Laboratory Procedure Manual

Analyte: **Caffeine and Caffeine Metabolites**

Matrix: **Urine**

Method: **UHPLC-ESI-MS/MS**

Method No: 4063.08

Revised: March 2018

as performed by: Nutritional Biomarkers Branch (NBB)
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Important Information for Users

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 testing the items listed in the following table.

This method file describes measurements of U2CF_H_R.

File Name	Variable Name	Analyte Description (and SI units)
U2CF_H_R	UR2AMU	5-acetylamino-6-amino-3-methyluracil (AAMU), Urine 2nd collection (umol/L)
	UR2MU1	1-methyluric acid, Urine 2nd Collection (umol/L)
	UR2MU2	3-methyluric acid, Urine 2nd Collection (umol/L)
	UR2MU3	7-methyluric acid, Urine 2nd Collection (umol/L)
	UR2MU4	1,3-Dimethyluric acid, Urine 2nd Collection (umol/L)
	UR2MU5	1,7-Dimethyluric acid, Urine 2nd Collection (umol/L)
	UR2MU6	3,7-Dimethyluric acid, Urine 2nd Collection (umol/L)
	UR2MU7	1,3,7-Trimethyluric acid, Urine 2nd Collection (umol/L)
	UR2MX1	1-methylxanthine, Urine 2nd Collection (umol/L)
	UR2MX2	3-methylxanthine, Urine 2nd Collection (umol/L)
	UR2MX3	7-methylxanthine, Urine 2nd Collection (umol/L)
	UR2MX4	1,3-dimethylxanthine (theophylline), Urine 2nd Collection (umol/L)
	UR2MX5	1,7-dimethylxanthine (paraxanthine), Urine 2nd Collection (umol/L)
	UR2MX6	3,7-dimethylxanthine (theobromine), Urine 2nd Collection (umol/L)
	UR2MX7	1,3,7-trimethylxanthine (caffeine), Urine 2nd Collection (umol/L)

1. 0verview

A. Clinical Relevance

Caffeine is an alkaloid that is known to have psychoactive stimulatory effects. Caffeine naturally occurs in plants (e.g., coffee beans, tea leaves, cocoa beans, cola nuts), and the dietary consumption of caffeine originates mainly from derivative beverages (e.g., coffee, tea, cola drinks) and foods (e.g., chocolate) [1-2]. Caffeine is also used as a food additive in beverages (e.g., caffeinated soft drinks, “energy” drinks) and as a drug either on its own or as an adjuvant in certain medications (e.g., analgesics) [3-5]. Given caffeine’s high prevalence in the worldwide diet at behaviorally active doses, significant scientific interest in the health effects of caffeine has developed. As a psychoactive stimulant, the behavioral effects of caffeine, such as its effect on mental alertness, have been studied extensively, and topics such as caffeine tolerance, addiction, and withdrawal have also been examined [2; 5-7]. Caffeine consumption has been studied as a risk factor for many diseases and conditions, including hypertension, bone health, cardiovascular diseases, various cancers, reproduction and developmental abnormalities, and mental and behavioral disorders [6;8-21]. In addition to assessing dietary exposure, the quantitation of caffeine and its urine metabolites provides a potential means of assessing differences in metabolic activity [22-23]. The liver serves as the primary site of caffeine metabolism. Caffeine undergoes an intricate series of reactions via several enzyme systems, primarily N-demethylations and C-8-hydroxylation, to yield a mixture of N-methylated xanthines, uric acids, and an acetylated uracil [24-26]. Cytochrome P450 1A2 (CYP1A2), CYP2A6, N-acetyltransferase 2 (NAT2) and xanthine oxidase (XO), are involved in caffeine metabolism [24-26]. Caffeine is a preferred metabolic probe for assessing CYP 1A2, CYP2A6, NAT2, and XO enzyme activities, all of these enzymes are involved in the activation or detoxification of various xenobiotic compounds [26-29]. The enzyme activity can be assessed by calculate the ratio of products and precursors (metabolic ratio). We can use our validated method to study these enzyme activities based on dietary caffeine intake with NHANES subjects [30-32].

B. Test Principle

Urine caffeine and its 14 metabolites are quantified with ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS) with stable isotope labeled internal standards. A 50-µL aliquot of urine is first diluted with 450 µL of water. 100 µL of the diluted urine is then combined with 120 µL of a 0.2 N NaOH solution containing stable isotope labeled internal standards. The mixture is allowed to incubate for at least 30 min at room temperature, facilitating the conversion of an unstable uracil metabolite (AFMU) into a more stable form (AAMU). Samples are then acidified 30 µL of 2.0 N HCl and 250 µL of a 10% methanol containing 0.1% formic acid such that the matrix of the sample is similar to the starting mobile phase composition of the initial analysis step. Samples are then filtered and analyzed by use of UHPLC-ESI-MS/MS with polarity switching. Quantitation is based on peak area ratios interpolated against an 11-point calibration curve derived from calibrators in synthetic urine. The following compounds are quantified:

Compound	Abbreviation	
	Scientific literature (including this document)	NHANES analyte code
1-methylxanthine	1X	MX1
3-methylxanthine	3X	MX2
7-methylxanthine	7X	MX3
1,3-dimethylxanthine (theophylline)	13X	MX4
1,7-dimethylxanthine (paraxanthine)	17X	MX5
3,7-dimethylxanthine (theobromine)	37X	MX6
1,3,7-trimethylxanthine (caffeine)	137X	MX7
1-methyluric acid	1U	MU1
3-methyluric acid	3U	MU2
7-methyluric acid	7U	MU3
1,3-dimethyluric acid	13U	MU4
1,7-dimethyluric acid	17U	MU5
3,7-dimethyluric acid	37U	MU6
1,3,7-trimethyluric acid	137U	MU7
5-acetylamino-6-amino-3-methyluracil	AAMU	AMU

The preparation of 60 patient samples, 11 calibrators, and quality control materials (QCs) generally takes 1.5 hours with an automated liquid processor (including 30 min for the alkaline conversion step). UHPLC-ESI-MS/MS analysis of each sample requires 9 min (6.5 min to run method) per sample.

2. Safety Precautions

Consider all urine specimens as potentially positive for infectious agents including HIV, hepatitis B and hepatitis C. We recommend the hepatitis B vaccination series for all analysts working with urine. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place all disposable plastic, glassware, and paper (pipet tips, auto sampler vials, gloves etc.) that contact urine in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Use disposable bench diapers during sample preparation and urine handling and discard after use. Also, wipe down all contaminated work surfaces with a 10% bleach solution when work is finished.

Handle acids and bases used in sample and reagent preparation with extreme care; they are caustic and toxic. Handle organic solvents only in a well-ventilated area or, as required, under a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Safety data sheets (SDSs) for all chemicals are readily available in the SDS section as hard copies in the laboratory. SDSs for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ossam/workplace-safety/safety-practices/chemical-safety/index.html.

3. Computerization and Data System Management

During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

The raw data file and respective batch file from the tandem mass spectrometer are collected using the instrument software and stored on the instrument workstation. The data file and batch file are copied to the network where the data file is processed into a results file that is saved on the CDC network. Results are typically generated by auto-integration, but may require manual integration in some cases. The results file (including analyte and internal standard names, peak areas, retention times, sample dilution factor, data file name, acquisition time, etc.) is imported into STARLIMS database for review of the data, statistical evaluation of QC/QA data, and approval of the results. See "**4063.08 SOP Computerization and Data System Management**" for a step-by-step description of data transfer, review, and approval.

For NHANES, data is transmitted electronically. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

Data files from the instrument workstation are typically copied to the CDC network on a run-by-run basis. This is the responsibility of the analyst under the guidance of the team lead and/or supervisor. Further data processing is typically conducted on a networked computer and saved directly to the CDC network. Files stored on the CDC network are automatically backed up nightly by ITSO support staff.

4. Specimen Collection, Storage, and Handling Procedures

We recommend that specimen donors fast prior to specimen collection, but fasting is not required. Specimens for caffeine and caffeine metabolite analysis are performed on fresh or frozen urine. One mL of urine is preferable to allow for repeat analyses. A volume of 50-µL is required for each analysis. The appropriate amount of urine is dispensed into a Nalgene 2.0 mL cryovial or other plastic screw-capped vial labeled with the participants ID. Specimens collected in the field are frozen, and then shipped on dry ice by overnight carrier. Frozen samples are stored at ≤ -20°C for short-term storage, and ≤ -70°C for long-term storage. Caffeine and its metabolites in urine appear to be stable over the course of at least 3 freeze/thaw cycles at ambient temperature. One of the caffeine metabolites (5-acetylamino-6-amino-3-methyluracil, AAMU) is light sensitive; excessive ambient light exposure (more than 2 hours) should be avoided, and preparation of AAMU standards should be performed under low-UV lighting.

Specimen handling conditions are outlined in the DLS Policies and Procedures Manual. The electronic copy of the file is located at \\cdc.gov\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA\DLS Policies and Procedures Manual). The protocol discusses collection and transport of specimens and the special equipment required. In general, urine should be transported and stored at -20°C. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of urine should be transferred into a sterile Nalgene cryovial labeled with the participant's ID.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this method.

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

A. Reagent Preparation

Prepare all solutions, samples and standards with 0.45 µm filtered deionized water with a resistance of at least 18 MΩ/cm, and HPLC-grade solvents and reagents. Use Class A volumetric glassware in all cases. Perform all steps involving concentrated acids, bases, and organic solvents in a chemical fume hood. Though each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare larger or smaller quantities if desired.

(1) 1.2 N NaOH solution

For 100 mL of solution, add approximately 50 mL of deionized water to a 100-mL volumetric flask. Quantitatively transfer 12 mL of 10M NaOH into the flask and mix the contents. Bring the solution up to volume with deionized water. Seal the volumetric flask and mix the contents by inversion. Transfer to a storage vessel. Prepare monthly and store at room temperature.

(2) HPLC mobile phase A (aqueous) -5% methanol/0.05% formic acid

For 2 L of solution, quantitatively transfer 1900 mL of deionized water, 100 mL of methanol and 1 mL of formic acid to a 2-L HPLC reservoir bottle. Cap the bottle and mix thoroughly, venting the bottle several times during mixing. Prepare every 7 days and store at room temperature.

(3) HPLC mobile phase B (organic) – 90% methanol/0.05% formic acid

For 500 mL of solution, quantitatively transfer 450 mL of methanol, 50 mL of deionized water and 250 µL of formic acid to a 1-L HPLC reservoir bottle. Cap the bottle and mix thoroughly, venting the bottle several times during mixing. Prepare every 7 days and store at room temperature.

(4) 2X HPLC mobile phase A (aqueous)

For 100 mL of solution, quantitatively transfer 90 mL of deionized water, 10 mL of methanol and 100 µL of concentrated formic acid to a storage vessel. Cap the vessel and mix thoroughly, venting the bottle several times during mixing. Prepare every 7 days and store at room temperature.

(5) 1X Synthetic Urine

For 1000 mL, quantitatively transfer 500 mL of deionized water to a 2 L beaker. Using a magnetic stir bar to mix the solution, add the following chemicals in the quantities and order specified:

- 3.8 g Potassium Chloride (KCl)
- 8.5 g Sodium Chloride (NaCl)
- 24.5 g Urea
- 1.03 g Magnesium Sulfate ($MgSO_4.7H_2O$)
- 1.03 g Citric Acid
- 0.34 g Ascorbic Acid
- 1.18 g Potassium Phosphate Dibasic (K_2HPO_4)
- 1.4 g Creatinine
- 0.64 g Sodium Hydroxide (add slowly)
- 0.47 g Sodium Bicarbonate ($NaHCO_3$)
- 0.28 mL Sulfuric Acid (conc.)

Once all compounds have dissolved in solution, transfer the mixture to a 1000 mL volumetric flask. Bring the solution up to volume with deionized water. Seal the volumetric flask and mix the contents by inversion, and transfer to a storage vessel. This solution can be stored at 4°C for up to

one year. The solution should be discarded and re-prepared if there is any visible evidence of precipitates, bacterial growth, or other changes in appearance.

B. Standards Preparation

A total of 11 calibrators (S1–S11), spanning the reportable range for each analyte, are prepared for this method. Target concentrations for the calibrators appear below (Table I), and the reportable ranges can be found in Section 9 (Table III).

Table I Final concentrations (µM) of analytes in calibrators (S1-S11)

Analyte	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
1X	10.0	25.0	100.0	200.0	5.0	400.0	1.0	300.0	50.0	0.1	0.03
3X	10.0	100.0	50.0	25.0	200.0	1.0	5.0	75.0	300.0	0.1	0.04
7X	600.0	10.0	5.0	400.0	50.0	1.0	100.0	200.0	2.5	0.1	0.02
1U	1.00	500.0	200.0	100.0	400.0	50.0	10.0	300.0	20.0	0.2	0.05
3U	6.0	10.0	0.8	0.6	8.0	2.00	1.00	4.00	15.00	0.4	0.1
7U	20.0	5.0	1.0	50.0	200.0	100.0	2.5	40.0	10.0	0.25	0.04
13X	20.0	10.0	0.4	0.25	5.00	15.0	1.0	0.6	2.5	0.05	0.01
17X	200.0	25.0	3.0	1.0	100.0	150.0	10.0	5.0	50.0	0.05	0.006
37X	25.0	250.0	3.0	50.0	10.0	1.0	150.0	5.0	100.0	0.05	0.004
13U	50.0	2.5	0.4	1.0	0.25	10.0	0.8	5.0	25.0	0.15	0.02
17U	5.0	50.0	10.0	100.0	3.0	1.0	250.0	20.0	300.0	0.25	0.02
37U	1.0	8.0	20.0	10.0	0.3	0.8	0.6	4.0	2.0	0.1	0.03
137X	2.5	0.6	0.3	40.0	20.0	1.0	5.0	10.0	50.0	0.05	0.003
137U	5.0	0.3	20.0	40.0	10.0	0.5	2.5	1.0	30.0	0.05	0.005
AAMU	100.0	1.0	500.0	3.0	5.0	200.0	400.0	30.0	50.0	0.4	0.1

Note: Special attention should be paid to the preparation of calibrators S10 and S11; they are low-level standards that are prepared in a different manner from calibrators S1–S9.

(1) Single-Analyte and Single-Internal Standard Stock Solutions

Separate stock solutions should be prepared for each analyte and stable isotope-labeled internal standard by dissolving an accurately known mass (±0.1 mg or less) of the pure solid compound in aqueous solution, targeting a final concentration of 1 mM based on the formula weight of the compound. The volume of solution prepared should be sufficiently large such that the determined mass of starting material has an imprecision of less than 1%. For example, to prepare 200 mL of a 1-mM stock solution of 1,3-dimethyluric acid (13U), weigh an accurately known mass (target 39.2 mg based on MW of 195.16 g/mol) of solid 1,3-dimethyluric acid into a glass weighing funnel. Carefully transfer the material to a 200-mL volumetric flask, rinsing the contents of the weighing funnel into the flask with deionized water. Partially fill the volumetric flask with deionized water and mix the contents by sonication until dissolved. Bring the solution up to volume with deionized water and mix by inversion. Aliquot the solution into 2-mL polypropylene cryovials (1 mL/vial), and store at -70°C.

Note: the following analytes require the addition of sodium hydroxide (final concentration of 1 mM NaOH in solution) for complete dissolution: 1X, 3X, 7X, 1U, 3U, 7U, 17U, 37U, and AAMU. The same is true for the stable isotope-labeled analogues of these analytes. All other analytes and internal standards can be prepared in deionized water.

Note: AAMU (5-acetylamino-6-amino-3-methyluracil) **is light sensitive**. The preparation of primary and intermediate stock solutions and the addition of this analyte to the standard mixture needs to be performed under low-UV yellow light.

Assignment of single-analyte stock solution concentration by use of UV-visible absorbance measurements and molar extinction coefficients is preferred. A list of recommended extinction coefficients appears in **Appendix C**. In the absence of reliable extinction coefficients, assignment of stock solution concentration by gravimetric measurement is acceptable.

(2) Intermediate Mixed-Analyte Stock Solutions – Preparation of Calibrators

The intermediate mixed-analyte stock solutions for S1–S9 are prepared by combining the single-analyte stock solutions according to the amounts specified in Table II. For example, to prepare 10 mL of intermediate mixed-analyte standard solution “S1”, use an air displacement pipette to transfer the defined amount single-analyte stock solution into a 10-mL polypropylene tube, the appropriate amount is confirmed by weight. Bring the solution up to volume with deionized water and mix thoroughly by inversion. Aliquot each calibrator (1 mL/vial) into 2-mL cryovials and store the calibrators at -70°C.

Table II: Volume of single-analyte stock solution (µL) required to prepare S1–S9

Analyte	S1	S2	S3	S4	S5	S6	S7	S8	S9
1X	93	234	935	1869	47	3738	**28***	2804	467
3X	93	935	467	234	1869	**28***	47	701	2804
7X	5505	92	46	3670	459	**28***	917	1835	23
1U	**29***	4854	1942	971	3883	485	97	2913	194
3U	57	94	**23***	**29***	75	**38***	**28***	38	142
7U	206	52	**21***	515	2062	1031	26	412	103
13X	202	101	**20***	**25***	51	152	**20***	**31***	25
17X	2000	250	30	20*	1000	1500	100	50	500
37X	250	2500	30	500	100	20*	1500	50	1000
13U	481	24	38*	**29***	**24***	96	23*	48	240
17U	52	515	103	1031	31	**21***	2577	206	3093
37U	**29***	78	196	98	29	**23***	**30***	39	20
137X	23	27	27*	360	180	**27***	45	90	450
137U	50	**30***	198	396	99	**25***	25	**30***	297
AAMU	877	**26***	4386	26	44	1754	3509	263	439
Water	54	187	1539	227	47	1034	1028	491	203

Note: Volumes denoted with * indicate that a 10x dilution of the single-analyte stock solution was used. The 10× dilution of the stock solution is necessary so that the volume of solutions being pipetted is >20 µL.

Note: The volumes provided in Table II assume all stock solution concentrations are exactly 1 mM. Actual stock solution concentrations should be used and pipetting volumes may be adjusted in order to obtain the target concentrations in Table I.

The intermediate mixed-analyte stock solutions for S10 and S11 are prepared by combining the single-analyte stock solutions according to the amounts specified in Table III. Because S10 and S11 are low-level calibrators, they are prepared using diluted stock solutions so that the volume of solution being pipetted is >20 µL.

Table III: Volume of single-analyte stock solution (µL) required to prepare S10 and S11

Analyte	S10 (using a 50 µM stock solution)	S11 (using a 20 µM stock solution)*†
1X	100	75
3X	100	100
7X	100	50
1U	200	125
3U	400	250
7U	250	100
13X	50	25
17X	50	**30***
37X	50	**20***
13U	150	50
17U	250	50
37U	100	75
137X	50	**30†**
137U	50	**25***
AAMU	400	250
Water	2700	3745

Note: Volumes denoted with * indicate that a 10 µM stock solution was used. Volumes denoted with a † indicate that a 20 µM stock solution was used.

(3) Preparation of Working Solution for Calibration Standards

Calibration standards are prepared by combining the appropriate intermediate mixed-analyte stock solution with 1× synthetic urine and deionized water in a relative proportion of 1:1:8 for S1 through S9. The same proportion applies to intermediate mixed-analyte stock solutions S10 and S11; however, these stock solutions need to be diluted 10× prior to use. All calibration standards are prepared in batches, and aliquoted as 125.0 µL/vial. An aliquot of 100 µL from each standard (S1 through S11) is required to set up a run. For example, to prepare enough calibration standards for approximately 1000 µL of diluted calibration standard "S1": combine 100 µL of "S1" stock solution, 100 µL of synthetic urine, and 800 µL of deionized water and mix thoroughly. Accurately aliquot the mixture into 1.5-mL micro-centrifuge vials (125.0 µL/vial) and store at -70 °C. The final concentrations (µM) of each analyte in S1–S11 are shown in Table I.

(4) Intermediate Mixed-Internal Standard Stock Solutions

Intermediate mixed-internal standard stock solutions are prepared by combining single internal standard stock solutions into a mixture containing 5 µM of each compound except for AAMU, which will have a concentration of 15 µM. Aliquot the solution into 2-mL polypropylene cryovials (0.5mL/vial or 0.2 ml/vial) and store at -70°C.

(5) Working Mixed-Internal Standard Solutions

Working mixed-internal standard stock solutions are prepared by diluting the intermediate mixed-internal standard stock solution by 5× with water.

C. Preparation of Quality Control Materials

Low, medium, and high quality control (QC) pools are prepared by selecting and pooling urine from anonymous volunteers. Urine samples from anonymous volunteers are first screened for their caffeine and metabolite concentrations and pooled to meet target concentrations for 1X, 17X, 137X, 1U, 17U and AAMU based on currently available reference data. A best-effort is made to meet target concentrations for the remaining analytes but this may not always be possible due to the total number of compounds being analyzed. For the low QC pool, urine samples are selected such that a pool can be generated with analyte concentrations approximating the 25th percentile population estimate. Similarly, the medium QC pool is prepared to approximate the 50th percentile and the high QC pool is prepared to approximate the 75th percentile. Each pool is stored in 500-µL aliquots in 2.0-mL Nalgene cryovials at -70 °C.

D. Other Materials

With some exceptions, a material listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of standards, internal standards, chemicals and reagents, the chemical and/or isotopic purity of the substituted must meet or exceed that of the listed product. In the case of the HPLC column and guard cartridge, equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures.

(1) General consumables

- Kinetex 1.7 µ XB-C18 column 100 x 3.0 mm, 100 Å pore (Phenomenex, Torrance, CA)
- Krudkatcher Ultra HPLC In-Line Filter 0.5 µ Depth Filter x 0.004 in ID (Phenomenex)
- 9” Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
- HPLC autosampler vials (2.0mL/12x32mm, National Scientific, Duluth, GA)
- 1-mL, 96-well plate, 31 mm (Nalgene, Rochester, NY)
- Pre-slit, silicone 96-well plate seal (Fisher Scientific, Suwanee, GA)
- Fisher brand nitrile examination gloves (Fisher Scientific, Suwanee, GA)
- Pipette tips, blue, 50-1000 µL, for Eppendorf pipette (Eppendorf, Hauppauge, NY)
- Pipette tips, yellow, 2-200 µL, for Eppendorf pipettes (Eppendorf)
- Positive displacement pipette tip, Combitip plus, 500 µL, 1mL, 2.5 mL, and 5 mL, for Eppendorf repeater pipette (Eppendorf)
- Hamilton high volume (1mL) tips without filter (Hamilton, Reno, NV)
- Hamilton standard volume (300µL) tips without filter (Hamilton)

- Costar Spin-X Centrifuge Tube filter (0.22 µm Nylon), polypropylene tune, non-sterile (Corning Incorporated, Corning, NY)
- AcroPrep 0.2-µm nylon, 96-well filter plate (Pall Life Sciences, Ann Arbor, MI)
- 2.0 mL Polypropylene cryovials (Nalgene)
- 10 mL Polypropylene T310-10A Cryovial with silicone washer seal (Simport, Beloeil, QC, Canada)
- 15 mL Falcon Tubes (Fisher Scientific, Suwanee, GA)
- 1.5mL micro centrifuge tubes (VWR, Suwanee, GA)
- Various glass beakers, volumetric flasks (Class A), graduated cylinders (Class A), and bottles (various suppliers)

(2) Chemicals and solvents

- Methanol, HPLC grade (Burdick & Jackson Laboratories, Muskegon)
- Water, 0.45 µm filtered, ≥ 18.0 MΩ resistance (in-house source, Aqua Solutions, Jasper, GA)
- Sodium hydroxide, 10N (Fisher Scientific Co., Fairlawn, NJ)
- Hydrochloric acid, 2N (Fisher Scientific)
- Formic acid (Sigma, St. Louis, MO)
- Potassium chloride (Sigma)
- Sodium chloride (Sigma)
- Urea (Sigma)
- Magnesium sulfate (MgSO4.7H2O) (Sigma)
- Citric acid (Sigma)
- Ascorbic acid (Sigma)
- Potassium phosphate (Sigma)
- Creatinine (Sigma)
- Sodium hydroxide (Sigma)
- Sodium bicarbonate (Sigma)
- Sulfuric acid, concentrated (Sigma)
- 1,3,7-trimethylxanthine (Sigma)
- 1,3 dimethylxanthine (Sigma)
- 1,7 dimethylxanthine (Sigma)
- 3,7 dimethylxanthine (Sigma)
- 1-methylxanthine (Sigma)
- 3-methylxanthine (Sigma)
- 7-methylxanthine (Sigma)
- 1,3,7-trimethyluric acid (Sigma)
- 1,3-dimethyluric acid (Sigma)
- 1,7-dimethyluric acid (Sigma)
- 3,7-dimethyluric acid (Sigma)
- 1-methyluric acid (Santa Cruz, Dallas, Texas)
- 3-methyluric acid (Toronto Research Chemicals, Toronto, ON, Canada)
- 7-methyluric acid (Toronto Research Chemicals)
- 5-acetylamino-6-amino-3-methyluracil (Toronto Research Chemicals)
- 1,3-dimethyl xanthine -13-(methyl-($^2H_3$)$_2$) (CDN Isotopes, Point Claire. QC, Canada)
- 1,3,7-trimethylxanthine -(1,3,7, -(methyl-($^2H_3$)$_3$) (CDN Isotopes, Point Claire. QC, Canada)

- 1,3,7-trimethyl xanthine -$^{2}H_{9}$ (CDN Isotopes)
- 1,3-dimethyl xanthine -$^{13}C_{4}{}^{15}N_{3}$ (IsoSciences, LLC, King of Prussia, PA)
- 1,7-dimethyl xanthine -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 3,7-dimethyl xanthine -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 1-methylxanthine -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 3-methylxanthine -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 7-methylxanthine -$^{13}C_{4}{}^{15}N_{2}$ (Iso Sciences)
- 1,3,7-trimethyluric acid -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 1,3-dimethyluric acid -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 1,7-dimethyluric acid -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 3,7-dimethyluric acid -$^{13}C_{4}{}^{15}N_{1}$ (Iso Sciences)
- 1-methyluric acid -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 3-methyluric acid -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 7-methyluric acid -$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)
- 5-acetylamino-6-amino-3-methyluracil-$^{13}C_{4}{}^{15}N_{3}$ (Iso Sciences)

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., UHPLC components, tandem quadrupole mass spectrometer) equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type.

(1) Agilent 1290 UHPLC system (Agilent Technologies, Palo Alta, CA), including:

- Model 4208A-Control Module
- Model G4220A-Binary pump
- Model G4226A-High Performance Autosampler
- Model G1330B-Autosampler Thermostat
- Model G1316C-Thermostatted Column Compartment

(2) AB Sciex 6500 triple quad mass spectrometer (AB Sciex, Foster City, CA), including:

- Turbo V Ion source, operated in ESI mode (AB Sciex)
- Analyst 1.6.2 software (AB Sciex)

(3) Hamilton Starlet 8-channel with auto-load arm (Hamilton), including:

- Two pipette tip carriers, TIP_CAR_480_A00
- Three sample vial carriers, SMP_CAR-32_A00
- One reagent carrier, RGT_CAR_5X50_G
- One plate carrier, PLT_CAR_L5AC_A00

(4) Other laboratory instrumentation:

- Harvard syringe pump (Harvard Apparatus, Inc., Holliston Massachusetts)
- Eppendorf pipette,100-1000µL (Eppendorf)
- Eppendorf pipette, 1-10 mL (Eppendorf)

- Eppendorf pipette, 20-200 µL (Eppendorf)
- Eppendorf pipette, 100 µL (Eppendorf)
- Eppendorf pipette, 10-100µL (Eppendorf)
- Eppendorf pipette, 2-20µL (Eppendorf)
- Eppendorf Repeater Plus pipette (Eppendorf)
- Vortexer (VWR)
- Accumet pH/mV meter (XL150, Fisher Scientific)
- Magnetic stirrer (Fisher Scientific)
- Eppendorf Centrifuge (5810R, Eppendorf)
- Analytical balance (AG104, Mettler Instrument Corp., Hightstown, NJ)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

Eleven calibrators (S1-S11) prepared in 0.1× synthetic urine are added to the reaction plate and processed as regular samples. These 11 calibrators are analyzed at the beginning of each run. The calibrators are re-analyzed as unknown samples at the end of each run. A quadratic calibration equation with 1/x weighting is used. Samples with the concentrations exceed the highest concentration of the calibrators are re-prepared with appropriate dilution. The measured concentrations of these calibrators should generally agree within 15% of their set values, although >15% agreement will be observed at concentrations approaching the LOD.

Reference materials are not available for urine caffeine and caffeine metabolites. Calibration verification is conducted as outlined in "**4063.08 SOP for Calibration and Calibration Verification**."

External proficiency testing programs currently do not exist for urine caffeine metabolites. An in-house proficiency testing program has been developed and is conducted at least twice a year, details of which can be found in "**4063.08 SOP for In-House Proficiency Testing**." For general information on the handling, analysis, review, and reporting of proficiency testing materials see "**NBB_SOP Proficiency Testing Procedure**."

Results from a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied are presented in **Appendix B.**

B. Instrument Calibration

(1) API 6500 Mass Spectrometer

The calibration of the mass spectrometer is scheduled on a semi-annual basis as part of a preventive maintenance program and is performed by the service engineer from Applied Biosystems. If necessary, the analyst can recalibrate using the calibration standards described below and by following the instructions contained in the operator's manual.

The tuning and mass calibration of the first (Q1) and third (Q3) quadrupoles of the API 6500 is performed using a solution of polypropylene glycol (PPG) by infusion and running the instrument in either Manual Tuning mode or using Automatic Mass Calibration. Please refer to the API 6500 User's Manual for additional details.

(2) Hamilton Microlab Starlet

Once a year, a qualified service engineer performs preventative maintenance, including volume verification at 10 µL and 1000 µL.

A volume verification of the various steps of the method can also be performed gravimetrically (e.g., using online gravimetric kit, Hamilton) by the user. Imprecision should be commensurate or exceed that obtained using manual pipettes.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A typical run (in the order in which they are injected into the LC-MS/MS) consists of a blank (with IS), a double blank (buffer only, without IS), 11 calibrators, 3 levels of bench QCs (low, medium, and high), patient samples (up to a maximum of 77), re-inject of 3 bench QCs (low, medium, and high), blanks, and calibrators.

A. Sample Preparation

(1) Manual Sample Preparation

(a) Sample dilution:

- Label one set of 1.5-mL micro-centrifuge tubes for all urine samples and two sets of bench QCs (dilution tubes).
- Quantitatively transfer 450 µL of water to each dilution tube. Quantitatively transfer 50 µL of each sample and QC to a dilution tube.
- Cap and mix all dilution tubes thoroughly by vortexing. Transfer 100 µL of the diluted urine to the second set of ependorf tubes (reaction tubes).

(b) Alkaline treatment:

- Label one set of 1.5-mL microcentrifuge tubes for all diluted samples and bench QCs from the previous step, plus additional tubes for a blank and calibrators which are pre-diluted (reaction tubes).
- Quantitatively transfer 80 µL of water, 20 µL of 1x internal standard, and 20 µL of 1.2 N NaOH to each reaction tube (alternatively, prepare a 4:1:1 mixture of these solutions and quantitatively transfer 120 µL of the mixture to each reaction tube).
- Quantitatively transfer 100 µL of each diluted sample, bench QC, blank and calibrator to each reaction tube. Cap and mix all reaction tubes and incubate at room temperature for at least 30 minutes.

(c) Acidification:

- Quantitatively transfer 30 µL of 2N HCl and 250 µL of 2× HPLC mobile phase A to each reaction tube (alternatively, prepare a 3:25 mixture of these solutions and quantitatively transfer 280 µL of the mixture to each reaction tube). Cap and mix all reaction tubes thoroughly by vortexing.

(d) Filtration:

- Label one set of 0.2-µm nylon microcentrifuge filter tubes for all samples, QCs, blanks and calibrators.
- Transfer the contents of each reaction tube to a microcentrifuge filter tube and centrifuge at 10,000 g for 5 min.

(e) HPLC Analysis:

- Label one set of HPLC vials for all samples, QC, blanks and calibrators.
- Transfer the filtered contents of each microcentrifuge filter to an HPLC vial with an insert.
- Cap all vials and gently tap each vial to ensure that there are no bubbles in the vial contents. The filtrate is ready for the analysis on HPLC (alternatively, transfer the filtered contents of each microcentrifuge filter, or use a 96-well filter plate to filter the samples directly into a 96-deep well plate and seal the plate with a pre-slit 96-well silicone sealing mat).

(2) Automated Sample Preparation

"**4063.08 SOP Automated Sample Preparation**" describes automated sample preparation using the Hamilton Starlet system. These steps directly mimic those described above for manual sample preparation with most pipetting actions being performed by the Hamilton Starlet. In brief: sample dilution steps (a) are performed in a 96-deep well plate; alkaline treatment (b) and acidification steps (c) are performed in a second 96-deep well plate; filtration steps (d) are performed using a 96-well 0.2 µm nylon centrifuge filter plate collecting into a 96-deep well plate; and HPLC analysis (e) is performed on the 96-well collection plate sealed with a pre-slit 96-well silicone sealing mat. All precautions observed in manual sample preparation should be observed when performing automated sample preparation.

The instructions given in the SOP reflect the custom program developed for performing sample preparation that is currently being used. Certain non-critical elements of this program (e.g., positions of samples, wording of user messages) may be modified and differ from the exact instructions given in the SOP. The user is strongly encouraged to be familiar with the exact program being used.

A liquid handling system other than the Hamilton Starlet may be used for this purpose provided that it is able to perform these steps with accuracy and precision that meets or exceeds that of the Hamilton Starlet.

B. Instrument Preparation

(1) UHPLC (Agilent 1290)

Fill all solvent bottles as follows. UHPLC Mobile Phase A (aqueous) (line A1) and Mobile Phase B (organic) (line B1) should be refilled with freshly prepared solvent before each run (see section 6.a. for preparation instructions). HPLC-grade water (line A2) and a solution of 50% HPLC-grade acetonitrile in water- (line B2) should be checked daily and refilled as needed. The solution of 60% methanol in water (needle wash) should be checked daily and refilled as needed. Clean or replace any solvent bottles, inlet filters or lines as needed.

Check the waste bottle to ensure that it will not overflow during the run. Dispose of all chemical waste according to procedures described in the CDC Hazardous Chemical Waste Management procedures.

Replace the guard column every 5 runs, or when the chromatographic performance has become poor, whichever occurs first.

Replace the HPLC column if chromatographic performance has become poor. Monitor chromatographic performance closely in terms of background noise and accuracy of S10 and S11if the column has been used for >1000 sample injections. Inspect all UHPLC tubing and tubing connections. Ensure that all connections are in place and tightened appropriately.

Using the hand-held control module, purge all solvent lines by running solvent through each line at 5 ml/min for at least 5 min. Purging is necessary if the instrument has been idle for one day or longer, or if air bubbles are visible in any of the solvent lines. Close the waste valve when done.

(2) Mass Spectrometer

Check the interface and turbo ion spray probe before each run to make sure that the needle height, probe height/width settings are correct. The probe position is optimized, and usually reset after preventative maintenance. In general, a test run containing standards and QCs is performed after maintenance to ensure that instrument performance (e.g., sensitivity, signal-to-noise ratio) is comparable with previous runs.

Clean the source, probe, and curtain plate interface every 2 full runs (caution: the interface may be very hot if the instrument was recently run). See the API 6500 User's Manual for specific guidance.

C. Sample Analysis

The UHPLC-MS/MS system is used to quantitate caffeine and caffeine metabolite levels in urine. See "**4063.08 SOP Sample Analysis**" for a detailed description of the sample analysis steps. UHPLC-MS/MS parameters are given in **Appendix D**. The following is an overview of the sample analysis process.

(1) Preliminaries

The user must first ensure that all instrumentation is turned on and ready for use. This entails starting Analyst software and ensuring the correct project and hardware configuration is selected and activated. Refer to "**4063.08 SOP for Sample Analysis**" for additional details.

(2) Building an Acquisition Batch

Because of the number of steps involved in building a new batch file, it is acceptable for the user to use a previous batch file and modify it to suit the current analysis by changing the necessary information (e.g., sample names, sample IDs, data file names, comments, etc..). In brief, the analyst must create a sample set to accommodate the following: the startup methods; equilibration injections, unknown samples, and shutdown method. These samples should be run in the order as presented above. Refer to "**4063.08 SOP for Sample Analysis**" for additional details.

(3) Instrument Equilibration

The instrument needs to be equilibrated for at least 30 minutes prior to starting an analysis. Though instrument equilibration is presented following the building of the acquisition batch, the acquisition batch can be built while the instrument is equilibrating.

This procedure assumes that the user is starting a new analysis after the instrument has successfully completed a previous analysis. The user may deviate from this procedure if special circumstances present themselves (e.g., restarting an instrument run that was interrupted).

Refer to "**4063.08 SOP for Sample Analysis**" for additional details.

(4) Submitting and Starting a Batch

Once the instrument has been properly equilibrated and the acquisition batch has been created and saved, the user may submit the batch to the analysis queue and start the analysis sequence. Refer to "**4063.08 SOP for Sample Analysis**" for additional details.

D. Quantitation and Data Review

The UHPLC- MS/MS system software (Analyst 1.6.2) is used for quantitating analysis data. Quantified results are then imported into Starlims for data review by the analyst and team lead, then finally by supervisor or quality assurance officer.

The quantitation of instrument results can be done either at the instrument computer or a different location (e.g., desktop PC) where the LC-MS/MS software is installed. In order to review data at a location other than the instrument, the user will have to create an identical project and copy all required files over to this location.

The following instructions assume that a complete analysis was performed. If the user is only interested in certain samples from an instrument run, the user may deviate from this procedure as necessary.

(1) Review Peak Integration

The quantitation method is set up to identify and integrate analyte and internal standard peaks based on specifications such as retention time windows and minimum peak area thresholds. The user should review all peak integrations and correct any integration errors where necessary. Refer to "**4063.08 SOP Starlims Data Review**"

(2) Review Calibration Curves

The analyst should review the calibration curve for each analyte, ensuring that the correct regression model and weighting are used in each case. If a calibration point appears to be erroneous, it may be removed from the curve in consultation with the team lead (Note: the analyst should be aware of the implications of removing the highest or lowest calibration point as this may affect the reportable range of values for an instrument run).

E. System Maintenance

Agilent UHPLC - Preventative maintenance is performed on an annual basis by a qualified service engineer. Routine maintenance should be performed as indicated in this document and in the Agilent User's Manual.

Applied Biosystems API 6500 MS/MS - Preventative maintenance, tuning and mass calibration is performed on an annual basis by a qualified service engineer. Routine maintenance should be performed as indicated in this document and in the Applied Biosystems User's Manual.

Hamilton Microlab Starlet - Preventative maintenance is performed on an annual basis by a qualified service engineer. Routine maintenance should be performed as indicated in the Hamilton User's Manual.

9. Reportable Range of Analytical Results

Table IV: Reportable Range of Analytical Results

Analyte	Reportable range (μM)
1X	0.03 – 400
3X	0.04 – 300
7X	0.02 – 600
13X	0.01 – 20
17X	0.006 – 200
37X	0.004 – 250
137X	0.003 – 50
1U	0.05 – 500
3U	0.1 – 15
7U	0.04 – 200
13U	0.02 – 50
17U	0.02 – 300
37U	0.03 – 20
137U	0.005 – 40
AAMU	0.1 – 500

Samples with concentrations exceeding the highest calibrator are diluted, re-prepared, and reanalyzed so that the measured value is within the range of the calibration. There is no known maximum acceptable dilution. When possible, avoid small volume pipetting and minimize use of serial dilutions when generating diluted samples. Changes in LOD or concentration of highest calibrator concentration will affect the reportable range.

10. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Nutritional Biomarkers Branch. These specimens are prepared at two levels so as to emulate the patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

Alternatively, open label blind QC specimens can be used where the analyst knows that the sample is a blind QC, but they do not know what pool the sample is from. Open label blind QCs are used only if they can be selected from at least 5 different pools and the analyte concentrations are similar to those found in patient samples.

B. Bench Quality Controls

Bench QC specimens are prepared from three urine pools that represent low, medium and high levels of urine caffeine and caffeine metabolites. Samples from these pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run using a multi-rule quality control system [33] based their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (S_w); the standard deviation associated with individual QC results (S_i); and the standard deviation associated with run mean QC results (S_m). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. In the case of three QC pools per run with two QC results per pool:

(1) If all three QC run means are within 2 S_m limits and individual results are within 2 Si limits, accept the run

(2) If one of the three QC run means is outside a 2 S_m limit, reject run if:

(a) 1 3S Rule—Run mean is outside a 3 S_m limit or

(b) 2 2S Rule—Two or more of the three run means are outside the same 2 S_m limit or

(c) 10 X bar Rule—Current and previous nine run means are on the same side of the characterization mean

(3) If one of the six QC individual results is outside a 2 S_i limit, reject run if:

(a) Outlier—One individual result is beyond the characterization mean ±4 S_i or

(b) R 4S Rule—two or more of the within-run ranges in the same run exceeds 4 S_w (i.e. 95 % range limit).

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material at least 20 consecutive runs and then are reevaluated periodically. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in STARLIMS. For the runs that are not imported into the database (i.e., R&D, troubleshooting, research-type runs), QC results are stored electronically in the analyte-specific folder on the DLS network. At the conclusion of studies, complete QC records are prepared for review by a DLS statistician.

C. Sample QC Criteria

Each individual sample result is checked against established sample QC criteria limits to assure data quality. The method also uses the following sample QC criteria:

- Relative retention time (retention time quantitation ion/retention time ISTD)
- Confirmation ion ratio (confirmation ion area/quantitation ion area)
- Percent difference of Individual ISTD area from within-run average

For additional details and criteria, see "**4063.08 SOP Sample QC Criteria**."

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

The following steps are provided as a general guideline for identifying possible problems resulting in "out of control" values for QC materials. The troubleshooting process should be done in consultation with the supervisor or team lead and may involve additional experiments beyond what is indicated below. Analytical results for runs not in statistical control should not be reported. The following remedial actions should be considered:

- Look for possible sample preparation errors, specimen, and reagents used, etc.
- Check whether the QC samples are handled properly.
- Check all HPLC reagents, any leaks or air bubbles in tube line.
- Check to make sure that the ESI probe position is correct and optimized, and other instrument hardware is functioning properly. Run PPGs in Q1 Scan to check the instrument calibration.
- Run standards in Q1 Scan to see if the molecular ion is detectable.
- Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator.

- Check the auto-sampler for evidence of correct sample injections.
- Check the calibrations of the pipettes and robotic liquid handler.

12. Limitations of Method; Interfering Substances and Conditions

The most common cause of poor method performance is a pipetting error. All buffers, reagents and mobile phases should be made fresh whenever possible and verified for performance. Occasionally, the concentration of caffeine or caffeine metabolites in urine will exceed the highest calibrator. In this case, a dilution run will be performed. When using a quadratic equation for calibration, care must be taken to minimize excessive "roll-over" of the curve at higher concentrations. This phenomenon is typically indicative of too much analyte being injected. If it is observed, reducing the sample injection volume is recommended.

This method has also undergone a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. A total of five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at a value substantially lower and higher than that specified in this method while holding all other experimental variables constant. The ruggedness testing findings for this method are presented in **Appendix B**. Please refer to Chapter 20 of the 2017 DLS Policies and Procedures Manual for further information on ruggedness testing.

13. Reference Ranges (Normal Values)

Reference ranges (2.5^{th}–97.5^{th} percentile) for the representative US population (NHANES 2009–10) [34] are as follows (Table V):

Table V Reference range of caffeine metabolites

Analyte	Percentile (μM)		
	2.5th	median	97.5th
1X	0.986	27.6	276
3X	0.693	30.9	305
7X	1.13	51.3	546
13X	<0.05	1.63	11.4
17X	<0.1	15.2	105
37X	0.406	20.3	186
137X	<0.01	3.39	33.8
1U	4.58	58.6	508
3U	<0.1	0.560	6.60
7U	0.368	15.5	182
13U	<0.05	6.42	62.9
17U	0.066	24.8	224
37U	<0.05	1.24	13.1
137U	<0.05	1.42	16.2
AAMU	0.339	49.9	539

14. Critical Call Results ("Panic Values")

There are no established critical values for urine caffeine and caffeine metabolites, i.e. there is no definition of a safe, normal or acceptable concentration of urine phytoestrogens versus one that would be considered abnormal or life-threatening.

15. Specimen Storage and Handling during Testing

Urine samples may be stored overnight in the refrigerator to expedite thawing prior to aliquotting. Samples should be allowed to warm to and be maintained at room temperature during preparation and testing and then returned to frozen storage (typically at ≤-70C) as soon as possible. Ambient light exposure should be avoided if the samples are kept on the working bench more than 2 hours.

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

There are no acceptable alternative methods for the analysis of urine caffeine and caffeine metabolites in the Nutritional Biomarker Branch. If the analytical system fails, we recommend that the specimens or prepared samples be stored (typically at ≤-70C) until the analytical system is restored to functionality.

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

Test results are reported to the collaborating agency at a frequency and by a method determined by the supervisor. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as a spreadsheet file (e.g., Microsoft Excel), either through email or via transfer to an ftp site.

For NHANES 1999+, all data are reported electronically on a periodic basis to Westat who in turn transfers the results to NCHS. For smaller studies, electronic copies of a data report are sent; a hard copy of the data report may also be sent if requested.

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

The LIMS is used to keep records and track specimens for all studies. For studies other than NHANES, additional records may be kept in Excel files on the network.

We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual urine from these analyses for non-NHANES studies are retained for at least 1 year after results have been reported and may then be returned or discarded at the request of the principal investigator. Very little residual material will be available after NHANES analyses are completed, however residual urine is retained for at least 2 years after results have been publicly released; at that point, samples with sufficient volume (>0.2 mL) are returned to NHANES and samples with insufficient may be autoclaved.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -80°C. The specimen ID is read off of the vial by a barcode reader used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the results file is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for documenting and keeping a record of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies. In general, these are documented using codes in LIMS.

19. Method performance documentation

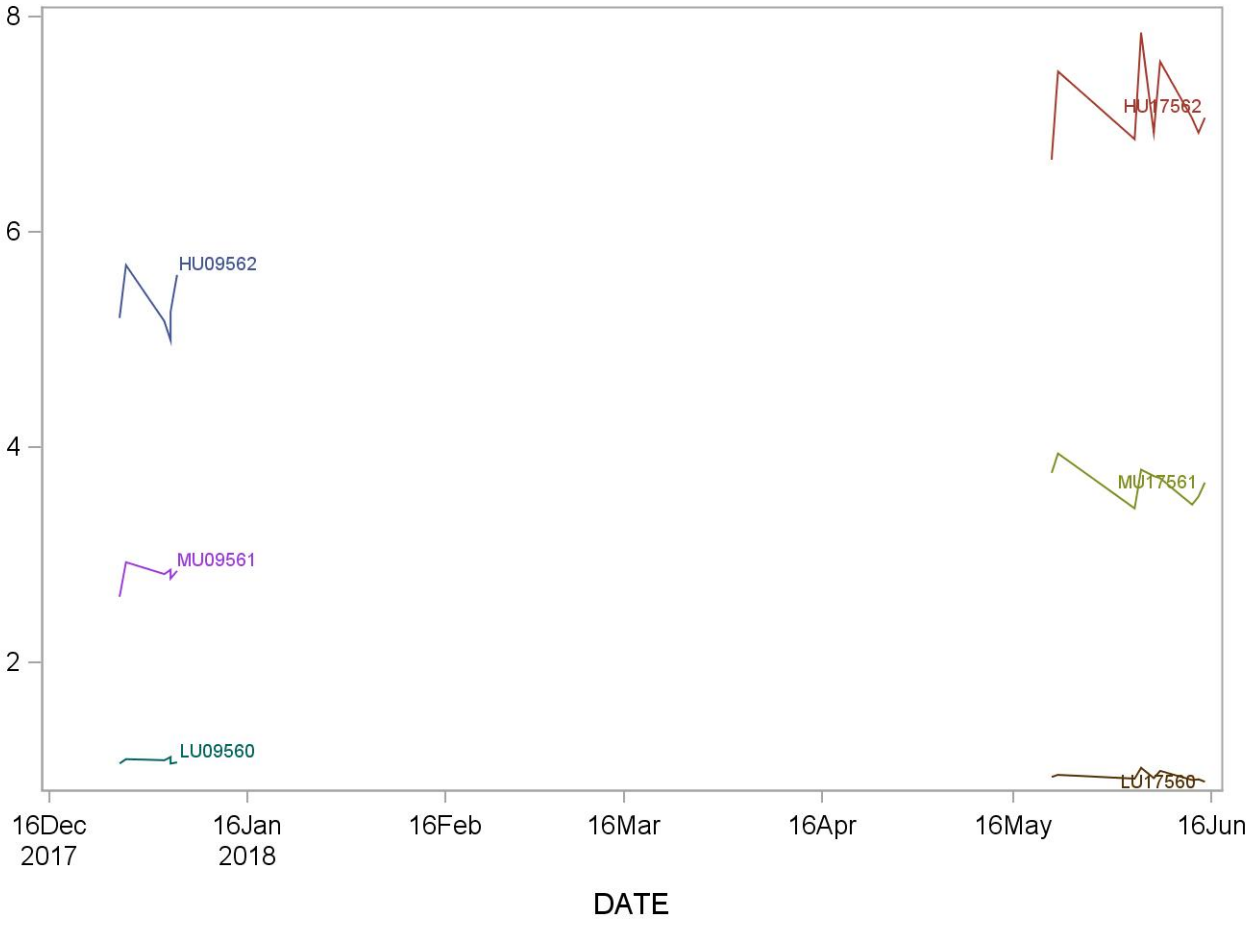
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in **Appendix A** of this method documentation. **The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.**

20. Summary Statistics and QC Graphs

See following pages.

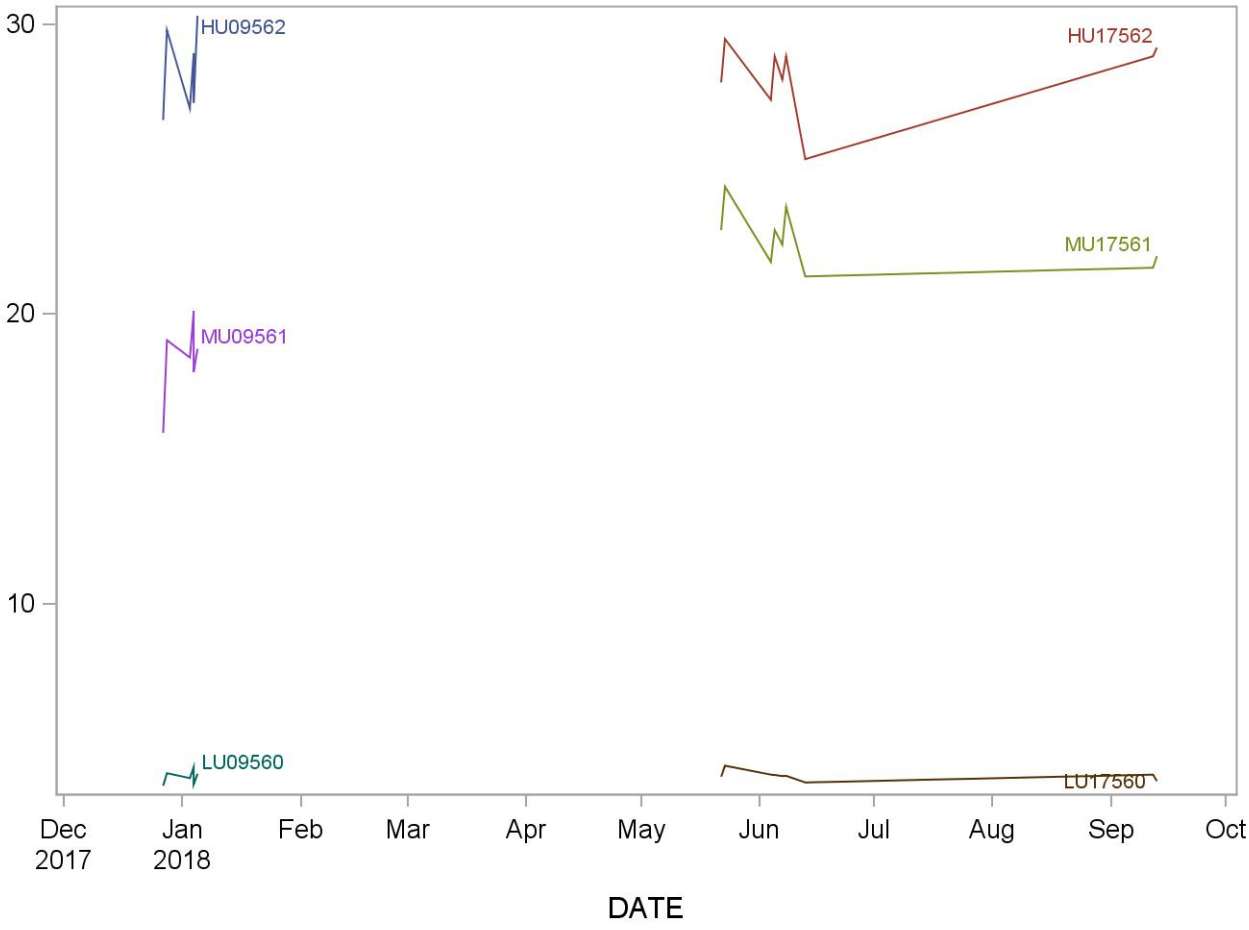
2014 Summary Statistics and QC Chart for 1,3,7-Trimethyluric acid, U 2nd C umol/L

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	5.3183	0.2701	5.1
LU09560	6	27DEC17	05JAN18	1.0833	0.0242	2.2
MU09561	6	27DEC17	05JAN18	2.8083	0.1091	3.9
HU17562	9	22MAY18	15JUN18	7.1561	0.3917	5.5
LU17560	9	22MAY18	15JUN18	0.9392	0.0421	4.5
MU17561	9	22MAY18	15JUN18	3.6706	0.1647	4.5



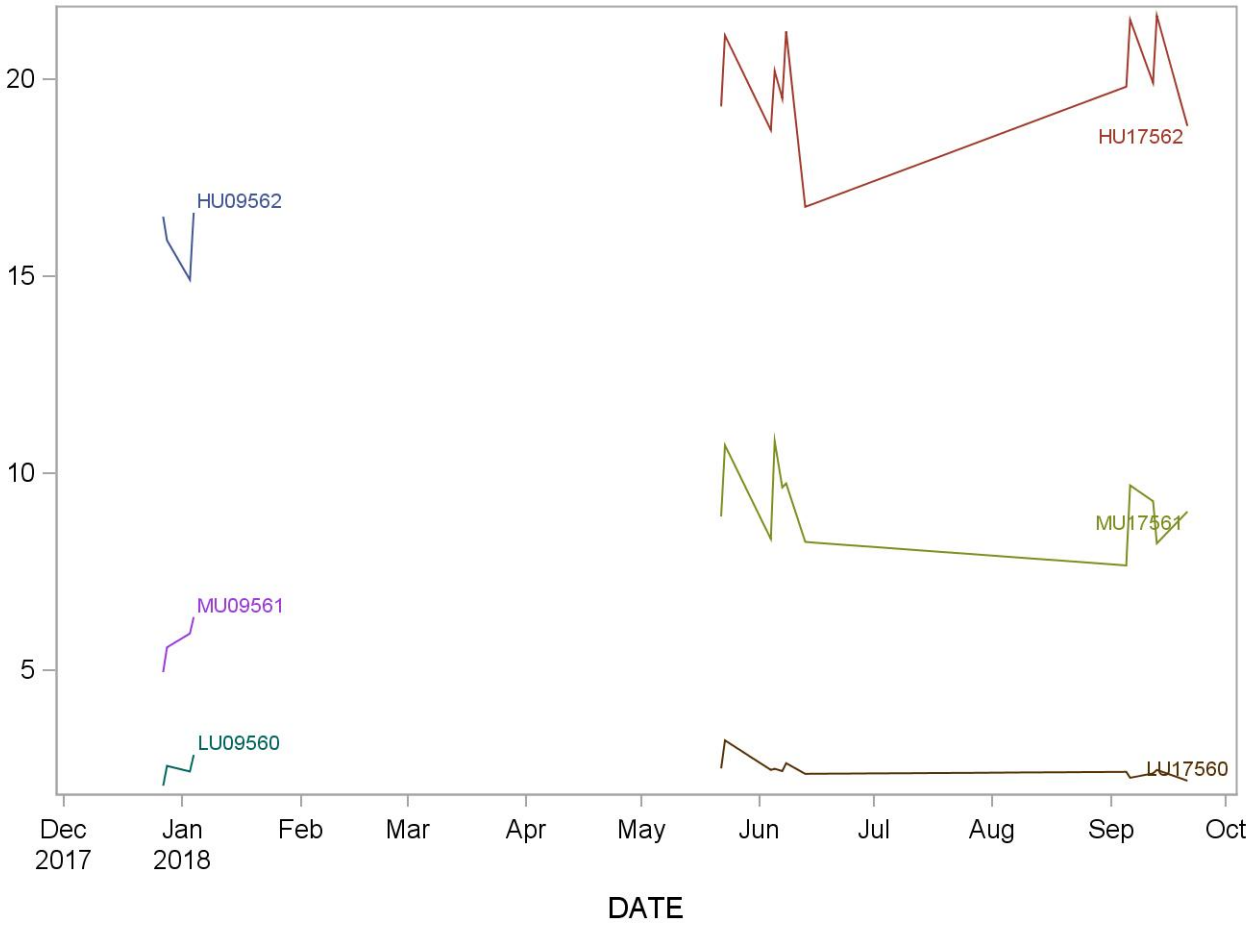
2014 Summary Statistics and QC Chart for 1,3,7-trimethylxanthine, U 2nd C umol/L

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	28.3667	1.5306	5.4
LU09560	6	27DEC17	05JAN18	4.0183	0.2444	6.1
MU09561	6	27DEC17	05JAN18	18.4000	1.4114	7.7
HU17562	9	22MAY18	13SEP18	28.2500	1.2723	4.5
LU17560	9	22MAY18	13SEP18	4.0611	0.1631	4.0
MU17561	9	22MAY18	13SEP18	22.5556	1.0212	4.5



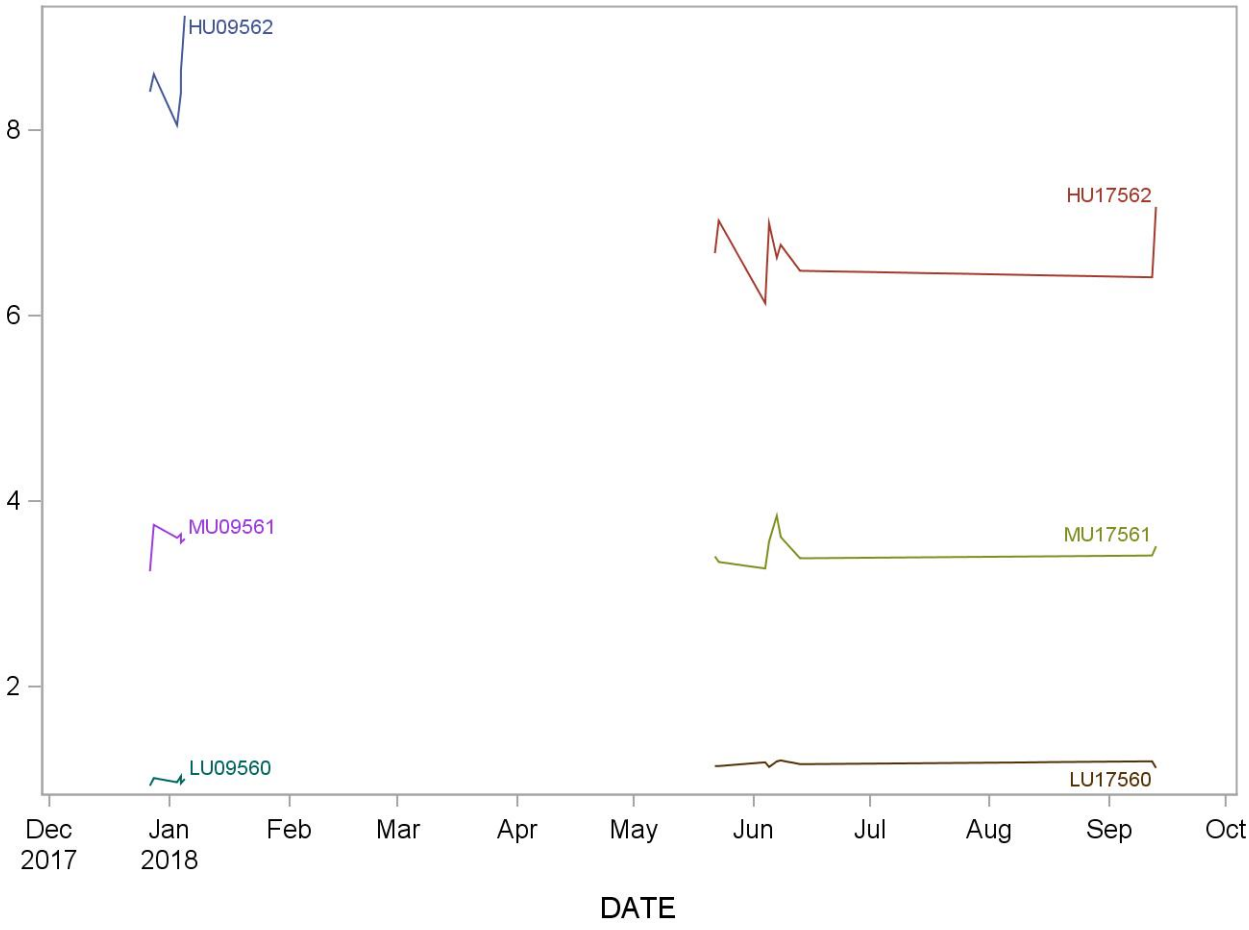
2014 Summary Statistics and QC Chart for 1,3-Dimethyluric acid, U 2nd Col(umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	4	27DEC17	04JAN18	15.9750	0.7805	4.9
LU09560	4	27DEC17	04JAN18	2.4700	0.3243	13.1
MU09561	4	27DEC17	04JAN18	5.6925	0.5922	10.4
HU17562	12	22MAY18	21SEP18	19.8625	1.4063	7.1
LU17560	12	22MAY18	21SEP18	2.4804	0.2569	10.4
MU17561	12	22MAY18	21SEP18	9.1792	0.9866	10.7



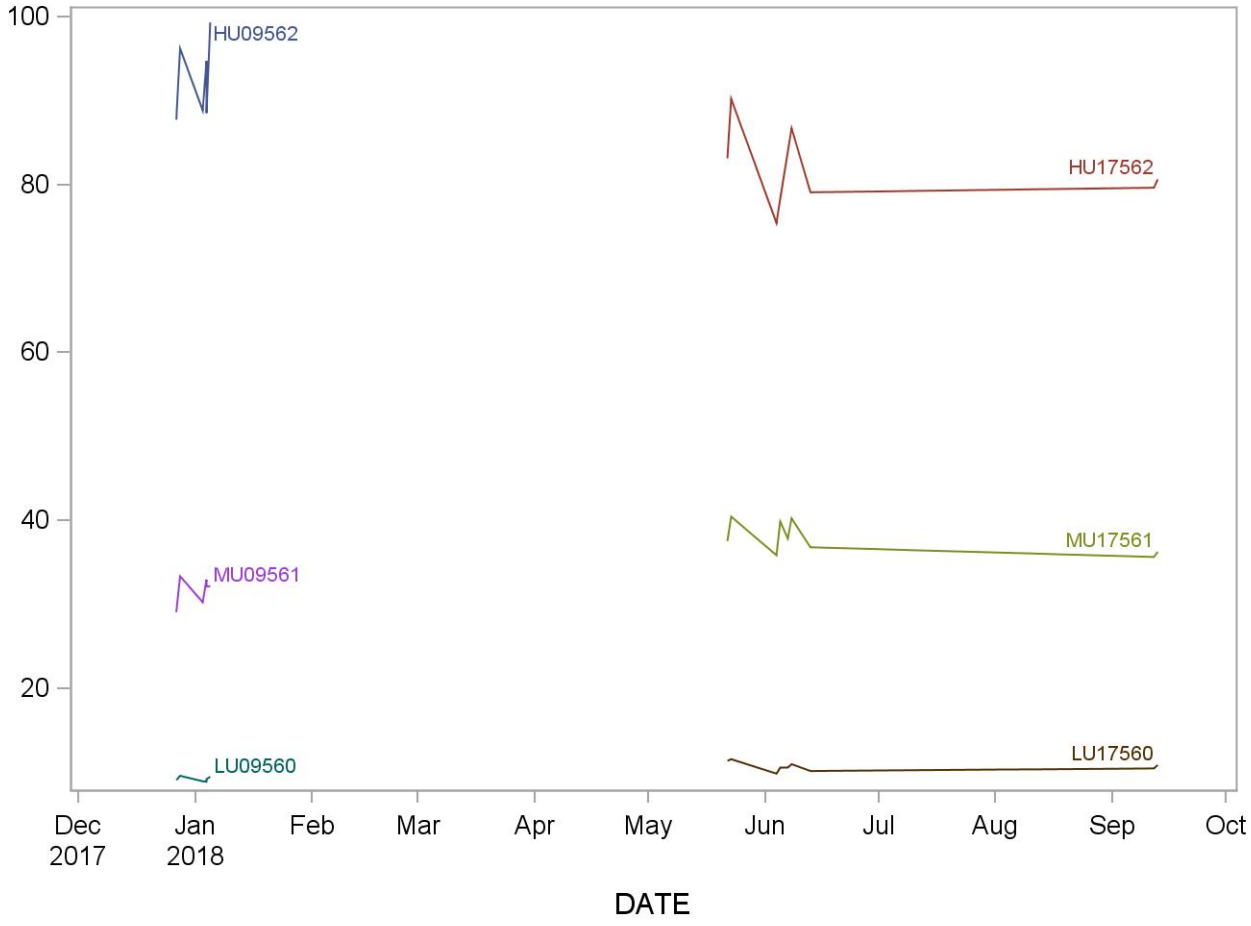
2014 Summary Statistics and QC Chart for 1,3-dimethylxanthine, Urn 2nd C (umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	8.5533	0.3907	4.6
LU09560	6	27DEC17	05JAN18	0.9812	0.0385	3.9
MU09561	6	27DEC17	05JAN18	3.5600	0.1696	4.8
HU17562	9	22MAY18	13SEP18	6.6944	0.3308	4.9
LU17560	9	22MAY18	13SEP18	1.1611	0.0298	2.6
MU17561	9	22MAY18	13SEP18	3.4800	0.1728	5.0



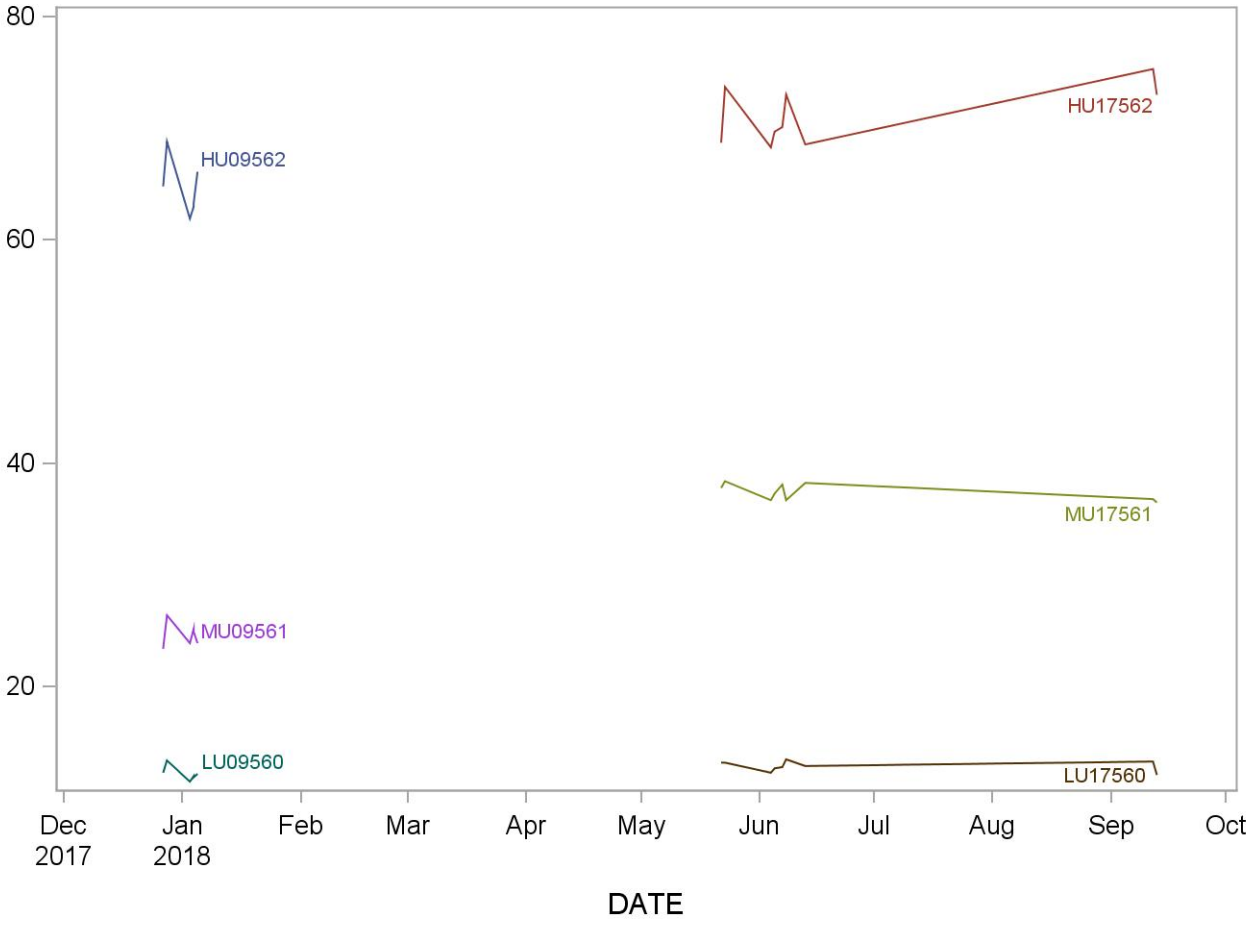
2014 Summary Statistics and QC Chart for 1,7-Dimethyluric acid, U 2nd Col(umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	92.5333	4.8475	5.2
LU09560	6	27DEC17	05JAN18	9.1233	0.2820	3.1
MU09561	6	27DEC17	05JAN18	31.6000	1.6613	5.3
HU17562	9	22MAY18	13SEP18	81.8722	4.5781	5.6
LU17560	9	22MAY18	13SEP18	10.6394	0.5511	5.2
MU17561	9	22MAY18	13SEP18	37.7833	1.9076	5.0



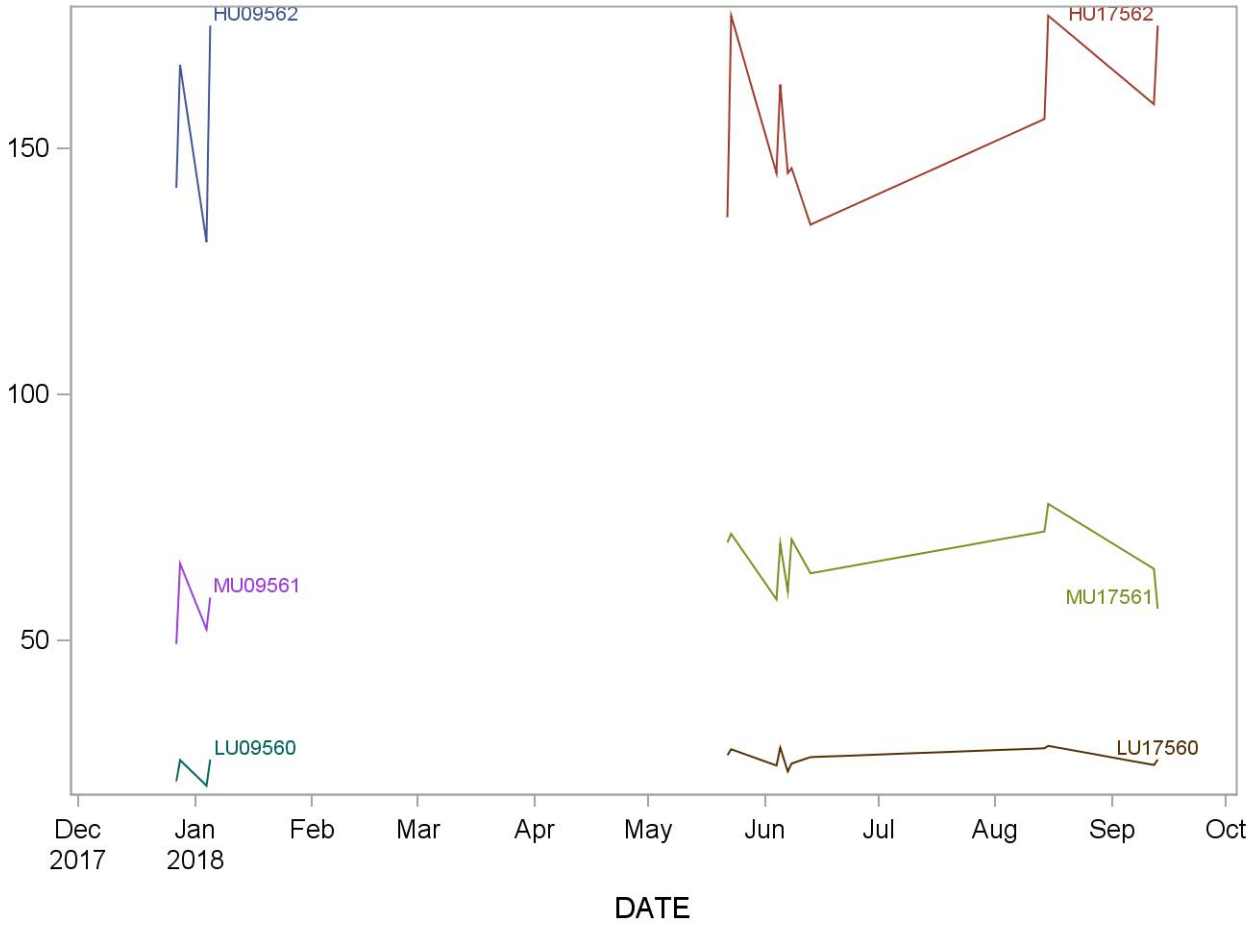
2014 Summary Statistics and QC Chart for 1,7-dimethylxanthine, Urn 2nd C (umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	64.6500	2.5082	3.9
LU09560	6	27DEC17	05JAN18	12.2167	0.6432	5.3
MU09561	6	27DEC17	05JAN18	24.6167	1.1053	4.5
HU17562	9	22MAY18	13SEP18	71.1500	2.6139	3.7
LU17560	9	22MAY18	13SEP18	12.8889	0.4676	3.6
MU17561	9	22MAY18	13SEP18	37.3944	0.7527	2.0



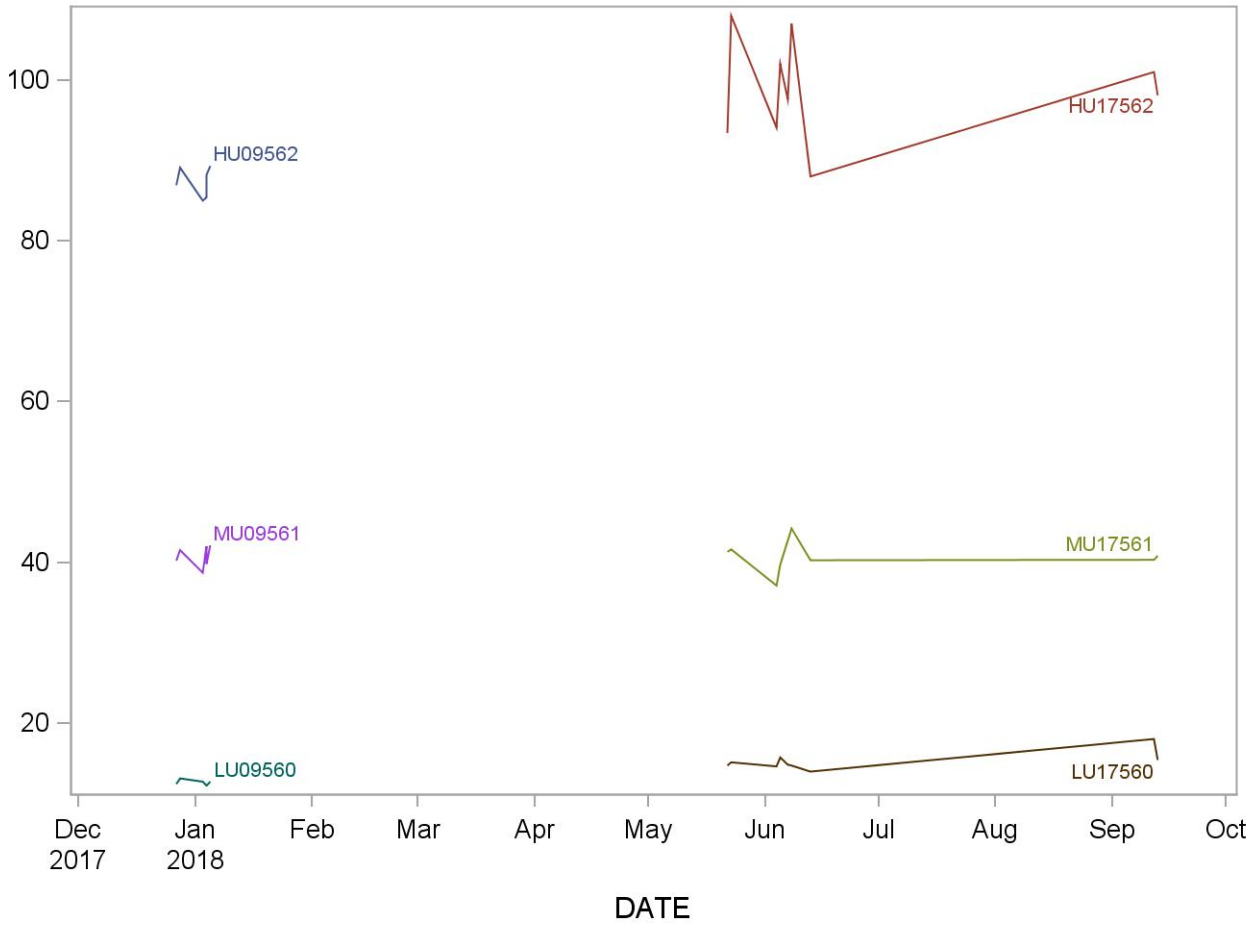
2014 Summary Statistics and QC Chart for 1-methyluric acid, Urine 2nd Col(umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	4	27DEC17	05JAN18	153.7500	20.6781	13.4
LU09560	4	27DEC17	05JAN18	23.2500	2.7958	12.0
MU09561	4	27DEC17	05JAN18	56.4250	7.2894	12.9
HU17562	11	22MAY18	13SEP18	155.7727	15.8262	10.2
LU17560	11	22MAY18	13SEP18	26.2000	1.7510	6.7
MU17561	11	22MAY18	13SEP18	66.7364	6.6677	10.0



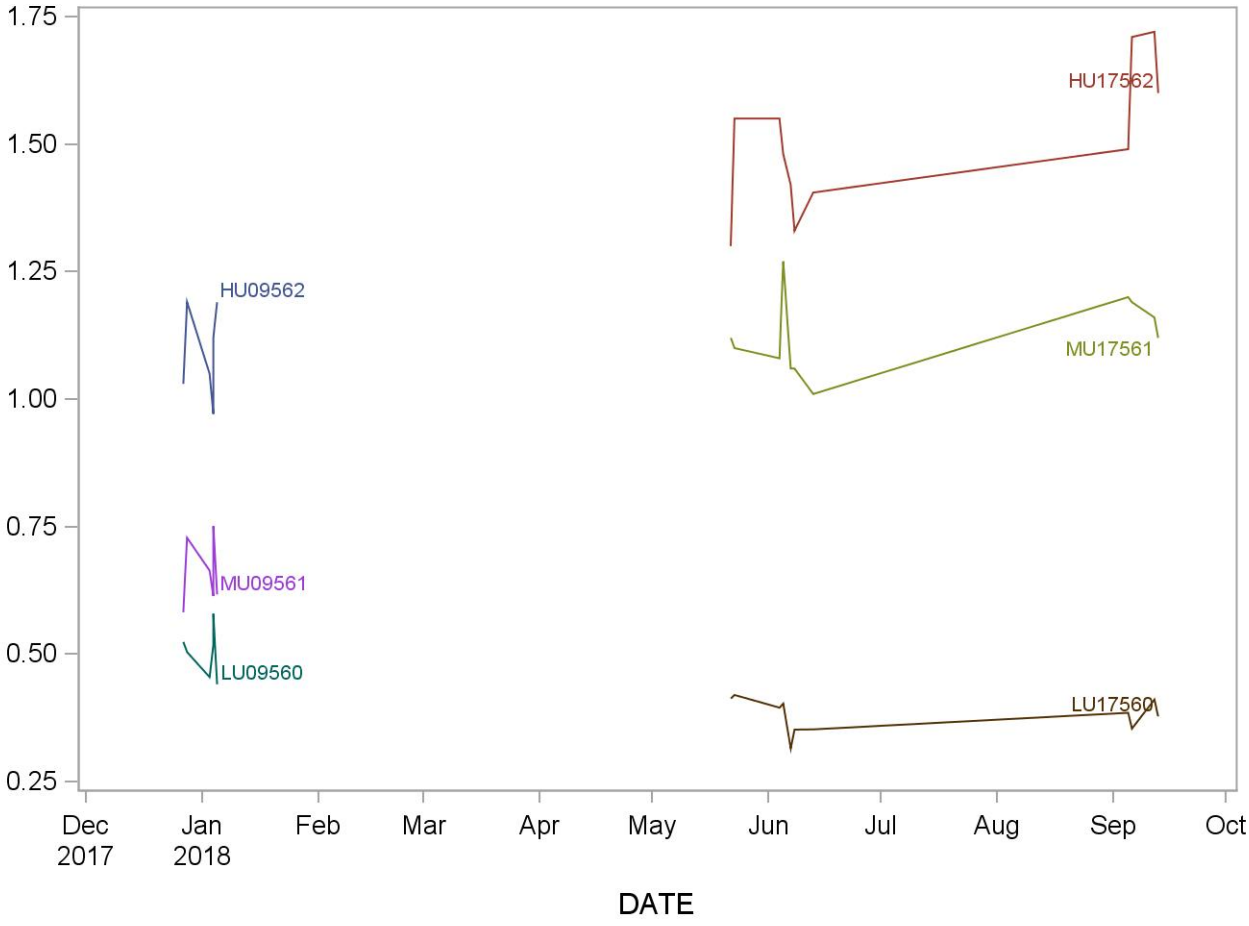
2014 Summary Statistics and QC Chart for 1-methylxanthine, Urine 2nd Col (umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	87.3167	1.8498	2.1
LU09560	6	27DEC17	05JAN18	12.5500	0.3507	2.8
MU09561	6	27DEC17	05JAN18	40.7167	1.3674	3.4
HU17562	9	22MAY18	13SEP18	98.8000	6.4883	6.6
LU17560	9	22MAY18	13SEP18	15.2167	1.1576	7.6
MU17561	9	22MAY18	13SEP18	40.8833	1.9827	4.8



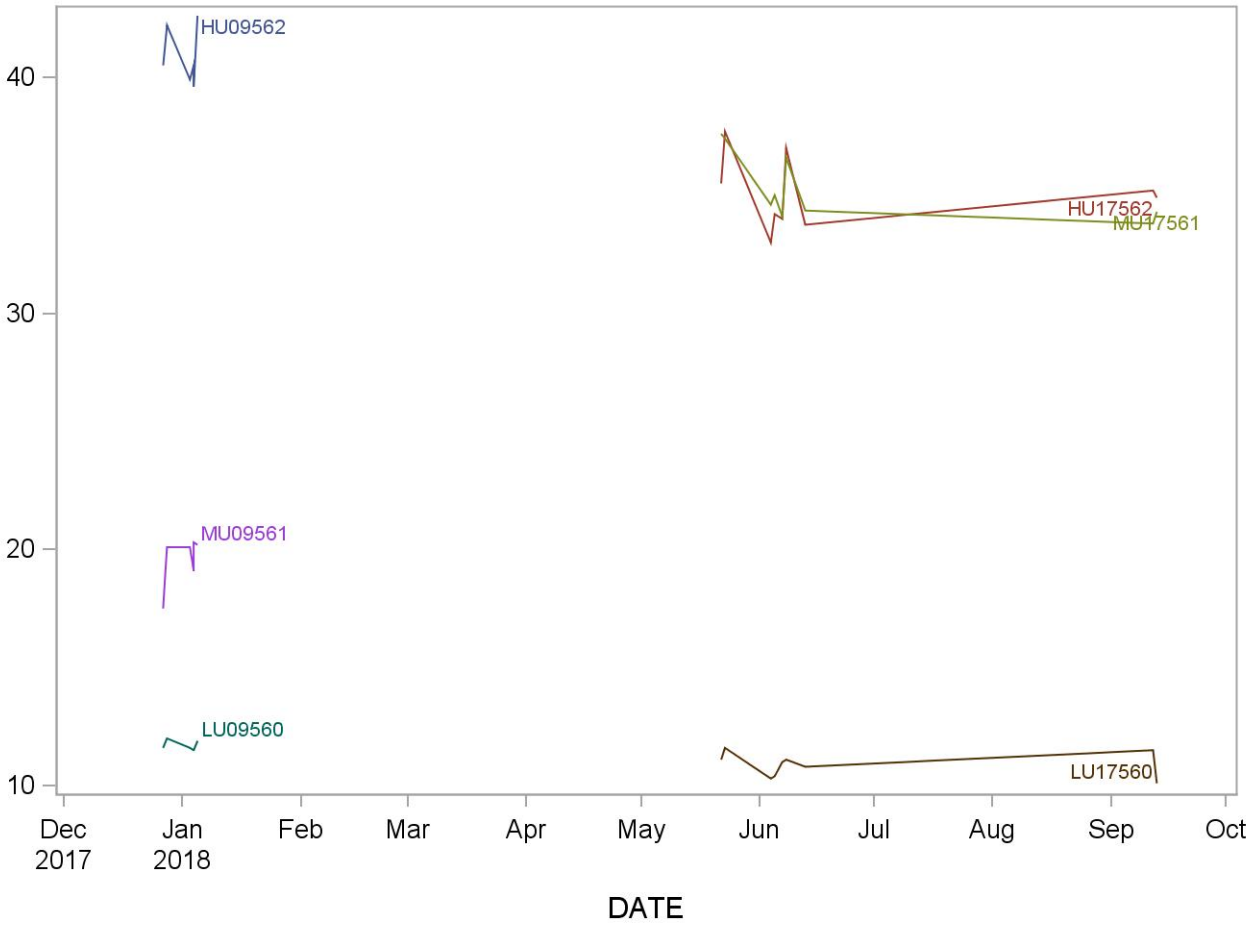
2014 Summary Statistics and QC Chart for 3,7-Dimethyluric acid, U 2nd Col(umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	1.0918	0.0897	8.2
LU09560	6	27DEC17	05JAN18	0.5035	0.0502	10.0
MU09561	6	27DEC17	05JAN18	0.6593	0.0678	10.3
HU17562	11	22MAY18	13SEP18	1.5050	0.1386	9.2
LU17560	11	22MAY18	13SEP18	0.3799	0.0330	8.7
MU17561	11	22MAY18	13SEP18	1.1245	0.0751	6.7



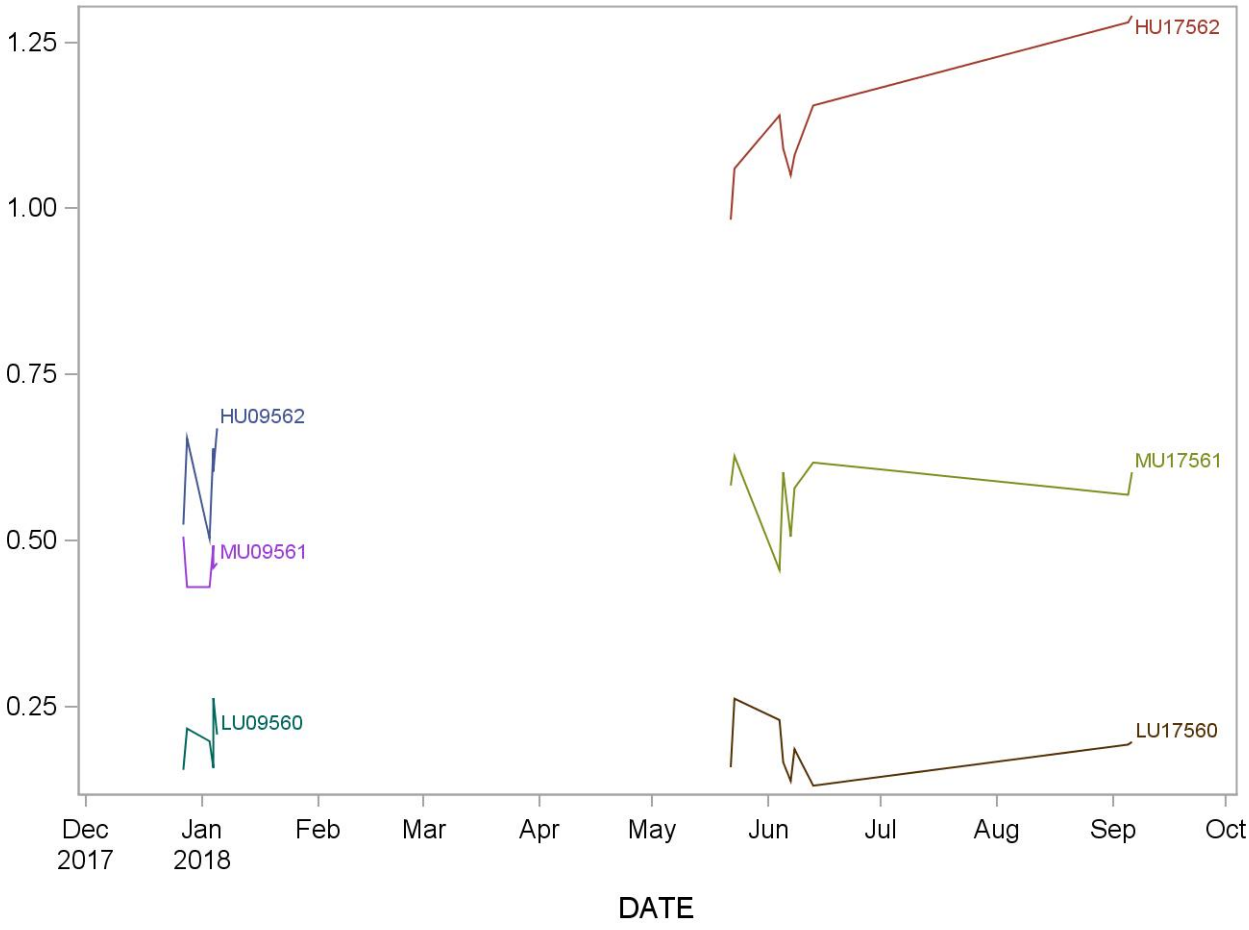
2014 Summary Statistics and QC Chart for 3,7-dimethylxanthine, Urn 2nd C (umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	40.8833	1.2319	3.0
LU09560	6	27DEC17	05JAN18	11.6833	0.2137	1.8
MU09561	6	27DEC17	05JAN18	19.5500	1.0950	5.6
HU17562	9	22MAY18	13SEP18	35.0278	1.5324	4.4
LU17560	9	22MAY18	13SEP18	10.8778	0.5239	4.8
MU17561	9	22MAY18	13SEP18	35.3056	1.4816	4.2



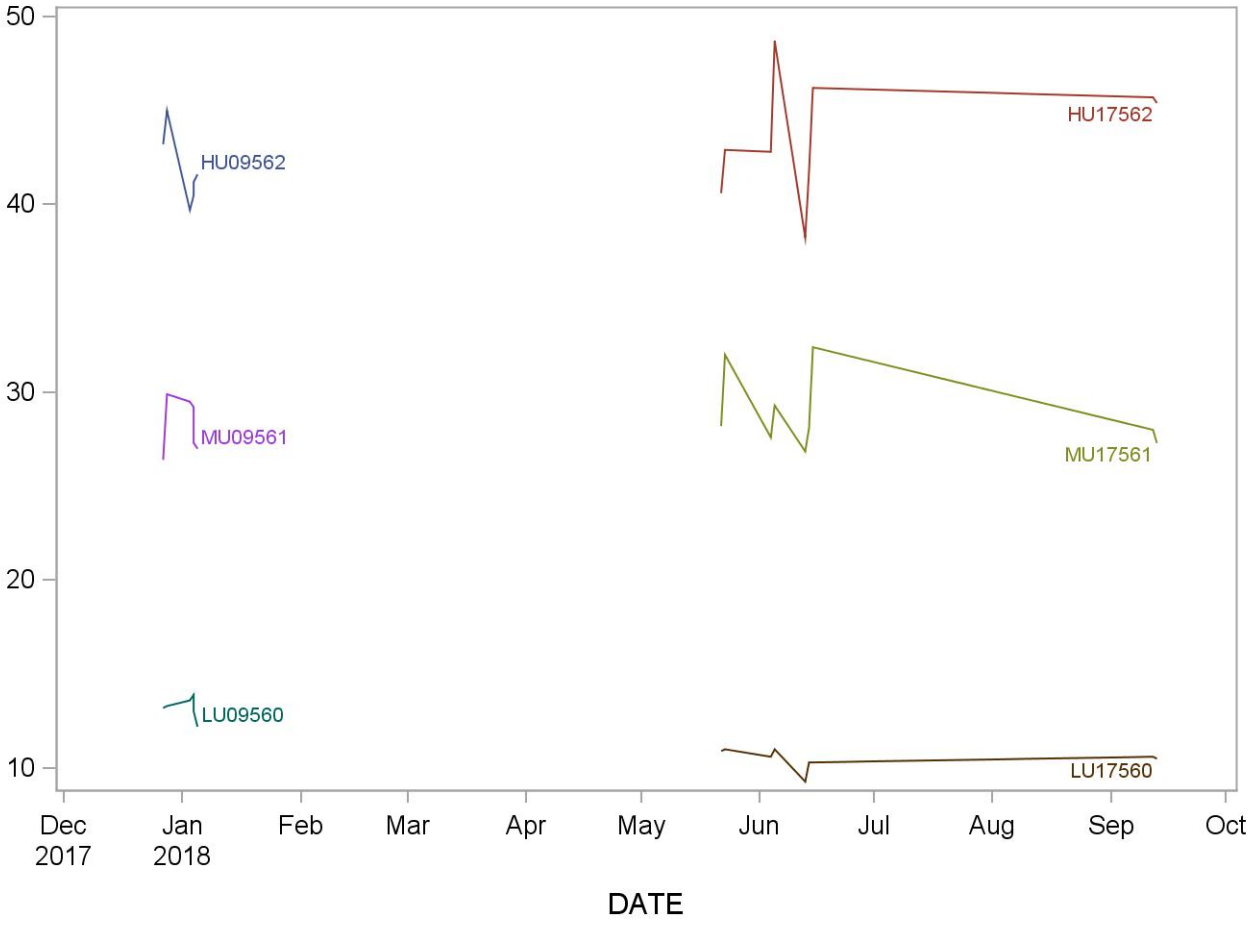
2014 Summary Statistics and QC Chart for 3-methyluric acid, Urine 2nd Col(umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	0.5988	0.0698	11.7
LU09560	6	27DEC17	05JAN18	0.1998	0.0403	20.2
MU09561	6	27DEC17	05JAN18	0.4640	0.0315	6.8
HU17562	9	22MAY18	06SEP18	1.1253	0.1035	9.2
LU17560	9	22MAY18	06SEP18	0.1847	0.0423	22.9
MU17561	9	22MAY18	06SEP18	0.5715	0.0559	9.8



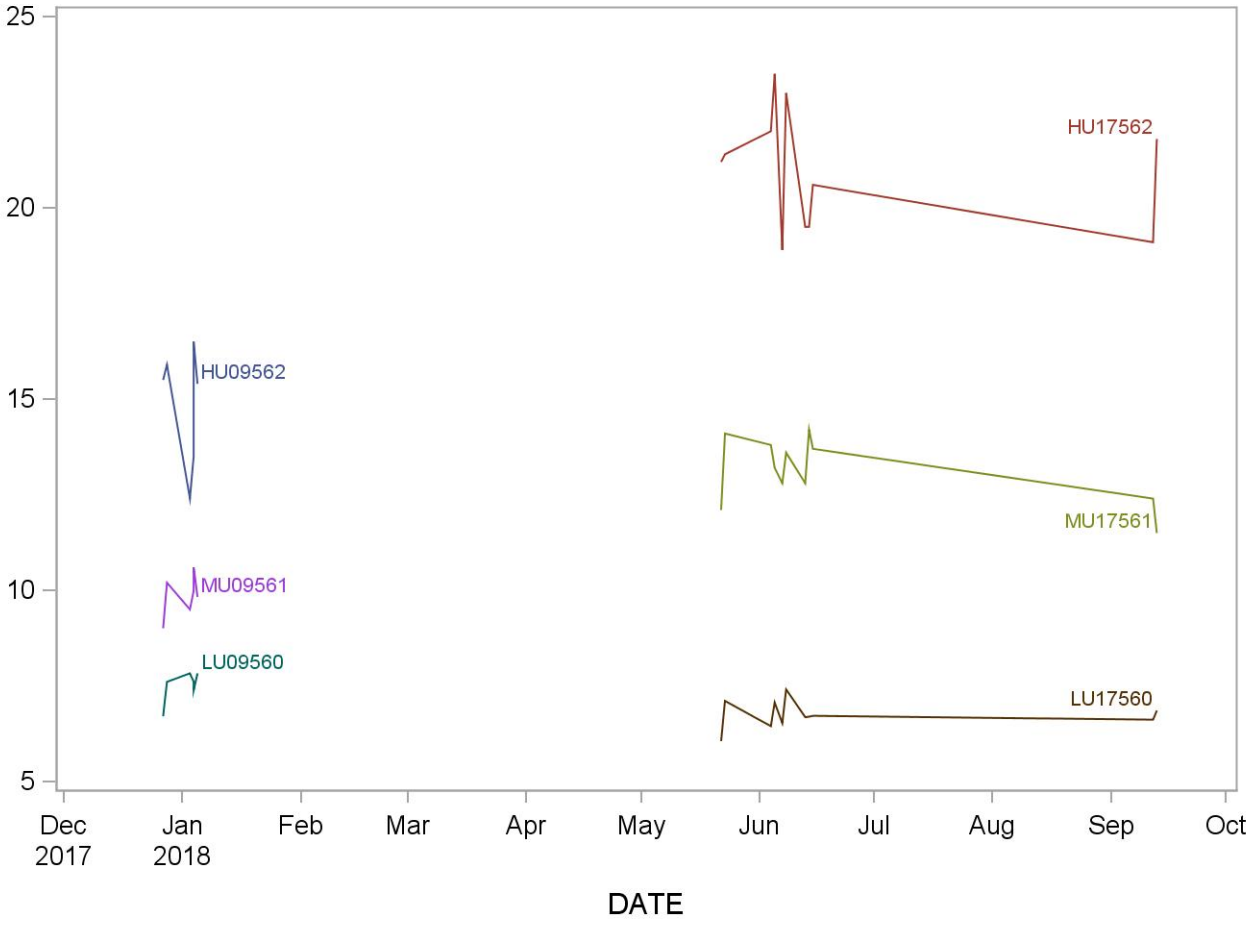
2014 Summary Statistics and QC Chart for 3-methylxanthine, Urine 2nd Col (umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	41.8667	1.9325	4.6
LU09560	6	27DEC17	05JAN18	13.2000	0.5831	4.4
MU09561	6	27DEC17	05JAN18	28.2167	1.4878	5.3
HU17562	9	22MAY18	13SEP18	43.5944	3.2096	7.4
LU17560	9	22MAY18	13SEP18	10.4972	0.5316	5.1
MU17561	9	22MAY18	13SEP18	28.8611	2.0124	7.0



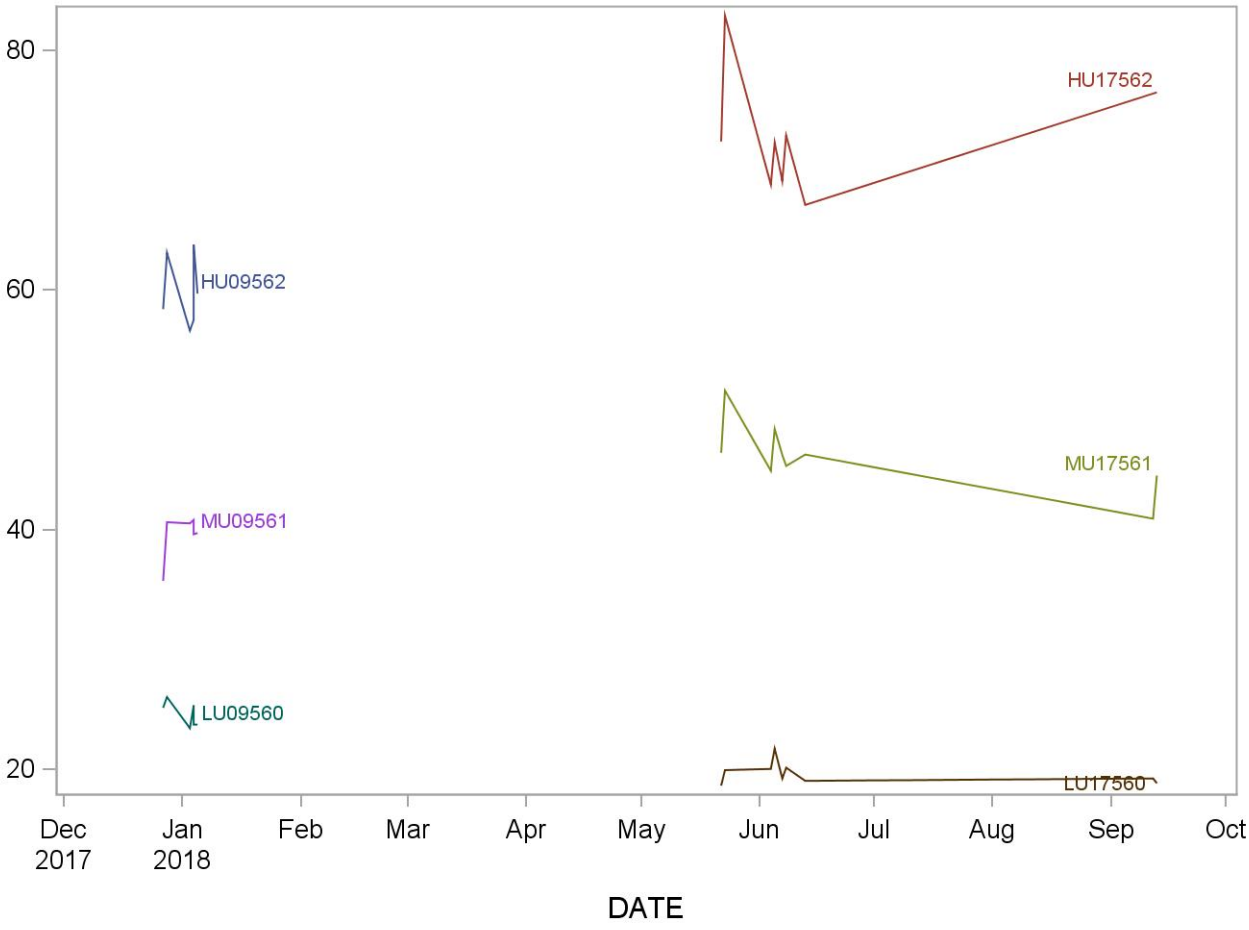
2014 Summary Statistics and QC Chart for 7-methyluric acid, Urine 2nd Col(umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	14.8667	1.5731	10.6
LU09560	6	27DEC17	05JAN18	7.4967	0.4191	5.6
MU09561	6	27DEC17	05JAN18	9.8567	0.5542	5.6
HU17562	11	22MAY18	13SEP18	20.9545	1.5731	7.5
LU17560	11	22MAY18	13SEP18	6.7468	0.3631	5.4
MU17561	11	22MAY18	13SEP18	13.1091	0.8689	6.6



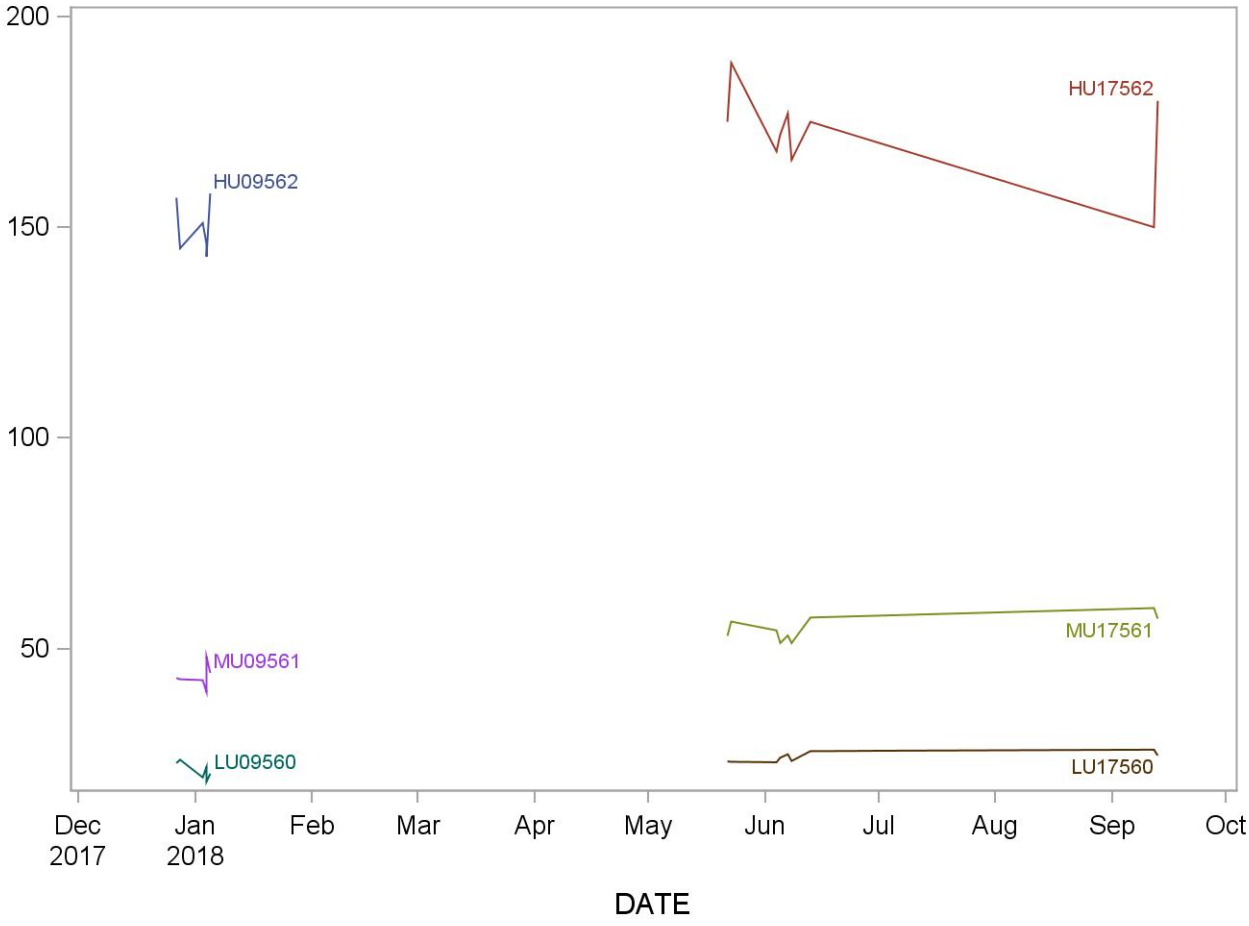
2014 Summary Statistics and QC Chart for 7-methylxanthine, Urine 2nd Col (umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	59.8500	2.9791	5.0
LU09560	6	27DEC17	05JAN18	24.5333	1.0708	4.4
MU09561	6	27DEC17	05JAN18	39.4833	1.9177	4.9
HU17562	9	22MAY18	13SEP18	73.1556	4.8750	6.7
LU17560	9	22MAY18	13SEP18	19.6111	0.9506	4.8
MU17561	9	22MAY18	13SEP18	46.0500	2.9009	6.3



2014 Summary Statistics and QC Chart for AAMU, Urine 2nd collection (umol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562	6	27DEC17	05JAN18	150.000	6.387	4.3
LU09560	6	27DEC17	05JAN18	21.067	2.029	9.6
MU09561	6	27DEC17	05JAN18	43.350	2.718	6.3
HU17562	9	22MAY18	13SEP18	172.444	10.783	6.3
LU17560	9	22MAY18	13SEP18	24.206	1.144	4.7
MU17561	9	22MAY18	13SEP18	54.828	2.921	5.3



Acknowledgements

We gratefully acknowledge the contributions of Ching-I Pao, Ph.D., Patrick Simon, B.S., Michael Rybak, Ph.D., and Christine Pfeiffer, Ph.D., who assisted in developing the methodology, performing the analyses for urine caffeine and caffeine metabolites, and preparing the manuscript for this chapter.

References

1. Ashihara H, Sano H, Crozier A. Phytochemistry. 2008 69:841-56.
2. Higdon JV, Frei B. Crit Rev Food Sci Nutr. 2006 46:101-23.
3. Ohta A, Sitkovsky M. Curr Opin Pharmacol. 2009 9:501.
4. Tunnicliffe JM, Erdman KA, Reimer RA, Lun V, Shearer J Appl Physiol Nutr Metab. 2008 33:1301.
5. Rogers PJ, Hohoff C, Heatherley SV, Mullings EL, Maxfield PJ, Evershed RP, Deckert J, Nutt DJ. Neuropsychopharmacology. 2010 35: 1973
6. Rottman BM, Ahn WK. Psychon Bull Rev Dec. 2009 16:1043-9.
7. Ferre S. J. Neurochem. 2008 105:1067
8. Cornelis MC, El-Sohemy A. Curr Opin Clin Nutr Metab Care. 2007 10:745.
9. Geleijnse JM. Vasc. Health Risk Manag. 2008 4:963.
10. Lopez-Garcia E, Rodriguez-Artalejo F, Rexrode KM, Logroscino G, Hu FB, van Dam RM. Circulation. 2009 119:1116.
11. Nettleton JA, Follis JL, Schabath MB. Am J Epidemiol. 2009 169:1445.
12. van Dam RM. Appl Physiol Nutr Metab. 2008 33:1269.
13. Tunnicliffe, JM, Shearer,J Appl Physiol Nutr Metab. 2008 33:1290
14. Lu PZ, Lai CY, Chan WH. Int J Mol Sci. 2008 9:698
15. Gressner OA. Hepatology. 2009 50:970.
16. Nkondjock A. Cancer Lett. 2009 277:121.
17. Montella M, Tramacere I, Tavani A, Gallus S, Crispo A, Talamini R, Dal Maso L, Ramazzotti V, Galeone C, Franceschi S, La Vecchia C. Nutr Cancer. 2009 61:76.
18. Tang N, Zhou B, Wang B, Yu R. Am J Obstet Gynecol. 2009 200:290.
19. Bakker R, Steegers EA, Obradov A, Raat H, Hofman A, Jaddoe VW. Am J Clin Nutr. 2010 91:1961.
20. Sin CW, Ho JS, Chung JW. J Clin Nurs. 2009 18:13-21.
21. Burgalassi A, Ramacciotti CE, Bianchi M, Coli E, Polese L, Bondi E, Massimetti G, Dell'osso L. Eat Weight Disord. 2009 14:212.
22. Miners JO, Birkett DJ. Gen. Pharmacol. 1996 27:245.
23. Kot M, Daniel WA. Pharmacol Rep. 2008 60:789.
24. Faber MS, Jetter A, Fuhr U. Basic Clin Pharmacol Toxicol. 2005 97:125.

25. Frye RF, Zgheib NK, Matzke GR, Chaves-Gnecco D, Rabinovitz M, Shaikh OS, Branch RA. Clin Pharmacol Ther. 2006 80:235.
26. Kh Hakooz NM. Curr Drug Metab. 2009 10:329.
27. Dorne JL, Walton K, Renwick AG. Food Chem Toxicol. 2005 43:206
28. Strolin Benedetti M, Whomsley R, Baltes E. Expert Opin Drug Metab Toxicol. 2006 2:895
29. Petersen MS, Halling J, Damkier P, et al. Eur. J Clin Pharmacol. 2006 62:1041.
30. Nyéki A, Biollaz J, Kesselring UW, Décosterd LA. J Chromatogr B Biomed Sci Appl. 2001 755:73
31. Nordmark A, Lundgren S, Cnattingius S, Rane A. Br J Clin Pharmacol. 1999 47:397
32. Jetter A, Kinzig-Schippers M, Illauer M, Hermann R, Erb K, Borlak J, Wolf H, Smith G, Cascorbi I, Sörgel F, Fuhr U. Eur J Clin Pharmacol. 2004 60:17
33. Caudill SP, Schleicher RL, Pirkle JL. Stat Med 2008 27:4094-4106.
34. Rybak, ME, Sternberg, MR, Pao, C-I, Ahluwalia, N, Pfeiffer, CM. J Nutr 2015 145: 766-774
35. Wong P, Villeneuve G, Tessier V, Banerjee K, Nedved H, Jean-Claude BJ, Leyland-Jones B. J Pharm Biomed Anal 2002 28: 693–700.
36. Johnson EA. Biochem J 1952 51: 133–138.
37. Gulland JM, Holiday ER, Macrae TF. J Chem Soc 1934 1639-1644.
38. Mann FG, Porter JWG. J Chem Soc 1945 751–760.
39. Fujii T, Saito T, Tamura K. Chem Pharm Bull 1991 39: 2855-2862.

Appendix A – Method Performance Validation

A. Accuracy

(1) AAMU

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	AAMU						AAMU						
		LU17560					MU17560						
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	25.0	22.7	24.7		0	49.3	59.0	56.1		86.9	3.7
	2		25.8	25.1				56.8	56.6				
	3		24.0	25.5				54.7	60.3				
Sample + Spike 1	1	15	36.5	39.3	37.6	86.2							
	2		37.4	36.2									
	3		37.2	39.1									
Sample + Spike 2	1	25	46.6	45.1	46.0	85.1	25	75.2	75.0	76.8	82.7		
	2		48.1	46.3				77.3	82.5				
	3		43.3	46.3				72.7	78.1				
Sample + Spike 3	1	50	76.6	74.8	71.1	92.8	50	94.6	101	99.9	87.6		
	2		65.9	72.5				99.2	104				
	3		65.0	71.6				96.7	104				

(2) 1-Methyluric Acid (1U)

Accuracy using Spike Recovery - fill in yellow shaded cells

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC

Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	1U						1U							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	24.7	26.9	25.6		0	59.7	62.6	64.4		103	4.93
	2		23.9	24.9				70.6	62.9				
	3		27.3	25.7				67.5	63.3				
Sample + Spike 1	1	10	35.1	37.1	36.0	104	15	76.7	79.3	80.9	110		
	2		36.8	34.3				84.2	80.5				
	3		37.6	34.8				86.3	78.5				
Sample + Spike 2	1	25	49.8	49.3	51.1	102	25	95.4	82.9	90.9	106		
	2		53.8	51.1				96.1	84.3				
	3		51.6	51.2				93.8	92.9				
Sample + Spike 3	1	50	76.2	69.8	73.3	95.4	50	112	116	115	100		
	2		77.8	72.1				121	103				
	3		70.7	72.9				119	117				

(3) 3-Methyluric Acid (3U)

Accuracy using Spike Recovery - fill in yellow shaded cells

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC

Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	3U						3U						
		LU17560					MU17560						
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.20	0.15	0.20		0	0.61	0.58	0.59		104	2.84
	2		0.21	0.17				0.54	0.65				
	3		0.28	0.18				0.52	0.62				
Sample + Spike 1	1	1	1.10	1.32	1.23	103	1	1.64	1.75	1.68	109		
	2		1.14	1.26				1.70	1.63				
	3		1.21	1.35				1.76	1.57				
Sample + Spike 2	1	2	2.24	2.04	2.21	101	2	2.72	2.69	2.67	104		
	2		2.22	2.22				2.52	2.94				
	3		2.28	2.28				2.46	2.69				
Sample + Spike 3	1	3	3.54	3.34	3.27	102	3	3.70	3.73	3.75	106		
	2		2.73	3.38				3.75	3.69				
	3		3.12	3.50				3.80	3.84				

(4) 7-Methyluric Acid (7U)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	7U						7U							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	7.52	6.95	7.19		0	13.3	14.7	14.3		101.0	3.73
	2		7.04	6.91				14.9	15.0				
	3		8.16	6.54				15.2	12.9				
Sample + Spike 1	1	3	10.0	9.81	10.1	96.4	10	25.4	25.1	24.9	106		
	2		10.8	9.25				25.8	24.7				
	3		10.9	9.71				25.1	23.3				
Sample + Spike 2	1	5	12.2	12.2	12.2	101	15	29.2	26.8	28.9	97.2		
	2		13.0	11.0				30.3	28.6				
	3		12.9	12.1				28.1	30.5				
Sample + Spike 3	1	10	18.0	18.5	17.6	104	25	44.3	37.8	39.7	102		
	2		18.3	17.8				38.3	36.6				
	3		17.7	15.5				40.0	41.3				

(5) 1,3-Dimethyluric Acid (13U)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	13U						13U						
		LU17560					MU17560						
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	2.17	2.55	2.47		0	8.34	8.81	9.26		103	4.39
	2		2.26	2.72				9.15	10.6				
	3		2.52	2.57				9.02	9.63				
Sample + Spike 1	1	2	4.45	4.55	4.45	99	5	14.4	15.5	14.8	112		
	2		4.25	4.65				14.7	14.5				
	3		4.13	4.65				15.0	14.9				
Sample + Spike 2	1	3	4.88	5.56	5.49	101	10	20.1	20.4	19.4	102		
	2		5.42	6.00				19.0	19.4				
	3		5.6	5.49				17.3	20.3				
Sample + Spike 3	1	5	6.81	8.27	7.54	101	15	22.3	26.3	24.7	103		
	2		6.87	8.14				24.9	25.4				
	3		7.45	7.68				22.6	26.7				

(6) 1,7-Dimethyluric Acid (17U)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	17U						17U							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	10.8	10.8	10.6		0	33.2	37.1	36.2		98.2	1.82
	2		10.0	11.1				35.8	37.2				
	3		10.8	10.3				36.4	37.2				
Sample + Spike 1	1	5	14.9	17.4	15.5	98.0	15	51.5	53.0	51.3	101		
	2		14.9	16.1				49.9	51.1				
	3		14.6	15.3				49.8	52.2				
Sample + Spike 2	1	10	19.7	21.4	20.4	97.2	25	61.4	63.7	61.2	100		
	2		20.2	20.6				58.5	60.4				
	3		19.9	20.3				59.4	63.6				
Sample + Spike 3	1	15	24.7	25.9	25.0	96.0	50	86.2	84.2	84.8	97		
	2		24.9	25.8				82.1	85.2				
	3		24.0	24.9				83.1	87.7				

(7) 3,7-Dimethyluric Acid (37U)

Accuracy using Spike Recovery - fill in yellow shaded cells

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC

Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	37U						37U							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.35	0.41	0.39		0	1.00	1.22	1.13		93.8	4.40
	2		0.36	0.45				1.21	1.13				
	3		0.38	0.35				1.19	1.05				
Sample + Spike 1	1	1	1.31	1.35	1.39	101	1	1.88	1.95	2.00	86.8		
	2		1.20	1.57				2.15	1.98				
	3		1.50	1.42				2.06	1.99				
Sample + Spike 2	1	2	2.36	2.32	2.25	93.1	2	3.3	3.13	3.01	94.0		
	2		2.01	2.39				3.13	2.89				
	3		2.05	2.35				2.76	2.87				
Sample + Spike 3	1	3	3.39	3.23	3.20	93.9	3	3.96	3.93	3.97	94.4		
	2		3.04	3.21				3.64	3.96				
	3		3.00	3.35				4.09	4.22				

(8) 1,3,7-Trimethyluric Acid (137U)

Accuracy using Spike Recovery - fill in yellow shaded cells

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC

Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	137U						137U							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.95	0.93	0.96		0	3.55	3.68	3.71		98.1	1.72
	2		0.92	1.00				3.67	3.84				
	3		0.98	0.99				3.74	3.78				
Sample + Spike 1	1	1	1.88	1.95	1.92	96.2	2	5.7	5.72	5.73	101.0		
	2		1.97	1.91				5.79	5.75				
	3		1.92	1.91				5.58	5.84				
Sample + Spike 2	1	2	2.92	2.82	2.91	97.2	3	6.73	6.68	6.67	98.7		
	2		2.96	2.84				6.7	6.6				
	3		2.97	2.92				6.63	6.69				
Sample + Spike 3	1	3	3.89	3.93	3.87	96.9	5	8.6	8.75	8.65	98.7		
	2		4.03	3.86				8.66	8.93				
	3		3.84	3.66				8.45	8.48				

(9) 1-Methylxanthine (1X)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	1X						1X							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	14.8	16.5	15.6		0	39.0	44.1	41.7		100	4.88
	2		14.7	16.2				41.3	42.2				
	3		15.2	16.4				38.6	45.2				
Sample + Spike 1	1	10	25.2	27.0	25.6	100	15	55.4	57.5	57.8	107		
	2		23.8	26.2				59.7	58.3				
	3		25.0	26.6				54.9	61.2				
Sample + Spike 2	1	15	28.9	30.9	29.7	93.6	25	66.8	69.2	67.4	103		
	2		29.5	29.2				62.8	68.9				
	3		30.2	29.3				65.5	71.4				
Sample + Spike 3	1	25	39.0	40.6	39.7	96.3	50	89.1	92.5	92.5	102		
	2		35.7	42.3				92.3	94.6				
	3		40.7	39.9				88.9	97.6				

(10) 3-Methylxanthine (3X)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	3X						3X							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	12.1	11.3	11.4		0	30.4	29.8	29.5		102	4.36
	2		11.6	10.6				29.6	28.0				
	3		11.5	11.1				28.5	30.7				
Sample + Spike 1	1	5	17.1	15.9	16.2	95.7	15	44.6	45.8	45.2	105		
	2		16.7	15.0				45.7	43.6				
	3		16.3	15.9				45.6	45.9				
Sample + Spike 2	1	10	21.6	21.3	21.8	104	25	60.6	53.9	56.5	108		
	2		21.8	21.0				57.0	52.7				
	3		22.7	22.2				57.0	57.6				
Sample + Spike 3	1	15	25.3	26.1	26.3	99.2	50	87.0	77.4	79.8	101		
	2		27.8	26.6				78.7	76.3				
	3		26.2	25.5				77.6	82.0				

(11) 7-Methylxanthine (7X)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	7X						7X							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	20.7	22.5	21.4		0	46.3	48.8	47.8		96.6	2.83
	2		21.1	21.5				48.1	49.0				
	3		22.0	20.4				48.2	46.1				
Sample + Spike 1	1	15	35.5	35.7	35.2	92.4	15	63.6	63.4	62.4	97.3		
	2		35.0	37.0				61.8	63.1				
	3		35.2	33.0				61.7	60.5				
Sample + Spike 2	1	25	44.8	42.4	44.9	94.3	25	74.0	71.5	72.3	98.1		
	2		43.0	46.2				69.4	73.8				
	3		46.1	47.1				69.8	75.1				
Sample + Spike 3	1	50	72.4	69.5	70.0	97.2	50	99.5	98.0	97.9	100		
	2		69.9	68.7				98.4	98.8				
	3		70.5	68.9				97.9	95.0				

(12) Theophylline (13X)

Accuracy using Spike Recovery - fill in yellow shaded cells

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC

Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

	13X		LU17560				13X	MU17560					
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	1.13	1.23	1.21		0	3.23	3.66	3.47		95.1	1.55
	2		1.13	1.27				3.46	3.54				
	3		1.23	1.26				3.40	3.53				
Sample + Spike 1	1	1	2.18	2.26	2.18	97.5	2	5.29	5.17	5.34	93.3		
	2		2.23	2.18				5.29	5.40				
	3		2.12	2.13				5.36	5.50				
Sample + Spike 2	1	2	2.99	3.34	3.13	95.9	3	6.56	6.22	6.29	94.1		
	2		2.98	3.18				6.20	6.40				
	3		3.16	3.11				6.25	6.13				
Sample + Spike 3	1	3	4.04	3.99	4.08	95.7	5	8.20	8.43	8.18	94.2		
	2		4.08	4.28				8.08	8.20				
	3		4.14	3.95				7.99	8.19				

(13) Paraxanthine (17X)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	17X						17X							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	14.7	15.4	15.1		0	37.4	40.6	40.0		99.3	4.04
	2		14.8	15.1				39.9	41.1				
	3		15.1	15.2				39.4	41.5				
Sample + Spike 1	1	5	20.1	21.1	20.3	105	15	54.4	56.2	54.5	96.9		
	2		19.6	21.0				52.9	55.1				
	3		19.6	20.5				53.9	54.6				
Sample + Spike 2	1	10	24.8	26.6	25.4	103	25	64.3	64.3	64.0	96.2		
	2		24.5	25.8				63	64.8				
	3		24.8	25.7				61.9	65.9				
Sample + Spike 3	1	15	29.9	30.7	29.9	98.9	50	87.5	87.0	87.7	95.5		
	2		29.0	31.1				88.4	87.3				
	3		28.7	29.9				87.4	88.7				

(14) Theobromine (37X)

Accuracy using Spike Recovery - fill in yellow shaded cells

Recovery = (final concentration – initial concentration)/added concentration

Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC

Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	37X						37X							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	11.8	11.6	11.7		0	36.4	37.4	37.4		99.4	1.18
	2		11.5	11.7				37.3	37.6				
	3		11.9	11.4				37.6	37.9				
Sample + Spike 1	1	5	16.5	17.0	16.6	99.0	15	53.6	51.0	52.3	99.6		
	2		16.5	16.5				51.3	52.7				
	3		16.6	16.5				50.7	54.5				
Sample + Spike 2	1	10	21.0	20.9	21.4	97.5	25	64.4	62.7	62.6	101		
	2		21.2	21.8				62.8	63.3				
	3		22.0	21.5				60.7	61.9				
Sample + Spike 3	1	15	26.5	26.9	26.7	100	50	88.1	88.3	87.0	99.3		
	2		26.7	27.0				85.6	85.6				
	3		26.5	26.3				84.7	89.9				

(15) Caffeine (137X)

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Samples: 2017 bench QC pool: low and medium QC
Run date: Dec 13, 2017 (day 1); Dec 15, 2017 (day 2)

Analyte:	137X						137X							
		LU17560					MU17560							
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	4.01	4.18	4.10		0	21.7	23.3	23.1		103	3.18
	2		4.01	4.14				23.1	23.6				
	3		4.15	4.13				23.4	23.4				
Sample + Spike 1	1	2	6.19	6.13	6.16	103	10	34.0	33.2	33.9	108		
	2		6.19	6.25				34.1	34.2				
	3		5.89	6.29				34.0	33.6				
Sample + Spike 2	1	5	9.08	9.35	9.11	100	15	38.7	38.6	38.7	104		
	2		9.09	9.01				39.6	37.8				
	3		9.09	9.05				38.2	39.1				
Sample + Spike 3	1	10	13.8	14.1	14.0	99.0	25	49.9	49.7	49.3	105		
	2		14.0	14.1				49.9	48.7				
	3		13.9	14.1				49.8	47.9				

A. Stability

(1) AAMU

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**AAMU**

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	45.1	51.9	48.1	52.9
Replicate 2	52.9	51.3	52.8	60.2
Replicate 3	54.8	46.4	54.4	57.6
Mean	50.9	49.9	51.8	56.9
% difference from initial measurement	--	**-2.09**	**1.64**	**11.7**

AAMU				
HU09562	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	155	145	173	198
Replicate 2	180	183	176	200
Replicate 3	174	169	164	187
Mean	170	166	171	195
% difference from initial measurement	--	**-2.36**	**0.79**	**14.9**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**AAMU**

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	52.5	44.8
Replicate 2	43.1	43.5
Replicate 3	44.2	38.2
Mean	46.6	42.2
% difference from initial measurement	--	**-9.51**

AAMU		
HU09562	**Initial measurement**	**Long-term stability**
Replicate 1	177	161
Replicate 2	180	159
Replicate 3	162	151
Mean	173	157
% difference from initial measurement	--	**-9.25**

(2) 1-Methyluric Acid (1U)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	1U

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	61.7	61.6	61.2	62.8
Replicate 2	65.6	64.0	63.6	61.6
Replicate 3	62.5	62.0	60.4	63.4
Mean	63.3	62.5	61.7	62.6
% difference from initial measurement	--	**-1.16**	**-2.42**	**-1.05**

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	153	151	160	164
Replicate 2	147	150	162	162
Replicate 3	153	147	152	150
Mean	151	149	158	159
% difference from initial measurement	--	**-1.10**	**4.64**	**5.08**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	1U

MU09561	Initial measurement	Long-term stability
Replicate 1	56.5	48.8
Replicate 2	50.4	52.3
Replicate 3	64.1	51.8
Mean	57.0	51.0
% difference from initial measurement	--	**-10.6**

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

HU09562	Initial measurement	Long-term stability
Replicate 1	127	123
Replicate 2	139	132
Replicate 3	156	136
Mean	141	130
% difference from initial measurement	--	**-7.35**

(3) 3-Methyluric Acid (3U)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Analyte:	3U

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	0.56	0.61	0.59	0.64
Replicate 2	0.59	0.54	0.71	0.77
Replicate 3	0.57	0.60	0.50	0.55
Mean	0.58	0.58	0.60	0.65
% difference from initial measurement	--	0.87	3.59	13.1

Analyte:	3U

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	1.06	1.28	1.11	1.09
Replicate 2	0.95	1.15	1.11	1.34
Replicate 3	1.04	1.24	0.98	1.14
Mean	1.02	1.22	1.07	1.19
% difference from initial measurement	--	20.3	4.89	17.0

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

Analyte:	3U

MU09561	Initial measurement	Long-term stability
Replicate 1	0.46	0.38
Replicate 2	0.44	0.52
Replicate 3	0.44	0.42
Mean	0.44	0.44
% difference from initial measurement	--	-0.98

Analyte:	3U

HU09562	Initial measurement	Long-term stability
Replicate 1	0.52	0.56
Replicate 2	0.81	0.53
Replicate 3	0.56	0.69
Mean	0.63	0.59
% difference from initial measurement	--	-5.95

(4) 7-Methyluric Acid (7U)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	7U

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	12.4	13.9	13.9	12.4
Replicate 2	13.9	14.1	14.1	12.4
Replicate 3	14.1	14.0	14.7	13.3
Mean	13.5	14.0	14.2	12.7
% difference from initial measurement	--	3.96	5.69	-5.69

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	21.1	21.9	24.3	22.5
Replicate 2	22.0	22.8	20.8	21.3
Replicate 3	22.6	21.8	21.3	20.6
Mean	21.9	22.2	22.1	21.5
% difference from initial measurement	--	1.22	1.07	-1.98

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	7U

	Initial Measurment		
	Replicate 1	Replicate 2	Replicate 3
	10/20/2015	10/22/2015	11/9/2015
	Long-term stability		
	Replicate 1	Replicate 2	Replicate 3
	11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	11.0	8.77
Replicate 2	10.4	9.81
Replicate 3	11.1	11.0
Mean	10.8	9.86
% difference from initial measurement	--	-8.98

HU09562	Initial measurement	Long-term stability
Replicate 1	14.3	15.2
Replicate 2	15.2	16.2
Replicate 3	16.0	14.1
Mean	15.2	15.2
% difference from initial measurement	--	0.00

(5) 1,3-Dimethyluric Acid (13U)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**13U**

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	8.68	8.81	8.26	8.41
Replicate 2	9.15	9.25	9.47	8.78
Replicate 3	9.80	8.92	9.33	8.36
Mean	9.21	8.99	9.02	8.52
% difference from initial measurement	--	**-2.35**	**-2.06**	**-7.53**

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	19.0	18.4	19.1	18.8
Replicate 2	18.9	19.6	19.6	18.1
Replicate 3	17.6	18.9	19.0	19.2
Mean	18.5	19.0	19.2	18.7
% difference from initial measurement	--	**2.52**	**3.96**	**1.08**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**13U**

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	5.28	5.13
Replicate 2	5.26	5.33
Replicate 3	5.59	4.96
Mean	5.38	5.14
% difference from initial measurement	--	**-4.40**

HU09562	Initial measurement	Long-term stability
Replicate 1	14.5	16.0
Replicate 2	15.8	15.8
Replicate 3	15.9	15.9
Mean	15.4	15.9
% difference from initial measurement	--	**3.25**

(6) 1,7-Dimethyluric Acid (17U)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**17U**

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	34.9	35.5	34.9	32.2
Replicate 2	35.7	35.9	36.3	35.4
Replicate 3	35.3	35.2	35.9	35.0
Mean	35.3	35.5	35.7	34.2
% difference from initial measurement	--	**0.66**	**1.13**	**-3.12**

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	80.6	81.1	79.4	81.8
Replicate 2	83.7	80.0	80.0	79.4
Replicate 3	80.2	77.9	80.0	79.4
Mean	81.5	79.7	79.8	80.2
% difference from initial measurement	--	**-2.25**	**-2.09**	**-1.60**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**37U**

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	0.66	0.61
Replicate 2	0.50	0.63
Replicate 3	0.76	0.63
Mean	0.64	0.62
% difference from initial measurement	--	**-2.40**

HU09562	Initial measurement	Long-term stability
Replicate 1	0.99	0.95
Replicate 2	1.10	0.91
Replicate 3	0.96	1.04
Mean	1.02	0.97
% difference from initial measurement	--	**-4.69**

(7) 3,7-Dimethyluric Acid (37U)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**37U**

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	1.09	1.13	1.07	0.989
Replicate 2	1.05	1.17	1.02	1.11
Replicate 3	0.97	0.99	1.24	1.13
Mean	1.04	1.10	1.11	1.08
% difference from initial measurement	--	**5.85**	**7.07**	**3.83**

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	1.51	1.31	1.48	1.44
Replicate 2	1.43	1.38	1.47	1.28
Replicate 3	1.41	1.33	1.37	1.45
Mean	1.45	1.34	1.44	1.39
% difference from initial measurement	--	**-7.59**	**-0.69**	**-4.14**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**17U**

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	**Initial measurement**	**Long-term stability**
Replicate 1	34.8	31.2
Replicate 2	32.0	29.9
Replicate 3	30.5	30.5
Mean	32.4	30.5
% difference from initial measurement	--	**-5.86**

HU09562	**Initial measurement**	**Long-term stability**
Replicate 1	91.2	90.9
Replicate 2	100	87.5
Replicate 3	95.1	88.8
Mean	95.4	89.1
% difference from initial measurement	--	**-6.67**

(8) 1,3,7-Trimethyluric Acid (137U)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**137U**

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	3.47	3.79	3.71	3.49
Replicate 2	3.68	3.70	3.63	3.66
Replicate 3	3.64	3.52	3.77	3.70
Mean	3.60	3.67	3.70	3.62
% difference from initial measurement	--	**2.04**	**2.97**	**0.56**

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	7.53	7.33	6.99	7.47
Replicate 2	7.18	7.37	7.61	7.52
Replicate 3	7.66	7.26	7.25	7.81
Mean	7.46	7.32	7.28	7.60
% difference from initial measurement	--	**-1.83**	**-2.32**	**1.92**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**137U**

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	2.78	2.71
Replicate 2	2.74	2.71
Replicate 3	2.82	2.78
Mean	2.78	2.73
% difference from initial measurement	--	**-1.68**

HU09562	Initial measurement	Long-term stability
Replicate 1	5.14	5.14
Replicate 2	5.24	5.06
Replicate 3	5.12	5.10
Mean	5.17	5.10
% difference from initial measurement	--	**-1.29**

(9) 1-Methylxanthine (1X)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Analyte:	1X

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	37.6	45.2	40.9	39.0
Replicate 2	40.1	38.7	38.7	43.2
Replicate 3	41.0	41.5	42.4	41.3
Mean	39.6	41.8	40.7	41.2
% difference from initial measurement	--	**5.64**	**2.78**	**4.04**

Analyte:	1X

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	93.8	106	99.1	93.4
Replicate 2	103	93.9	97.8	98.8
Replicate 3	97.7	98.2	102	101
Mean	98.2	99.4	99.6	97.7
% difference from initial measurement	--	**1.22**	**1.49**	**-0.44**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

Analyte:	1X

MU09561	Initial measurement	Long-term stability
Replicate 1	40.2	34.9
Replicate 2	41.9	39.9
Replicate 3	39.1	37.4
Mean	40.4	37.4
% difference from initial measurement	--	**-7.43**

Analyte:	1X

HU09562	Initial measurement	Long-term stability
Replicate 1	78.7	80.9
Replicate 2	90.3	84.3
Replicate 3	95.1	85.8
Mean	88.0	83.7
% difference from initial measurement	--	**-4.96**

(10) 3-Methylxanthine (3X)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	3X

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	27.9	28.1	30.1	28.8
Replicate 2	28.7	28.2	28.9	30.2
Replicate 3	29.8	28.6	29.7	31.6
Mean	28.8	28.3	29.6	30.2
% difference from initial measurement	--	-1.74	2.66	4.86

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	45.2	48.1	45.5	54.1
Replicate 2	50.5	42.9	50.0	49.6
Replicate 3	44.8	47.2	50.8	49.2
Mean	46.8	46.1	48.8	51.0
% difference from initial measurement	--	-1.64	4.13	8.83

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	3X

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	26.9	28.0
Replicate 2	26.7	28.1
Replicate 3	30.5	28.9
Mean	28.0	28.3
% difference from initial measurement	--	1.07

HU09562	Initial measurement	Long-term stability
Replicate 1	41.9	42.0
Replicate 2	39.7	39.1
Replicate 3	40.6	40.7
Mean	40.7	40.6
% difference from initial measurement	--	-0.33

(11) 7-Methylxanthine (7X)

Stability - fill in yellow shaded cells
Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	7X

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	43.3	47.8	49.3	45.7
Replicate 2	46.7	47.8	49.0	48.4
Replicate 3	47.4	48.8	50.0	49.2
Mean	45.8	48.1	49.4	47.8
% difference from initial measurement	--	5.09	7.93	4.29

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	76.5	78.2	80.1	77.4
Replicate 2	77.0	78.4	77.4	77.6
Replicate 3	76.5	77.2	81.6	76.7
Mean	76.7	77.9	79.7	77.2
% difference from initial measurement	--	1.65	3.96	0.74

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: QC samples stored at -80°C for 2 years
All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	7X

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	37.4	38.6
Replicate 2	39.5	38.2
Replicate 3	39.5	39.3
Mean	38.8	38.7
% difference from initial measurement	--	-0.26

HU09562	Initial measurement	Long-term stability
Replicate 1	59.5	60.4
Replicate 2	63.3	58.8
Replicate 3	57.5	62.2
Mean	60.1	60.5
% difference from initial measurement	--	0.61

(12) Theophylline (13X)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	13X

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	3.12	3.47	3.63	3.18
Replicate 2	3.50	3.51	3.50	3.37
Replicate 3	3.62	3.56	3.52	3.32
Mean	3.41	3.51	3.55	3.29
% difference from initial measurement	--	**2.93**	**4.00**	**-3.61**

13X				
HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	6.56	6.80	6.90	6.38
Replicate 2	6.54	6.93	6.57	6.58
Replicate 3	6.54	6.64	6.77	6.11
Mean	6.55	6.79	6.75	6.36
% difference from initial measurement	--	**3.72**	**3.05**	**-2.90**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	13X

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	4.29	3.36
Replicate 2	4.07	3.30
Replicate 3	2.62	3.32
Mean	3.66	3.33
% difference from initial measurement	--	**-9.11**

13X		
HU09562	Initial measurement	Long-term stability
Replicate 1	7.76	8.11
Replicate 2	8.63	7.91
Replicate 3	7.91	7.94
Mean	8.10	7.99
% difference from initial measurement	--	**-1.40**

(13) Paraxanthine (17X)

Stability - fill in yellow shaded cells
Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	17X

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	36.7	40.0	40.2	36.8
Replicate 2	40.6	40.0	40.7	38.7
Replicate 3	39.3	40.0	41.2	38.1
Mean	38.9	40.0	40.7	37.9
% difference from initial measurement	--	2.92	4.72	-2.57

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	75.3	75.7	76.2	70.3
Replicate 2	76.3	75.9	75.3	71.8
Replicate 3	77.1	75.3	74.9	69.3
Mean	76.2	75.6	75.5	70.5
% difference from initial measurement	--	-0.79	-1.01	-7.56

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: QC samples stored at -80°C for 2 years
All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	17X

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

MU09561	Initial measurement	Long-term stability
Replicate 1	23.4	23.5
Replicate 2	23.7	24.6
Replicate 3	24.4	24.4
Mean	23.8	24.2
% difference from initial measurement	--	1.40

HU09562	Initial measurement	Long-term stability
Replicate 1	58.7	66.2
Replicate 2	65.3	65.2
Replicate 3	63.0	66.5
Mean	62.3	66.0
% difference from initial measurement	--	5.83

(14) Theobromine (37X)

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.

Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)

Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours

Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler

Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**37X**

MU09561	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	32.8	36.5	36.7	33.6
Replicate 2	34.4	36.5	36.1	36.3
Replicate 3	35.8	35.9	35.9	36.3
Mean	34.3	36.3	36.2	35.4
% difference from initial measurement	--	**5.73**	**5.53**	**3.11**

37X

HU09562	Initial measurement	Three freeze-thaw cycles	Bench-top stability	Processed sample stability
Replicate 1	35.7	36.7	36.5	35.6
Replicate 2	37.2	36.4	37.0	37.3
Replicate 3	36.7	36.8	36.3	35.9
Mean	36.5	36.6	36.6	36.3
% difference from initial measurement	--	**0.27**	**0.18**	**-0.73**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis

Describe condition: example: QC samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Caffeine and Metabolites in Urine
Method #:	4063
Matrix:	Urine
Units:	µmol/L
Analyte:	**37X**

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

37X

MU09561	Initial measurement	Long-term stability
Replicate 1	19.5	19.1
Replicate 2	21.0	19.0
Replicate 3	20.5	18.4
Mean	20.3	18.8
% difference from initial measurement	--	**-7.38**

HU09562	Initial measurement	Long-term stability
Replicate 1	37.8	41.4
Replicate 2	43.3	40.5
Replicate 3	40.7	40.6
Mean	40.6	40.8
% difference from initial measurement	--	**0.57**

(15) Caffeine (137X)

Stability - fill in yellow shaded cells
Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions.
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 1 month

All stability sample results should be within ±15% of nominal concentration.

Run date
Data for Initial, three freeze-thaw, and bench-top stability were run on 11/16/2017
Data for processed sample stability was run on 12/07/2017

Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L

Analyte: **137X**

MU09561					**HU09562**				
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**		**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	20.9	22.2	22.2	21.5	Replicate 1	30.2	30.1	30.2	29.5
Replicate 2	22.8	22.4	22.4	23.2	Replicate 2	29.8	29.8	29.7	29.9
Replicate 3	22.4	22.1	22.3	25.2	Replicate 3	29.5	30.0	29.4	30.0
Mean	22.0	22.2	22.3	23.3	Mean	29.8	30.0	29.8	29.8
% difference from initial measurement	--	**0.91**	**1.21**	**5.75**	**% difference from initial measurement**	--	**0.45**	**-0.22**	**-0.11**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: QC samples stored at -80°C for 2 years
All stability sample results should be within ±15% of nominal concentration

Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L

Initial Measurment		
Replicate 1	Replicate 2	Replicate 3
10/20/2015	10/22/2015	11/9/2015
Long-term stability		
Replicate 1	Replicate 2	Replicate 3
11/14/2017	11/15/2017	11/16/2017

Analyte: **137X**

MU09561			**HU09562**		
	Initial measurement	**Long-term stability**		**Initial measurement**	**Long-term stability**
Replicate 1	18.0	15.9	Replicate 1	26.6	26.1
Replicate 2	17.6	16.7	Replicate 2	28.2	25.9
Replicate 3	17.8	16.1	Replicate 3	27.3	26.6
Mean	17.8	16.2	Mean	27.4	26.2
% difference from initial measurement	--	**-8.80**	**% difference from initial measurement**	--	**-4.26**

A. Precision

(1) AAMU

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

AAMU - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	59.9	57.8	58.9	1.10	1.10	6927
2	59.2	52.6	55.9	10.9	10.9	6250
3	54.9	68.0	61.5	42.9	42.9	7552
4	56.6	56.1	56.4	0.06	0.06	6351
5	61.5	61.3	61.4	0.01	0.01	7540
6	56.1	60.2	58.2	4.20	4.20	6763
7	53.6	57.8	55.7	4.41	4.41	6205
8	57.7	60.9	59.3	2.56	2.56	7033
9	53.8	56.6	55.2	1.96	1.96	6094
10	47.7	55.0	51.4	13.32	13.32	5274
Grand sum	1147	Grand mean	57.4			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	163	16.3	4.04	7.03
Between Run	173	19.2	1.21	2.10
Total	336		4.21	**7.34**

AAMU - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	201	176	189	156	156	71065
2	186	175	181	30.3	30.3	65161
3	197	192	195	6.25	6.25	75661
4	204	185	195	90.3	90.3	75661
5	180	188	184	16.0	16.0	67712
6	204	190	197	49.0	49.0	77618
7	165	195	180	225	225	64800
8	189	189	189	0.00	0.00	71442
9	178	175	177	2.25	2.25	62305
10	183	180	182	2.25	2.25	65885
Grand sum	3732	Grand mean	187			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1155	116	10.7	5.76
Between Run	916	102	0.00	0.00
Total	2071		10.7	**5.76**

(2) 1-Methyluric Acid (1U)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

1U - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	68.3	72.5	70.4	4.41	4.41	9912
2	68.3	72.5	70.4	4.41	4.41	9912
3	62.6	71.7	67.2	20.7	20.7	9018
4	65.9	70.7	68.3	5.76	5.76	9330
5	68.0	64.9	66.5	2.40	2.40	8831
6	66.7	69.3	68.0	1.69	1.69	9248
7	67.0	76.2	71.6	21.2	21.2	10253
8	61.3	66.5	63.9	6.76	6.76	8166
9	67.1	89.6	78.4	127	127	12277
10	55.4	60.0	57.7	5.29	5.29	6659
Grand sum	1365	Grand mean	68.2			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	398	39.8	6.31	9.25
Between Run	514	57.2	2.94	4.31
Total	913		6.96	**10.2**

1U - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	167	159	163	16.0	16.0	53138
2	155	160	158	6.25	6.25	49613
3	155	175	165	100	100	54450
4	148	158	153	25.0	25.0	46818
5	152	170	161	81.0	81.0	51842
6	168	174	171	9.00	9.00	58482
7	146	147	147	0.25	0.25	42925
8	169	197	183	196	196	66978
9	142	146	144	4.00	4.00	41472
10	140	162	151	121	121	45602
Grand sum	3190	Grand mean	160			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1117	112	10.6	6.63
Between Run	2514	279	9.16	5.74
Total	3631		14.0	**8.77**

(3) 3-Methyluric Acid (3U)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

3U - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.587	0.617	0.602	0.00	0.00	0.72
2	0.688	0.727	0.708	0.00	0.00	1.00
3	0.662	0.448	0.555	0.01	0.01	0.62
4	0.686	0.598	0.642	0.00	0.00	0.82
5	0.627	0.479	0.553	0.01	0.01	0.61
6	0.566	0.671	0.619	0.00	0.00	0.77
7	0.556	0.732	0.644	0.01	0.01	0.83
8	0.409	0.492	0.451	0.00	0.00	0.41
9	0.545	0.620	0.583	0.00	0.00	0.68
10	0.604	0.601	0.603	0.00	0.00	0.73
Grand sum	11.9	Grand mean	0.596			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.066	0.007	0.081	13.7
Between Run	0.085	0.009	0.037	6.26
Total	0.151		0.090	**15.0**

3U - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.29	1.24	1.27	0.00	0.00	3.20
2	1.24	1.26	1.25	0.00	0.00	3.13
3	1.05	1.14	1.10	0.00	0.00	2.40
4	1.37	1.05	1.21	0.03	0.03	2.93
5	1.14	0.92	1.03	0.01	0.01	2.13
6	1.29	1.10	1.20	0.01	0.01	2.86
7	1.12	1.19	1.16	0.00	0.00	2.67
8	1.28	1.32	1.30	0.00	0.00	3.38
9	1.05	1.03	1.04	0.00	0.00	2.16
10	1.24	0.99	1.12	0.02	0.02	2.49
Grand sum	23.3	Grand mean	1.17			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.13	0.013	0.115	9.90
Between Run	0.16	0.018	0.047	3.99
Total	0.29		0.124	**10.7**

(4) 7-Methyluric Acid (7U)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

7U - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	12.1	13.1	12.6	0.25	0.25	318
2	14.4	15.0	14.7	0.09	0.09	432
3	13.7	12.7	13.2	0.25	0.25	348
4	15.8	13.5	14.7	1.32	1.32	429
5	14.5	12.5	13.5	1.00	1.00	365
6	13.7	14.4	14.1	0.12	0.12	395
7	13.7	13.2	13.5	0.06	0.06	362
8	14.3	13.5	13.9	0.16	0.16	386
9	12.9	12.6	12.8	0.02	0.02	325
10	13.7	13.3	13.5	0.04	0.04	365
Grand sum	273	Grand mean	13.6			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	6.64	0.66	0.81	5.98
Between Run	9.04	1.00	0.41	3.03
Total	15.7		0.91	**6.70**

7U - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	22.1	18.7	20.4	2.89	2.89	832
2	23.1	22.6	22.9	0.06	0.06	1044
3	23.9	22.1	23.0	0.81	0.81	1058
4	23.6	23.8	23.7	0.01	0.01	1123
5	20.3	21.3	20.8	0.25	0.25	865
6	24.2	23.4	23.8	0.16	0.16	1133
7	21.7	21.2	21.5	0.06	0.06	920
8	22.3	23.8	23.1	0.56	0.56	1063
9	20.5	21.2	20.9	0.12	0.12	869
10	21.1	22.4	21.8	0.42	0.42	946
Grand sum	443	Grand mean	22.2			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	10.7	1.07	1.03	4.67
Between Run	28.7	3.19	1.03	4.65
Total	39.4		1.46	**6.59**

(5) 1,3-Dimethyluric Acid (13U)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

13U - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	8.90	9.39	9.15	0.06	0.06	167
2	9.67	9.93	9.80	0.02	0.02	192
3	8.48	9.10	8.79	0.10	0.10	155
4	8.83	9.31	9.07	0.06	0.06	165
5	9.12	8.20	8.66	0.21	0.21	150
6	9.38	9.33	9.36	0.00	0.00	175
7	8.77	8.97	8.87	0.01	0.01	157
8	10.20	9.78	9.99	0.04	0.04	200
9	8.63	9.72	9.18	0.30	0.30	168
10	9.84	9.48	9.66	0.03	0.03	187
Grand sum	185	Grand mean	9.25			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1.65	0.17	0.41	4.39
Between Run	3.56	0.40	0.34	3.67
Total	5.22		0.53	**5.73**

13U - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	22.0	20.5	21.3	0.56	0.56	903
2	19.2	18.7	19.0	0.06	0.06	718
3	20.9	21.9	21.4	0.25	0.25	916
4	21.0	19.9	20.5	0.30	0.30	836
5	17.9	18.5	18.2	0.09	0.09	662
6	20.5	20.4	20.5	0.00	0.00	836
7	20.2	19.2	19.7	0.25	0.25	776
8	22.5	22.6	22.6	0.00	0.00	1017
9	20.9	19.1	20.0	0.81	0.81	800
10	20.3	19.8	20.1	0.06	0.06	804
Grand sum	406	Grand mean	20.3			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	4.79	0.48	0.69	3.41
Between Run	27.9	3.10	1.15	5.64
Total	32.7		1.34	**6.59**

(6) 1,7-Dimethyluric Acid (17U)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

17U - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	35.9	37.0	36.5	0.30	0.30	2657
2	36.0	35.7	35.9	0.02	0.02	2570
3	34.7	33.7	34.2	0.25	0.25	2339
4	36.2	37.9	37.1	0.72	0.72	2745
5	38.1	37.7	37.9	0.04	0.04	2873
6	36.7	37.6	37.2	0.20	0.20	2760
7	35.3	36.6	36.0	0.42	0.42	2585
8	38.3	41.1	39.7	1.96	1.96	3152
9	36.0	31.7	33.9	4.62	4.62	2292
10	36.4	36.1	36.3	0.02	0.02	2628
Grand sum	728.7	Grand mean	36.4			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	17.1	1.71	1.31	3.59
Between Run	52.0	5.77	1.42	3.91
Total	69.1		1.93	**5.31**

17U - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	82.4	71.4	76.9	30.3	30.3	11827
2	86.0	78.8	82.4	13.0	13.0	13580
3	81.2	80.4	80.8	0.16	0.16	13057
4	84.8	83.8	84.3	0.25	0.25	14213
5	82.6	82.4	82.5	0.01	0.01	13613
6	84.3	79.8	82.1	5.06	5.06	13464
7	80.2	83.5	81.9	2.72	2.72	13399
8	85.3	86.8	86.1	0.56	0.56	14809
9	80.5	75.4	78.0	6.50	6.50	12152
10	79.5	87.2	83.4	14.8	14.8	13894
Grand sum	1636.3	Grand mean	81.8			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	147	14.7	3.83	4.68
Between Run	135	15.0	0.41	0.50
Total	282		3.85	**4.71**

(7) 3,7-Dimethyluric Acid (37U)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

37U - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.18	1.10	1.14	0.00	0.00	2.60
2	1.15	1.10	1.13	0.00	0.00	2.53
3	1.20	1.04	1.12	0.01	0.01	2.51
4	1.05	1.11	1.08	0.00	0.00	2.33
5	1.10	1.11	1.11	0.00	0.00	2.44
6	1.20	1.28	1.24	0.00	0.00	3.08
7	1.13	1.14	1.14	0.00	0.00	2.58
8	1.15	1.20	1.18	0.00	0.00	2.76
9	1.11	1.05	1.08	0.00	0.00	2.33
10	1.01	0.98	0.99	0.00	0.00	1.98
Grand sum	22.4	Grand mean	1.12			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.026	0.003	0.051	4.55
Between Run	0.075	0.008	0.053	4.77
Total	0.10		0.074	**6.59**

37U - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.62	1.53	1.58	0.00	0.00	4.96
2	1.61	1.20	1.41	0.04	0.04	3.95
3	1.67	1.27	1.47	0.04	0.04	4.32
4	1.56	1.37	1.47	0.01	0.01	4.29
5	1.36	1.22	1.29	0.00	0.00	3.33
6	1.49	1.31	1.40	0.01	0.01	3.92
7	1.36	1.41	1.39	0.00	0.00	3.84
8	1.35	1.37	1.36	0.00	0.00	3.70
9	1.53	1.41	1.47	0.00	0.00	4.32
10	1.41	1.43	1.42	0.00	0.00	4.03
Grand sum	28.5	Grand mean	1.42			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.22	0.022	0.15	10.4
Between Run	0.11	0.012	0.00	0.00
Total	0.33		0.15	**10.4**

(8) 1,3,7-Trimethyluric Acid (137U)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

137U - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	3.70	3.61	3.66	0.00	0.00	26.7
2	3.85	3.75	3.80	0.00	0.00	28.9
3	3.75	3.82	3.79	0.00	0.00	28.7
4	3.63	3.76	3.70	0.00	0.00	27.3
5	3.72	3.71	3.72	0.00	0.00	27.6
6	3.66	3.65	3.66	0.00	0.00	26.7
7	3.65	3.83	3.74	0.01	0.01	28.0
8	3.65	3.71	3.68	0.00	0.00	27.1
9	3.63	3.68	3.66	0.00	0.00	26.7
10	3.62	3.60	3.61	0.00	0.00	26.1
Grand sum	74.0	Grand mean	3.70			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.040	0.0040	0.063	1.70
Between Run	0.067	0.0075	0.042	1.14
Total	0.107		0.076	**2.04**

137U - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	7.45	7.44	7.45	0.00	0.00	111
2	7.90	7.48	7.69	0.04	0.04	118
3	7.62	7.84	7.73	0.01	0.01	120
4	8.02	7.54	7.78	0.06	0.06	121
5	7.55	7.59	7.57	0.00	0.00	115
6	7.42	7.17	7.30	0.02	0.02	106
7	7.48	7.68	7.58	0.01	0.01	115
8	7.56	7.58	7.57	0.00	0.00	115
9	7.18	7.34	7.26	0.01	0.01	105
10	7.14	7.33	7.24	0.01	0.01	105
Grand sum	150	Grand mean	7.52			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.31	0.031	0.18	2.35
Between Run	0.71	0.079	0.15	2.05
Total	1.02		0.23	**3.12**

(9) 1-Methylxanthine (1X)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

1X - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	36.7	41.1	38.9	4.84	4.84	3026
2	39.9	40.7	40.3	0.16	0.16	3248
3	44.5	38.2	41.4	9.92	9.92	3420
4	40.7	40.6	40.7	0.00	0.00	3305
5	44.8	44.4	44.6	0.04	0.04	3978
6	39.2	44.4	41.8	6.76	6.76	3494
7	40.8	42.2	41.5	0.49	0.49	3445
8	42.5	42.6	42.6	0.00	0.00	3621
9	40.4	37.8	39.1	1.69	1.69	3058
10	41.5	40.8	41.2	0.12	0.12	3387
Grand sum	824	Grand mean	41.2			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	48.1	4.81	2.19	5.32
Between Run	49.3	5.48	0.58	1.41
Total	97.4		2.27	**5.51**

1X - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	102	103	103	0.25	0.25	21013
2	106	97	102	18.9	18.9	20665
3	104	107	106	2.25	2.25	22261
4	105	104	105	0.25	0.25	21841
5	103	105	104	1.00	1.00	21632
6	112	102	107	25.0	25.0	22898
7	101	103	102	1.00	1.00	20808
8	108	104	106	4.00	4.00	22472
9	100	91	96	18.1	18.1	18260
10	101	97	99	4.62	4.62	19543
Grand sum	2055	Grand mean	103			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	151	15.1	3.88	3.78
Between Run	219	24.4	2.16	2.10
Total	370		4.44	**4.32**

(10) 3-Methylxanthine (3X)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

3X - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	26.4	24.1	25.3	1.32	1.32	1275
2	28.8	27.3	28.1	0.56	0.56	1574
3	30.5	30.3	30.4	0.01	0.01	1848
4	29.3	28.3	28.8	0.25	0.25	1659
5	29.2	30.0	29.6	0.16	0.16	1752
6	27.8	26.8	27.3	0.25	0.25	1491
7	29.8	29.0	29.4	0.16	0.16	1729
8	28.1	30.3	29.2	1.21	1.21	1705
9	27.7	25.9	26.8	0.81	0.81	1436
10	28.8	26.7	27.8	1.10	1.10	1540
Grand sum	565	Grand mean	28.3			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	11.7	1.17	1.08	3.82
Between Run	42.5	4.73	1.33	4.72
Total	54.2		1.72	**6.08**

3X - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	46.3	44.9	45.6	0.49	0.49	4159
2	48.1	44.5	46.3	3.24	3.24	4287
3	49.9	48.2	49.1	0.72	0.72	4812
4	46.5	46.1	46.3	0.04	0.04	4287
5	46.5	45.1	45.8	0.49	0.49	4195
6	42.2	43.7	43.0	0.56	0.56	3689
7	47.5	48.5	48.0	0.25	0.25	4608
8	46.9	46.7	46.8	0.01	0.01	4380
9	45.8	45.9	45.9	0.00	0.00	4204
10	46.5	50.5	48.5	4.00	4.00	4705
Grand sum	930	Grand mean	46.5			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	19.6	1.96	1.40	3.01
Between Run	54.5	6.05	1.43	3.08
Total	74.1		2.00	**4.30**

(11) 7-Methylxanthine (7X)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

7X - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	47.3	48.0	47.7	0.12	0.12	4541
2	47.1	46.3	46.7	0.16	0.16	4362
3	47.5	46.4	47.0	0.30	0.30	4409
4	45.4	46.8	46.1	0.49	0.49	4250
5	47.4	47.4	47.4	0.00	0.00	4494
6	49.2	48.3	48.8	0.20	0.20	4753
7	48.9	50.1	49.5	0.36	0.36	4901
8	48.2	47.6	47.9	0.09	0.09	4589
9	46.8	48.3	47.6	0.56	0.56	4522
10	45.4	44.7	45.1	0.12	0.12	4059
Grand sum	947	Grand mean	47.4			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	4.83	0.48	0.69	1.47
Between Run	28.9	3.21	1.17	2.47
Total	33.7		1.36	**2.87**

7X - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	86.5	79.5	83.0	12.3	12.3	13778
2	79.4	72.6	76.0	11.6	11.6	11552
3	75.2	83.3	79.3	16.4	16.4	12561
4	80.0	72.9	76.5	12.6	12.6	11689
5	75.8	75.4	75.6	0.04	0.04	11431
6	84.5	75.6	80.1	19.8	19.8	12816
7	74.8	77.6	76.2	1.96	1.96	11613
8	76.8	74.5	75.7	1.32	1.32	11446
9	78.1	74.9	76.5	2.56	2.56	11705
10	70.6	70.3	70.5	0.02	0.02	9926
Grand sum	1538	Grand mean	76.9			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	157	15.7	3.96	5.15
Between Run	198	22.0	1.78	2.31
Total	355		4.34	**5.65**

(12) Theophylline (13X)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

13X - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	3.63	3.41	3.52	0.01	0.01	24.8
2	3.53	3.54	3.54	0.00	0.00	25.0
3	3.56	3.47	3.52	0.00	0.00	24.7
4	3.53	3.48	3.51	0.00	0.00	24.6
5	3.45	3.77	3.61	0.03	0.03	26.1
6	3.41	3.46	3.44	0.00	0.00	23.6
7	3.48	3.68	3.58	0.01	0.01	25.6
8	3.37	3.48	3.43	0.00	0.00	23.5
9	3.42	3.33	3.38	0.00	0.00	22.8
10	3.25	3.38	3.32	0.00	0.00	22.0
Grand sum	69.6	Grand mean	3.48			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.12	0.012	0.11	3.15
Between Run	0.15	0.017	0.05	1.43
Total	0.27		0.12	**3.46**

13X - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	7.00	6.96	6.98	0.00	0.00	97
2	7.43	6.90	7.17	0.07	0.07	103
3	7.24	7.48	7.36	0.01	0.01	108
4	7.27	7.12	7.20	0.01	0.01	104
5	7.17	7.57	7.37	0.04	0.04	109
6	7.52	7.10	7.31	0.04	0.04	107
7	7.21	7.27	7.24	0.00	0.00	105
8	6.92	6.27	6.60	0.11	0.11	87
9	6.53	6.01	6.27	0.07	0.07	79
10	6.21	5.85	6.03	0.03	0.03	73
Grand sum	139	Grand mean	6.95			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.76	0.076	0.28	3.97
Between Run	4.20	0.47	0.44	6.36
Total	4.96		0.52	**7.50**

(13) Paraxanthine (17X)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

17X - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	38.0	37.4	37.7	0.09	0.09	2843
2	38.2	37.4	37.8	0.16	0.16	2858
3	36.2	35.9	36.1	0.02	0.02	2599
4	37.1	37.4	37.3	0.02	0.02	2775
5	38.4	40.2	39.3	0.81	0.81	3089
6	38.0	38.7	38.4	0.12	0.12	2941
7	38.5	38.8	38.7	0.02	0.02	2988
8	37.7	37.8	37.8	0.00	0.00	2850
9	38.0	37.3	37.7	0.12	0.12	2835
10	38.3	38.2	38.3	0.00	0.00	2926
Grand sum	757.5	Grand mean	37.9			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2.76	0.28	0.52	1.39
Between Run	13.6	1.52	0.79	2.08
Total	16.4		0.95	**2.50**

17X - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	73.6	68.1	70.9	7.56	7.56	10039
2	74.1	70.7	72.4	2.89	2.89	10484
3	73.5	72.9	73.2	0.09	0.09	10716
4	71.8	69.8	70.8	1.00	1.00	10025
5	70.7	71.7	71.2	0.25	0.25	10139
6	73.7	69.2	71.5	5.06	5.06	10210
7	69.2	71.0	70.1	0.81	0.81	9828
8	73.6	70.3	72.0	2.72	2.72	10354
9	70.9	71.1	71.0	0.01	0.01	10082
10	69.4	69.6	69.5	0.01	0.01	9661
Grand sum	1424.9	Grand mean	71.2			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	40.8	4.08	2.02	2.84
Between Run	20.9	2.33	0.00	0.00
Total	61.7		2.02	**2.84**

(14) Theobromine (37X)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

37X - medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	35.9	35.2	35.6	0.12	0.12	2528
2	38.5	37.1	37.8	0.49	0.49	2858
3	35.7	34.2	35.0	0.56	0.56	2443
4	36.4	37.1	36.8	0.12	0.12	2701
5	33.3	34.5	33.9	0.36	0.36	2298
6	35.7	37.1	36.4	0.49	0.49	2650
7	37.3	35.8	36.6	0.56	0.56	2672
8	36.0	34.9	35.5	0.30	0.30	2513
9	35.6	35.1	35.4	0.06	0.06	2499
10	35.8	34.8	35.3	0.25	0.25	2492
Grand sum	716	Grand mean	35.8			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	6.65	0.67	0.82	2.28
Between Run	21.6	2.40	0.93	2.60
Total	28.2		1.24	**3.46**

37X - high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	38.1	36.1	37.1	1.00	1.00	2753
2	39.8	37.4	38.6	1.44	1.44	2980
3	34.8	37.9	36.4	2.40	2.40	2643
4	37.0	35.8	36.4	0.36	0.36	2650
5	33.7	33.1	33.4	0.09	0.09	2231
6	38.3	35.5	36.9	1.96	1.96	2723
7	35.0	36.1	35.6	0.30	0.30	2528
8	36.5	33.7	35.1	1.96	1.96	2464
9	35.0	35.1	35.1	0.00	0.00	2457
10	35.2	36.2	35.7	0.25	0.25	2549
Grand sum	720	Grand mean	36.0			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	19.5	1.95	1.40	3.88
Between Run	35.7	3.96	1.00	2.78
Total	55.2		1.72	**4.77**

(15) Caffeine (137X)

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)
Method name: Caffeine and Metabolites in Urine
Method #: 4063
Matrix: Urine
Units: µmol/L
Data Source: 2017 bench QC characterization
6/13/2017 to 8/25/2017

137X - medium bench QC

Quality material 1 -medium bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	21.6	20.5	21.1	0.30	0.30	886
2	24.5	24.5	24.5	0.00	0.00	1201
3	22.9	23.3	23.1	0.04	0.04	1067
4	23.4	24.2	23.8	0.16	0.16	1133
5	22.1	22.5	22.3	0.04	0.04	995
6	22.3	21.6	22.0	0.12	0.12	964
7	22.3	23.3	22.8	0.25	0.25	1040
8	23.2	23.3	23.3	0.00	0.00	1081
9	22.6	25.2	23.9	1.69	1.69	1142
10	22.7	22.4	22.6	0.02	0.02	1017
Grand sum	458.4	Grand mean	22.9			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	5.26	0.53	0.73	3.16
Between Run	18.7	2.08	0.88	3.84
Total	24.0		1.14	**4.98**

137X - high bench QC

Quality material 2 -high bench QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	28.6	28.1	28.4	0.06	0.06	1607
2	32.1	30.8	31.5	0.42	0.42	1978
3	29.4	31.0	30.2	0.64	0.64	1824
4	30.4	30.6	30.5	0.01	0.01	1861
5	28.3	27.6	28.0	0.12	0.12	1562
6	28.9	25.9	27.4	2.25	2.25	1502
7	28.2	28.8	28.5	0.09	0.09	1625
8	30.6	28.2	29.4	1.44	1.44	1729
9	29.9	32.2	31.1	1.32	1.32	1928
10	28.8	28.1	28.5	0.12	0.12	1619
Grand sum	586.5	Grand mean	29.3			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	13.0	1.30	1.14	3.88
Between Run	35.3	3.92	1.15	3.90
Total	48.2		1.61	**5.51**

B. LOD, Specificity, and Fit for Intended Use

LOD, specificity and fit for intended use - fill in yellow shaded cells			
Method name:	Caffeine and Metabolites in Urine		
Method #:	4063		
Matrix:	Urine		
Units:	µmol/L		

Analytes	Limit of Detection (LOD)(µM)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
AAMU	0.10	yes	yes
1U	0.05	yes	yes
3U	0.10	yes	yes
7U	0.04	yes	yes
13U	0.02	yes	yes
17U	0.02	yes	yes
37U	0.03	yes	yes
137U	0.005	yes	yes
1X	0.03	yes	yes
3X	0.04	yes	yes
7X	0.02	yes	yes
13X	0.01	yes	yes
17X	0.006	yes	yes
37X	0.004	yes	yes
137X	0.003	yes	yes

Appendix B: Ruggedness testing

A. Principles and Proposals

(1) Conversion of AFMU to AAMU

Principle: 5-acetylamino-6-formylamino-3-methyluracil (AFMU) is an unstable product of caffeine metabolism that will gradually decompose into 5-acetylamino-6-amino-3-methyluracil (AAMU). In order to accurately quantify this metabolite, the conversion of AFMU to AAMU is forced to completion via alkaline sample treatment during sample preparation.

Proposal: The incubation time (i.e., time allowed for alkaline sample treatment) and the concentration of HCl used in the re-acidification of the sample following alkaline treatment were varied.

(2) Sample filtration

Principle: All samples, calibrators and quality control materials are filtered using a 0.2 µm nylon filter prior to analysis. Filtration removes particulate matter that may interfere with HPLC-MS/MS measurements or cause problems (e.g., reduced HPLC column life).

Proposal: Filtration with a 0.2 µm nylon filter was compared with removal of particulates by centrifugation.

(3) Sample matrix and mobile phase composition

Principle: Samples are prepared in a buffer that matches the matrix of the starting mobile phase composition. The injection of samples in which the sample matrix differs significantly from the mobile phase may lead to poor chromatographic performance.

Proposal: The relative strength (in terms of formic acid and methanol content) of the buffer solution in which samples were prepared was varied. The formic acid content of the HPLC mobile phases was also varied.

B. Findings

(1) Conversion of AFMU to AAMU

Analyte	Effect of alkaline treatment incubation time on concentration (µM)			Effect of HCl concentration in re-acidification on concentration (µM)		
	Method specification (30 min)	Low test condition (10 min)	High test condition (60 min)	Method specification (2 N)	Low test condition (1 N)	High test condition (3 N)
1X	41.2	39.1	42.5	41.2	44.3	44.4
3X	27.5	27.4	28.4	27.5	29.2	28.7
7X	36.0	37.0	37.5	36.0	38.4	38.5
13X	3.6	3.8	3.9	3.6	3.9	3.8
17X	25.0	23.8	24.6	25.0	25.0	25.3
37X	21.3	20.0	21.1	21.3	22.0	21.9
137X	16.3	15.9	16.9	16.3	16.7	17.6
1U	48.5	48.0	48.7	48.5	52.7	53.0
3U	0.4	0.4	0.4	0.4	0.4	0.4
7U	11.2	10.8	11.1	11.2	11.8	12.0
13U	5.6	5.4	5.7	5.6	6.2	5.8
17U	33.1	31.5	34.0	33.1	36.0	34.1
37U	0.8	0.8	0.8	0.8	0.8	0.9
137U	2.9	2.7	2.7	2.9	3.2	3.3
AAMU	39.2	37.5	37.4	39.2	41.0	39.6

No changes were observed for AAMU, caffeine, or any other caffeine metabolite when incubation time and HCl concentration were varied within the range tested.

(2) Sample filtration:

Analyte	Effect of sample filtration on concentration (µM)	
	Method specifies (filtered)	Alternative condition (centrifuge)
1X	41.2	41.3
3X	27.5	27.3
7X	36.0	37.1
13X	3.6	3.9
17X	25.0	25.7
37X	21.3	21.1
137X	16.3	17.0
1U	48.5	50.2
3U	0.4	0.4
7U	11.2	11.4
13U	5.6	5.7
17U	33.1	32.0
37U	0.8	0.8
137U	2.9	2.9
AAMU	39.2	38.1

No changes were observed when particulates were removed from samples using centrifugation versus filtration.

(3) Sample matrix and mobile phase composition

Analyte	Effect of sample dilution buffer strength (relative strength to mobile phase) on concentration (µM)	Effect of formic acid in mobile phase on concentration

	Method specifies (1X)	Low condition (0.5X)	High condition (2X)	Method specifies (0.05%)	Low condition (0.01%)	High condition (0.1%)
1X	41.2	42.0	41.2	41.2	40.8	40.3
3X	27.5	27.9	28.5	27.5	27.3	27.9
7X	36.0	37.5	36.5	36.0	36.2	36.5
13X	3.6	3.8	3.7	3.6	4.2	4.1
17X	25.0	24.5	24.4	25.0	22.4	24.1
37X	21.3	21.5	20.9	21.3	21.0	20.1
137X	16.3	16.8	16.4	16.3	16.4	16.6
1U	48.5	50.5	51.2	48.5	50.2	48.4
3U	0.4	0.4	0.4	0.4	0.4	0.4
7U	11.2	11.3	11.6	11.2	11.5	10.9
13U	5.6	5.6	5.8	5.6	5.3	5.6
17U	33.1	33.5	33.0	33.1	31.3	32.9
37U	0.8	0.8	0.8	0.8	0.8	0.9
137U	2.9	2.9	2.9	2.9	2.8	3.0
AAMU	39.2	38.4	37.4	39.2	37.6	39.7

Samples are prepared in a buffer that matches the matrix of the starting mobile phase composition. The injection of samples in which the sample matrix differs significantly from the mobile phase may lead to poor chromatographic performance.

Appendix C: Extinction Coefficients

Analyte	Extinction coefficient ($m^{-1}cm^{-1}$)	Wavelength (nm)	pH	Reference
AAMU	18000	264	<7.0	35
1U	11400	284	3.0	36
3U	11100	287	3.0	36
7U	11400	286	3.0	36
13U	11600	287	3.0	36
17U	11000	286	3.0	36
1X	10200	266	5.0	37
3X	10000	271	5.0	37
7X	9600	269	5.0	37
13X	10407	270	6.0	37
17X	9549	267	2	38
37 X	10100	273	7.0	39
137X	9900	273	7.0	39

Appendix D: Analysis Parameters

MS/MS transitions*

Compound	Positive ion mode†				Negative ion mode‡			
	RT (min)	MRM transition (m/z)		MS parameter (V)	RT (min)	MRM transition (m/z)		MS parameter (Volt)
		Precursor	Product	CE		Precursor	Product	CE
1X	1.86	167	110	24	**1.86**	**165**	**108**	**-24**
3X	1.69	167	124	24	**1.69**	**165**	**122**	**-24**
7X	**1.46**	**167**	**150**	**24**	–	–	–	–
		167	124	24	–	–	–	–
13X	–	–	–	–	**4.19**	**179**	**164**	-26
	–	–	–	–		179	122	-28
17X	**3.98**	**181**	**124**	**24**	–	–	–	–
		181	96	32	–	–	–	–
37X	**2.93**	**181**	**138**	**24**	–	–	–	–
		181	163	24	–	–	–	–
137X	**6.36**	**195**	**138**	**24**	–	–	–	–
		195	110	32	–	–	–	–
1U	–	–	–	–	**1.50**	**181**	**138**	**-22**
	–	–	–	–		181	110	-24
3U	–	–	–	–	**1.00**	**181**	**138**	**-20**
	–	–	–	–		181	110	-26
7U	–	–	–	–	**1.28**	**181**	**138**	**-20**
	–	–	–	–		181	110	-24
13U	–	–	–	–	**2.59**	**195**	**110**	**-30**
	–	–	–	–		195	180	-24
17U	–	–	–	–	**3.74**	**195**	**137**	**-32**
	–	–	–	–		195	180	-24
37U	–	–	–	–	1.82	195	124	-26
	–	–	–	–		195	180	-24
137U	–	–	–	–	**5.13**	**209**	**194**	**-24**
	–	–	–	–		209	137	-32
AAMU	–	–	–	–	**0.74**	**197**	**140**	**-16**
	–	–	–	–		197	127	-20

* MS/MS transition used for quantitation appears in bold.

† For positive ion mode, the following global conditions were used: ionization voltage = 1850 V; interface temperature = 700 °C; entrance potential = 10V; declustering potential = 25; cell exit potential = 11.

‡ For negative ion mode, the following global conditions were used: ionization voltage = -1850V; interface temperature = 700 °C; entrance potential = -10V; declustering potential = -25; cell exit potential = -16.

Internal standard MS/MS transitions

Compound (IS)	(IS) Positive ion mode§				(IS) Negative ion mode**			
	RT (min)	MRM transition (m/z)		MS parameter (V)	RT (min)	MRM transition (m/z)		MS parameter (V)
		Precursor	Product	CE		Precursor	Product	CE
1X (IS)	1.86	174	115	24	**1.86**	**172**	**113**	**-24**
3X (IS)	1.69	174	129	24	**1.69**	**172**	**127**	**-24**
7X (IS)	**1.46**	**173**	**128**	**24**	–	–	–	–
13X (IS)	–	–	–	–	**4.08**	**185**	**125**	**-28**
17X (IS)	**3.98**	**188**	**129**	**24**	–	–	–	–
37X (IS)	**2.93**	**187**	**143**	**24**	–	–	–	–
137X (IS)	**6.32**	**204**	**144**	**24**	–	–	–	–
1U (IS)	–	–	–	–	1.50	188	143	-22
3U (IS)	–	–	–	–	1.00	188	143	-20
7U (IS)	–	–	–	–	1.28	188	143	-20
13U (IS)	–	–	–	–	2.59	202	114	-30
17U (IS)	–	–	–	–	3.74	202	142	-32
37U (IS)	–	–	–	–	1.82	199	127	-26
137U (IS)	–	–	–	–	5.13	216	142	-32
AAMU (IS)	–	–	–	–	0.74	204	130	-20

§ For (IS) positive ion mode, the following global conditions were used: ionization voltage = 1850 V; interface temperature = 700°C; entrance potential = 10V; declustering potential = 25; cell exit potential = 11.

** For (IS) negative ion mode, the following global conditions were used: ionization voltage = -1850V; interface temperature = 700°C; entrance potential = -10V; declustering potential = -25; cell exit potential = -16.