of fit of the hypothesized models to the data by several fit indices, including comparative fit index (CFI) ≥0.95 and root mean square error approximation (RMSEA) ≤0.05. (Hu and Bentler, 1999; Browne and Cudeck, 1992).

3. Results

Table 1 presents main characteristics of the study population. About one half of the study population was female (51.2%). The majority of the study population was non-Hispanic White (71.1%). The arithmetic mean of serum cotinine was 64.1 ng/mL. Table 2 presents selected percentiles of urinary OH-PAH biomarkers.

The main estimates of the study are presented in Table 3. Individual polycyclic aromatic hydrocarbons were positively associated with self-reported CVD. However, only 1-hydroxynaphalene, 2-hydroxynaphalene, 2-hydroxyfluorene and 3-hydroxyfluorene were statistically robust.

Fig. 1 presents the baseline conceptual model regressing the latent construct of CVD level as the response variables on the level of PAH exposure, measured by eight urinary PAH biomarkers, controlling for age (years) and sex. Results indicated a positive association between PAHs and CVD ($\beta = 0.12$; 95% CI: 0.07, 0.17). The factor loadings were all ≥0.4 suggesting a strong correlation between the measures and each corresponding construct.

Table 4 presents the association of PAH exposure level and CVD level constructs additionally adjusting for ESI, education, PIR, alcohol drinking, serum cotinine, total cholesterol and BMI. Our results did not change appreciably ($\beta = 0.12$; 95% CI: 0.03, 0.20), suggesting a positive association between PAHs and CVD independent of the potential confounders studied here. Our results were consistent using either MLR or WLSMV estimator. Table 4 also presents the association of PAH and CVD constructs, stratified by sex, ESI and smoking status. Overall, PAH was associated with CVD in the stratified subgroups, although some associations were not statistically robust. We did not detect subgroup variations in the estimates.

Table 1
Baseline characteristics of the study population ($n = 7301$). Data for the United States adults ≥20 years of age based on the National Health and Nutrition Examination Survey 2001–2010.

Characteristics	Mean values ± std error of mean or sample size (weighted percentages)
Age (years)	46.2 ± 0.3
Sex (%)	
Male	3550 (48.8)
Female	3751 (51.2)
Ethnic self-identification (%)	
Non-Hispanic White	3693 (71.1)
Non-Hispanic Black	1378 (10.7)
Hispanics	1919 (12.8)
All others	311 (5.4)
Education (%)	
Less than high school	2079 (18.3)
High school	1773 (25.1)
Above high school	3449 (56.6)
Income-poverty ratio < 1	1325 (12.6)
Past-year alcohol drinking (%)	4527 (67.6)
Body mass index (kg/m^2)	
<25	2184 (32.7)
25–29.9	2542 (33.4)
≥30	2575 (33.9)
Serum cotinine (ng/mL)	64.1 ± 2.8
Total cholesterol (mg/dL)	198.8 ± 0.8
Any CVD (%)	732 (7.9)
Coronary heart disease (%)	333 (3.7)
Angina (%)	218 (2.4)
Heart attack (%)	307 (3.3)
Stroke (%)	235 (2.4)

Table 2
Selected percentiles of urinary biomarkers of polycyclic aromatic hydrocarbons (ng/g creatinine) among participants included in the final analysis. Data for the United States adults ≥20 years of age based on the National Health and Nutrition Examination Survey 2001–2010.

Biomarkers	10th percentile	Q1	Median	Q3	90th percentile
1-Hydroxynaphthalene	515	901	2081	6941	17,532
2-Hydroxynaphthalene	957	1576	3114	7454	15,717
2-Hydroxyfluorene	103	142	227	609	1630
3-Hydroxyfluorene	33	50	86	279	909
1-Hydroxyphenanthrene	62	90	141	231	361
2-Hydroxyphenanthrene	26	39	61	102	178
3-Hydroxyphenanthrene	36	53	85	158	297
1-Hydroxypyrene	26	44	83	159	313

4. Discussion

Exposure to polycyclic aromatic hydrocarbons measured by eight urinary PAH biomarkers exhibited a positive association with cardiovascular disease in adults ≥20 years of age independent of serum cotinine a marker of nicotine exposure as well as other potential confounders. Our results are consistent with findings from previous occupational studies which reported positive associations between exposure to PAHs, and ischemic heart diseases and cardiovascular mortality in occupations that include likely exposure to PAHs (Brucker et al., 2014; Burstyn et al., 2005; Thériault et al., 1988; Tüchsen et al., 1996). Xu et al. used NHANES 2001–04 and reported a positive association between PAH biomarkers and self-reported CVD using logistic regression modes (Xu et al., 2010). In the current study, a latent CVD construct approach was selected in addition to utilizing the standard binary approach (0: no event occurrence/1: occurrence of at least one of the 4 clinical events) to allow for consideration of the shared pathogenesis in the development of CHD, angina, heart attack and stroke by means of a main common mechanism, namely atherosclerosis (Soler and Ruiz, 2010). A latent PAH variable was also used to account for the interdependencies of the PAH biomarkers as humans are usually exposed to mixtures of PAHs (Elovaara et al., 1995). Due to the high cost of detecting parent PAH levels in humans, the most commonly used biomarkers of PAH exposure are urinary OH-PAH biomarkers. Urinary PAH biomarkers have been previously found to correlate well with levels of exposure to PAHs in the general population (Castano-Vinyals et al., 2004).

The mechanisms underlying the positive association of PAH exposure and CVD remain unknown. Upon exposure to PAHs, detoxification

Table 3
The association of PAH biomarkers and cardiovascular disease. Data for the United States adults ≥20 years of age based on the National Health and Nutrition Examination Survey 2001–2010.

OH-PAH (ng/g creatinine)	Age-sex adjusted odds ratio (95% CI)	Multivariable-adjusted odds ratio (95% CI)[a]
1-Hydroxynaphthalene	1.13 (1.07, 1.21)	1.11 (1.04, 1.18)
2-Hydroxynaphthalene	1.31 (1.19, 1.45)	1.22 (1.08, 1.38)
2-Hydroxyfluorene	1.31 (1.18, 1.45)	1.27 (1.12, 1.43)
3-Hydroxyfluorene	1.21 (1.11, 1.33)	1.18 (1.06, 1.32)
1-Hydroxyphenanthrene	1.12 (1.00, 1.25)	1.07 (0.94, 1.21)
2-Hydroxyphenanthrene	1.22 (1.09, 1.37)	1.11 (0.98, 1.26)
3-Hydroxyphenanthrene	1.13 (1.00, 1.27)	1.10 (0.96, 1.25)
1-Hydroxypyrene	1.20 (1.08, 1.33)	1.12 (1.00, 1.26)

[a] Adjusted for age (years), sex, ESI (non-Hispanic White, non-Hispanic Black, Hispanic and all others), education (less than high school, high school and above high school), PIR, past-year alcohol drinking, BMI (normal, overweight and obese), total cholesterol (mg/dL) and serum cotinine (ng/mL).

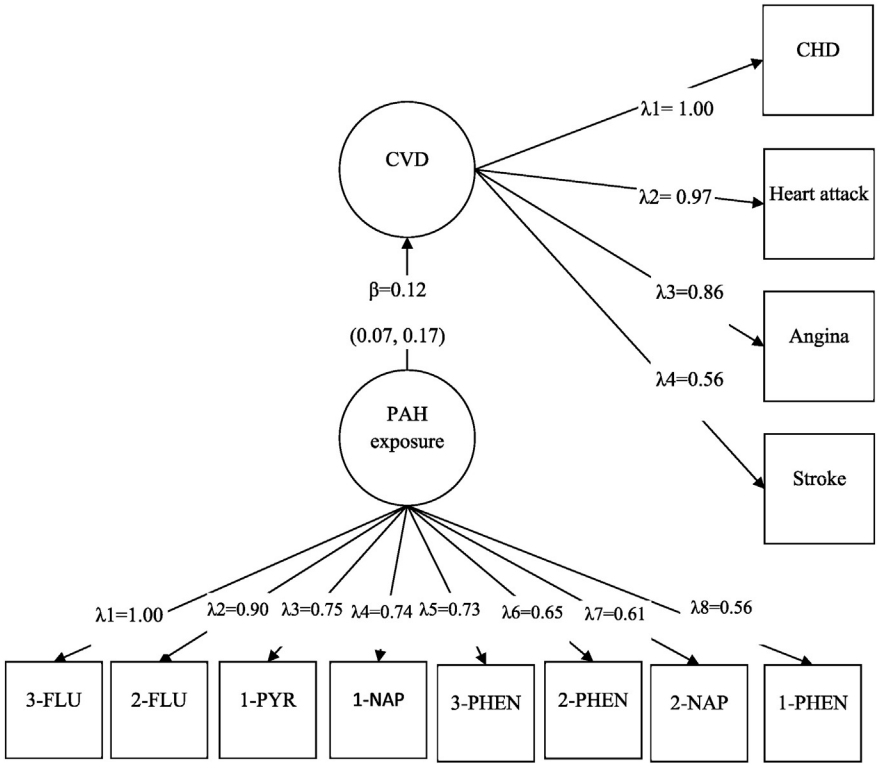

Fig. 1. Model depicting the hypothesized PAH exposure-CVD association. Data for the National Health and Nutrition Examination Survey 2001–2010 ($n = 7301$)

occurs leading to the formation of highly reactive intermediates that can interact with the DNA (Curfs et al., 2004). Such effects of PAHs exposure on plaque buildup in animals were found to be dose dependent (Penn et al., 1981). Pre-clinical studies have also suggested that PAHs might also exert their atherogenic effect via stimulation of an inflammatory process involving an increased influx of proinflammatory cells into these plaques (Curfs et al., 2005). The role of inflammation as a risk factor for CVD development has been well established (Pearson et al., 2003; Goff et al., 2013). Population based studies supported an association between PAH and inflammation (Alshaarawy et al., 2013; Clark et al., 2012). A recent study also suggested an association between PAHs and a number of obesity-related cardiometabolic health risk factors (Ranjbar et al., 2015).

Table 4
Estimated association of polycyclic aromatic hydrocarbon exposure on cardiovascular disease level in adults (≥20 + years), stratified by participant characteristics. Data for the United States based on the National Health and Nutrition Examination Survey 2001–2010.

Characteristics	Age-sex adjusted[a] β (95% CI)	Multivariable adjusted[b] β (95% CI)
Full sample	0.12 (0.07, 0.17)	0.12 (0.03, 0.20)
Sex		
Male	0.06 (−0.01, 0.13)	0.08 (−0.02, 0.17)
Female	0.18 (0.11, 0.25)	0.16 (0.07, 0.26)
ESI		
NH-White	0.13 (0.07, 0.19)	0.13 (0.03, 0.23)
NH-Black	0.10 (0.01, 0.19)	0.15 (0.04, 0.27)
Hispanic	−0.04 (−0.12, 0.04)	0.01 (−0.10, 0.13)
Smoking		
Never	0.08 (−0.04, 0.20)	0.11 (−0.01, 0.23)
Former	0.03 (−0.09, 0.15)	0.02 (−0.10, 0.14)
Recently active	0.24 (0.09, 0.39)	0.25 (0.13, 0.37)

A WLSMV estimator was used (probit regression are estimated). Model fit for each of the three models: RMSEA < 0.05; CFI ≥ 0.95.
[a] Adjusted for age and sex, except for stratified variable.
[b] Adjusted for age, sex, ESI, education, PIR, alcohol drinking, serum cotinine, total cholesterol and BMI, except for stratified variable.

Several of the study limitations merit attention. Of main concern is the cross sectional nature of NHANES which does not allow to draw temporal or causal inferences regarding the association of PAHs and CVD. In addition, our study does not have the data to estimate the sources of exposure to PAHs. Urinary biomarker measurements reflect recent PAH exposure as non-smoking sources of PAHs can vary day-to-day in the general population. Finally, CVD was ascertained by self-report. Accordingly, some recall bias may exist. However, these biases are likely to be non-differential biases, which would minimize any observed association. Notwithstanding these limitations, the study findings are of interest because of the inclusion of a relatively large nationally representative multiethnic sample, the NHANES standardized data collection approaches, and the ability to adjust for potential confounders.

In conclusion, this epidemiological evidence from the current study tends to confirm what prior research found – namely, a positive association between PAH exposure and CVD. Any claim of atherogenic effects of PAH exposure is premature, but given increased prevalence of PAH exposure and CVD in the U.S. and elsewhere, there is a reason to study their linkages. More probing experimentation of a clinical translational character is needed, including research on potential mechanisms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2016.02.006.

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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**

Revised: **05/31/2012**

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.

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.

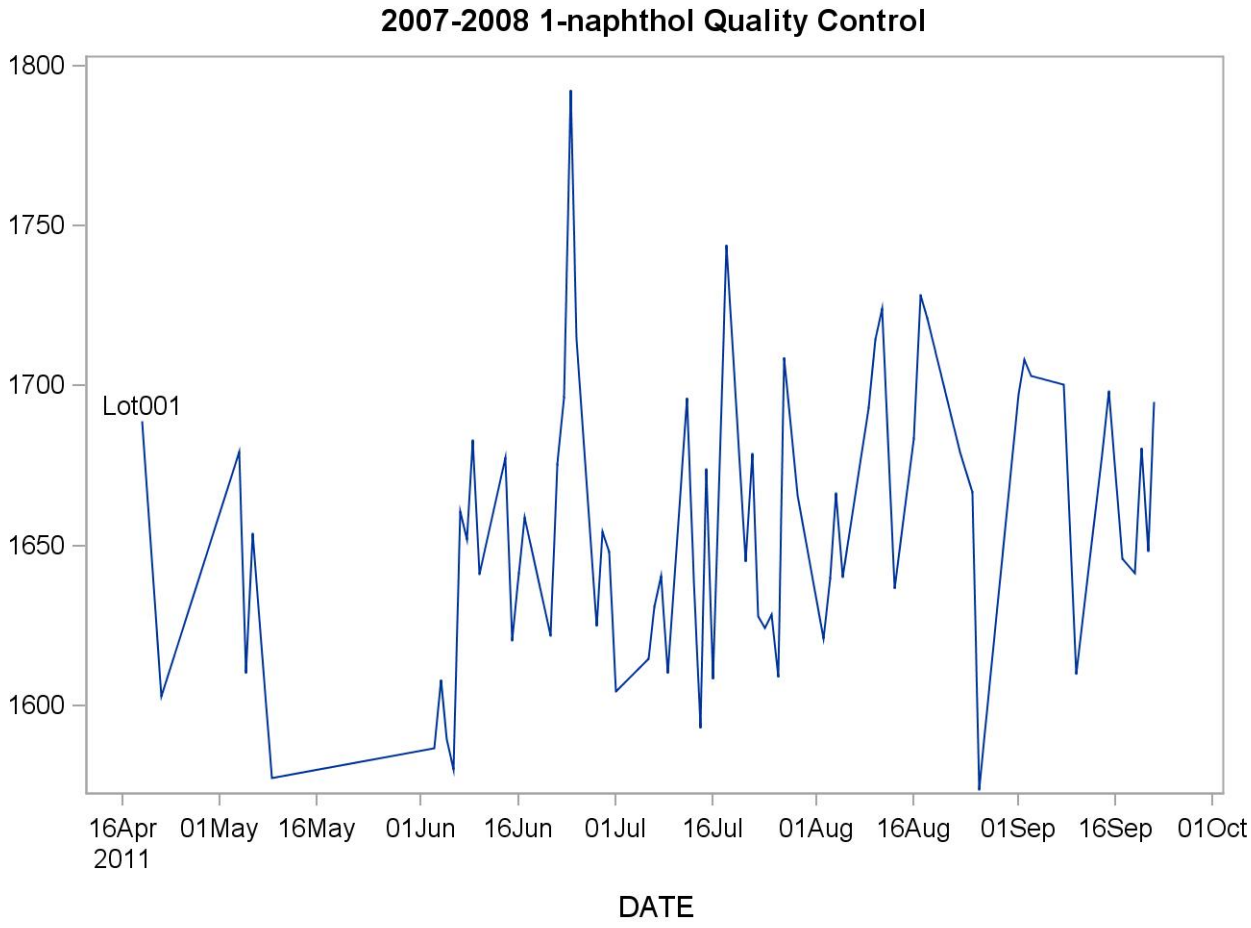
Standard record keeping (e.g., database, notebooks, and data files) is used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Transfer is carried out through the DLS Samples Logistic Group. Specimens may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

19. Summary Statistics and QC Graphs

See following pages.

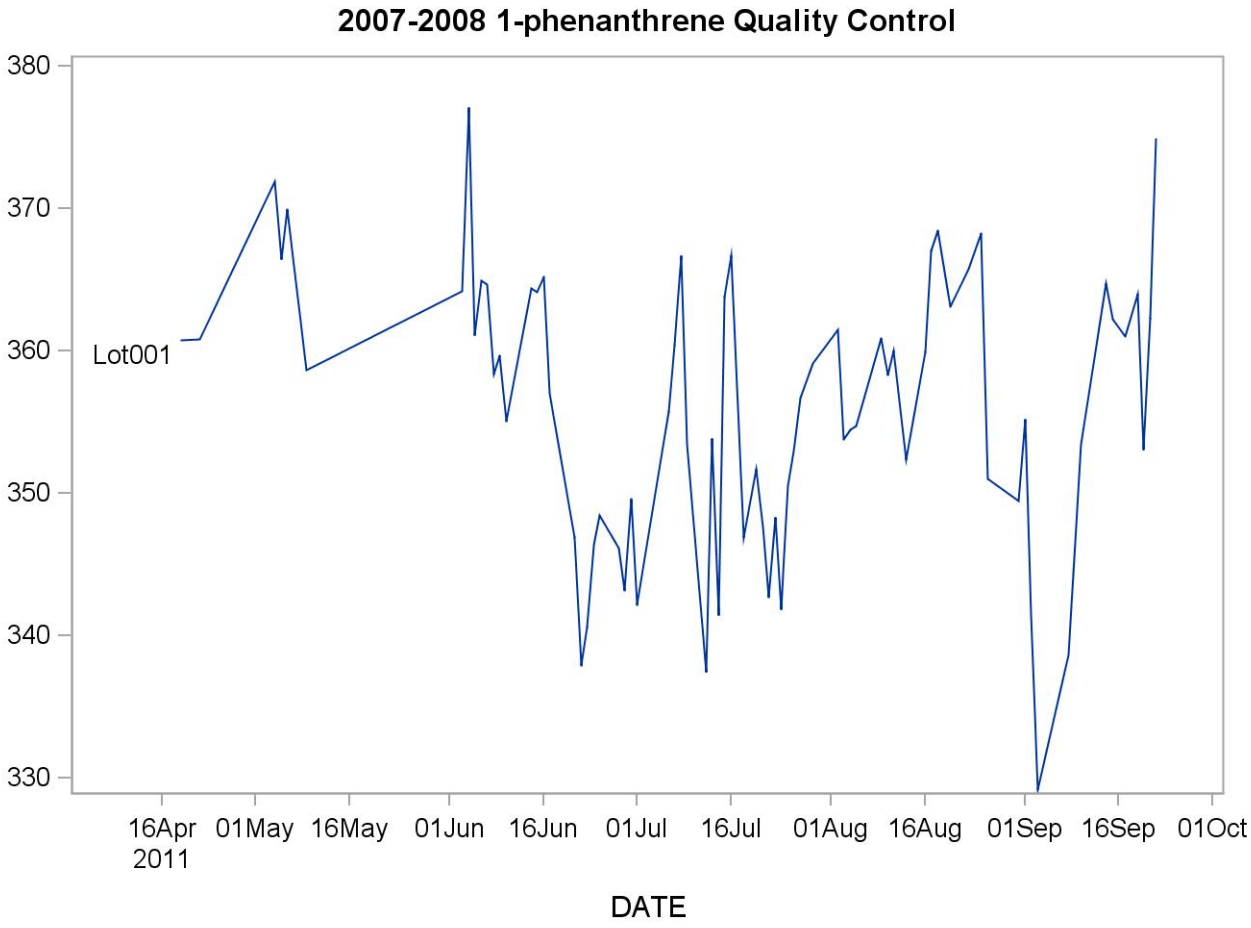
Summary Statistics for 1-naphthol

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



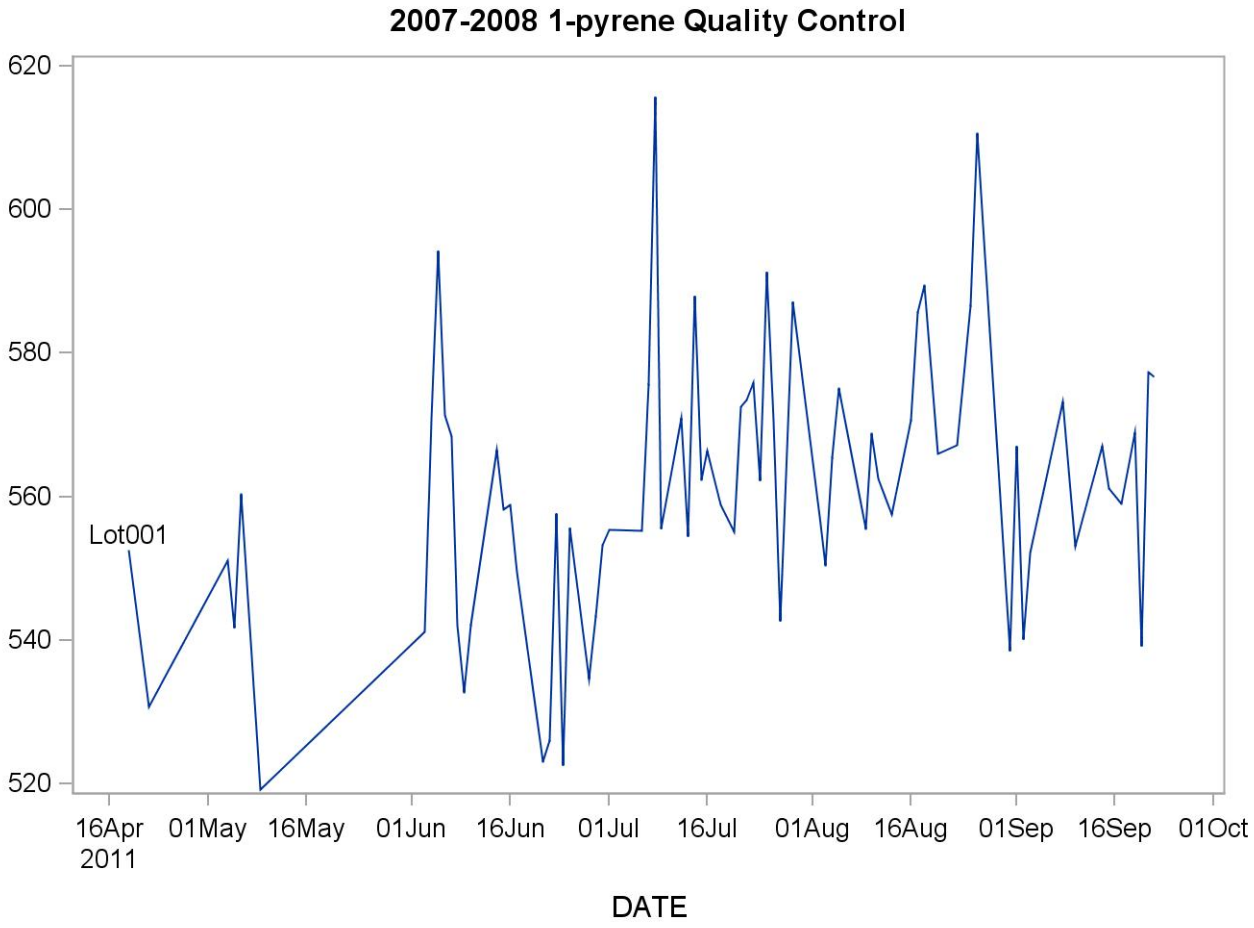
Summary Statistics for 1-phenanthrene

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



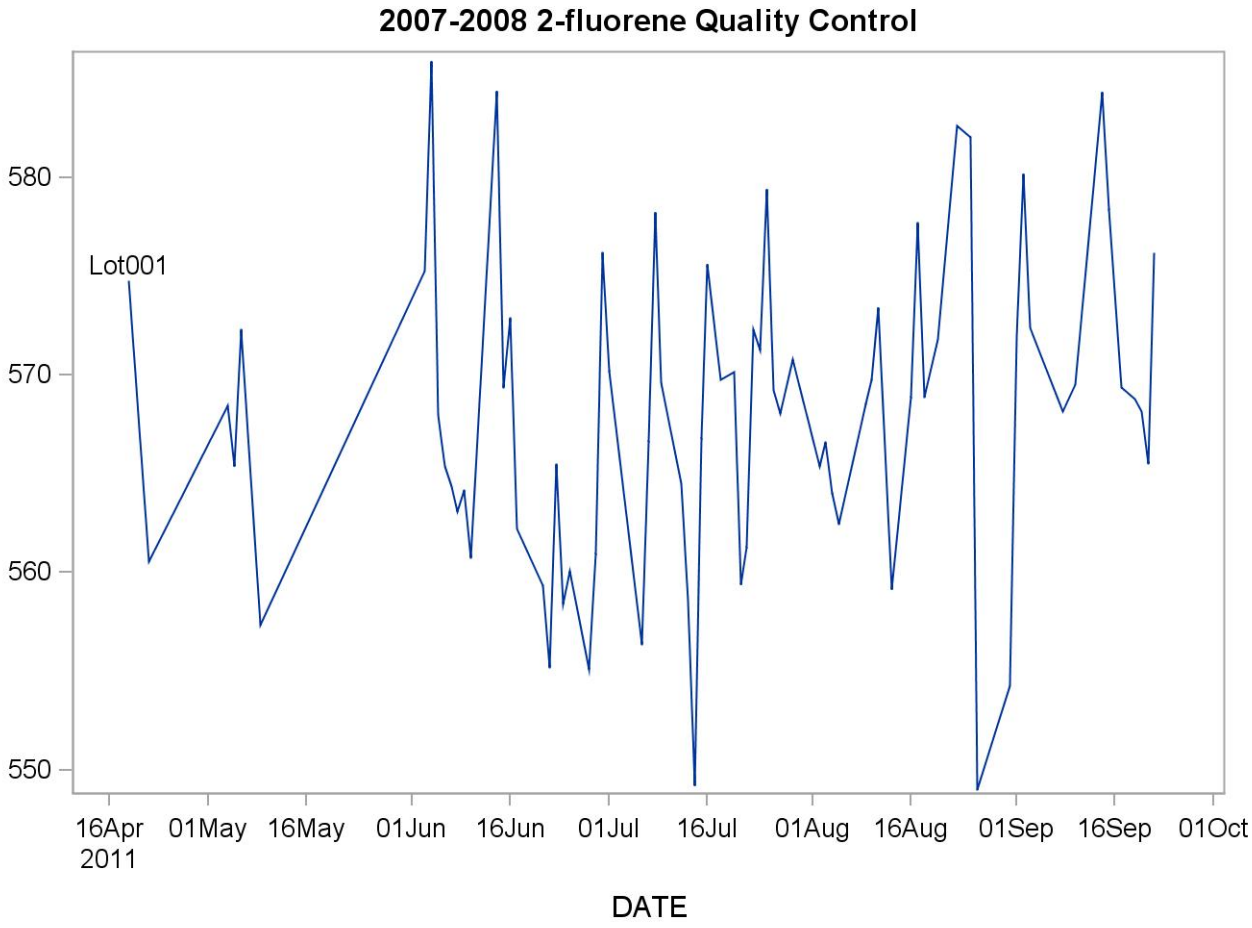
Summary Statistics for 1-pyrene

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



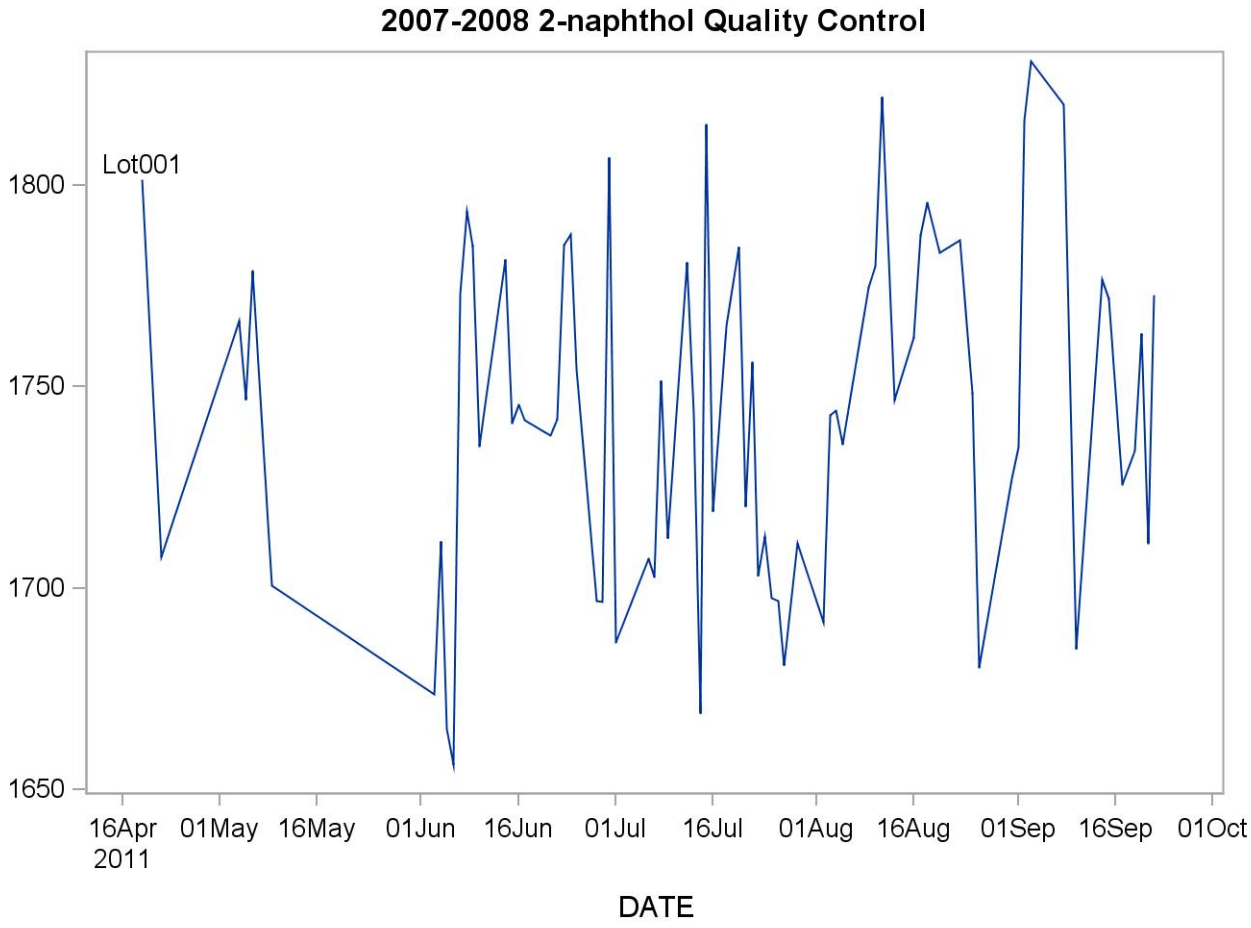
Summary Statistics for 2-fluorene

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



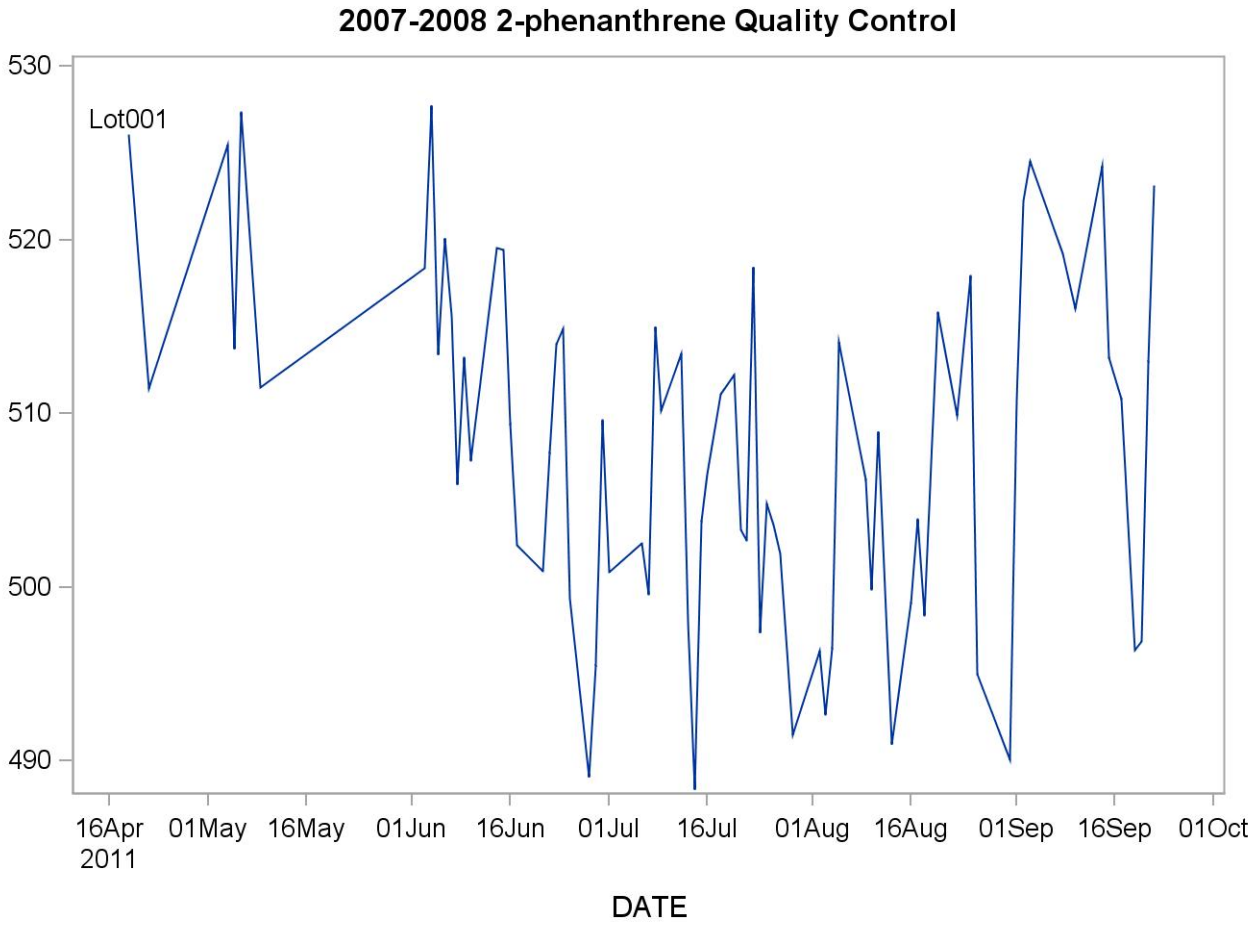
Summary Statistics for 2-naphthol

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



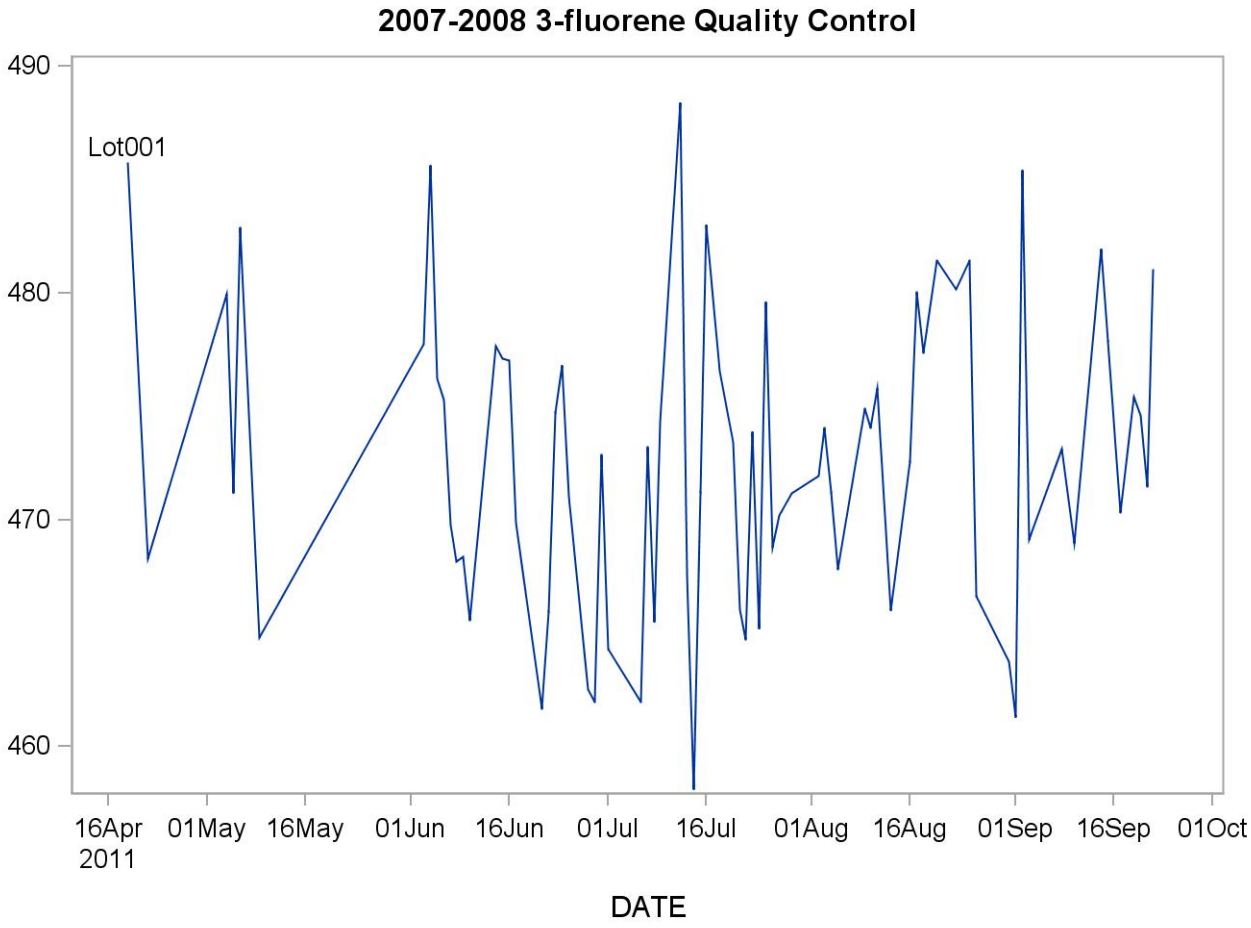
Summary Statistics for 2-phenanthrene

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



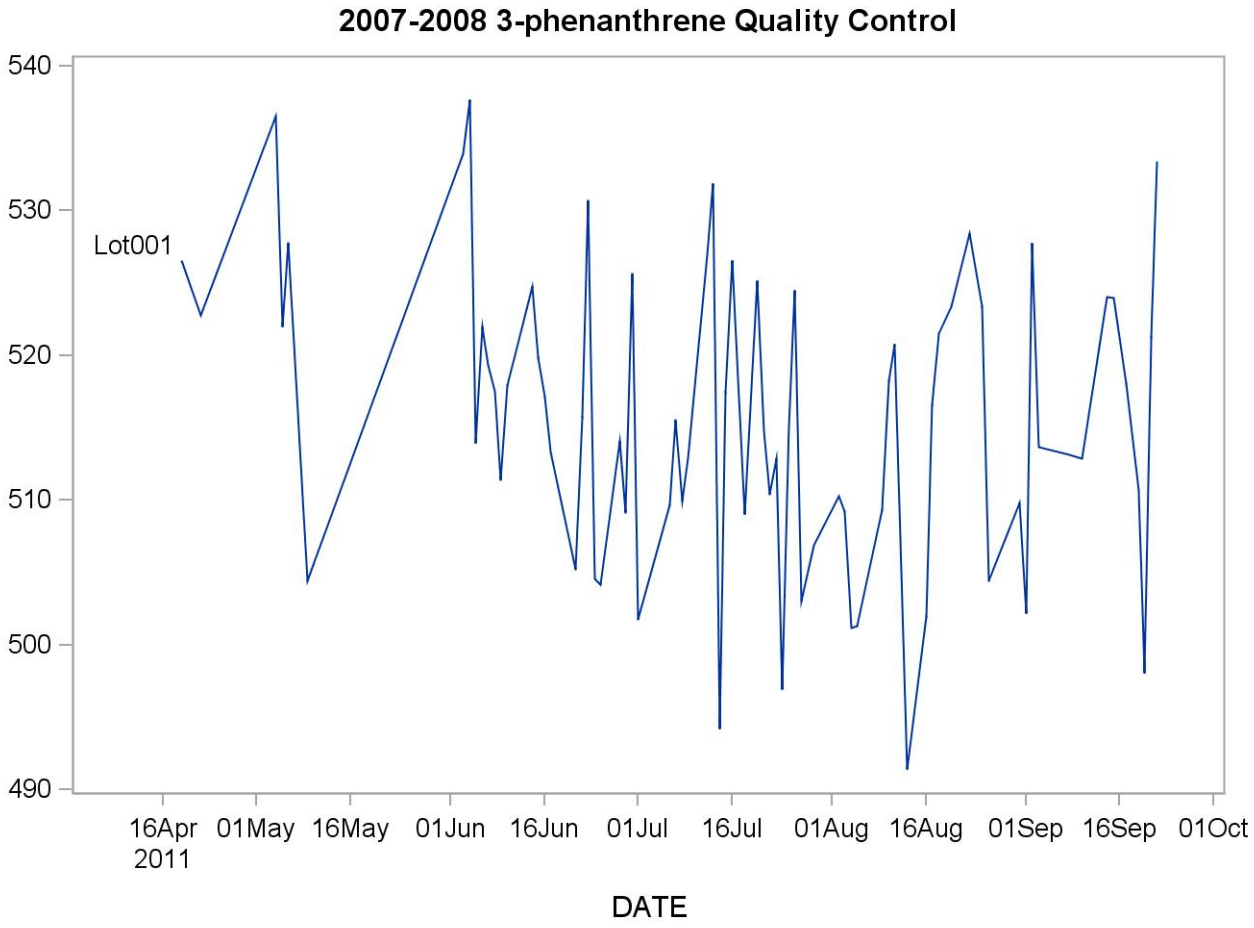
Summary Statistics for 3-fluorene

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



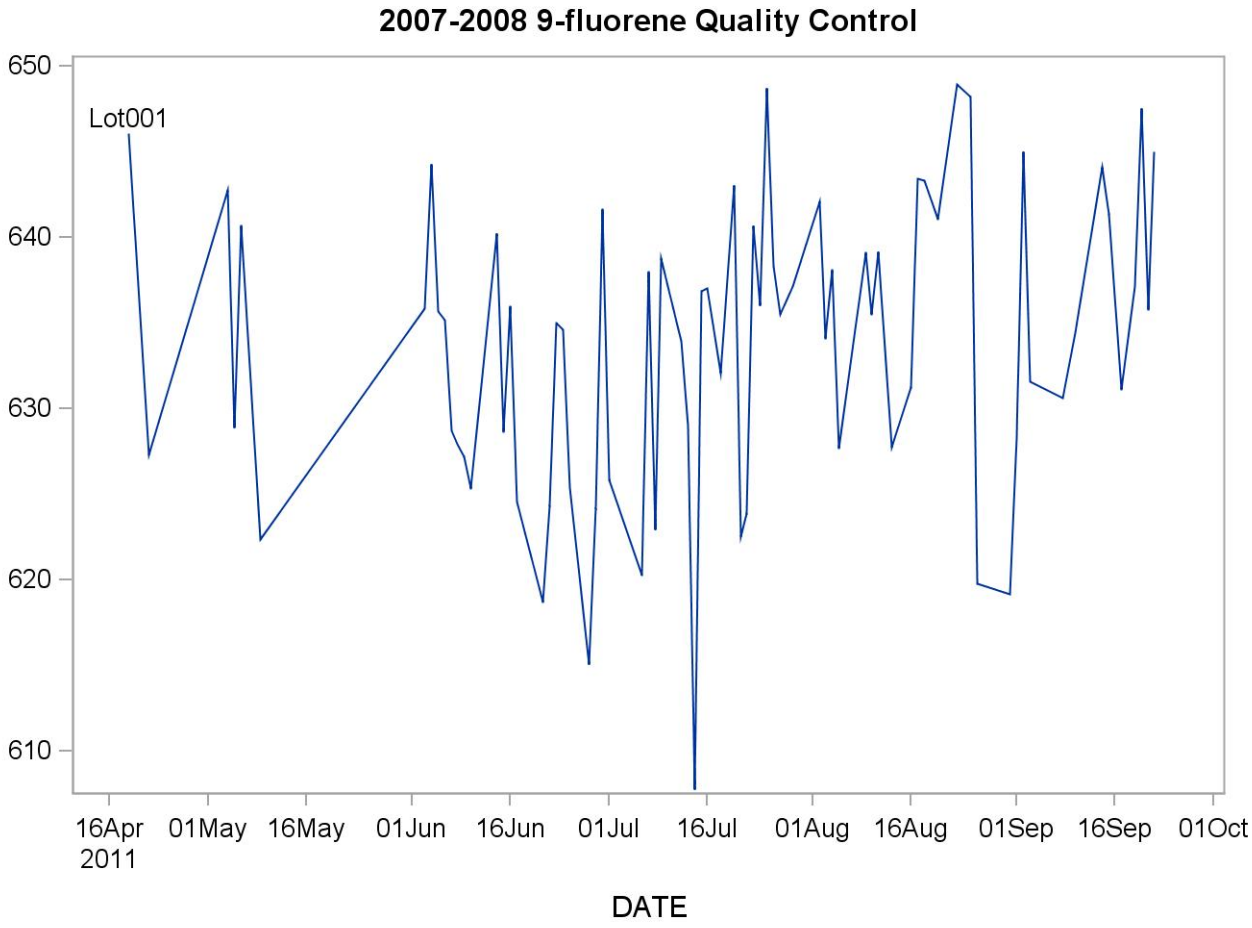
Summary Statistics for 3-phenanthrene

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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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
Division of Laboratory Sciences
National Center for Environmental Health

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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 2009–2010 data.
A tabular list of the released analytes follows:

Data File Name	Variable Name	SAS Label
PAH_F	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 ºC 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 ºC 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.

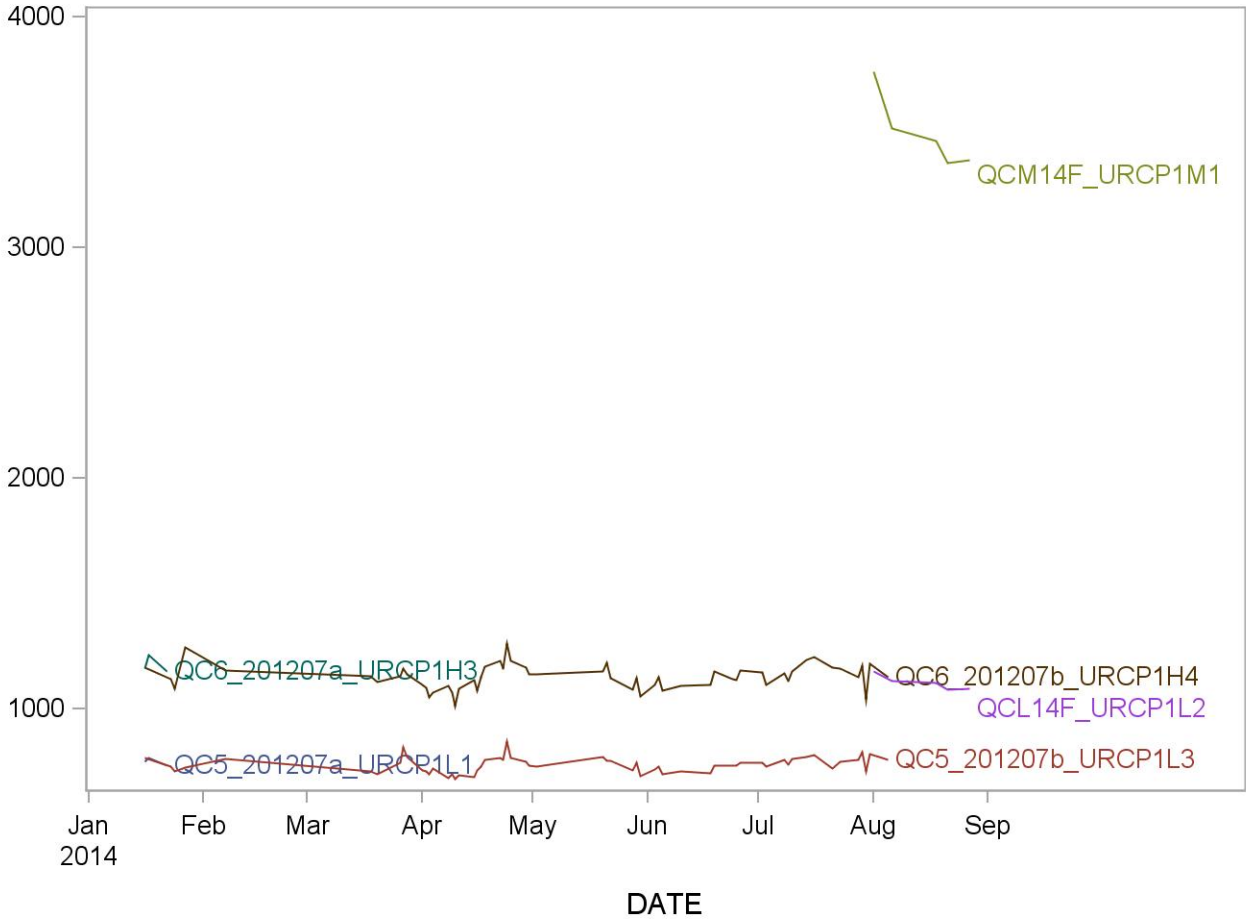
Standard record keeping (e.g., database, notebooks, and data files) is used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Transfer is carried out through the DLS Samples Logistic Group. Specimens may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

19. Summary Statistics and QC Graphs

See following pages

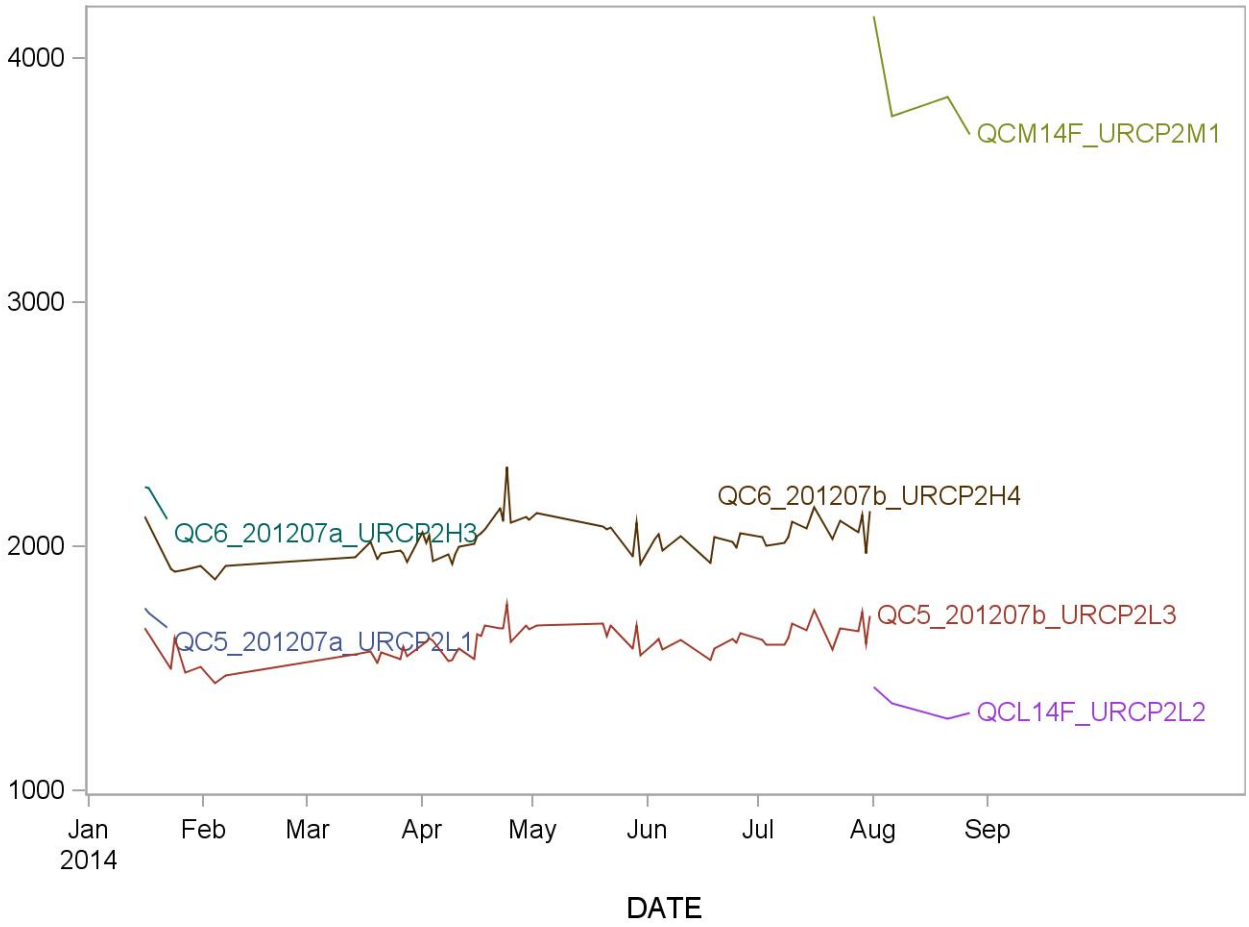
2009-2010 Summary Statistics and QC Chart for 1-naphthol

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URCP1H3	4	16JAN14	22JAN14	1181.38	44.79	3.8
QC6_201207b_URCP1H4	77	16JAN14	05AUG14	1139.10	52.95	4.6
QC5_201207a_URCP1L1	4	16JAN14	22JAN14	766.25	25.38	3.3
QC5_201207b_URCP1L3	77	16JAN14	05AUG14	755.16	36.90	4.9
QCL14F_URCP1L2	5	01AUG14	27AUG14	1112.20	31.33	2.8
QCM14F_URCP1M1	5	01AUG14	27AUG14	3496.60	159.83	4.6



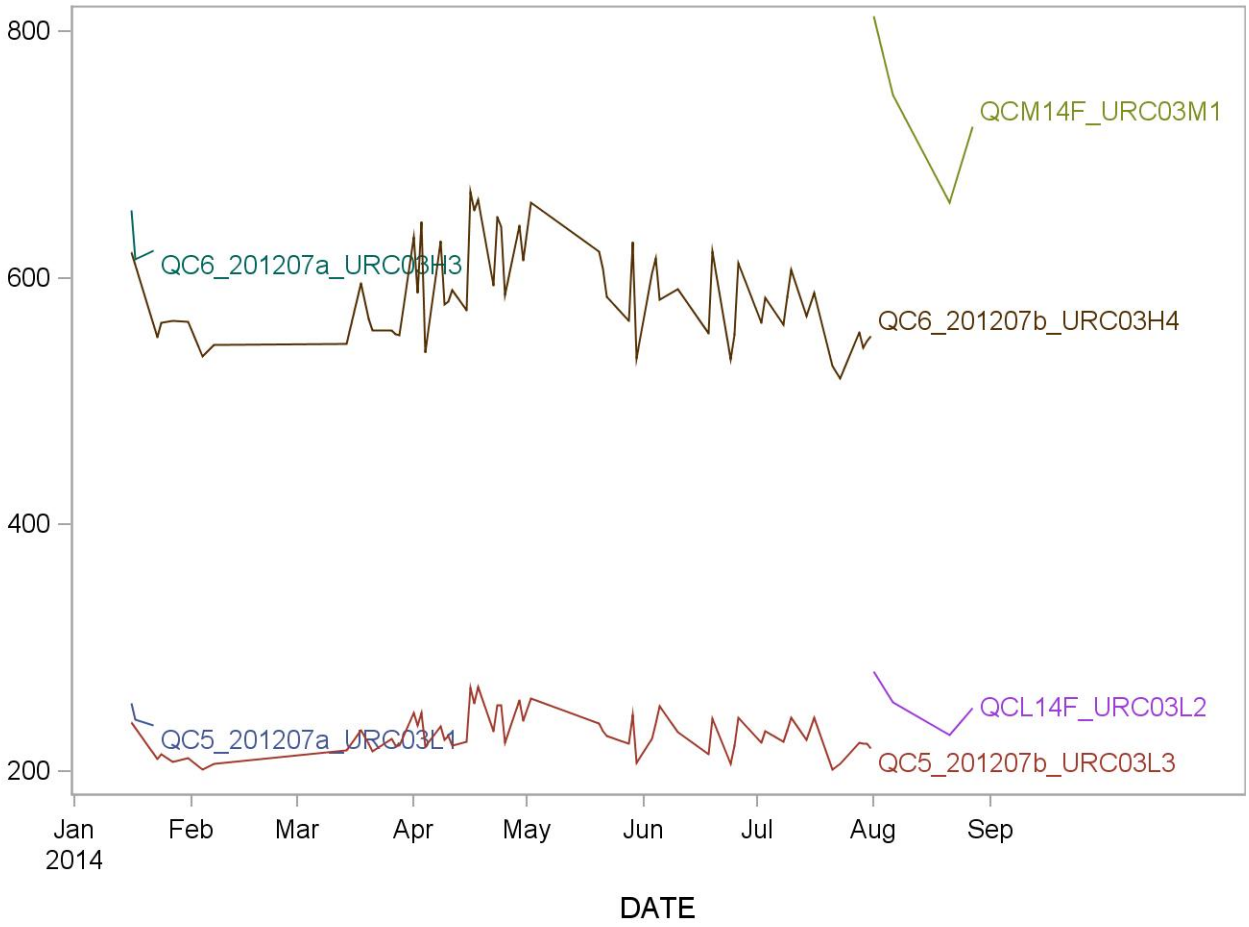
2009-2010 Summary Statistics and QC Chart for 2-naphthol

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URCP2H3	4	16JAN14	22JAN14	2178.63	136.27	6.3
QC6_201207b_URCP2H4	80	16JAN14	31JUL14	2028.63	88.38	4.4
QC5_201207a_URCP2L1	4	16JAN14	22JAN14	1704.50	97.11	5.7
QC5_201207b_URCP2L3	80	16JAN14	31JUL14	1607.63	70.57	4.4
QCL14F_URCP2L2	4	01AUG14	27AUG14	1350.13	56.62	4.2
QCM14F_URCP2M1	4	01AUG14	27AUG14	3866.00	214.16	5.5



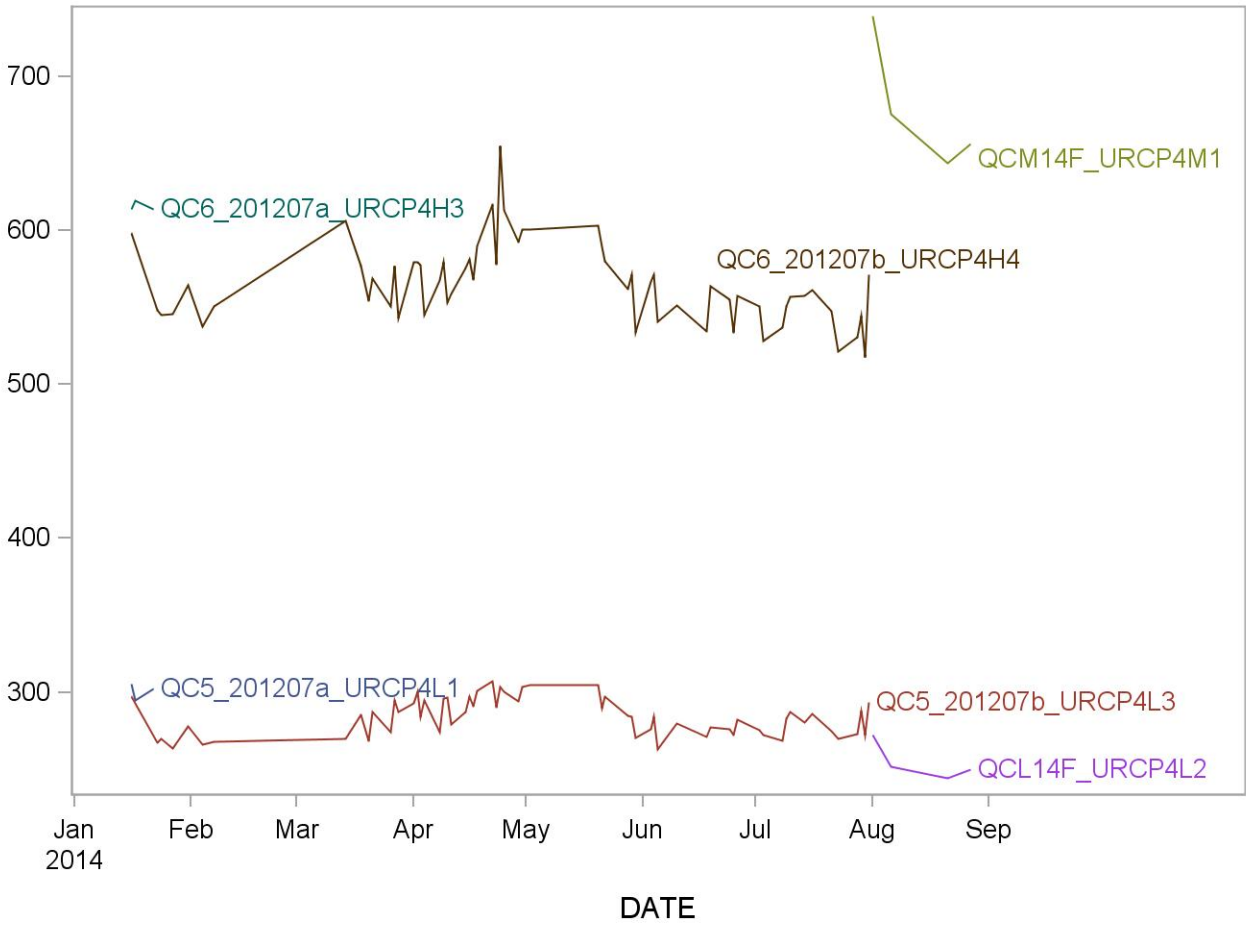
2009-2010 Summary Statistics and QC Chart for 3-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URC03H3	4	16JAN14	22JAN14	628.88	28.32	4.5
QC6_201207b_URC03H4	80	16JAN14	31JUL14	586.10	41.78	7.1
QC5_201207a_URC03L1	4	16JAN14	22JAN14	243.25	15.25	6.3
QC5_201207b_URC03L3	80	16JAN14	31JUL14	229.97	17.31	7.5
QCL14F_URC03L2	4	01AUG14	27AUG14	254.63	20.93	8.2
QCM14F_URC03M1	4	01AUG14	27AUG14	736.25	62.72	8.5



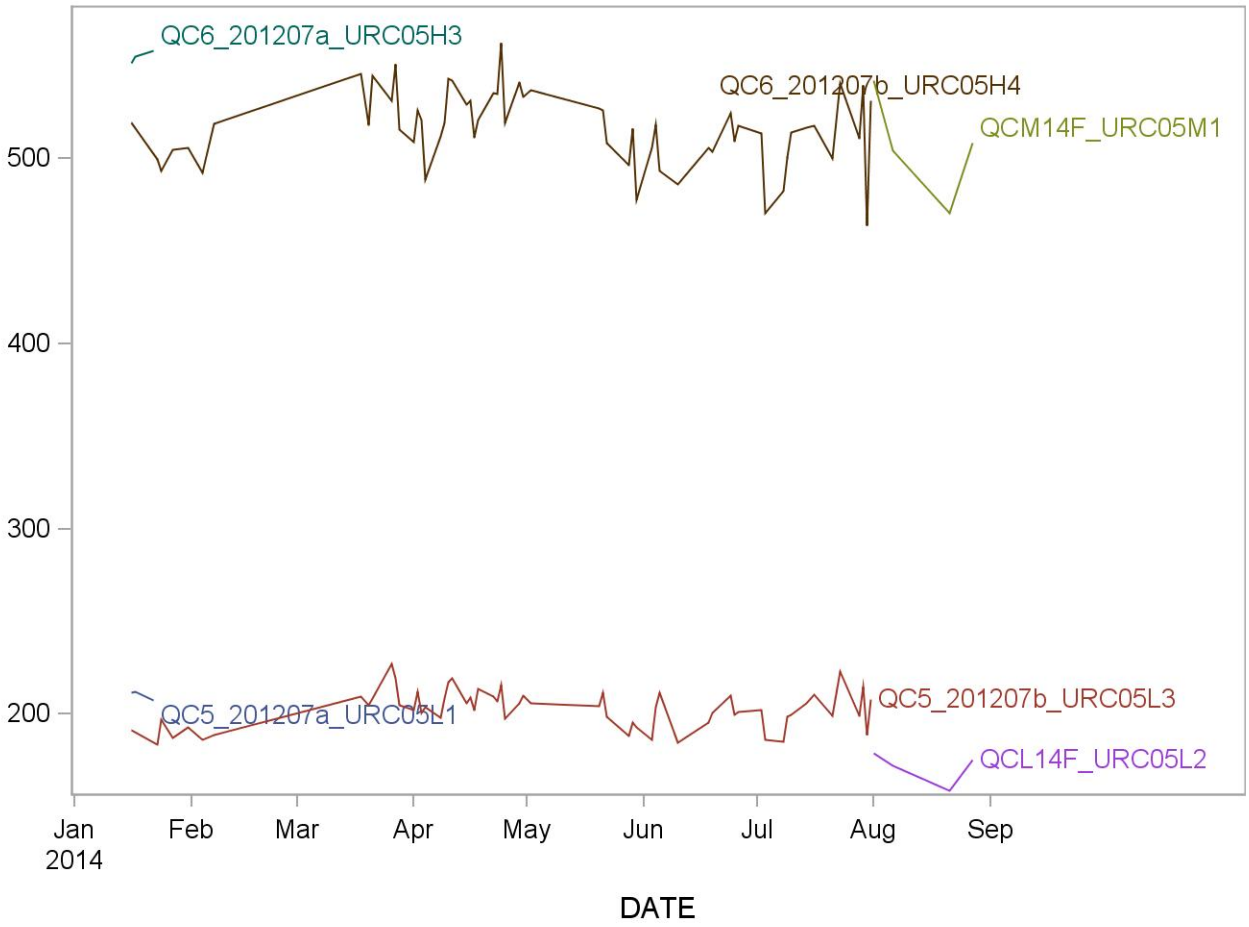
2009-2010 Summary Statistics and QC Chart for 2-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URCP4H3	4	16JAN14	22JAN14	615.38	15.35	2.5
QC6_201207b_URCP4H4	80	16JAN14	31JUL14	565.26	27.33	4.8
QC5_201207a_URCP4L1	4	16JAN14	22JAN14	301.13	10.74	3.6
QC5_201207b_URCP4L3	80	16JAN14	31JUL14	283.73	13.37	4.7
QCL14F_URCP4L2	4	01AUG14	27AUG14	254.25	12.25	4.8
QCM14F_URCP4M1	4	01AUG14	27AUG14	679.00	42.43	6.2



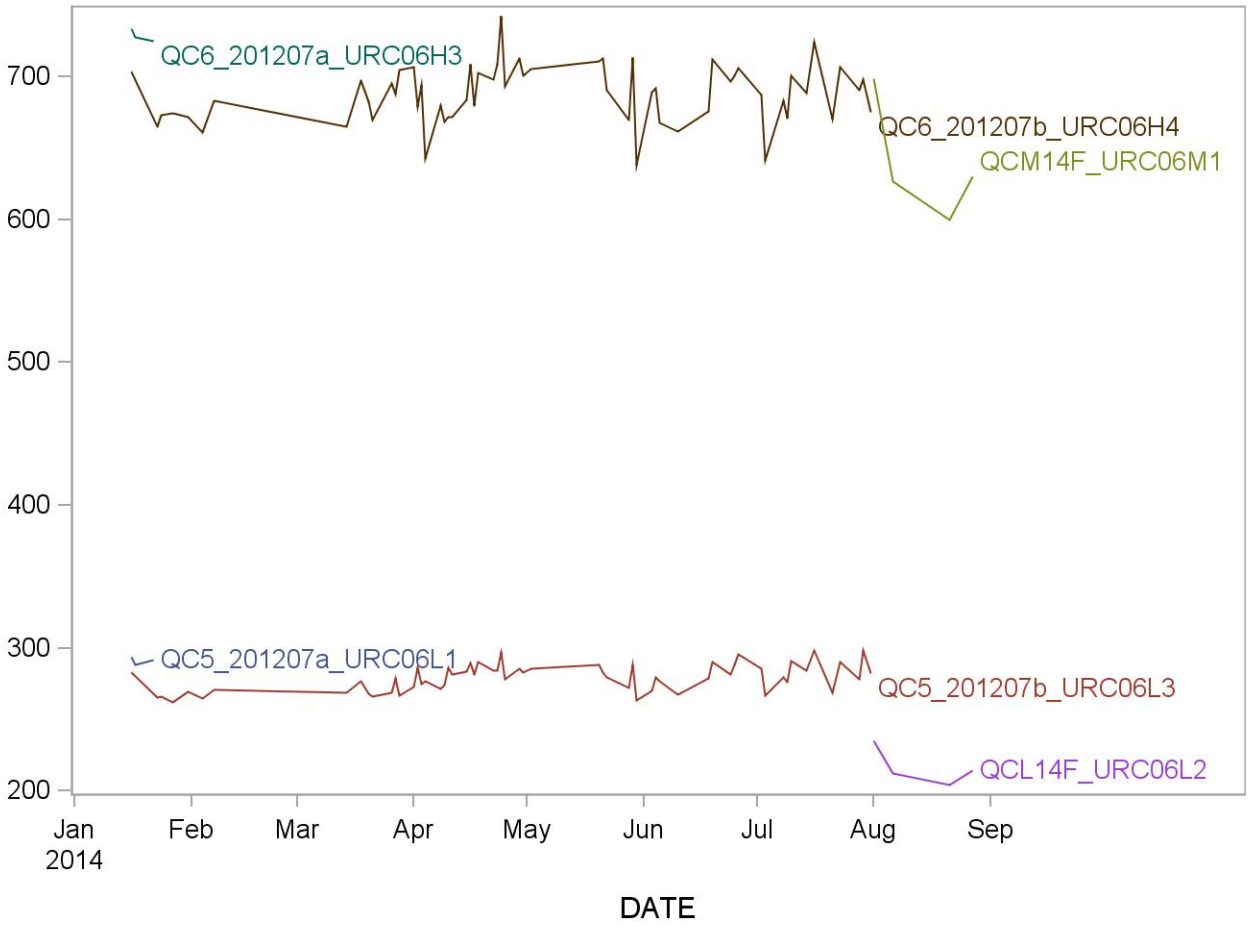
2009-2010 Summary Statistics and QC Chart for 3-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URC05H3	4	16JAN14	22JAN14	555.75	3.71	0.7
QC6_201207b_URC05H4	79	16JAN14	31JUL14	516.72	20.84	4.0
QC5_201207a_URC05L1	4	16JAN14	22JAN14	209.50	4.67	2.2
QC5_201207b_URC05L3	79	16JAN14	31JUL14	201.82	10.54	5.2
QCL14F_URC05L2	4	01AUG14	27AUG14	171.13	8.89	5.2
QCM14F_URC05M1	4	01AUG14	27AUG14	506.38	29.24	5.8



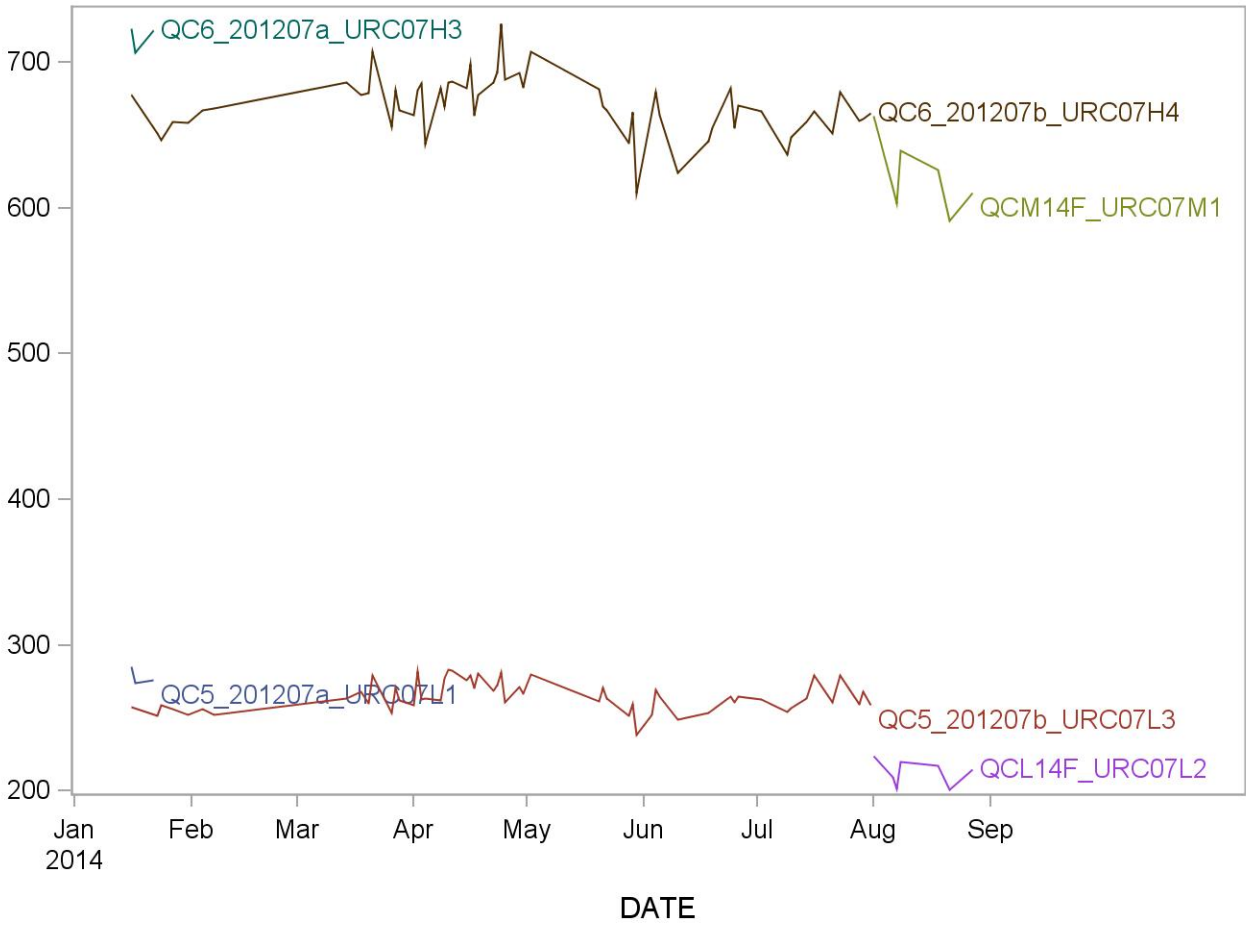
2009-2010 Summary Statistics and QC Chart for 1-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URC06H3	4	16JAN14	22JAN14	728.00	9.93	1.4
QC6_201207b_URC06H4	79	16JAN14	31JUL14	687.87	23.01	3.3
QC5_201207a_URC06L1	4	16JAN14	22JAN14	291.38	3.38	1.2
QC5_201207b_URC06L3	79	16JAN14	31JUL14	279.09	10.57	3.8
QCL14F_URC06L2	4	01AUG14	27AUG14	216.50	13.15	6.1
QCM14F_URC06M1	4	01AUG14	27AUG14	638.88	41.98	6.6



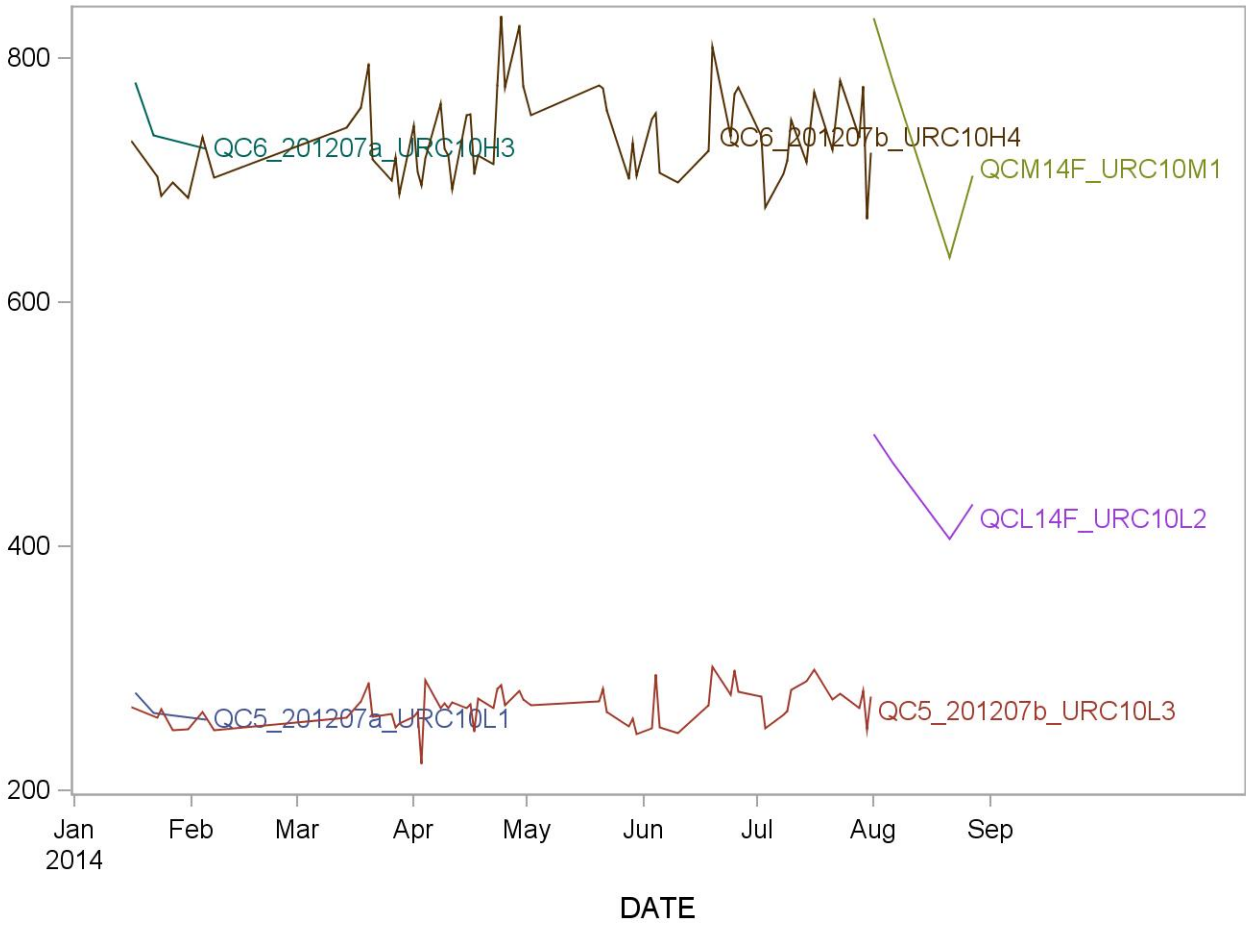
2009-2010 Summary Statistics and QC Chart for 2-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URC07H3	4	16JAN14	22JAN14	718.25	7.88	1.1
QC6_201207b_URC07H4	76	16JAN14	31JUL14	669.76	22.02	3.3
QC5_201207a_URC07L1	4	16JAN14	22JAN14	277.88	5.98	2.2
QC5_201207b_URC07L3	76	16JAN14	31JUL14	264.05	10.85	4.1
QCL14F_URC07L2	7	01AUG14	27AUG14	212.50	9.05	4.3
QCM14F_URC07M1	7	01AUG14	27AUG14	621.14	24.12	3.9



2009-2010 Summary Statistics and QC Chart for 1-pyrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207b_URC10H4	80	16JAN14	31JUL14	737.85	42.66	5.8
QC5_201207b_URC10L3	80	16JAN14	31JUL14	269.15	16.79	6.2
QC6_201207a_URC10H3	4	17JAN14	05FEB14	745.25	34.30	4.6
QC5_201207a_URC10L1	4	17JAN14	05FEB14	266.63	16.36	6.1
QCL14F_URC10L2	4	01AUG14	27AUG14	450.63	37.60	8.3
QCM14F_URC10M1	4	01AUG14	27AUG14	739.13	86.28	11.7

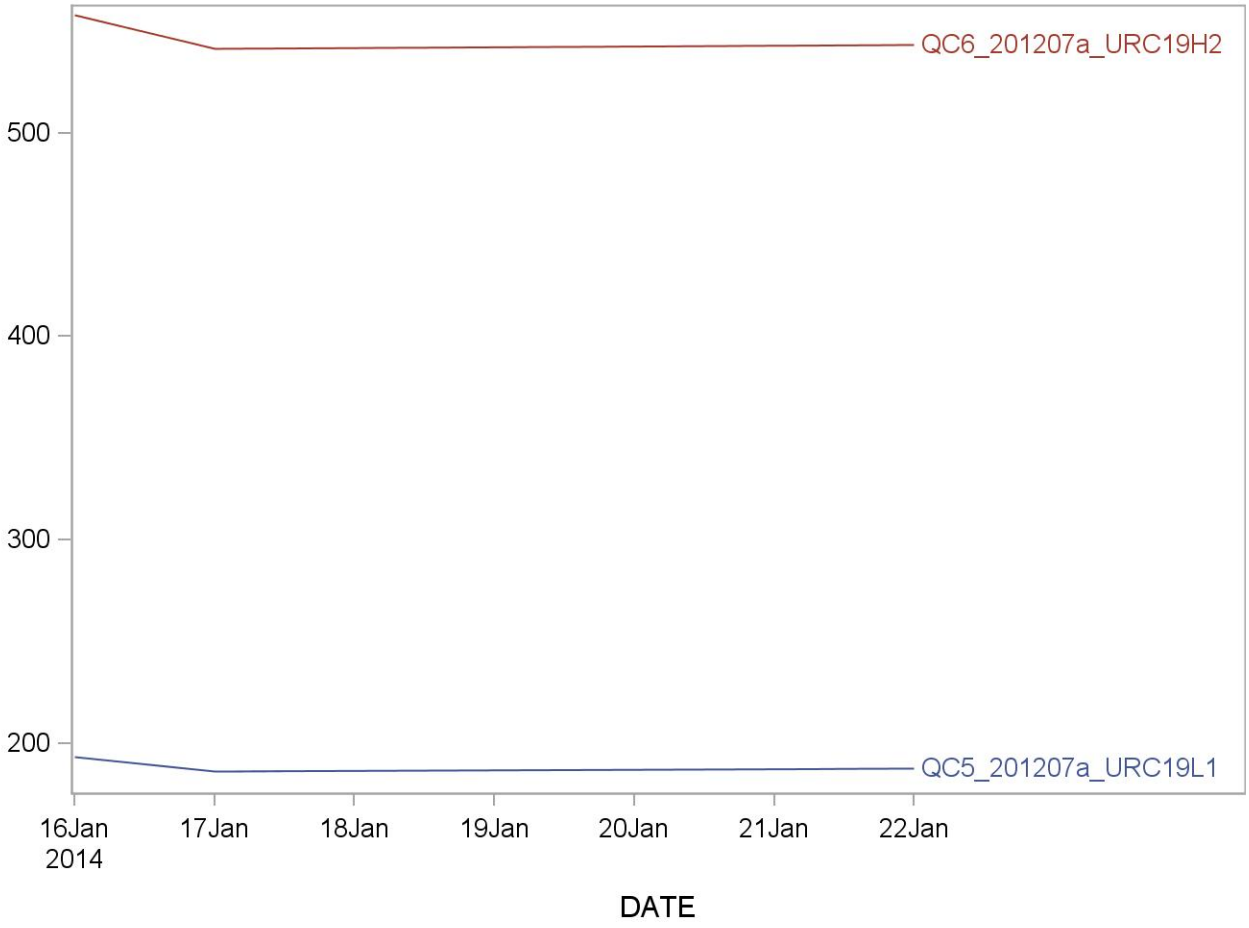


2009-2010 Summary Statistics and QC Chart for 9-fluorene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URC17H3	4	16JAN14	22JAN14	672.75	43.08	6.4
QC6_201207b_URC17H4	80	16JAN14	31JUL14	646.03	33.69	5.2
QC5_201207a_URC17L1	4	16JAN14	22JAN14	405.63	20.29	5.0
QC5_201207b_URC17L3	80	16JAN14	31JUL14	387.01	16.89	4.4
QCL14F_URC17L2	5	01AUG14	27AUG14	207.10	8.50	4.1
QCM14F_URC17M1	5	01AUG14	27AUG14	560.60	46.65	8.3

2009-2010 Summary Statistics and QC Chart for 4-phenanthrene

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC6_201207a_URC19H2	4	16JAN14	22JAN14	546.63	7.91	1.4
QC5_201207a_URC19L1	4	16JAN14	22JAN14	188.50	3.11	1.6



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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

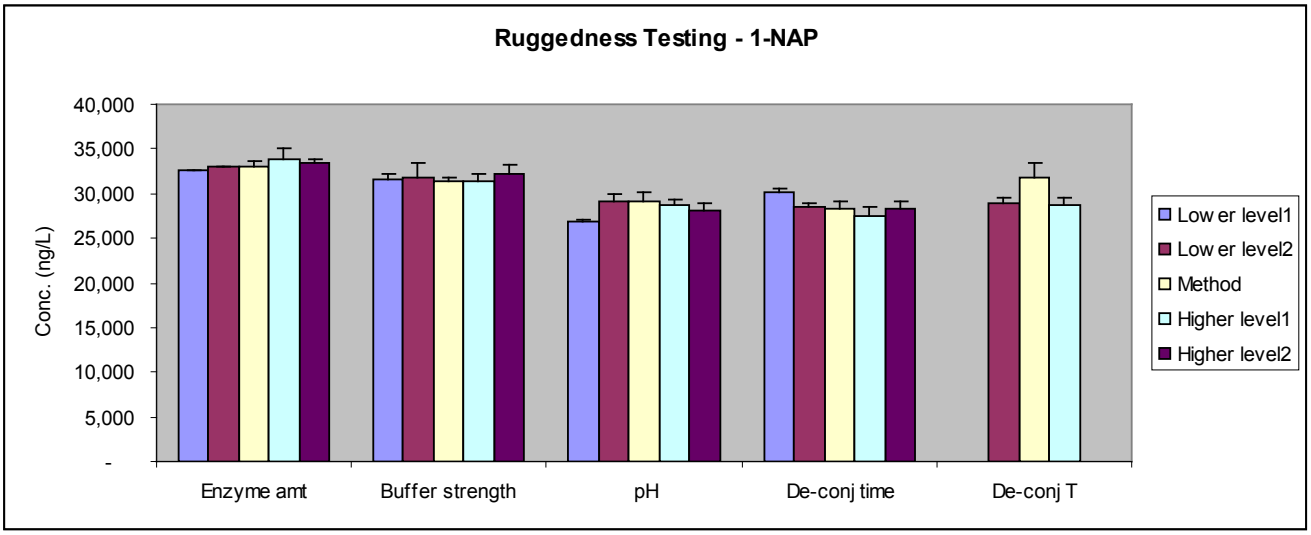
Buffer strength (M)		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		average concentration (pg/mL)									
Lower level1	0.5M	31599	35592	5706	1732	3087	492	1851	1735	1363	1221
Lower level2	0.9M	31708	36069	5684	1746	3028	452	1860	1711	1369	1231
Method	1M	31289	35629	5764	1753	3070	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

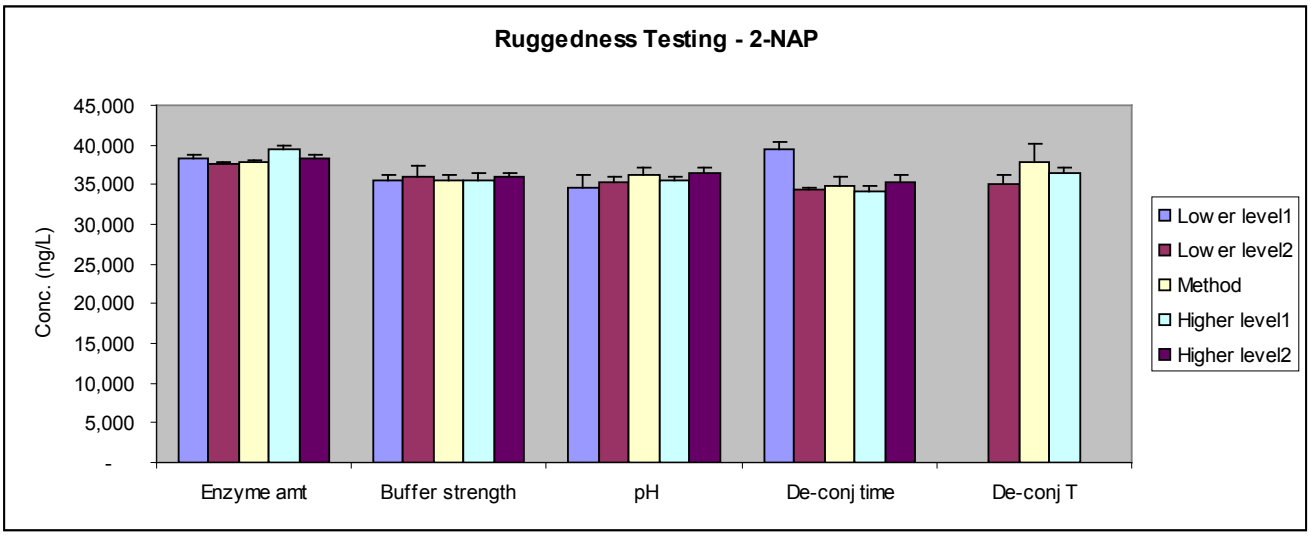
Buffer pH		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		Average concentration (pg/mL)									
Lower level1	pH4.5	26966	34660	5554	1774	2943	442	1878	1699	1374	1188
Lower level2	pH5.3	29123	35246	5757	1746	3061	401	1837	1671	1364	1290
Method	pH5.5	29221	36267	5773	1750	3101	467	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

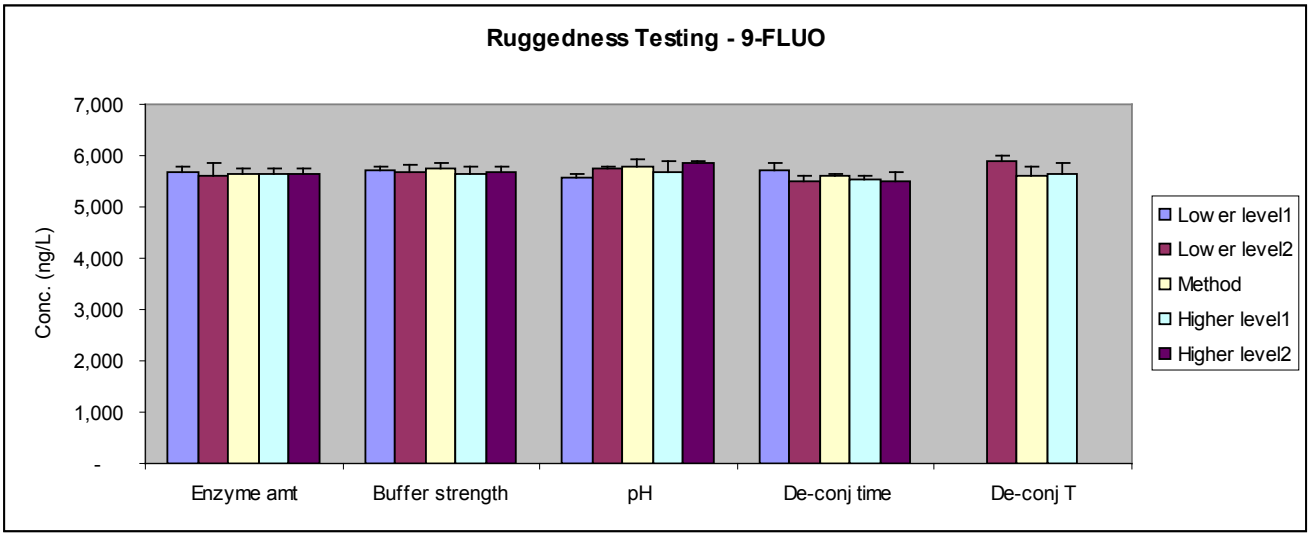
De-conjugation time (hours)		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		Average concentration (pg/mL)									
Lower level1	4 hr	30121	39503	5721	1766	3111	478	1846	1690	1377	1246
Lower level2	17 hr	28590	34326	5516	1740	2982	429	1741	1631	1257	1226
Method	18 hr	28293	34936	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

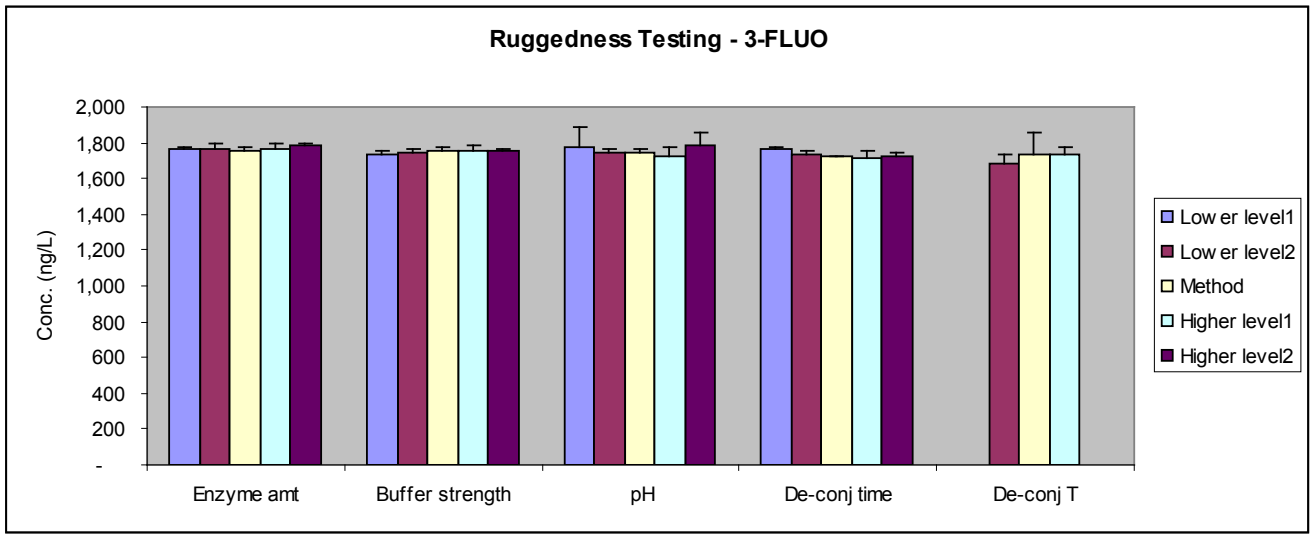
De-conjugation temperature (ºC)		1-NAP	2-NAP	9-FLUO	3-FLUO	2-FLUO	4-PHEN	3-PHEN	1-PHEN	2-PHEN	1-PYR
		Average concentration (pg/mL)									
Lower level	32ºC	28842	35031	5882	1681	3065	495	1752	1640	1355	1223
Method	37ºC	31709	37941	5601	1738	2985	416	1727	1568	1273	1196
Higher level	42ºC	28793	36394	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

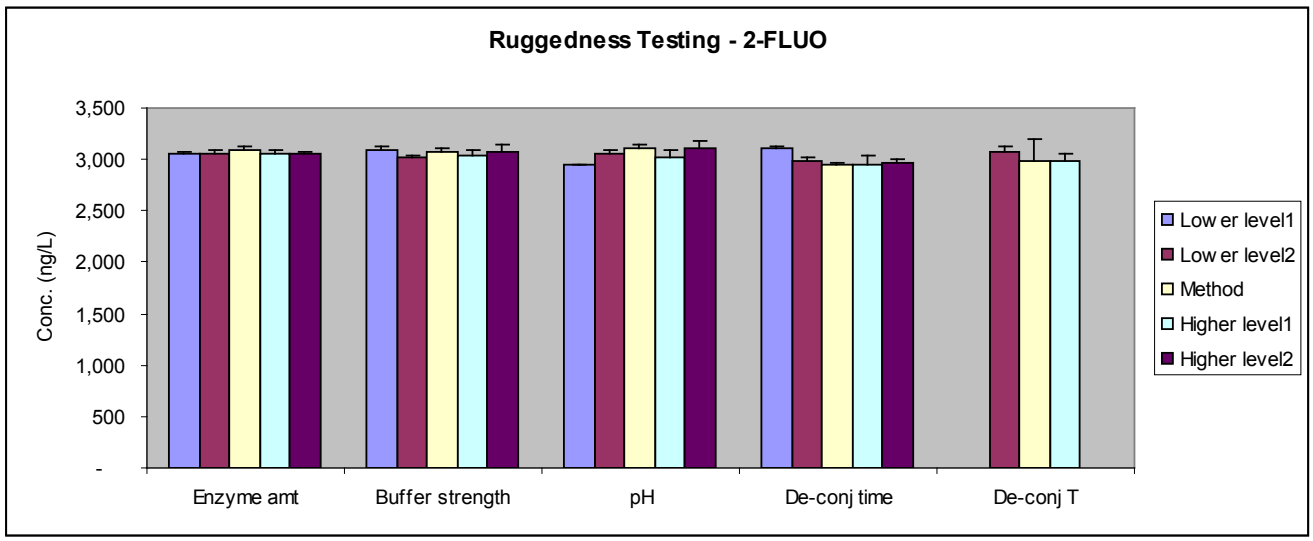
Results in Graphs

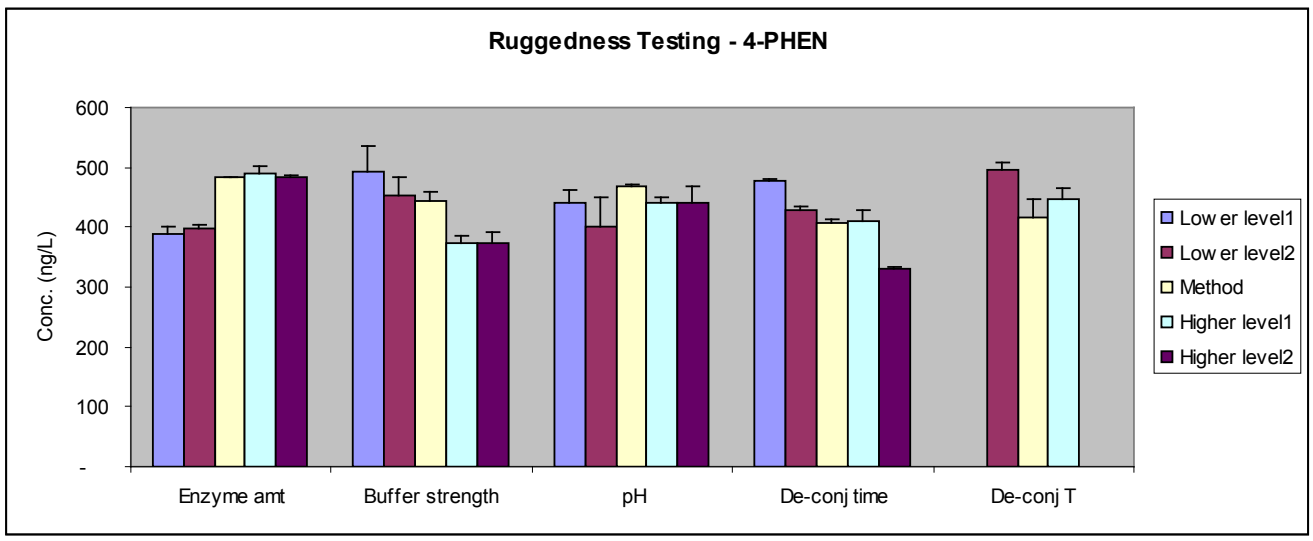


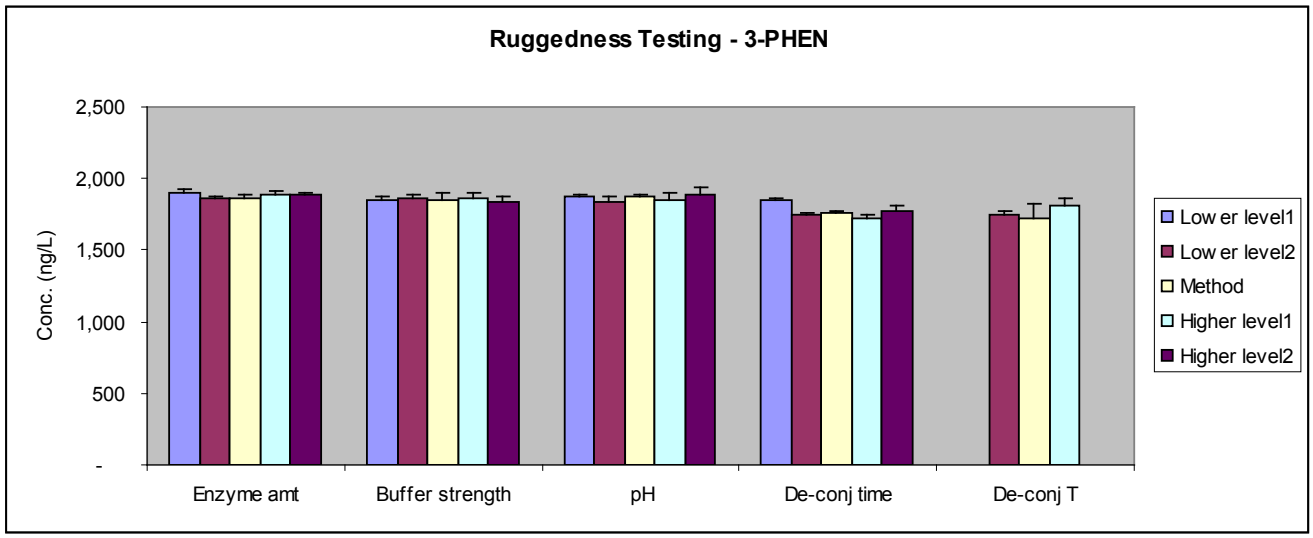


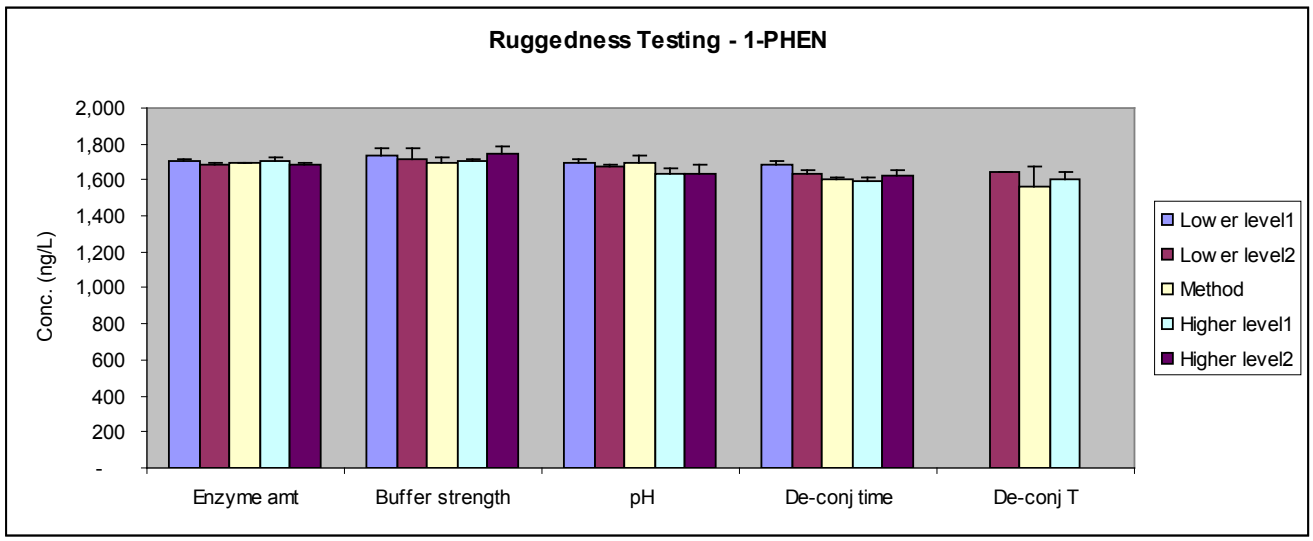


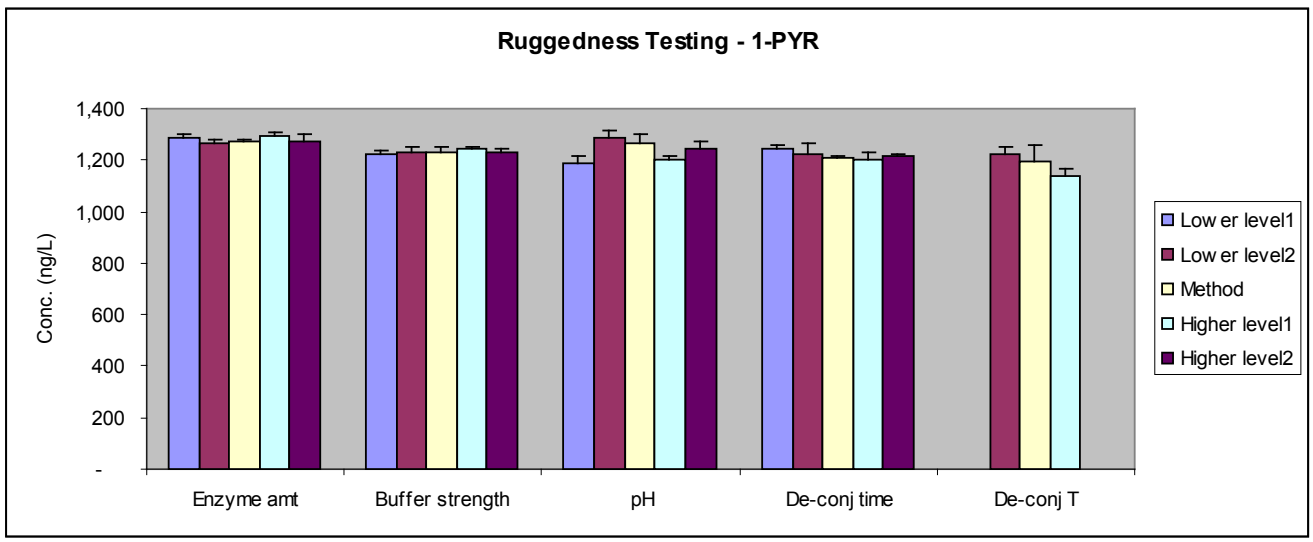

Ruggedness Testing - 3-FLUO
Conc. (ng/L)
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
-
Enzyme amt
Buffer strength
pH
De-conj time
De-conj T
Low er level1
Low er level2
Method
Higher level1
Higher level2


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

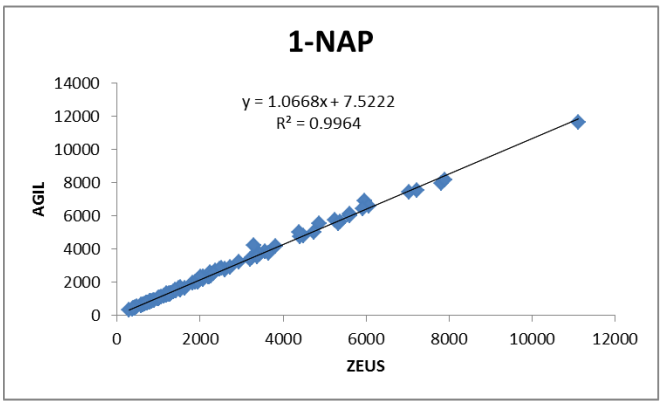
% difference compared to urine-spiked curve

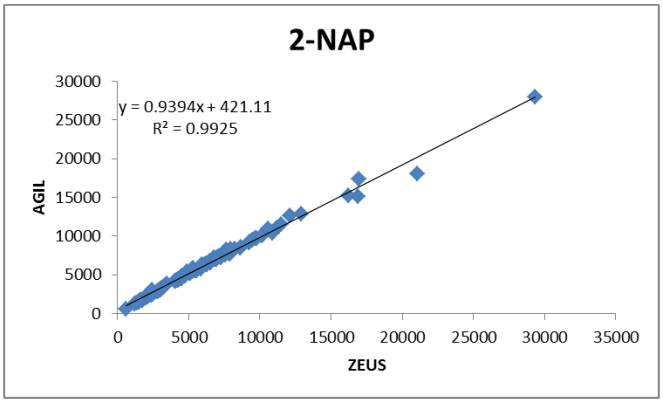
Solvent	3.5%	0.5%	1.4%	1.5%	1.5%	3.7%	-0.3%	0.6%	0.4%	3.5%
Water	1.4%	0.6%	-0.4%	-0.1%	0.4%	4.2%	-0.1%	-0.7%	0.2%	-0.6%
Syn-urine	3.2%	0.7%	-0.1%	0.2%	0.2%	3.7%	0.3%	-0.9%	0.4%	-0.7%
Urine	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

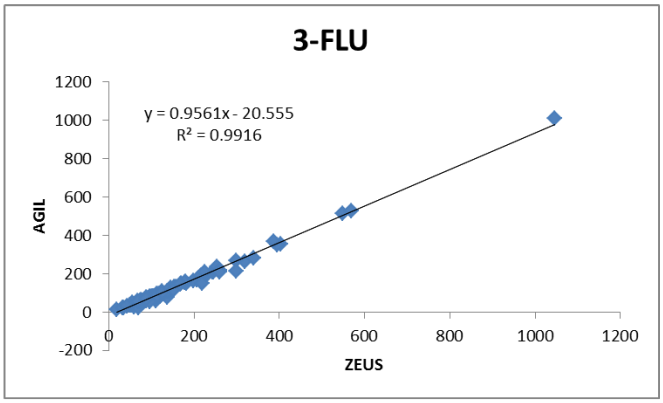
Appendix D – Method Comparison Graphs

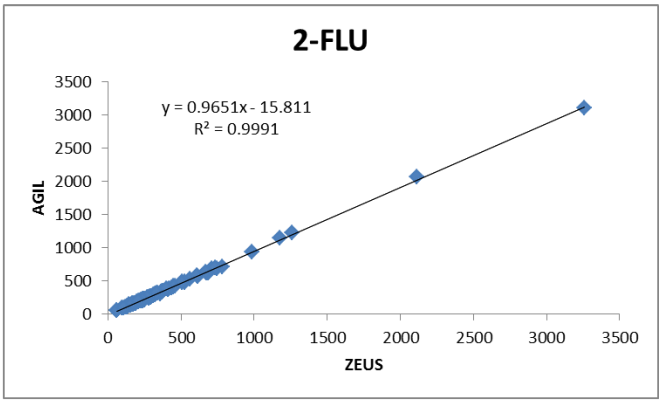
Method comparison was performed by running 100 clinical samples using the previous method by Thermo Quantum GC/MS/MS (Method #6703.03) and the updated method (Method #6703.04) by Agilent GC/QQQ 7000 GC/MS/MS. The results are evaluated by both the linear regression plots between the Thermo ("ZEUS") and 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.

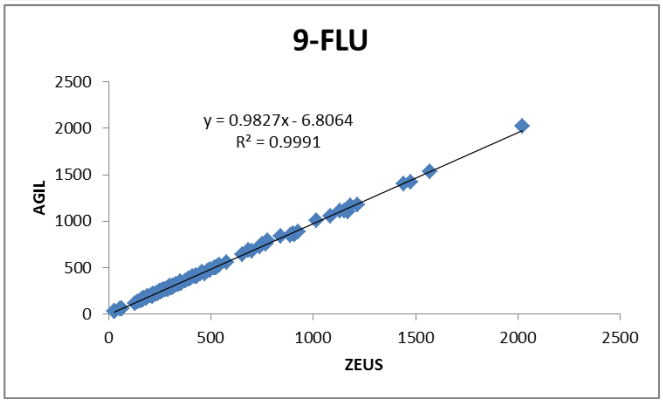
Linear regression plots: New method "AGIL" vs. old method "ZEUS"

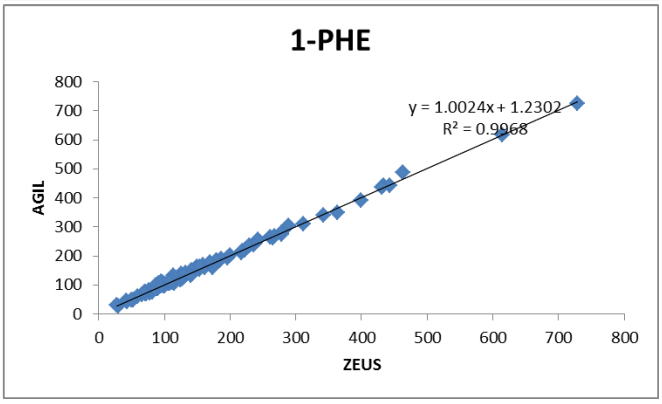


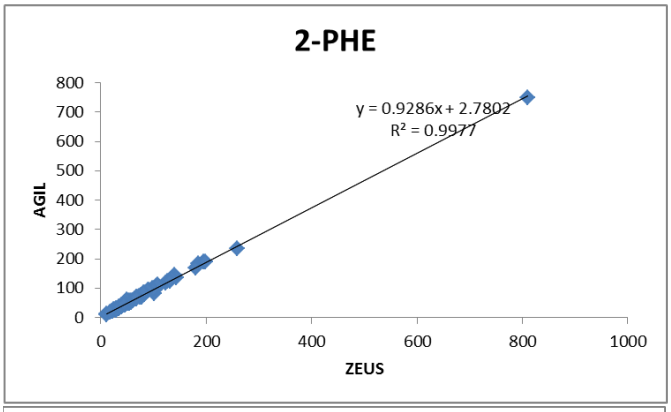


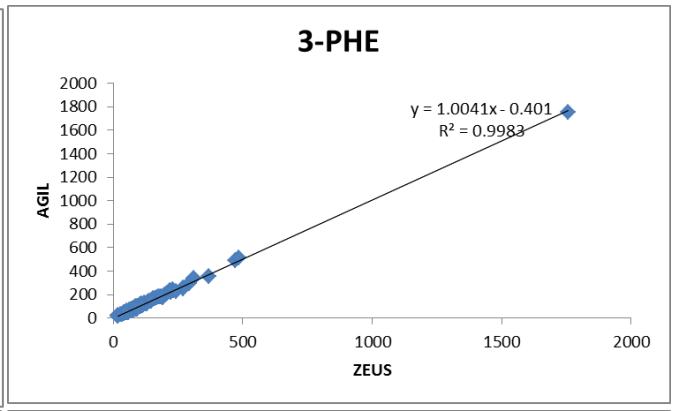


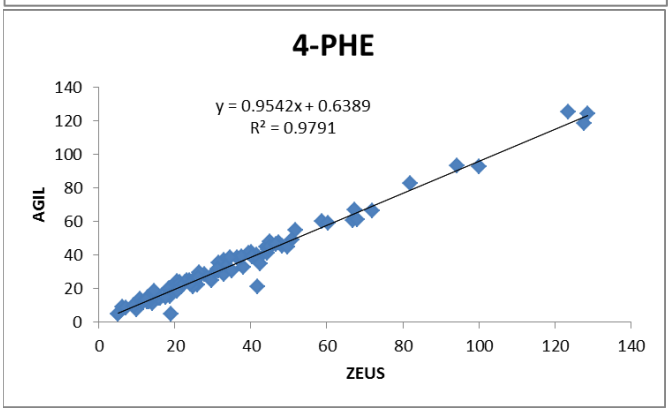


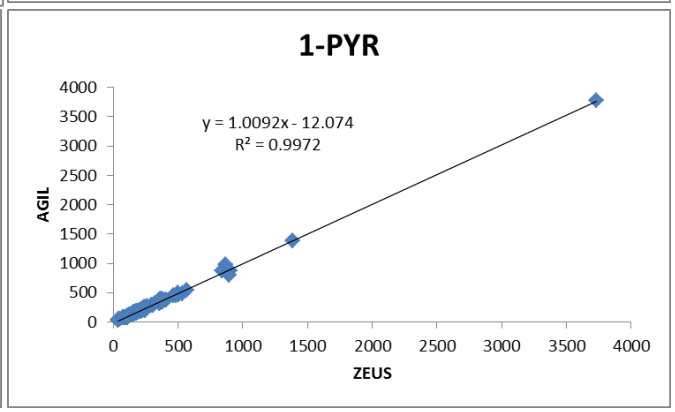




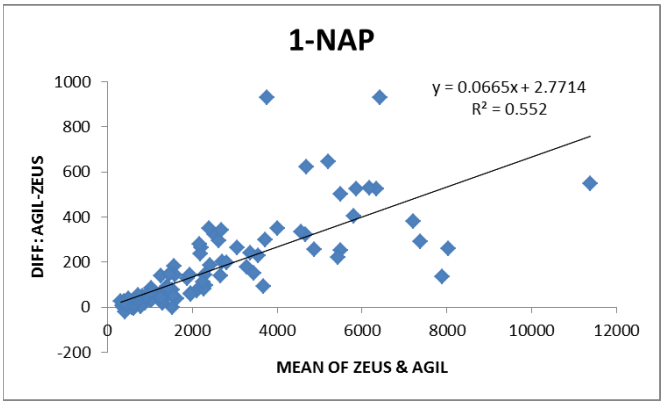


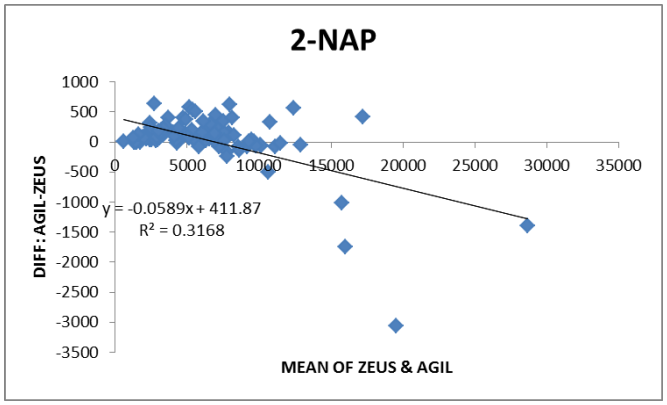


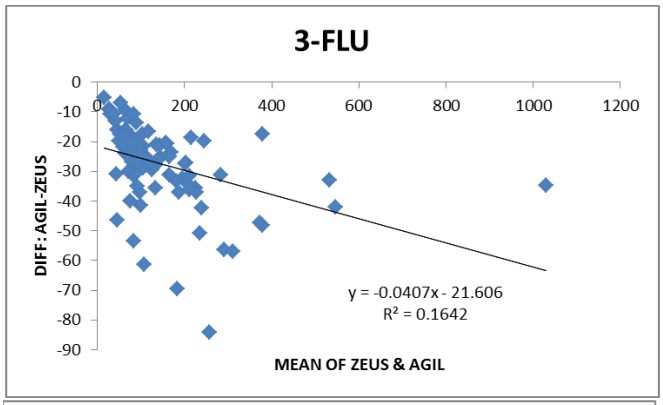


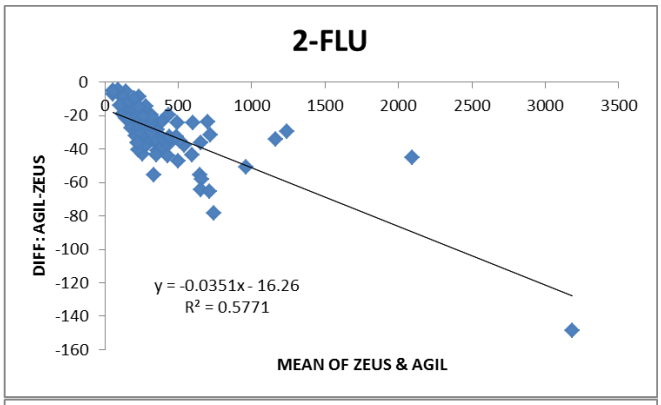


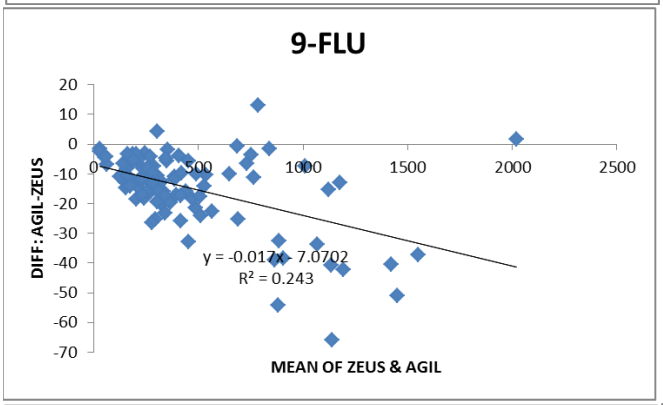
Bland-Altman plots: Difference (AGIL-ZEUS) vs. Mean of AGIL and ZEUS

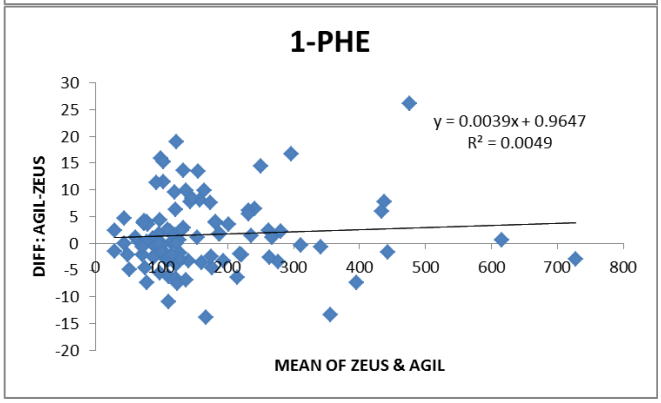


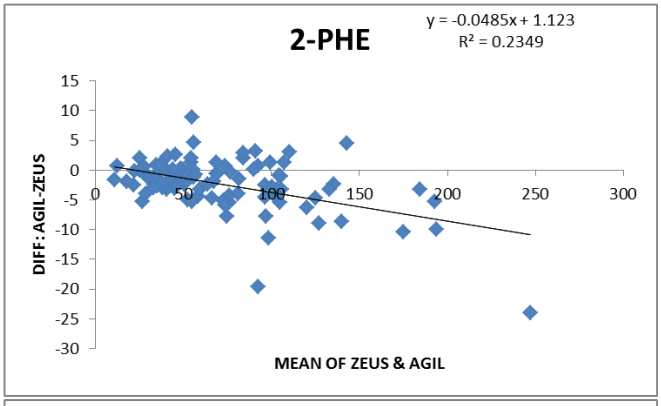


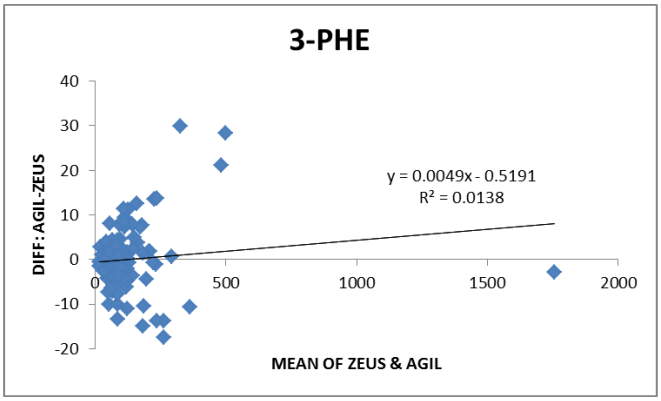

3-FLU
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.0407x - 21.606
R² = 0.1642

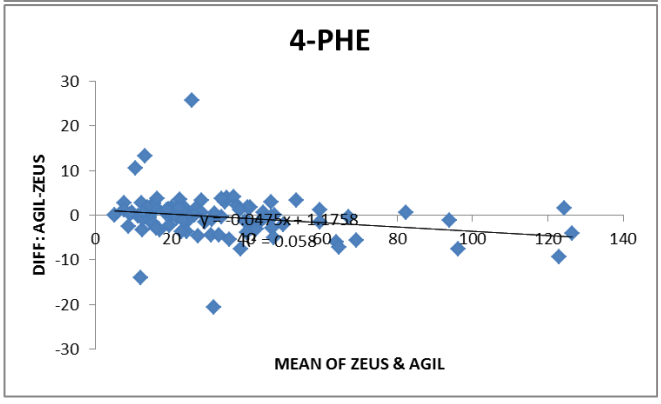

2-FLU
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.0351x - 16.26
R² = 0.5771

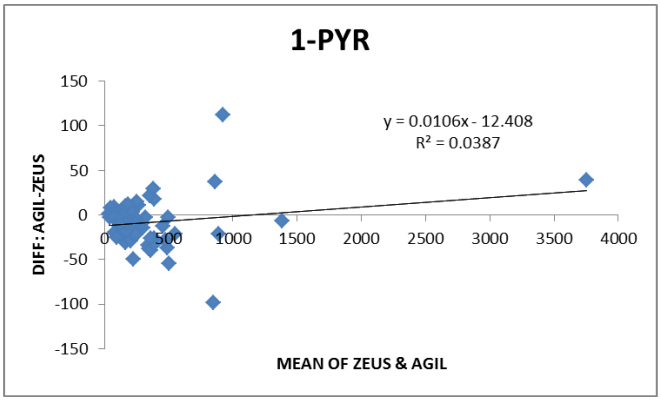

9-FLU
DIFF: AGIL-ZEUS
MEAN OF ZEUS & AGIL
y = -0.017x - 7.0702
R² = 0.243


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


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

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: **09-OD**

Revised: **10/03/2006**

as performed by:

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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.

OH-PAH in Urine
NHANES 2003-2004

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

Data File Name	Variable Name	SAS Label
l31pah_c	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
	URXP19	4-phenanthrene

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). 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 (2, 3). 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) (4, 5). 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-FLUO
4	Fluorene	2-hydroxyfluorene	2-FLUO
5	Fluorene	3-hydroxyfluorene	3-FLUO
6	Phenanthrene	1-hydroxyphenanthrene	1-PHEN
7	Phenanthrene	2-hydroxyphenanthrene	2-PHEN
8	Phenanthrene	3-hydroxyphenanthrene	3-PHEN
9	Phenanthrene	4-hydroxyphenanthrene	4-PHEN
11	Fluoranthene	3-hydroxyfluoranthene	3-FLRAN

12	Pyrene	1-hydroxypyrene	1-PYR
No.	**Parent PAH**	**Metabolite/Analyte**	**Abbreviation**
13	Benzo[c]phenanthrene	1-hydroxybenzo[c]phenanthrene	1-BCP
14	Benzo[c]phenanthrene	2-hydroxybenzo[c]phenanthrene	2-BCP
15	Benzo[c]phenanthrene	3-hydroxybenzo[c]phenanthrene	3-BCP
16	Benz[a]anthracene	1-hydroxybenz[a]anthracene	1-BAA
17	Benz[a]anthracene	3-hydroxybenz[a]anthracene	3-BAA
18	Benz[a]anthracene	9-hydroxybenz[a]anthracene	9-BAA
19	Chrysene	1-hydroxychrysene	1-CHRY
20	Chrysene	2-hydroxychrysene	2-CHRY
21	Chrysene	3-hydroxychrysene	3-CHRY
22	Chrysene	4-hydroxychrysene	4-CHRY
23	Chrysene	6-hydroxychrysene	6-CHRY
24	Benzo[a]pyrene	3-hydroxybenzo[a]pyrene	3-BAP
25	Benzo[a]pyrene	7-hydroxybenzo[a]pyrene	7-BAP

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. Laboratorians 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 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

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
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
$^{13}C_6$ 1-NAP	Synthesized in house, CDC
$^{13}C_6$ 1-PYR, $^{13}C_6$ 3-FLRAN,	TerraChem, Merriam, KS
$^{13}C_6$ 3-PHEN, $^{13}C_6$ 6-CHRY, $^{13}C_6$ 1-BAA, $^{13}C_6$ 3-BAP, $^{13}C_6$ 4-OH-PCB79	Cambridge Isotope Laboratories, Andover, MA
Reagent	**Manufacturers***
$^{13}C_6$ 2-FLUO, $^{13}C_6$ 3-BCP, $^{13}C_6$ 3-CHRY	ChemSyn, Lenexa, KS
$^{13}C_6$ 3-FLUO, $^{13}C_6$ 9-FLUO, $^{13}C_6$ 2-PHEN	Los Alamos National Laboratory, Los Alamos, NM
1-NAP, 2-NAP, 2-FLUO, 3-FLUO, 9-FLUO, 1-PYR	Sigma-Aldrich Chemicals, St. Louis, MO

1-PHEN, 2-PHEN, 3-PHEN, 4-PHEN	Promochem, Wesel, Germany
3-FLRAN, 1-BCP, 2-BCP, 3-BCP, 1-BAA, 3-BAA, 9-BAA, 1-CHRY, 2-CHRY, 3-CHRY, 4-CHRY, 6-CHRY, 3-BAP, 7-BAP	Midwest Research Institute, NCI, Kansas City, MO

* Equivalent products from other manufacturers may be used.

b. Preparation of Reagents

1) 1 mol/L Sodium Acetate Buffer

Place 41 g sodium acetate in a 500-mL volumetric flask and add about 300 mL de-ionized water (D.I. H_2O). Swirl solution to dissolve sodium acetate. Fill flask to the 500 mL line with D.I. H_2O. Adjust the pH to 5.5 with glacial acetic acid.

2) 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 microreaction vessel 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 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

1) Stock Solutions of Individual Analytes (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 -70 ºC freezer with an inert argon atmosphere in the vials.

2) Working Standard Solution of 24 native OH-PAH mix (W.S.A)

Combine 400 μL from each of the 24 native OH-PAH standard stock solutions in a silanized screw cap amber vial to generate the working standard solution (W.S.A, 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 at -70 ºC until needed.

3) Working Standard Solution of 13 C13-labeled OH-PAH mix (W.S.I)

Combine 3.76 mL of acetonitrile and 480 μL of each of the 13 ^{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, 4 ng/μL for each of the 13 ^{13}C-labeled OH-PAHs). Homogenize the mixture by gentle swirling and vortexing. Displace air in the vial with argon, cap the vials, and stored them at -70 ºC 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. Concentrations for ^{13}C-labeled internal standards are 100 pg/μL in all calibration standards.

Table 3. Preparation of external calibration standards (ECS)

Standard No.	Analytes, excluding 1- & 2-NAP		1- and 2- NAP	
	Concentration (pg/μL)	Equivalent concentration in urine(pg/mL)	Concentration (pg/μL)	Equivalent concentration in urine (pg/mL)
0	0.5	5	2	20
1	1	10	4	40
2	2	20	8	80
3	5	50	20	200
Standard No.	**Analytes, excluding 1- & 2-NAP**		**1- and 2- NAP**	
	Concentration (pg/μL)	**Equivalent concentration in urine (pg/mL)**	**Concentration (pg/μL)**	**Equivalent concentration in urine (pg/mL)**
4	10	100	40	400
5	50	500	200	2,000
6	100	1,000	400	4,000
7	500	5,000	2,000	20,000
8	1000	10,000	4,000	40,000
9	2000	20,000	8,000	80,000

10	4000	40,000	16,000	160,000

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

Measure 5.0 mL of W.S.I into a 200-mL silanized volumetric flask. Dilute the solution with toluene to the 200-mL line to get the internal qualification standard (I.Q.S). The concentration of each ^{13}C-labeled OH-PAH is 100 pg/μL. Aliquot 1.7 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 (3 mL in each tube) and store them at -70 ± 10 °C until use.

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

Approximately 3 mg of neat $^{13}C_6$ 4-OH-PCB79 is weighed into a silanized screw cap amber vial. Add 3 mL of toluene into the vial and record the exact weight of the solvent. Allow the standard to dissolve by gentle swirling or placing in an ultrasonic bath. Methylate the standard using dioazomethane to obtain its methylated derivative $^{13}C_6$ 4-MeH-PCB79. Dilute the methylated standard solution using toluene to 200 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 the 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 (3 mL in each tube) 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 J&W DB-5 30-m fused silica capillary column. The

column ID is 0.25-mm and the film thickness is 0.25-micron (J&W, # 22-5022 or equivalent). The temperature program lasts a total of 23 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. GC Temperature Program

Time (min.)	Temperature (ºC)
0	95
2	95
6	160
Time (min.)	**Temperature (ºC)**
20	295
21	320
25	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 °C
Conversion dynode voltage	Positive
Electron multiplier voltage	1.45 – 2.25 kV (or 10^6 gain)

Table 7. Analyte Masses

Analyte	Molecular Ion [$M^{\cdot+}$]	Confirmation Ion [$M^{\cdot+}$-15]
1-NAP, 2-NAP	216.0970	201
2-FLUO, 3-FLUO, 9-FLUO	254.1127	239
1-PHEN, 2-PHEN, 3-PHEN, 4-PHEN	266.1127	251
3-FLRAN, 1-PYR	290.1127	275
3-BAP	340.1283	325
Analyte	**Molecular Ion [$M^{\cdot+}$]**	**Confirmation Ion [$M^{\cdot+}$-15]**
1-BCP, 2-BCP, 3-BCP, 1-BAA, 3-BAA, 9-BAA, 1-CHRY, 2-CHRY, 3-CHRY, 4-CHRY, 6-CHRY	316.1283	301
$^{13}C_6$ 1-NAP	222.1172	207
$^{13}C_6$ 2-FLUO, $^{13}C_6$ 3-FLUO, $^{13}C_6$ 9-FLUO	260.1328	245
$^{13}C_6$ 3-PHEN, $^{13}C_6$ 2-PHEN	272.1328	257
$^{13}C_6$ 3-FLRAN, $^{13}C_6$ 1-PYR	272.1328	257

$^{13}C_6$ 3-CHRY, $^{13}C_6$ 6-CHRY, $^{13}C_6$ 1-BAA, $^{13}C_6$ 3-BCP	296.1328	281

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 calibration curve, using eight ECS with concentration ranging from 1 to 1000 pg/μL, is generated using the ratio of the peak area of the analyte to the labeled internal standard. The concentrations in ECS correspond to 10 - 10,000 pg/mL (40-40,000 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, three E.C.S from a different batch are analyzed every six months. 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 were 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 10 μL of I.Q.S, 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. Turn the test tube gently several times to mix well. Place the caps on the tube and incubate the sample at 37 ºC overnight.

2) Automated Liquid-Liquid Extraction

Add D.I. water (2 mL) to the sample, and extract the sample twice with pentane using the Gilson 215 liquid handler (Gilson Inc., Middleton, WI) for automation. Place the sample in the automix tray and initiate the extraction procedure. The automated procedure starts with adding pentane (5 mL) to the sample and mixing the sample by rotating it in the automix for 5 minutes (20 rpm). Then the procedure is paused and the sample is centrifuged at 2800 rpm on an AllegraTM 6 centrifuge (Beckman Coulter Inc., Fullerton, CA) for 20 minutes. After returning the samples to the automix tray, resume the procedure and a probe transfers the organic phase to clean collection tubes. This extraction procedure is repeated again with additional pentane (5 mL).

3) Evaporation

Sample extract is spiked with 5 μL dodecane and placed in a TurboVap LV evaporator. Under a stream of N_2 and in a 40 ºC water bath, the sample is evaporated to ~5 μL within 10-15 minutes. Spike 20 μL toluene and 5 μL R.S.S into the sample tube, vortex the sample, and transfer the contents to clear 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 ºC for 30 minutes. The samples are then ready for analysis on the mass spectrometer. The samples may also be stored in a refrigerator for up to 1 week.

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	NHANES0304.met
GC	pah_deriv.mcr
MID	pah_deriv.mid
Autosampler	pah_1ul.mcs
Program	**Method**
ICL	Sleep90.icl 5
Process	NHANES0304.pmd

Filenames conform to the following format: PMYYNNN or KMYYNNN 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; 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 "NHANES0304.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 an RBase database located on the Q:\ share drive.

3-BAA and 9-BAA co-elute in the GC chromatograms, so they are reported together as 3_9-BAA. 3-FLRAN had an interfering peak in the GC chromatograms; therefore, 3-FLRAN is not reported.

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-NAPH and 2-NAPH is 5 pg/mL to 40,000 pg/mL. Calibration curves for 1-NAPH and 2-NAPH are extended to 160,000 ng/L, because their high concentrations detected from unknown samples. Therefore, the linear range for 1-NAPH and 2-NAPH 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 higher 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 (0.5 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 3. The detection limits determined for the analytes are presented in Table 9.

Table 9. Limits of Detection (LODs)

Analyte	LOD (pg/mL)
1-NAP	18
2-NAP	12
9-FLUO	3.9
2-FLUO	4.5
3-FLUO	6.9
1-PHEN	2.6
2-PHEN	3.8
3-PHEN	2.6
4-PHEN	3.7

1-PYR	4.9
1-BCP	2.8
1-BAA	2.6
3_9-BAA	5.2
1-CHRY	2.6
2-CHRY	2.6
3-CHRY	2.6
4-CHRY	2.6
6-CHRY	2.6
3-BAP	2.6
7-BAP	2.6

c. Precision

The precision of the method is reflected in the variance of quality control samples analyzed over time. The coefficients of variation (CV) of the method over analyses of six batches of QC samples (N=42) are listed in Table 10 below. These QC samples were prepared over 3 weeks by four different analysts using two automated liquid handlers and two GC/HRMS instruments.

Table 10. Mean Concentration and CV for 42 QC samples

Analyte	Mean (pptr)	CV (%)
1-NAP	1584	2.9
2-NAP	2912	3.3
9-FLUO	596	2.9
2-FLUO	559	3.0
3-FLUO	485	2.8
1-PHEN	391	4.4
2-PHEN	606	3.5
3-PHEN	533	3.2
4-PHEN	531	3.9
1-PYR	542	3.4

1-BCP	925	8.0
2-BCP	559	3.3
3-BCP	522	3.0
1-BAA	528	2.7
3_9-BAA	843	10
1-CHRY	496	11
2-CHRY	565	4.4
3-CHRY	510	3.3
4-CHRY	468	6.7
6-CHRY	509	4.5
3-BAP	686	6.6
7-BAP	772	8.5

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. The observation of confirmation ions (M-15 ions) confirms the presence of analytes of interest. 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) Establishing QC limits

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. Typical QC characterization statistics for OH-PAH analytes are listed in Table 11.

Table 11. QC Characterization Statistics

Analyte	Mean - 3σ (pptr)	Mean - 2σ (pptr)	Mean (pptr)	Mean + 2σ (pptr)	Mean + 3σ (pptr)
1-NAP	2950	3146	3536	3927	4123
2-NAP	2201	2359	2677	2995	3153
2-FLUOR	563	583	624	664	684
3-FLUOR	444	460	493	527	543
9-FLUOR	575	599	647	695	719
1-PHEN	336	357	398	439	459
2-PHEN	489	507	542	577	595
3-PHEN	492	515	560	606	628
4-PHEN	433	468	537	606	641
1-PYR	489	507	544	581	599
2-BCP	420	458	534	611	649
3-BCP	460	476	508	540	557
1-BAA	451	473	517	561	583
3-9-BAA	446	592	885	1178	1324
2-CHRY	359	399	479	559	599
3-CHRY	457	477	517	557	577

4-CHRY	399	435	506	577	613
6-CHRY	402	433	497	560	592
3-BAP	228	328	528	727	827
7-BAP	99	291	676	1060	1253

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 (6):

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

b) 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.

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

Four samples of unknown PT concentrations are analyzed twice a year using the same method described for unknown samples. Proficiency testing must be

performed a minimum of once every 6 months. 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 pipeting 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 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 for 23 OH-PAH analytes were determined from a subset of National Health and Nutritional Survey (NHANES) 2001-2002 urine samples. The reference values are presented in Table 12.

Table 12. Reference values from NHANES 2001-2002 (95% confidence interval in parentheses)

Analyte	N	Urine Concentration (ng/L)		Creatinine Adjusted Concentration (ng/g crea.)	
		Median	95th	Median	95th
1-NAP	2748	2050 (1790-2340)	22300 (18000-26600)	1910 (1700-2160)	17800 (14500-20800)
2-NAP	2748	2470 (2110-2890)	26000 (22500-29700)	2310 (1980-2300)	16700 (14100-19200)
2-FLUO	2745	318 (277-366)	2820 (2340-3450)	298 (361-340)	1890 (1590-2290)
3-FLUO	2745	134 (115-155)	1620 (1390-1900)	125 (108-144)	1060 (909-1290)
9-FLUO	2745	219 (191-251)	1090 (950-1300)	205 (183-229)	852 (736-1120)
Analyte	**N**	**Urine Concentration (ng/L)**		**Creatinine Adjusted Concentration (ng/g crea.)**	
		Median	**95th**	**Median**	**95th**
1-PHEN	2741	140 (125-158)	684 (581-763)	132 (118-147)	464 (404-539)
2-PHEN	2742	54.0 (46.0-63.5)	332 (299-377)	50.6 (43.3-59.2)	231 (206-275)
3-PHEN	2741	105 (92.5-118)	649 (542-747)	98.0 (87.4-110)	428 (360-621)
4-PHEN	2741	41.9 (31.3-56.0)	280 (250-339)	39.2 (29.6-52.0)	347 (293-411)
1-PYR	2747	49.6 (43.4-56.7)	349 (290-400)	46.4 (40.9-52.8)	243 (206-269)
1-BCP	2732	<LOD	34.0 (16.0-54.0)	<LOD	41.3 (21.0-70.8)
2-BCP	2748	<LOD	13.0 (6.00-20.0)	<LOD	20.3 (15.9-23.3)
3-BCP	2748	<LOD	11.0 (8.00-16.0)	<LOD	15.9 (14.9-16.7)
1-BAA	2748	<LOD	30.0 (22.0-36.0)	<LOD	27.0 (19.6-32.4)
3_9-BAA	2748	<LOD	24.0 (19.0-31.0)	<LOD	31.8 (28.4-35.5)
1-CHRY	2748	<LOD	105 (85.0-138)	<LOD	89.2 (71.4-104)
2-CHRY	2748	<LOD	31.0 (14.0-45.0)	<LOD	27.8 (20.9-35.0)

3-CHRY	2748	<LOD	42.0 (36.0-46.0)	<LOD	56.8 (47.4-62.5)
4-CHRY	2748	<LOD	<LOD	<LOD	<LOD
6-CHRY	2736	<LOD	77.0 (57.0-101)	<LOD	61.5 (47.7-76.4)
3-BAP	2748	<LOD	179 (139-251)	<LOD	184 (147-248)

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 ºC 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.

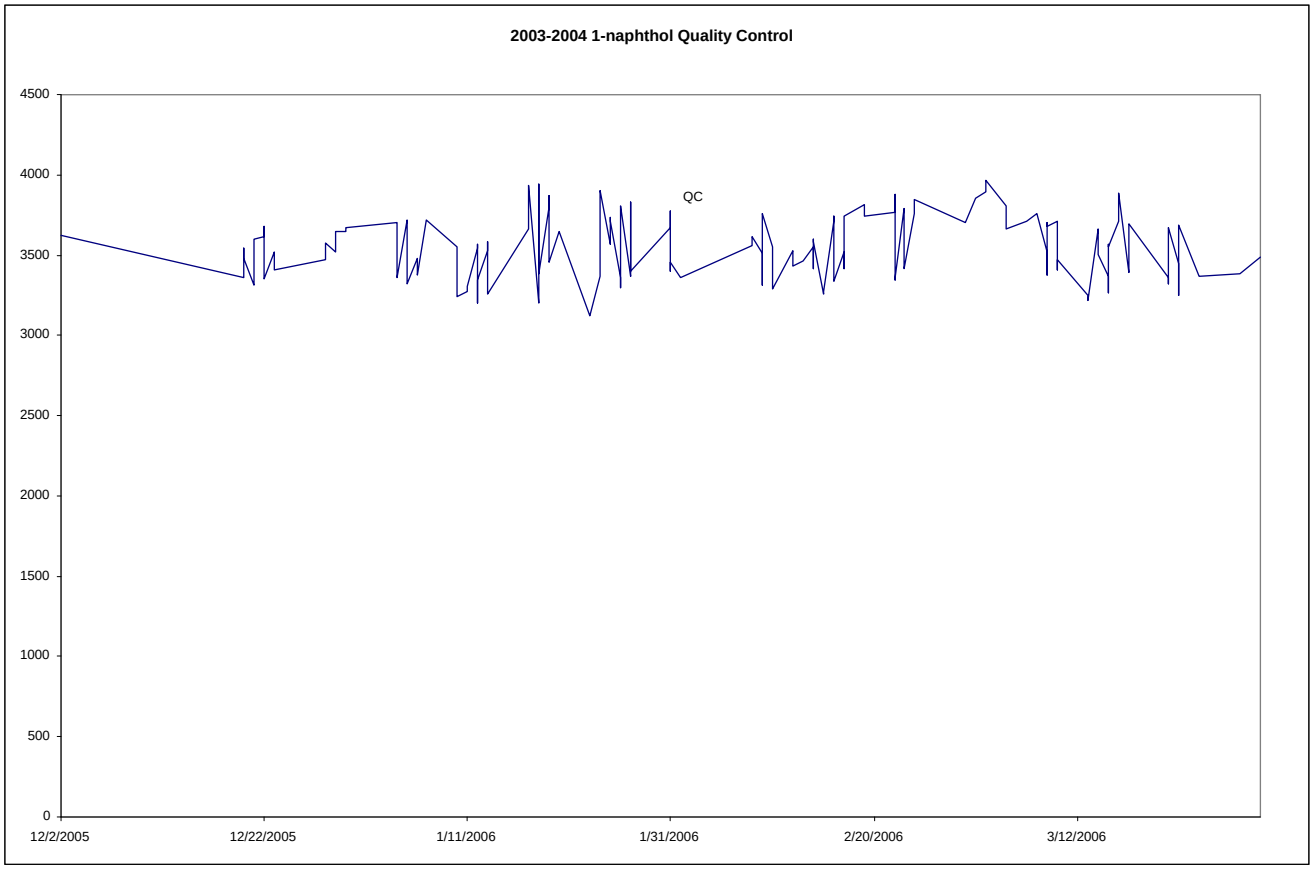
Standard record keeping (e.g., database, notebooks, and data files) is used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Transfer is carried out through the DLS Samples Logistic Group. Specimens may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

19. Summary Statistics and QC Graphs

A. 1-naphthol

Summary Statistics for 1-naphthol by Lot

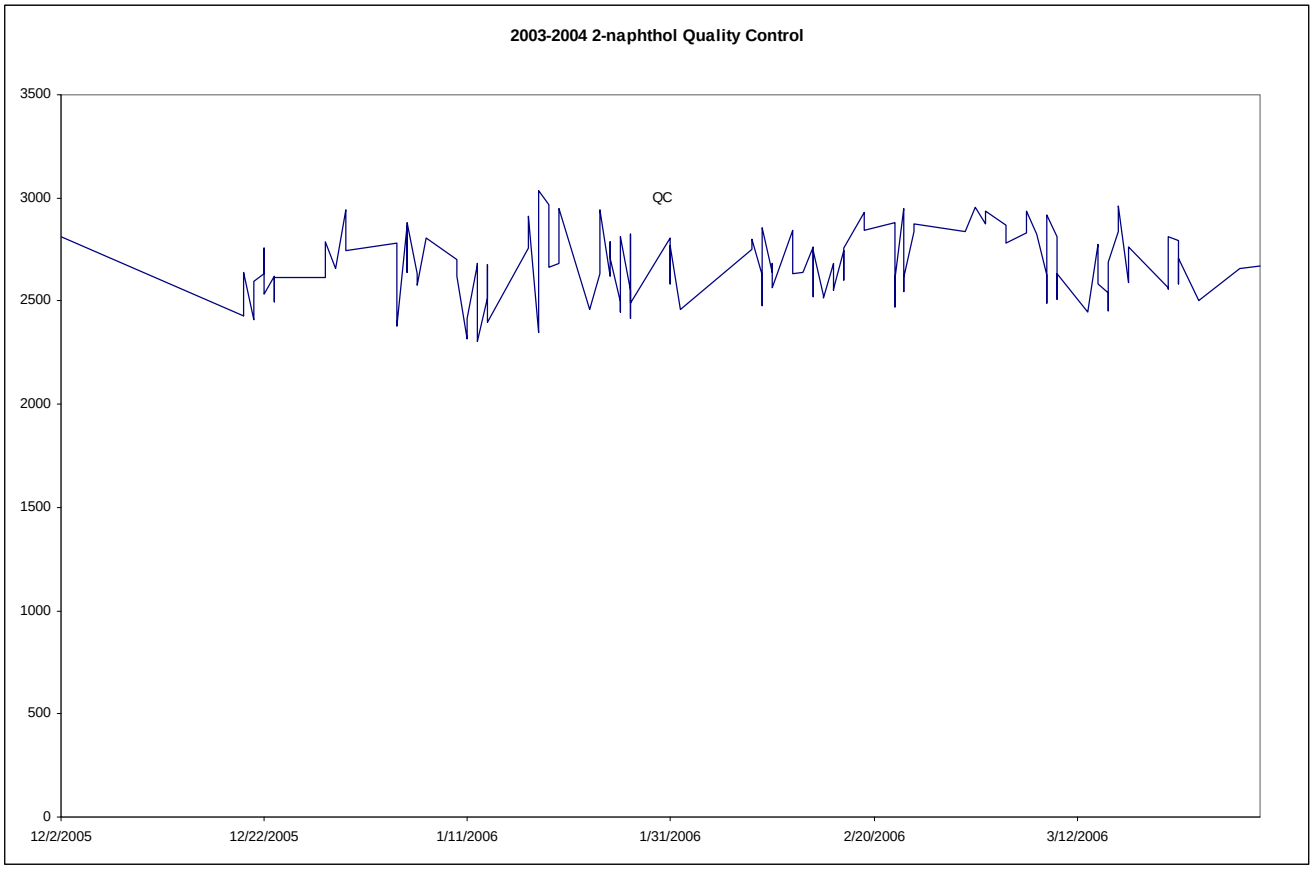
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	157	12/2/2005	3/30/2006	3540.24	188.83	5.3



B. 2-naphthol

Summary Statistics for 2-naphthol by Lot

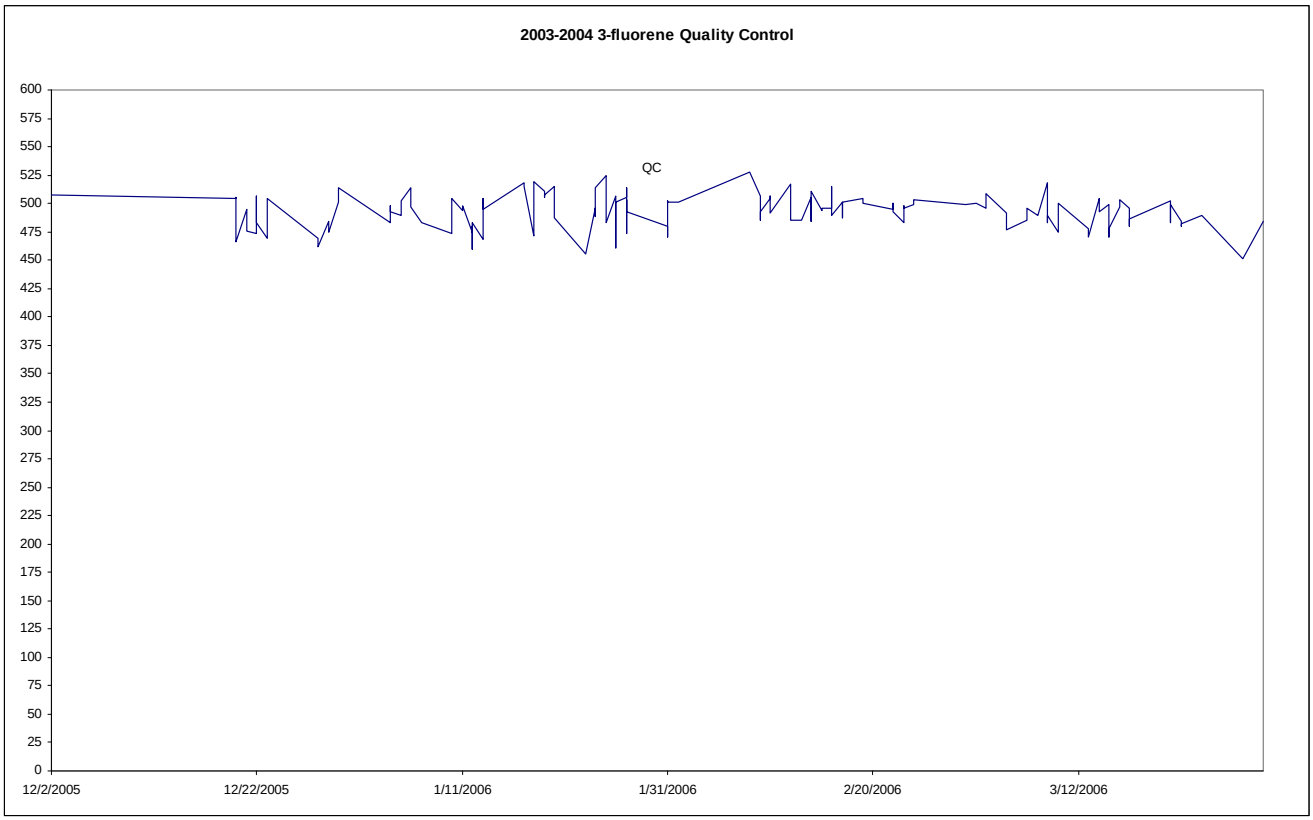
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	156	12/2/2005	3/30/2006	2676.16	157.34	5.9



C. 3-fluorene

Summary Statistics for 3-fluorene by Lot

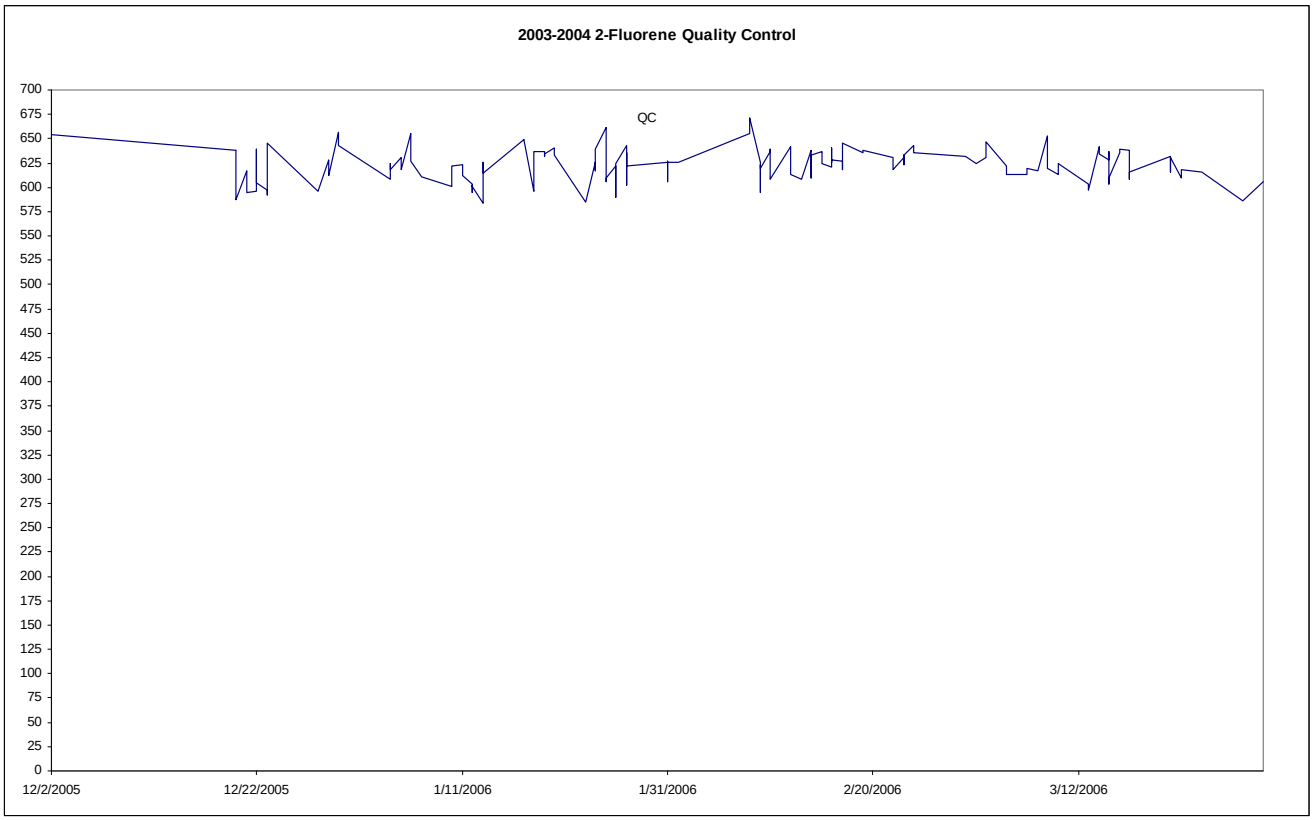
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	153	12/2/2005	3/30/2006	492.08	14.53	3.0



D. 2-fluorene

Summary Statistics for 2-fluorene by Lot

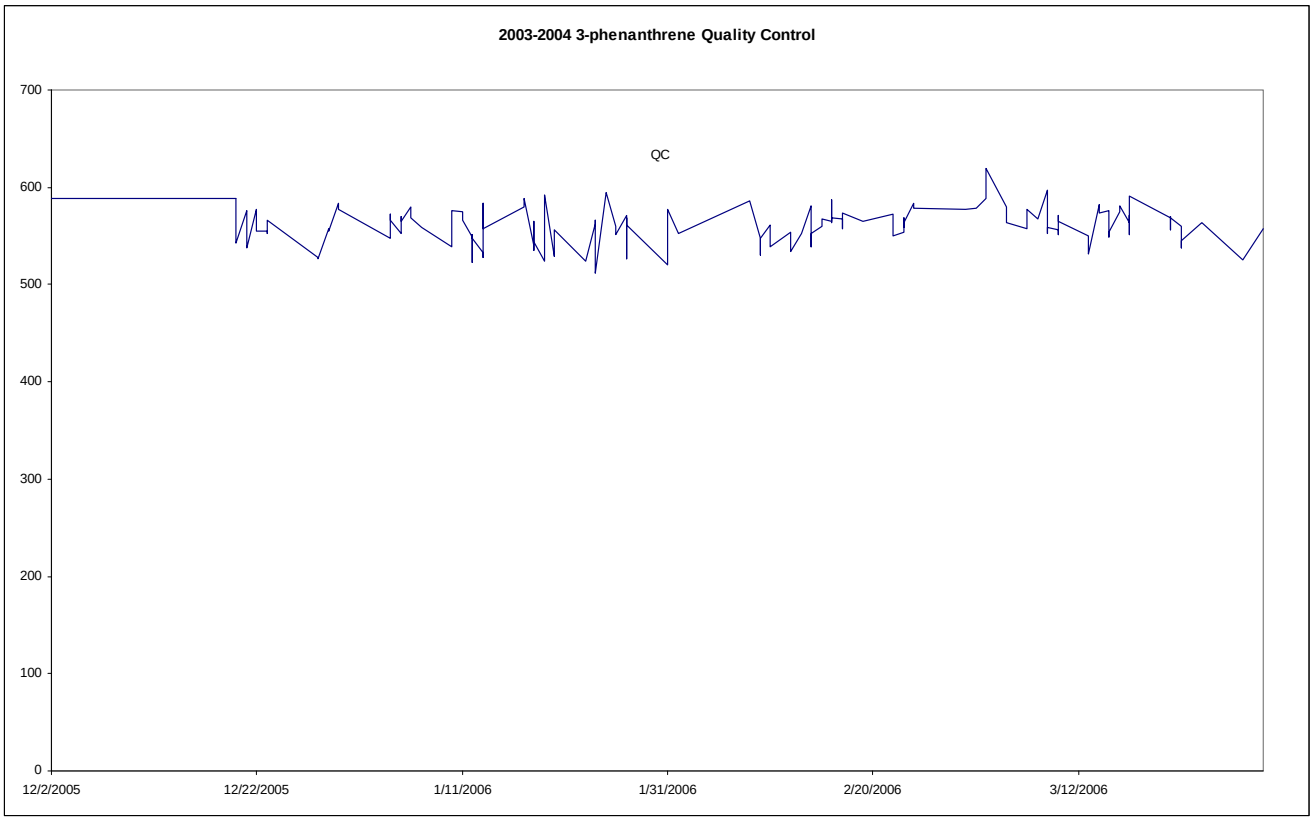
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	153	12/2/2005	3/30/2006	621.75	17.1	2.7



E. 3-phenanthrene

Summary Statistics for 3-phenanthrene by Lot

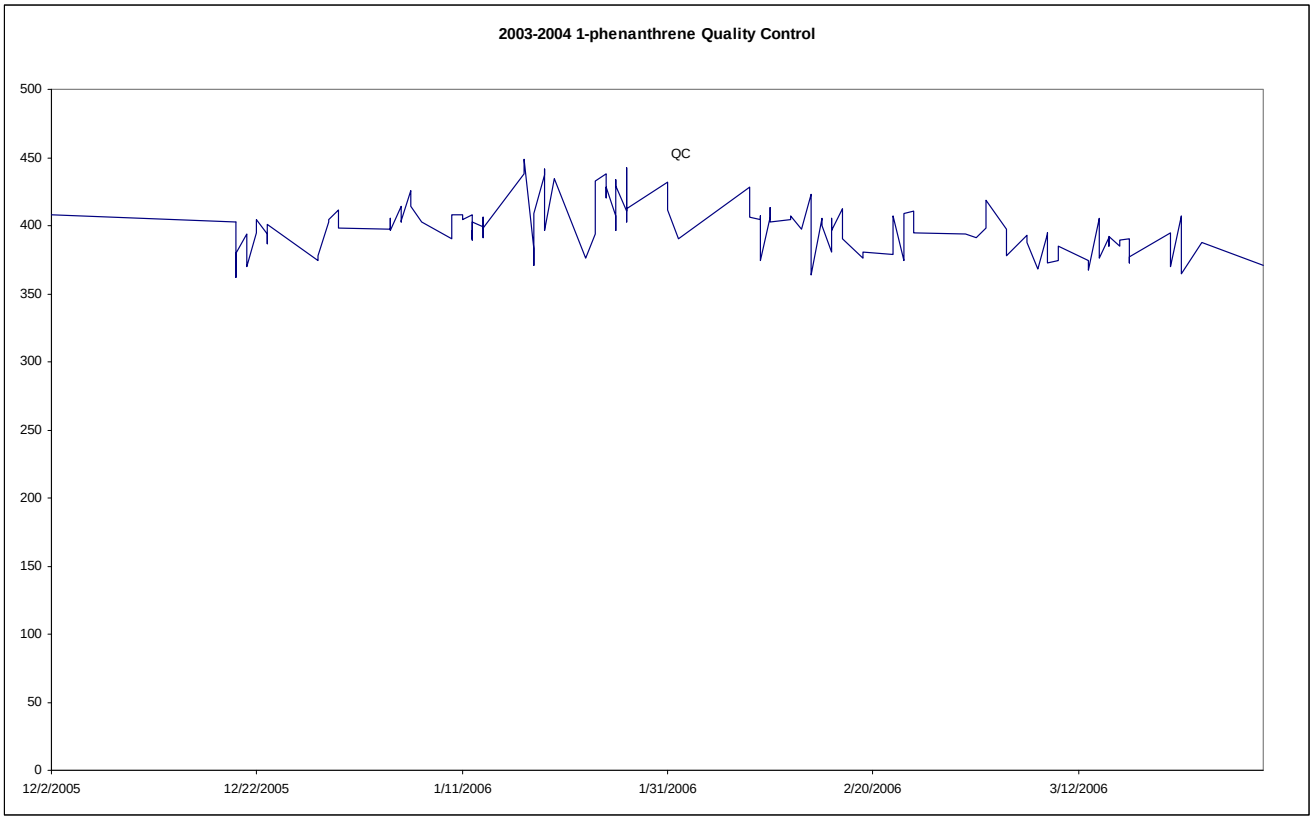
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	148	12/2/2005	3/30/2006	560.38	18.91	3.4



F. 1-phenanthrene

Summary Statistics for 1-phenanthrene by Lot

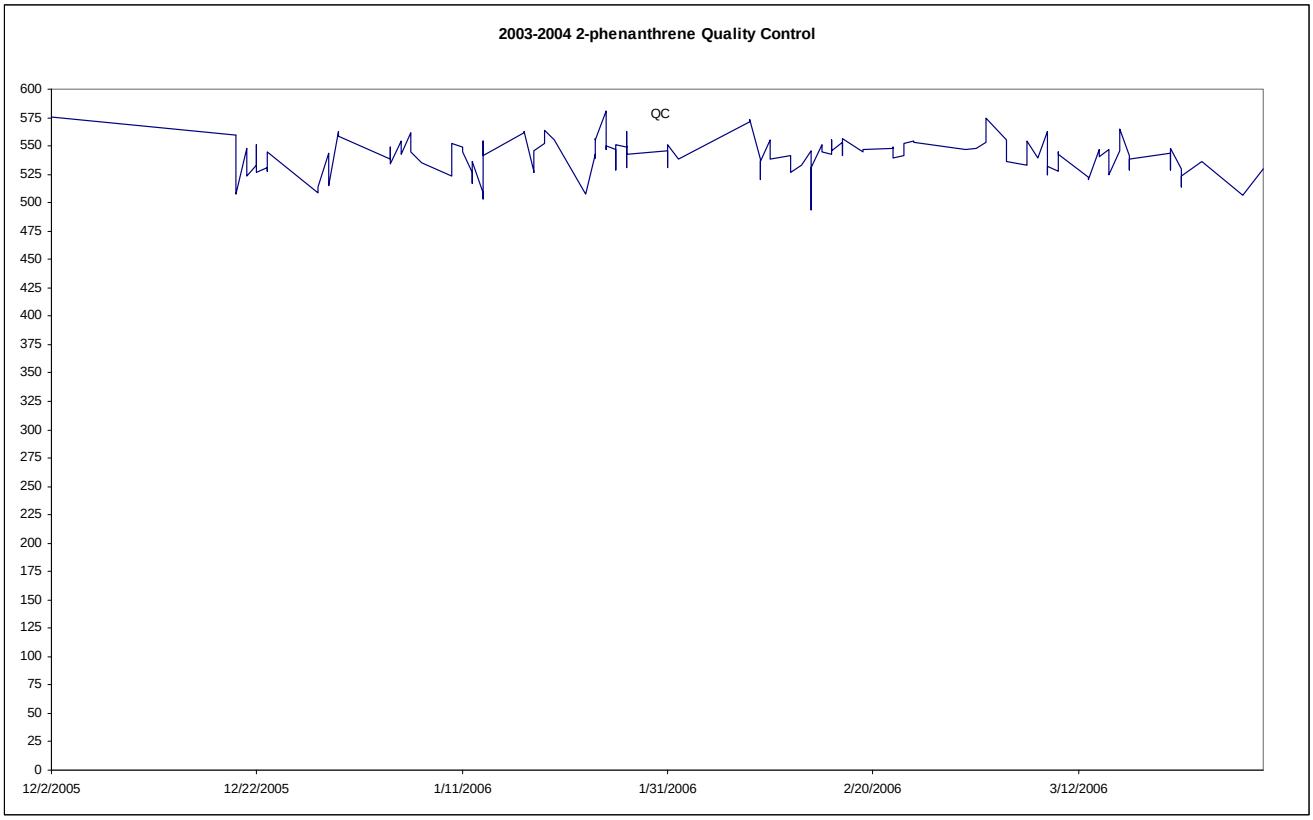
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	150	12/2/2005	3/30/2006	397.58	18.16	4.6



G. 2-phenanthrene

Summary Statistics for 2-phenanthrene by Lot

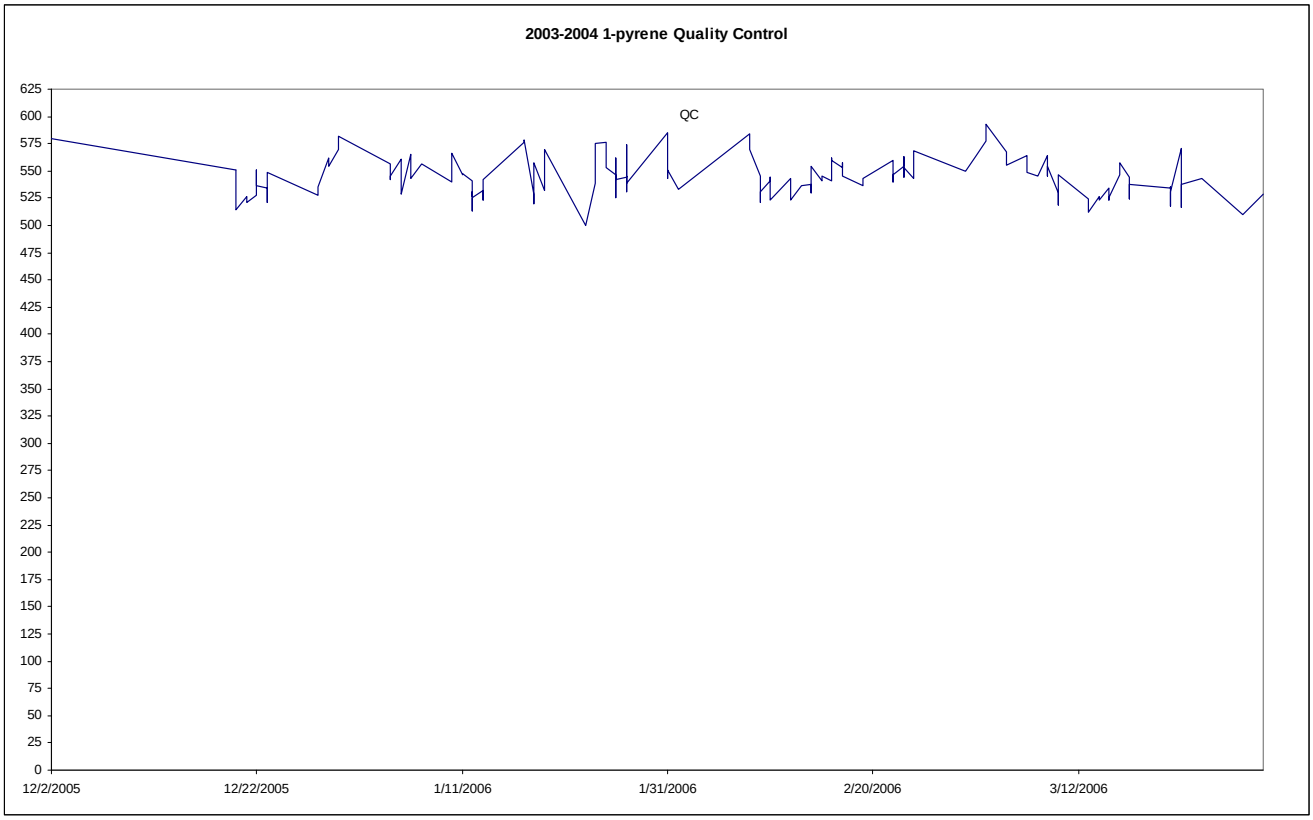
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	152	12/2/2005	3/30/2006	541.17	15.22	2.8



H. 1-pyrene

Summary Statistics for 1-pyrene by Lot

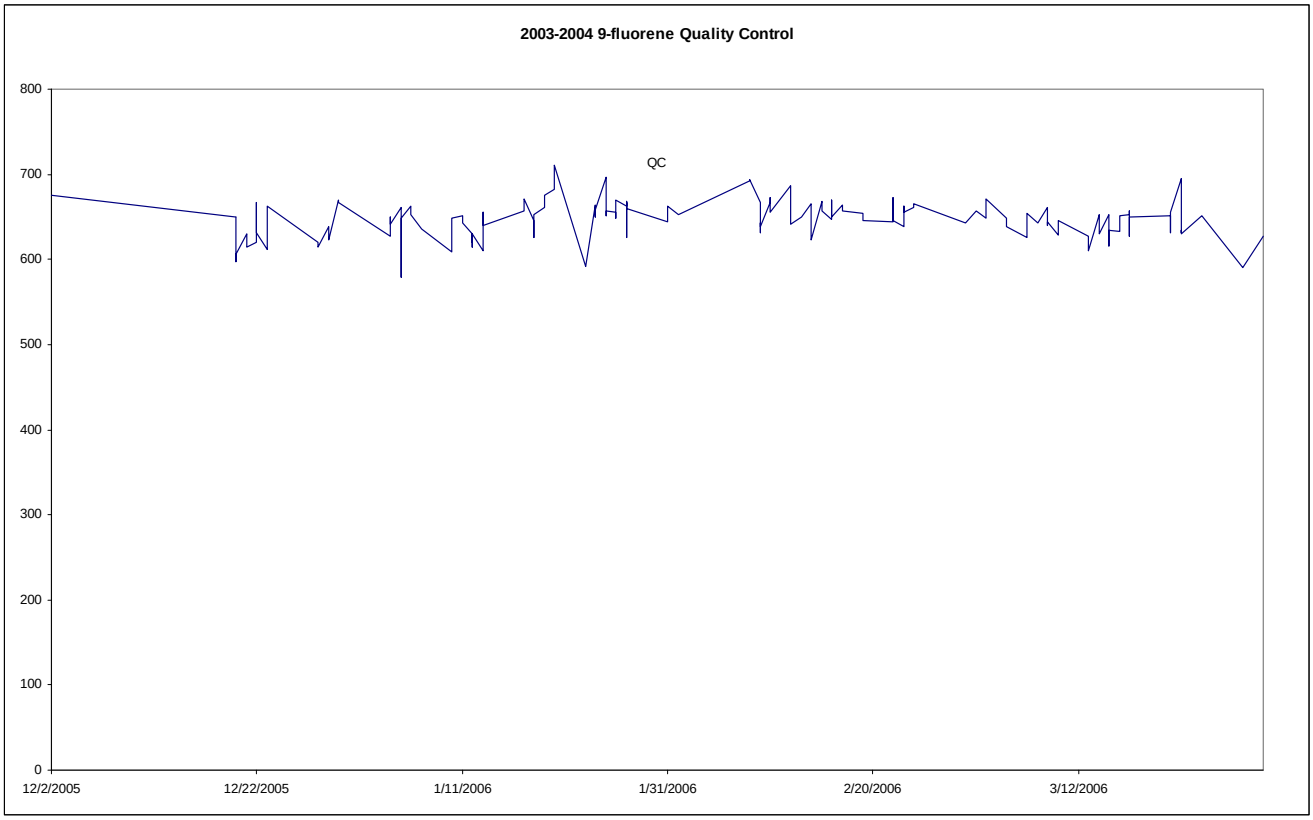
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	152	12/2/2005	3/30/2006	544.21	17.79	3.3



I. 9-fluorene

Summary Statistics for 9-fluorene by Lot

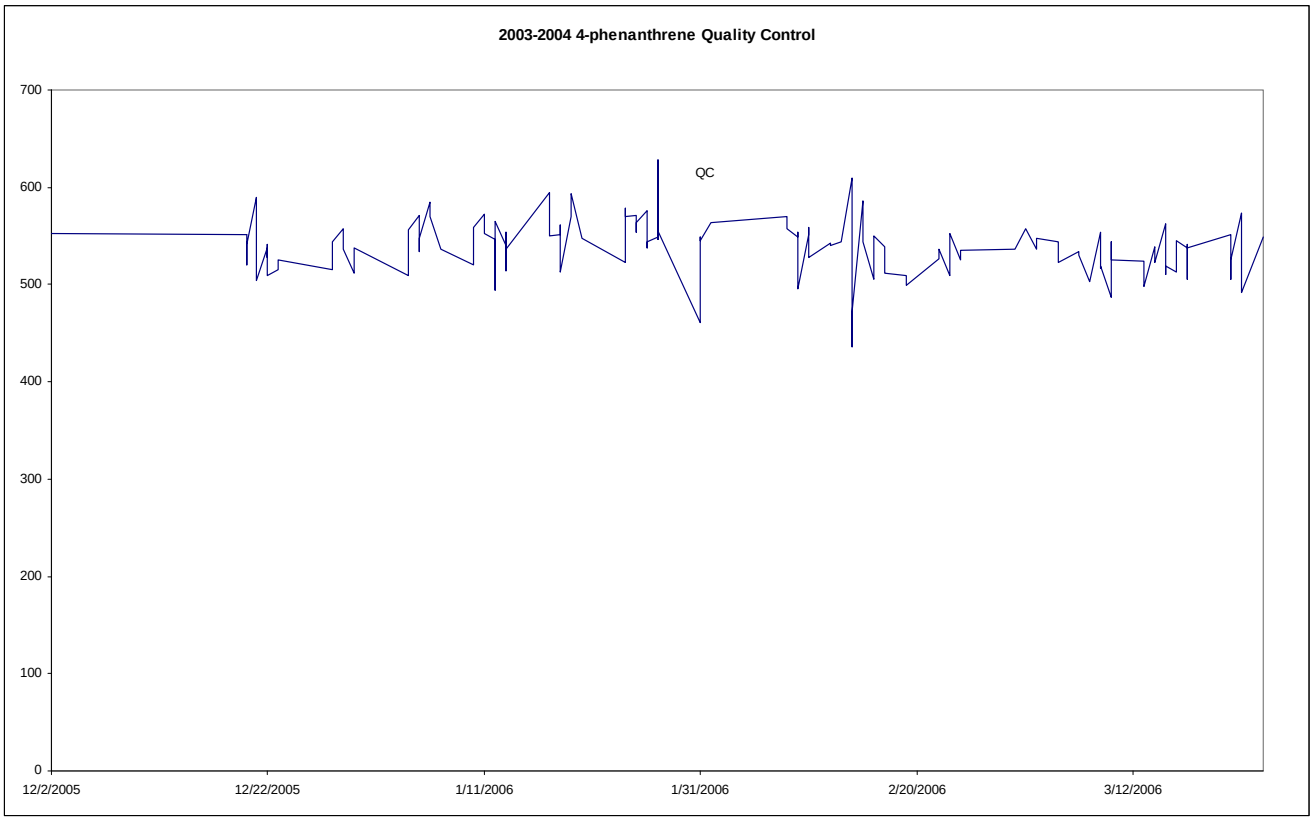
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	152	12/2/2005	3/30/2006	646.52	21.14	3.3



J. 4-phenanthrene

Summary Statistics for 4-phenanthrene by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
QC	149	12/2/2005	3/24/2006	538.52	27.18	5.0



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