Laboratory Procedure Manual

Analytes: **Urinary metabolites of Benzene, Furfural, 5-Hydroxymethylfurfural, and N-Methyl-2-pyrrolidone**

Matrix: **Urine**

Method: **UPLC-ESI-MS/MS**

Method No: **2105.02**

as performed by:

Tobacco and Volatiles Branch
Division of Laboratory Sciences
National Center for Environmental Health

contact:

Dr. Deepak Bhandari
Phone: 770-488-0939
Fax: 770-488-0181
Email: xwo1@cdc.gov

Dr. James Pirkle, Director
Division of Laboratory Sciences

Important Information for Users

The Centers for Disease Control and Prevention (CDC) periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

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
UVOC2_K	URXMUCA	*trans, trans*-Muconic acid (ng/mL)
	URXPHMA	Phenylmercapturic acid (ng/mL)

1. CLINICAL RELEVANCE AND SUMMARY OF TEST PRINCIPLE

a) Clinical Relevance

Exposure to volatile organic compounds (VOCs) such as benzene, N-methyl-2-pyrrolidone, furfural, and 5-hydroxymethylfurfural are associated with numerous known health risks (**Table 1**). Exposure can occur via enteral and parenteral routes from various sources including tobacco smoke, e-cigarette aerosol, automobile exhaust, industrial applications, and food products (**Table 2**). VOCs can be metabolized prior to urinary excretion, so VOC exposure can be assessed by measuring their metabolites in urine. **Table 3** shows the urinary VOC metabolites measured using this method.

Table 1. Health risks of selected VOCs

Parent compound	Health effects
Benzene	Acute exposure: Respiratory toxicity (hemorrhage, edema, bronchitis), hepatotoxic. Chronic exposure: Hematological toxicity, immunotoxic, reproductive toxicity (decreases sperm motility, disturbs menstrual cycle and various reproductive hormones, induces miscarriage, birth defects), potentially neurotoxic, respiratory toxicity, endocrine disrupting chemical (EDC), possible cardiovascular effects (hypertension)[1], Group 1 carcinogen[2].
Furfural	Acute exposure can cause cough, labored breathing, skin redness/pain, abdominal pain, diarrhea, vomiting[4].
5-Hydroxymethylfurfural	Potentially carcinogenic[5].
N-Methyl-2-pyrrolidone	Embryolethal, teratogenic, can cause intrauterine growth restriction[6,7].

Table 2. Sources of VOC exposure

Parent compound	Exposure sources
Benzene	Cigarette smoke, automobile exhaust, occupational exposure due to industrial applications, crude oil, gasoline[2].
Furfural	Coffee[9], animal feed, resins, flavoring, antacids, inks, fungicides, nematicides, adhesives[10], whiskey[11], prepared carbohydrate and sugar-containing foods[12].
5-Hydroxymethylfurfural	Preparation of carbohydrate-containing foods, food flavoring, dried fruits, coffee, caramel products[5].
N-Methyl-2-pyrrolidone	Industrial applications, solvent, paint stripper, graffiti remover, spinning agent for PVC, penetration enhancer in transdermal therapies[13].

Table 3. VOC metabolites and their parent compounds

Parent compound	VOC metabolite	Abbreviation
Benzene	*trans,trans*-Muconic acid	MUCA
	N-Acetyl-S-phenyl-L-cysteine[14]	PMA
Furfural	N-2-Furoylglycine[9,16]	N2FG
5-Hydroxymethylfurfural	5-Hydroxymethyl-2-furancarboxylic acid	HMFA
	5-Hydroxymethyl-2-furoylglycine[5]	HMFG
N-Methyl-2-pyrrolidone	5-Hydroxy-N-methyl-2-pyrrolidone[13,17]	5HMP

b) Test Principle

This method is a quantitative procedure to measure urinary metabolites of benzene, furfural, 5-hydroxymethylfurfural, and N-methyl-2-pyrrolidone using ultra performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). Chromatographic separation of diluted urine is achieved by using a Waters Acquity UPLC HSS fluoro-phenyl (PFP) column using a Solvent A (0.02% formic acid) and a Solvent B (methanol). The eluate from the column is ionized using electrospray ionization, which is used to generate and transmit ions into the mass spectrometer. Comparison of relative response factors (ratio of native analyte to stable isotope labeled internal standard) with known standard concentrations yields individual analyte concentrations in each sample.

2. SAFETY PRECAUTIONS

a) Reagent Toxicity or Carcinogenicity

Observe Universal Precautions. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis according to CDC/EHLS guidelines for disposal of hazardous waste.

Follow special precautions while handling methanol and formic acid.

Methanol and acetonitrile are toxic flammable liquids. **If methanol or acetonitrile comes in contact with any part of the body, quickly wash with water for at least 15 minutes and remove contaminated clothing.**

Formic acid is very hazardous if in contact with skin or eyes, and may produce burns. **If formic acid comes into contact with any part of the body, flush with water for at least fifteen minutes and remove contaminated clothing. In the case of serious skin contact, wash with disinfectant soap, cover the contaminated skin, and seek medical attention immediately.**

b) Radioactive Hazards

This method does not use radioactive materials and is not associated with radioactive hazards.

c) Microbiological Hazards

This assay uses human urine samples. Universal precautions must be followed to minimize biological hazards. Analysts working directly with the specimens must use proper technique and avoid direct contact with the samples. A Hepatitis *B* vaccination series is recommended for all laboratory analysts who may get exposed to human fluids and tissues.

d) Mechanical Hazards

There are minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should read and follow the manufacturer's information regarding the safe operation of the equipment. Avoid direct contact with the mechanical and electronic components of the mass spectrometer unless all power to the instrument is off. Generally, only qualified technicians should perform mechanical and electronic maintenance and repair. The UPLC and the mass spectrometer contain a number of areas that are hot enough to cause burns. High voltages are found in certain areas of mass spectrometers and care must be taken when working in these areas.

e) Protective Equipment

Follow standard safety precautions when performing this procedure, including the use of a lab coat/disposable gown, safety glasses, appropriate gloves, and chemical fume hood. Refer to the laboratory Chemical Hygiene Plan and CDC Division of Laboratory Sciences (DLS) safety policies and procedures for details related to specific activities, reagents, or agents.

f) Training

Analysts are required to demonstrate safe and proper techniques in performing the method, and generate data with acceptable accuracy and precision based on their calibration curves, quality control (QC) and proficiency testing (PT) samples. Educational and specific training information are maintained for all laboratory analysts certified to work on this method.

g) Disposal of Waste

All laboratory waste disposal must be in compliance with DLS policy. Dispose of solvents and reagents in an appropriate container clearly marked for waste products and temporarily stored in a chemical fume hood. Place all disposable items that come in direct contact with the biological specimens in a biohazard autoclave bag that is kept in appropriate containers until autoclaved. Immediately place unshielded needles, pipette tips and disposable syringes into a sharps container and autoclave when this container becomes full.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a) Software and Knowledge Requirements

This method has been validated using a Waters or Shimadzu UPLC system coupled to a Sciex 5500 triple quadrupole mass spectrometer. The UPLC-MS system is controlled with Sciex Analyst software with a Waters Acquity module. Data analysis is performed using Sciex MultiQuant software and the quantitation reports are stored as .qsession and ASCII files and entered into the STARLIMS database. Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

b) Sample Information

Typically, an analytical run contains up to 96 samples. Corresponding batch files contain information such as run ID, sample ID, sample file number, date of analysis, injection volume, standard and internal standard lot, and special notes and observations for each run. Information pertaining to particular specimens is entered into the batch file either manually or electronically.

c) Data Maintenance

All analytical data sets are checked prior to being entered into the STARLIMS database for transcription errors and overall validity. The database is routinely backed up locally onto a computer hard drive and through the standard practices of the NCEH network. The local area network manager should be contacted for emergency assistance.

d) Information Security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the individual laboratories, buildings, and site. Confidentiality of the results is protected by use of blind coded ID numbers only (no clinical specimen are labeled with personal identifiers).

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

a) No special instructions such as fasting or special diets are required.
b) The sample type is urine.
c) An aliquot of 50 µL is needed per assay. A volume of 250 µL is required to allow for repeated analysis. If the sample volume is below 250 µL, a second urine specimen should be requested.
d) Acceptable containers include sterile polystyrene cryovials or polypropylene centrifuge tubes.
e) Specimens suspected of being contaminated due to improper collection procedures or collection devices are considered unacceptable.
f) Specimen handling conditions are outlined in the DLS protocol for urine collection and handling (copies available in Branch, Laboratory and Special Activities specimen handling offices). Collection, transport, and special requirements are discussed. Urine specimens should be transported and stored chilled or frozen at ≤ -15 ºC. Once received, the samples can be frozen at ≤ -60 ºC until time of analysis. Portions of the sample that remain after analytical aliquots are withdrawn should be refrozen at ≤ -60ºC.

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

Not applicable to this procedure

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

a) Reagent Sources

Reagents used during the development, validation and application of this method and their sources are listed in **Table 4**. All chemical reagents are used without further purification**.**

Table 4. Suggested reagent sources

Reagent	Abbreviation	Suggested source
Solvents		
Acetonitrile (LC-MS grade)		Fisher Scientific, Fairlawn, NJ
Ammonium Formate (LC-MS grade)		Fisher Scientific, Fairlawn, NJ
Formic Acid (LC-MS grade)		Fisher Scientific, Fairlawn, NJ
Methanol (LC-MS grade)		Fisher Scientific, Fairlawn, NJ
Isopropyl alcohol (LC-MS grade)		Fisher Scientific, Fairlawn, NJ
Water (HPLC grade)		Fisher Scientific, Fairlawn, NJ
Native Standards Calibration and Control Materials		
5-Hydroxy-N-methyl-2-pyrrolidone	5HMP	Toronto Research Chemicals, Toronto, Canada
5-Hydroxymethyl-2-furancarboxylic Acid	HMFA	Santa Cruz Biotechnology, Dallas, Texas
5-Hydroxymethyl-2-furoylglycine	HMFG	Toronto Research Chemicals, Toronto, Canada
N-2-Furoylglycine	N2FG	Toronto Research Chemicals, Toronto, Canada
trans,trans-Muconic acid	MUCA	Toronto Research Chemicals, Toronto, Canada
N-Acetyl-S-phenyl-L-cysteine	PMA	Toronto Research Chemicals, Toronto, Canada
Isotopically Labeled Internal Standards		
5-Hydroxy-N-methyl-2-pyrrolidone-d_3	5HMP-d_3	Toronto Research Chemicals, Toronto, Canada
5-Hydroxymethyl-2-furancarboxylic Acid-$^{13}C_6$	HMFA-$^{13}C_6$	Toronto Research Chemicals, Toronto, Canada
5-Hydroxymethyl-2-furoylglycine-^{13}C,^{15}N	HMFG-^{13}C,^{15}N	Toronto Research Chemicals, Toronto, Canada
N-2-Furoyglycine-d_3	N2FG-d_3	Toronto Research Chemicals, Toronto, Canada
trans,trans-Muconic acid-$^{13}C_6$	MUCA-$^{13}C_6$	Sigma Chemicals, St. Louis, MO
N-Acetyl-S-phenyl-L-cysteine -$^{13}C_6$	PMA-$^{13}C_6$	Cambridge Isotopes, Andover, MA

b) Reagent Preparation

1) Sample preparation buffer: 5 mM ammonium formate aqueous with 0.15% formic acid —pH 2.89-2.93
    - The buffer is prepared by mixing 50 mL of 200 mM ammonium formate solution, 3 mL of formic acid, and 1.947 L of water. A 200 mM ammonium formate solution is prepared by adding 3.15 g of neat ammonium formate to 250 mL HPLC grade water (stored at 2-8° C). The pH of the sample preparation buffer should be checked to make sure it is within range, and it can be stored at room temperature.
2) Mobile phase A: water + 0.02% formic acid.
3) Mobile phase B: methanol
4) Strong needle wash: An organic rinse such as 25/25/25/25% (v/v/v/v) isopropanol/methanol/acetonitrile/water
5) Seal wash: A mostly aqueous rinse such as 90/10% (v/v) water/methanol or 90/10% (v/v) water/isopropanol
6) Weak needle washes: water and/or water + 0.02% formic acid

c) Standard solutions

The primary stock solutions can be prepared by outside sources. The preparation of the working standard solutions should follow the following criteria: (a) concentration at each level should be separated from the next level by a maximum factor of √10, and (b) the lowest concentration is to be equal to or less than the LOD. The standards should be prepared in water. The suggested target standard concentrations are shown in **Table 5**.

Table 5. Suggested target standard concentrations in source vial (ng/mL)

Analyte	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	STD 9
5HMP	0.253	0.400	0.800	2.53	8.00	25.3	80.0	253	800
HMFA	23.7	37.5	75.0	237	750	2370	7500	23700	75000
HMFG	10.0	15.8	31.6	99.9	316	999	3160	9990	31600
N2FG	39.5	62.5	125	395	1250	3950	12500	39500	125000
MUCA	7.59	12.0	24.0	75.9	240	759	2400	759	N/A
PMA	0.0949	0.150	0.300	0.949	3.00	9.49	30.0	94.9	N/A

d) Isotopically labeled internal standard solutions

Stable isotope labeled internal standards used in this method are listed in **Table 4**. Internal standard compounds of at least 98% purity can be used without further purification. Isotopically labeled compounds are dissolved in methanol or water and checked for any spectral overlap with corresponding native analogs before use. Other isotopic analogs may be used when there is availability or cost limitation as long as the internal standard is stable and there are no chromatographic or mass spectral interferences. All internal standard solutions are stored at ≤ -60°C prior to use.

e) Proficiency Testing Materials

Proficiency Testing (PT) materials at four (native analyte concentration) levels can be prepared by external PT providers. Aliquots are stored in cryovials at ≤ -60°C until use. PT samples are run at least two times a year and run following any major maintenance on instrumentation. A proficiency testing coordinator, independent from the sample analysis team, blind-codes the PT stock vials and verifies the accuracy of quantified results of four PT samples at each of the four concentration levels and one sample at any of the four different levels.

f) Quality Control Materials

Quality Control (QC) materials are prepared at two concentration levels, QC low (Q_L) and QC high (Q_H), in urine. Q_L and Q_H are made from two urine pools with spiked analytes to obtain desired concentrations. Aliquots of Q_L and Q_H are stored separately in cryovials at ≤ -60°C until use. Each vial is thawed once, and the remaining solution is discarded after use. At least 20 separate QC samples are analyzed using different sample

runs and instruments to characterize the QCs and to determine the mean values and coefficient of variation (CV) for individual analytes.

g) Instrumentation and Other Equipment

- Ultra performance liquid chromatography system and autosampler (e.g., Waters Acquity UPLC®)
- Triple quadrupole mass spectrometer (e.g., Sciex 5500)
- Thawing rack
- Robotic liquid handling system
- Rugged rotator
- Desktop computer

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

All calibration standards are prepared in the sample preparation buffer. Matrix validation experiments are performed to verify that the slopes of the calibration curves resulting from the calibrators mixed in the calibration solution are comparable to those mixed in urine. These results validate the use of non-urine based calibrators for quantifying analytes in urine samples. The difference in slopes from matrix-matched urine calibrators and non-matrix-matched calibrators meets DLS Policies and Procedures Manual (PPM) requirements of ≤ 5% difference. The slopes of analytes in urine and water matrix are shown in **Table 6.**

Table 6. Slopes of urine matrix and water matrix concentration plots.

	Average Slope		
Analyte	Urine Matrix	Water Matrix	Percent Difference
5HMP	0.3443	0.3556	3.2%
HMFA	0.0040	0.0041	2.1%
HMFG	0.0048	0.0049	2.1%
N2FG	0.0036	0.0036	0.7%
MUCA	0.0161	0.0161	0.0%
PMA	0.1702	0.1707	0.3%

a) Instrument Response Calibration

A set of nine calibration solutions is analyzed twice, bracketing unknowns and QC materials in an analytical run. These calibration results are combined and used for the quantification of analytes in all samples and QC materials from that batch. Calibration curves are constructed for each analyte from the peak response ratio of standards to internal standards as the nine different concentration levels. The slope (response factor) and intercept are determined by linear least squares of 1/x weighted data. Calibration curve statistics are evaluated for each analyte to ensure that the coefficient of determination (R^2) value of the curve is equal to or greater than 0.98. The highest calibrator on the calibration curve is above the expected range of results for non-occupationally exposed subjects and the lowest calibrator is near or below the measurable detection limit. A calibrator can be omitted from a calibration curve as long

as there are at least five standard levels that span the range of all detectable unknown samples.

b) Calibration Verification

Calibration is verified by analyzing a full set of calibrators with every batch of unknown samples as outlined in Section 7a. Absolute accuracy is verified by using proficiency testing samples at least twice per year.

8. PROCEDURE OPERATION INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

An analytical run consists of solvent blanks, blanks with internal standard, calibration standards, low level QC, high level QC and unknown urine samples.

a) Sample preparation

1) Thaw urine samples, standards, internal standards, and QCs at room temperature using a thawing rack.
2) Homogenize urine samples using rugged rotator or equivalent for 15 minutes.
3) Prepare samples on 96 well plate using Hamilton robotic liquid handling system or equivalent. Urine samples and QCs are diluted 1:10 in sample preparation buffer with internal standard (25 µL internal standard, 50 µL sample, 425 µL sample preparation buffer). The samples are mixed by the liquid handling system.
4) Transfer the samples into LC autosampler and perform UPLC-MS/MS analysis.

b) UPLC-MS/MS Analysis

1) Ultra Performance Liquid chromatography (UPLC)

Chromatographic separation of the analytes is achieved with a UPLC system (e.g., Waters Acquity or Shimadzu LC-40) fitted with a reversed phase pentafluoro-phenyl column (e.g., Waters Acquity UPLC® HSS PFP). The separation conditions are optimized to obtain good resolution among VOC metabolites. Before each run, the column is equilibrated with the initial mobile phase composition until the pressure has stabilized. After each sample injection, the needle is cleaned with a strong wash and a weak wash. At the end of each run, the column is washed preferably with an aqueous

solution of water and methanol, and is stored in methanol (shutdown method). The suggested UPLC parameters are shown in **Table 7**.

Table 7. Suggested UPLC parameters

Parameter	Details
Column	Waters Acquity UPLC® HSS PFP (2.1 x 100 mm, 1.8 µm)
Mobile Phase A	0.02% formic acid
Mobile Phase B	Methanol
Weak Wash	0.02 % formic acid
Strong Wash	25/25/25/25% water/acetonitrile/methanol/isopropanol
Seal Wash	90/10% water/methanol or 90/10% water/isopropanol
Gradient (flow rate of 500 µL/min):	
Time, % Solvent B	initial, 3 1.7 min, 25 3.15 min, 35 3.20 min, 43 4.20 min, 68 4.35 min, 3
Column Temperature	30 °C
Sample Manager Temperature	25 °C
Injection volume	2 µL

2) Mass spectrometry (MS)

A triple quadrupole mass spectrometer (e.g., Sciex 5500) with an electrospray ion source is used for the detection of urinary VOC metabolites. The mass spectrometer is operated using Scheduled Multiple Reaction Monitoring (SMRM). The instrument parameters are optimized to obtain the maximum signal intensity, dynamic range, and signal to noise ratio (S/N). Compounds (native analytes and internal standards) are optimized individually to select transitions and associated mass spectrometric parameters (e.g., declustering potential, collision energy, etc.) for maximum selectivity and signal intensity. These parameters can be re-optimized when transferring the method to a new instrument. Ideally, the *m/z* value for the precursor ion should match between the quantitation and the confirmation ions whenever possible. Similarly, the internal standard transition should correspond to the quantitation ion transition to reduce quantitation bias. **Table 8** lists suggested transitions for the VOC metabolites measured by this method.

Table 8. Suggested MRM transitions for VOC metabolites

Analyte	Transition (*m/z*)		Internal Standard	Transition (*m/z*)
	Quan. ions[a]	Conf. ions[b]		
5HMP	116.1/85.2	116.1/57.0	5HMP-d_3	119.1/85.2
HMFA	141.1/97.1	141.1/69.0	HMFA-$^{13}C_6$	147.1/102.1
HMFG	198.0/97.0	198.0/124.0	HMFG-^{13}C,^{15}N	201.0/97.0
N2FG	168.0/66.9	168.0/124.1	N2FG-d_3	171.0/70.0
MUCA	141.1/53.2	141.1/97.1	MUCA-$^{13}C_6$	147.0/102.0
PMA	238.1/109.1	None	PMA-$^{13}C_6$	244.2/115.1

[a]Quantitation ions. [b]Confirmation ions.

It is recommended that mass spectrometers are tuned monthly and after any major repair. The curtain plate is cleaned as needed to remove any deposition from previous runs. The performance of the instrument is checked before every scheduled run by measuring the intensity of a blank (internal standard) and the signal to noise ratio of a low standard.

Suggested MS parameters are shown in **Tables 9a and 9b**.

Table 9a. Suggested MS source parameters

Parameter	Settings
Scan type	Scheduled MRM
Ionization	ESI
Polarity	Positive / Negative
IonSpray Voltage (IS)	3500 V / -4500 V
Entrance Potential (EP)	10 V / -10 V
Curtain gas (CUR)	50
Collision gas (CAD)	7
Heater Temperature	500 °C
Nebulizing gas (GS1)	55
Heater gas (GS2)	65

Table 9b. Suggested MS parameters (compound specific)

Analytes	DP (volts)	CE (volts)	CXP (volts)
5HMP	50	16.8	10
5HMPC	50	30	15
5HMP-d_3	55	18	12
HMFA	-45	-6	-9
HMFAC	-45	-16	-9
HMFA-$^{13}C_6$	-45	-12	-9
HMFG	-45	-18	-8
HMFGC	-45	-17	-8
HMFG-$^{13}C_2$,^{15}N	-45	-18	-8
N2FG	-60	-6	-10
N2FGC	-60	-9	-10
N2FG-d_3	-60	-14	-10
MUCA	-40	-5	-3
MUCAC	-40	-10	-9
MUCA-$^{13}C_6$	-40	-5	-9
PMA	-50	-22	-9
PMA-$^{13}C_6$	-50	-22	-9

DP=declustering potential; CE=collision energy; CXP=cell exit potential

c) Robotic Liquid Handling System

All calibration standards, QCs, and urine samples are aliquoted, prepared, and mixed by a robotic liquid handling system such as Hamilton Microlab Star. **Table 10** exemplifies a sample preparation protocol.

Table 10. Example of the sample preparation protocol

Sample	Vol. of sample (µL)	Vol. of IS (µL)	Vol. of ammonium formate buffer (µL)
Double blank	0	0	500
Blank	0	25	475
Calibration standard	50	25	425
Quality control	50	25	425
Urine	50	25	425
Proficiency testing	50	25	425

d) Data Processing

1) Peak Integration

Chromatograms are processed individually after the corresponding samples are run. Each target peak is confirmed by retention time and MRM transition. Peaks are integrated using multianalyte data processing software such as MultiQuant. Each peak is visually inspected and peak integration is corrected if the integrator erroneously integrates a peak. The integration approach for all samples in an

analytical run is consistent for each analyte and the use of manual integration is minimized.

2) Excluding calibrators
Calibrator data are excluded only if it can be determined that the calibrator data is biased independently of the samples in the analytical batch. Scenarios that might only affect a single calibrator are rare, however may be due to improper amount of internal standard addition, detector saturation, and/or contaminated autosampler vials. Higher calibrators (calibrations solutions 8 and/or 9) can be excluded if the calibration curve is nonlinear over this range and all QCs and unknowns fall between calibrators 1 and 7.

3) Excluding sample data
Sample data are excluded if there is no or very low internal standard signal. Absolute response from internal standards is evaluated for consistency among the standards, QCs, and unknowns. An unusually high internal standard level can occur if the internal standard is added twice. A low or no response is observed if internal standard is not added.

e) Formal Quality Control Material Evaluation

Following data analysis and import of data into a database, QC results are formally evaluated by an independent QC officer. The QC samples are evaluated against the QC characterized means and standard deviation limits are approved by the QC officer. QC samples are evaluated using modified Westgard rules as specified by DLS SAS program and the PPM. Any failure of QC rules for an analyte disqualifies the corresponding data for that analyte for that specific run. Once the source of QC problem is identified, the samples are subsequently reanalyzed with new QC samples.

f) Additional Quality Assurance Data Evaluation

Other quality parameters such as quantitation/confirmation ion ratios, QC blank samples, and adequate internal standard response are evaluated for acceptable precision and accuracy.

9. REPORTABLE RANGE OF RESULTS

a) Reportable Limits

Sample results above the LOD and that pass sample and batch QC evaluation are marked as reportable, otherwise they are marked as not reportable. The upper reportable limit corresponds to the concentration of the highest linear standard. If the analyte level exceeds the upper calibration range, the sample is diluted and reanalyzed.

b) Limit of Detection

The analytical limits of detection are based on the method described in the DLS PPM.

c) Accuracy

The absolute accuracy is evaluated by blind analysis of independently prepared and certified PT materials (Section 10c). Absolute accuracy may also be verified using spiked urine samples. The percent accuracy must be within DLS PPM guidelines. Relative accuracy is evaluated upon comparison of characterized QC mean values with those obtained on each run. Error in relative accuracy should not exceed the precision of the characterized QC samples as defined in the DLS procedures.

d) Precision

Method precision is reflected in the variance of quality control samples analyzed over time (Section 10b).

e) Analytical Specificity

Specificity of this method is conferred through the hyphenation of two established analytical approaches. The analytical specificity in liquid chromatography is defined by the retention time in a chromatogram, while tandem mass spectrometry adds another dimension in analytical specificity by monitoring ion-transitions specific to an analyte. Further assurance of peak identity is provided by estimating appropriate ion ratios between quantitation and confirmation ion-transitions when applicable and monitoring the presence of co-eluting interferences in QC blanks.

f) Ruggedness Testing

Ruggedness testing was performed to evaluate the potential variables that affect analytical results. The variables examined were sample dilution factor, injection volume, column temperature, mixing time on a rugged rotator, and the pH of the urine sample. The parameters were changed to conditions that could realistically happen when running samples, hence the changes in the parameters should not have a substantial influence on the final analytical results. When compared to the final method results, the results of the lower and higher level parameters had a percent accuracy of ≤15 %. Ruggedness testing results for all eight analytes are shown in **Tables 11a-11f.**

Table 11a. Ruggedness data for 5HMP

Five tested parameters	Final method level	Result (ng/mL)	Lower level	Result at lower level (ng/mL)	Higher level	Result at higher level (ng/mL)
Sample dilution factor	10x	437	5x	416	25x	401
Injection volume	2 µL	437	1.8 µL	415	2.2 µL	418
Column temperature	30 °C	430	28 °C	416	32 °C	421
Rugged rotator time	15 min	50.9	5 min	53.1	25 min	52.1
Sample pH	pH 6.4	15.7	pH 4.8	16.2	pH 7.8	16.2

Table 11b. Ruggedness data for HMFA

Five tested parameters	Final method level	Result (ng/mL)	Lower level	Result at lower level (ng/mL)	Higher level	Result at higher level (ng/mL)
Sample dilution factor	10x	6470	5x	6426	25x	5938
Injection volume	2 µL	6470	1.8 µL	6439	2.2 µL	6160
Column temperature	30 °C	5858	28 °C	6335	32 °C	5963
Rugged rotator time	15 min	1943	5 min	1960	25 min	2020
Sample pH	pH 6.4	1189	pH 4.8	1285	pH 7.8	1226

Table 11c. Ruggedness data for HMFG

Five tested parameters	Final method level	Result (ng/mL)	Lower level	Result at lower level (ng/mL)	Higher level	Result at higher level (ng/mL)
Sample dilution factor	10x	5489	5x	5456	25x	5108
Injection volume	2 µL	5489	1.8 µL	5071	2.2 µL	5113
Column temperature	30 °C	5178	28 °C	4949	32 °C	5164
Rugged rotator time	15 min	734	5 min	771	25 min	760
Sample pH	pH 6.4	168	pH 4.8	189	pH 7.8	193

Table 11d. Ruggedness data for N2FG

Five tested parameters	Final method level	Result (ng/mL)	Lower level	Result at lower level (ng/mL)	Higher level	Result at higher level (ng/mL)
Sample dilution factor	10x	8817	5x	9242	25x	8740
Injection volume	2 µL	8817	1.8 µL	9153	2.2 µL	9126
Column temperature	30 °C	9414	28 °C	8962	32 °C	8910
Rugged rotator time	15 min	4728	5 min	4883	25 min	4654
Sample pH	pH 6.4	237	pH 4.8	217	pH 7.8	207

Table 11e. Ruggedness data for MUCA

Five tested parameters	Final method level	Result (ng/mL)	Lower level	Result at lower level (ng/mL)	Higher level	Result at higher level (ng/mL)
Sample dilution factor	10x	1178	5x	1186	25x	1151
Injection volume	2 µL	1178	1.8 µL	1211	2.2 µL	1189
Column temperature	30 °C	1197	28 °C	1198	32 °C	1211
Rugged rotator time	15 min	134	5 min	136	25 min	139
Sample pH	pH 6.4	162	pH 4.8	162	pH 7.8	170

Table 11f. Ruggedness data for PMA

Five tested parameters	Final method level	Result (ng/mL)	Lower level	Result at lower level (ng/mL)	Higher level	Result at higher level (ng/mL)
Sample dilution factor	10x	20.0	5x	21.3	25x	21.2
Injection volume	2 µL	20.0	1.8 µL	21.7	2.2 µL	21.0
Column temperature	30 °C	21.3	28 °C	21.1	32 °C	21.7
Rugged rotator time	15 min	1.96	5 min	2.00	25 min	1.76
Sample pH	pH 6.4	13.8	pH 4.8	13.7	pH 7.8	14.2

10. QUALITY ASSESSMENT AND PROFICIENCY TESTING

a) Quality Assessment

Quality assessment procedures follow standard practices. Daily experimental checks are made on the stability of the analytical system. Blanks and standards, as well as QC materials, are included in each run sequence. At least three quality assessment sample types are analyzed in each analytical run that include a QC blank and QCs at two different concentrations. In addition, solvent blanks are prepared to monitor carryover.

b) Quality Control Procedures

1) Establishing QC limits
   Precision is evaluated using the QC sample results. Up to two different pools of quality control material are used—typically, one at a low and the other at a high concentration. Expected precision ranges for the QC samples are established for a new QC batch using modified Westgard rules as specified by DLS SAS program. Different variables are included in the characterization analyses (e.g. different analysts, days, batches, and columns) to capture realistic assay variation over time. The mean, standard deviation, coefficient of variation, and confidence limits are calculated from this QC characterization data set. Individual quality control charts for the characterization runs are created, examined, and quality control limits are used to verify assay precision and accuracy for each run.

2) Quality control evaluation
   After the completion of an analytical run, the calculated results from the analyses of QC samples are compared to the established QC limits to determine if the run is "in control." The quality control rules apply to the average of the beginning and ending analyses of each of the QC pools. The quality control results are evaluated using modified Westgard rules as specified by DLS SAS program.
   If a QC result is declared "out of control," the results for all patient samples analyzed during that run are invalid.

c) Proficiency Testing (PT)

PT materials may be purchased from outside sources. The PT scheme for this method is administered by an in-house proficiency testing coordinator, who prepares and blind-codes the samples. The samples are analyzed and the results evaluated by the in-house PT coordinator.

1) Frequency of PT
   Five of four different levels of PTs are analyzed at least twice a year using the same method as for unknown samples.

2) Documentation of PT results
Analytical PT results are reviewed by the analyst and laboratory supervisor and submitted to the in-house PT Coordinator electronically. The PT results are evaluated by the PT Coordinator; the analysis passes proficiency testing if $\geq$ 80% of the results deviate $\leq$ 25% from the known value. A summary report of the PT evaluation is maintained by the laboratory supervisor. If the assay fails proficiency testing then the sample preparation and instrumentation are thoroughly examined to identify and correct the source of assay error. Unknown specimens cannot be analyzed, or analytical results reported, until the method successfully passes proficiency testing.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a) Internal Reference Area Counts

Internal standards are used to compensate for sample loss such as those caused by matrix effects. Since sample matrices vary for the standards, QCs, and among urine samples, the differences in internal standard signal intensity can vary by approximately three-fold. If the intensity drop for an internal standard is on the order of a factor of 5 relative to the median response among the other samples of similar matrices, the sample may not have been prepared properly or may need to be diluted. The cause of this decrease in response should be investigated, determined, and resolved.

b) Analyte in Blank Material Only

If analyte signal intensity is abnormally high in the blank, but not in other samples, there could be a possible contamination of the blank. The source of the contamination should be determined to prevent biasing of sample results.

c) Analyte in all Samples

There is likely a continual source of contamination when an unexpected amount of analyte is observed in all samples. Steps should be taken to identify and eliminate the source of contamination. Contamination specific to analytes could come from new lots of chemical reagents, sample collection tubes, and other sample processing materials.

d) QC Sample Outside the Confidence Limits

If results for a QC sample fails the QC criteria described in section 10b(2), data for the failed analyte are not reported for the run. The cause for the QC failure is to be investigated, isolated, and solved. No analytical results are reported for runs that are not in statistical control. Note that in all cases, the supervisor should be consulted for the appropriate corrective actions.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

This method uses isotope dilution UPLC-MS/MS, widely regarded as the definitive method for the measurement of toxicants in human body fluids. Alteration of this method may bias analytical results. Care should be taken to reduce the risk of contaminating standard, quality control, and blank samples. The quantification range and LODs are to be determined as described in section 9.

13. REFERENCE RANGES (NORMAL VALUES)

Reference ranges for these compounds are not available.

14. CRITICAL CALL RESULTS ("PANIC" VALUES)

There are no critical call values for the analytes included in this assay.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens must be stored at ≤ -60 °C until analysis; however, they may be kept at ambient temperature during analysis. If the measurement is delayed to the next day, samples must be frozen at ≤ -60 °C.

16. ALTERNATE METHODS FOR PERFORMING TEST AND STORING SPECIMENS IF TEST SYSTEM FAILS

Alternate methods have not been evaluated or validated.

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

Analytical results are reportable once the validity of the data is established by the DLS QC/QA policies and procedures, and are verified by a DLS statistician. One hardcopy and one electronic copy (ASCII format) of the data will be generated. This data, a cover letter, and a table of method specifications will be routed through the appropriate channels for approval (i.e., supervisor, branch chief, division director). A report is sent to the contact person who requested the analyses upon approval.

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

If greater than 250 µL of sample remains after analysis, this material should be returned to storage at ≤ -60 °C in case reanalysis is required. These samples shall be retained until valid results have been obtained and reported and sufficient time has passed for review of the results.

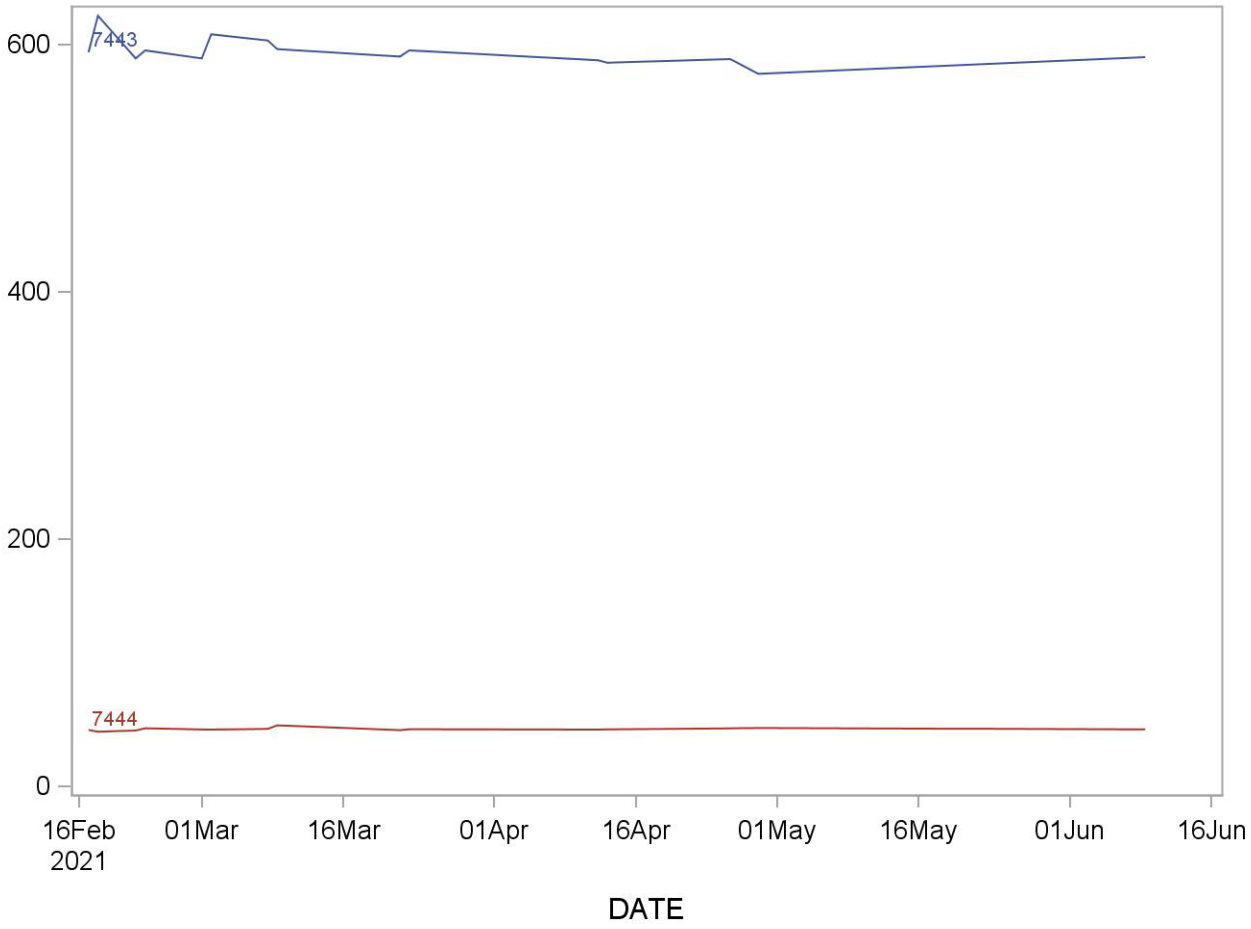
Standard record keeping (e.g., sample ID, database, notebooks, and data files) is used for tracking specimens. Records are maintained for 3 years, including related QA/QC data, and duplicate records will be kept off-site in electronic format. Study subject confidentiality is protected by providing personal identifiers only to the medical officer.

19. Summary Statistics and QC Graphs

Please see follow page.

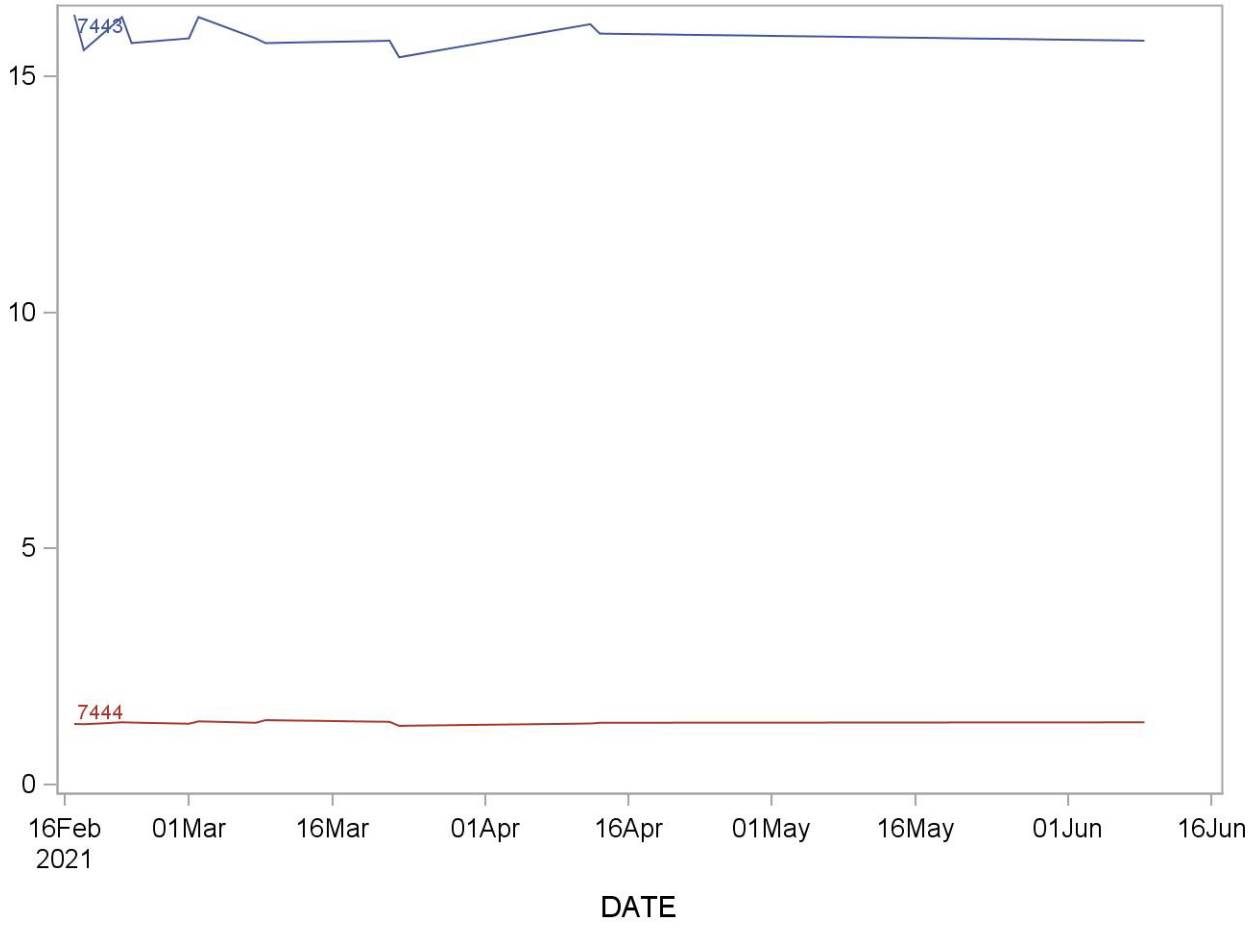
2019-2020 Summary Statistics and QC Chart
URXMUCA (Muconic acid)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
7443	15	17FEB21	09JUN21	594.2333	11.0516	1.9
7444	15	17FEB21	09JUN21	46.3467	1.1438	2.5



2019-2020 Summary Statistics and QC Chart
URXPHMA (Phenylmercapturic acid (PMA))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
7443	13	17FEB21	09JUN21	15.8654	0.2809	1.8
7444	13	17FEB21	09JUN21	1.3081	0.0301	2.3



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APPENDIX A: Method Performance Documentation

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

A1. Accuracy using spiked samples

Analyte: **5HNMP**

		Sample 1 (Mass Spec Gold Urine)					Sample 2 (Urine Pool)					Mean recovery (%)	SD (%)
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1 (H18109)	Day 2 (H18110)	Mean	Recovery (%)	Spike concentration	Day 1 (H18109)	Day 2 (H18110)	Mean	Recovery (%)		
Sample (L0)	1	0.00	3.42	3.33	3.36		0.00	16.0	14.7	14.8		101.7	3.7
	2		3.49	3.11				13.9	14.6				
	3		3.30	3.50				15.2	14.4				
Sample + Spike 1 (L1)	1	12.65	16.2	16.5	16.6	107.0	12.65	25.2	27.3	25.9	99.6		
	2		17.4	16.5				26.2	25.5				
	3		16.8	16.0				25.2	26.2				
Sample + Spike 2 (L2)	1	40.00	43.8	43.9	43.4	100.8	40.00	56.7	56.3	55.6	105.6		
	2		43.6	42.3				55.8	56.1				
	3		43.5	43.0				54.1	54.4				
Sample + Spike 3 (L3)	1	400.00	410	382	403	100.0	400.00	392	415	403	97.4		
	2		412	398				410	401				
	3		409	408				396	405				

Analyte: **HMFA**

		Sample 1 (Mass Spec Gold Urine)					Sample 2 (Urine Pool)					Mean recovery (%)	SD (%)
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1 (H18155)	Day 2 (H18157)	Mean	Recovery (%)	Spike concentration	Day 1 (H18155)	Day 2 (H18157)	Mean	Recovery (%)		
Sample (L0)	1	0.00	0.00	0.00	0.0		0.00	1219	1413	1220		103.8	4.2
	2		0.00	0.00				1286	1191				
	3		0.00	0.00				1117	1094				
Sample + Spike 1 (L1)	1	158.11	169	178	163	103.3	1581.14	2459	2906	2786	106.7		
	2		169	164				2842	2790				
	3		153	147				2642	3075				
Sample + Spike 2 (L2)	1	500.00	532	520	512	102.5	5000.00	5986	5920	5960	97.2		
	2		550	419				5932	6013				
	3		494	560				6024	5884				
Sample + Spike 3 (L3)	1	5000.00	5786	4655	5479	109.6	10000.00	11490	12095	11330	103.5		
	2		4510	6121				10565	9926				
	3		5067	6734				11995	11910				

Analyte: **HMFG**

		Sample 1 (Mass Spec Gold Urine)					Sample 2 (Urine Pool)					Mean recovery (%)	SD (%)
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1 (H18278)	Day 2 (H18283)	Mean	Recovery (%)	Spike concentration	Day 1 (H18278)	Day 2 (H18283)	Mean	Recovery (%)		
Sample (L0)	1	0.00	1.91	0.00	1.1		0.00	151	188	164.9		101.8	4.4
	2		1.88	0.00				162	180				
	3		2.79	0.00				152	155				
Sample + Spike 1 (L1)	1	158.11	165	177	166.4	104.6	1581.14	1570	1863	1690.2	97.5		
	2		157	172				1479	1722				
	3		162	166				1712	1795				
Sample + Spike 2 (L2)	1	500.00	497	589	547.2	109.2	5000.00	4607	5389	5169.5	100.4		
	2		538	570				5156	5560				
	3		540	549				4944	5360				
Sample + Spike 3 (L3)	1	5000.00	4872	5145	4992	99.8	10000.00	8872	10920	10042	99.1		
	2		5057	5173				10590	10110				
	3		4371	5332				9756	10005				

Analyte: **N2FG**

		Sample 1 (Mass Spec Gold Urine)					Sample 2 (Urine Pool)					Mean recovery (%)	SD (%)
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1 (H18155)	Day 2 (H18157)	Mean	Recovery (%)	Spike concentration	Day 1 (H18155)	Day 2 (H18157)	Mean	Recovery (%)		
Sample (L0)	1	0.00	0.00	0.00	0.0		0.00	808	867	821.6		104.5	2.8
	2		0.00	0.00				777	780				
	3		0.00	0.00				782	916				
Sample + Spike 1 (L1)	1	158.11	151	163	160	101.0	1581.14	2629	2418	2449.0	108.1		
	2		163	148				2309	2627				
	3		162	171				2310	2402				
Sample + Spike 2 (L2)	1	500.00	522	543	528	105.6	5000.00	5403	6239	5956.1	104.3		
	2		527	529				5659	6626				
	3		522	524				5404	6405				
Sample + Spike 3 (L3)	1	5000.00	4548	5323	5325	106.5	10000.00	10706	10598	10788	101.3		
	2		5363	5863				10311	11036				
	3		5340	5510				10737	11340				

Analyte: **MUCA**

		Sample 1 (Mass Spec Gold Urine)					Sample 2 (Urine Pool)					Mean recovery (%)	SD (%)
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1 (H18109)	Day 2 (H18110)	Mean	Recovery (%)	Spike concentration	Day 1 (H18109)	Day 2 (H18110)	Mean	Recovery (%)		
Sample (L0)	1	0.00	1.11	0.93	0.9		0.00	13.2	16.2	14.7		94.2	3.7
	2		0.65	0.89				15.5	13.7				
	3		0.51	1.34				14.2	15.7				
Sample + Spike 1 (L1)	1	37.95	37.8	36.9	37.5	96.8	37.95	45.9	46.2	46.3	87.0		
	2		40.5	35.7				45.1	49.0				
	3		36.8	37.6				43.8	47.7				
Sample + Spike 2 (L2)	1	120.00	114	117	113.7	94.1	120.00	138	135	128.8	96.3		
	2		115	117				123	134				
	3		112	108				122	121				
Sample + Spike 3 (L3)	1	1200.00	1142	1174	1163	96.8	1200.00	1146	1130	1146	94.4		
	2		1178	1160				1165	1177				
	3		1150	1171				1130	1129				

Analyte: **PMA**

	Replicate	Sample 1 (Mass Spec Gold Urine) Spike concentration	Measured concentration Day 1 (H18109)	Day 2 (H18110)	Mean	Recovery (%)	Sample 2 (Urine Pool) Spike concentration	Measured concentration Day 1 (H18109)	Day 2 (H18110)	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample (L0)	1	0.00	0.00	0.00	0.0		0.00	0.00	0.00	0.0		93.9	4.8
	2		0.00	0.04				0.00	0.00				
	3		0.00	0.00				0.00	0.00				
Sample + Spike 1 (L1)	1	0.63	0.49	0.62	0.6	90.1	0.63	0.53	0.50	0.5	86.1		
	2		0.60	0.62				0.48	0.66				
	3		0.53	0.60				0.52	0.57				
Sample + Spike 2 (L2)	1	2.00	1.89	1.91	1.9	96.7	2.00	2.13	1.99	2.0	97.8		
	2		1.93	2.06				1.87	2.14				
	3		1.84	2.01				1.82	1.79				
Sample + Spike 3 (L3)	1	20.00	19.5	19.0	20	97.9	20.00	18.7	19.0	19	94.9		
	2		20.0	19.5				19.2	18.8				
	3		19.9	19.6				19.1	19.0				

A2. Precision

Matrix: Urine, Units: ng/mL. Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Analyte: **5HNMP**

Quality material 1 (L1)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	26.9	26.7	26.8	0.01	0.01	1434
H18109	27.4	27.8	27.6	0.04	0.04	1519
H18110	26.6	27.2	26.9	0.09	0.09	1446
H18113	27.3	28.7	28.0	0.47	0.47	1567
H18114	28.2	28.1	28.1	0.00	0.00	1584
H18115a	27.7	28.1	27.9	0.03	0.03	1554
H18122	29.9	27.6	28.8	1.27	1.27	1654
H18124	28.3	27.7	28.0	0.10	0.10	1568
H18127	28.2	29.2	28.7	0.25	0.25	1649
H18135	28.7	29.6	29.2	0.19	0.19	1701
Grand sum	560	Grand mean	28.0			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	4.91	0.491	0.7	2.50
Between Run	10.8	1.20	0.6	2.13
Total	15.7		0.9	**3.29**

Quality material 2 (L3)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	423	419	421	3.44	3.44	354979
H18109	401	429	415	207	207	344403
H18110	424	426	425	0.902	0.902	361675
H18113	441	432	436	20.7	20.7	380803
H18114	418	412	415	8.70	8.70	344201
H18115a	430	426	428	3.80	3.80	366625
H18122	434	464	449	216	216	403202
H18124	435	433	434	1.32	1.32	376278
H18127	422	422	422	0.00	0.00	355354
H18135	430	453	442	135	135	390021
Grand sum	8573	Grand mean	429			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1193	119	10.9	2.55
Between Run	2323	258	8.33	1.94
Total	3516		13.7	**3.20**

Analyte: **HMFA**

Quality material 1 (L1)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18122	1777	1585	1681	9216	9216	5651522
H18124	1572	1787	1680	11556	11556	5641441
H18127	1679	1566	1623	3232	3232	5265608
H18135	1724	1642	1683	1681	1681	5664978
H18136	1581	1666	1624	1806	1806	5271505
H18142	1551	2019	1785	54756	54756	6372450
H18150	1585	1409	1497	7744	7744	4482018
H18155	1228	1706	1467	57237	57237	4304183
H18157	1758	1512	1635	15086	15086	5346509
H18170	1761	1446	1604	24821	24821	5142933

Grand sum 32554 **Grand mean** 1628

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	374272	37427	193	11.89
Between Run	153822	17091	0.00	0.00
Total	528094		193	**11.89**

Quality material 2 (L3)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18122	6573	7855	7214	410881	410881	104083592
H18124	6320	6050	6185	18225	18225	76508450
H18127	6315	6647	6481	27514	27514	84016388
H18135	6694	6951	6823	16512	16512	93093013
H18136	6092	6668	6380	82944	82944	81408800
H18142	7040	7171	7106	4290	4290	100976261
H18150	5760	7403	6582	674862	674862	86632285
H18155	6354	6778	6566	45066	45066	86225483
H18157	7082	6037	6560	273059	273059	86059595
H18170	6302	6936	6619	100515	100515	87623100

Grand sum 133029.2835 **Grand mean** 6651.464175

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	3307738	330774	575	8.65
Between Run	1787452	198606	0.00	0.00
Total	5095190		575	**8.65**

Analyte: **HMFG**

Quality material 1 (L1)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	387	383	385	3.10	3.10	296206
H18109	420	400	410	96.0	96.0	335708
H18110	413	421	417	15.2	15.2	347945
H18113	446	353	399	2167	2167	319121
H18114	422	347	385	1403	1403	296219
H18115a	497	376	437	3666	3666	381676
H18122	438	337	388	2550	2550	300313
H18124	512	435	474	1475	1475	448973
H18127	463	437	450	165	165	405390
H18135	436	402	419	292	292	351122

Grand sum 8327 **Grand mean** 416.3

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	23665	2366	48.6	11.68
Between Run	16045	1783	0.0	0.00
Total	39710		48.6	**11.68**

Quality material 2 (L3)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	5079	5528	5303	50482	50482	56254191
H18109	6332	5694	6013	101761	101761	72312338
H18110	5891	6296	6094	41006	41006	74261485
H18113	5294	5891	5593	89102	89102	62552113
H18114	5114	5353	5234	14280	14280	54779045
H18115a	6204	5325	5765	193160	193160	66458921
H18122	6250	5458	5854	156816	156816	68538632
H18124	5772	4922	5347	180625	180625	57180818
H18127	5165	5803	5484	101720	101720	60140393
H18135	5015	6387	5701	470596	470596	65002802

Grand sum 112772 **Grand mean** 5639

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2799097	279910	529	9.38
Between Run	1601643	177960	0.00	0.00
Total	4400740		529	**9.38**

Analyte: **N2FG**

Quality material 1 (L1)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18122	4669	4522	4596	5402	5402	42237241
H18124	4453	4361	4407	2116	2116	38843298
H18127	4724	4707	4715	64.7	64.7	44471218
H18135	4444	4734	4589	21025	21025	42117842
H18136	4640	4790	4715	5625	5625	44462450
H18142	4708	4221	4465	59292	59292	39863521
H18150	4745	4880	4813	4556	4556	46320313
H18155	4355	4389	4372	275	275	38228624
H18157	4957	4782	4870	7609	7609	47427660
H18170	3862	4588	4225	131575	131575	35700075
Grand sum	91531	**Grand mean**	4577			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	475082	47508	218	4.76
Between Run	774726	86081	139	3.03
Total	1249808		258	**5.65**

Quality material 2 (L3)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18122	9447	10930	10189	549822	549822	207611065
H18124	9466	9755	9611	20880	20880	184723421
H18127	9316	9261	9289	751	751	172557410
H18135	9342	9608	9475	17689	17689	179551250
H18136	9591	10010	9801	43890	43890	192099601
H18142	8694	9665	9180	235710	235710	168526441
H18150	9001	10480	9741	546860	546860	189754681
H18155	9305	8358	8831	223840	223840	155988672
H18157	9241	9293	9267	693	693	171751138
H18170	8909	8263	8586	104351	104351	147424392
Grand sum	187934	**Grand mean**	9397			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	3488974	348897	591	6.29
Between Run	4026350	447372	222	2.36
Total	7515324		631	**6.71**

Analyte: **MUCA**

Quality material 1 (L1)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	51.8	48.3	50.0	3.06	3.06	5009
H18109	50.0	50.0	50.0	0.00	0.00	4994
H18110	50.0	50.1	50.0	0.00	0.00	5005
H18113	46.6	52.4	49.5	8.56	8.56	4898
H18114	55.3	52.8	54.0	1.60	1.60	5840
H18115a	52.2	53.7	53.0	0.58	0.58	5607
H18122	57.6	54.6	56.1	2.24	2.24	6296
H18124	51.1	47.7	49.4	2.86	2.86	4885
H18127	50.7	52.4	51.6	0.79	0.79	5317
H18135	51.7	48.3	50.0	2.82	2.82	4996

Grand sum 1027 **Grand mean** 51.4

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	45.0	4.5	2.12	4.13
Between Run	93.7	10.4	1.72	3.35
Total	138.7		2.73	**5.32**

Quality material 2 (L3)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	1239	1222	1230	71	71	3027542
H18109	1231	1231	1231	0	0	3030722
H18110	1224	1226	1225	1	1	3001250
H18113	1214	1207	1211	12	12	2930621
H18114	1218	1228	1223	25	25	2991458
H18115a	1201	1219	1210	81	81	2928200
H18122	1249	1370	1310	3660	3660	3429581
H18124	1185	1210	1198	156	156	2868013
H18127	1262	1249	1255	42	42	3150904
H18135	1244	1224	1234	100	100	3045512

Grand sum 24652 **Grand mean** 1233

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	8298	830	28.8	2.34
Between Run	17627	1959	23.8	1.93
Total	25925		37.3	**3.03**

Analyte: **PMA**

Quality material 1 (L1)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	0.509	0.583	0.546	0	0	0.597
H18109	0.447	0.518	0.482	0	0	0.465
H18110	0.539	0.420	0.479	0	0	0.460
H18113	0.595	0.624	0.609	0	0	0.743
H18114	0.638	0.514	0.576	0	0	0.663
H18115a	0.592	0.536	0.564	0	0	0.636
H18122	0.566	0.694	0.630	0	0	0.794
H18124	0.584	0.576	0.580	0	0	0.672
H18127	0.649	0.678	0.664	0	0	0.881
H18135	0.621	0.608	0.614	0	0	0.755
Grand sum	11.5	**Grand mean**	0.575			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.031	0.003	0.055	9.65
Between Run	0.065	0.007	0.045	7.89
Total	0.095		0.072	**12.46**

Quality material 2 (L3)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
H18106	19.9	19.7	19.8	0	0	786
H18109	19.8	19.9	19.8	0	0	786
H18110	20.5	20.1	20.3	0	0	824
H18113	20.3	19.8	20.1	0	0	804
H18114	19.9	19.5	19.7	0	0	778
H18115a	18.9	20.2	19.5	0	0	764
H18122	20.9	22.7	21.8	1	1	946
H18124	21.5	21.4	21.5	0	0	922
H18127	21.3	22.0	21.6	0	0	935
H18135	20.7	21.4	21.0	0	0	883
Grand sum	410	**Grand mean**	20.5			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	3.18	0.318	0.564	2.75
Between Run	13.4	1.48	0.764	3.72
Total	16.5		0.949	**4.63**

A3. Stability

Stability

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, 24 hrs between each cycle)
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 1 day
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 room temperature for 1 day with cap mat
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: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration
All stability samples were run on the same day

Method name:	VOCM II
Method #:	2105
Matrix:	Urine
Units:	ng/mL
Run:	H18114
Analyte:	5HNMP

Quality material 1 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	25.8	26.1	25.8	26.0	25.8	26.3	25.8	TBD
Replicate 2	26.1	25.0	26.1	25.3	26.1	25.2	26.1	TBD
Replicate 3	25.5	26.7	25.5	25.4	25.5	27.0	25.5	TBD
Mean	25.8	25.9	25.8	25.5	25.8	26.2	25.8	#DIV/0!
% accuracy from control measurement	--	0.5	--	-1.0	--	1.5	--	#DIV/0!

Quality material 2 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	404	411	403.8	404	403.8	397	403.8	TBD
Replicate 2	410	396	410.1	430	410.1	401	410.1	TBD
Replicate 3	401	403	400.7	432	400.7	399	400.7	TBD
Mean	405	404	405	422	405	399	405	#DIV/0!
% accuracy from control measurement	--	-0.3	--	4.2	--	-1.5	--	#DIV/0!

Analyte: **HMFA**

Quality material 1 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	1474	1786	1474	1828	1474	1793	1474	TBD
Replicate 2	1753	1727	1753	1702	1753	1718	1753	TBD
Replicate 3	1653	1922	1653	1892	1653	1808	1653	TBD
Mean	1627	1812	1627	1807	1627	1773	1627	#DIV/0!
% accuracy from control measurement	--	**11.4**	--	**11.1**	--	**9.0**	--	**#DIV/0!**

Quality material 2 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	7211	6961	7211	7073	7211	7030	7211	TBD
Replicate 2	6854	6850	6854	7910	6854	7396	6854	TBD
Replicate 3	7541	7069	7541	7866	7541	6693	7541	TBD
Mean	7202	6960	7202	7616	7202	7040	7202	#DIV/0!
% accuracy from control measurement	--	**-3.4**	--	**5.8**	--	**-2.3**	--	**#DIV/0!**

Analyte: **HMFG**

Quality material 1 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	356	373	356	360	356	400	356	TBD
Replicate 2	395	378	395	354	395	389	395	TBD
Replicate 3	387	366	387	402	387	461	387	TBD
Mean	379	372	379	372	379	417	379	#DIV/0!
% accuracy from control measurement	--	**-1.8**	--	**-1.9**	--	**9.9**	--	**#DIV/0!**

Quality material 2 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	5147	5851	5147	5336	5147	5481	5147	TBD
Replicate 2	4975	4843	4975	6235	4975	4612	4975	TBD
Replicate 3	5239	4615	5239	5542	5239	5240	5239	TBD
Mean	5120	5103	5120	5704	5120	5111	5120	#DIV/0!
% accuracy from control measurement	--	**-0.3**	--	**11.4**	--	**-0.2**	--	**#DIV/0!**

Analyte: **N2FG**

Quality material 1 (spiked urine sample)	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	4502	4763	4502	4487	4502	4622	4502	TBD
Replicate 2	4570	4603	4570	4459	4570	4378	4570	TBD
Replicate 3	4610	4630	4610	4464	4610	4602	4610	TBD
Mean	4561	4665	4561	4470	4561	4534	4561	#DIV/0!
% accuracy from control measurement	--	2.3	--	-2.0	--	-0.6	--	#DIV/0!

Quality material 2 (spiked urine sample)	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	9828	9796	9828	9534	9828	10020	9828	TBD
Replicate 2	9548	9701	9548	9524	9548	9524	9548	TBD
Replicate 3	9190	9206	9190	10190	9190	9556	9190	TBD
Mean	9522	9568	9522	9749	9522	9700	9522	#DIV/0!
% accuracy from control measurement	--	0.5	--	2.4	--	1.9	--	#DIV/0!

Analyte: **MUCA**

Quality material 1 (spiked urine sample)	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	48.5	49.7	48.5	48.5	48.5	45.7	48.5	TBD
Replicate 2	47.8	53.5	47.8	45.1	47.8	47.3	47.8	TBD
Replicate 3	45.1	47.1	45.1	47.1	45.1	49.4	45.1	TBD
Mean	47.1	50.1	47.1	46.9	47.1	47.5	47.1	#DIV/0!
% accuracy from control measurement	--	6.3	--	-0.5	--	0.7	--	#DIV/0!

Quality material 2 (spiked urine sample)	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	1158	1118	1158	1127	1158	1170	1158	TBD
Replicate 2	1143	1135	1143	1203	1143	1146	1143	TBD
Replicate 3	1154	1137	1154	1255	1154	1119	1154	TBD
Mean	1152	1130	1152	1195	1152	1145	1152	#DIV/0!
% accuracy from control measurement	--	-1.9	--	3.8	--	-0.6	--	#DIV/0!

Analyte: **PMA**

Quality material 1 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	0.474	0.577	0.474	0.489	0.474	0.533	0.474	TBD
Replicate 2	0.563	0.531	0.563	0.564	0.563	0.481	0.563	TBD
Replicate 3	0.470	0.513	0.470	0.577	0.470	0.431	0.470	TBD
Mean	0.502	0.540	0.502	0.544	0.502	0.482	0.502	#DIV/0!
% accuracy from control measurement	--	7.6	--	8.2	--	-4.0	--	#DIV/0!

Quality material 2 (spiked urine sample)								
	Control measurement	Three freeze-thaw cycles	Control measurement	Bench-top stability	Control measurement	Processed sample stability	Control measurement	Long-term stability
Replicate 1	18.8	19.0	18.8	18.6	18.8	19.1	18.8	TBD
Replicate 2	18.7	19.1	18.7	19.8	18.7	18.7	18.7	TBD
Replicate 3	19.5	19.2	19.5	19.9	19.5	18.7	19.5	TBD
Mean	19.0	19.1	19.0	19.4	19.0	18.8	19.0	#DIV/0!
% accuracy from control measurement	--	0.6	--	2.4	--	-0.7	--	#DIV/0!

A4. LOD, specificity

Analytes	**Limit of Detection (LOD)** (Std 2 - To be revised at a later time)	**3So Taylor LOD** (not used because too low)	**Interferences successfully checked in at least 50 human samples** (H18071)	**Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use**	**Quant/Qual agreement threshold** (ROC analysis of TNBB samples from runs H18071, H18261, Q18310)
5HMP	0.300	0.274	yes	yes	3.8
HMFA	36.1	13.1	yes	yes	80
HMFG	16.0	6.71	yes	yes	210
N2FG	64.4	7.20	yes	yes	1200
MUCA	9.81	1.20	yes	yes	36
PMA	0.150	0.0975	yes	yes	N/A