Laboratory Procedure Manual

Analyte:	**Cotinine and Hydroxycotinine (Total)**
Matrix:	**Urine**
Method:	**UHPLC/MS/MS**
Method No:	**2024.02**
Branch:	**Tobacco and Volatiles Branch**
Revised:	**August 08, 2020**
As performed by:	Tobacco and Volatiles Branch Division of Laboratory Sciences National Center for Environmental Health
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Important Information for Users

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

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
UCOT_J	URXCOTT	Cotinine, urine (ng/mL)
	URXHCTT	Hydroxycotinine, urine (ng/mL)

1. Clinical Relevance and Summary of Test Principle.

Analyte (-)-Cotinine. 1-methyl-5-(3-pyridyl)-2-pyrrolidinone; N–methyl-2-(3-pyridyl)-5- pyrrolidone. $C_{10}H_{12}N_2O$; Mol Wt 176.2; m.p. 40-42^oC.

Analyte (-)-*trans*-3'-Hydroxycotinine. 1-methyl-3-hydroxy-5-(3-pyridyl)-2-pyrrolidinone. $C_{10}H_{12}N_2O_2$; Mol Wt 192.2; m.p. 103-106^oC.

a. Clinical Relevance

Cotinine (COT) and *trans*-3'-hydroxycotinine (HC) are the primary metabolites of nicotine. The concentrations of COT and HC in body fluids can be used as markers for active smoking and as indices for secondhand smoke (SHS) exposure. Because their concentrations are greater and their elimination half-lives significantly longer, these metabolites are generally preferred over nicotine itself as biomarkers, and COT, the primary proximal metabolite of nicotine, is generally regarded as the marker of choice (**1-2**). The estimated elimination half-life of COT is about 15-20 hr (**3-5**); by contrast, the half-life of nicotine is only 0.5-3 hr (**5-7**). The half-life of HC is approximately 5-6 hr (**8-9**), but when HC is generated from COT, its elimination half-life becomes similar to that of COT.

In urine HC is two to four times more concentrated than COT (**10-13**). This method is primarily aimed at measuring COT and HC in the urine of nonsmokers for purposes of estimating SHS exposures.

The ratio of HC to COT is called the nicotine metabolite ratio (NMR). It is highly correlated with the rate of nicotine metabolism in smokers (**14-15**). It is believed that the severity of nicotine dependence is related to an individual's rate of nicotine metabolism – the higher the NMR, the faster the metabolism of nicotine and hence the more dependent on nicotine the individual is (**16**). The conversion of nicotine to COT, as well as the conversion of COT to HC is largely mediated by the liver enzyme cytochrome P450 2A6 (CYP2A6**) (17-18**). Thus, the NMR provides a convenient measure to phenotype individuals for CYP2A6 activity. CYP2A6 is also responsible for metabolic activation of carcinogenic tobacco-specific nitrosamines (**19-23**). Therefore, the NMR may be used to estimate tobacco-related disease risk, and it can be helpful in the development of individual pharmacotherapies for nicotine dependence. Total nicotine equivalents, TNE, refers to the molar sum of nicotine and its metabolites measured in urine. The number of analytes included in the TNE sum depends on the analysis method. The two most abundant analytes are COT and HC, and their sum is called TNE2.

b. Test Principle

COT and HC are measured by an isotope-dilution high-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (ID HPLC-APCI MS/MS) method. Briefly, the urine sample is spiked with methyl-D_3-COT and methyl-D_3-HC as internal standards. The sample is first hydrolyzed with β-glucuronidase to free the bound analytes, and then it is basified and applied to a supported liquid extraction (SLE) plate. The analytes are extracted with 5% (v/v) isopropanol in methylene chloride. The organic extract is concentrated, and the residue is injected onto a C18 UHPLC column. The eluant from these injections is monitored by APCI-MS/MS. The m/z 98 product ion from the m/z 177 quasi-molecular ion is quantitated for COT and the m/z 80 product ion from the m/z 193 quasi-molecular ion is quantitated for HC. Additional ions for the internal standards and for confirmation transitions are also monitored for the respective compounds. Concentrations are derived from the ratios of native-to-labeled compounds in the sample by comparisons to standard curves.

Note: The “free" form of COT and HC can be measured by the same ID HPLC-APCI MS/MS. The only difference is that the samples are not hydrolyzed prior to analysis, all other steps in the sample preparation are the same.

Special Precaution: Because of the nature of these assays, all analysts involved in this study must be nonsmokers.

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

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

b. Radioactive Hazards

None.

c. Microbiological Hazards

This assay involves human samples. Universal precautions must be followed. Analysts working directly with the specimens must use proper technique and avoid any direct contact with the sample. Wear a lab coat, gloves, and

protective eyewear (as required) while handling the specimens and perform all sample aliquoting in a biological safety cabinet.

d. Mechanical Hazards

The robotic arm that is part of the automated sample preparation system used in this assay is a very powerful instrument and can potentially be a hazard if interfered with during operation. The plastic doors that enclose the robot arm's access area need to be utilized during operation to prevent contact with the arm. Follow all standard safety practice procedures.

e. Protective Equipment

Standard chemical laboratory personal safety equipment is required including lab coats, safety glasses, and appropriate gloves.

f. Training

Training for sample preparation, sample handling, and instrument operation is required.

g. Personal Hygiene

Follow standard precautions and comply with all established laboratory safety practices. Care needs to be taken when handling chemicals to avoid inhalation or dermal exposure. Lab coat, gloves and safety glasses need to be worn when handling standards or samples.

h. Disposal of Wastes

Dispose of all waste materials in compliance with laboratory, federal, state, and local regulations. Always place solvents and reagents in an appropriate container that has been clearly marked for waste products. Place disposable laboratory supplies such as vials, pipette tips, syringes, etc. that directly contact samples in a biohazard autoclave bag or similar approved storage container.

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

Proficiency is required in the analytical software package of the HPLC and mass spectrometer used in the analysis. For the AB Sciex API 6500 mass

spectrometer this package is Analyst. Statistical analysis of results requires proficiency in a standard statistical analysis software package. The Statistical Analysis System (SAS Institute, Cary, NC) is one such package. Sample cleanup is accomplished using an automated sample preparation system which requires knowledge of the operating software (currently i-Link and Maestro). Proficiency is required in the software that automatically integrates the sample chromatograms (currently ASCENT from Indigo BioSystems, Indianapolis, IN).

b. Sample Information

During sample clean-up, individual sample ID's are entered into a spreadsheet electronically using a handheld or automated barcode reader. If necessary the ID's can be entered manually. Other information is recorded on a hard-copy runsheet which includes the run ID, SLE plate lot number, dilution factor, and any other information not associated with the LC/MS/MS analysis. This information is stored as the runsheet for those samples. Any unusual observations made by the analyst during sample clean-up can be recorded on the runsheet.

The sample ID's from the spreadsheet are transferred to the LC/MS/MS instrument in a sequence file. This information is transmitted, along with the LC/MS/MS response data for each sample and the associated calibrators, QCs, and blanks, to the data cloud where the automated data analysis software is hosted.

c. Data Maintenance

The data files containing the raw and processed data are automatically backed up each week through the TEBL shared network drive.

d. Information Security

The information management systems including the instrument workstation and database server containing the raw data and 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 campus, buildings, and individual laboratories.

4. Procedures for Collecting, Storing, and Handling Samples; Criteria for Sample Rejection

a. Special Instructions

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

b. Sample Collection

Urine can be collected by using standard equipment; mix the sample well before aliquoting, and then freeze the urine aliquot in polypropylene cryogenic, screw-cap vials.

The laboratory needs to be contacted before samples are collected to confirm the suitability of any equipment used to collect, process or store samples intended for these analyses. Some materials can provide significant contamination sources; only equipment that has been prescreened and found to be acceptable by this laboratory can be used for collecting samples.

c. Sample Handling

Specimen handling and transport needs to be conducted according to standard protocols. Ensure that samples remain in the frozen state during shipment and subsequent storage. Store samples in low-temperature freezers at or below approximately -60^{o}C.

d. Sample Quantity

A minimum of 1.0 mL of urine is needed for this assay to provide sufficient volume for a repeat analysis if indicated.

e. Unacceptable Specimens

Currently, there is no evidence that atypical specimen characteristics influence the HPLC/MS/MS analysis of COT or HC. However, record unusual sample characteristics on the runsheet for tracking purposes.

5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides

Not applicable for this procedure.

6. Preparation of Reagents, Calibrators (standards), Controls, and Other

Materials; Equipment and Instrumentation

Note: Use class-A glassware, such as pipets and volumetric flasks, unless otherwise stated. The accuracy of balances, automated pipets and other measuring equipment needs to be confirmed and documented at least annually.

a. Reagents, Materials and Sources

Reagents and sources used in this method are listed below. All reagents are used without further purification. Equivalent sources may be used.

Reagent	Grade	Source	Catalog #
acetic acid, glacial	HPLC	Tedia, Fairfield, OH	AS-1102
methanol	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	A456
ammonium acetate	99.999%	Sigma-Aldrich, St. Louis, MO	37,233-1
ammonium hydroxide, concentrated (14.8N)	ACS Plus	Fisher Scientific, Pittsburgh, PA	A669
2-propanol	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	A461
ß-glucuronidase from Helix pomatia, type H-1 [or ß-glucuronidase from Haliotis rufescens (Red Abalone)]	≥300,000 units/g [or ≥100,000 units/mL]	Sigma-Aldrich, St. Louis, MO [or United Chemical Technologies (UCT), Bristol PA]	G0751 [or BETA-Gluc-10]
methylene chloride	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	D151
potassium hydroxide	85-90% reagent	Fisher Scientific, Pittsburgh, PA	P-250
water	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	W6
Isolute SLE+ 400 mg extraction plate	n/a	Biotage, Charlotte, NC	820-0400-P01
Acetonitrile (ACN)	LC grade	Fisher Scientific, Pittsburgh, PA	A955-4

b. Reagent Preparation

Prepare the following solutions on an as-needed basis.

1) 5N and 0.2N Potassium Hydroxide (KOH) Solutions
Fisher P-250, 85-90% reagent, FW = 56.11, stored at room temperature. To prepare 1000 mL of 5N KOH 5 mols or 280.55 g is required. Using the percentage purity listed on the bottle, calculate the required weight by dividing 280.55 by the (decimal) purity. For example, if the reagent is 85%, then the required weight of KOH is 280.55 /0.85 = 333.06 grams. Weigh out the indicated amount of KOH, dissolve in 500-600 mL HPLC-grade water, transfer to a 1000-mL volumetric flask, and dilute to volume. Label the flask with the preparation date and preparer's initials. To prepare 0.2N KOH, dilute the 5N KOH solution 1:25 with HPLC-grade water.

2) HPLC Mobile Phase Buffer A: 6 mM Ammonium Acetate
Sigma-Aldrich Chemical Co. (St.Louis, MO) # 73594 (≥99.0%), FW = 77.08. Weigh out 0.47 g ammonium acetate and dissolve in a solution made up of 970 mL HPLC-grade water and 30 mL acetonitrile. Store in a labeled, capped, glass bottle.

3) Enzyme Buffer: Ammonium Acetate, 0.5M, pH 5.1
Sigma-Aldrich Chemical Co. (St.Louis, MO) # 73594 (≥99.0%), FW = 77.08. Weigh out 19.47 g ammonium acetate and place in a 500 mL graduated cylinder, add HPLC-grade water to the 500mL line. Adjust the pH of the solution to 5.1 using glacial acetic acid. Store the buffer in a glass stoppered flask in the refrigerator at about 4ºC. This solution expires one year after preparation

4) *ß*-Glucuronidase Enzyme Solution

Helix pomatia Enzyme
We use 40 µL containing 400 units of the enzyme from Sigma per 200 µL urine. This requires an enzyme concentration of 10,000 units/mL. To make 25 mL of enzyme solution (=250,000 units) you need to take into account the labeled activity of the enzyme lot that you have. For example, if the lot is labeled to contain 486,000 units/g and you need 250,000 units then you need to dissolve 0.52 g (=250,000 units / 486,000 units/g) in 25 mL enzyme buffer (0.5M ammonium acetate, pH 5.1). Add 40 µL (400 units) of this solution to each sample for hydrolysis. Store the solution in a glass stoppered flask in the refrigerator at about 4ºC. This solution expires 10 days after preparation.

Haliotis rufescens Enzyme

We use a 50 µL aliquot containing 800 units of the enzyme from United Chemical Technologies per 200 µL urine, which requires an enzyme concentration of 800/0.05 = 16,000 units/mL. To make 25 mL of enzyme solution at 16,000 units/mL add 4 mL enzyme liquid (100,000 units/mL) to 21 mL enzyme buffer (0.5M ammonium acetate, pH 5.1). Add 50 µL (800 units) of this solution to each sample for hydrolysis. Store the solution in a glass stoppered flask in the refrigerator at approximately 4°C. This solution expires 12 months after preparation.

c. Standard Material

Prepare one complete set of 16 calibration standards ranging in concentration from 0 to 400 ng/mL from the compounds given in the table below. Equivalent sources may be used. Use an analytical balance to weigh out the solids. Record weight to at least 4 decimal places.

Reagent	Purity	Source	Catalog #
cotinine perchlorate	>99% by ^{1}H NMR, elemental analysis	recrystallized in-house from Toronto Research Chemicals, Toronto, Ontario, Canada (**24**)	C725015
cotinine-methyl-D_3	>98% by ^{1}H NMR	Cambridge Isotope Laboratories, Andover, MA	DLM-1819
trans-3'-hydroxycotinine	>99% by ^{1}H NMR, elemental analysis	Toronto Research Chemicals, Toronto, Ontario, Canada	H924500
trans-3'-hydroxycotinine-methyl-D_3	>98% by ^{1}H NMR	Toronto Research Chemicals, Toronto, Ontario, Canada	H924510
Water	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	W6

1) Original Stocks of Native Standards

- Solution COT-A. Dissolve 0.0340 g of cotinine perchlorate in water, q.s. to 100 mL. Nominal final Cotinine concentration yield = 216.55 µg/mL COT. [To calculate the free COT concentration from the perchlorate salt, multiply by 176/276 (the ratio of the FW of COT to the FW of cotinine perchlorate).]

- Solution COT-B. Take 4.617 mL of Solution COT-A and q.s. to 100 mL with water. Nominal final concentration = 10 µg/mL COT.

- Solution OHCOT-A. Dissolve 0.0334 g of *trans*-3'-hydroxycotinine in water, q.s. to 100 mL. Nominal final hydroxycotinine concentration is 334.0 µg/mL HC.

- Solution OHCOT-B. Take 2.994 mL of Solution OHCOT-A and q.s. to 100 mL with water. Nominal final concentration = 10 µg/mL HC.

2) Working Solutions of Native Standards

- Solution COT-C. Dilute Solution COT-B 1:10 (10 mL into a 100 mL volumetric). Nominal final concentration = 1000 ng/mL COT.

- Solution COT-D. Dilute Solution COT-C 1:10 (10 mL into a 100 mL volumetric). Nominal final concentration = 100 ng/mL COT.

- Solution OHCOT-C. Dilute Solution OHCOT-B 1:10 (10 mL into a 100 mL volumetric). Nominal final concentration = 1000 ng/mL HC.

- Solution OHCOT-D. Dilute Solution OHCOT-C 1:10 (10 mL into a 100 mL volumetric). Nominal final concentration = 100 ng/mL HC.

3) Original Stocks of Labeled Standards

- Solution D3COT-A. Dissolve 20.3 mg of D_3-COT in water, q.s. to 100 mL. Nominal final corrected concentration = 199 µg/mL D_3-COT.

- Solution D3OHCOT-A. Dissolve 9.8 mg of D_3-HC in water, q.s. to 100 mL. Nominal final concentration = 98 µg/mL D_3-HC.

4) Working Solutions of Labeled Standards

- Solution D3COT-B. Dilute 2.513 mL Solution D3CA and q.s. to 100 mL with water. Nominal final concentration = 5 µg/mL D_3-COT

- Solution D3OHCOT-B. Dilute 5.102 mL Solution D3OHCOT-A and q.s. to 100 mL with water. Nominal final concentration = 5 µg/mL D_3-HC.

5) Standards Preparation Table

Prepare the 16 standard solutions using the volumes listed in the table below, q.s. to 100 mL with water. The nominal concentration of both D3-COT and D3-HC in the standards is 5 ng/mL.

Analyze the standards for two weeks to confirm their suitability. Seal the standards in approximately 3mL aliquots in pre-cleaned (using acetone, methanol and water), 5mL amber ampules. Store them at approximately 4°C.

Std #	Conc in Std* (ng/mL)	Conc in Sample** (ng/mL)	Stocks Native both anlytes (stock used) (mL)	Solution D3-B ISTD both analytes (mL)
1	0	0	0 (Stock D)	0.2
2	0.05	0.005	0.1 (Stock D)	0.2
3	0.1	0.01	0.2 (Stock D)	0.2
4	0.2	0.02	0.4 (Stock D)	0.2
5	0.5	0.05	1 (Stock D)	0.2
6	1	0.1	2 (Stock D)	0.2
7	2	0.2	4 (Stock D)	0.2
8	5	0.5	1 (Stock C)	0.2
9	10	1	2 (Stock C)	0.2
10	20	2	4 (Stock C)	0.2
11	50	5	1 (Stock B)	0.2
12	100	10	2 (Stock B)	0.2
13	150	15	3 (Stock B)	0.2
14	200	20	4 (Stock B)	0.2
15	300	30	6 (Stock B)	0.2
16	400	40	8 (Stock B)	0.2

* Conc in Std is the actual concentration in the standard solution.
** Conc in Sample is the calculated concentration of analyte if 1 mL of sample is analyzed.

6) Internal Standard Spiking Solution

- Place 6 mL D3COT-B and 6 mL D3OHCOT-B into a 3 L volumetric flask, q.s. to 3 L with water. Seal this solution in approximately 3mL aliquots in pre-cleaned (rinsed 3 times with methylene chloride), 5mL amber ampules. Store the ampules at approximately 4^{o}C. Nominal final concentration = 10 ng/mL D_3-COT and 10 ng/mL D_3-HC. Add 50 µL of the spiking solution to each sample. The amount of ISTD per sample is 0.5 ng for both ISTDs.

This ISTD spiking solution should be within ± 1% of the concentration of the ISTDs in the standards.

7) Standards Acceptance Criteria

Analyze the standards in the forward and backward direction 20 times. Use linear regression with 1/x weighting. In order to accept the standards, the following must be true:

- Correlation coefficient $R^2 \geq 0.9990$. No more than two out of the 20 calibration curves can have an $R^2 < 0.9990$.
- Back-calculated standard value = nominal concentration ± 15%*. No more than one standard per calibration curve can fall outside these limits.

  *30% for standards 2 to 4 in the standard set

Note: We do not use Standard #1, the standard with ISTD only, in the calibration curve

d. Quality Control (QC) Materials

Prepare four QC urine pools; two pools with low concentrations and two pools with high concentrations of the two analytes. Each analytic run will be classified as either high or low. Each run will include two vials of either low or high QC pools.

Prepare each of the QC pools from two stock pools of human urine, as required: a low stock pool from nonsmokers with minimum exposure to SHS, and a high stock pool from users of tobacco. Add a calculated amount of the high concentration stock pool to a measured volume of the low concentration stock pool to make the QC pools with the targeted concentrations as given in the table below.

Pool	Approximate Target Concentration	
	COT (ng/mL)	HC (ng/mL)
Low urine 1	0.5	1
Low urine 2	4	8
High urine 1	50	100
High urine 2	500	1000

Stir the resulting pools overnight at approximately 4°C. The next day, mix the pools at room temperature for about 5 hours, then with continuous

stirring, dispense into labeled 2mL cryovials. Store the vials at or below -60°C.

e. Major Instrumentation and Other Equipment

Automated Sample Preparation System. PerkinElmer Staccato Systems Robotics containing one Caliper Life Sciences Sciclone G3 automated liquid handling workstation, one Mitsubishi RV-6SDL robotic arm, one Hettich Rotanta 460 Robotic centrifuge, one Biotage Turbovap 96 evaporator, two FLuidX vial decappers, one FluidX 2D barcode reader, one Inheco DWP incubator-shaker, one Inheco Hotel-incubator, one Thermo Scientific ALPS 3000 microplate heat sealer, and iLink Pro and Maestro software.

Liquid Handler. Hamilton Star automated liquid handler.

HPLC. Shimadzu Nexera UHPLC modular system, containing one CBM-20A control module, two LC-30AD pumps, one SIL-30ACMP 6-MTP autosampler, one DGU-20A5R degasser, and one CTO-20AC column oven.

Mass Spectrometer. AB Sciex API 6500 Triple Quadrupole mass spectrometer with APCI interface, Peak Scientific Instruments Ltd gas generator, and Analyst version 1.6 software.

7. Calibration and Calibration Verification

a. Creation of Calibration Curve

Base the calibration curve for this assay on the analysis of the standards described above in Section **6c**. Each day analyze the standards in order from 0-40 ng/mL sample. Repeat the analysis in reverse order (from 40-0 ng/mL sample). Use both standard sets data points, to generate one calibration curve using the ratio of the peak area of the analyte to the labeled internal standard. Determine the slope, intercept and R-squared value using 1/x weighted linear regression and use these data to quantitate the day's samples.

b. Usage of Curve

Quantification can only be reported for values that fall within the calibration range (between highest and lowest calibrator points).

For sample results that are higher than the highest calibrator, the analysis can be repeated with a smaller amount of sample to bring the result within the calibration range.

Evaluate the standards using the following criteria:

(A) Standard calculated value = nominal concentration ± 15%*
(B) R-squared value ≥ 0.9990

* 30% for standards less than 0.05 ng/mL

Up to 4 standards with values falling outside these limits can be excluded from the calibration curve.

Note: We do not use Standard #1, the standard with ISTD only, in the calibration curve.

c. Calibration Verification

QC is analyzed in every analytical run verifying that calibration is within acceptable limits.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of a rack of 24 vials that contains the following: one blank in position 1, two QC samples, one in position 2 and one in a random position in the rack, and 21 unknown samples. Smoker samples are analyzed with high QC samples, nonsmoker samples are analyzed with low QC samples. A batch consists of four runs worked up together on one 96-well SLE plate. All four runs are analyzed with one set of 32 calibration standards. Sometimes 2 plates are analyzed at the same time, if so the 2 plates and the standards set comprise a batch.

a. Sample Preparation (These instructions are for sample preparation using the Hamilton Star liquid handler and the PerkinElmer Staccato System robot. If you have different equipment you may need to modify these procedures.)

(1) Remove samples from the freezer and let thaw at room temperature. The sample racks may be placed in a few inches of cool water in the

sink to facilitate thawing. During the week, the next day's samples are generally placed in the refrigerator the night before to thaw.

(2) Rotate the samples for at least 15 min on a rotary mixer.

(3) Prepare one set of at least 6 labels with the Run IDs and label the mixing plate, collection plate, and storage box with the Run IDs.

Use the Hamilton Star for Steps 4-11:

(4) Load each of the four Hamilton Star tube carriers with 24 samples from the sample racks (positions 1-24) – a blank in position 1, a QC in position 2, another QC in a random position, and 21 unknown samples in the remaining positions. This will result in four runs with a total of 84 unknown samples, 4 blanks, and 8 QCs per plate.

(5) Remove ISTD spiking solution ampules (as many ampules as needed for the batch) from the refrigerator, let warm to room temperature. Open the ampules and dispense the ISTD spiking solution from all ampules into column 1 of a 12-column reservoir using a disposable plastic or glass transfer pipette. Place a sufficient volume of prepared enzyme solution in the 12 column position of the same reservoir. (If the analysis is for "free" analytes do not use enzyme solution, use water instead.). Place the 12-channel plate in position 4 with the ISTD on the left.

(6) Place the labeled 1.1mL 96-well mixing plate on the Hamilton Star deck in position 3 of the plate carrier.

(7) Scan in the barcodes from the vials and mixing plate to make the sequence file and fill out the runsheet. The sequence file will be stored on the Hamilton Star's computer where it, including the sample ID's, can be copied and transferred to the LC/MS/MS system for sample analysis

(8) Add 50 µL ISTD spiking solution to each well of mixing plate.

(9) Mix the contents of each vial by aspirating and dispensing three times.

(10) Pipette 200 µL of each vial (50 µL for high samples) to one well of the mixing plate.

(11) Add 50 µL enzyme solution to each well of mixing plate. (For “free” analysis add 50 µL water to each well.)

(12) Note the date and plate ID on the Hamilton Instrument Log. Enter the SLE Plate lot #, cleanup analyst initials and any pertinent notes on the run sheet.

(13) Seal the plate and vortex. Place the plate into a 60±2^{o}C incubator for at least 6 hours (usually overnight) if using the United Chemical Technologies enzyme solution. Place the plate into a 37^{o}C incubator for at least 21 hours (usually overnight) if using the Sigma enzyme. Record the water bath temperature on the daily run. (For “free” analysis skip this step.)

Use the PerkinElmer Staccato System robot for Steps 14-27:

(14) Remove the foil sealing cover and place the mixing plate containing the samples and a mixing plate with the corresponding run ID onto the Caliper Que rack.

(15) Add 50 µL 0.2N KOH to each well of the mixing plate.

(16) For high samples, add 150 µLHPLC grade water to each well of the mixing plate.

(17) Mix together the sample, ISTD, KOH, and water (for high samples) in the mixing plate by aspirating and then dispensing with a pipette tip 5 times.

(18) Place the 400mg 96-well Isolute SLE+ extraction plate onto the labeled 2mL 96-well collection plate on the Caliper deck.

(19) Pipette the contents of each well of the mixing plate to the corresponding wells of the extraction plate.

(20) Use nitrogen to gently push the sample mixture onto the extraction plate packing. Allow to equilibrate for 5 min.

(21) Add 0.9 mL of the solvent mixture, 5% isopropanol in methylene chloride, to the extraction plate. Allow to elute by gravity for 5 min into the collection plate then pushing gently with nitrogen at 5 psi for 35 s. Repeat.

(22) Evaporate the solvents under nitrogen at 13-18 psi for 70 min at 40^{o}C using the Turbovap.

(23) Reconstitute the eluted samples by adding 0.1 mL HPLC-grade water to each well of the collection plate.

(24) Seal the collection plate.

(25) Record Plate ID and date on the Caliper Instrument Log.

(26) Record enzyme lot and preparation date, Isolute plate lot #, analytical column lot #s after a change and any anomalies in the cleanup or in the appearance or behavior of the samples as a note on the runsheet.

(27) Use the HamiltonToAnalyst Macro to make a sequence file for the LC/MS instrument. The following fields need to be filled out:

Assay (this will determine whether a high or low run and thus the volume/dilution factor to use)
Initials of LC/MS operator or CDC UserID
Number of samples
Plate Type (Deep Well MTP 96)
Plate Position (the position in the LC autosampler where the plate will sit during analysis)
Run IDs
Input File (this is the sequence file that is copied from the Hamilton Star, which contains the sample ID's)

b. LC/MS/MS Analysis

Listed below are the conditions and settings for the Shimadzu Nexera UHPLC and AB Sciex API 6500 mass spectrometer. If you have different instrumentation, then you will need to optimize the conditions and settings for your equipment.

1) HPLC Conditions and Settings

Analytical column:	Phenomenex Luna Omega 1.6µm C18 100Å, 100 x 2.1 mm (Part # 00D-4742-AN)
Injection volume:	5 µL

HPLC Settings	
Pump A	Buffer A
Pump B	Acetonitrile (ACN)
Flow rate	0.8 mL/min
Pressure limits	Min = 0, Max = 17000
Column Oven Settings	
Temperature	55ºC
Min/Maximum temp	39- 57ºC
Injection Settings	
Sampling speed	5.0 µL/sec
Cooler temp	4ºC
Measuring line purge vol	600 µL
Air gap vol	0.5 µL
Rinse type	External
Rinse Settings	
Rinsing speed	35.0 µL/sec
Rinse port liquid	R2
Rinsing vol	300 µL
Rinse mode	After aspiration
Rinse dip time	1 sec
Rinse method	Rinse pump only
Rinse time	1 sec
Purge settings	
R1	5.0 min
R2	0.5 min
R3	5.0 min

Solvent Program			
Time (min)	Module	Event	Parameter
0.01	Pumps	Pump B Conc	0.1
0.3	Pumps	Pump B Conc	5.0
1.5	Pumps	Pump B Conc	6.0
1.51	Pumps	Pump B Conc	18.0
2.6	Pumps	Pump B Conc	22.0
2.61	Pumps	Pump B Conc	98.0
4.10	Pumps	Pump B Conc	98.0
4.11	Pumps	Pump B Conc	0.1
5.0	Controller	Stop	

2) Mass Spectrometry Conditions and Settings

Mass Spec Settings	
Exp	1
SC type	MRM
Polarity	Positive
Duration	5.0
Delay	0
MCA	no
Optimized Gas, Temp & Voltage Settings*	
CAD	11
CUR	50
GS1	25
NC	3.0
TEM	650
Res Q1	Unit
Res Q3	Unit
Pause between mass range	5.007
Dwell	30
DP	Transition dependent
EP	11
CE (COT)	54
CE (HC)	30
CXP (COT)	9
CXP (HC)	11

*Note: This is just one example of optimized settings. Different instruments may have different values for these settings.

3) Tuning Procedure

The AB Sciex API 6500 mass spectrometer is tuned approximately once a year or as needed. If you are using a different instrument, follow the manufacturer's procedure for tuning.

(1) Obtain PPG tuning solution from AB Sciex: Pos PPG $2x10^{-7}$ M Buffer solution (P/N 4405233), store at 2-8^{o}C.

(2) Fill a glass syringe with PPG tuning solution. Place on the integrated Harvard Apparatus syringe pump.

(3) Put the IS (IonSpray) probe in the interface. Connect the PPG syringe to the IS probe. Change the configuration to MS only. Set the instrument to the API Instrument project and open the most current tuning file for "LM Q1 Pos PPGs" mode. Choose the Tune icon button. Turn on the flow (0.6 mL/hr) to the syringe pump and start the tuning method by choosing the start hot key.

(4) Once the instrument has run 10 scans, look to the bottom left to ensure the total ion count is sufficient (around 1×10^8) and stable. Right-click on the lower right chromatogram and open that field. A series of eight scan chromatograms will be seen on the next screen.

(5) Ensure that the 906 peak has at least 2×10^7 counts for sensitivity (adjust sensitivity with detector CEM voltage setting). Then verify that the peaks are not overly jagged in appearance and that the peak shape is consistent in appearance to previous tune records.

(6) Click the calculate button at the top and on the next screen; check that all the peaks are chosen for calibration. Choose calibrate. A screen will appear showing the acceptable range and average for both peak shift and width. Peak width must be between 0.6 and 0.8. Peak shift must be less than 0.1 in either direction but with a practical target of 0.05 or less. If the shift is off, it is first advisable to either update the calibration if it is fairly close or replace it if it is not – always replace if unsure. Keep rerunning the tunes and repeating this while updating calibration until the shifts fall close to the zero line. Once the calibration is sufficient, adjust the peak widths by altering the offsets settings. To obtain wider peaks, lower the offsets (less resolution) or vice versa for narrower peaks (more resolution). When all the parameters appear to be within specifications, print out the results of each screen and place the printouts in a tuning log binder. Repeat the same procedure for Q3 positive.

(7) After completing the tunes, close the configuration for MS only and remove the IS probe. Clean the interface skimmer plate and the surrounding area with methanol. Clean the IS probe with methanol and put the APCI probe back in. Clean out the syringe with methanol, and wash the buffer bottle and replace with fresh buffer. Return the configuration to LM LCMS and the project to the latest current project setting. Analyze a set of standards up and down (see Daily Procedure, below) to verify the instrument is running correctly.

4) Analytical Procedure

The following is the procedure for running the API 6500 mass spectrometer using Analyst software. If you have different instrumentation then you need to modify these procedures.

(1) Make fresh Buffer A on a regular basis (weekly is recommended).

(2) Whenever problems with sensitivity or contamination occur, clean the front end of the MS. Cleaning may be done on a weekly basis as a preventative measure. The following is the procedure for cleaning: Remove the APCI assembly from the front of the MS. Remove the skimmer plate from the orifice area. Clean the plate with soap and water. Dry it thoroughly and then rinse with methanol. Take a low lint paper towel and clean the inside cavity of the APCI with methanol on the towel making sure to wipe the needle off. Do the same with the orifice plate being careful not to allow the small orifice hole to become blocked with towel fibers. Replace the skimmer plate and reassemble the APCI interface making certain the shorter probe is installed in the ceramic area for APCI analysis (the longer probe is for IS only and is used to run tunes).

(3) Check to make sure the mobile phase bottles are full enough to complete the runs planned for the day. It takes approximately 400 mL of Buffer A and 300 mL of acetonitrile to analyze 32 standards and one plate of 96 samples. Verify the waste bottle is not too full to accept the needle wash flow volume for the day. Purge both LC pumps to make certain there are no air bubbles in the lines.

(4) Record the following in the electronic instrument log:

a. Vacuum readings before and after starting gas flow. Be sure the readings have stabilized before recording them.
b. The three pressure gauges on the Peak gas generator.
c. HPLC Pump A pressure readings at injection.

d. The run ID numbers of the standards and samples for that day, the instrument analyst, and notes on cleaning or repair made to the instrument.
e. Record peak height of the hydroxycotinine internal standard and the signal to noise for hydroxycotinine quant peak of the #2 standard for column life verification.

Electronic copies of the instrument log are kept on the instrument desktop. Any repairs which are made to the instrument are also recorded in an electronic file that is kept on the desktop of the instrument computer for quick reference.

(5) Prepare a batch file for the standards and samples by using the Batch Uploader Template files and then uploading this file after selecting “Build Acquisition Batch”.

(6) Connect the LC line to the APCI and run several test standards to check for a stable retention time. Also check that the peak height for the HC ISTD is greater than 100,000 cps. Record this height on the daily sheet.

(7) Submit the standards and samples from the batch file, and then submit the Wash/Shutdown batch to condition the column and to shut the instrument down after the run. (Wash the column with methanol for at least 30 min before shutting down.)

(8) Prepare the current set of samples to be analyzed by placing the standard vials and the sealed sample plate into the LC autosampler.

c. Uploading Data to Indigo Ascent

The following is a description of how to transfer data to the server for automated integration and QA review by Indigo Ascent software. If you are using different integration software you will need to modify these instructions.

(1)Obtain data from your instrument computer
a. Obtain the sequence file for your run
Export this from the batch file
b. Obtain the wiff and wiff.scan files for your run
Find these in the data folder of your instrument computer

You can either copy the files to a flask drive or transfer them directly via ISEL internal network.

(2) Open a Web browser (Google Chrome)

(3) Access Indigo Ascent's webpage at https://cdc.poweredbyascent.net/

a. Type in your username and password

(4) Convert your sequence file into a .csv file with Indigo Ascent's accessioning feature

a. From Indigo's home page, click on "Accessioning" in the top left corner
b. Choose your assay from the drop down list: "2024-UCOH-4"
c. Locate your sequence file and drag/drop it into the box containing the words
"Currently Viewing [filename]"

If your sequence file is on your flash drive, click on the folder icon, then click on the "Removable Disk" that represents your flash drive.

Once you've dropped your sequence file into the box, it should turn green and your data should appear below. Remember the name located in the box titled "Batch" as this will be the name of your .csv file. Now, enter your instrument name into the open space. (If you've done this before, just double-click in the space and it will appear.)

d. Make sure dilution factors are correct and that blanks, standards, and unknowns are labeled properly.
e. Scroll to the bottom of the data and click the "ascent" button on the bottom right.
f. If you are prompted, select "Save as" and continue.

(5) Finish by putting the newly created .csv file and your wiff and wiff.scan files for your run into the Indigo Dropbox

a. Click on the folder icon on the bottom left of your screen to open a new window
b. In the new window, locate the folder labeled "+NCEH_DLS_Indigo_Dropbox-FC" and click on it so that it is highlighted.

c. Right-click and select "New → Folder". A blank folder will appear. Inside the folder, type the name of the batch you are uploading.
d. Once your folder is created, you will need to put the .csv file for this run into the folder. To do this, click on the windows icon on the bottom left of your screen. Then type the name of the "Batch" that you converted.

This is the name you should remember from step 5d

c. Your search should open a new window with the .csv file in it. Drag/drop this file into the folder you created in the Indigo Dropbox.
d. Now, locate your wiff and wiff.scan files

If you are using a flash drive, you will have to click on the "Removable Disk" that represents your flash drive again.

e. Select all of the wiff and wiff.scan files associated with the run you are uploading and drag/drop them into the folder you created in the Indigo Dropbox.

This is the same folder where you just put the .csv file

(6) You are finished. The folder you created will eventually upload to the server and no longer appear in this folder as Indigo Ascent processes the data.

d. Quality Assurance (QA) Review in Indigo Ascent

Indigo Ascent automatically integrates the chromatograms, makes a calibration curve from the standards data, quantifies the analytes in the samples, and performs an initial QA review based on the QA rules that are chosen for the assay. After uploading the data, the analyst reviews the quality of the peak integration and the linearity of the calibration curve.

Open the batch file by clicking on the batch name, then click review in the upper left corner of the screen.

(1) Check the calibration curves for both analytes.

- R2 needs to be >0.9990
- All concentration deviations need to be < 15% except standards ≤ 0.05 ng/mL need to have concentration deviations < 30%

If these criteria are not met, the chromatogram peaks can often be corrected. If peaks are properly integrated and these criteria are still not met, up to 4 standards from each set of standards can be excluded. If this does not bring the calibration within the specifications then the batch will need to be repeated.

(2) Check peak integrations.
Inspect all chromatogram peaks to make sure the peaks are integrated properly and the correct peak is chosen. Correct peak integrations if necessary.

(3) Check QA flags
Click “show flags” and double check each chromatogram that was flagged. Make a note in the comment column if there is something wrong with the result (interference, bad peak shape, no ISTD peak, low recovery, etc).

(4) Set batch status to “Reviewed”.

(5) The QA certifier repeats steps 1 to 3 above and then sets the batch to “Certified”.

e. Calculations

Indigo Ascent calculates all sample concentrations using the calibration curve associated with the run. The software reports results in ng/mL and uses the dilution factor for each sample. The only manual calculation that is needed is to blank subtract the concentration of the water blank from each sample result.

Subtract the calculated blank result for the run from the sample results as measured on the calibration curve, i.e. before correcting for sample volume. For example if the COT blank is 0.002 ng/mL and the sample is calculated to be 0.214 ng/mL using 0.2mL sample volume, then the blank-subtracted result is:

(0.214*0.2-.002)/0.2 = 0.204 ng/mL

Sometimes blank subtraction results in a negative number for the concentration. Replace all negative numbers with a zero for the result.

9. Reportable Range of Results

a. Limit of Detection

The method detection limits are defined as 3 times S_0, where S_0 is the estimate of the standard deviation at zero analyte concentration. The value of S_0 is taken as the y-intercept of a linear regression of standard deviation

versus concentration as specified by Taylor (**26**). See Appendix A for LOD calculation.

b. Accuracy

Accuracy was tested on at least four days using at least three determinations per concentration level for five concentrations that ranged across the calibration curve. The analytes were spiked into nonsmoker urine and the samples were worked up the usual way. The unspiked urine was analyzed at least three times each day to determine the mean background concentrations of COT and HC; these were then subtracted from the spiked sample results.

Acceptable results were obtained at all concentration levels for both analytes. The mean value was within ± 11% of the theoretical value at all levels.

See Appendix A for accuracy results.

c. Precision

Within-day precision was measured using the accuracy samples described above. Acceptable results were obtained at all concentration levels for both analytes. The coefficient of variation (CV) did not exceed 11% for either analyte at any concentration level except at the LOD where it did not exceed 26%.

Between-day precision was also measured using the accuracy samples described above. Acceptable results were obtained at all concentration levels for both analytes. The CV did not exceed 10% for either analyte at any concentration level except at the LOD where it did not exceed 36%.

See Appendix A for precision results.

d. Matrix Effects and Recovery

Matrix effects (ME) and extraction recoveries (RE) were measured according to the method of Matuszewski (**27**). We compared the instrumental response for the following three cases:

(A) the ISTD directly injected in the mobile phase (result A)
(B) the same amount of ISTD added to the already extracted sample (result B)
(C) the same amount of ISTD added to the sample before extraction (result C)

The Case A is just the ISTD injected with the standards. Result A is the average of all the standards analyzed the same day. Results B and C were measured in five different nonsmoker serums in quadruplicate and the mean results were compared. All results were measured on the same day. (Results A, B and C are measured as peak areas.)

ME = B/A*100
RE = C/B*100

Overall COT had an average ME of 101% for these 5 serum samples and HC had an average ME of 104%. Average extraction recoveries were 44% and 41% respectively.

See Appendix A for matrix effects results.

e. Linearity Limits

The lower reportable limit is the LOD. The upper reportable limit is the highest standard concentration which is 40 ng/mL for a sample volume of 0.2 mL and 800 ng/mL for a sample volume of 0.05 mL. Samples with analyte concentrations greater than the highest standard are analyzed again using a smaller sample volume to bring the measured concentration below the concentration of the highest standard.

f. Ruggedness Testing

Method ruggedness for the assay was tested by varying the following parameters: KOH concentration, volume of KOH solution, N2 evaporation pressure, N2 evaporation temp, N2 evaporation time, volume of sample, enzyme amount, hydrolysis temperature, and hydrolysis time. Each parameter, except enzyme type, was tested at the method level and at a lower and higher level using QC pools.

See Appendix A for ruggedness testing results.

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

This assay measures two analytes, COT and HC. The QC evaluation considers each analyte independent of the other. A run may be out of control for one analyte and in control for the other analyte. For example if COT is found to be out of control due to a QC pool or blank outlier, but all HC QC and

blank samples are in control, then the HC results for the samples in the run will be acceptable, however the samples will need to be reanalyzed in a repeat run for COT.

The preparation of the QC materials was described previously in Section **6d**. Prior to releasing a set of data, all samples are subjected to a final evaluation according to the following criteria:

1) QC results. Confirm all QC results for the mean and range values using the current DLS QC rules based on the division SAS QC program (**28**).

2) Blanks. Reject low run if COT blank > 0.030 ng/mL or if HC blank > 0.030 ng/mL. Also reject low run if the blank batch average > 0.015 ng/mL for either analyte. Reject high run if COT blank > 0.050 ng/mL or if HC blank > 0.050 ng/mL.

3) Relative retention times. If the retention time difference between the quantitation and ISTD ions is more than 3 sec, inspect the chromatogram carefully for any possible interferences. If the identity of the peak cannot be confirmed, then the sample is marked as invalid.

4) Confirmation ratios. Calculate the confirmation ratio for each analyte by dividing the confirmation ion area by the quantitation ion area. The ion transitions are given below.

   COT Confirmation ion = m/z 177 → 80
   COT Quantitation ion = m/z 177 → 98
   HC Confirmation ion = m/z 193 → 134
   HC Quantitation ion = mz 193 → 80

   The confirmation ion ratio range is determined from the mean of the standards for that day with concentrations ≥ 0.02 ng/mL. Because of low ion counts for the confirmation ion, these evaluations are limited to samples with a calculated concentration ≥ 0.2 ng/mL. Select those samples for further evaluation that have a calculated concentration ≥ 0.2 ng/mL and a confirmation ratio greater than 25% from the mean.

5) Linear range. Make certain that the values are within the linear range of the calibration curve; in general, that means that the actual measured value for both analytes (prior to correction for dilution) must be no greater than 40 ng/mL. Select samples with (uncorrected) values greater than 40 ng/mL for repeat analysis at a greater dilution.

6) Recoveries. Estimate the mean recovery of each sample from the raw ion counts observed for the ISTD relative to the mean observed for all of the standards (generally n=24) assayed that day for both analytes. Reanalyze any sample with an estimated recovery of less than 20% if sufficient residual sample is available. However, low recovery alone is not grounds for rejecting a sample.

7) Other checks

- Examine the chromatograms carefully for indications of possible problems
- Check runsheet for analyst notes of potential problems with samples or run
- Compare results of repeat analyses for consistency

b. Establishing QC Limits

Acceptable QC concentration limits are calculated initially from at least 20 analyses of the QC pools over a period of at least two weeks. These data may then be updated periodically based on additional runs. The process of limits calculation is performed using the laboratory database and the SAS division QC characterization program (**28**).

c. Proficiency Testing

Prepare four urine proficiency testing (PT) pools for this assay. Aliquot the pools into 2mL coded cryovials, and freeze the vials at or below approximately -60^{o}C. Characterize the pools in at least 20 analytical runs over a period of at least two weeks.

Use the characterized PT pools to conduct PT assays at least semi-annually. PT results are reviewed by the analyst, the supervisor, and a DLS statistician. To pass PT at least 80% of the results must agree with the target value or characterized mean ± 25%. If the assay fails PT, all analyses are stopped and the source of the error is investigated. No assays will resume until the problem has been resolved and a repeat PT assay has been passed.

PT samples are handled and analyzed in the same way as patient samples.

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

a. Internal Standard Response

If the peak height of the HC ISTD in the standards falls below 100,000 cps, this indicates the instrumental sensitivity has fallen below acceptable limits. The following steps need to be taken.

- Clean the mass spectrometer front end including curtain gas plate, orifice, and needle.
- Break vacuum and clean the Q-Jet and Q0 of the mass spectrometer.
- Call for service of the mass spectrometer.

b. Calibration Regression

If the calibration curve becomes nonlinear, first determine if the problem is the LC, the MS or the standards.

- Standards checks: analyze the standards on another instrument. If the standards have become unsuitable prepare new standards.
- HPLC checks: look for leaks, make sure the pumps are delivering the correct volumes, look for high backpressure, make fresh mobile phase, replace the analytical column, make sure the needle rinse program is working properly.
- MS checks: clean the front end, recalibrate the instrument, do a PM.

c. Analyte in Standards or QC Materials

If an unexpectedly large amount of analyte is measured in one of the calibration standards or QC materials, but this is not seen in the remainder of the samples, this indicates a contamination of this particular sample. The source of this incident needs to be investigated to prevent repeat occurrences, but no further action is required.

d. Analyte in All Samples

If an unexpectedly large amount of analyte is present in all measurements for a particular day, it is likely that the source of contamination is in the reagents, the SLE plate, the collection plate, and/or the instrument. These need to be tested to identify the source of contamination. Reagents can be replaced. Plate lots can be replaced. The instrument can be cleaned or parts replaced.

Note: We have seen contamination when the bottle containing the needle rinse solvent becomes dry. The fix is to monitor the solvent level. Contamination has also occurred when the needle rinse program was not working properly. This was caused by a miscommunication between the

software running the LC (Shimadzu) and the software running the MS (Analyst). This problem was solved by updating Analyst software to 1.6.2. To avoid contamination and carryover confirm that the rinse program is working properly by monitoring the solvent levels in the rinse solvent bottles.

e. QC Sample Outside of Control Limits

If an analytical run is deemed to be out of QC control by the division QC program, no results can be reported from that run. Repeat the run. Most out of control QC issues are resolved with one repeat run. If several runs in a row are found to be out of control, analyses need to be suspended while the source of the problem is investigated. Possible sources of error are the liquid handler is out of calibration or the ISTD spiking solution has concentrated due to evaporation or it is contaminated. Check the calibration of the liquid handler. Check the ISTD spiking solution by injecting the solution directly into the LC/MS and comparing the area counts to the ISTD area counts in the standards and look for contamination in the quant ion channels. Test plates for contamination. Wipe down the lab bench area where samples are prepared.

12. Limitation of Method; Interfering Substances and Conditions

In some studies other nicotine metabolites (e.g., HC) and physiological substances (e.g., caffeine) have interfered with immunoassay or chromatographic assays of COT. However, we are aware of no known interferents for this tandem mass spectrometric method for either COT or HC. There can be a small amount of *cis*-3'-hydroxycotinine present in some urine samples; it is usually less than 5% of the *trans* isomer concentration (**29-30**). We detected the *cis* isomer when we analyzed a *cis* standard and found it to have an earlier retention time with nearly baseline separation from the *trans* isomer. We conclude that the presence of the *cis* isomer should have little or no impact on the quantification of HC. The presence of other interfering substances in a particular sample should be indicated by a deviation in the expected confirmation ratio for that sample.

13. Reference Range (Normal Values)

Since the population includes both smokers and nonsmokers, the range of COT and HC levels in urine is quite broad, from < 0.1 ng/mL to > 10,000 ng/mL.

The distribution of serum COT in the nonsmoker U.S. population for the period 1988 to 1991 was established from the analyses conducted as part of NHANES III, Phase 1. Those results have been published (**31**). Subsequent evaluations

have indicated a decline in the median level of serum COT among nonsmokers (**32**). The most recent NHANES survey, for which serum cotinine data have been published (survey years 2011-2012), found the median and 95th percentile level in nonsmokers to be 0.020 and 1.30 ng/mL, respectively (**33-34**)

Total urine cotinine concentrations are about 5 to 10 times higher than in serum (**35**), and total HC concentrations are about two to four times total COT concentrations (**9,13**).

Benowitz et. al (**36**) recommended a serum COT value of 3 ng/mL to distinguish smokers from nonsmokers in the US population. This result was based on NHANES data from 1999-2002. Using this updated cutpoint for serum COT, a cutpoint for total urine COT is estimated to be 30 ng/mL. Others have recommended a total urine COT value of 50 ng/mL to distinguish smokers from nonsmokers (**37**).

14. Critical-Call Results ("Panic" Values)

Not applicable to this procedure.

15. Sample Storage and Handling during Testing

Samples are stored frozen at or below approximately -60^{o}C until they are analyzed. Remove the rack of frozen samples from the freezer and allow them to thaw overnight in a refrigerator. Bring the samples to room temperature on the morning of the analysis. After analysis, replace the residual samples in the racks, and re-freeze them at or below approximately -20^{o}C. Samples can be kept at approximately -20^{o}C during analysis and final QA review before dense packed and stored at approximately -60^{o}C.

16. Alternative Methods for Performing Test and Storing Specimens if Test System Fails

There is no alternate method that is sensitive enough to measure these analytes at the low levels expected for nonsmokers.

a. Length of Time Samples May be Stored

If there is a problem with the method, samples may be stored until the problem is resolved. Samples that have been extracted and reconstituted can be analyzed after one day at room temperature or after one week at -20^{o} C.

b. Proper Storage Procedures

Extracted, reconstituted samples need to be sealed and can be kept at room temperature for 24 hours or at -20^{o} C for one week before assaying.

17. Test-Result Reporting System; Protocol for Reporting Critical Calls (if applicable)

Analytical results are reported as ng/mL for each sample. Final results that meet all QC/QA criteria are then reviewed by a DLS statistician, and formally released by the Director of DLS to the indicated recipient. Data that have successfully completed all review and validation processes may also be provided in electronic file format.

Critical-call reporting is not applicable for this method.

18. Procedures for Specimen Accountability and Tracking

Standard record keeping (e.g., sample ID, notebooks, data files, database, etc.) is used for sample tracking. All records are maintained in accordance with the HHS Records Management guidance. (See: http://www.hhs.gov/open/records/index.html)

19. Method Performance Documentation

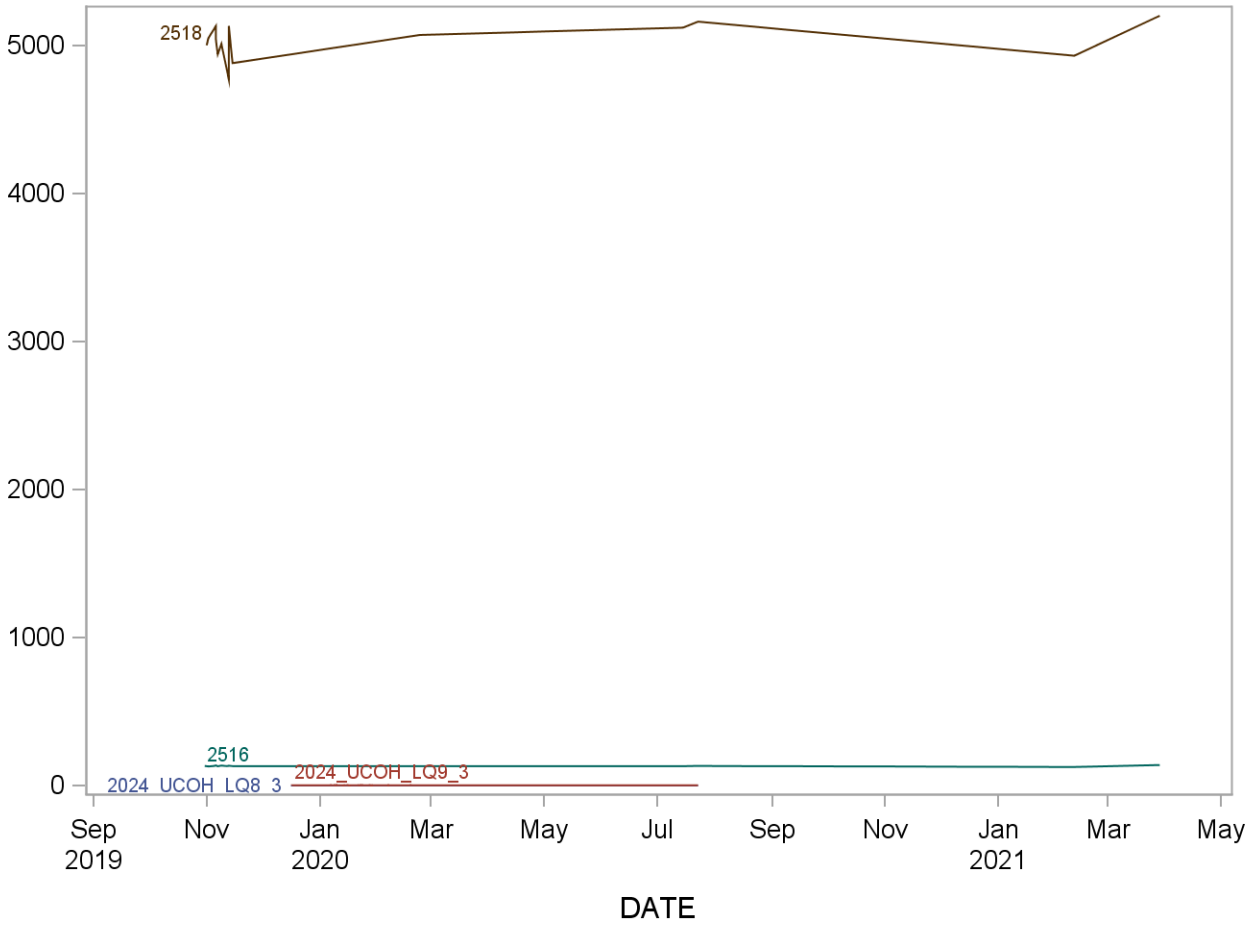
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in Appendix A of this method documentation. **The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote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

20. Summary Statistics and QC Graphs

Please see follow page.

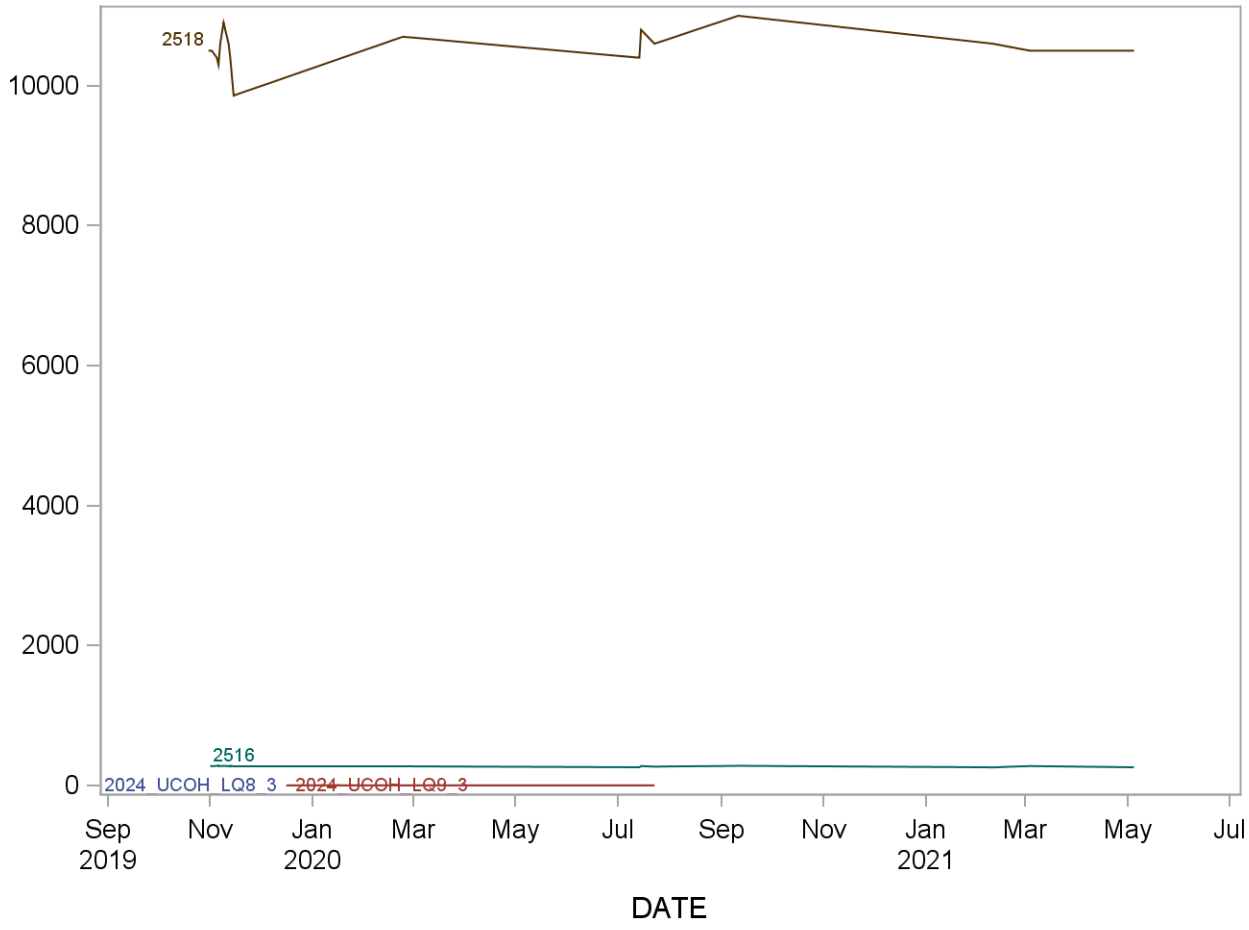
2017-2018 Summary Statistics and QC Chart
URXCOTT (Total Cotinine, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2516	14	31OCT19	29MAR21	132.9286	3.1736	2.4
2518	14	01NOV19	29MAR21	5030.714	119.4516	2.4
2024_UCOH_LQ9_3	108	17DEC19	23JUL20	2.4207	0.0877	3.6
2024_UCOH_LQ8_3	108	17DEC19	23JUL20	0.7971	0.0335	4.2



2017-2018 Summary Statistics and QC Chart
URXHCTT (Total Hydroxycotinine, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	17	31OCT19	05MAY21	10538.82	254.2116	2.4
2516	17	01NOV19	05MAY21	276.8235	7.7236	2.8
2024_UCOH_LQ9_3	107	17DEC19	23JUL20	5.8008	0.2080	3.6
2024_UCOH_LQ8_3	107	17DEC19	23JUL20	1.8831	0.0795	4.2



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Appendix A: Method Performance Documentation

Accuracy using Spike Recovery

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

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

Method name:	Total Cotinine and Hydroxycotinine in Urine
Method #:	2024
Matrix:	urine
Units:	µg/L
Analyte:	**cotinine**

		Sample 1						Sample 2							
	Replicate	spike solution	Spike concentration	Measured concentration Day 1 (UM372-3) (UM384 &UM388)	Measured concentration Day 1 (UM372-3) (UM384 &UM388)	Mean	Recovery (%)	spike solution	Spike concentration	Measured concentration Day 1 (UM372-3)	Measured concentration Day 2 (UM376-7R)	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	UR1	0	0.031	0.014			UR2	0	0.029	0.028				
	2			0.022	0.016	0.018				0.022	0.036	0.028		106.0	3.1
	3			0.014	0.013					0.028	0.024				
Sample	1	1	0.2582	0.303	0.298			1	0.2582	0.272	0.300				
+	2			0.293	0.287	0.298	108.4			0.294	0.291	0.288	100.7		
Spike 1	3			0.294	0.315					0.291	0.279				
Sample	1	2	7.672	8.46	8.14			2	7.672	8.26	8.55				
+	2			8.55	8.43	8.37	108.8			7.96	7.81	8.12	105.5		
Spike 2	3			8.36	8.26					8.01	8.14				
Sample	1	3	139.5	155	150			3	139.5	157	151				
+	2			153	152	151	108.2			142	144	146	104.6		
Spike 3	3			150	145					139	144				

Method name:	Total Cotinine and Hydroxycotinine in Urine
Method #:	2024
Matrix:	urine
Units:	µg/L
Analyte:	**trans-3'-hydroxycotinine**

		Sample 1						Sample 2							
				Measured concentration						Measured concentration					
	Replicate	spike solution	Spike concentration	Day 1 (UM372-3) (UM384 &UM388)	Day 1 (UM372-3) (UM384 &UM388)	Mean	Recovery (%)	spike solution	Spike concentration	Day 1 (UM372-3)	Day 2 (UM376-7R)	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	UR1	0	0.003	0.006	0.006		UR2	0	0.033	0.030	0.032		105.9	2.8
	2			0.014	0.006					0.027	0.043				
	3			0.006	0.003					0.031	0.031				
Sample	1	1	0.2024	0.233	0.231	0.225	108.1	1	0.2024	0.233	0.249	0.237	101.3		
	2			0.226	0.224					0.239	0.239				
	3			0.217	0.220					0.231	0.234				
Sample	1	2	6.015	6.76	6.51	6.55	108.9	2	6.015	6.30	6.99	6.45	106.7		
	2			6.44	6.59					6.25	6.58				
	3			6.39	6.63					6.25	6.35				
Sample	1	3	109.4	109	120	116	106.3	3	109.4	120	119	114	104.4		
	2			119	119					111	118				
	3			113	118					109	110				

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Total Cotinine and Hydroxycotinine in Urine
Method #:	2024
Matrix:	urine
Units:	µg/L
Analyte:	**cotinine**

Quality material 1	**LQ2 (BB868,BB869,BB870,BB871,BB872,UL257,UL258,UL259,UL260,UL275)**					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	**3.201**	**3.595**	3.40	0.038646934	0.038646934	23.0925844
2	**3.990**	**3.917**	3.95	0.001320823	0.001320823	31.26099335
3	**3.905**	**3.823**	3.86	0.001648235	0.001648235	29.86207156
4	**3.853**	**3.879**	3.87	0.000175475	0.000175475	29.88873331
5	**3.988**	**4.059**	4.02	0.001279166	0.001279166	32.37947278
6	**3.381**	**3.859**	3.62	0.057346256	0.057346256	26.20854999
7	**3.429**	**3.743**	3.59	0.024548655	0.024548655	25.72050063
8	**3.709**	**3.671**	3.69	0.000357826	0.000357826	27.22780988
9	**3.636**	**3.720**	3.68	0.001749629	0.001749629	27.05703165
10	**4.199**	**4.321**	4.26	0.003742755	0.003742755	36.297528
Grand sum	75.8781828	**Grand mean**	3.793909138			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.26163151	0.026163151	0.161750273	4.26		
Between Run	1.12034456	0.124482729	0.22172007	5.84		
Total	1.38197607		0.274450251	**7.23**		

Quality material 2	LQ4 (BB868,BB869,BB870,BB871,BB872,UL257,UL258,UL259,UL260,UL275)					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	**0.482**	**0.499**	0.49	7.57143E-05	7.57143E-05	0.481487943
2	**0.628**	**0.611**	0.62	7.71132E-05	7.71132E-05	0.767791278
3	**0.557**	**0.553**	0.55	5.09773E-06	5.09773E-06	0.615998835
4	**0.553**	**0.599**	0.58	0.000523447	0.000523447	0.6628329
5	**0.612**	**0.611**	0.61	8.93892E-08	8.93892E-08	0.748254165
6	**0.557**	**0.550**	0.55	1.22263E-05	1.22263E-05	0.613118612
7	**0.559**	**0.560**	0.56	3.27861E-07	3.27861E-07	0.625384496
8	**0.560**	**0.578**	0.57	7.50549E-05	7.50549E-05	0.647655982
9	**0.616**	**0.565**	0.59	0.000646377	0.000646377	0.696813652
10	**0.683**	**0.654**	0.67	0.000208798	0.000208798	0.893243096
Grand sum	11.5861141	**Grand mean**	0.579305707			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.00324849	0.000324849	0.018023574	3.11		
Between Run	0.04067891	0.004519879	0.045798636	7.91		
Total	0.04392741		0.04921752	**8.50**		

Method name:	Total Cotinine and Hydroxycotinine in Urine
Method #:	2024
Matrix:	urine
Units:	µg/L
Analyte:	**trans-3'-hydroxycotinine**

Quality material 1	**LQ2 (BB868,BB869,BB870,BB871,BB872,UL257,UL258,UL259,UL260,UL275)**					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	**7.488**	**8.289**	7.89	0.160202045	0.160202045	124.4586618
2	**8.673**	**8.474**	8.57	0.009888154	0.009888154	147.024081
3	**8.750**	**8.476**	8.61	0.018726294	0.018726294	148.3632701
4	**8.649**	**8.555**	8.60	0.00223667	0.00223667	147.9858935
5	**8.664**	**8.985**	8.82	0.025757214	0.025757214	155.7439761
6	**7.589**	**8.322**	7.96	0.134375867	0.134375867	126.5885509
7	**7.792**	**8.162**	7.98	0.034282124	0.034282124	127.2726758
8	**8.342**	**8.089**	8.22	0.015960191	0.015960191	134.987425
9	**7.994**	**8.078**	8.04	0.00176354	0.00176354	129.1403586
10	**8.854**	**8.761**	8.81	0.002155335	0.002155335	155.1455309
Grand sum	166.9866743	**Grand mean**	8.349333715			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.810694866	0.081069487	0.284727039	3.41		
Between Run	2.482954193	0.275883799	0.312101196	3.74		
Total	3.293649059		0.422464961	**5.06**		

Quality material 2	LQ4 (BB868,BB869,BB870,BB871,BB872,UL257,UL258,UL259,UL260,UL275)					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	**1.154**	**1.112**	1.13	0.000426968	0.000426968	2.566668039
2	**1.299**	**1.265**	1.28	0.000289153	0.000289153	3.285798533
3	**1.282**	**1.256**	1.27	0.000173845	0.000173845	3.220373159
4	**1.203**	**1.294**	1.25	0.002081929	0.002081929	3.117006324
5	**1.259**	**1.274**	1.27	5.72439E-05	5.72439E-05	3.206583901
6	**1.166**	**1.168**	1.17	9.51721E-07	9.51721E-07	2.724835135
7	**1.265**	**1.273**	1.27	1.66598E-05	1.66598E-05	3.219778071
8	**1.281**	**1.282**	1.28	9.64676E-09	9.64676E-09	3.284768143
9	**1.248**	**1.202**	1.22	0.000518345	0.000518345	3.001207374
10	**1.261**	**1.244**	1.25	7.17925E-05	7.17925E-05	3.139085516
Grand sum	24.78708773	**Grand mean**	1.239354386			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	0.007273796	0.00072738	0.026969976	2.18		
Between Run	0.046118299	0.005124255	0.046887503	3.78		
Total	0.053392094		0.054090826	**4.36**		

Stability						
Freeze and thaw stability =	**Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions**					
Describe condition:	3 times frozen at ≤ -70°C and then thawed (3 freeze-thaw cycles)					
Bench-top stability =	**Assess short-term stability for length of time needed to handle study samples (typically at room temperature)**					
Describe condition:	original samples (not yet prepared for instrument analysis) stored at room temperature for 1 week for LQ2, 1 day for LQ3					
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 4°C for 1 day					
Long-term stability =	**Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis**					
Describe condition:	example: samples stored at -80°C for 6 years					
All stability sample results should be within ±15% of nominal concentration						
Method name:	Total Cotinine and Hydroxycotinine in Urine					
Method #:	2024					
Matrix:	urine					
Units:	µg/L					
Analyte:	**cotinine**					

Quality material 1	LQ2					
	Initial measurement1 (UL060, -063, -065)	3 freeze-thaw cycles (UL065)	Bench-top stability (UL086)	Initial measurement 2 (UM366)	Processed sample stability (UM368)	Long-term stability (UM366)
Replicate 1	3.83	4.09	4.30	4.08	4.06	4.08
Replicate 2	3.97	4.17	4.46	4.10	4.04	4.10
Replicate 3	4.12	3.88	4.40	4.17	4.04	4.17
Mean	3.97	4.05	4.39	4.12	4.05	4.12
% difference from initial measurement	--	**1.9**	**10.5**	--	**-1.6**	**3.6**

Quality material 2	LQ3					
	Initial measurement1 (UL060, -063, -065)	3 freeze-thaw cycles (UL065)	Initial measurement2 (UM366)	Bench-top stability (UM371)	Processed sample stability (UM368)	Long-term stability (UM366)
Replicate 1	66.3	65.7	62.5	64.9	60.7	62.5
Replicate 2	66.4	67.2	61.7	64.0	62.0	61.7
Replicate 3	67.4	64.7	64.6	67.6	66.4	64.6
Mean	67	66	63	65	63	63
% difference from initial measurement	--	**-1.3**	--	**4.0**	**0.2**	**-5.6**

Stability

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

Describe condition: 3 times frozen at ≤ -70°C and then thawed (3 freeze-thaw cycles)

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

Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 week for LQ2, 1 day for LQ3

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 4°C for 1 day

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

Describe condition: example: samples stored at -80°C for 6 years

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

Method name:	Total Cotinine and Hydroxycotinine in Urine
Method #:	2024
Matrix:	urine
Units:	µg/L
Analyte:	**trans-3'-hydroxycotinine**

Quality material 1	LQ2					
	Initial measurement1 (UL060, -063, -065)	3 freeze-thaw cycles (UL065)	Bench-top stability (UL086)	Initial measurement2 (UM366)	Processed sample stability (UM368)	Long-term stability (UM366)
Replicate 1	8.72	9.15	9.05	9.27	9.56	9.27
Replicate 2	9.23	9.40	8.98	9.92	9.70	9.92
Replicate 3	8.89	9.29	9.25	9.42	9.93	9.42
Mean	8.95	9.28	9.09	9.54	9.00	9.54
% difference from initial measurement	--	**3.7**	**1.6**	--	**-5.6**	**6.6**

Quality material 2	LQ3					
	Initial measurement1 (UL060, -063, -065)	3 freeze-thaw cycles (UL065)	Initial measurement2 (UM366)	Bench-top stability (UM371)	Processed sample stability (UM368)	Long-term stability (UM366)
Replicate 1	161	155	163	152	163	163
Replicate 2	148	152	148	151	161	148
Replicate 3	156	158	144	151	155	144
Mean	155	155	152	151	160	152
% difference from initial measurement	--	**0.0**	--	**-0.2**	**5.3**	**-2.3**

LOD, specificity and fit for intended use - fill in yellow shaded cells

Method name:	Total Cotinine and Hydroxycotinine in Urine
Method #:	2024
Matrix:	urine
Units:	µg/L

Analytes	Matrix	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
Cotinine	urine	0.03	yes	yes
Hydroxy-cotinine	urine	0.03	yes	yes

COTT and HCTT specificity in urine	
UM261-264 on 9/20/17	
COTT range: 0.028 to 4.31 ng/mL (not blank subtracted)	1 sample out of a total of 83 samples with interference in COTT quant
HCTT range: 0.018 to 17.2 ng/mL (not blank subtracted)	no samples out of a total of 83 with interference in HCTT quant

Appendix B: Matrix Calibration Curve Comparison

Calibrators should be in the same matrix as unknown samples to be analyzed. However there are no sources of urine that do not have residual COT and HC in them. Therefore we have chosen to make our calibration curve in water. In order to test the equivalency of our water calibration curve to a calibration curve in the matrix, we created two calibration curves: one in urine and one in water. Then we compared the slopes of the two calibration curves. The division policy is if the slopes differ by no more than 5% then the calibration curves can be considered equivalent and the alternate matrix (in this case water) can be used for the assay calibration curve.

We followed the procedure outlined below:

1. Ten matrix calibrators were prepared by spiking known amounts of COT and HC into urine. Calibrators were spaced across the measurement range. A second set of calibrators was prepared by spiking the same amounts of COT and HC into water. See the table below for the calibrator concentrations.

2. The two sets of calibrators were analyzed on the instrument four times. The urine calibrators were worked up as if they were samples before they were analyzed on the instrument. The water calibrators were injected directly into the LC/MS/MS.

3. Calibration curves were constructed by averaging the four instrument responses (Quant area/ISTD area) for each calibrator and plotting those averages against the expected calibrator concentrations. The slopes for the two calibration curves were calculated using 1/x weighting and compared for each analyte. A percent difference less than or equal to 5% is acceptable to demonstrate equivalency of slopes. See table below for all data. Our slope differences were less than 5%.

Calibrator Concentrations for Matrix Calibration Curve

Calibrator #	COT Conc (ng/mL)	HC Conc (ng/mL)
1	0.0128	0.01
2	0.096	0.075
3	0.638	0.5
4	1.275	1
5	6.375	5
6	12.75	10
7	30	23.49
8	60	47.05
9	100	78.31
10	192.1	150.6

Matrix Calibration Curve Slope Comparison

Analyte	Curve	Weight	Slope	Intercept	R2	slope diff (%)
HC	water	1/x	0.44483	0.00496	0.9998	3.8
HC	urine	1/x	0.42814	0.10925	0.991	
COT	water	1/x	0.54895	0.01062	0.9994	4.5
COT	urine	1/x	0.52456	0.0802	0.9991	

Appendix C: Ruggedness Testing

Method ruggedness was tested by varying the following parameters:

1. Enzyme amount (all N=3)
2. Hydrolysis temperature (all N=3)
3. Hydrolysis time (all N=3)
4. KOH concentration (N=4, 4 and 2)
5. N2 evaporation pressure (all N=3)
6. N2 evaporation temp (all N=3)
7. N2 evaporation time (all N=3)
8. Volume of sample (all N=24)
9. Percent IPA in MeCl2 (N=16, 16 and 15)

The first three parameters were tested using urine pools, the other parameters were tested using serum pools, since the sample preparation of serum and urine to extract cotinine and hydroxycotinine are the same, after the urine has been hydrolyzed with enzyme. Each parameter was tested at the method level and at a lower and higher level using QC pools.

Ruggedness Testing Results

Parameter	Pool	Method Level	Lower level	Upper Level	COT Method level (ng/mL)	COT Lower level (ng/mL)	COT Upper Level (ng/mL)	HC Method level (ng/mL)	HC Lower level (ng/mL)	HC Upper Level (ng/mL)
Enzyme amount	LQ6	480 units	320 units	640 units	3.09	3.11	3.08	6.65	6.69	6.68
Hydrolysis temp	LQ2	37°C	35°C	39°C	3.89	3.84	4.05	8.54	8.54	8.82
Hydrolysis temp	LQ4	37°C	35°C	39°C	0.590	0.571	0.621	1.27	1.27	1.27
Hydrolysis temp	LQ3	37°C	35°C	39°C	66.5	65.9	68.6	152	155	156
Hydrolysis temp	LQ5	37°C	35°C	39°C	550	552	550	1246	1254	1257
Hydrolysis time	LQ2	20 hr	18 hr	22 hr	4.08	3.92	3.98	8.75	8.40	8.66
Hydrolysis time	LQ4	20 hr	18 hr	22 hr	0.712	0.572	0.595	1.41	1.19	1.20
Hydrolysis time	LQ3	20 hr	18 hr	22 hr	67.7	69.3	63.4	155	158	145
Hydrolysis time	LQ5	20 hr	18 hr	22 hr	544	557	533	1227	1270	1251
KOH conc	806	0.2N	0.1N	0.5N	1.07	1.05	1.09	0.235	0.23	0.246
N2 pressure	806	15 psi	10 psi	20 psi	1.16	1.17	1.15	0.254	0.252	0.251
N2 temp	806	60ºC	45ºC	75ºC	1.16	1.17	1.21	0.254	0.259	0.263
N2 time	806	40 min	35 min	45 min	1.16	1.15	1.14	0.254	0.247	0.255
sample vol	804	200 uL	150 uL	250 uL	1.38	1.27	1.37	0.37	0.351	0.374
IPA in MeCl2	804	5%	3%	8%	1.3	1.28	1.33	0.394	0.394	0.377

Appendix D: Method Validation

a. Accuracy and Precision

Accuracy was tested on at least four days using at least three determinations per concentration level for five concentrations that ranged across the calibration curve. The analytes were spiked into nonsmoker urine and the samples were worked up the usual way. The unspiked urine was analyzed at least three times each day to determine the mean background concentrations of COT and HC; these were then subtracted from the spiked sample results. The mean value was within ± 11% of the theoretical value at all levels.

Within-day precision was measured using the accuracy samples described above. The coefficient of variation (CV) did not exceed 11% for either analyte at any concentration level except at the LOD where it did not exceed 26%.

Between-day precision was also measured using the accuracy samples described above. The CV did not exceed 10% for either analyte at any concentration level except at the LOD where it did not exceed 36%.

To be acceptable the mean value should be within ±15% of the theoretical value except at LLOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% coefficient of variation (CV), except for LLOQ, where it should not exceed 20% CV.

All results were within the acceptable limits.

Accuracy and Between-Day Precision Results

Level	COT Conc (ng/mL)	HC Conc (ng/mL)	Vol (mL)	COT expected	3OH expected	COT acc	OH acc	COT cv	OH cv	N
AC_1	0.038	0.029	0.2	**0.0382**	**0.03**	99	97	29	36	18
AC_2	0.418	0.302	0.2	**0.382**	**0.3**	109	101	10	9	20
AC_3	9.84	7.32	0.2	**10.2**	**8**	96	91	6	8	20
AC_4	29.5	21.6	0.2	**28.8**	**22.6**	103	96	5	5	18
AC_5	181	134	0.2	**192**	**151**	94	89	3	2	20

Within-Day Precision Results

Run	Ac_1 OH	Ac_1 COT	Ac_2 OH	Ac_2 COT	Ac_3OH	Ac_3 COT	Ac_4 OH	Ac_4 COT	Ac_5 OH	Ac_5 COT
BB863	0.033	0.033	0.272	0.397	7.28	10.3	20.9	28.6	136	182
BB863	0.039	0.026	0.332	0.415	7.48	10.3	19.9	28.3	135	180
BB863	0.028	0.029	0.296	0.402	6.78	9.11	22.2	31.3	131	187
BB863	0.031	0.033	0.259	0.365	7.35	10.1	21.6	30.0	136	184
BB863	0.018	0.036	0.320	0.421	7.25	9.90	22.3	30.7	139	187
Mean	0.030	0.031	0.296	0.400	7.23	9.94	21.4	29.8	135	184
SD	0.008	0.004	0.031	0.022	0.264	0.488	0.994	1.28	2.91	3.08
cv	25.8	12.6	10.5	5.5	3.7	4.9	4.6	4.3	2.2	1.7

b. Carryover

Carryover was tested by injecting a high concentration COT solution (approx. 100 ng/mL) followed by 5 injections of Standard #1 (ISTD solution only). This was done in triplicate. Then a high concentration HC solution (approx. 100ng/mL) was injected followed by 5 injections of Standard #1, the whole thing in triplicate. Carryover was detected however it was minimal and it will not affect calculated results.

Carryover Results

Sample	COT Quant area	COT calc conc	HC Quant area	HC calc conc
High solution	122864000	101	79575400	104
Blank1	15139	0.002	4272	0.000
Blank2	7183	0.000	2608	0.000
Blank3	6061	0.000	3132	0.000
Blank4	2173	0.000	774	0.000
Blank5	6859	0.000	2337	0.000
High solution	128359000	103	78849800	102
Blank1	13298	0.001	5459	0.000
Blank2	7273	0.000	3795	0.000
Blank3	6423	0.000	2917	0.000
Blank4	8235	0.000	948	0.000
Blank5	2145	0.000	1547	0.000
High solution	132396000	106	79410200	106
Blank1	13756	0.001	5639	0.001
Blank2	10971	0.001	4489	0.000
Blank3	10176	0.000	2389	0.000
Blank4	8992	0.000	4025	0.000
Blank5	3386	0.000	3689	0.000

c. Stability

Stability was tested three ways.

(1) To test analyte integrity after freezing and thawing, the analytes were measured in a two QC pools that had been through three freeze-thaw cycles. The concentrations were compared to the concentrations in QC samples that had only been through one freeze-thaw cycle (the usual case). The means were tested using Student's T test with all $p>0.05$.

Freeze-Thaw Results

Pool	Mean (SD) (ng/mL)	N	Analyte	FT cycles
LQ2	4.047 (0.148)	3	COT	3
LQ2	4.043 (0.214)	18	COT	1
LQ2	9.282 (0.126)	3	HC	3
LQ2	9.218 (0.341)	18	HC	1
LQ3	65.85 (1.26)	3	COT	3
LQ3	68.85 (7.18)	16	COT	1
LQ3	155 (2.8)	3	HC	3
LQ3	160 (14.1)	16	HC	1

(2) To test the storage stability of processed samples, worked up samples were analyzed the same day, the following day after storing the extracts at room temperature, and after storing the extracts at approximately -20°C for four weeks. The results were tested using the Paired T test and the differences were found to be not significant for either storage time period.

Stability of Extracted Samples

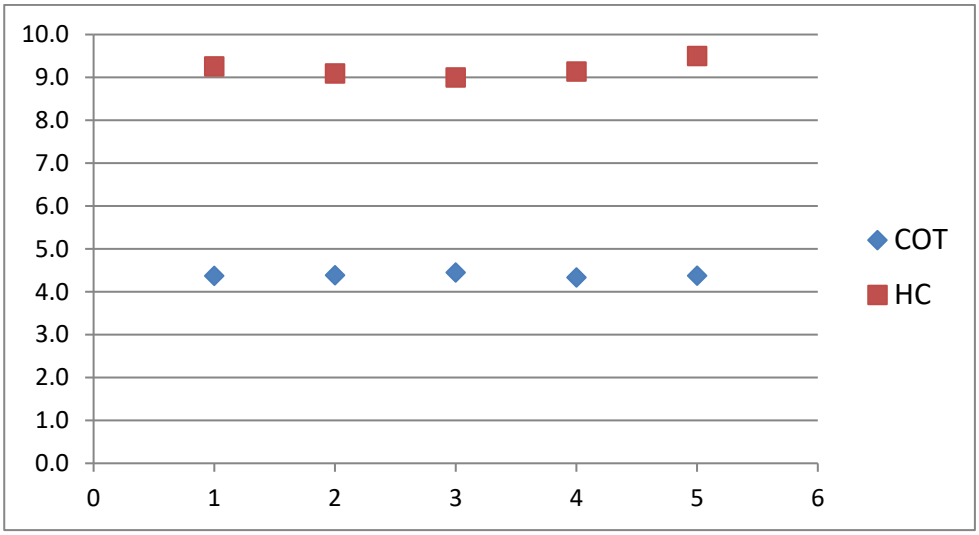
Pool	Cot[1]	Cot[2]	Cot[3]	Cot[4]	OH[1]	OH[2]	OH[3]	OH[4]
LQ2	3.973	3.825	3.624	3.732	9.231	9.210	9.051	9.273
LQ4	0.610	0.621	0.516	0.488	1.277	1.340	1.303	1.325
UPT1	2.848	2.829	2.729	2.812	6.437	6.300	6.598	6.455
UPT2	10.241	10.283	10.374	10.265	24.235	23.534	24.542	25.033
UPT3	37.644	34.663	31.571	30.894	85.395	84.588	80.043	80.911
UPT4	59.034	57.502	52.726	55.652	136.015	138.762	133.741	132.145
UPT5	103.547	100.609	96.659	100.063	247.744	250.373	234.365	245.694
LQ3	66.413	66.808	53.050	57.364	148.088	158.090	139.122	139.034
LQ5	515.117	512.005	520.693	514.997	1288.920	1230.780	1286.340	1376.53

1 = same day, 2 = after 1 day at room temp, 3 = same day, 4 = after 4 weeks at -20 °C
(1 is compared to 2, and 3 is compared to 4)

(3) An accelerated stability study was also performed. Samples were held at room temp and in a water bath at 37°C for up to two weeks then analyzed in triplicate along with samples that had remained in the -70°C freezer for the two week period. Results are in the table and chart below. The data show no decrease in analyte values.

Accelerated Stability Study

Sample Type (LQ2)	Point	Mean COT (ng/mL)	Mean HC (ng/mL)
Frozen (-70°C)	1	4.373	9.258
Room Temp 1 week	2	4.389	9.094
Room Temp 2 week	3	4.449	8.999
37°C 1 week	4	4.333	9.137
37°C 2 week	5	4.376	9.500



Note: The stability studies were done for the previous assay, Method 2012.

d. Matrix Effects

We measured the effect of the matrix according to the method of Matuszewski (**27**). We compared the instrumental response for the following three cases:

(A) the ISTD directly injected in the mobile phase (result A)
(B) the same amount of ISTD added to the already extracted sample (result B)
(C) the same amount of ISTD added to the sample before extraction (result C)

The Case A is just the ISTD injected with the standards. Result A is the average of all the standards analyzed the same day. Cases B and C were

measured in five different nonsmoker urines in triplicate and the mean results were compared. (Results A, B and C are measured as peak areas.)

ME = B/A*100 = Matrix Effect
If ME = 100 no matrix effect is present
if ME > 100 there is a signal enhancement
if ME < 100 there is a signal suppression.

RE = C/B*100 = recovery of the extraction procedure

All results are in the table below. Overall COT had an average ME of 101% for these 5 urine samples and HC had an average ME of 104%. Average extraction recoveries were 44% and 41% respectively.

Matrix Effects Results

Urine	HC ISTD Case B	COT ISTD Case B	HC ISTD Case C	COT ISTD Case C	HC ME (%)	COT ME (%)	HC RE (%)	COT RE (%)
1	444081	892566	170517	350407	101	99	38	39
2	469238	909644	185227	384796	107	101	39	42
3	453526	887710	187576	372110	104	99	41	42
4	436671	899121	184217	407465	100	100	42	45
5	474263	942324	207775	497930	108	105	44	53
Average	455556	906273	187062	402541	104	101	41	44
Standards = Case A	438044	897689						

Appendix E: Method 2024 LOD Estimate by New DLS LOD Determination

Estimate of the Limit of Detection (LOD)

We used DLS's new method to estimate the LOD that allows for a maximum 5% false negative rate. We followed the procedure outlined below.

1. A water blank and four low concentration urine pools (B1, B2, UL and UN) were analyzed in at least 40 runs over a period > 2 months (analysis date range: 11/1/2013-5/26/2015).
2. Outliers were removed and the means and standard deviations for each pool and the water blank were calculated.
3. A linear relationship between the means and standard deviations resulted in a regression line with slope=A and intercept=B.
4. The collected values were substituted into Equation (1) below to calculate the estimated concentration for the LOD.

Equation (1): $Conc_{LOD} = [Mean_b + 1.645*(S_b + B)]/(1-1.645*A)$

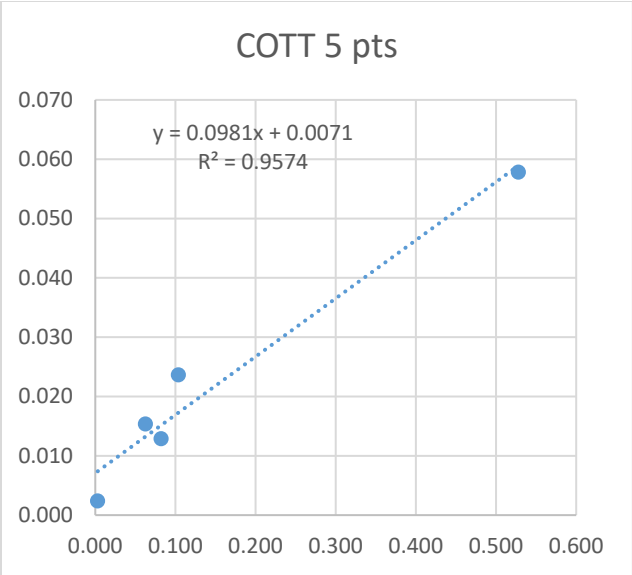
Where $Mean_b$ = mean of blank and S_b = std dev of blank

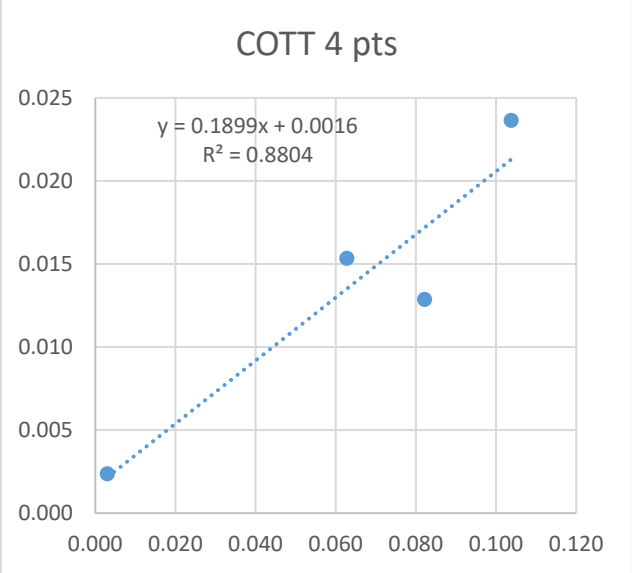
LOD Data

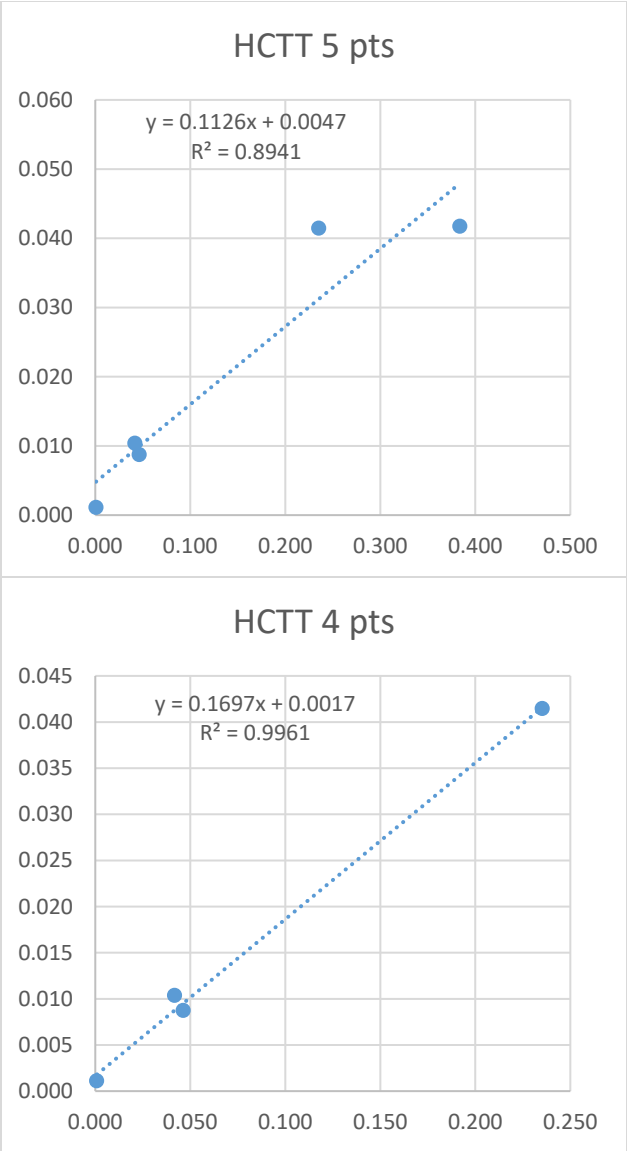
Below is a table of the means and standard deviations for all urine pools and the water blank.

Pool	Analyte	Mean	Std Dev	N
BLK	COTT	0.00305	0.00236	32
B2	COTT	0.0628	0.0153	31
UL	COTT	0.0822	0.0129	31
B1	COTT	0.1038	0.0236	35
UN	COTT	0.5280	0.0578	33
BLK	HCTT	0.00080	0.00110	33
UL	HCTT	0.0418	0.0104	31
B2	HCTT	0.0463	0.0087	30
B1	HCTT	0.2353	0.0415	33
UN	HCTT	0.3838	0.0417	29

Pool means were plotted against standard deviations and a regression line was obtained for each analyte:


COTT 5 pts
y = 0.0981x + 0.0071
R² = 0.9574
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
0.000
0.100
0.200
0.300
0.400
0.500
0.600


COTT 4 pts
y = 0.1899x + 0.0016
R² = 0.8804
0.025
0.020
0.015
0.010
0.005
0.000
0.000
0.020
0.040
0.060
0.080
0.100
0.120

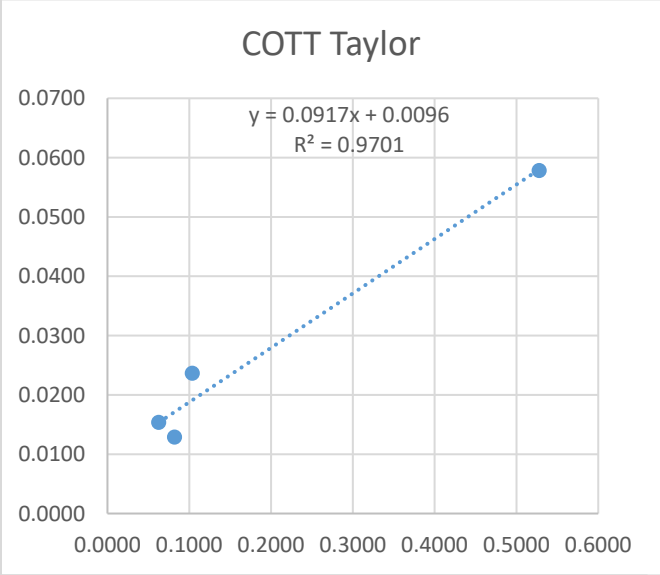


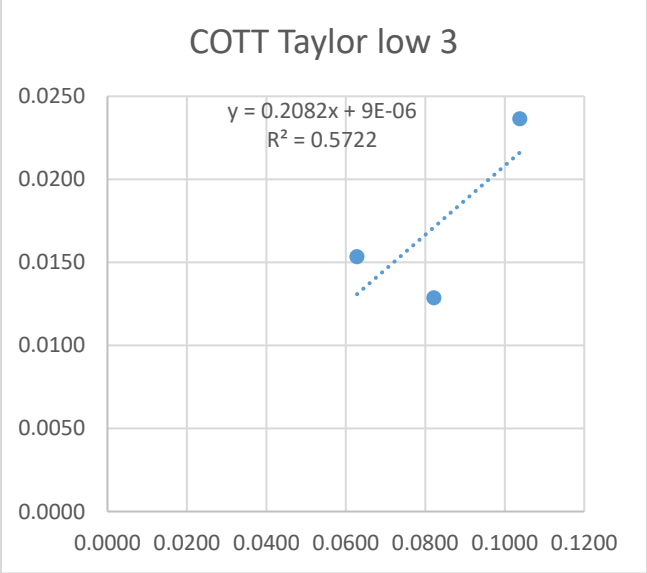
$Conc_{LOD}$ was obtained using the regression parameters and Equation (1).

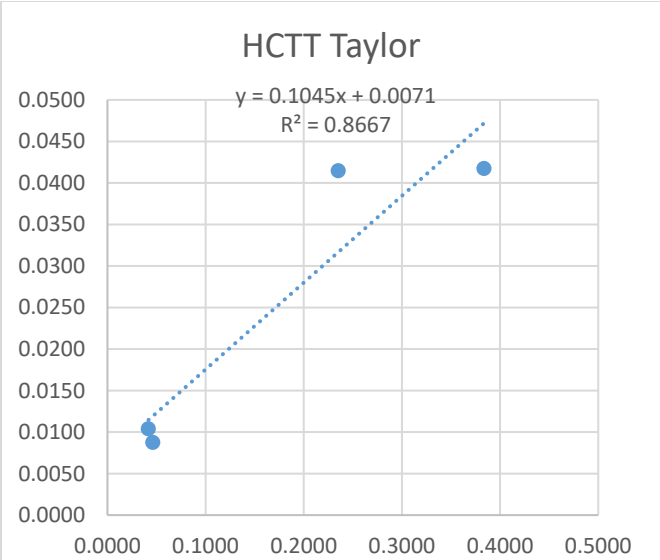
Analyte	A=slope	B=inter	$Conc_{LOD}$	#pts
COTT	0.0981	0.0071	**0.022**	all 5
COTT	0.1899	0.0016	**0.014**	low 4
HCTT	0.1126	0.0047	**0.013**	all 5
HCTT	0.1697	0.0017	**0.007**	low 4

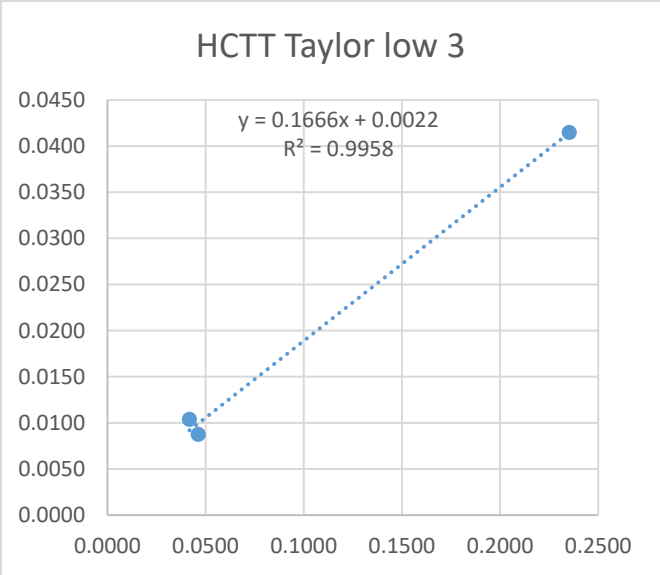
We will round up these numbers and continue to use 0.030 ng/mL as the LOD for both analytes.

For comparison, the same data was used to calculate the LOD by Taylor's method yielding very similar results.









Analyte	Taylor ($3S_0$)	#pts
COTT	**0.029**	4
COTT	**0.000**	3
HCTT	**0.021**	4
HCTT	**0.007**	3