Laboratory Procedure Manual

Analyte: **Caffeine and caffeine metabolites**

Matrix: **Urine**

Method: **HPLC-ESI-MS/MS**

Method No: 4063.03

Revised:

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
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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.

Public Release Data Set Information

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

Data File Name	Variable Name	SAS Label
CAFE_F	URXMX1	1-methylxanthine
	URDMX2	3-methylxanthine
	URXMX3	7-methylxanthine
	URXMX4	1,3-dimethylxanthine; theophylline
	URXMX5	1,7-dimethylxanthine; paraxanthine
	URXMX6	3,7-dimethylxanthine; theobromine
	URXMX7	1,3,7-trimethylxanthine; caffeine
	URXMU1	1-methyluric acid
	URDMU2	3-methyluric acid
	URXMU3	7-methyluric acid
	URXMU4	1,3-dimethyluric acid
	URXMU5	1,7-dimethyluric acid
	URXMU6	3,7-dimethyluric acid
	URXMU7	1,3,7-trimethyluric acid
	URXAMU	5-acetylamino-6-amino-3-methyluracil

1. Summary of Test Principle and Clinical Relevance

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 naturally occurring caffeine originates principally 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 disease, 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 urinary metabolites provides a potential means of assessing differences in metabolic activity [22-23]. The liver serves as the primary site of caffeine metabolism and 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]. Caffeine metabolism is dominated by cytochrome P450 1A2 (CYP1A2) although other P450 enzyme systems, as well as N-acetyltransferase 2 (NAT2) and xanthine oxidase (XO), play a role in the metabolism of caffeine and/or its metabolites [24-26]. Caffeine is a preferred metabolic probe for assessing CYP 1A2, NAT 2, and XO enzyme activities, all of which are involved in the activation or detoxification of various xenobiotic compounds [26-29]. It has been demonstrated that uncontrolled dietary caffeine consumption can be used as a means of assessing enzyme activity [30-32].

B. Test principle

Caffeine and 14 of its metabolites are quantified in urine by use of high performance liquid chromatography-electrospray ionization-tandem quadrupole mass spectrometry (HPLC-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 into a more stable form. Samples are then acidified 30 µL of 2.0 N HCl and 250 µL of a 1:9 methanol/water solution containing 0.1% formic acid such that the matrix of the sample is similar to the starting mobile phase composition of the analysis step. Samples are then filtered and analyzed by HPLC-ESI-MS/MS in both positive and negative ionization modes. 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 with calibrators and quality control materials takes approximately 1.5 hours when performed by an automated liquid handler (including 30 min for the alkaline conversion step). HPLC-ESI-MS/MS analysis of each sample requires 10.5 min in positive ion mode and 6 min in negative ion mode.

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, autosampler 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. Material safety data sheets (MSDSs) for all chemicals are readily available in the MSDS section as hard copies in the laboratory. MSDSs for other chemicals can be viewed at http://www.ilpi.com/msds/index.html or at http://intranet.cdc.gov/ohs.

3. Computerization; 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 transferred via USB flash drive to the network where the data file is processed into a results file that is also saved on the CDC network. Results are typically generated by auto-integration, but may require in some cases manual integration. 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 a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See "**4063.02 SOP for 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; Criteria for Specimen Rejection

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.

1 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. Excessive freeze/thaw cycles might result in degradation of caffeine or its metabolites in urine, however, caffeine and caffeine metabolites in urine appear to be stable over the course of three freeze/thaw cycles.

Specimens generally arrive frozen. Refrigerated samples may be used provided they are kept cold and brought promptly (within 2 hours) from the site of collection.

Specimen handling conditions are outlined in the 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 no more than -20°C. Samples thawed and refrozen less than five times are not compromised. 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 fumehood. 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 M NaOH solution

For 100 mL of solution, add approximately 50 mL of deionized water to a 100-mL volumetric flask. Quantitatively transfer 20.0 mL of 6 M 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) 2 M HCl solution

For 100 mL of solution, add approximately 50 mL of deionized water to a 100-mL volumetric flask. Quantitatively transfer 20.0 mL of 10 M HCl into the flask and carefully 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.

(3) HPLC mobile phase A (aqueous)

For 1 L of solution, quantitatively transfer 950 mL of deionized water, 50 mL of methanol and 500 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 5 days and store at room temperature.

(4) HPLC mobile phase B (organic)

For 1 L of solution, quantitatively transfer 900 mL of methanol, 100 mL of deionized water and 500 µL of concentrated formic acid to a 1-L HPLC reservoir bottle. Cap the bottle and mix thoroughly,

venting the bottle several times during mixing. Prepare every 5 days and store at room temperature.

(5) 2× 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 5 days and store at room temperature.

(6) 2× Synthetic Urine

For 500 mL, quantitatively transfer 250 mL of deionized water to a 1 L beaker. Using a magnetic stir bar to agitate the solution, add the following chemicals in the quantities and order specified:

- 3.8 g Potassium Chloride
- 8.5 g Sodium Chloride
- 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
- 1.4 g Creatinine
- 0.64 g Sodium Hydroxide (add slowly)
- 0.47 g Sodium Bicarbonate
- 0.28 mL Sulfuric Acid (conc.)

Once all compounds have dissolved in solution, quantitatively transfer the mixture to a 500 mL volumetric flask. Bring the solution up to volume with deionized water. Seal the volumetric flask and mix the contents by inversion. Transfer to a storage vessel. This solution can be stored at 4 °C for up to one year.

B. Standards Preparation

(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.

Assignment of single-analyte stock solution concentrations by use of UV-visible absorbance measurements and molar extinction coefficients is preferred. In the absence of reliable extinction coefficients, assignment of stock solution concentration by gravimetric measurement is acceptable. Please refer to "**4063.03 SOP for Spectrophotometric Determination of Single-Analyte Stock Solution Concentrations**" for additional information.

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.

(2) Intermediate Mixed-Analyte Stock Solutions

Intermediate mixed-analyte stock solutions are prepared by combining the single-analyte stock solutions according to the amounts specified below.

Analyte	Amount of 1 mM single analyte stock solution used (µL)										
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
1X	969.9	53.3	97.0	485.0	242.5	3879.7	9.7	2909.8	1939.9	50.0	100.0
3X	10.2	2040.8	1020.4	56.1	765.3	255.1	102.0	510.2	3061.2	50.0	100.0
7X	6198.3	1033.1	2066.1	4132.2	103.3	516.5	10.3	56.8	258.3	50.0	100.0
1U	515.5	5154.6	108.2	2061.9	4123.7	1030.9	206.2	3092.8	10.3	50.0	100.0
3U	0.10	103.09	5.15	10.31	0.57	25.77	2.58	1.03	51.55	50.0	800.0
7U	12.5	5.0	499.0	249.5	20.0	49.9	998.0	99.8	748.5	100.0	500.0
13X	49.5	2.5	148.5	24.8	99.0	3.7	198.0	9.9	5.0	50.0	100.0
17X	1509.1	10.1	251.5	1006.0	503.0	100.6	30.2	2012.1	50.3	50.0	100.0
37X	254.8	509.7	1019.4	1529.1	30.6	51.0	2548.4	10.2	101.9	50.0	100.0
13U	102.1	408.6	10.2	1.0	51.1	510.7	25.5	5.6	204.3	80.0	500.0
17U	29.9	49.9	10.0	99.8	2495.0	499.0	1497.0	249.5	2994.0	50.0	100.0
37U	3.0	100.1	40.0	5.0	80.1	20.0	10.0	200.2	1.0	80.0	500.0
137X	97.9	391.8	2.0	195.9	5.9	9.8	489.7	49.0	24.5	50.0	100.0
137U	4.9	24.7	49.4	9.9	3.0	197.4	98.7	1.0	394.9	50.0	100.0
AAMU	137.0	9.1	4566.2	27.4	1369.9	2739.7	3653.0	684.9	45.7	90.0	500.0
Subtotal (µl)	**9894.9**	**9896.3**	**9893.1**	**9893.8**	**9892.8**	**9889.9**	**9879.4**	**9892.8**	**9891.2**	**905**	**376.3**
Water (µl)	**105.1**	**103.7**	**106.9**	**106.2**	**107.2**	**110.1**	**120.6**	**107.2**	**108.8**	**95**	**623.7**

For example, to prepare 10 mL of intermediate mixed-analyte stock solution "S1", use a positive displacement pipette to transfer the amount specified in the table above for each 1 mM single-analyte stock solution into a 10-mL volumetric flask. 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: Values highlighted in the table indicate occurrences where the pipetted amount is less than 10 µL. In these cases the single-analyte stock solution should be diluted to allow a volume larger than 10 µL to be pipetted without exceeding the total allowable volume for the mixed intermediate standard (10 mL). For standards S10 and S11, the 1 mM single-analyte stock solutions are diluted 10× prior to use since all pipetting would involve volumes of 10 µL or less with the undiluted single-analyte stock solutions.

Note: As an alternative to using a volumetric flask, a glass or polypropylene vessel can be used and the calculated amount of deionized water needed to bring the solution up to volume can be added by use of a positive displacement pipette or by gravimetric measurement.

(3) Calibration Standards

Calibration standards are prepared by combining the appropriate intermediate mixed-analyte stock solution with 2× synthetic urine and deionized water in a relative proportion of 2:1:17 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. Calibration standards are prepared in batches to last over several runs. Each run requires a complete set of calibration standards (S1 through S11) with 125.0 µL of calibration standard in each vial.

For example, to prepare enough calibration standards for approximately 1000 µL of calibration standard “S1” (enough for 9 runs), combine 100.0 µL of intermediate mixed-analyte stock solution “S1”, 50.0 µL of 2× synthetic urine, and 850 µL of deionized water and mix thoroughly. Accurately aliquot the solution into 1.5-mL microcentrifuge vials (100.0 µL/vial) and store at -70 °C.

The following table indicates the final concentrations of each analyte in each calibration standard, assuming a 1mM nominal concentration in all single-analyte stock solutions. Actual concentrations will vary depending on the exact concentration of the single-analyte stock solutions.

Analyte	Concentration (µmol/L)										
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**
1X	97.0	5.3	9.7	48.5	24.2	388.0	1.0	291.0	194.0	0.05	0.01
3X	1.0	204.1	102.0	5.6	76.5	25.5	10.2	51.0	306.1	0.05	0.01
7X	619.8	103.3	206.6	413.2	10.3	51.7	1.0	5.7	25.8	0.05	0.01
1U	51.5	515.5	10.8	206.2	412.4	103.1	20.6	309.3	1.0	0.05	0.01
3U	0.0	10.3	0.5	1.0	0.1	2.6	0.3	0.1	5.2	0.05	0.08
7U	1.2	0.5	49.9	25.0	2.0	5.0	99.8	10.0	74.9	0.1	0.05
13X	5.0	0.2	14.9	2.5	9.9	0.4	19.8	1.0	0.5	0.05	0.01
17X	150.9	1.0	25.2	100.6	50.3	10.1	3.0	201.2	5.0	0.05	0.01
37X	25.5	51.0	101.9	152.9	3.1	5.1	254.8	1.0	10.2	0.05	0.01
13U	10.2	40.9	1.0	0.1	5.1	51.1	2.6	0.6	20.4	0.08	0.05
17U	3.0	5.0	1.0	10.0	249.5	49.9	149.7	25.0	299.4	0.05	0.01
37U	0.3	10.0	4.0	0.5	8.0	2.0	1.0	20.0	0.1	0.08	0.05
137X	9.8	39.2	0.2	19.6	0.6	1.0	49.0	4.9	2.4	0.05	0.01
137U	0.5	2.5	4.9	1.0	0.3	19.7	9.9	0.1	39.5	0.05	0.01
AAMU	13.7	0.9	456.6	2.7	137.0	274.0	365.3	68.5	4.6	0.1	0.05

(4) Intermediate Mixed-Internal Standard Stock Solutions

Intermediate mixed-internal standard stock solutions are prepared by combining single internal-standard stock solutions (1 mM) 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 is 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. An example of QC pool target concentrations is given in **Appendix A**.

Each pool is stored in 500-µL aliquots in 2.0-mL Nalgene cryovials at -70 °C. Under these storage conditions the QC pools are stable for at least 3 years.

Characterization limits are established by analyzing duplicates of each pool for at least 20 consecutive runs.

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

- Synergi Fusion-RP column, 100 × 3.0 mm, 2.5 µm particle, 100 Å pore (Phenomenex, Torance, CA)
- Security Guard cartridge, C_{18}, 4.0 × 2.0 mm (Phenomenex)
- 9” Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
- HPLC autosampler vials (2.0mL/12x32mm, National Scientific, Duluth, GA)
- HPLC autosampler vial snap-caps, 11mm (National Scientific)
- HPLC autosampler vial inserts, 200µL (Kimble Glass)
- 1-mL, 96-well plate, 31 mm (Nalgene, Rochester, NY)
- Pre-slit, silicone 96-well plate seal (Fisher Scientific, Suwanee, GA)
- N-Dex nitrile examination gloves (Best Manufacturing Corporation, Menlo, GA)
- Pipette tips, blue, 50-1000 µL, for Eppendorf pipette (Brinkmann Instruments Inc., Westbury, NY)
- Pipette tips, yellow, 2-200 µL, for Eppendorf pipettes (Brinkmann)
- Positive displacement pipette tip, Combitip plus, 500 µL, for Eppendorf repeater pipette (Brinkmann)
- 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)
- 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, Muskegan)
- Water, 0.45 µm filtered, ≥ 18.0 MΩ resistance (in-house source, Aqua Solutions, Jasper, GA)
- Sodium hydroxide, 6N (Fisher Scientific Co., Fairlawn, NJ)
- Hydrochloric acid, 10N (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 (Sigma)
- 3-methyluric acid (Sigma)
- 7-methyluric acid (Sigma)
- 5-acetylamino-6-amino-3-methyluracil (Toronto Research Chemicals, Toronto, ON, Canada)
- 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-$^2H_9$ (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., HPLC 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 1200 HPLC system (Agilent Technologies, Palo Alta, CA), including:

- Model 4208A Control Module
- Model G1379B-Degasser
- Model G1312A-Binary pump
- Model G1367B-High Performance Autosampler
- Model G1316A-Thermostatted Column Compartment

(2) AB Sciex API 5000 tandem quadrupole mass spectrometer (AB Sciex, Foster City, CA), including:

- Turboionspray ion source (TIS), operated in ESI mode
- Analyst 1.5.1 software (AB Sciex, Foster City, CA)
- Parker-Balston model LCMS-5001NTNA Tri-Gas Generator (Parker-Balston, Haverhill, MA)

(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 (Brinkmann)
- Eppendorf pipette, 10-100µL (Brinkmann)
- Eppendorf pipette, 2-20µL (Brinkmann)
- Eppendorf Repeater Plus pipette (Brinkmann)
- Vortexer (VWR)
- Magnetic stirrer (Fisher Scientific)
- Eppendorf Centrifuge (5810R, Brinkmann)
- 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. At the end of each run, the calibrators are re-analyzed as unknown samples. The measured concentrations of these calibrators should agree within 15% of their set values. A quadratic calibration equation with 1/x weighting is used.

Method accuracy and specificity can be accessed by amending low and medium QC pools with each of the analytes and calculating the recovery of the exogenous analyte addition. Spike recovery

Reference materials are not available for urinary caffeine metabolites. Calibration verification is conducted as outlined in "**4063.02 SOP for Calibration and Calibration Verification**."

External proficiency testing programs currently do not exist for urinary 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 "**4063.02 SOP for In-House Proficiency Testing**."

Method figures of merit are presented in **Appendix B**.

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 C.**

B. Instrument Calibration

(1) API 5000 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 Bio systems. 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 and third quadrupoles of the API 5000 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 5000 User's Manual for additional details.

(2) Hamilton Microlab Starlet

Twice a year a Hamilton service engineer performs a 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 (no IS), 11 calibrators, 3 bench QCs (low, medium, and high), patient samples (up to a maximum of 77), a second set of 3 bench QCs (low, medium, and high), and reinjection of the blanks and calibrators.

A. Sample Preparation

(1) Manual Sample Preparation

(a) Sample dilution:

- Label one set of 1.5-mL microcentrifuge 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 Eppendorf tubes (reaction tubes).

(b) Alkaline treatment:

- Label one set of 1.5-mL microcentrifuge tubes for all diluted samples 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 working mixed 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-split 96-well silicone sealing mat).

(2) Automated Sample Preparation

"**4063.02 SOP for 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-split 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) HPLC

Fill all solvent bottles as follows. HPLC Mobile Phase A (aqueous) (line A1) and HPLC 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 HPLC-grade acetonitrile (line B2) should be checked daily and refilled as needed. 60% methanol (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 cartridge every 3 full instrument runs or if chromatographic performance has become poor, whichever occurs first.

Replace the HPLC column if chromatographic performance has become poor. Monitor chromatographic performance closely if the column has been used for >1500 sample injections.

Inspect all HPLC tubing and tubing connections. Ensure that all connections are in place and tightened appropriately.

Open the waste valve on the front of the HPLC. Using the hand-held control module, purge all solvent lines by running solvent through each line at 5 ml/min for 5 min. Purging is necessary if the instrument has been idle for one day or longer. Close the waste valve when done.

(2) Mass Spectrometer

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

Clean the interface and curtain plate if needed (caution: the interface may be very hot if the instrument was recently run). See the API 5000 User’s Manual for specific guidance.

C. Sample Analysis

The HPLC-MS/MS system is used to quantitate caffeine and caffeine metabolite levels in urine. See "**4063.02 SOP for Sample Analysis**" for a detailed description of the sample analysis steps. HPLC-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 "**4063.02 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 sample sets to accommodate the following: the startup methods; equilibration injections; analysis of samples in positive ionization mode; analysis of samples in negative ionization mode; and shutdown methods. These sample sets should be run in the order presented above. Refer to "**4063.02 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 "**4063.02 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 "**4063.02 SOP for Sample Analysis**" for additional details.

D. Quantitation and Data Review

The HPLC- MS/MS system software is used for quantitating analysis data. Quantified results are then imported into a LIMS database for data review by the analyst, project lead, quality assurance officer and supervisor.

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 of samples in both positive and negative ion modes was performed. If a run was performed only in one ionization mode or if the user is only interested in certain samples from an instrument run, the user may deviate from this procedure as necessary. Refer to “**4063.02 SOP for Quantitation and Data Review**” for additional details.

(1) Create a Results Table

A data file will be created for each sample set submitted for analysis. The user will have to create a results file for each data file being processed. This will typically be the done only for the analysis sets of samples in positive and negative ion modes. Refer to “**4063.02 SOP for Quantitation and Data Review**” for additional details.

(2) 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 “**4063.02 SOP for Quantitation and Data Review**” for additional details.

(3) 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). Refer to “**4063.02 SOP for Quantitation and Data Review**” for additional details.

(4) Importing Results into LIMS Database

The results file is imported into a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. Refer to “**4063.02 SOP for Computerization and Data System Managemen**t” for additional details.

E. System Maintenance

(1) Agilent HPLC

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.

(2) Applied Biosystems API 5000 MS/MS

Preventative maintenance, tuning and mass calibration is performed on a semi-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.

(3) 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.

(4) CDC Modifications.

N/A. This manuscript is a description of an original method and has been submitted for publication.

9. Reportable Range of Results

The reportable range of results for each of the analytes is as follows:

Analyte	Reportable range (µmol/L)
1X	0.01 – 400
3X	0.05 – 300
7X	0.05 – 600
13X	0.05 – 20
17X	0.1 – 200
37X	0.05 – 250
137X	0.1 – 50
1U	0.04 – 500
3U	0.1 – 10
7U	0.1 – 100
13U	0.05 – 50
17U	0.05 – 300
37U	0.05 – 20
137U	0.05 – 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. 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 can be inserted into the mix of patient specimens. These QC specimens are generally prepared at two levels that would be encountered in 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.

B. Bench Quality Controls

Bench QC specimens are prepared from three urine pools that represent low, medium and high levels of urinary 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 Xbar 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 exceed 4 S_w (i.e. 95 per cent 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 in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in a LIMS database. For 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. A hardcopy of the QC results from each run is also maintained by the analyst.

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 and may involve additional experiments beyond what is indicated below. Analytical results for runs not in statistical control should not be reported.

- Check to make sure that the hardware is functioning properly. Make sure the Mass spectrometer calibrations are proper. Run PPGs in Q1 Scan to check the instrument calibration.
- Run standards in Q1 Scan to see if molecular ion is detected.
- Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator.
- Look for sample preparation errors, i.e., if the analyst forgot to add internal standard, specimen, etc.
- Check the calibrations of the pipettes.

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 smaller aliquot of urine can be used as described earlier. 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.

13. Reference Ranges (Normal Values)

No reference ranges currently exist for urinary caffeine and caffeine metabolite concentrations in the U.S. population. These data will be available upon completion of the NHANES 2009-2010 analyses for urinary caffeine and caffeine metabolites.

In the absence of representative population data, our laboratory collected urine specimens (n = 115) from anonymous human participants on-site in a manner consistent with an internal review board approved sampling protocol. Urinary caffeine and caffeine metabolite concentrations were quantified using the present method. Reference ranges (2.5^{th} – 97.5^{th} percentile) and selected percentiles for this subset are as follows:

Analyte	Percentile (µmol/L)								
	2.5th	5th	10th	25th	50th	75th	90th	95th	97.5th
1X	0.34	1.61	3.36	7.00	27.5	61.6	129	159	205
3X	<LOD	1.36	3.12	10.2	21.6	42.5	82.5	112	145
7X	<LOD	1.99	4.04	14.2	36.8	67.7	138	202	218
13X	<LOD	<LOD	0.16	0.46	1.58	4.53	8.03	10.0	11.9
17X	<LOD	0.4	1.3	6.6	15.6	35.8	88.0	100	117
37X	<LOD	0.85	2.44	8.23	18.7	36.3	75.14	92.79	107.45
137X	<LOD	<LOD	0.2	1.2	5.0	17.7	30.3	37.6	41.8
1U	2.24	4.02	6.92	13.5	40.0	90.3	185	233	284
3U	<LOD	<LOD	<LOD	0.1	0.3	0.8	1.3	2.5	3.5
7U	<LOD	0.5	1.5	3.7	12.5	20.0	43.4	66.0	74.9
13U	<LOD	0.17	0.40	1.04	3.97	9.27	24.8	31.1	32.8
17U	<LOD	0.41	1.48	4.78	19.1	46.9	112	130	167
37U	<LOD	<LOD	0.08	0.34	0.82	1.66	3.49	6.09	7.40
137U	<LOD	<LOD	0.05	0.49	1.64	4.41	8.94	11.3	13.5
AAMU	<LOD	1.07	3.68	11.6	31.9	77.4	167	269	410

14. Critical Call Results (“Panic Values”)

There is currently insufficient data to correlate urinary caffeine and caffeine metabolite concentrations with serious health effects. Therefore, critical call values have not been established.

15. Specimen Storage and Handling During Testing

Urine samples may be stored overnight in the refrigerator to expedite thawing prior to aliquoting. Samples should be allowed to warm to and be maintained at room temperature during preparation and testing.

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

There are no acceptable alternative methods for the analysis of urinary caffeine and caffeine metabolites in the Nutritional Biomarker Branch. If the analytical system fails, we recommend that the specimens or prepared samples be stored at -80°C 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 study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically on a periodic basis to the Westat who in turn transfers the results to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

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

The LIMS database is used to keep records and track specimens for NHANES 1999+. If urinary caffeine and caffeine metabolite analyses are used for smaller, non-NHANES studies, 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 may be discarded at the request of the principal investigator, or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

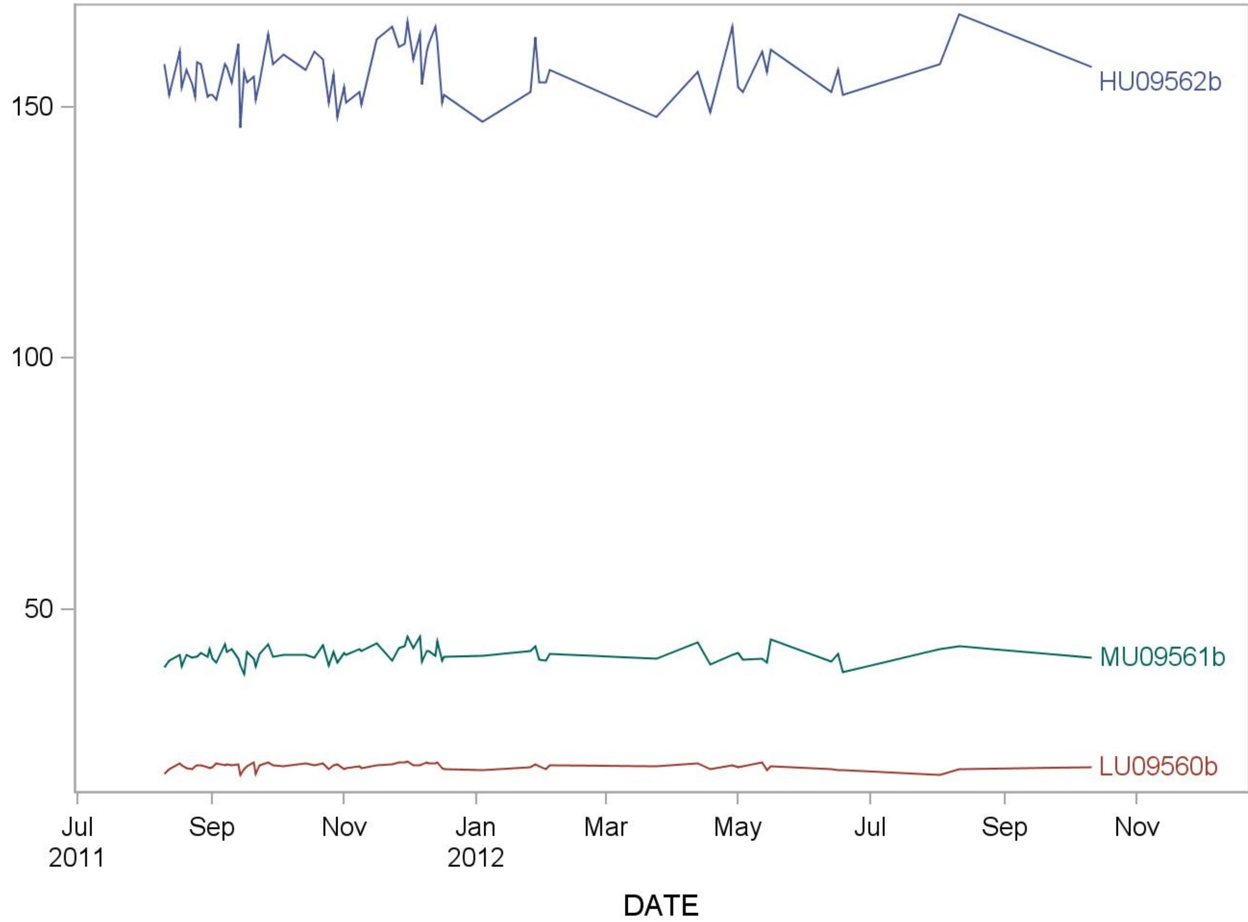
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the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

19. Summary Statistics and QC Graphs

See following pages.

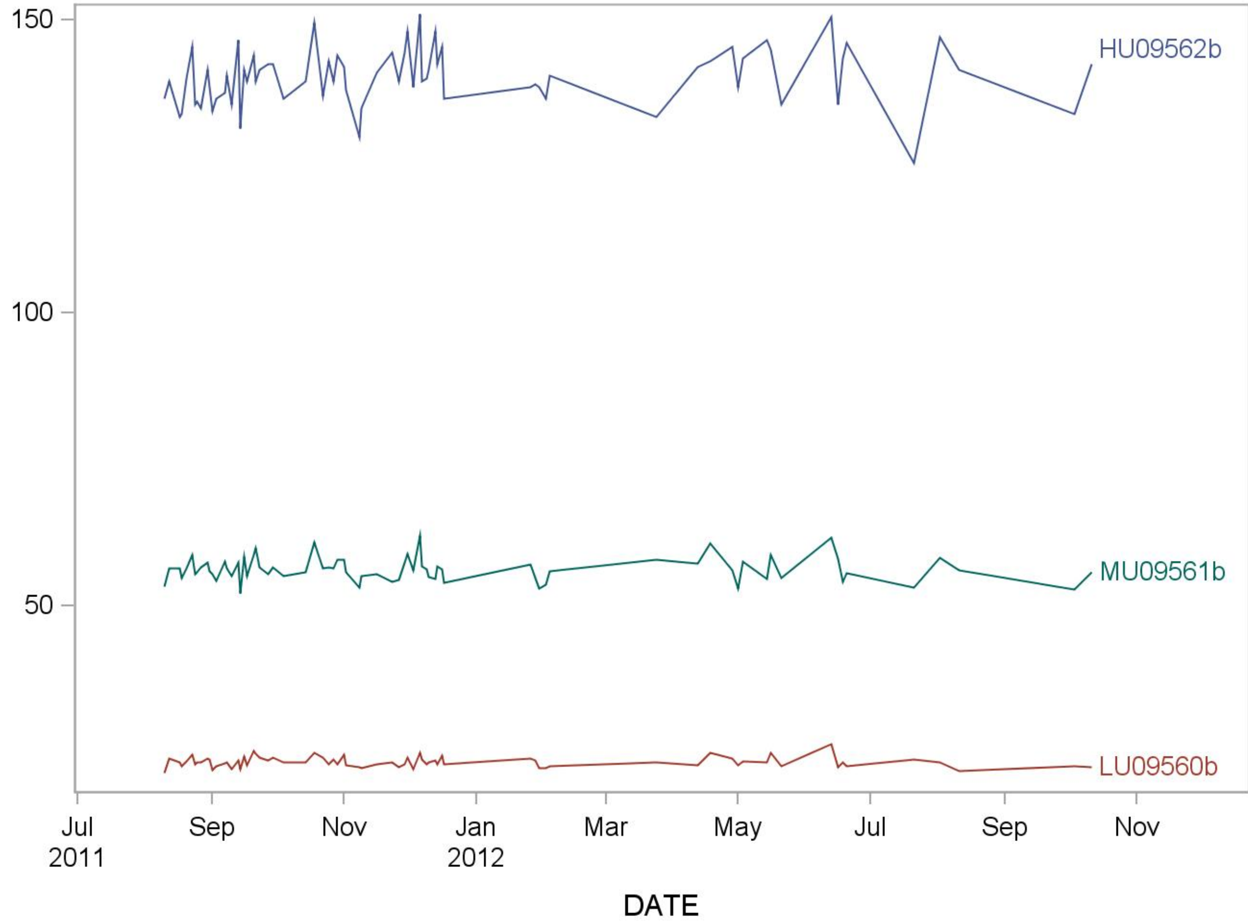
2009-2010 Summary Statistics and QC Chart for 5-acetylamino-6-amino-3-methyluracil

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	71	10AUG11	11OCT12	156.866	5.197	3.3
LU09560b	71	10AUG11	11OCT12	18.812	0.611	3.3
MU09561b	71	10AUG11	11OCT12	41.034	1.545	3.8



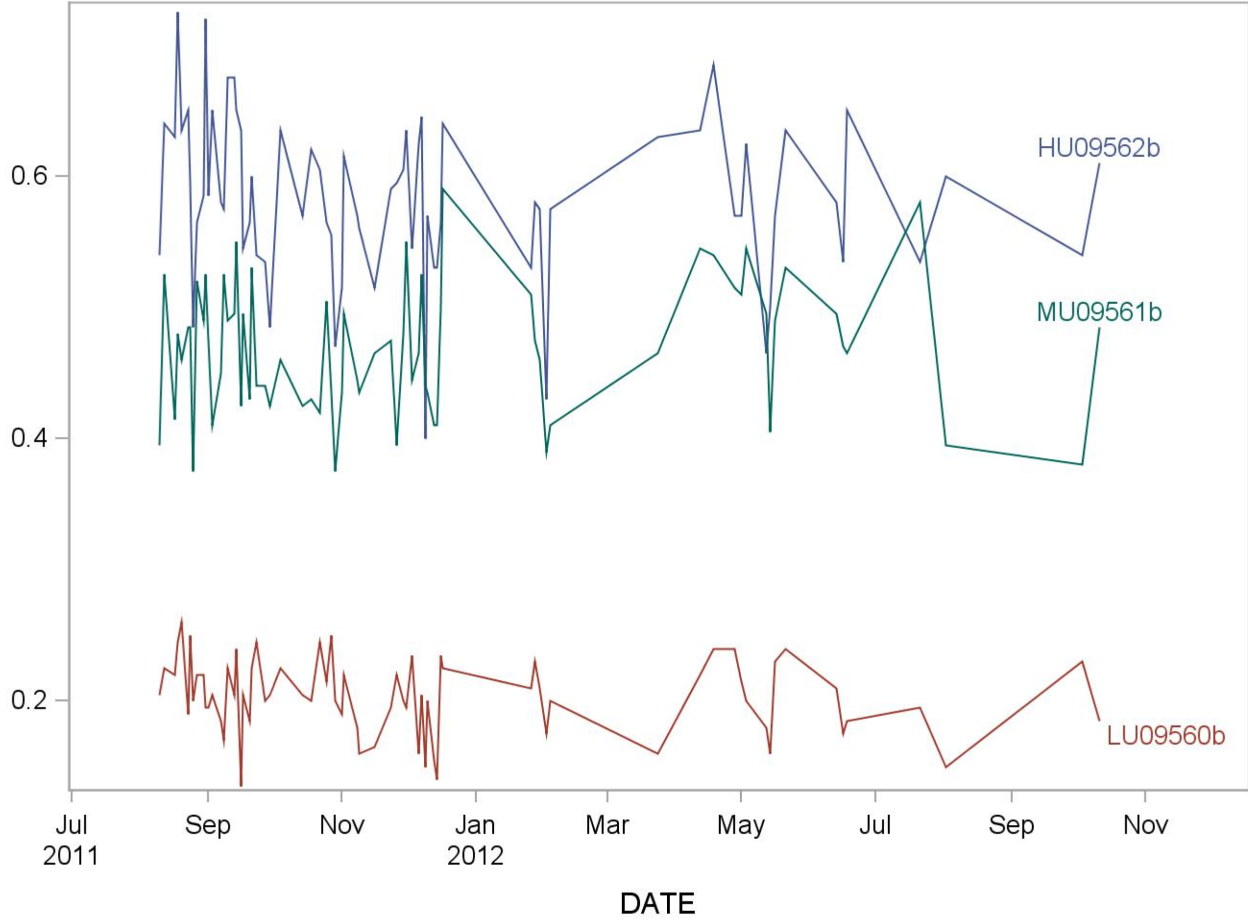
2009-2010 Summary Statistics and QC Chart for 1-methyluric acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	73	10AUG11	11OCT12	140.212	4.863	3.5
LU09560b	73	10AUG11	11OCT12	23.279	0.909	3.9
MU09561b	73	10AUG11	11OCT12	56.184	2.045	3.6



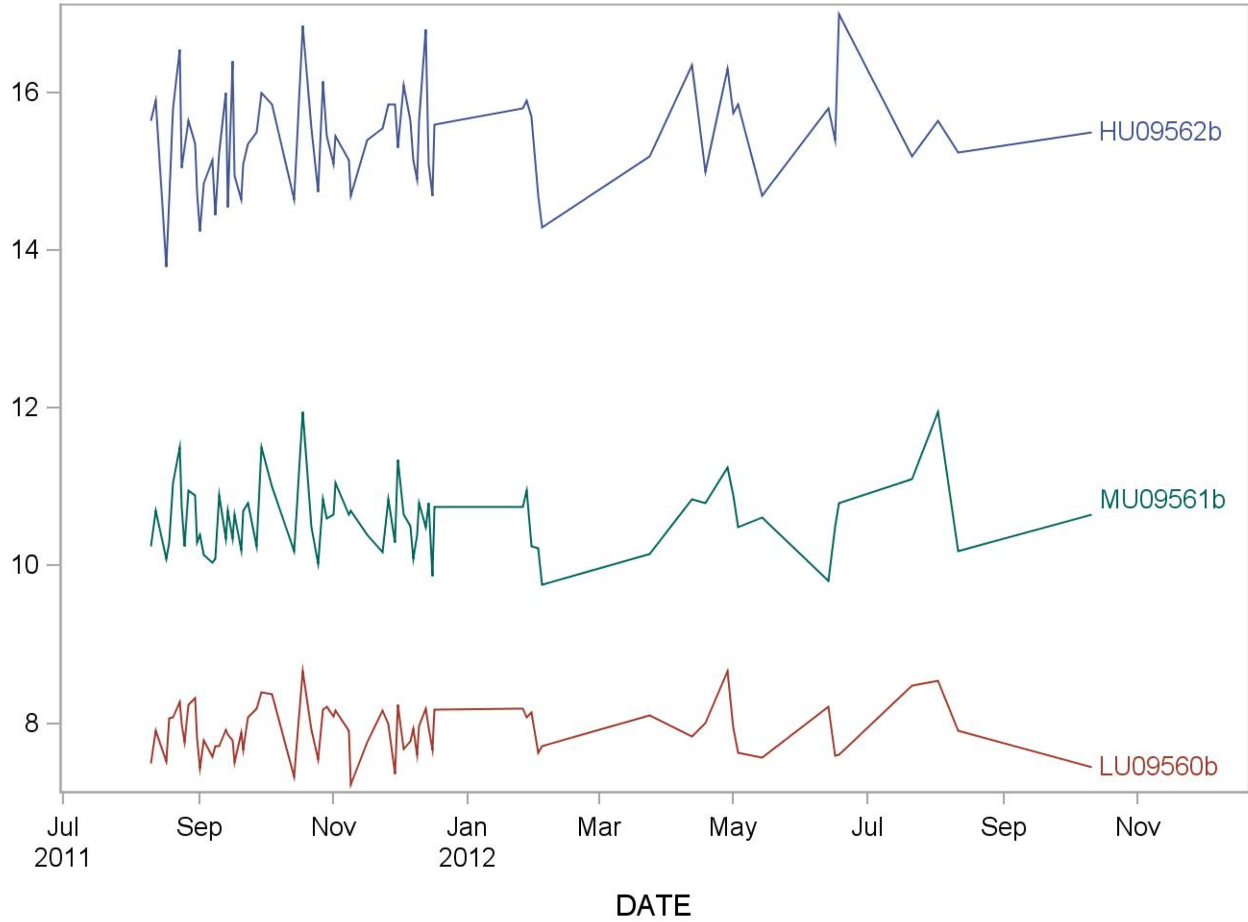
2009-2010 Summary Statistics and QC Chart for 3-methyluric acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	72	10AUG11	11OCT12	0.583	0.062	10.6
LU09560b	72	10AUG11	11OCT12	0.204	0.029	14.1
MU09561b	72	10AUG11	11OCT12	0.469	0.051	10.8



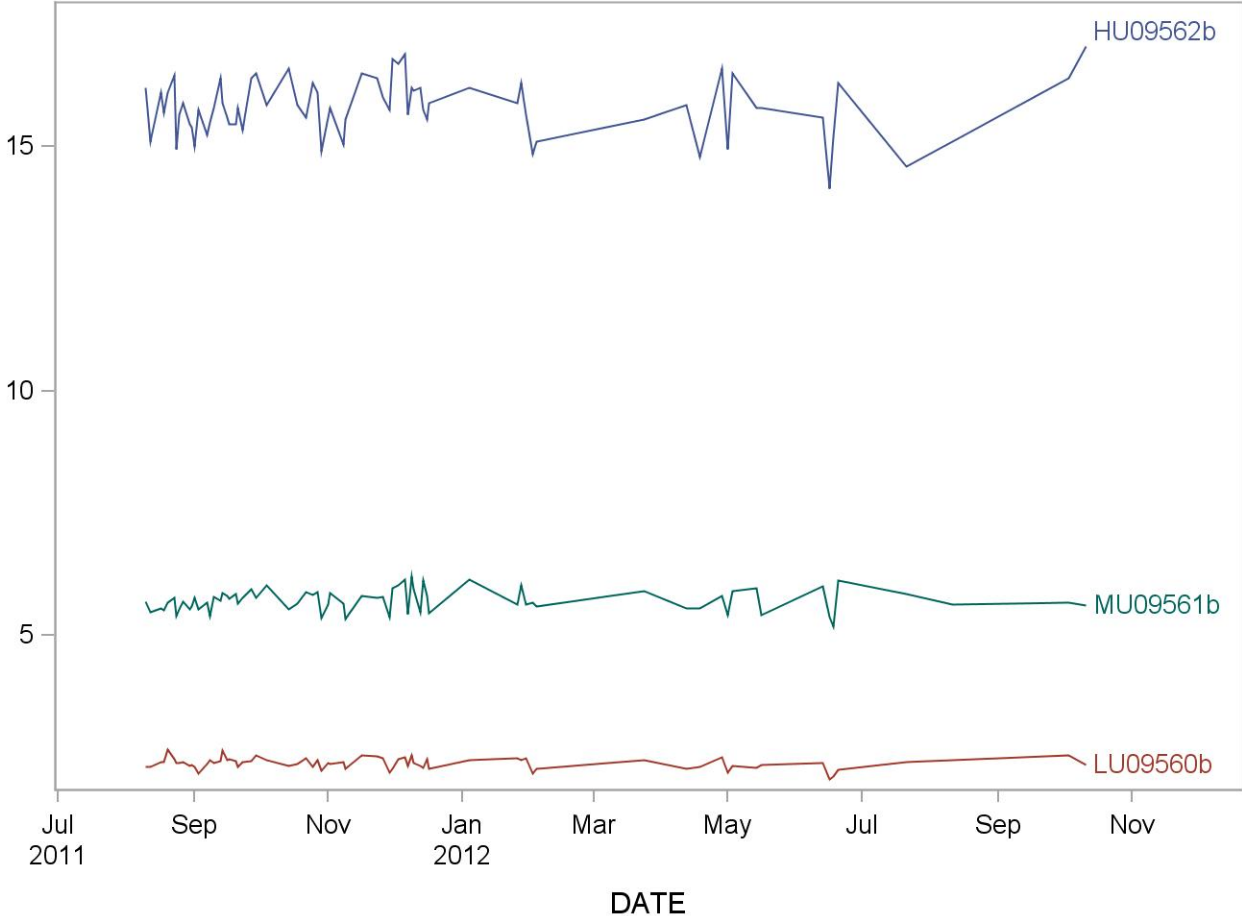
2009-2010 Summary Statistics and QC Chart for 7-methyluric acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	69	10AUG11	11OCT12	15.413	0.638	4.1
LU09560b	69	10AUG11	11OCT12	7.918	0.322	4.1
MU09561b	69	10AUG11	11OCT12	10.604	0.448	4.2



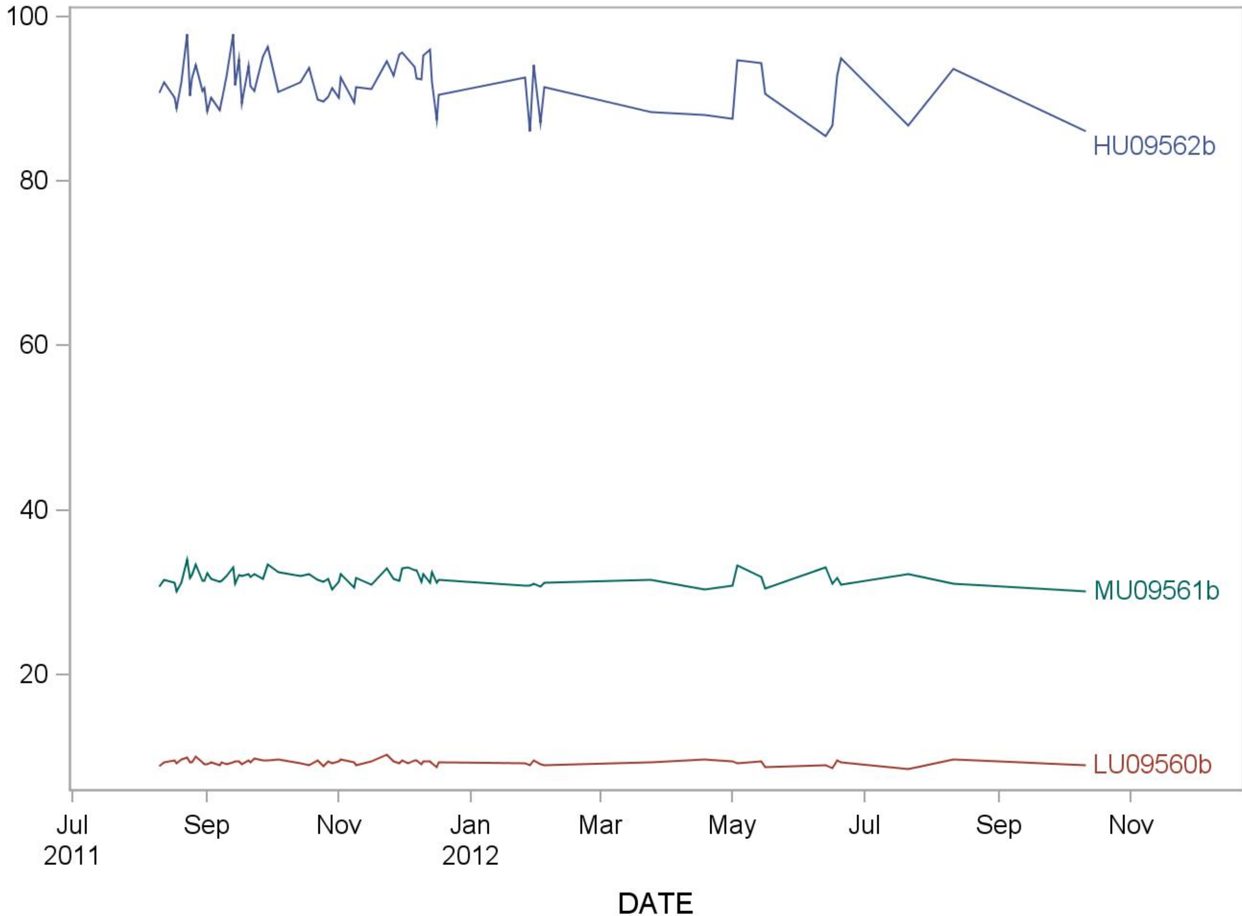
2009-2010 Summary Statistics and QC Chart for 1,3-dimethyluric acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	72	10AUG11	11OCT12	15.792	0.581	3.7
LU09560b	72	10AUG11	11OCT12	2.390	0.115	4.8
MU09561b	72	10AUG11	11OCT12	5.718	0.224	3.9



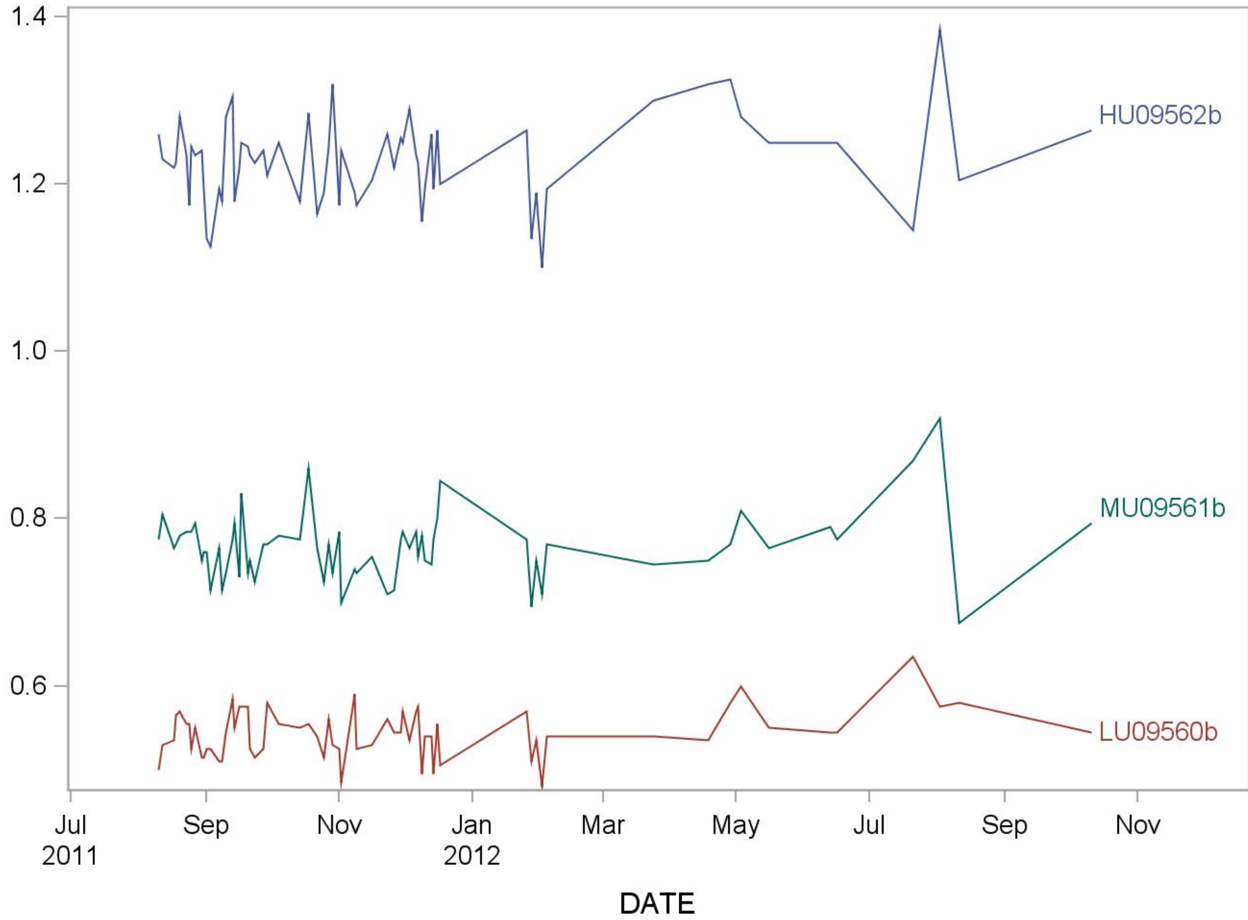
2009-2010 Summary Statistics and QC Chart for 1,7-dimethyluric acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	68	10AUG11	11OCT12	91.653	2.891	3.2
LU09560b	68	10AUG11	11OCT12	9.350	0.311	3.3
MU09561b	68	10AUG11	11OCT12	31.725	0.849	2.7



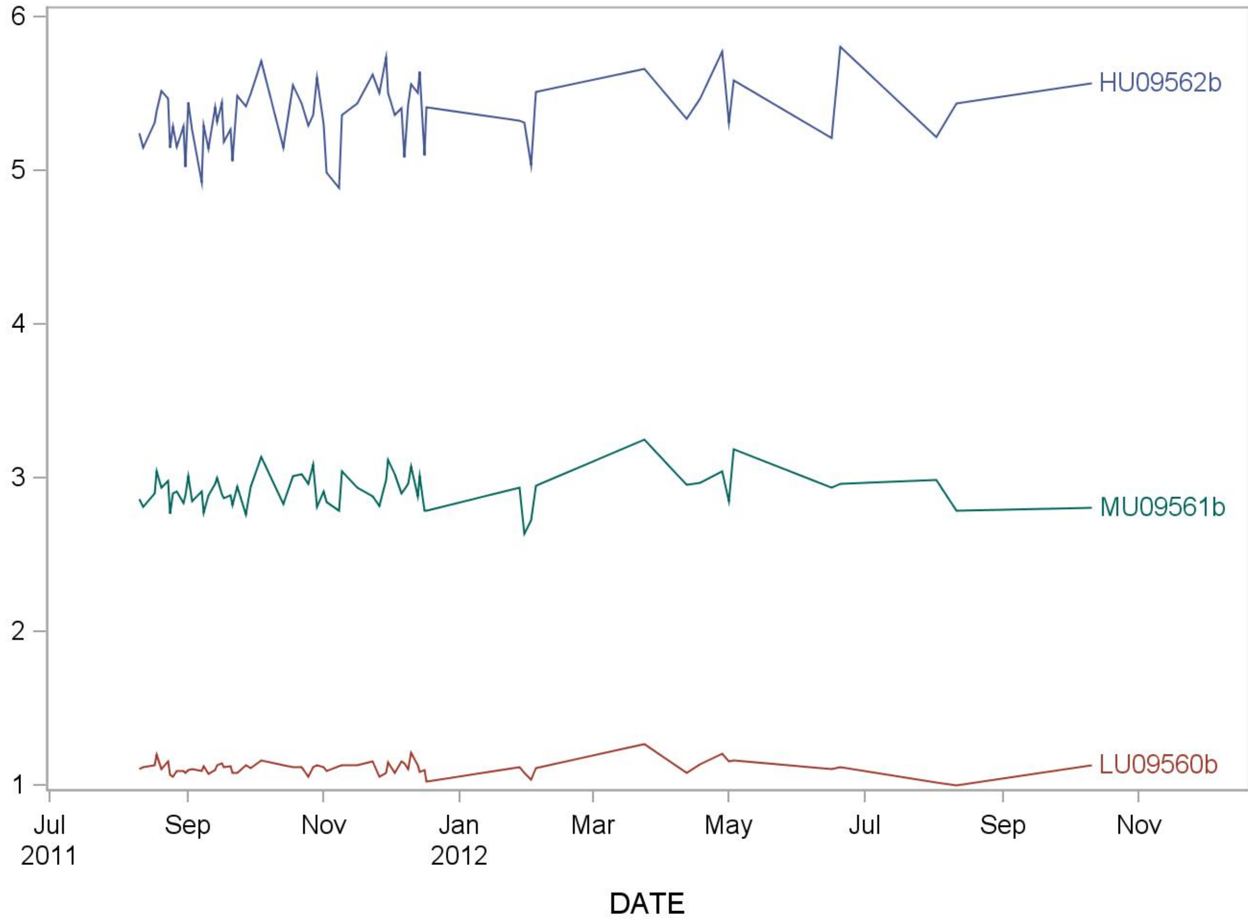
2009-2010 Summary Statistics and QC Chart for 3,7-dimethyluric acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	66	10AUG11	11OCT12	1.228	0.052	4.3
LU09560b	66	10AUG11	11OCT12	0.544	0.029	5.3
MU09561b	66	10AUG11	11OCT12	0.767	0.041	5.3



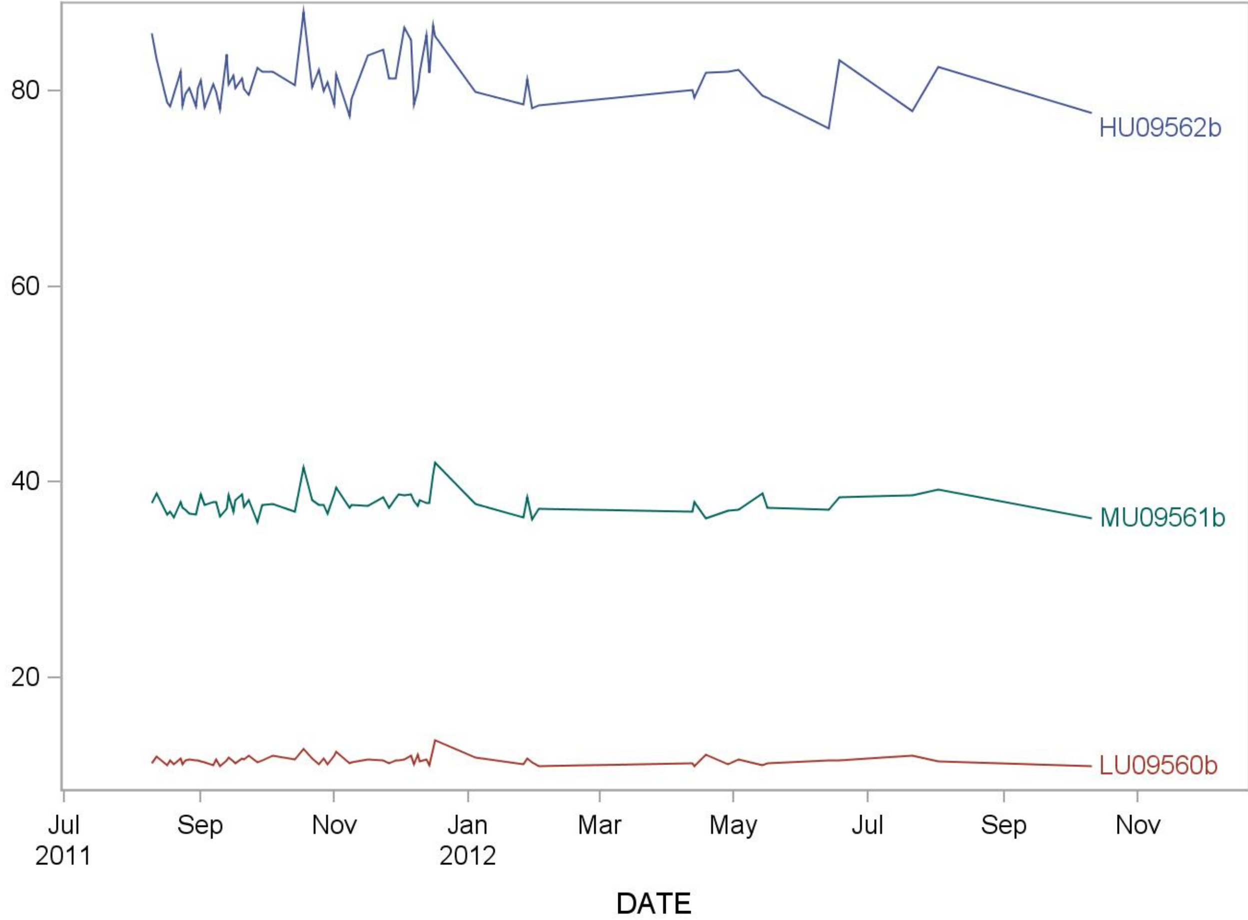
2009-2010 Summary Statistics and QC Chart for 1,3,7-trimethyluric acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	65	10AUG11	11OCT12	5.365	0.205	3.8
LU09560b	65	10AUG11	11OCT12	1.115	0.045	4.0
MU09561b	65	10AUG11	11OCT12	2.922	0.112	3.8



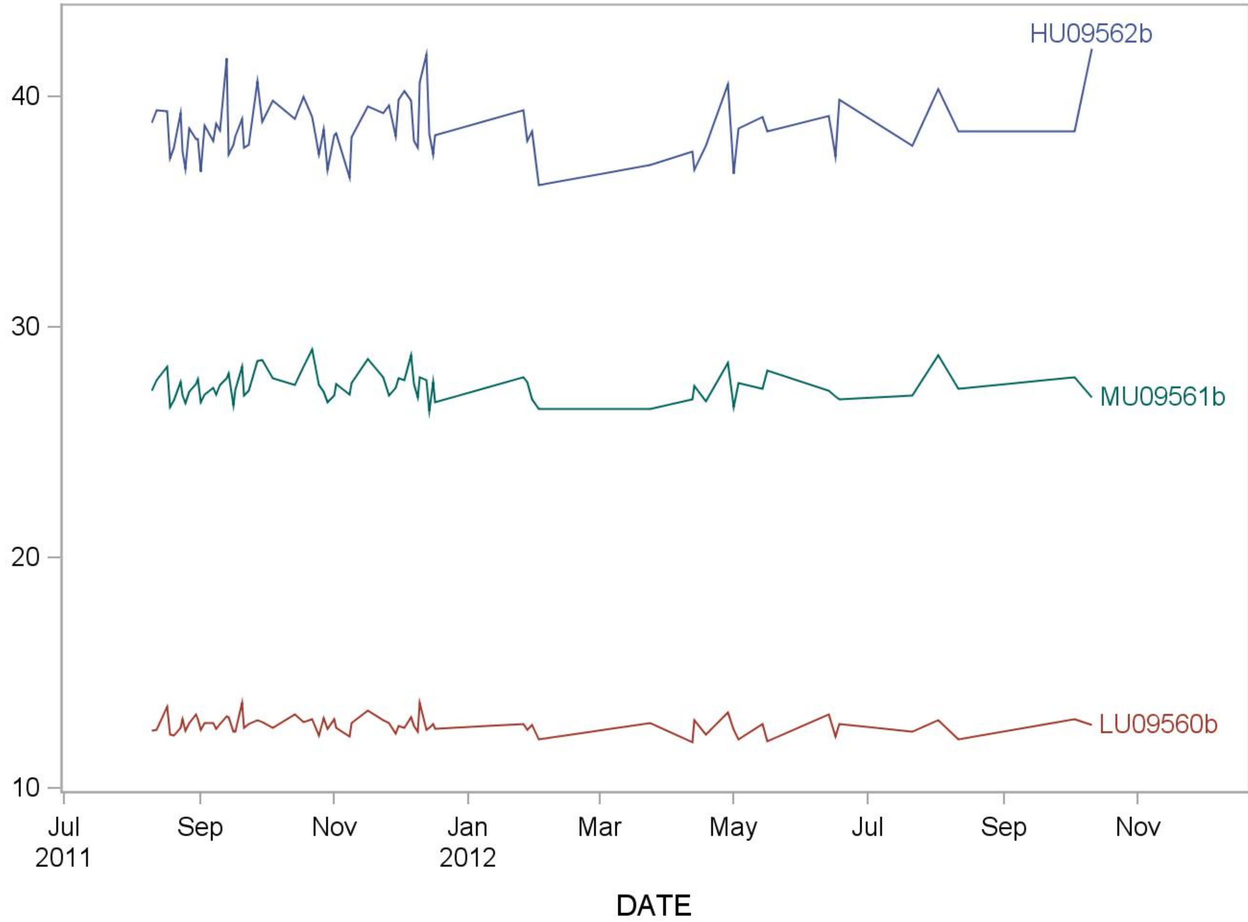
2009-2010 Summary Statistics and QC Chart for 1-methyl1xanthine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	67	10AUG11	11OCT12	81.075	2.459	3.0
LU09560b	67	10AUG11	11OCT12	11.540	0.461	4.0
MU09561b	67	10AUG11	11OCT12	37.839	1.116	2.9



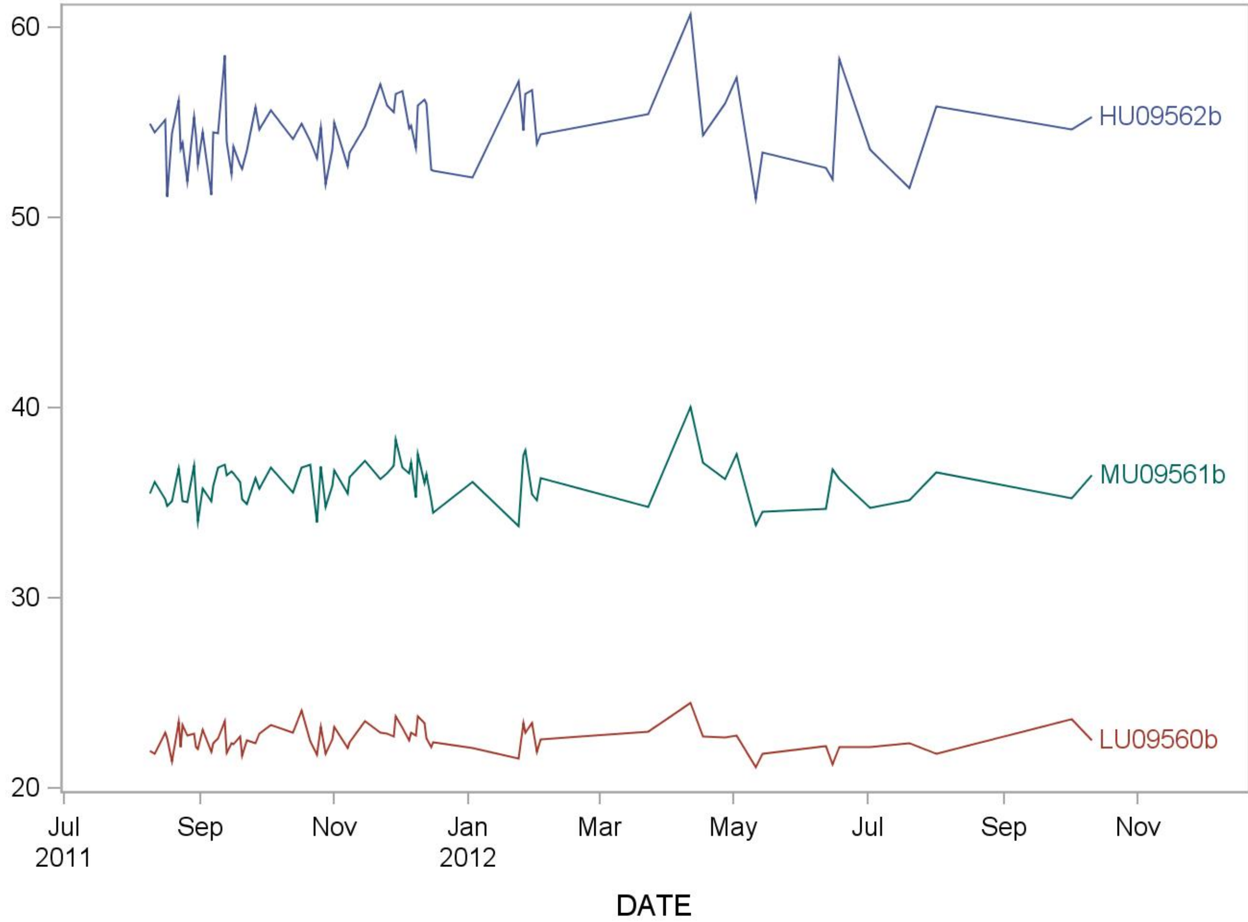
2009-2010 Summary Statistics and QC Chart for 3-methylxanthine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	71	10AUG11	11OCT12	38.646	1.242	3.2
LU09560b	71	10AUG11	11OCT12	12.746	0.362	2.8
MU09561b	71	10AUG11	11OCT12	27.452	0.620	2.3



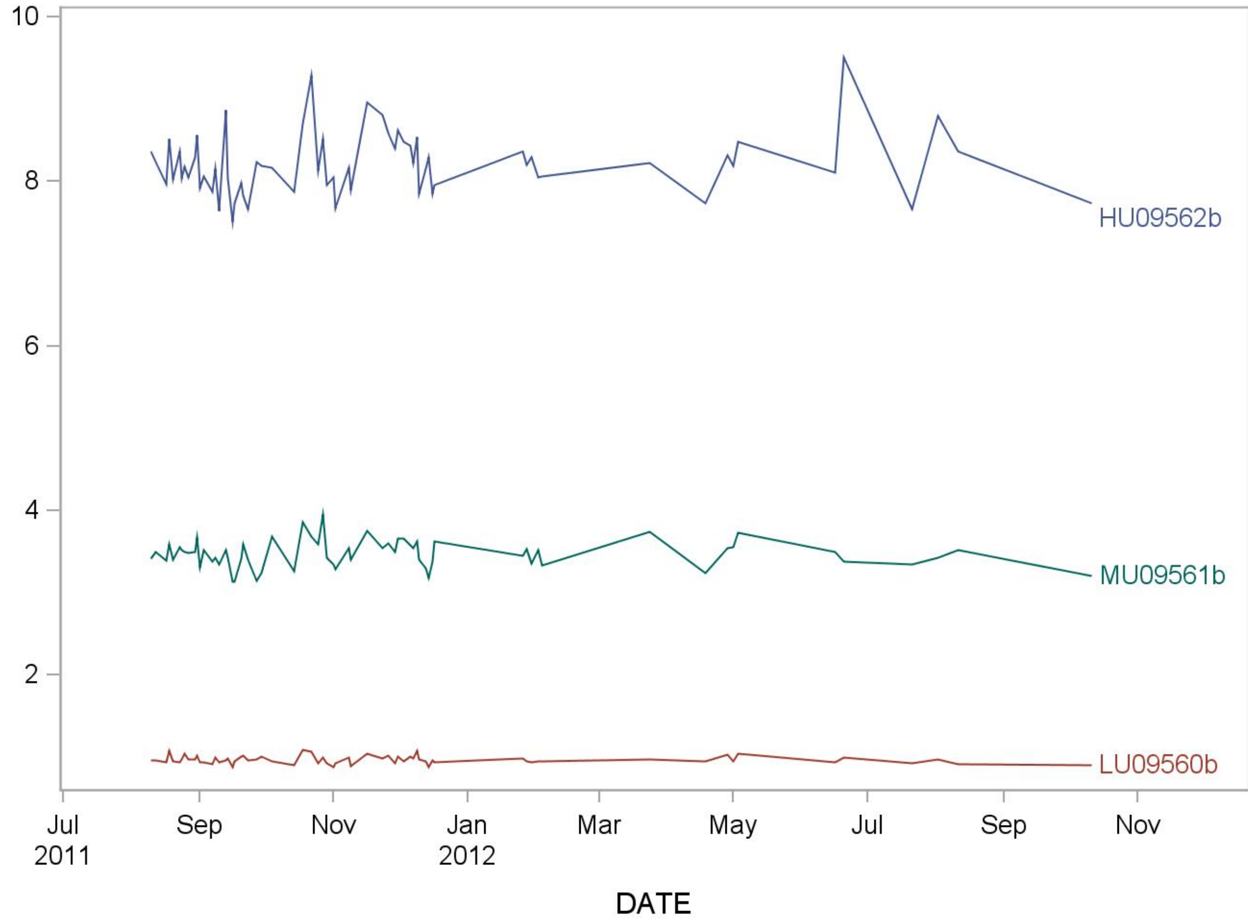
2009-2010 Summary Statistics and QC Chart for 7-methylxanthine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	72	09AUG11	11OCT12	54.503	1.844	3.4
LU09560b	72	09AUG11	11OCT12	22.634	0.678	3.0
MU09561b	72	09AUG11	11OCT12	36.030	1.114	3.1



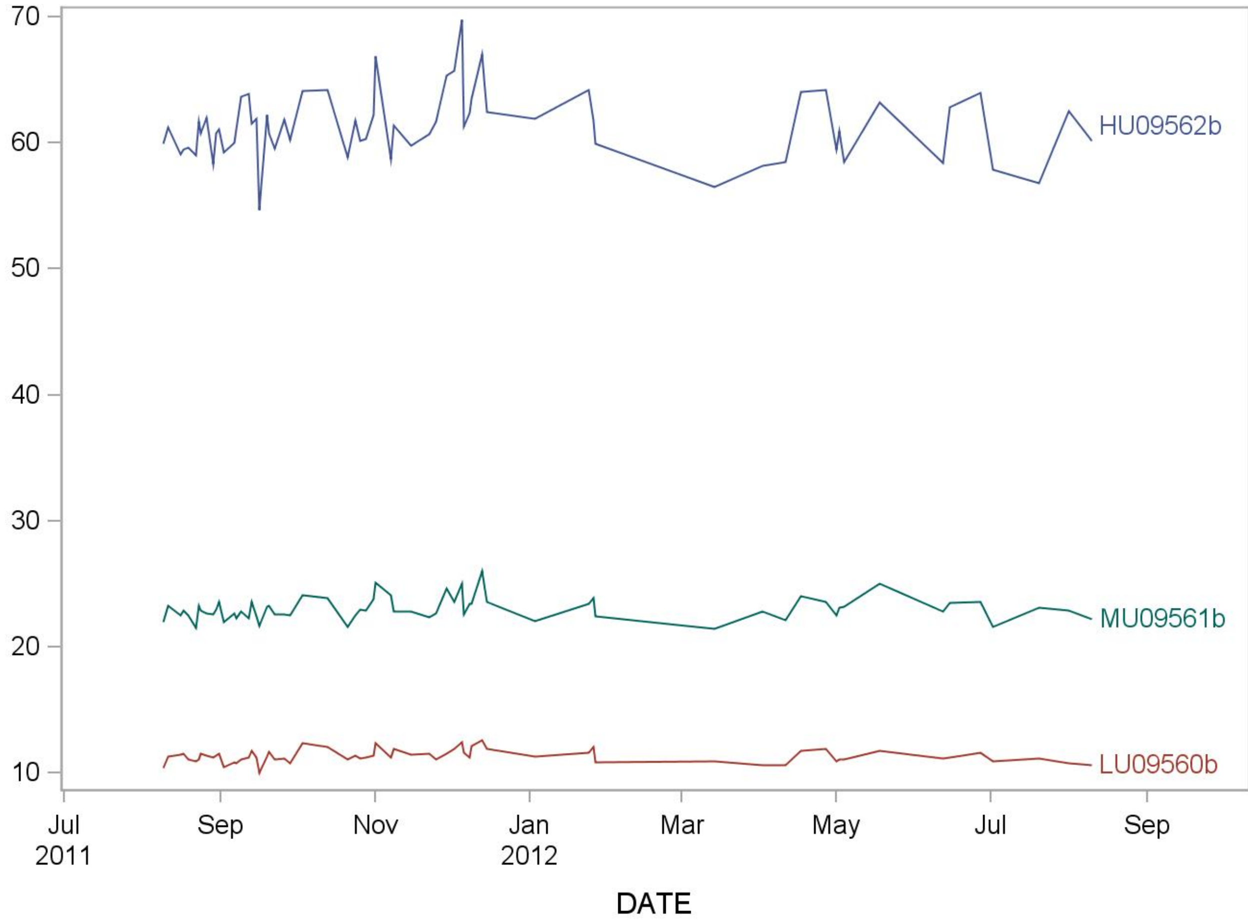
2009-2010 Summary Statistics and QC Chart for 1,3-dimethylxanthine; theophylline

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	66	10AUG11	11OCT12	8.213	0.383	4.7
LU09560b	66	10AUG11	11OCT12	0.970	0.048	5.0
MU09561b	66	10AUG11	11OCT12	3.474	0.168	4.8



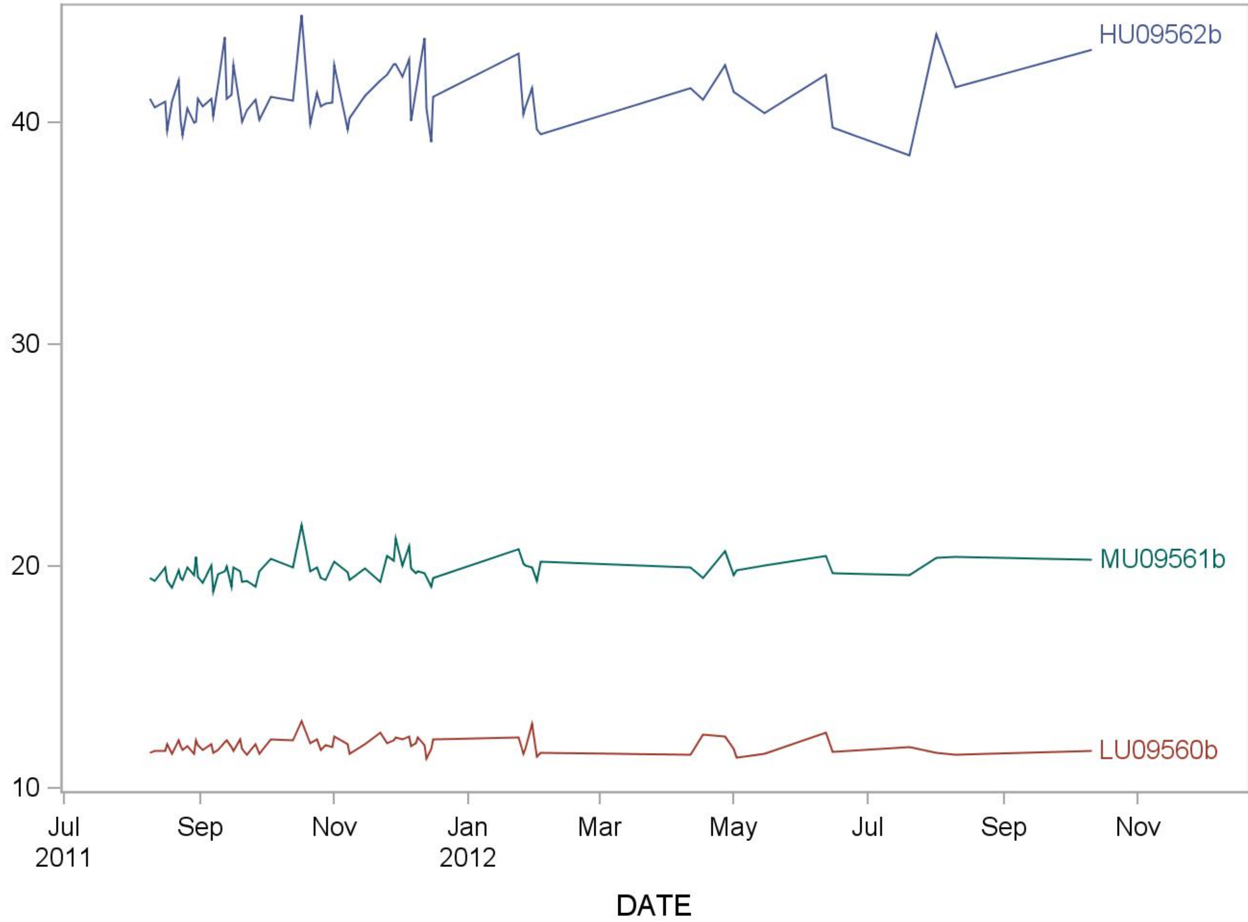
2009-2010 Summary Statistics and QC Chart for 1,7-dimethylxanthine; paraxanthine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	66	09AUG11	10AUG12	61.300	2.617	4.3
LU09560b	66	09AUG11	10AUG12	11.333	0.512	4.5
MU09561b	66	09AUG11	10AUG12	23.033	0.900	3.9



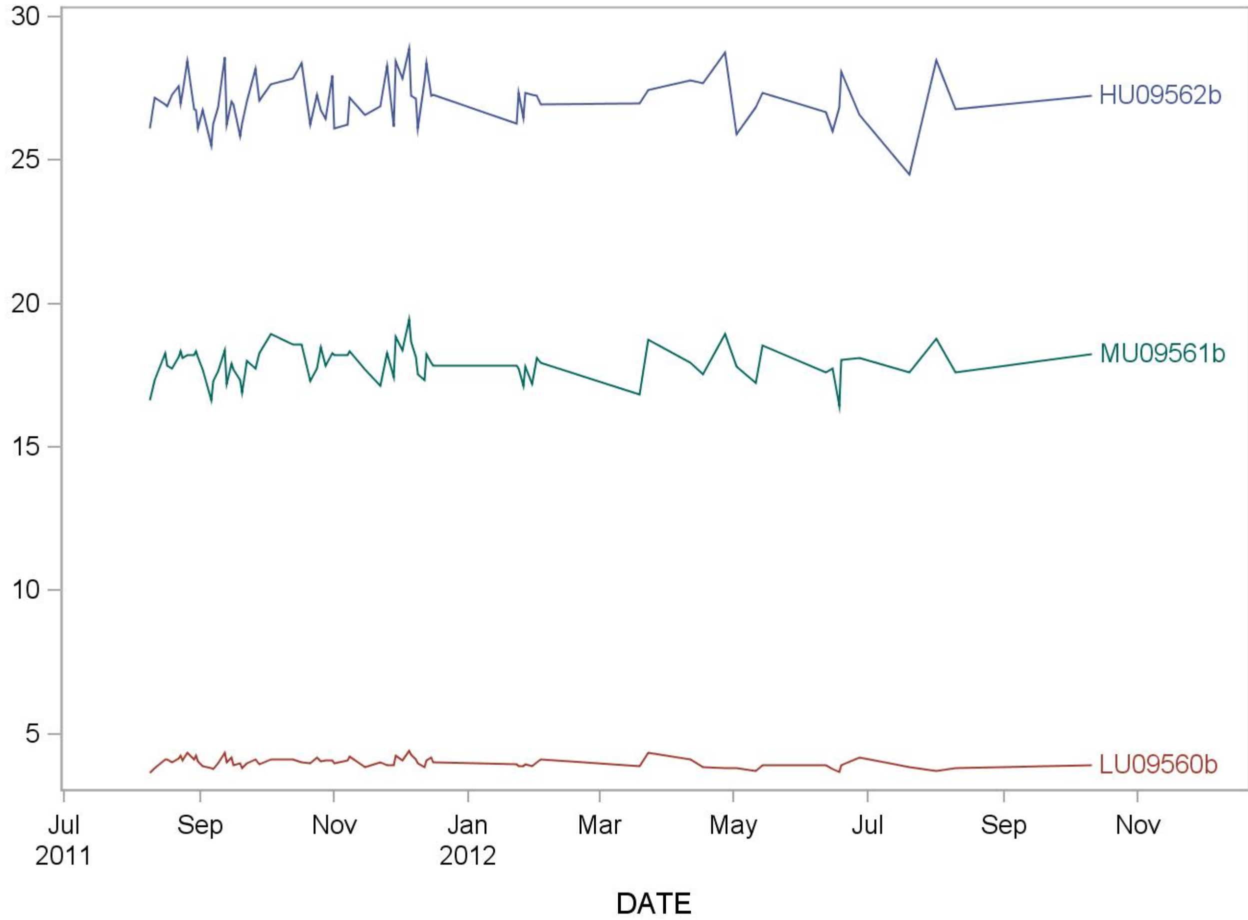
2009-2010 Summary Statistics and QC Chart for 3,7-dimethylxanthine; theobromine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	68	09AUG11	11OCT12	41.199	1.232	3.0
LU09560b	68	09AUG11	11OCT12	11.934	0.345	2.9
MU09561b	68	09AUG11	11OCT12	19.857	0.528	2.7



2009-2010 Summary Statistics and QC Chart for 1,3,7-trimethylxanthine; caffeine

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HU09562b	74	09AUG11	11OCT12	27.093	0.827	3.1
LU09560b	74	09AUG11	11OCT12	4.015	0.171	4.3
MU09561b	74	09AUG11	11OCT12	17.913	0.581	3.2



Acknowledgements

We gratefully acknowledge the contributions of Ching-I (Jenny) Pao, Ph.D., Michael Rybak, Ph.D., and Christine Pfeiffer, Ph.D., who assisted in developing the methodology, performing the analyses for urinary 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 June 2 [Epub ahead of print].

[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.e1-9.

[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. 2008. Multi-rule quality control for the age-related eye disease study. Stat Med 27:4094-4106.