Laboratory Procedure Manual

Analyte: 1-Methyl-9H-pyrido[3,4-b]indole (Harman), 9H-Pyrido[3,4-b]indole (Norharman), 2-amino-9H-pyrido[2,3-b] indole (AC), 2-amino-3-methyl-9H-pyrido[2,3-b] indole (MeAC), 3-amino-1, 4-dimethyl-5H-pyrido [4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-A:3',2'-D]imidazole (Glu-P-1), 2-aminodipyrido[1,2-a:3',2-D]imidazole (Glu-P-2), 2-amino-3-methyl-3H-imidazo[4,5-f]quinolone (IQ) and2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

Matrix: **Urine**

Method: **HPLC API Tandem Mass Spectrometry**

Method No: **2015**

Revised:

As performed by:

Tobacco and Volatiles 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.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table

Data File Name	Variable Name	SAS Label
HCAA_H	URXAAC	A-α-C (pg/mL)
	URXGLP1	Glu-P1 (pg/mL)
	URXGLP2	GLU-P2 (pg/mL)
	URXHM	Harman (pg/mL)
	URXIQ	IQ (pg/mL)
	URXMAAC	MeA-α-C (pg/mL)
	URXNHM	Norharman (pg/mL)
	URXPHIP	Ph1P (pg/mL)
	URXTRP1	Trp-P-1 (pg/mL)
	URXTRP2	Trp-P-2 (pg/mL)

1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

a. *Analyte.*

1-Methyl-9H-pyrido[3,4-b]indole (Harman), 9H-Pyrido[3,4-b]indole (Norharman), 2-amino-9H-pyrido[2,3-b] indole (AC), 2-amino-3-methyl-9H-pyrido[2,3-b] indole (MeAC), 3-amino-1, 4-dimethyl-5H-pyrido [4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-A:3',2'-D]imidazole (Glu-P-1), 2-aminodipyrido[1,2-a:3',2-D]imidazole (Glu-P-2), 2-amino-3-methyl-3H-imidazo[4,5-f]quinolone (IQ) and2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

b. *Clinical Relevance.*

Heterocyclic aromatic amines (HCAA) are an important class of carcinogens formed during combustion of cigarettes and cooking meats at high temperature (1,2). IARC considered a number of HCAAs as possible and probable carcinogens that include 2-amino-9H-pyrido[2,3-b] indole (AC), 2-amino-3-methyl-9H-pyrido[2,3-b] indole (MeAC), 3-amino-1, 4-dimethyl-5H-pyrido [4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-A:3',2'-D]imidazole (Glu-P-1), 2-aminodipyrido[1,2-a:3',2-D]imidazole (Glu-P-2), 2-amino-3-methyl-3H-imidazo[4,5-f]quinolone (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (3,4). Harman and norharman are the most abundant beta-carboline HCAA in cigarette condensate, though they are not carcinogenic (1, 5). Biomonitoring of urinary carcinogenic HCAA as well as harman and norharman provides useful information on exposure to HCAA related to tobacco smoking and impact of HCAA on human health.

c. *Assay Principle.*

AC, MeAC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, IQ, PhIP, harman and norharman in urine are measured by an isotope-dilution high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (ID HPLC-ESI MS/MS). For "total" HCAA assays, the urine samples are fortified with internal standards (ISTD), and hydrolyzed in basic condition at 70°C for 5 hours. The samples are then extracted by solid phase extraction, after which the analytes are eluted and analyzed by LC/MS/MS. The analyte specific ion transitions are monitored for HCAA quantitation, confirmation and ISTD respectively. The concentrations are derived from their respective ratios of native to isotope-labeled ions in the samples by comparing to their standard curves.

d. *Special Precaution.*

Because of the nature of these assays, all analysts involved in this study must be non-tobacco users, and measurements must be performed in a smoke-free building environment.

2 SAFETY PRECAUTIONS

Safety glasses, gloves and clothing must be worn during the extraction and processing of samples by this method.

a. *Reagent Toxicity / Carcinogenicity.*

Some of the reagents used in this procedure are toxic and some of the analytes themselves are toxic. Universal safety precautions must be taken to avoid inhalation or dermal exposure to assay reagents or analytical standards.

b. *Radioactive Hazards.*

This procedure does not use radioactive materials and there are no radioactive hazards associated with it.

c. *Biological Hazards.*

This assay involves human urine samples. Universal precautions must be followed. Analysts working directly with the specimens must use proper technique and avoid direct contact with the sample. Lab coats, gloves and protective eyewear (as required) should be worn while handling the specimens.

d. *Chemical Hazards.*

Reagents and solvents are used in this method including those listed below in Section 6.a. MSDSs for these chemicals are readily accessible on the internet (e.g., http://www.msdssearch.net/MSDSSearch.asp, or http://msds.ehs.cornell.edu/msdssrch.asp). Hardcopies are maintained on file.

e. *Mechanical hazards.*

There are no unusual mechanical hazards associated with this method. Analysts should know and follow the manufacturer's recommendations concerning the safe handling of instruments and other equipment. High voltages are found within certain areas of the mass spectrometer and care must be taken when working in those areas. Safety interlocks on instruments such as the mass spectrometer, LC autosampler and centrifuge covers, etc. should not be defeated during normal operations.

f. *Protective equipment.*

Standard safety precautions should be followed when performing this procedure including the use of a lab coat/disposable gown, safety glasses, appropriate gloves, and the use of biological safety cabinets and chemical fume hoods as needed. Refer to the laboratory Chemical Hygiene Plan and standard CDC / DLS safety policies and procedures guidelines for details related to specific activities or reagents.

g. ***Training.***

Method specific training in the use of tandem mass spectrometry is required. All analysts must be CLIA-certified and demonstrate proficiency in the analysis before handling samples. Educational and specific training information is maintained for all analysts certified to work on this method.

h. ***Disposal of Wastes.***

All waste disposals must be in compliance with DLS policy. Discard solvents and other waste reagents into an appropriate container marked for waste handling and store it in a chemical fume hood. Place all disposable items that come in contact with biological specimens in a biohazard autoclave bag which is maintained in an appropriate covered container until autoclaved. Unshielded needles, pipette tips and disposable syringes with attached needles must be placed in a sharps container and autoclaved when the container is full. Wipe down all surfaces potentially exposed to biological samples with a freshly prepared bleach solution (10% dilution of commercial sodium hypochlorite or the equivalent) each day. Non-disposable glassware or other equipment that comes into contact with biological samples must be rinsed with bleach before cleaning and reuse.

3 COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. ***Software and knowledge requirements.***

This method has been validated using a two-step of solid-phase extraction (SPE) sample preparation procedure on automation systems, followed by liquid chromatography ESI-tandem mass spectrometry utilizing AB Sciex API5500 with Analyst software. Analyte concentrations can be calculated by Analyst. The results with additional information (retention times, area counts, etc.) are exported to local laboratory information management system STARLIMS. The final results and QC evaluation are performed in STARLIMS. Knowledge of, and experience with these software packages is required in performing these functions.

The sample master database is maintained in STARLIMS. Sample sequence is generated on automation systems, such as Hamilton liquid handler and Caliper Staccato systems. The analyst should upload the sequence file to STARLIMS where it is merged with the analytical results data. Contact the supervisor for emergency assistance with any custom files and databases used in this method; contact the DLS LAN manager for assistance with any DLS network problems.

b. ***Sample information.***

All samples are analyzed in runs of, typically, 96 samples including 3 blank, 6 QCs and unknowns. Each run is recorded in STARLIMS that contains such information as Run and sample ID, date of analysis, analyst, internal standard, and special notes and observations for each run. STARLIMS containing this information has been developed by this DLS and is maintained on the intranet.

c. ***Data maintenance.***

Following the analytical (LC/MS/MS) analysis, the standards and samples are processed using Analyst software and information for each run includes sample file number, sample I.D., date and time assayed, integrated peak area counts, retention times, quantitated results, etc. These files are transferred and uploaded to STARLIMS where data are checked and QCs are evaluated. The final data are stored in STRALIMS for reporting.

d. ***Information security.***

Information security is provided at multiple levels. The data systems (such as STARLIMS) used in this work are accessed via computers that require individual login and passwords and that default to locked conditions during extended periods of nonuse. In addition, all systems and equipment are located on the Chamblee campus of CDC which has restricted access with security personnel approving all entry. Furthermore, the individual laboratory building has multiple levels of controlled access including the requirement for card keys to access the building itself, and also the individual floors where the equipment is located. Confidentiality of the results is protected by use of blind coded ID numbers only (no personal identifiers are ever used).

4 COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. ***Special requirements.***

There are no special requirements such as fasting or adherence to special diets for this assay.

b. ***Sample collection.***

The specimen for these analyses is human urine. Samples are collected in standard urine collection cups or vials (note: cups and all other materials contacting samples should be pre-screened and approved by this laboratory before they are used to avoid background contamination issues). Samples should be refrigerated as soon as possible after collection, and frozen for longer term storage. The sample should be well-mixed, and placed in an appropriate vial (e.g. Nalgene Cryogenic Tube, sterile, 5.0mL), and the tubes capped securely. Be careful not to overfill the tube (maximum volume of approximately 4 mL for 5 mL vials) to allow for expansion in the freezer.

c. ***Sample handling.***

Specimen handling conditions for urine samples including general collection and transport requirements are outlined in the DLS protocol for urine collection and handling (available from this laboratory or the DLS specimen handling activity). In general, urine samples should be frozen at approximately –20ºC and shipped with dry ice by overnight air. A packing list must be included with the samples, and the laboratory (or the specimen handling group) should be notified before shipment. Unless special arrangements are made, shipment schedules should avoid having samples arrive at CDC on the weekends or holidays since sample handling at those times may not be appropriate. After receipt, samples are stored frozen at approximately –20ºC, or in some cases at –70ºC for long-term storage.

d. ***Sample quantity.***

A minimum of 1 ml of urine is needed for the analysis. The optimum volume is approximately 1.5 ml of urine for accurate dispensing by robotic automation system.

e. ***Unacceptable specimens.***

Criteria for defining a sample as unacceptable include (1) use of improper collection materials or techniques leading to elevated background contamination; (2) sample volumes less than the required minimum; or (3) improper shipment or storage of samples leading to thawing, leaking, sleeve cracking or similar problems. All samples are logged in at receipt and problems with storage or shipment are identified at that point. Inadequate volumes will generally be identified when the samples are thawed for analysis. If a sample must be rejected as unacceptable, a description of the problem must be entered into the database and associated with that sample.

f. ***Long-term stability.***

Long-term stability results are currently under investigation for these analytes. Until more information is available, samples should be stored dry at ≤ –50ºC and protected from light for long term storage.

5 PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6 PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

Note: Class A glassware such as pipettes and volumetric flasks are used for preparation of methanol stock solutions including native HCAA and isotopically labeled HCAA methanol stock solutions. Class A plastic volumetric flasks and calibrated automatic pipettes are used for preparation of all of standard solutions, QC and PT samples that are dissolved in the media with over 50% (v/v) of water, and the prepared solutions should be stored in polypropylene plastic containers. The accuracy of balances, and automated pipettes should be confirmed at least annually.

a. ***Solvents and reagents handling.***

(1) HCAA are probable or possible human carcinogens and suitable protective clothing, gloves and eye/face protection must be utilized. It can be harmful if inhaled, swallowed or absorbed through the skin, and should only be used in a chemical safety hood. If contact occurs, flush area immediately with copious amounts of water.

(2) Sodium Hydroxide – this is a very caustic base, corrosive to all tissues. It is used to adjust the pH of urine samples. It generates considerable heat when mixed with water or an acid. It is nonflammable but would be harmful if inhaled or swallowed. Safety glasses and gloves must be worn while working with this reagent.

(3) Formic Acid - this is used to adjust the pH of the washing solution for sample cleanup. It will burn skin tissue and is harmful if inhaled or swallowed. If exposure occurs, flush the area with copious amounts of water. Always wear protective clothing and safety glasses when working with this reagent.

(4) Methylene chloride - this solvent is chemically stable and relatively unreactive. It poses a relatively low hazard. It is not flammable, but the vapor can be irritating to the eyes, nose and throat, and skin or eye contact with the liquid should be avoided. Flush copiously with water if any contact should occur. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator, or in a chemical fume hood.

(5) Methanol - this solvent is used to pre-condition SPE columns. It is toxic by ingestion, inhalation and skin absorption. It may cause acidosis, blindness and death. It is also flammable. Evaporation of significant volumes of this solvent must be performed in the Turbovap, Savant evaporator or in a chemical fume hood. Safety glasses and gloves must be worn when handling this solvent.

(6) Acetonitrile - used as a mobile phase for LC. It is toxic by ingestion, inhalation and skin absorption, and can be a source of cyanide toxicity. It is also flammable. Evaporation of significant volumes of this solvent must be performed in the Turbovap, Savant evaporator, or in a chemical fume hood. Wear appropriate protective clothing to prevent skin exposure.

(7) Ammonium Hydroxide - this is a caustic base, corrosive to all tissues. It is used to adjust pH of methanol solution for sample cleanup.

(8) Biotage Evolute CX 96-well SPE plate, 30 mg

(9) Biotage Isolute 96-well SLE plate, 400 µL

b. ***Stock reagent preparation***

See appendix A

c. ***Calibration materials***

See appendix B

d. ***Controls.***

1. ***Quality control materials.*** **(QCL, QCH)** There are two quality control pools for the urinary HCAA assay. Pools QCL and QCH represent the low and high HCAA quality control pools for HCAA analysis. Pools QCL and QCH were prepared in house by spiking blank urine with HCAA standards. Pools were pooled, mixed well and 4.0 ml aliquots were dispensed into appropriate sample tubes and frozen at approximately -70°C.

2. ***Proficiency testing materials.*** **(Level 1, Level 2 and Level 3.)** These materials are prepared from synthetic urine (for harman and norharman) or blank urine pool (for other HCAA) spiked at known levels with HCAAs. Three pools are prepared at known concentration levels with target amounts of approximately 40, 100 and 150 pg/ml for AC, MeAC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, IQ, PhIP; 400, 1000, 1500 pg/ml for harman and norharman. The pools are stored at approximately –70°C and labeled with blind-coded sample ID. Five aliquots of all 3 pools are analyzed by the standard procedure at least twice a year. The coded results are reviewed by DLS personnel not involved in the analysis to confirm acceptable method performance.

e. ***Major Instrumentation and Other Equipment.***

Liquid Handler. Hamilton MicroLab STAR, 8-channel, deepwell plate carrier.

Automation system. Caliper Staccato: Sciclone, 2D Fluidx barcode reader, Fluidx decapper, Hettich centrifuge, Inheco IVD, plate sealer, Biotage Turbovap.

HPLC. Shimadzu LC-30AD module which consists of degasser, binary pump, SIL-30AC autosampler, LC-20AD pump and CTO-30A column oven.

Mass Spectrometer. AB Sciex API 5500 Triple Quad Tandem Mass Spectrometer with electrospray interface.

Data System. Dell Optiplex 960 or the equivalent using Windows XP and AB Sciex Analyst software v. 1.6 or higher version.

7 CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. *Cleaning and instrument tune procedure.*

For automation systems (Hamilton STAR and Caliper Staccato), calibration is performed periodically following manufacturer's procedure. For LC/MS/MS instrument, the inlet skimmer plate is removed and the system front end is cleaned at the sign of the buildup of stain. Periodically the system calibration is confirmed by infusing a polypropylene glycol (PPG) tune solution provided by AB Sciex. Overall performance and complete mass calibrations with PPGs are conducted at each preventive maintenance (2/year), following significant repairs or other changes to the instrument, and on other occasions as indicated.

b. *Calibration Curve*

A calibration curve for this assay is based on the analysis of the standard set described in Section 6.c. A set of eleven standards ranging in values from zero to approximately 4 ng/mL for AC, MeAC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, IQ, PhIP and zero to 40 ng/ml for harman and norharman are analyzed in duplicate in the forward and the reverse direction prior to the start of each sample run. After adjusting for dilution, the corresponding urine calibration curves are tabulated in the following table. The calibration ranges for this method cover ranges of values from the limit of detection (LOD) to concentrates highlighted in urine calibration curves (Following Table).

	Level 0	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7	Level 8	Level 9	Level 10
Concentrations in water standard (pg/ml)											
AC	0	9.83	19.7	39.3	98.3	196.6	393.2	983	1474.5	1966	3932
MeAC	0	10	20	40	100	200	400	1000	1500	2000	4000
Trp-P-1	0	7.79	15.58	31.16	77.9	155.8	311.6	779	1168.5	1558	3116
Trp-P-2	0	6.73	13.45	26.91	67.27	134.5	269.1	673	1009	1345	2691
Glu-P-1	0	7.34	14.68	29.36	73.4	146.8	293.6	734	1101	1468	2936
Glu-P-2	0	6.63	13.26	26.52	66.3	132.6	265.2	663	994.5	1326	2652
IQ	0	10	20	40	100	200	400	1000	1500	2000	4000
PhIP	0	10	20	40	100	200	400	1000	1500	2000	4000
Harman	0	100	200	400	1000	2000	4000	10000	15000	20000	40000
Norharman	0	100	200	400	1000	2000	4000	10000	15000	20000	40000
Corresponding concentrations in urine (pg/ml)											
AC	0	0.983	1.97	3.93	9.83	19.7	39.3	98.3	147	197	393
MeAC	0	1	2	4	10	20	40	100	150	200	400
Trp-P-1	0	0.78	1.56	3.12	7.79	15.58	31.16	77.90	116.85	155.80	311.6
Trp-P-2	0	0.67	1.35	2.69	6.73	13.45	26.91	67.27	100.90	134.53	269.1
Glu-P-1	0	0.73	1.47	2.94	7.34	14.68	29.36	73.40	110.10	146.80	293.6
Glu-P-2	0	0.66	1.33	2.65	6.63	13.26	26.52	66.30	99.45	132.60	265.2
IQ	0	1	2	4	10	20	40	100	150	200	400
PhIP	0	1	2	4	10	20	40	100	150	200	400
Harman	0	10	20	40	100	200	400	1000	1500	2000	4000
Norharman	0	10	20	40	100	200	400	1000	1500	2000	4000

c. *Verification*

Initial. The initial accuracy of this method was established by analyzing a series of pure standards prepared as described above. The ratio of native and labeled HCAA area counts was regressed on concentration ratio using 1/X weighting using AB Sciex system software Analyst. The resulting calibration curves were linear up to the highest concentrations, and R-squared values were typically > 0.98.

Daily. Prior to assaying each run of unknowns, the results from standard analyses are reviewed for acceptable accuracy, precision and instrument sensitivity. The results from the 22 calibration standards analyzed prior to each run are reviewed daily. Acceptable back-calculated values for standards above the detection limit are typically in the range of nominal concentration ± 10% for high standards (Level 6-10) and ± 20% for low standards (Level 1-5).

8 PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

When the run is prepared, each sample or control is assigned an alphanumeric laboratory ID with a seven-character prefix, and a three-digit suffix in the format XXXXXXX-nnn, where XXXXXXX is the run designation (e.g., UHAA001) and -nnn is the sample's position in the run (e.g., 096). These working ID numbers are linked to the original IDs (sample barcode) in the sample database. Three of Water blanks, the low bench QCs and the high bench QCs are included in each run. Water blanks, the low bench QCs, the high bench QCs and unknown samples are distributed in 96 well plate in the following format:

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A	*BLK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*QCH*	*UNK*	*UNK*	*UNK*	*BLK*	*UNK*
B	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*
C	*UNK*	*UNK*	*QCL*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*QCL*	*UNK*	*UNK*	*UNK*
D	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*
E	*QCH*	*UNK*	*UNK*	*UNK*	*BLK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*
F	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*
G	*UNK*	*UNK*	*UNK*	*UNK*	*QCL*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*QCH*	*UNK*
H	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*	*UNK*

BLK: water blank
QCH: high bench QC
QCL: the low bench QC
UNK: unknown samples

Standards run with the samples will be named corresponding to the study. Standards IDs are HAA_STD_level XX (where XX ranges from 0 to 10) in STARLIMS. Each run should have two different standards sets associated with it. Both the sample run number and standards run numbers should be recorded on the daily instrument log.

a. *Sample Preparation*

See appendix C

b. ***LC/MS/MS Analysis***

1. The LC mobile phase A contains 0.05% ammonium hydroxide in water (pH = 10.5). The mobile phase B is 100% acetonitrile. Prepare the mobile phase A stock as needed and discard old stock after one week. Equilibrate the column at least 15 minutes prior to starting the run for the day.

2. Operate the ESI source of the API 5500 mass spectrometer in the positive ion mode with the following parameters: IonSpray voltage at 2000 V, source heater temperature at 650 °C, curtain gas at 35 psi, ion Source gas 1 at 60 psi, ion Source gas 2 at 70 psi, and collision gas at high level. Record all LC/MS/MS data in MRM (multiple reaction monitoring) mode. Data of following ion transitions are recorded. The total run time is 12 min. Set the analytical quadrupole to unit resolution. Optimize the compound-related mass spectrometric parameters for each individual ion transition as listed below.

		Precursor ions (m/z)	Product ions (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Harman	Quantitation ion	183.1	115.0	260	10	68	14
	Confirmation ion	183.1	88.9	260	10	63	14
	Internal standard ion	186.1	117.1	260	10	68	14
NorHarman	Quantitation ion	169.1	115.1	170	10	82	14
	Confirmation ion	169.1	88.9	170	10	105	10
	Internal standard ion	176.2	120.0	170	10	82	14
AC	Quantitation ion	184.1	167.1	51	10	31	22
	Confirmation ion	184.1	140.0	51	10	43	20
	Internal standard ion	187.0	169.1	51	10	31	22
MeAC	Quantitation ion	198.1	181.1	36	10	31	16
	Confirmation ion	198.1	127.1	36	10	53	12
	Internal standard ion	201.1	184.1	36	10	31	16
IQ	Quantitation ion	199.1	184.1	56	10	35	18
	Confirmation ion	199.1	157.1	56	10	47	12
	Internal standard ion	202.2	184.2	56	10	35	18
Trp-P-1	Quantitation ion	212.1	167.1	41	10	55	16
	Confirmation ion	212.1	195.0	41	10	32	16
	Internal standard ion	215.1	168.0	41	10	55	16

Trp-P-2	**Quantitation ion**	**198.1**	**181.1**	**36**	**10**	**31**	**16**
	Confirmation ion	**198.1**	**127.0**	**36**	**10**	**53**	**12**
	Internal standard ion	**201.1**	**183.1**	**36**	**10**	**31**	**16**
Glu-P-1	**Quantitation ion**	**199.1**	**92.0**	**121**	**10**	**45**	**12**
	Confirmation ion	**199.1**	**172.1**	**121**	**10**	**34**	**12**
	Internal standard ion	**202.0**	**92.0**	**121**	**10**	**45**	**12**
Glu-P-2	**Quantitation ion**	**185.1**	**168.1**	**126**	**10**	**35**	**10**
	Confirmation ion	**185.1**	**78.0**	**126**	**10**	**49**	**10**
	Internal standard ion	**188.1**	**171.1**	**126**	**10**	**35**	**10**
PhIP	**Quantitation ion**	**225.1**	**210.1**	**111**	**10**	**39**	**28**
	Confirmation ion	**225.1**	**115.0**	**111**	**10**	**61**	**10**
	Internal standard ion	**228.2**	**210.1**	**111**	**10**	**39**	**28**

CE = collision energy; CXP = collision cell exit potential; DP = declustering potential; EP = entrance potential. (Note: the parameters for Harman and NorHarman have been detuned to avoid saturating detector)

3. HPLC separation is achieved using a Zorbax Eclipse Plus C18 2.1×100 mm 3.5 µm or the equivalent, purchased from Agilent (Santa Clara, CA). The column is eluted with the following linear gradient of H_2O (0.05% Ammonium hydroxide; eluent A) and ACN (eluent B) at a flow rate of 0.4 ml/min. The third pump is 100% acetonitrile with flow rate 0.20ml/min. An injection volume of 10 uL of extracted samples is applied on LC/MS/MS for analysis. The injection volumes of water standards are listed in the appended table at Page 31.

4. A typical LC gradient program is as follows:

   Flow rate: 0.4 ml/min

Time	Eluent A	Eluent B
min	%	%
0	95	5
5	64	36
6.5	64	36
7.5	2	98
10	2	98
10.01	95	5
12	95	5

5. Prior to performing a run, inject the 2nd HCAA standard to determine accurate

system operation and suitable sensitivity.

6. Immediately after the run, wash the LC column using 100 % acetonitrile for overnight and place the system in standby.

c. *Data Processing*

1. The peak integration is automatically performed by Analyst software.
2. After the sample is quantitated by Analyst software, check each peak integration result and manually re-integrate where it is needed.
3. After the quantitation result file is generated, export the file to STARLIMS where blank mean subtraction is performed and QCs are evaluated. Sample repeats are also generated in STARLIMS.

d. *Calculations*

The results are reported in pg/mL urine or it can be expressed relative to the urine creatinine value when available: HCAA (pg/ml)/Creatinine (ng/ml) = HCAA pg/ng creatinine.

9 REPORTABLE RANGE OF RESULTS

a. *Linearity Limits*

Samples are obtained from both smokers and non-smokers. Therefore, a broad range of urine HCAA levels can be expected. If the value of a sample exceeds the highest concentrations of calibration ranges, the sample is repeated using a smaller sample aliquot and re-analyzed.

b. *Limit of Detection*

The LOD of AC, MeAC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, IQ, PhIP and harman and norharman were determined by analyzing 4 to 5 low urine pools at the following concentrations for each analyte. Each pool was analyzed over 30 times. The standard deviation was plotted against the concentrations. LOD are calculated according to Policies and Procedures Manual of DLS.

	Low urine pools (pg/ml)				
AC	0.49	0.983	1.97	3.93	9.83
MeAC	0.5	1	2	4	10
Trp-P-1	-	0.78	1.56	3.12	7.79
Trp-P-2	-	0.67	1.35	2.69	6.73
Glu-P-1	-	0.73	1.47	2.94	7.34
Glu-P-2		0.66	1.33	2.65	6.63
IQ	0.5	1	2	4	10
PhIP	0.5	1	2	4	10

Harman *	-	10	20	40	100
Norharman *	-	10	20	40	100

*Harman and norharman were prepared in the synthetic urine because of high endogenic harman and norharman in the urine pools.

The estimated LOD are summarized as follows:

	LOD (pg/ml)
AC	0.62
MeAC	0.33
Trp-P-1	0.79
Trp-P-2	0.63
Glu-P-1	0.31
Glu-P-2	0.83
IQ	0.37
PhIP	0.34
Harman	4.59
Norharman	12.6

c. ***Precision***

Short-term precision was estimated by the repetitive analysis of three levels of spiked urine samples. 4-8 replicates were used to evaluate intra-day precision, and the inter-day precision was estimated over a period of 3 -6 days with daily analyses of the same urine pools. The precision study was performed for total HCAA analysis. (Appendix D)

d. ***Accuracy***

Accuracy was estimated by the repetitive analysis of three levels of spiked urine samples. 4-8 replicates were used to evaluate intra-day accuracy, and the inter-day accuracy was estimated over a period of 3-6 days with daily analyses of the same urine pools. The accuracy study was performed for total HCAA analysis. (Appendix D)

e. ***Analytical Specificity***

Analytical specificity in this method is mainly conferred through the use of tandem mass spectrometry which is a very specific technique. Further assurance of the identity of the analyte in unknowns is provided by comparison of retention times of the unknowns in HPLC with that observed with standards; by coelution of the analyte with the labeled internal standard; and by the calculation of appropriate confirmation ion ratios.

f. *Carryover*

Carryover effects in urine samples were evaluated by injections of urine samples at the highest quantification levels followed by blank urine samples. No carryover was observed between samples in these evaluations. Samples higher than the highest quantification levels are subject to be repeated in dilution. The following sample will be repeated too.

g. *Freeze-Thaw and Storage Stability*

Freeze (−70 °C)–thaw (room temperature) stability was estimated by exposing of spiked urine samples to four freeze–thaw cycles before sample preparation. Stability at room temperature was evaluated by keeping urine samples at room temperature for 24 hrs before sample preparation. Stability of extracted samples in autosampler was estimated by keeping extracted samples in 15 °C autosampler for 24 hrs before analysis. Stability study demonstrated no significant degradation of HCAA following four freeze-thaw cycles, at room temperature for 24 hrs and at autosampler for 24 hrs. (see Appendix E).

h. *Ruggedness validation*

The following four factors that may influence the accuracy of the method were tested for ruggedness validation:

1. % of ammonium hydroxide in LC mobile phase. The % of ammonium hydroxide was examined at 0.03%, 0.05%, 0.08%. 0.05% ammonium hydroxide was used in the final method (see Appendix F).
2. The incubation time course for HCAA hydrolysis. A lower level and an upper level of incubation time were chosen to examine their influence to the analysis. (see Appendix F).
3. % of methanol in the washing solution for MCX step. 20, 30%, or 40% methanol in water with 2% ammonia hydroxide were prepared and 30% methanol in water with 2% ammonia hydroxide was used in the final method (see Appendix F).
4. % of Formic acid in the washing solution for MCX step. 1% 2%, and 3% formic acid in methanol were prepared and 2% formic acid in methanol was used in in the final method (see Appendix F).

i. *Influence of urine matrix on calibration curve*

The calibration curves built with urine matrix after sample extraction were run in parallel with calibration curves built with water. The averages of the slopes of calibration curve for urine matrix or water matrix were compared to evaluate the influence of matrix effect. The difference is less than 5% for the slopes of calibration curves between two different matrixes, which indicates that urine matrix would not influence the quantification of HCAA by using calibration curve prepared in the water (see Appendix G)

10 Quality Assessment and Proficiency Testing

Two human urine pools containing high level and low level of HCAA are used as QC in this analysis. All pools were subjected to an initial characterization run series with at least 20 replicates over more than 2-week of period. The preparation of these pools was described previously in section 6.1. In addition, water blanks are included in all sample runs.

a. *QUALITY CONTROL (QC) PROCEDURES*

1. Relatively low concentrations of harman and norharman are detected in water blanks (<20 pg/ml) due to trace of harman and norharman in distilled water used for sample preparation. Other HCAAs in water blank are typically negative or below LOD for the method (non-detectable). If a calculated concentration of any amount is obtained, the blank value will be subtracted from the sample value.

2. For the bench QC, the run is rejected if any pool is outside of the 3-sigma limits of the mean, or if any two pools are outside of 2-sigma limits in the same direction. All data are periodically batched and analyzed by using the Division of Laboratory Science SAS-QC program incorporating standard criteria for analysis. Any run failing the DLS SAS QC analysis is repeated if sufficient sample volumes exist; otherwise, no quantitative results for the samples analyzed in that run are reported.

3. HCAA concentrations are checked to make certain the values are within the range of the method. The actual measured concentration must be no greater than the quantification range in standard curve; if above that limit, the sample must be diluted and reanalyzed if sufficient volume exists. In addition, expected ion ratios for confirmation and quantitation ions, the expected retention times, etc. are checked for each sample.

b. *Proficiency Testing*

1. Proficiency testing is performed at least semi-annually. Currently, no external source of PT materials is available and there are no blank urine pools that are free of harman and norharman. Therefore, PT assays for harman and norharman are conducted using synthetic urine spiked with known amounts of harman and norharman; PT assays for other HCAA are conducted using blank urine pools spiked with known amounts of HCAA.

2 Analytical PT results are reviewed by the analyst and the supervisor. Acceptable results require that > 80% of the results agree with the target value ± 25%. If the assay fails PT, all analyses are stopped and the source of error is investigated. No assays will resume until the problem has been resolved and a repeat PT assay has been passed.

11 REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET

ACCEPTABLE CRITERIA

a. ***Calibration.***

System calibration and general readiness is assessed on a daily basis from a review of the instrument's operating conditions, the values for the water blanks, and the results of the pre-run standard (e.g. Internal standard area counts and calculated concentration). When corrective actions are indicated, they are performed and the system is re-evaluated with additional standards until acceptable results are obtained before any unknowns are analyzed.

b. ***Quality Control.***

If the results from analysis of QC samples are outside the acceptable limits and a reason is identified for the apparent problem, it is indicated and the run is scheduled for repeat sample preparation and analysis for samples that have sufficient quantity. If the problem is not identified, sample preparation and analysis is suspended until the problem or problems are discovered and corrected for each analyte. Any questionable sample identified by QC or individual sample evaluation that cannot be confirmed by repeat analysis is not included for the affected analyte in the reportable database of results.

12 LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Some plastic materials, solvents, air and water may provide trace amounts of HCAAs, which could contribute as a contaminant to the level measured in the urine sample. Besides, some interfering substances do exist in some urine samples. However, this issue can be resolved by monitoring the confirmation ratio. In addition, the specificity of LC/MS/MS detection helps to avoid background chemical interferences with the quantitation ion.

13 REFERENCE RANGES (NORMAL VALUES)

Previous study showed that urinary AC levels were about 20 ng/g creatinine and 7 ng/g creatinine in smokers and non-smokers, respectively; Urinary harman levels were around 300-1400 ng/g creatinine in both smokers and non-smokers; Urinary norharman levels were around 1000-2500 ng/g creatinine in both smokers and non-smokers; Urinary PhIP levels were around 4-6 ng/g creatinine in both smokers and non-smokers; Urinary IQ levels were around 5-10 ng/g creatinine; Urinary MeAC, Glu-P-1 and Glu-P-2 could not be detected in both smokers and non-smokers (6). Since limited number of human subjects were involved in the previous study, the reference ranges of urinary HCAA may be revised based on future large scale biomonitoring studies. For our method, expected levels for urinary HCAA are currently under investigation.

14 CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable for this procedure.

15 SPECIMEN STORAGE AND HANDLING DURING TESTING

Samples are received frozen and typically stored frozen at or below –20 °C until analysis. After samples are aliquoted, the remainder of the samples is returned to freezer at -70°C until duplicate analysis is completed or for repeat analysis if required.

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

If a problem with the method exists, samples are held in the freezer until the problem can be resolved. If necessary, filtered and extracted samples ready for analysis can be stored, well-sealed, at –70 °C for at least 1 month before they are assayed.

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

Not applicable at this time.

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

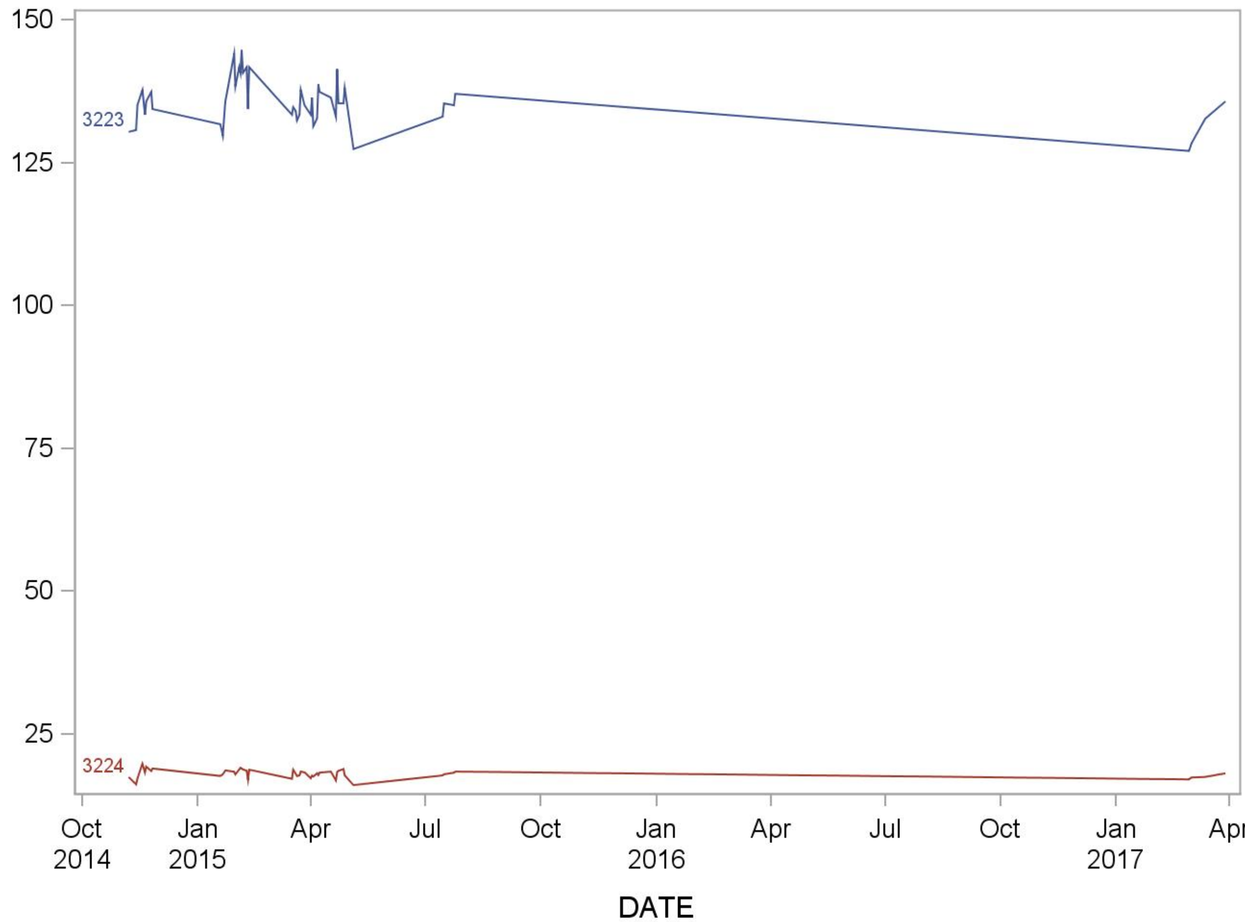
Following analysis, residual samples, if available, are held in storage at approximately -70°C in Chamblee buildings.

19 SUMMARY STATISTICS AND QC GRAPHS

See following pages.

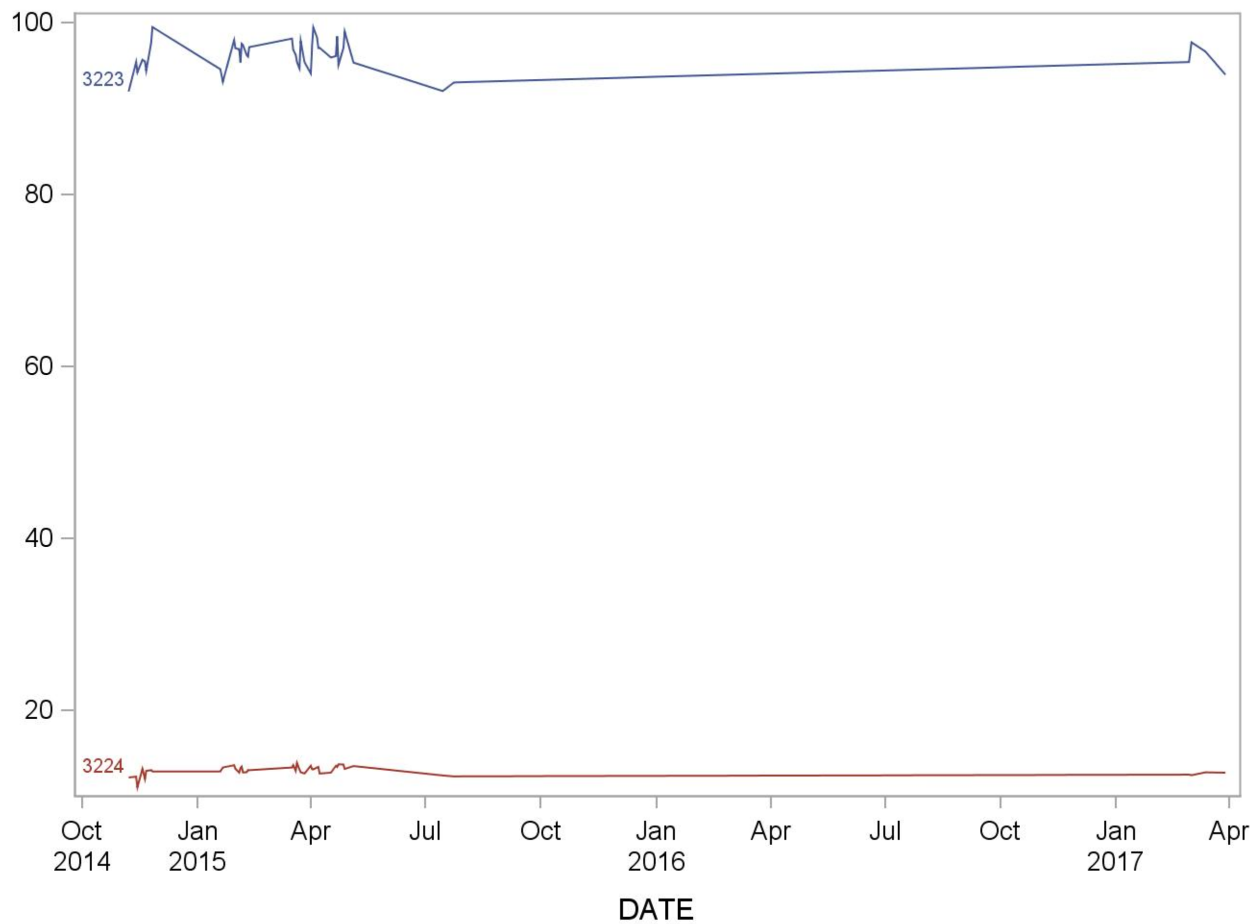
2013-2014 Summary Statistics and QC Chart for A-a-C (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	48	07NOV14	29MAR17	135.4097	4.05240	3.0
3224	48	07NOV14	29MAR17	17.99191	0.76085	4.2



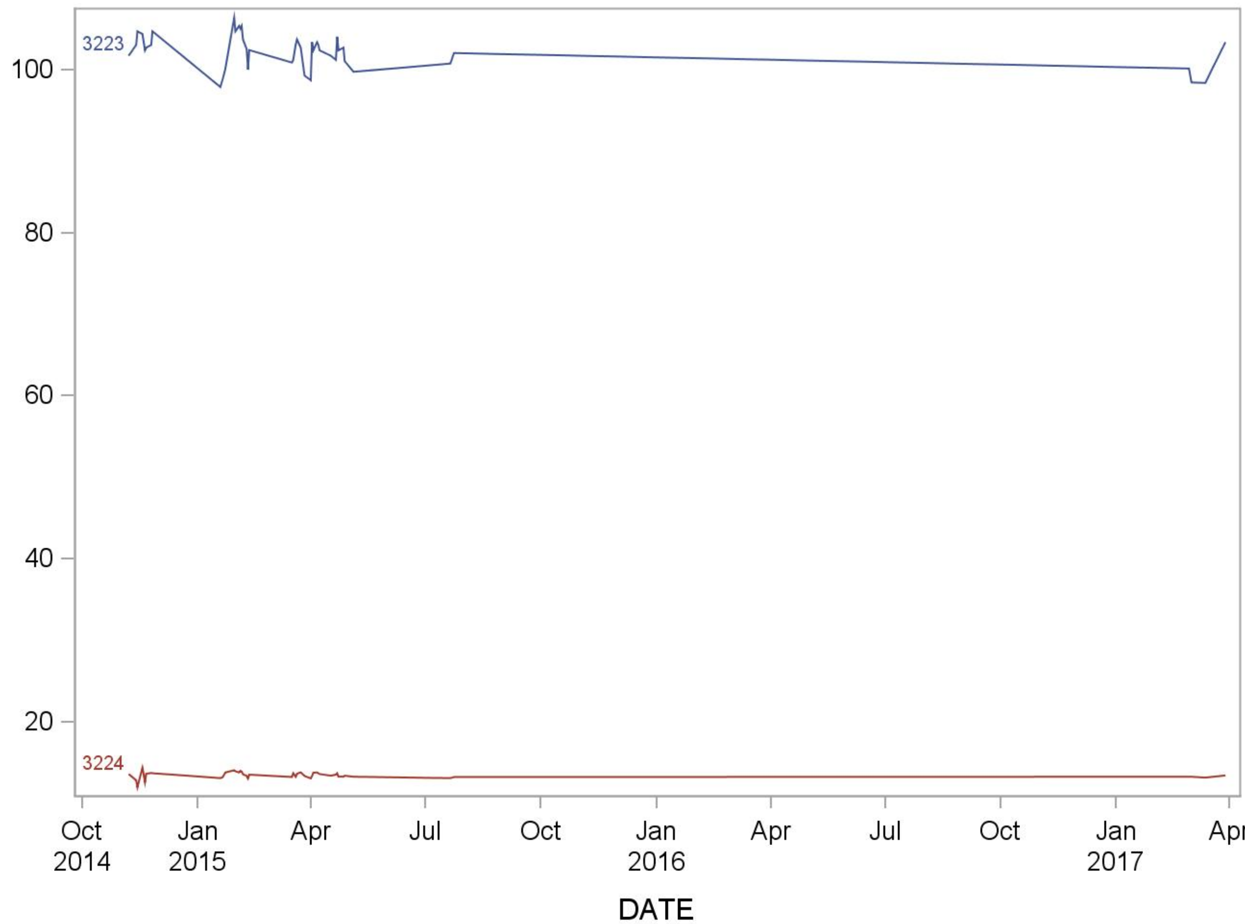
2013-2014 Summary Statistics and QC Chart for GLU-P2 (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	45	07NOV14	29MAR17	96.1474	1.8061	1.9
3224	45	07NOV14	29MAR17	12.9709	0.5330	4.1



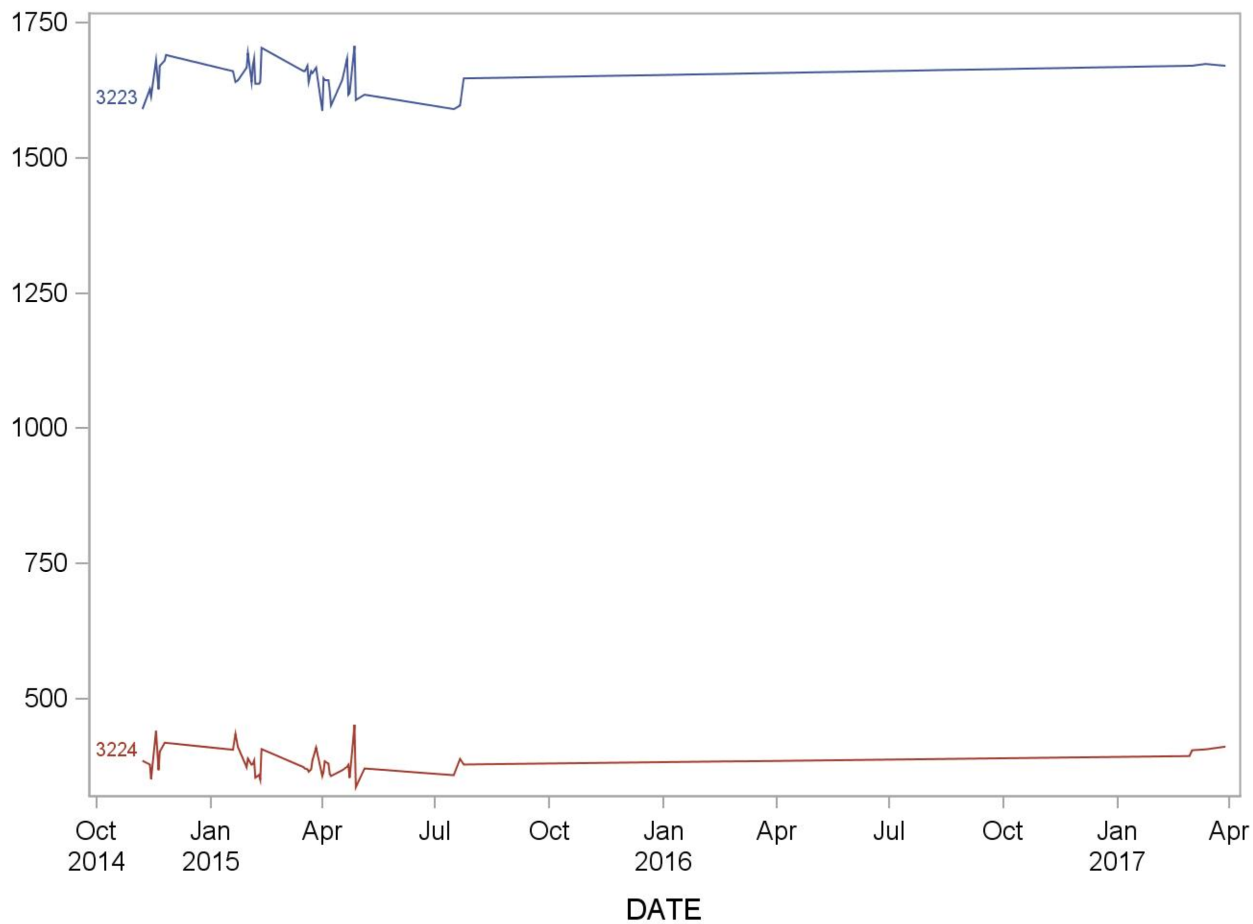
2013-2014 Summary Statistics and QC Chart for Glu-P1 (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	46	07NOV14	29MAR17	102.1775	2.0666	2.0
3224	46	07NOV14	29MAR17	13.4274	0.4044	3.0



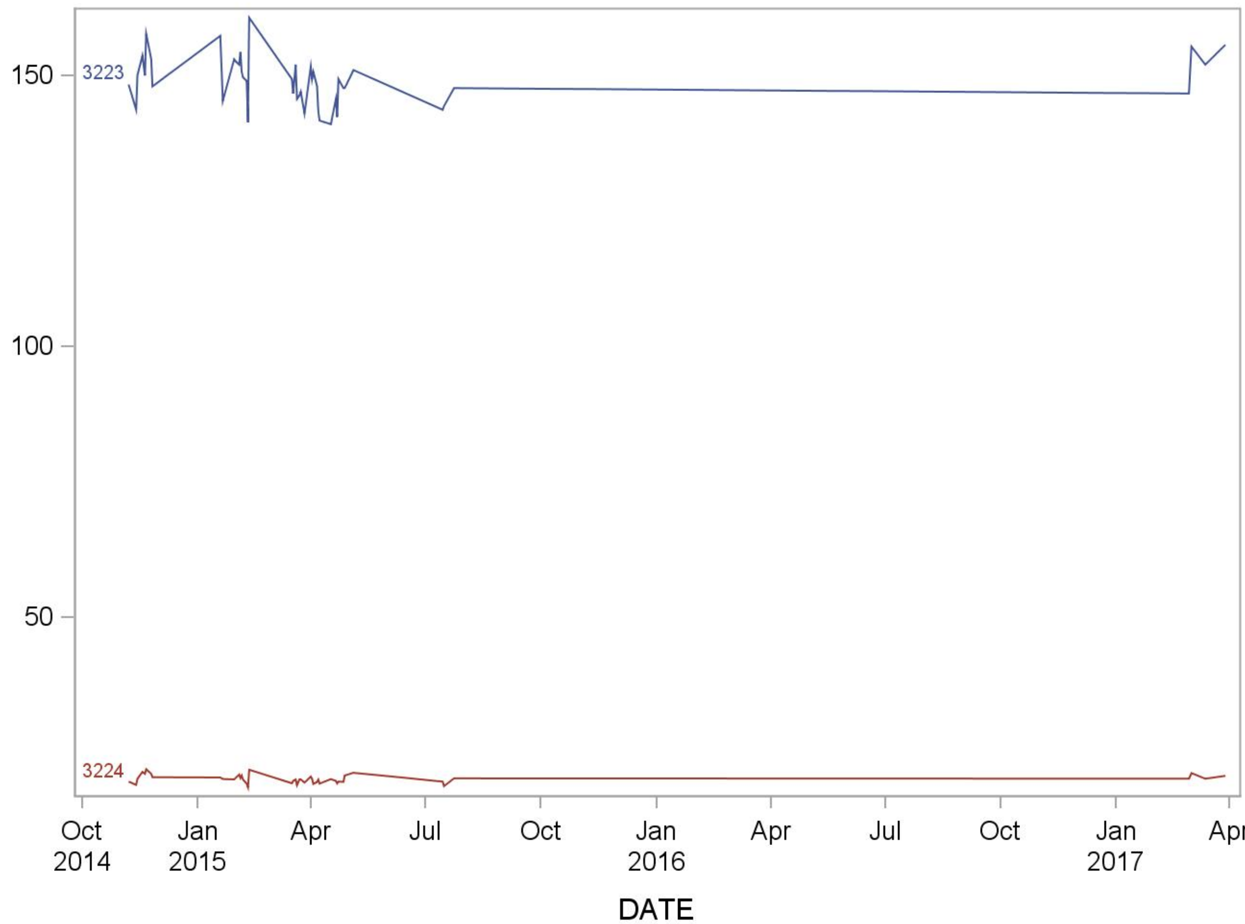
2013-2014 Summary Statistics and QC Chart for Harman (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	47	07NOV14	29MAR17	1647.943	30.68110	1.9
3224	47	07NOV14	29MAR17	383.2267	25.23367	6.6



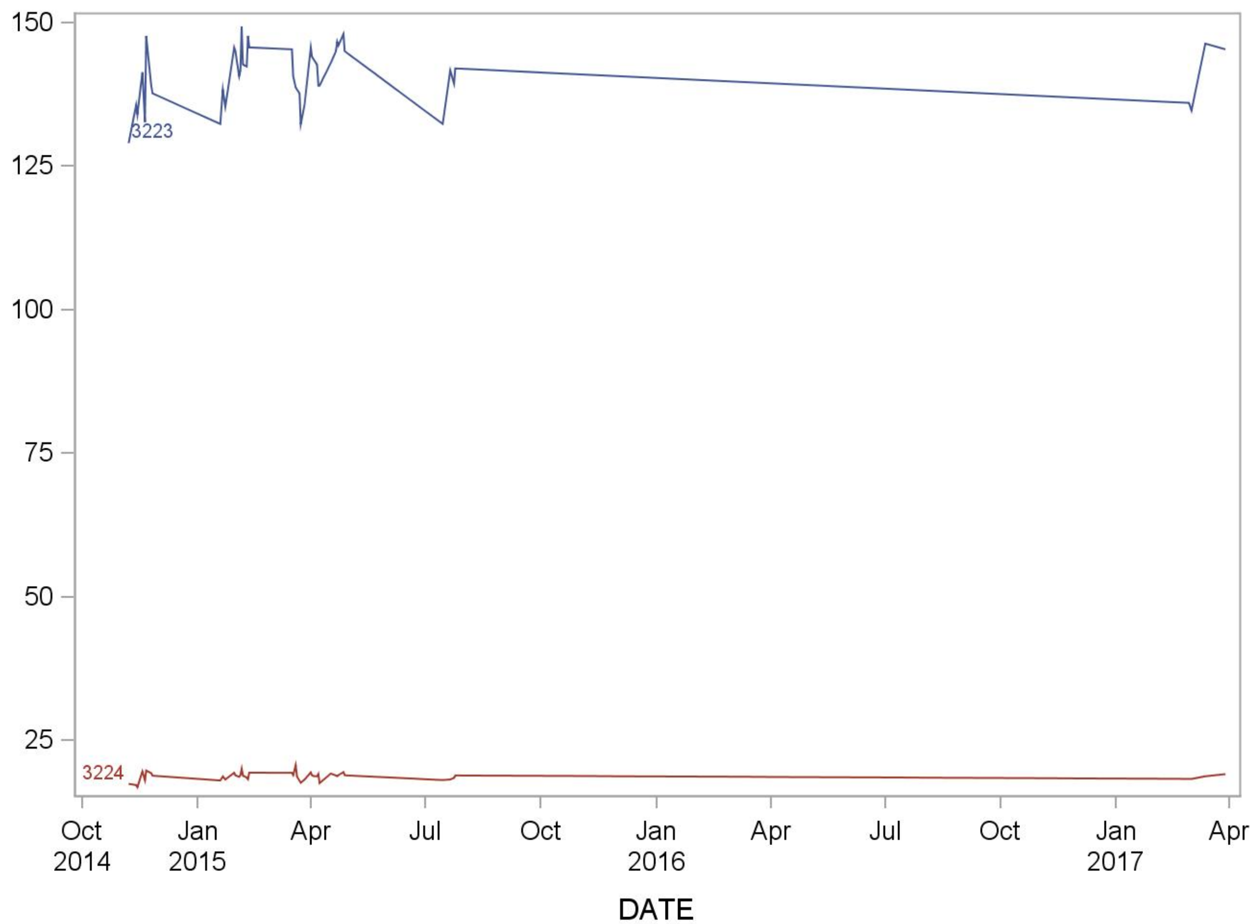
2013-2014 Summary Statistics and QC Chart for IQ (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	46	07NOV14	29MAR17	149.0217	4.57173	3.1
3224	46	07NOV14	29MAR17	20.07382	0.77000	3.8



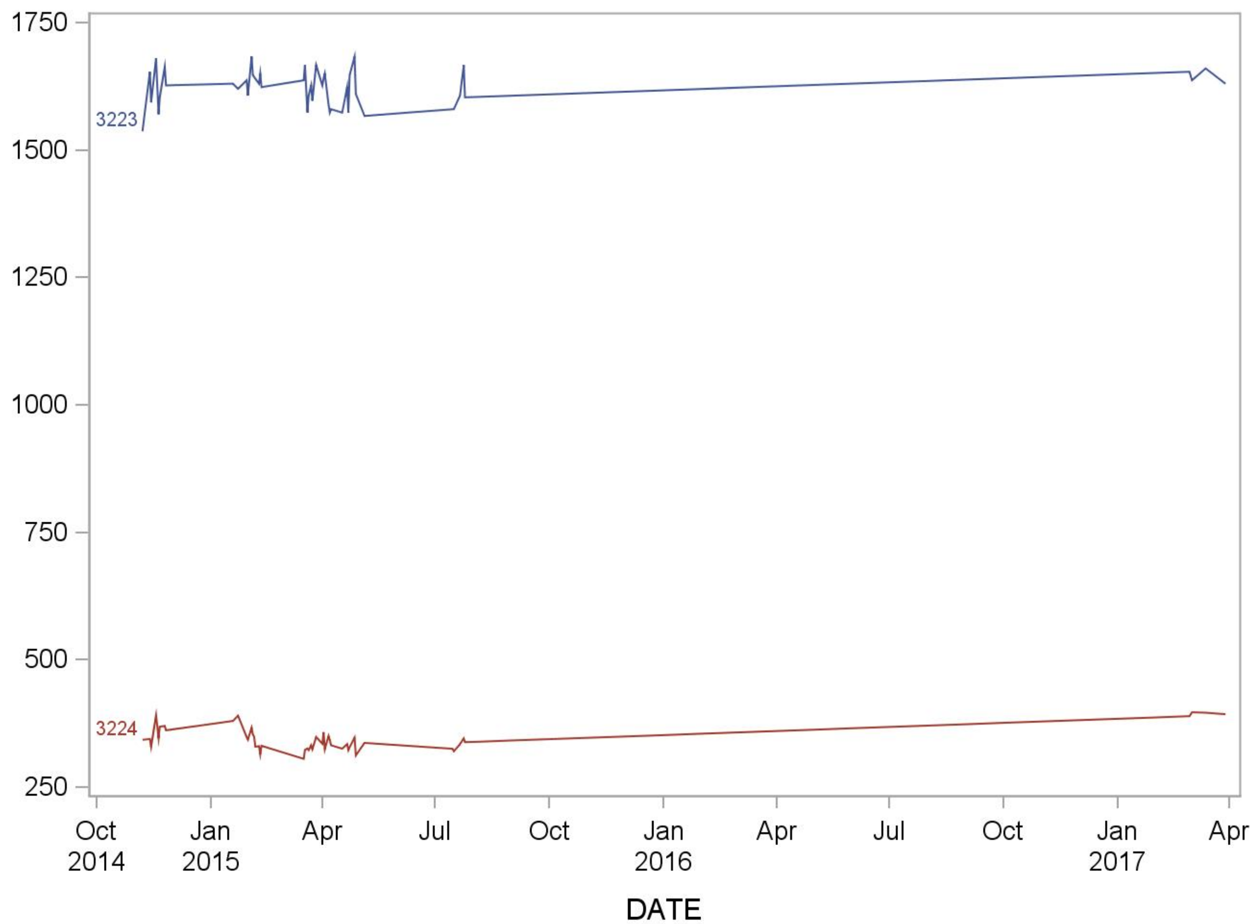
2013-2014 Summary Statistics and QC Chart for MeA-a-C (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	47	07NOV14	29MAR17	140.8014	5.0436	3.6
3224	47	07NOV14	29MAR17	18.6840	0.7308	3.9



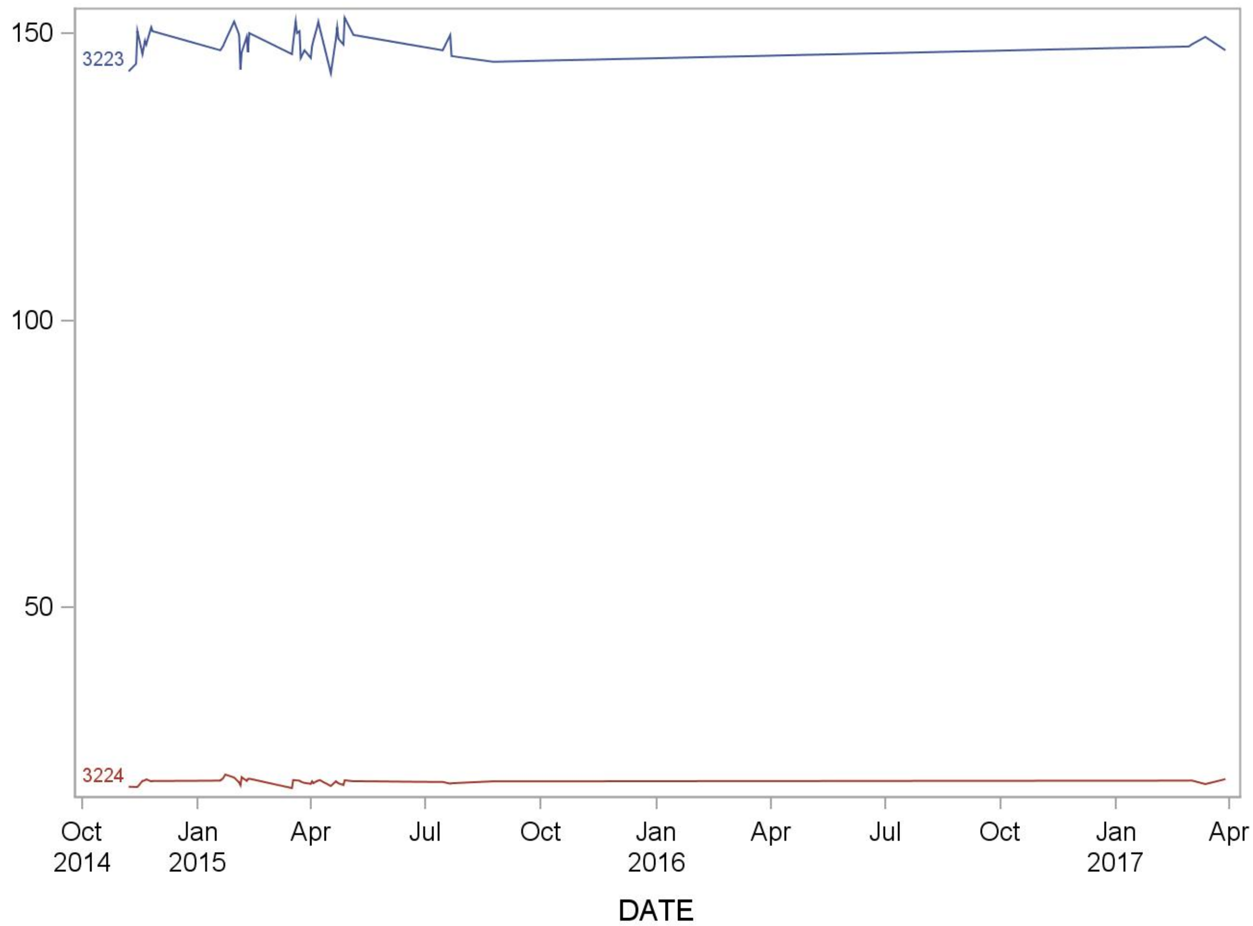
2013-2014 Summary Statistics and QC Chart for Norharman (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	48	07NOV14	29MAR17	1622.153	35.32015	2.2
3224	48	07NOV14	29MAR17	345.1531	23.88361	6.9



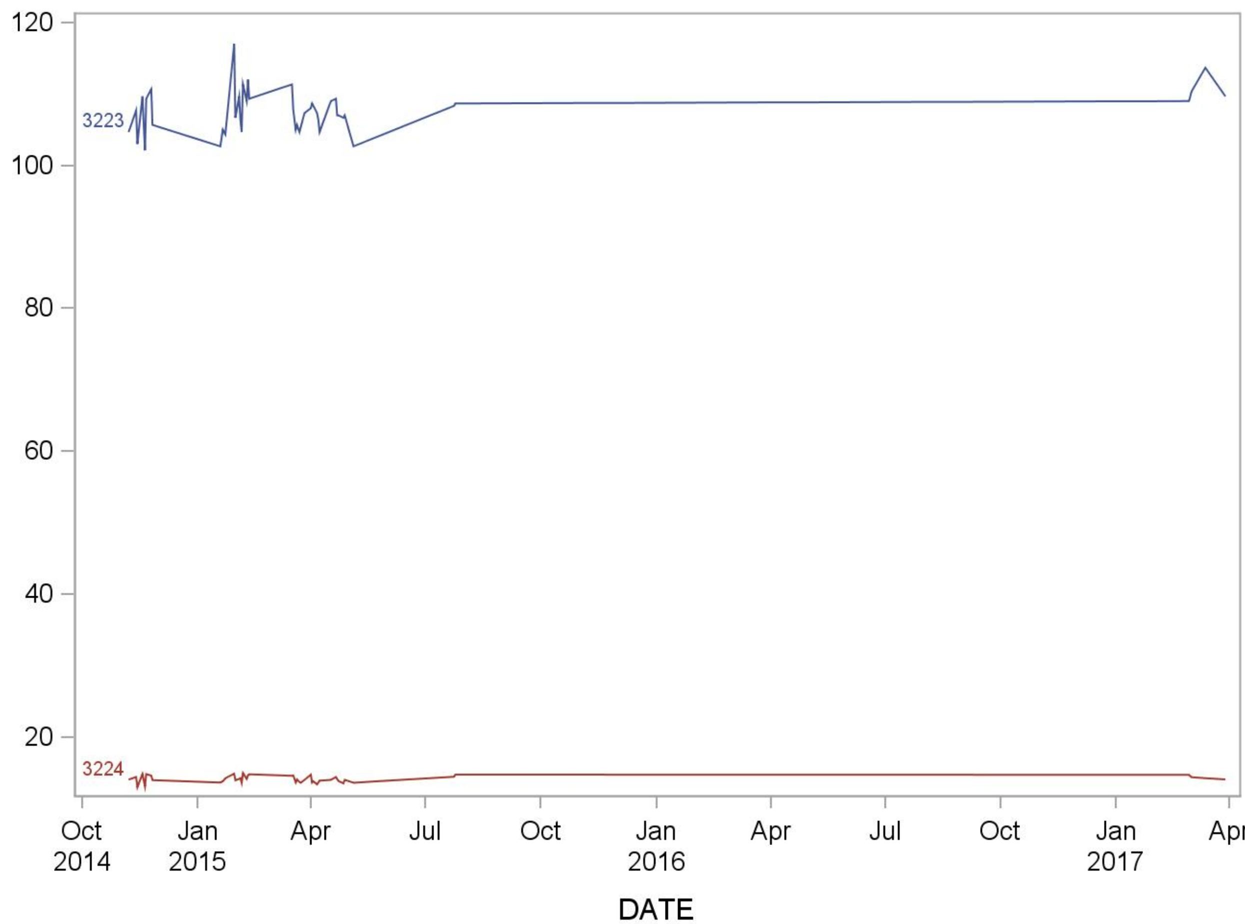
2013-2014 Summary Statistics and QC Chart for Ph1P (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	46	07NOV14	29MAR17	148.2971	2.3823	1.6
3224	46	07NOV14	29MAR17	19.6873	0.4772	2.4



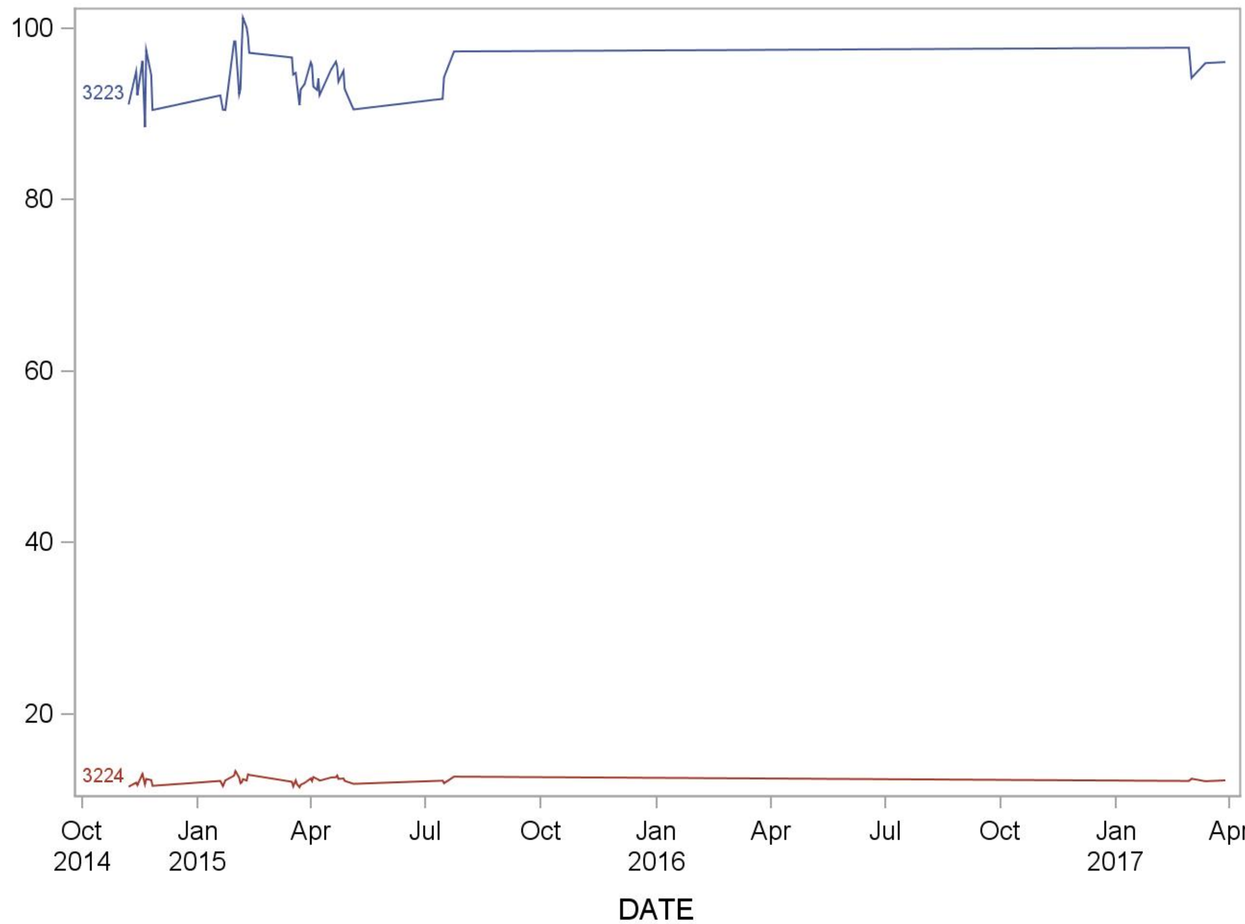
2013-2014 Summary Statistics and QC Chart for Trp-P-1 (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	46	07NOV14	29MAR17	107.6022	2.9742	2.8
3224	46	07NOV14	29MAR17	14.0716	0.4887	3.5



2013-2014 Summary Statistics and QC Chart for Trp-P-2 (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3223	47	07NOV14	29MAR17	94.5277	2.7922	3.0
3224	47	07NOV14	29MAR17	12.2255	0.4259	3.5



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Appendix A

Stock reagent preparation

1. Stock solutions for sample preparation

5% Ammonia hydroxide in Methanol (V/V) –

Combine 25mL of ammonium hydroxide (Optima™, A470-250, Fisher Chemical; NH_3 in H_2O w/w=21, or equivalent) with 475mL of methanol using a graduated cylinder. Store in fume hood.

2% Formic Acid in Methanol (V/V) –

Combine 10mL formic acid with 490 mL of methanol using a graduated cylinder. Store in fume hood

30% methonal in water (V/V) with 2% Ammonia hydroxide (V/V) –

Combine 10mL of ammonium hydroxide (Optima™, A470-250, Fisher Chemical; NH_3 in H_2O w/w=21, or equivalent) with 150mL of methanol and bring up to 500 mL with water by using a graduated cylinder. Store in fume hood.

2. Stock solution for HPLC mobile phase

0.05% Ammonia hydroxide in water (V/V) –

Combine 0.5 mL ammonium hydroxide solution (Sigma, 44273-10X1ML, NH_3 in H_2O w/w=25) with 1000 mL of water using a graduated cylinder. Store in fume hood.

Appendix B

Calibration materials

The methanol stock solutions were prepared for use in March, 2013 and stored in approximately -20 ^{o}C freezer in 103/3318. The internal standard spike solutions and working standards are prepared as needed. A total of 11 levels of calibration standards and internal standard spiking solution were prepared in 10% methanol in water containing 0.1% formic acid, and both stored in approximately -70 ^{o}C freezer.

Standard name		Purity	Method for purity	Catalog #
1-Methyl-9H-pyrido[3,4-b]indole	(Harman)	>99%	^{1}H NMR, MS & elemental analysis	H105000¶
9H-Pyrido[3,4-b]indole	(NorHarman)	>99%	^{1}H NMR, MS & elemental analysis	N700000¶
3-amino-1, 4-dimethyl-5H-pyrido [4,3-b]indole	(Trp-P-1·CH3COOH)	>99%	^{1}H NMR, MS & elemental analysis	A607500¶
3-amino-1-methyl-5H-pyrido [4,3-b] indole	(Trp-P-2· CH3COOH)[A]	>99%	^{1}H NMR, MS & elemental analysis	A618001¶
2-amino-9H-pyrido[2,3-b] indole	(AC) [B]	>99%	^{1}H NMR, MS & elemental analysis	A629000¶
2-amino-3-methyl-9H-pyrido[2,3-b] indole	(MeAC)	>99%	^{1}H NMR, MS & elemental analysis	A617500¶
2-amino-6-methyldipyrido[1,2-A:3',2'-D]imidazole	(Glu-P-1·HCl) [C]	95.3%	^{1}H NMR, MS & elemental analysis	A616100¶
2-aminodipyrido[1,2-a:3',2-D]imidazole	(Glu-P-2·2HCl) [D]	>99%	^{1}H NMR, MS & elemental analysis	A608801¶
2-amino-3-methyl-3H-imidazo[4,5-f]quinolone	(IQ)	>99%	^{1}H NMR, MS & elemental analysis	A616500¶
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine	(PhIP)	>99%	^{1}H NMR, MS & elemental analysis	A617000¶

A: with12.06% water content
B: with 1.7% water content
C: with 8.72% water content
D: with 7.47% water content

Internal standard name		Purity	Method for purity	Catalog #
1-Methyl-9H-pyrido[3,4-b]indole-13C2,15N	(Harman-13C2,15N)	97%	^{1}H NMR & MS	H105002¶
9H-Pyrido[3,4-b]indole-d7	(NorHarman-d7)	98%	^{1}H NMR & MS	N700002¶
3-amino-1, 4-dimethyl-5H-pyrido [4,3-b]indole - 13C2,15N	(Trp-P-1·CH3COOH -13C2,15N)	98%	^{1}H NMR & MS	A607502¶
3-amino-1-methyl-5H-pyrido [4,3-b] indole - 13C2,15N	(Trp-P-2· CH3COOH -13C2,15N)	97%	^{1}H NMR & MS	A618002¶
2-amino-9H-pyrido[2,3-b] indole-15N3	(AC-15N3)	98%	^{1}H NMR & MS	A629002¶
2-amino-3-methyl-9H-pyrido[2,3-b] indole-d3	(MeAC-d3)	98%	^{1}H NMR & MS	A617502¶
2-amino-6-methyldipyrido[1,2-A:3',2'-D]imidazole - 13C3	(Glu-P-1·HCl·H2O - 13C3)	95%	^{1}H NMR & MS	A616102¶
2-aminodipyrido[1,2-a:3',2-D]imidazole -13C2,15N	(Glu-P-2·HCl - 13C2,15N)	93.4%	^{1}H NMR & MS	SC-358861§
2-amino-3-methyl-3H-imidazo[4,5-f]quinolone-d3	(IQ-d3)	98%	^{1}H NMR & MS	A616720¶
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-d3	(PhIP-d3)	99%	^{1}H NMR & MS	A617350¶

¶ :Toronto Research Chemicals
§ : Santa Cruz Biotechnology

<u>Original stocks</u>:

Accurately measure appropriate amount of HCAA powders and dissolve them in methanol as the following table to obtain the original stock solution:

			MeOH	Conc.
Standard name	M.W.	mg	mL	µg/ml
Harman	182.22	10.094	25	403.76
NorHarman	168.19	10.215	25	408.60
Trp-P-1·CH3COOH	271.31	10.222	25	408.88
Trp-P-2· CH3COOH [a]	257.29	10.12	25	404.80
AC [b]	183.2	10.035	25	401.40
MeAC	197.24	10.057	25	402.28
Glu-P-1·HCl [c]	234.7	10.086	25	403.44
Glu-P-2·2HCl [d]	257.12	5.019	25	200.76
PhIP	224.26	10.055	25	402.20

a: with12.06% water content
b: with 1.7% water content
c: with 8.72% water content
d: with 7.47% water content

For IQ, 97.63 mg of powder is dissolved in 100 mL of methanol. 75 mL of 100 mL solution is further diluted with methanol into 250 mL final stock solution at 292.89 µg/ml as the following tables

			MeOH	Conc.
Standard name	M.W.	mg	mL	µg /ml
IQ	198.22	97.63	100	976.30

Dilution

		976.30 µg /ml	MeOH	Conc.
Standard name	M.W.	mL	mL	µg /ml
IQ	198.22	75	250	292.89

Appropriate amount of isotope-labeled HCAA are dissolved in methanol to obtain original stock solutions of isotope-labeled internal standards as follows:

ISTD	Original Stock Conc
	ug/ml
Harman	60.93
NorHarman	21.73
AC	22.50
MeAC	11.02
Trp-P-1·CH3COOH	8.80
Trp-P-2· CH3COOH	62.09
Glu-P-1·HCl·H2O	48.76

Glu-P-2·2HCl	42.34
IQ	46.88
PhIP	58.18

Working Stocks:

Dilute HCAA original stock solution at appropriate ratio with 30% methanol in water (with 0.1% formic acid) to obtain Solution A:

	Original Stock Conc	Volume of Original Stock	Final volume	Solution A
	ug/ml	mL	mL	ug/ml
Harman	403.76	N/A	N/A	403.76
NorHarman	408.6	N/A	N/A	408.6
AC	401.4	5	100	20.07
MeAC	402.28	5	100	20.114
Trp-P-1·CH3COOH	408.88	5	100	20.444
Trp-P-2· CH3COOH	404.8	5	100	20.24
Glu-P-1·HCl·H2O	403.44	5	100	20.172
Glu-P-2·2HCl	200.76	10	100	20.076
IQ	292.89	7	100	20.503
PhIP	402.2	5	100	20.11

Mix and dilute Solution A of all HCAA at appropriate ratio with 30% methanol in water (with 0.1% formic acid) to obtain Solution B:

	Solution A Conc	Volume of Solution A	Final volume	Solution B Conc
	µg/ml	mL	mL	µg/ml
Harman	403.76	2.477	100	10
NorHarman	408.6	2.447	100	10
AC	20.07	4.983	100	1
MeAC	20.114	4.972	100	1
Trp-P-1·CH3COOH	20.444	4.891	100	1
Trp-P-2· CH3COOH	20.24	4.941	100	1
Glu-P-1·HCl·H2O	20.172	4.957	100	1
Glu-P-2·2HCl	20.076	4.981	100	1
IQ	20.503	4.877	100	1
PhIP	20.11	4.973	100	1

Dilute Solution B at ratio of 10:1 with 30% methanol in water (with 0.1% formic acid) to obtain Solution C:

	SolutionB Conc	Volume of Original Stock	Final volume	Solution C
	ug/ml	mL	mL	ug/ml
Harman	10	10.0	100	1
NorHarman	10	10.0	100	1
AC	1	10.0	100	0.1
MeAC	1	10.0	100	0.1
Trp-P-1·CH3COOH	1	10.0	100	0.1
Trp-P-2· CH3COOH	1	10.0	100	0.1
Glu-P-1·HCl·H2O	1	10.0	100	0.1
Glu-P-2·2HCl	1	10.0	100	0.1
IQ	1	10.0	100	0.1
PhIP	1	10.0	100	0.1

Dilute Solution C at ratio of 10:1 with 30% methanol in water (with 0.1% formic acid) to obtain Solution D:

	Solution C Conc	Volume of Solution C	Final volume	Solution D Conc
	µg/ml	mL	mL	ng/ml
Harman	1	10.0	100	100
NorHarman	1	10.0	100	100
AC	0.1	10.0	100	10
MeAC	0.1	10.0	100	10
Trp-P-1·CH3COOH	0.1	10.0	100	10
Trp-P-2· CH3COOH	0.1	10.0	100	10
Glu-P-1·HCl·H2O	0.1	10.0	100	10
Glu-P-2·2HCl	0.1	10.0	100	10
IQ	0.1	10.0	100	10
PhIP	0.1	10.0	100	10

Dilute HCAA (ISTD) original stock solution at appropriate ratio with 30% methanol in water (with 0.1% formic acid) to obtain Solution A (ISTD):

ISTD	Original Stock Conc	Volume of Original Stock	Final volume	Solution A ISTD

	µg/ml	mL	mL	µg/ml
Harman	60.93	2	10	12.19
NorHarman	21.73	5	10	10.87
AC	22.50	5	100	1.13
MeAC	11.02	10	100	1.10
Trp-P-1·CH3COOH	8.80	10	100	0.88
Trp-P-2· CH3COOH	62.09	2	100	1.24
Glu-P-1·HCl·H2O	48.76	3	100	1.46
Glu-P-2·2HCl	42.34	3	100	1.27
IQ	46.88	3	100	1.41
PhIP	58.18	2	100	1.16

Mix and dilute Solution A (ISTD) of all HCAA at appropriate ratio with 30% methanol in water (with 0.1% formic acid) to obtain <u>Solution B (ISTD):</u>

ISTD	Solution A ISTD	Volume of Original Stock	Final volume	Solution B ISTD
	ug/ml	mL	mL	ug/ml
Harman	12.19	2.46	100	0.30
NorHarman	10.87	2.76	100	0.30
AC	1.13	4.44	100	0.05
MeAC	1.10	4.54	100	0.05
Trp-P-1·CH3COOH	0.88	5.68	100	0.05
Trp-P-2· CH3COOH	1.24	4.03	100	0.05
Glu-P-1·HCl·H2O	1.46	6.84	100	0.10
Glu-P-2·2HCl	1.27	11.81	100	0.15
IQ	1.41	2.13	100	0.03
PhIP	1.16	2.15	100	0.025

Dilute Solution B (ISTD) solution at ratio of 100:1 with 10% methanol in water (with 0.1% formic acid) to obtain <u>internal standard spike solution</u>

ISTD	Solution B ISTD	Volume of Solution B (ISTD)	Final volume	Spike solution ISTD
	ng/ml	mL	mL	ng/ml
Harman	300	1	100	3
NorHarman	300	1	100	3
AC	50	1	100	0.5
MeAC	50	1	100	0.5
Trp-P-1·CH3COOH	50	1	100	0.5
Trp-P-2· CH3COOH	50	1	100	0.5
Glu-P-1·HCl·H2O	100	1	100	1

Glu-P-2·2HCl	150	1	100	1.5
IQ	30	1	100	0.3
PhIP	25	1	100	0.25

Below is a complete list of the HCAA standards:

Combine appropriate volumes of HCAA and ISTD working solution and dilute them with 30% methanol in water (with 0.1% formic acid) to obtain X10 HCAA calibrators:

	Working solution	Volume of	Final volume
		Working solutions	
		ml	mL
x10 Level 0	NA	0	50
x10 Level 1	Solution D	0.5	50
x10 Level 2	Solution D	1	50
x10 Level 3	Solution D	2	50
x10 Level 4	Solution C	0.5	50
x10 Level 5	Solution C	1	50
x10 Level 6	Solution C	2	50
x10 Level 7	Solution B	0.5	50
x10 Level 8	Solution B	0.75	50
x10 Level 9	Solution B	1	50
x10 Level 10	Solution B	2	50
ISTD in each Level	Solution B (ISTD)	5	50

Dilute X10 Calibrators with 10% methanol in water (with 0.1% formic acid) at ratio of 10: 1 to obtain X1 HCAA calibrators:

	X10 Calibrator	Volume	Final volume	Injection volume
		X10 Calibrator		X1 Calibrator
		ml	mL	µL
Level 0	x10 Level 0	10	100	10
Level 1	x10 Level 1	10	100	10
Level 2	x10 Level 2	10	100	10
Level 3	x10 Level 3	10	100	10
Level 4	x10 Level 4	10	100	10
Level 5	x10 Level 5	10	100	10

Level 6	x10 Level 6	10	100	10
Level 7	x10 Level 7	10	100	10
Level 8	x10 Level 8	10	100	5
Level 9	x10 Level 9	10	100	5
Level 10	x10 Level 10	10	100	2.5

Appendix C

Sample Preparation

HCAA sample clean-up procedure

1 .Sample hydrolysis

A. Remove samples from freezer and allow them to thaw completely at room temperature.
B. Pipet 50 µL of 10 N NaOH in to each well on a deep 96-well collection plate (CP#1).
C. Pipet 0.5 mL urine sample into each well
D. Pipet 50 µl ISTD into each well
E. Place CP#1 in oven that is set to 70 °C for 5 hours to hydrolyze

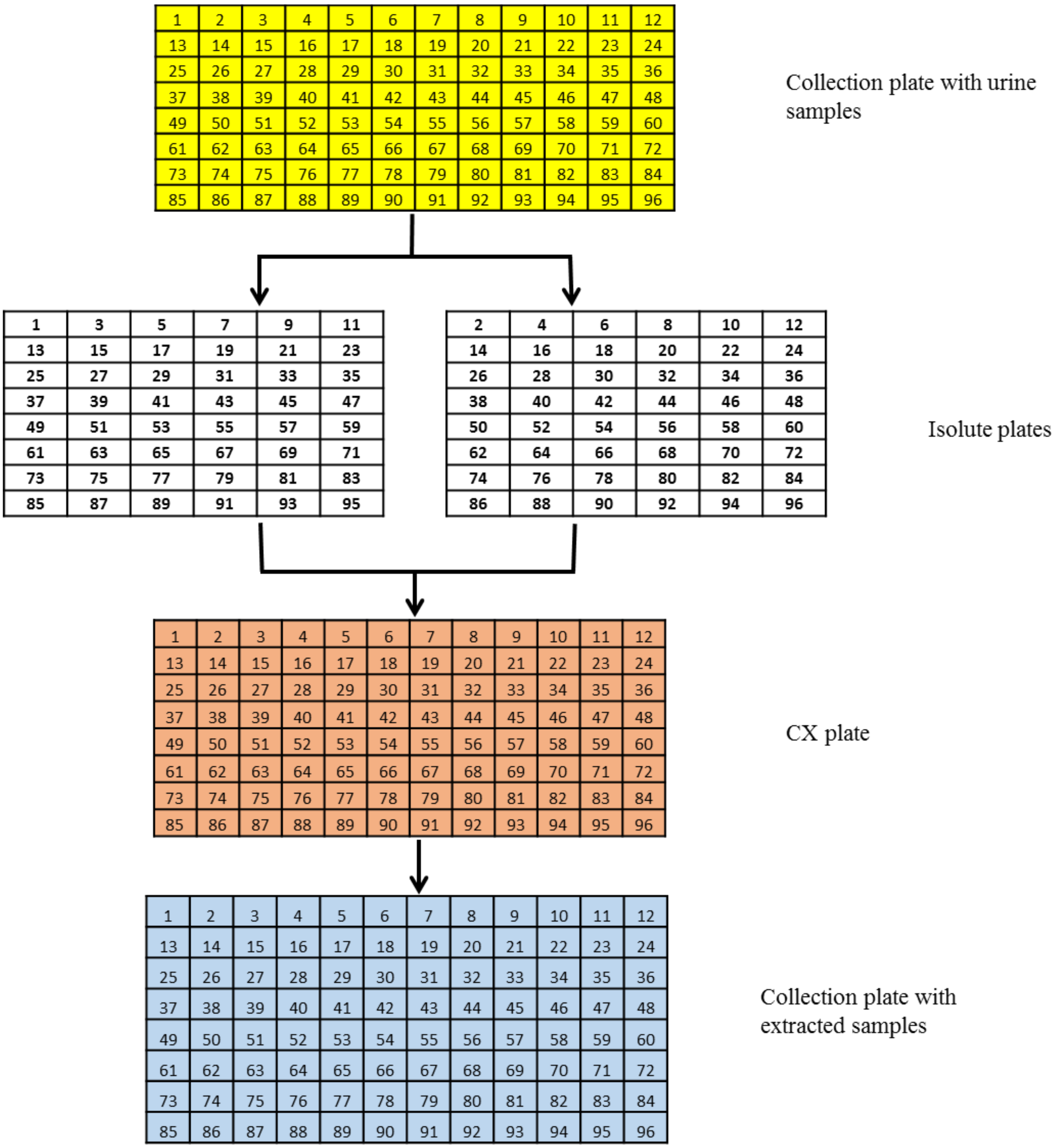
2. SPE by Isolute (Biotage)

A. Load samples on Isolute 96-well plate (300 µl to each of two adjacent wells)
B. Apply positive pressure to initiate loading and wait for sample to be completely absorbed.
C. Add 3 x 0.75 mL (for 96-well plate) of dichloromethane (DCM) to the SPE. Allow to be eluted by positive pressure. Collect DCM fraction in new Deep 48-well pate (CP#2)
D. Add 40 µl of Formic acid in to DCM in each well of CP#2 and Mix well (aspirate and dispense 3X).

3. SPE by CX (Biotage)

A. Condition the CX 96-well plate with 1 ml of 5% ammonia hydroxide in methanol, followed by 1 mL of methanol and 1 mL 2% formic acid in methanol.
B. Transfer acidified DCM collected in CP#2 on CX 96-well plate. Adjust the pressure so that the sample is drawn through the plate at a rate of about 0.5 ml/min.
C. Once all to the samples have been loaded, wash the SPE CX with 1 mL of 2% formic acid in methanol, 1 mL of water, and 1 mL of 30% methanol in water with 2% ammonia. Dry the SPE CX under positive N_2 pressure on SPE.
D. The analytes are eluted at a rate of about 0.5 ml/min with 1 mL of 5% ammonia hydroxide in methanol and apply under positive N_2 pressure on SPE to complete elution
E. Dry down methanol in CP#3 and the residue is reconstituted with 50 µl of 10% methanol in water with 0.1% formic acid.

Schematic procedure of sample preparation



Appendix D

Precision (RSD%) and accuracy (% of Nominal concentrations)

Analyte		Intraday						Interday				
	Nominal Concentration (pg/mL)	Determined Concentration (pg/mL)			Accuracy (%)	RSD (%)	Nominal Concentration (pg/mL)	Determined Concentration (pg/mL)			Accuracy (%)	RSD (%)
		n=8						n=6				
AC	150	151	±	2.3	101	1.5	150	148	±	5.7	98	3.9
	40	41	±	1.6	103	4.0	40	40	±	1.8	100	4.5
	10	10	±	0.2	101	1.7	10	10	±	0.5	100	5.0
MeAC	150	150	±	1.1	100	0.8	150	144	±	6.9	96	4.8
	40	40	±	0.8	101	2.0	40	38	±	2.1	96	5.5
	10	10	±	0.1	98	0.8	10	10	±	0.4	95	4.2
IQ	150	152	±	1.8	102	1.2	150	152	±	11.2	102	7.3
	40	42	±	1.0	105	2.4	40	41	±	3.2	103	7.7
	10	11	±	0.3	106	3.2	10	10	±	0.8	102	7.8
Trp-P-1	117	117	±	2.1	100	1.8	117	112	±	4.9	96	4.3
	31	32	±	1.8	101	5.7	31	30	±	1.7	96	5.7
	7.8	7.5	±	0.2	97	3.2	7.8	7.2	±	0.7	93	9.9
Trp-P-2	101	104	±	2.4	103	2.3	101	100	±	4.1	99	4.1
	27	28	±	1.2	104	4.3	27	26	±	1.5	98	5.8
	6.7	6.6	±	0.2	98	2.8	6.7	6.4	±	0.4	95	6.3
Glu-P-1	105	104	±	0.8	98	0.8	105	104	±	1.4	99	1.3
	28	28	±	0.2	100	0.8	28	28	±	0.4	98	1.4
	7.0	7.0	±	0.2	100	2.7	7.0	6.8	±	0.2	98	2.3
Glu-P-2	107	106	±	0.8	99	0.7	107	105	±	2.1	98	2.0
	29	29	±	0.2	100	0.8	29	28	±	0.7	97	2.6
	7.2	7.4	±	0.3	103	4.1	7.2	7.1	±	0.3	99	4.2
PhIP	150	149	±	1.8	100	1.2	150	147	±	3.9	98	2.7
	40	40	±	0.5	99	1.3	40	39	±	1.0	97	2.6
	10	10	±	0.2	100	2.0	10	10	±	0.4	96	4.2
		Intraday n=4						Interday n=3				
Harman	3530	3825	±	205.3	108	5.4	3530	3583	±	302.8	102	8.5
	313	335	±	18.1	107	5.4	313	341	±	32.6	109	9.6
NorHarman	2132	2120	±	37.4	99	1.8	2132	2171	±	118.9	102	5.5
	662	690	±	27.3	104	4.0	662	710	±	63.0	107	8.9
Harman [a]	4000	3816	±	205.2	95	5.4	4000	3832	±	176.8	96	4.6
	400	401	±	6.9	100	1.7	400	389	±	17.5	97	4.5
	100	100	±	2.0	100	2.0	100	97	±	5.5	97	5.7
NorHarman [a]	4000	4024	±	96.0	101	2.4	4000	4073	±	137.0	102	3.4
	400	416	±	3.6	104	0.9	400	418	±	16.8	104	4.0
	100	105	±	1.3	105	1.2	100	104	±	5.1	104	4.9

a: prepared in synthetic urine

Appendix E

Stability of HCAA

	Nominal concentrations (pg/ml)	Determined concentrations (pg/ml)								
		Room temperature for 24 hrs			Four freeze–thaw cycles			Auto-sampler for 24 hrs		
AC	150	149.7	±	3.1	142.7	±	2.9	152.7	±	1.5
	20	21.1	±	0.5	18.9	±	0.4	21.0	±	0.7
MeAC	150	151.3	±	2.1	139.0	±	2.0	163.7	±	3.2
	20	20.5	±	0.5	18.8	±	0.1	21.2	±	0.2
IQ	150	155.0	±	2.6	152.7	±	1.5	156.7	±	2.5
	20	21.6	±	0.7	21.1	±	0.2	20.8	±	0.7
Trp-P-1	150	149.3	±	2.5	149.3	±	3.8	152.3	±	2.1
	20	19.6	±	0.9	18.8	±	0.6	20.5	±	0.5
Trp-P-2	150	149.3	±	2.5	148.0	±	1.0	148.7	±	2.1
	20	19.6	±	0.2	18.6	±	0.2	19.5	±	0.3
Glu-P-1	150	151.7	±	6.1	148.3	±	4.2	153.3	±	2.1
	20	21.0	±	0.2	19.3	±	0.2	21.2	±	0.5
Glu-P-2	150	148.7	±	2.3	146.0	±	2.6	150.0	±	1.7
	20	19.9	±	0.6	20.1	±	0.5	21.1	±	0.5
PhIP	150	152.3	±	4.9	149.7	±	2.1	151.3	±	4.7
	20	21.2	±	0.5	20.7	±	0.5	20.0	±	0.2
Harman	1487	1563	±	21	1507	±	23	1493	±	15.3
	257	282	±	9.5	266	±	9.5	261	±	8.5
NorHarman	1493	1580	±	10	1537	±	5.8	1477	±	15.3
	287	278	±	2.9	297	±	11	272	±	5.1

Appendix F

Ruggedness Tests

PhIP

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	147	159	153		158	153	160		154	154	159
	153	151	152		158	157	165		138	156	155
	161	149	165		156	157	160		153	157	154
Mean	154	153	157	Mean	157	156	162	Mean	148	156	156
SD	7	5	7	SD	1	2	3	SD	9	2	3

PhIP

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	42.9	41	36.9		43	42	40		36.5	41.6	42.9
	41.4	41.5	43.4		41	43	43		41.6	42.1	42.6
	41.8	39	38.9		42	41	40		40.1	39.9	41.2
Mean	42	41	40	Mean	42	42	41	Mean	39	41	42
SD	0.8	1.3	3.3	SD	1.1	0.8	1.6	SD	2.6	1.2	0.9

Glu-P-2

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	100	105	104		103	102	104		104	99.5	104
	100	105	103		104	103	103		104	104	105
	103	105	105		105	100	101		101	102	103
Mean	101	105	104	Mean	104	102	103	Mean	103	102	104
SD	2	0	1	SD	1	2	2	SD	2	2	1

Glu-P-2

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	26.6	26.8	27.8		27	26	28		27.2	26.3	26.9
	27	27	27.8		26	27	27		27.6	27	26.6
	27	28.2	27.7		28	28	27		27.7	27.2	27.2
Mean	27	27	28	Mean	27	27	27	Mean	28	27	27
SD	0.2	0.8	0.1	SD	0.7	1.0	0.4	SD	0.3	0.5	0.3

Glu-P-1

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	107	110	111		111	110	110		110	108	112
	100	108	108		113	107	113		108	109	109
	106	108	111		110	110	109		108	110	109
Mean	104	109	110	Mean	111	109	111	Mean	109	109	110
SD	4	1	2	SD	2	2	2	SD	1	1	2

Glu-P-1

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	26.9	27.7	28.6		29	29	29		28.6	27.7	27.9
	28.2	28.4	29.4		29	29	29		28.8	28.6	28.2
	27.8	28.7	28.5		29	29	29		28.9	29.5	28.3
Mean	28	28	29	Mean	29	29	29	Mean	29	29	28
SD	0.7	0.5	0.5	SD	0.4	0.2	0.2	SD	0.2	0.9	0.2

Trp-P-2

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	103	113	108		110	115	116		114	111	111
	101	112	112		114	110	112		119	124	109

	109	115	116		121	108	110		113	113	108
Mean	104	113	112	Mean	115	111	113	Mean	115	116	109
SD	4	2	4	SD	6	4	3	SD	3	7	2

Trp-P-2

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	30.8	26.6	33.9		29	30	29		28.8	28.6	30
	29.6	29.9	29.4		29	30	30		29.8	29.7	29.1
	28.6	30.9	30.1		30	30	28		29.7	29.7	28.1
Mean	30	29	31	Mean	29	30	29	Mean	29	29	29
SD	1.1	2.3	2.4	SD	0.5	0.3	0.9	SD	0.6	0.6	1.0

Trp-P-1

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	114	113	113		110	114	112		114	116	114
	101	111	124		107	123	114		98.2	116	114
	108	113	124		119	112	108		117	116	116
Mean	108	112	120	Mean	112	116	111	Mean	110	116	115
SD	7	1	6	SD	6	6	3	SD	10	0	1

Trp-P-1

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	31.5	28.4	27.2		29	32	31		29.6	30.4	26.9
	29	28.3	30.4		30	30	31		30	27.7	30.9
	29.3	29.9	29.5		31	29	29		31.4	29.1	28.7
Mean	30	29	29	Mean	30	30	30	Mean	30	29	29
SD	1.4	0.9	1.7	SD	1.0	1.4	0.8	SD	0.9	1.4	2.0

IQ

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	146	155	149		162	164	163		156	130	168
	135	136	149		171	136	138		144	164	150
	163	165	140		144	152	164		148	135	136
Mean	148	152	146	Mean	159	151	155	Mean	149	143	151
SD	14	15	5	SD	14	14	15	SD	6	18	16

IQ

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	39.8	36.8	39.9		42	42	39		40	37.2	37.3
	39.6	37.3	38.9		38	38	41		40	39.2	37.8
	40	43.2	42.2		45	44	38		38.2	43	43.3
Mean	40	39	40	Mean	42	41	39	Mean	39	40	39
SD	0.2	3.6	1.7	SD	3.3	3.0	1.5	SD	1.0	2.9	3.3

MeAC

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3
	pg/ml				pg/ml				pg/ml		
	143	148	150		147	145	146		152	158	146
	151	152	147		149	149	160		151	150	154
	141	140	162		160	155	150		144	153	155
Mean	145	147	153	Mean	152	150	152	Mean	149	154	152
SD	5	6	8	SD	7	5	7	SD	4	4	5

MeAC

	Time for hydrolysis (hr)				% MeOH in H_2O for washing				% FA in MeOH for washing		
	4	5	6		20	30	40		1	2	3

	pg/ml		
	39.1	38.2	44.3
	37.8	40.7	39.8
	38.9	35.2	38
Mean	39	38	41
SD	0.7	2.8	3.2

	pg/ml		
	39	40	41
	40	42	42
	38	38	41
Mean	39	40	41
SD	1.0	1.9	0.6

	pg/ml		
	38.9	41.7	42.4
	41	40.2	40.6
	38.3	38.9	37.9
Mean	39	40	40
SD	1.4	1.4	2.3

AC

	Time for hydrolysis (hr)		
	4	5	6
	pg/ml		
	143	148	141
	140	143	143
	138	145	146
Mean	140	145	143
SD	3	3	3

	% MeOH in H_2O for washing		
	20	30	40
	pg/ml		
	144	150	148
	148	140	146
	151	145	144
Mean	148	145	146
SD	4	5	2

	% FA in MeOH for washing		
	1	2	3
	pg/ml		
	147	142	151
	162	144	143
	141	141	141
Mean	150	142	145
SD	11	2	5

AC

	Time for hydrolysis (hr)		
	4	5	6
	pg/ml		
	36.5	37.8	35
	38.2	38.4	39.4
	37.6	36.2	39.6
Mean	37	37	38
SD	0.9	1.1	2.6

	% MeOH in H_2O for washing		
	20	30	40
	pg/ml		
	37	37	38
	37	39	37
	36	37	37
Mean	37	38	37
SD	0.5	1.1	0.2

	% FA in MeOH for washing		
	1	2	3
	pg/ml		
	36.6	36.9	36.8
	37.7	36.5	36.1
	37	37	36
Mean	37	37	36
SD	0.6	0.3	0.4

Norharman

	Time for hydrolysis (hr)		
	4	5	6
	pg/ml		
	1640	1750	1670
	1560	1650	1800
	1840	1840	1680
Mean	1680	1747	1717
SD	144	95	72

	% MeOH in H_2O for washing		
	20	30	40
	pg/ml		
	1610	1630	1590
	1570	1420	1490
	1500	1560	1580
Mean	1560	1537	1553
SD	56	107	55

	% FA in MeOH for washing		
	1	2	3
	pg/ml		
	1550	1430	1650
	1460	1590	1560
	1540	1390	1430
Mean	1517	1470	1547
SD	49	106	111

Norharman

	Time for hydrolysis (hr)		
	4	5	6
	pg/ml		
	686	676	716
	651	639	677
	649	694	715
Mean	662	670	703
SD	20.8	28.0	22.2

	% MeOH in H_2O for washing		
	20	30	40
	pg/ml		
	510	494	470
	462	465	508
	492	515	487
Mean	488	491	488
SD	24.2	25.1	19.0

	% FA in MeOH for washing		
	1	2	3
	pg/ml		
	479	457	468
	470	472	471
	456	490	499
Mean	468	473	479
SD	11.6	16.5	17.1

Harman

	Time for hydrolysis (hr)		
	4	5	6
	pg/ml		
	1700	1770	1790
	1670	1720	1780
	1720	1730	1810
Mean	1697	1740	1793
SD	25	26	15

	% MeOH in H_2O for washing		
	20	30	40
	pg/ml		
	1460	1500	1530
	1520	1450	1480
	1520	1460	1450
Mean	1500	1470	1487
SD	35	26	40

	% FA in MeOH for washing		
	1	2	3
	pg/ml		
	1500	1460	1520
	1510	1460	1490
	1440	1480	1480
Mean	1483	1467	1497
SD	38	12	21

Harman

	Time for hydrolysis (hr)		
	4	5	6
	pg/ml		
	688	672	724
	698	700	731
	685	703	718
Mean	690	692	724
SD	6.8	17.1	6.5

	% MeOH in H_2O for washing		
	20	30	40
	pg/ml		
	463	467	466
	455	461	465
	463	459	465
Mean	460	462	465
SD	4.6	4.2	0.6

	% FA in MeOH for washing		
	1	2	3
	pg/ml		
	454	457	456
	473	466	459
	473	467	454
Mean	467	463	456
SD	11.0	5.5	2.5

% of ammonium hydroxide in LC mobile phase

High concentrations

	PhIP			Glu-P-2			Glu-P-1			Trp-P-2			Trp-P-1		
	NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O		
	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08
	ng/ml			ng/ml			ng/ml			ng/ml			ng/ml		
Mean	1.52	1.50	1.51	1.07	1.08	1.08	1.17	1.18	1.17	1.13	1.15	1.13	1.18	1.18	1.17
SD	0.02	0.02	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.03	0.02	0.02	0.02	0.01

	IQ			MeAC			AC			Norharman			Harman		
	NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O		
	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08
	ng/ml			ng/ml			ng/ml			ng/ml			ng/ml		
Mean	1.52	1.50	1.50	1.51	1.50	1.51	1.52	1.50	1.49	15.10	15.03	14.80	15.13	14.97	15.00
SD	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.10	0.25	0.17	0.55	0.32	0.36

Low concentrations

	PhIP			Glu-P-2			Glu-P-1			Trp-P-2			Trp-P-1		
	NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O		
	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08
	ng/ml			ng/ml			ng/ml			ng/ml			ng/ml		
Mean	0.20	0.20	0.20	0.14	0.14	0.14	0.16	0.16	0.16	0.15	0.15	0.15	0.16	0.16	0.16
SD	0.002	0.003	0.003	0.002	0.003	0.003	0.001	0.002	0.002	0.001	0.008	0.008	0.002	0.001	0.001

	IQ			MeAC			AC			Norharman			Harman		
	NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O			NH4OH % in H2O		
	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08	0.03	0.05	0.08
	ng/ml			ng/ml			ng/ml			ng/ml			ng/ml		
Mean	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	1.97	1.92	2.02	2.01	1.96	2.02
SD	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.029	0.012	0.012	0.050	0.020	0.020

Appendix G

Urine VS Water calibration curves

	Linearity ranges	Urine concentration ranges		Calibration curves	Difference in slope
	on-column (pg)	pg/ml	R^2		%
AC	0-40	0-400	0.999	Water: y=0.975x+0.00172 Urine: y=1.00x+0.00392	2.5
MeAC	0-20	0-200	0.999	Water: y=0.857x+0.00031 Urine: y=0.873x-9.39e-5	1.8
IQ	0-20	0-200	0.999	Water: y=1.24x+0.00568 Urine: y=1.25x+0.0033	0.8
Trp-P-1	0-20	0-200	0.999	Water: y=0.984x+0.00153 Urine: y=0.989x+0.00062	0.5
Trp-P-2	0-20	0-200	1.000	Water: y=0.924x+0.00085 Urine: y=0.955x+0.00023	3.3
Glu-P-1	0-20	0-200	1.000	Water: y=1.22x+0.00167 Urine: y=1.23x+0.00246	0.81
Glu-P-2	0-20	0-200	1.000	Water: y=1.10x+0.00027 Urine: y=1.11x-0.00464	0.9
PhIP	0-20	0-200	0.998	Water: y=1.04x+0.00982 Urine: y=1.07x+0.00983	2.8
Harman	1-400	10 ~4000	1.000	Water: y=1.14 x+0.00458 Urine: y=1.17x+4.05	2.6
NorHarman	1-400	10 ~4000	0.999	Water: y=1.43x+0.00875 Urine: y=1.42x+2.03	0.7