Laboratory Procedure Manual

Analyte: **Cotinine and Hydroxycotinine**
Matrix: **Serum**
Method: **HPLC - APCI Tandem Mass Spectrometry**
Method No: **2017.05**
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as performed by:

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

contact:

Tiffany Seyler, Ph.D.
Phone: 770-488-4527
Email: tvh2@cdc.gov

Ben Blount, Ph.D.
CLIA 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
COT_L	LBXCOT	Cotinine, serum (ng/mL)
	LBXHCOT	Hydroxycotinine, serum (ng/mL)

1. Clinical Relevance and Summary of Test Principle

Analytes:

- (-)-Cotinine. 1-methyl-5-(3-pyridyl)-2-pyrrolidinone; N–methyl-2-(3-pyridyl)-5-+ pyrrolidone. $C_{10}H_{12}N_2O$; Mol Wt 176.21; m.p. 40-42 ^oC.
- (-)-*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 (HCT) are the primary metabolites of nicotine. The concentrations of COT and HCT 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. 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 HCT is approximately 5-6 hr [8, 9].

COT and HCT can be measured in serum, urine, and saliva—the half-life of cotinine in all three fluids is essentially the same [3]. COT concentrations tend to be three to eight times higher in urine than in serum; however, plasma or serum is the fluid of choice for studies requiring a quantitative assessment of exposure [1]. For that reason, serum was chosen as the matrix for the National Health and Nutrition Examination Survey (NHANES) COT analyses. In serum HCT concentrations tend to be two to four times lower than COT concentrations [9].

The ratio of HCT to COT is called the nicotine metabolite ratio (NMR). It is highly correlated with the rate of nicotine metabolism in smokers [10, 11]. 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 [12]. The conversion of nicotine to COT, as well as the conversion of COT to HCT is largely mediated by the liver enzyme cytochrome P450 2A6 (CYP2A6) [13, 14]. 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 [15-19]. 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.

b. Test Principle

COT and HCT are measured by an isotope-dilution high-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometric (ID HPLC-APCI MS/MS) method [20-22]. Briefly, the serum sample is spiked with methyl-D_3-COT and methyl-D_3-HCT as internal standards. The sample is basified and then applied to a supported liquid extraction (SLE) plate. The analytes are extracted with an isopropanol/methylene chloride mixture, the organic extract is evaporated and reconstituted with water, and the reconstituted elution is injected onto a C18 HPLC column. The eluent from these injections is monitored by APCI-MS/MS. The m/z 80 product ion from the m/z 177 quasi-molecular ion is measured for COT and the m/z 80 product ion from the m/z 193 quasi-molecular ion is measured for HCT. Additional ions for the internal standards and for confirmation are also monitored for the respective compounds. Analyte concentrations are derived from the area ratios of native-to-labeled compounds in the sample by comparisons to a standard curve.
Serum cotinine and hydroxycotinine are measured by low (for nonusers) and high (for users) analytical runs based on a cutoff value of 25 ng/mL so that the markedly different levels of nicotine exposure biomarkers can be optimally quantified in both users and non-users. Unknown samples were assigned to low or high runs based on self-report of tobacco use status or by a test with a cotinine strip test [23] to determine the appropriate assay to be used for quantitative analysis.

c. Special Precaution

Because of the nature of these assays, all analysts involved in this study must be nonsmokers. All analysts answer no to using tobacco products.

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.

d. Mechanical Hazards

The sample preparation system used in this assay is a powerful instrument and can potentially be a hazard if interfered with during operation. 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. The latest version of the APM is kept in the instrument folder.

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. Expiration date of waste is indefinite if stored under proper storage conditions. Determine the suitability for waste pickup through inspection of physical properties (color, viscosity, granularity, presence of particulate matter, etc.).

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 and for the 7500 system this package is OS. 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 liquid handler system; these require knowledge of the operating software (Instinct V and SW Venus). 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 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 workstations and database servers 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

The specimen for these analyses is serum. Sample processing does not require anticoagulants, special preservatives, or unusual sterility procedures. Blood can be collected from a venipuncture by using standard equipment, e.g., red top (no anticoagulant) Vacutainer® tubes. Allow the blood to clot for a minimum of 30 minutes and up to 2 hours to create maximum serum yield. Transfer the serum to polypropylene cryogenic, screw-cap vials and freeze.

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 need to be conducted according to standard protocols. Ensure that samples remain in the frozen state during shipment and subsequent storage. Store samples in freezers (-10 to -50°C) or ultralow freezers (-50 to -100°C).

d. Sample Quantity

A minimum of 1.0 mL of serum 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, such as hemolysis or lipemia, influence the HPLC/MS/MS analysis of COT or HCT. However, unusual sample characteristics are recorded on the runsheet for tracking purposes.

5. Procedures for Microscopic Examinations

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

Reagent/Material	Grade	Source	Catalog #
ammonium acetate	≥99.0%	Sigma-Aldrich, St. Louis, MO	73594
ammonium hydroxide, concentrated (14.8N)	ACS Plus	Fisher Scientific, Pittsburgh, PA	A669
2-propanol	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	A461
methanol	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	A456
methylene chloride	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	D151
potassium hydroxide	Certified ACS	Fisher Scientific, Pittsburgh, PA	P-250
water	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	W7SK-4
Isolute SLE+ 400 mg extraction plate	n/a	Biotage, Charlotte, NC	820-0400-P01
Cotinine (COT) Rapid Test Cassette, 50ng/mL	n/a	Acro Biotech	DCT-102

b. Reagent Preparation

Prepare the following solutions on an as-needed basis.

- **5N and 0.2N Potassium Hydroxide (KOH) solutions**
  Fisher P-250, Certified ACS, FW = 56.11, stored at room temperature (+15 to +30 °C). 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. This solution expires after 1 year.

- **HPLC Mobile Phase Buffer A: 6 mM Ammonium Acetate**
  Sigma-Aldrich Chemical Co. (St. Louis, MO) # 73594 (≥99.0%), FW = 77.08, stored at room temperature. For concentrated stock (600mM), weigh out 46.26 g ammonium acetate and dissolve in 1000 mL HPLC-grade water, the solution expires 1 year after prep date. For each liter of buffer, aliquot 10ml of concentrated stock in 940ml water and 50 mL methanol. Store concentrated stock (600mM) in a labeled, capped, glass bottle in the fridge (2-10°C). Buffer solution expires after 1 month.

c. Standards

Prepare two sets of calibration standards from the compounds given in the table below. Equivalent sources may be used. Use an analytical balance to weigh out the solids. Record weights to at least 4 decimal places. Use class A volumetric pipets for volumes greater than 4 mL. Use a Rainin pipettor, or equivalent, for volumes 4 mL and less.

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

The first set of standards, the low serum standards, has analyte concentrations consistent with those found in nonsmokers. The second set of standards, the high serum standards, has analyte concentrations consistent with those found in smokers. Unknown samples were built to low (nonusers) or high (users of tobacco-products)

runs based on self-report of tobacco use status or by a cotinine strip test (Urinary Cotinine Strip Test, DLS Method #2028). If analytes are found to have concentrations above 25 ng/mL, the samples will be repeated as user samples.

1. Original Stock Solutions of Native Standards

- Stock COT_A. Dissolve 31.4 mg of cotinine perchlorate in water, q.s. to 100 mL. Nominal final concentration = 200 µg/mL free 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).)

- Stock COT_B. Dilute Stock COT_A. 1:20 by taking 5 mL of Stock COT_A and q.s. to 100 mL with water. Nominal final concentration = 10 µg/mL COT.

- Stock COT_C. Dilute Stock COT_B. 1:10 by taking 10 mL of Stock COT_B and q.s. to 100 mL with water. Nominal final concentration = 1000 ng/mL COT.

- Stock COT_D. Dilute Stock COT_C. 1:10 by taking 10 mL of Stock COT_C and q.s. to 100 mL with water. Nominal final concentration = 100 ng/mL COT.

- Stock HCT_A. Dissolve 20.4 mg of HCT in water, q.s. to 100 mL. Nominal final concentration = 20.4 * 0.98 (% purity) = 200 µg/mL HCT.

- Stock HCT_B. Dilute Stock HCT_A 1:20 by taking 5 mL of Stock HCT_A and q.s. to 100 mL with water. Nominal final concentration = 10 µg/mL HCT.

- Stock HCT_C. Dilute Stock HCT_B 1:10 by taking 10 mL of Stock HCT_B and q.s. to 100 mL with water. Nominal final concentration = 1000 ng/mL HCT.

- Stock HCT_D. Dilute Stock HCT_C 1:10 by taking 10 mL of Stock HCT_C and q.s. to 100 mL with water. Nominal final concentration = 100 ng/mL HCT.

2. Original Stock Solutions of Labeled Standards

- Stock COTD3_A. Dissolve 25 mg of D_3-COT in water, q.s. to 100 mL. Nominal final concentration = 250 µg/mL D_3-COT.

- Stock COTD3_B. Dilute Stock COTD3_A 1:50 with water by taking 2 mL of Stock COTD3_A and q.s. to 100 mL with water. Nominal final concentration = 5 µg/mL (5000 ng/mL) D_3-COT.

- Stock HCTD3_A. Dissolve 25 mg of D_3-HCT in water, q.s. to 100 mL. Nominal final concentration = 250 µg/mL D_3-HCT.

- Stock HCTD3_B. Dilute Stock D3HCT_A 1:50 with water by taking 2 mL of Stock HCTD3_A and q.s. to 100 mL with water. Nominal final concentration = 5 µg/mL (5000 ng/mL) D_3-HCT.

3. Standards Preparation Tables

Prepare the two standard sets using the volumes listed in the tables below. Bring all standard solutions up to 200 mL with water. The nominal concentration of both D3-COT and D3-HCT in the standard solutions is 5 ng/mL.

Low Serum Standards Preparation Table

Std #	Conc in Std[a] (ng/mL)	Conc in Sample[b] (ng/mL)	Stock COT_D (mL)	Stock COT_C (mL)	Stock COTD3_B (mL)	Stock HCT_D (mL)	Stock HCT_C (mL)	Stock HCTD3_B (mL)	Conc D3_COT and D3_HCT[c] (ng/mL)	Conc in diluted sample[d] (ng/mL)
1	0	0	0	n/a	0.2	0	n/a	0.2	5	0
2	0.02	0.002	0.04	n/a	0.2	0.04	n/a	0.2	5	0.01
3	0.05	0.005	0.1	n/a	0.2	0.1	n/a	0.2	5	0.025
4	0.1	0.01	0.2	n/a	0.2	0.2	n/a	0.2	5	0.05
5	0.2	0.02	0.4	n/a	0.2	0.4	n/a	0.2	5	0.1
6	0.5	0.05	1	n/a	0.2	1	n/a	0.2	5	0.25
7	1	0.1	2	n/a	0.2	2	n/a	0.2	5	0.5
8	2	0.2	4	n/a	0.2	4	n/a	0.2	5	1
9	5	0.5	n/a	1	0.2	n/a	1	0.2	5	2.5
10	10	1	n/a	2	0.2	n/a	2	0.2	5	5
11	20	2	n/a	4	0.2	n/a	4	0.2	5	10
12	50	5	n/a	10	0.2	n/a	10	0.2	5	25

[a]Conc in Std is the actual concentration in the standard solution.
[b]Conc in Sample is the calculated concentration of analyte if 1 mL of sample is analyzed.
[c]Conc D3_COT and D3_HCT are actual concentrations in the standard solution.
[d]Conc of in diluted sample is the calculated concentration of analyte if sample vol = 200 µL.

High Serum Standards Preparation Table

Std #	Conc in Std[a] (ng/mL)	Conc in Sample[b] (ng/mL)	Stock COT_C (mL)	Stock COT_B (mL)	Stock COTD3_B (mL)	Stock HCT_C (mL)	Stock HCT_B (mL)	Stock HCTD3_B (mL)	Conc D3_COT and D3_HCT[c] (ng/mL)	Conc in diluted sample[d] (ng/mL)
1	0	0	0	n/a	0.2	0	n/a	0.2	5	0
2	1	0.1	0.2	n/a	0.2	0.2	n/a	0.2	5	2
3	2	0.2	0.4	n/a	0.2	0.4	n/a	0.2	5	4
4	5	0.5	1	n/a	0.2	1	n/a	0.2	5	10
5	10	1	2	n/a	0.2	2	n/a	0.2	5	20
6	20	2	4	n/a	0.2	4	n/a	0.2	5	40
7	50	5	n/a	1	0.2	n/a	1	0.2	5	100
8	100	10	n/a	2	0.2	n/a	2	0.2	5	200
9	150	15	n/a	3	0.2	n/a	3	0.2	5	300
10	200	20	n/a	4	0.2	n/a	4	0.2	5	400
11	300	30	n/a	6	0.2	n/a	6	0.2	5	600
12	400	40	n/a	8	0.2	n/a	8	0.2	5	800

[a]Conc in Std is the actual concentration in the standard solution.
[b]Conc in Sample is the calculated concentration of analyte if 1 mL of sample is analyzed.
[c]Conc D3_COT and D3_HCT are actual concentrations in the standard solution.
[d]Conc of in diluted sample is the calculated concentration of analyte if sample vol = 50 µL.

Analyze the standards for two weeks to confirm their suitability (see section 7 below for standards acceptance criteria). Seal the standards in approximately 3 mL aliquots in pre-cleaned (rinsed 3 times with methylene chloride), 5 mL amber ampules. Store them in refrigerator (2-10°C).

4. Internal Standard Spiking Solution

Place 6 mL COTD3_B and 6 mL HCTD3_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 one time each with acetone, methanol and water), 5mL amber ampules. Store the ampules in the refrigerator. Nominal final concentration = 10 ng/mL D_3-COT and 10 ng/mL D_3-HCT. 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.

d. 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% (30% for standards 2 to 4 in the low serum standard set). No more than one standard per calibration curve can fall outside these limits.

e. Quality-Control (QC) Materials

Prepare four QC serum pools to use in this assay: two pools with analyte concentrations consistent with smoker levels and two pools with analyte concentrations consistent with nonsmoker levels. Each analytic run will be classified as either high or low and will include one vial of each QC pool of the appropriate analyte concentration.

Prepare each of the QC pools from two stock pools of human serum, 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. If necessary, targeted concentrations can be obtained by spiking the serum pools with solutions of analytes.

QC Pool Target Concentrations

Pool	Approximate Target Concentration	
	COT (ng/mL)	HCT (ng/mL)
Low serum 1	0.1	0.05
Low serum 2	1	0.5
High serum 1	75	200
High serum 2	200	55

Stir the resulting pools overnight in a refrigerator. The next day, mix the pools at room temperature for about 2-5 hours, then with continuous stirring, dispense into labeled 2mL cryovials. Store vials in freezers.

f. Major Instrumentation and Other Equipment

Automated Liquid Handler: Hamilton Vantage automated liquid handler and Instinct V/SW Venus Software

HPLC: Waters UHPLC I-Class System, containing one SM-FTN Sample Manager, one BSM Solvent Manager, and one APH column oven.

Mass Spectrometers:
Low Concentration Analysis- Sciex API 7500 Triple Quadrupole mass spectrometer with APCI interface, Peak Scientific Instruments gas generator, and OS version 2.2.0 software.
High Concentration Analysis- Sciex API 6500 Triple Quadrupole mass spectrometer with APCI interface, Peak Scientific Instruments gas generator, and Analyst version 1.7.2 software.

7. Calibration and Calibration Verification

a. Creation of Calibration Curve

Base the calibration curves for this assay on the analysis of the standards described above in Section **6c**. For low runs, use the low serum standard set (N=12 standards). for high runs, use the high serum standard set (N=12 standards). Each day analyze the standards in order from Standard 1 to Standard 12. Repeat the analysis in reverse order, from Standard 12 to Standard 1. Use both standard sets, 24 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.

Note: We use Standard #1 in the low curve. We do not use Standard # 1 in the high curve. Standard #1 contains ISTD only.

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 responses 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% (30% for standards less than 0.05 ng/mL)
(B) R-squared value > 0.9990 for the low curve; R-squared value > 0.98 for the high curve.

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

c. Calibration Verification

QCs are 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 1-21 unknown samples. Unknown samples are built in low or high run depending on smoking status. Samples from participants who answered ‘yes’ to smoking question are built in high runs. Samples from participants who answered ‘no’ or did not answer to smoking question are strip tested to determine cotinine concentration. Negative strip tested samples are built in low run, and positive strip tested samples are built in high runs. High runs are analyzed with high QC samples, and low runs are analyzed with low QC samples. A batch consists of four runs worked up together on one 96-well plate. All four runs are analyzed with one set of 20 calibration standards for high runs or 24 calibration standards for low runs.

a. Strip Test

To screen cotinine concentration in unknown samples, we follow the Urinary Cotinine Strip Test analytical procedure (DLS-2028). We use one low and one high QC per set. Samples with a self-report nonsmoking status or no status are strip tested to determine cotinine concentration. The cotinine cutoff concentration of the strip is 50 ng/mL. Each strip test uses 80 µL from individual samples. Samples are then sorted by high or low concentration depending on status of the strip test. Sorted samples are then built into runs.

b. Sample Preparation

(These instructions are for sample preparation using the Hamilton Vantage liquid handler. If you have different equipment you may need to modify these procedures.)

Vantage performance check:

1. For daily use, instrument performance check includes initialization. There is no other performance check required. The manufacturer recommendation is preventative maintenance is performed at the appropriate intervals, 1 PM per year.

2. For NCE such as humidity and/or temperature out of range, we need to stop and wait for humidity and/or temperature to be back in range. The instrument performance is checked and confirmed during the PM.

(Note: From the Hamilton Vantage User Manual- 3.9.1 Instrument Life Cycle The instrument has been designed for a life cycle of 7 years under the assumption that the user maintenance and preventative maintenance is performed at the appropriate intervals. Limiting conditions: Life cycles are related to 5 days a week and 8h a working day.)

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 15 min on a rotary mixer.

3. Prepare one set of at least 9 labels with the Run IDs and label the mixing plate, collection plate, and storage box with the Run IDs.

Use the Hamilton Vantage for Steps 4-23:

4. Load the Hamilton Vantage tube carriers (positions 1-24) – a blank in position 1, a QC in position 2, another QC in a random position, and 1-21 unknown samples in the remaining positions. A full sample set 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 the reservoir on the deck in position 3 on the right most plate carrier (rows 69-74).

6. Place the labeled 1.1 mL 96-well mixing plate on the Hamilton Vantage deck in position 4 of the right most 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 Vantage'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 two times.

10. Pipette 200 µL of each vial (50 µL for high samples, 20 µl for extra high samples) to one well of the mixing plate.

11. Enter the SLE Plate lot #, cleanup analyst initials and any pertinent notes on the run sheet.

12. Place the mixing plate containing the samples and ISTD in position 5 on the middle plate carrier (rows 35-40).

13. Add 50 µL 0.2N KOH to each well of the mixing plate.

14. For high samples, add 150 µL HPLC grade water to each well of the mixing plate.

15. Mix together the sample, ISTD, KOH, and water (for high samples) in the mixing plate by aspirating and then dispensing with a pipette tip 3 times.

16. Place the 400 mg 96-well Isolute SLE+ extraction plate on the middle plate carrier (rows 35-40) in position 1 and the 96-well collection plate in position 2 of the same plate carrier.

17. Place the 400mg 96-well Isolute SLE+ extraction plate into the MPE

18. Pipette the contents of each well of the mixing plate to the corresponding wells of the extraction plate.

19. Use nitrogen at 10 psi for 10 seconds to gently push the samples mixture onto the extraction plate packing. Allow to equilibrate for 5 min.

20. Place the 96-well collection plate into the MPE and then place the 96-well Isolute SLE+ extraction plate on top the collection plate

21. Add 0.6 mL of the solvent mixture, 5% isopropanol in methylene chloride, to the extraction plate. Allow to elute by gravity for 1 min into the collection plate then pushing gently with nitrogen at 0.5 psi for 120s. Repeat two more times increasing the pressure elution time to 240s.

22. Evaporate the solvents under nitrogen at 50 psi for 35 min at 40°C using the MPE.

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 any anomalies in the cleanup or in the appearance or behavior of the samples as a note on the runsheet.

26. Use the batch file generator 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)
- Number of samples
- Reinjection checkbox, if checked enter reinjection number
- Run IDs
- Standard Set Names
- Input File (this is the sequence file that is copied from the Hamilton Vantage, which contains the sample ID's)

c. LC/MS/MS Analysis

Listed below are the conditions and settings for the Waters Acquity UPLC and Sciex 6500 and 7500 mass spectrometers. If you have different instrumentation, then you will need to optimize the conditions and settings for your equipment. One analytical column has been validated for this method and are listed below.

1. HPLC Conditions and Settings

Analytical columns: Hypersil Gold C18 Selectivity, 50 x 3 mm, 1.9 µm particle size from Thermo Scientific (catalog # 2500-053030)

Injection volume: 5 uL

Sample Manager (FTN)	
Wash Solvent	Methanol
Purge Solvent	Methanol
Pre-Inject Wash	6 sec for low runs, 0 sec for high runs
Post-Inject Wash	6 sec
Column Temperature	50ºC ± 5ºC
Sample Temperature	15ºC ± 5ºC
Active Preheater	enabled
Solvent Manager (BSM)	
A1 Solvent	6mM Ammonium Acetate 5% MeOH
B1 Solvent	Methanol
Max Pressure	18000
Seal Wash Time	5 min

Solvent Program	Time	Flow (ml/min)	%A	%B	Curve
1	*Initial*	1	100	0	*Initial*
2	0.2	1	100	0	6
3	2	1	68	32	6
4	2.4	1	68	32	6
5	2.41	1	0	100	11
6	3.4	1	0	100	6
7	3.41	1	100	0	11
8	6	1	100	0	11

2. Mass Spectrometry Conditions and Settings

Mass Spec Settings	**6500**	**7500**
SC type	MRM	MRM
Polarity	Positive	Positive
Duration	4.006	4.01
Delay	0	N/A
MCA	no	N/A
Optimized Gas, Temp & Voltage Settings*		
CAD	10	10
CUR	50	50
GS1	35	35
NC	3.0	3
TEM	650	625
Res Q1	Unit	Unit
Res Q3	Unit	Unit
Pause between mass range	5.007	5.007
Dwell	20	20
EP COT (Quant, Qual, IS)	9 for all	9 for all
EP HCT (Quant, Qual, IS)	7 for all	7 for all
COT CE (Quant, Qual, IS)	31, 30, 31	32, 29, 32
HCT CE (Quant, Qual, IS)	45, 25, 45	42, 27, 42
COT CXP (Quant, Qual, IS)	10, 17, 10	9, 16, 10
HCT CXP (Quant, Qual, IS)	9, 10, 9	9, 10, 12
COT DP (Quant, Qual, IS)	60 for all	N/A
HCT DP (Quant, Qual, IS)	62 for all	N/A

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

3. Analytical Procedure

The following is the procedure for running the Sciex 6500 and 7500 mass spectrometers using Analyst and OS software. If you have different instrumentation, then you need to modify these procedures.

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

(2) Whenever problems with sensitivity or contamination occur, clean the front end of the MS. Cleaning may be done monthly as a preventative measure. The following is the procedure for cleaning: Remove the APCI assembly from the front of the MS. Remove the curtain 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. Be careful to not allow the small orifice hole to become blocked with towel fibers. Replace the curtain 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 ESI 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 methanol to analyze 24 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) Instrument Performance Check: record the following in the daily sample log:

a. Vacuum readings before and after starting gas flow. Be sure the readings have stabilized before recording them.
b. Curtain, Source and Exhaust gas readings.
c. HPLC Pump A pressure readings after approximately 30 sec of flow.
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. The sensitivity of the HCT and COT native and ISTD peak heights in start-up runs and/or standards
f. Note any changes in buffer, instrument cleaning, maintenance, purging, etc.
g. Electronic copies of the daily sample log are kept in an electronic .xls file on the instrument computer 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.
h. For NCE such as humidity and/or temperature out of range, we need to stop and wait for humidity and/or temperature to be back in range. The instrument performance is checked and confirmed during the PM.

(5) Prepare a batch file for the standards and samples by using the output file from the Hamilton Vantage. Use the batch file generator macro to create batch files for the instrument and for eventual Indigo upload.

(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 HCT ISTD is greater than 100,000 cps.

(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 water for 10 minutes and 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.

d. 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
2. Obtain the batch file that was created prior to the run.
3. Obtain the wiff and wiff.scan files for your run
   a. *Find these in the data folder of your instrument computer and transfer them directly via ISEL internal network.*
4. Open a Web browser (Google Chrome)
5. Access Indigo Ascent's webpage at https://cdc.poweredbyascent.net/
6. Type in your username and password
7. Convert your sequence file into a .csv file with Indigo Ascent's accessioning feature
8. From Indigo's home page, click on "Accessioning" in the top left corner
9. Choose your assay from the drop-down list: "2017-SCOH-low" for low run or "2017-SCOH-high" for high run
10. Locate your batch file and drag/drop it into the box containing the words "Currently Viewing [filename]"
11. 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.)
12. Make sure dilution factors are correct and that blanks, standards, and unknowns are labeled properly.
13. Scroll to the bottom of the data and click the "ascent" button on the bottom right.
14. If you are prompted, select "Save as" and continue.
15. Copy the newly created .csv file and your wiff and wiff.scan files for your run into the Indigo Dropbox
16. Click on the folder icon on the bottom left of your screen to open a new window
17. In the new window, locate the folder labeled "+NCEH_DLS_Indigo_Dropbox-FC" and click on it so that it is highlighted.
    a. Right-click and select "New → Folder". A blank folder will appear. Inside the folder, type the name of the batch you are uploading.
    b. 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.
    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. 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. (Note: the OS software creates 2 extra file types during analysis do not include these in the upload). *This is the same folder where you just put the .csv file*

18. 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.

e. 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.
   a. R2 needs to be >0.9990
   b. All concentration deviations need to be < 15% except standards ≤ 0.05 ng/mL need to have concentration deviations < 30%
   c. 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.
   a. 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
   a. 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”.

f. Calculations

Indigo Ascent calculates all sample concentrations using the calibration curve associated with the run. The software reports results in ng/mL and uses a corresponding dilution factor for each sample. Final reported results are blank subtracted.

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*0.2-0.002)/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. Method Performance Specifications

Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in Appendix A of this method documentation. The electronic approvals of the Branch Chief and the CLIA Laboratory Director of the Division of Laboratory Sciences denote that the method performance is fit for the intended use of the method

a. Accuracy

Accuracy was tested on at least two days using at least four determinations per concentration level for nine concentrations that ranged across both calibration curves (low and high). The analytes were spiked into nonsmoker serum and the samples were worked up the usual way. The unspiked serum was analyzed at least four times each day to determine the mean background concentrations of COT and HCT; 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 ±12% of the theoretical value at all levels except at the LLOQ where it was within ±15% of the theoretical values. See Appendix A for accuracy results.

b. Precision

To calculate within-run, between run and total precision, two QC materials were tested 20 times in 10 different analytical runs (2 analyses per run). Relative standard deviation was under 5% for both QC materials. See Appendix A for precision results.

c. Analytical Sensitivity

See Appendix A for limit of detection (LOD) calculation. [25]

d. Analytical Specificity

A high degree of analytical specificity is achieved with this UPLC/MS/MS method; however, there is always a possibility that a sample will have an unknown interference. The specificity of the assay was established by analyzing serum samples from 84

nonsmokers. No interferences were seen in the Quant or ISTD chromatograms for either analyte in any of the samples.

Specificity is monitored by checking that the confirmation ion ratios are within established limits. See section 10a for how to establish the limits. The confirmation ion ratio ranges are determined using standards data from all standards for that day with concentrations ≥ 0.02 ng/mL. The confirmation ion ratio range is applied to all samples for that day with calculated concentrations ≥ 0.1 ng/mL. If a sample does not meet the confirmation ratio limits then it is repeated. If it fails again then the result is not reported; there is likely a contaminant in the sample. See Appendix A for specificity results

e. Stability

Stability testing ensures the measured concentration of analytes is not altered by conditions that the sample is likely to encounter during analysis. Multiple stability tests were evaluated with three replicates of a low and high QC. The mean values from the replicates were within 15% of the established value for each QC. See Appendix A for stability results.

f. Reportable Range of Test Results

The lower reportable limit is the LOD. The upper reportable limit is the highest standard concentration which is 25 ng/mL for a sample volume of 0.2 mL and 400 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.

g. Reference Range

Since the population includes both smokers and nonsmokers, the range of COT values is quite broad. The distribution of serum COT in U.S. nonsmokers for the period 1988 to 1991 was established from the analyses conducted as part of NHANES III, Phase 1. Those results have been published [26]. Subsequent evaluations have indicated a decline in the median level of serum COT among nonsmokers [22]. A NHANES survey, for which serum cotinine data have been published (survey years 2017-2018), found the 50th and 95th percentile level in nonsmokers to be 0.016 and 0.305 ng/mL, respectively [27-30].

To distinguish smokers from nonsmokers, Jarvis et al. [31] estimated a cutoff value of 13.7 ng/mL for plasma cotinine levels as measured by gas chromatography, and Benowitz et al. [4] suggested a cutoff of approximately 10 ng/mL. More recently Benowitz et al [32] 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. A NHANES survey, for which serum cotinine data have been published (survey years 2017-2018), found the 50th and 95th percentile level in smokers to be 211 and 494 ng/mL, respectively [33].

h. Matrix Effects and Recovery

Matrix effects (ME) and extraction recoveries (RE) were measured according to the method of Matuszewski [34]. 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 eighteen different nonsmoker serums in duplicate 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 100% for these 18 serum samples and HCT had an average ME of 99.1%. Average extraction recoveries were 74.4% and 79.4% respectively. See Appendix A for matrix effects results.

i. Ruggedness test

Method ruggedness for the serum assay was tested by varying the following parameters: %IPA in DCM, elution pressure, N2 evaporation pressure, N2 evaporation temp and N2 evaporation time. Each parameter was tested at the method level using a low bench QC pool. See Appendix A for ruggedness testing results.

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

This assay measures two analytes, COT and HCT. The QC evaluation considers each analyte independently 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 HCT QC and blank samples are in control, then the HCT 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 6e. 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 [35].

2. Blanks. For a low run, reject run if COT blank > 0.0086 ng/mL or if HCT blank > 0.0010 ng/mL. For a high run, reject high run if COT blank > 0.050 ng/mL or if HCT 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.

   a. COT Confirmation ion = m/z 177 → 98
   b. COT Quantitation ion = m/z 177 → 80
   c. HCT Confirmation ion = m/z 193 → 134
   d. HCT Quantitation ion = m/z 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 ≥ 0.1 ng/mL. Select those samples for further evaluation that have a calculated concentration ≥ 0.1 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 5 ng/mL for the low serum assay and no greater than 20 ng/mL for the high serum assay. (If the sample volume is 0.2 mL for a low sample, then the calculated result is valid if it is no greater than 25 ng/mL. If the sample volume is 0.05 mL for a high sample, then the calculated result is valid if it is no greater than 400 ng/mL.) Select samples with values greater than these limits 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 [35].

c. Proficiency Testing

Prepare four serum proficiency testing (PT) pools for this assay. Aliquot out the pools into 2mL coded cryovials and freeze the vials in freezers. 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 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 Systems Fail to Meet Acceptable Criteria: Nonconforming Events (NCEs)

a. Internal Standard Response

If the peak height of the HCT 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.

1. Clean the mass spectrometer front end including curtain gas plate orifice, and needle.
2. 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.

1. Standards checks: analyze the standards on another instrument. If the standards have become unsuitable prepare new standards.

2. 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.
3. 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.

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.

f. Limitations of Method; Interfering Substances and Conditions

In some studies, other nicotine metabolites (e.g., HCT) 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 HCT. The presence of other interfering substances in a particular sample should be indicated by a deviation in the expected confirmation ratio for that sample.

12. Specimen Storage and Handling During Testing

Store samples in freezers or ultralow freezers until they are analyzed. Remove the 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 rack/boxes with run ID on the boxes. Keep samples in freezers during QA review, and after dense packing.

13. Alternate 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 in freezers until the problem is resolved. Samples that have been extracted and reconstituted can be kept at room temperature for up to two years.

b. Proper Storage Procedures

Extracted, reconstituted samples need to be sealed and can be kept at room temperature for up to two years.

14. 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 reviewed by the data team and then 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.

15. Procedures for Specimen Accountability and Tracking

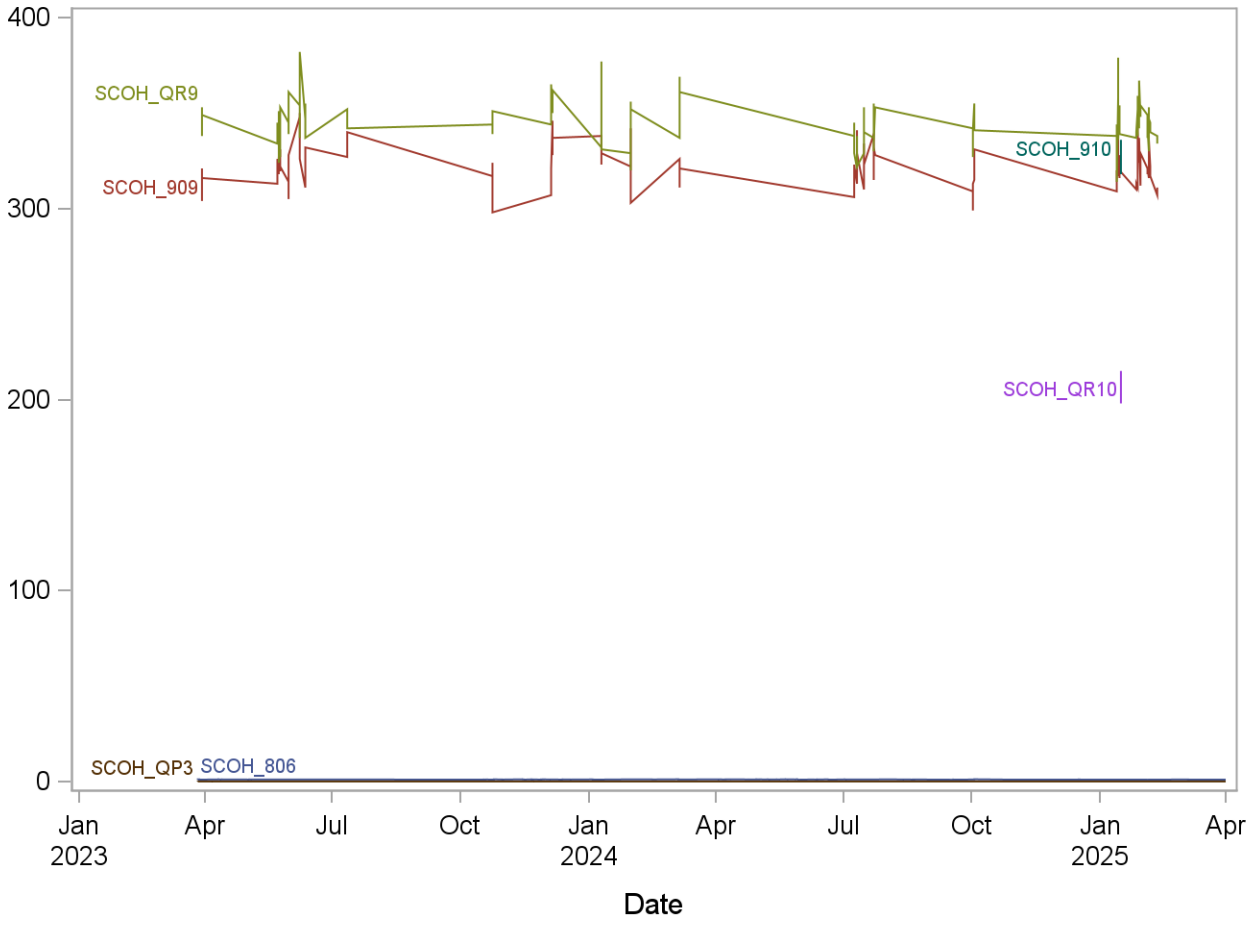
Standard record keeping (e.g., sample ID, notebooks, data files, databases, etc.) is used for sample tracking. All records are maintained in accordance with the HHS Records Management guidance. (See: http://www.hhs.gov/open/records/index.html)

16. Summary Statistics and QC Graphs

Please see following pages.

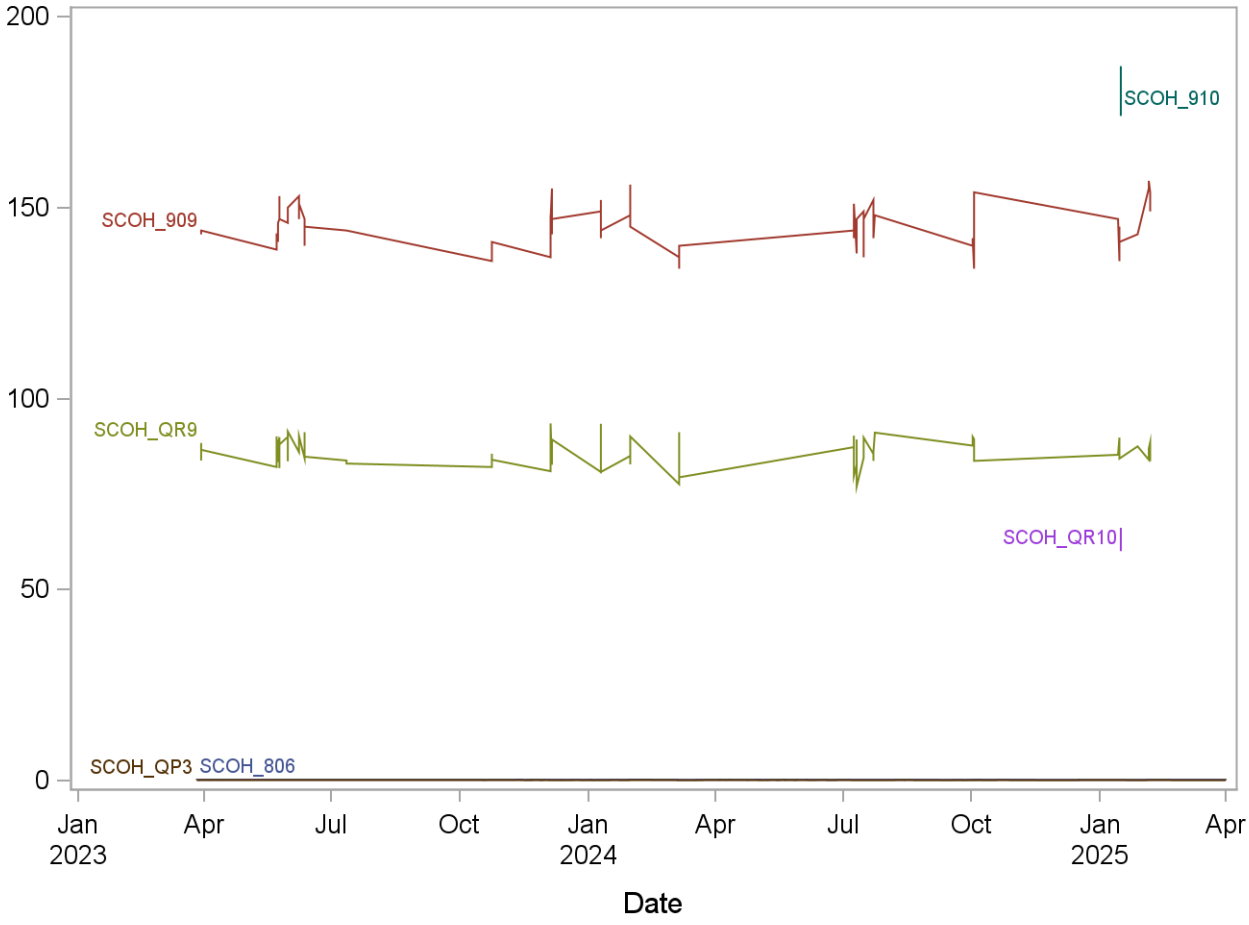
August 2021-August 2023 Summary Statistics and QC Chart
LBXCOT (Cotinine, Serum (ng/mL))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
SCOH_806	326	27MAR23	01APR25	1.1451	0.0495	4.3
SCOH_909	116	30MAR23	11FEB25	322.7586	10.7190	3.3
SCOH_910	4	16JAN25	16JAN25	329.0000	7.7028	2.3
SCOH_QP3	326	27MAR23	01APR25	0.1235	0.0056	4.5
SCOH_QR10	4	16JAN25	16JAN25	206.5000	7.2342	3.5
SCOH_QR9	116	30MAR23	11FEB25	344.5690	12.2838	3.6



August 2021-August 2023 Summary Statistics and QC Chart
LBXHCOT (Hydroxycotinine, Serum (ng/mL))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
SCOH_806	349	27MAR23	01APR25	0.2565	0.0125	4.9
SCOH_909	82	30MAR23	06FEB25	145.3415	5.2263	3.6
SCOH_910	4	16JAN25	16JAN25	179.5000	6.1373	3.4
SCOH_QP3	349	27MAR23	01APR25	0.1501	0.0059	4.0
SCOH_QR10	4	16JAN25	16JAN25	63.1000	2.5073	4.0
SCOH_QR9	82	30MAR23	06FEB25	86.2341	3.5175	4.1



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18. Appendix A: Method Validation and Performance Documentation

a. 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:	Cotinine and Hydroxycotinine in Serum
Method #:	2017
Matrix:	serum
Units:	µg/L
Analyte:	**cotinine**

		Human Serum						Synthetic Serum					
				Measured concentration						Measured concentration			
	Replicate	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)
Sample	1	LL0	0.000	0.215	0.389	0.315		LL0	0.000	0.123	0.303	0.221	
	2			0.214	0.401					0.117	0.327		
	3			0.206	0.466					0.155	0.299		
Sample + Spike 1	1	H1	25.000	24.935	26.113	25.073	99.0	H1	25.000	26.485	24.824	24.963	99.0
	2			24.464	24.361					25.567	23.094		
	3			25.078	25.486					25.969	23.838		
Sample + Spike 2	1	H2	100.00	101.10	93.15	99.02	98.7	H2	100.00	103.72	96.26	101.21	101.0
	2			98.20	104.09					103.81	98.42		
	3			96.84	100.73					106.81	98.24		
Sample + Spike 3	1	H3	200.0	211.5	202.2	200.5	100.1	H3	200.0	193.3	192.9	198.3	99.1
	2			195.2	200.9					210.1	198.7		
	3			199.6	193.9					195.2	199.8		

*Day 1: BE528-531, first three replicates reported.

**Day 2: BE512-515, first three replicates reported.

first three LL0 values reported

Mean recovery (%)	SD (%)
99.5	0.9

		Human Serum					Synthetic Serum				
			Measured concentration					Measured concentration			
	Replicate	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)
Sample	1	LL0	0.000	0.120	0.162	0.136		LL0	0.000	0.014	0.016	0.015	
	2			0.117	0.134					0.011	0.012		
	3			0.136	0.146					0.009	0.026		
Sample + Spike 1	1	L1	0.500	0.623	0.630	0.638	100.5	L1	0.500	0.491	0.540	0.522	101.4
	2			0.624	0.671					0.513	0.562		
	3			0.628	0.654					0.517	0.507		
Sample + Spike 2	1	L2	5.00	5.28	5.30	5.25	102.3	L2	5.00	4.78	5.01	4.95	98.7
	2			5.35	5.16					4.86	5.12		
	3			5.09	5.32					4.93	5.01		
Sample + Spike 3	1	L3	15.0	16.0	16.8	16.3	107.9	L3	15.0	15.0	15.6	15.7	104.4
	2			16.5	16.7					15.6	16.4		
	3			15.8	16.2					15.8	15.7		

*Day 1: BE528-531, first three replicates reported.

**Day 2: BE512-515, first three replicates reported.

first three LL0 values reported

Mean recovery (%)	SD (%)
102.5	3.2

Method name:	Cotinine and Hydroxycotinine in Serum
Method #:	2017
Matrix:	serum
Units:	µg/L
Analyte:	**trans-3'-hydroxycotinine**

		Human Serum						Synthetic Serum					
				Measured concentration						Measured concentration			
	Replicate	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)
Sample	1	LL0	0.000	0.002	0.034	0.024		LL0	0.000	0.000	0.000	0.000	
	2			0.005	0.040					0.000	0.000		
	3			0.000	0.065					0.000	0.000		
Sample + Spike 1	1	H1	25.000	25.845	27.072	26.160	104.5	H1	25.000	28.756	28.151	27.496	110.0
	2			26.237	26.362					28.494	24.799		
	3			24.875	26.567					27.910	26.869		
Sample + Spike 2	1	H2	100.00	104.29	105.92	107.60	107.6	H2	100.00	113.62	113.53	111.62	111.6
	2			108.61	113.93					109.10	108.53		
	3			103.77	109.10					113.21	111.74		
Sample + Spike 3	1	H3	200.0	213.4	226.2	217.7	108.8	H3	200.0	218.5	219.0	222.4	111.2
	2			217.3	213.3					237.1	222.0		
	3			220.1	216.0					216.8	220.9		

*Day 1: BE528-531, first three replicates reported.

**Day 2: BE512-515, first three replicates reported.

first three LL0 values reported

Mean recovery (%)	SD (%)
109.0	2.6

		Human Serum						Synthetic Serum					
				Measured concentration						Measured concentration			
	Replicate	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)	spike solution	Spike conc.	Day 1*	Day 2**	Mean	Recovery (%)
Sample	1	LL0	0.000	0.057	0.062	0.058		LL0	0.000	0.000	0.001	0.001	
	2			0.056	0.062					0.000	0.001		
	3			0.053	0.060					0.000	0.003		
Sample + Spike 1	1	L1	0.500	0.610	0.638	0.619	112.1	L1	0.500	0.543	0.576	0.556	111.1
	2			0.603	0.649					0.552	0.544		
	3			0.610	0.602					0.558	0.564		
Sample + Spike 2	1	L2	5.00	5.39	5.34	5.20	102.7	L2	5.00	5.54	5.64	5.58	111.7
	2			5.15	5.07					5.44	5.63		
	3			5.10	5.12					5.63	5.63		
Sample + Spike 3	1	L3	15.0	14.0	14.6	14.4	95.4	L3	15.0	15.9	16.5	16.6	111.0
	2			14.5	14.5					17.4	16.6		
	3			14.0	14.6					16.5	16.9		

*Day 1: BE496-499R2, first three replicates reported.

**Day 2: BE500-503, first three replicates reported.

Mean recovery (%)	SD (%)
107.3	6.8

b. Precision

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

Method name:	Cotinine and Hydroxycotinine in Serum
Method #:	2017
Matrix:	serum
Units:	µg/L
Analyte:	**cotinine**

Quality material 1 **806***

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1.22	1.25	1.23	0.000289	0.000289	3.045734
2	1.20	1.17	1.19	0.000202	0.000202	2.817036
3	1.20	1.23	1.22	0.000195	0.000195	2.953228
4	1.11	1.15	1.13	0.000396	0.000396	2.565611
5	1.16	1.15	1.15	0.000031	0.000031	2.662924
6	1.21	1.19	1.20	0.000043	0.000043	2.884081
7	1.14	1.16	1.15	0.000132	0.000132	2.631080
8	1.17	1.16	1.17	0.000074	0.000074	2.714590
9	1.16	1.14	1.15	0.000061	0.000061	2.636015
10	1.14	1.14	1.14	0.000003	0.000003	2.592752

Grand sum 23.44399 **Grand mean** 1.1721995

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.002851111	0.000285111	0.016885234	1.44
Between Run	0.022017317	0.002446369	0.032872918	2.80
Total	0.024868428		0.036955917	**3.15**

*Runs: BE504-505, BE516-517, BE524-525, BE492-493, BE496-497R2, BE500-501, BE538-539, BD132-133R1, BD136-137R1, BD140-141R1

Quality material 2 **QP3***

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	0.129	0.130	0.13	2.21E-07	2.21E-07	0.033303
2	0.133	0.134	0.13	2.30E-07	2.30E-07	0.035495
3	0.129	0.129	0.13	4.90E-09	4.90E-09	0.033117
4	0.135	0.130	0.13	5.76E-06	5.76E-06	0.035155
5	0.133	0.129	0.13	4.00E-06	4.00E-06	0.034285
6	0.134	0.125	0.13	1.69E-05	1.69E-05	0.033564
7	0.124	0.124	0.12	1.09E-07	1.09E-07	0.030831
8	0.129	0.131	0.13	1.77E-06	1.77E-06	0.033790
9	0.131	0.128	0.13	2.45E-06	2.45E-06	0.033605
10	0.124	0.127	0.13	1.74E-06	1.74E-06	0.031400

Grand sum 2.58612 **Grand mean** 0.129306

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	6.644E-05	6.6438E-06	0.0025776	1.99
Between Run	0.0001446	1.6062E-05	0.0021701	1.68

Total	0.000211	0.0033694	**2.61**

*Runs: BE516-517, BE526-527, BE492-493, BE496-497, BE500-501, BE538-539, BE542-543, BD132-133, BD134-135, BD136-137

Quality material 3 **909***

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	332.4	322.3	327.33	25.29	25.29	214285
2	324.0	319.3	321.66	5.69	5.69	206932
3	313.9	334.2	324.06	102.43	102.43	210030
4	323.2	330.1	326.65	11.60	11.60	213402
5	324.1	312.4	318.24	34.31	34.31	202550
6	305.3	320.6	312.97	58.74	58.74	195906
7	334.2	338.7	336.45	4.95	4.95	226399
8	331.8	336.3	334.06	5.11	5.11	223198
9	332.5	323.1	327.82	22.01	22.01	214935
10	346.7	324.2	335.44	127.12	127.12	225035

Grand sum 6529.36952 **Grand mean** 326.468476

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	794.5075801	79.45075801	8.913515469	2.73
Between Run	1038.276465	115.3640517	4.23752839	1.30
Total	1832.784046		9.869518978	**3.02**

*Runs: BE528-529, BE512-513, BE532-533R1, BE550-551, BE554-555, BE558-559, BE569-570, BE577-578, BE581-582, BE585-586

Quality material 4 **QR9***

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	350.8	340.3	345.55	27.45	27.45	238814
2	335.8	328.6	332.21	13.07	13.07	220729
3	347.8	343.9	345.84	3.73	3.73	239208
4	342.8	355.3	349.06	38.79	38.79	243688
5	325.0	331.8	328.37	11.49	11.49	215650
6	335.5	339.4	337.47	3.79	3.79	227769
7	346.7	350.9	348.79	4.35	4.35	243310
8	354.2	350.3	352.24	3.87	3.87	248148
9	364.9	364.4	364.65	0.04	0.04	265933
10	333.3	328.3	330.81	6.28	6.28	218875

Grand sum 6869.9809 **Grand mean** 343.49904

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	225.69522	22.5695223	4.7507391	1.38
Between Run	2291.8311	254.647903	10.772149	3.14
Total	2517.5263		11.77322	**3.43**

*Runs: BE512-513, BE530-531, BE532-533, BE534-535, BE550-551, BE554-555, BE569-570, BE581-582, BE585-586, BE590-591R2

Method name:	Cotinine and Hydroxycotinine in Serum
Method #:	2017
Matrix:	serum
Units:	µg/L
Analyte:	**trans-3'-hydroxycotinine**

Quality material 3	**806***					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.260	0.276	0.27	0.000063	0.000063	0.143782
2	0.275	0.267	0.27	0.000017	0.000017	0.147110
3	0.253	0.279	0.27	0.000169	0.000169	0.141007
4	0.253	0.263	0.26	0.000029	0.000029	0.133169
5	0.270	0.267	0.27	0.000002	0.000002	0.144152
6	0.257	0.248	0.25	0.000018	0.000018	0.127614
7	0.253	0.250	0.25	0.000003	0.000003	0.126520
8	0.264	0.258	0.26	0.000008	0.000008	0.136440
9	0.248	0.237	0.24	0.000029	0.000029	0.117875
10	0.253	0.258	0.26	0.000007	0.000007	0.130315

Grand sum	5.18941	**Grand mean**	0.2594705

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.000687568	6.87568E-05	0.008291971	3.20
Between Run	0.001484992	0.000164999	0.006936942	2.67
Total	0.00217256		0.01081101	**4.17**

*Runs: BE504-505, BE516-517, BE524-525, BE492-493, BE496-497R2, BE500-501, BE538-539, BD132-133R1, BD136-137R1, BD140-141R1

Quality material 2	**QP3***					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.154	0.147	0.15	1.36E-05	1.36E-05	0.04541
2	0.154	0.154	0.15	1.30E-07	1.30E-07	0.04741
3	0.151	0.159	0.15	1.85E-05	1.85E-05	0.04794
4	0.160	0.153	0.16	1.03E-05	1.03E-05	0.04906
5	0.157	0.153	0.16	3.72E-06	3.72E-06	0.04822
6	0.162	0.156	0.16	9.92E-06	9.92E-06	0.05075
7	0.158	0.158	0.16	3.24E-08	3.24E-08	0.04997
8	0.154	0.157	0.16	2.28E-06	2.28E-06	0.04851
9	0.152	0.160	0.16	1.54E-05	1.54E-05	0.04855
10	0.155	0.157	0.16	1.40E-06	1.40E-06	0.04849

Grand sum	3.11195	**Grand mean**	0.1555975

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.0001506	1.5056E-05	0.0038802	2.49
Between Run	9.686E-05	1.0762E-05	0	0.00
Total	0.0002474		0.0038802	**2.49**

*Runs: BE516-517, BE526-527, BE492-493, BE496-497, BE500-501, BE538-539, BE542-543, BD132-133, BD134-135, BD136-137

Quality material 2	909*					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	146.0	144.2	145.10	0.78	0.78	42109
2	140.9	145.5	143.18	5.32	5.32	40998
3	148.3	143.2	145.74	6.56	6.56	42483
4	144.4	144.2	144.29	0.01	0.01	41637
5	145.3	150.7	147.96	7.23	7.23	43786
6	134.9	149.6	142.22	53.98	53.98	40456
7	142.5	145.1	143.83	1.70	1.70	41375
8	147.6	139.5	143.56	16.39	16.39	41216
9	148.4	147.1	147.77	0.43	0.43	43670
10	147.4	139.0	143.21	17.65	17.65	41016

Grand sum	2893.71321	**Grand mean**	144.6856605

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	220.0961422	22.00961422	4.691440527	3.24
Between Run	68.44288281	7.604764757	0	0.00
Total	288.539025		4.691440527	**3.24**

*Runs: BE528-529, BE512-513, BE532-533R1, BE550-551, BE554-555, BE558-559, BE569-570, BE577-578, BE581-582, BE585-586

Quality material 4	QR9*					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	87.1	84.3	85.70	2.04	2.04	14690
2	84.3	77.8	81.06	10.83	10.83	13141
3	81.6	83.7	82.67	1.13	1.13	13669
4	77.8	84.0	80.90	9.50	9.50	13089
5	84.3	83.7	84.00	0.10	0.10	14112
6	84.1	86.7	85.37	1.65	1.65	14578
7	86.1	86.3	86.19	0.02	0.02	14858
8	89.2	87.6	88.38	0.62	0.62	15623
9	88.4	87.9	88.16	0.07	0.07	15543
10	82.9	85.7	84.29	1.88	1.88	14209

Grand sum	1693.4472	**Grand mean**	84.672361

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	55.667986	5.56679855	2.3594064	2.79
Between Run	123.33204	13.7035604	2.0170228	2.38
Total	179.00003		3.1040586	**3.67**

*Runs: BE512-513, BE530-531, BE532-533, BE534-535, BE550-551, BE554-555, BE569-570, BE581-582, BE585-586, BE590-591R2

c. Stability

Stability was tested three ways.

(a) To test analyte integrity after freezing and thawing, the analytes were measured in a two QC pools in serum that had been through four 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). All N = 3. The means were tested using Student's T test and found to be the same (with all p>0.05).

Freeze-Thaw Results

Pool	Matrix	Mean (SD) (ng/mL)	Std Dev (ng/mL)	Analyte	FT cycles
806	serum	1.09	0.014	COT	1
806	serum	1.06	0.039	COT	4
806	serum	0.223	0.004	HC	1
806	serum	0.223	0.010	HC	4
QR2	serum	193	0.645	COT	1
QR2	serum	193	1.37	COT	4
QR2	serum	54.4	0.273	HC	1
QR2	serum	54.6	0.739	HC	4

(b) 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^{o}C for one week. All N = 3. The means were tested using a one-way anova (by Proc GLM in SAS) and found to be the same for all storage periods (all p > 0.05).

Stability of Extracted Samples

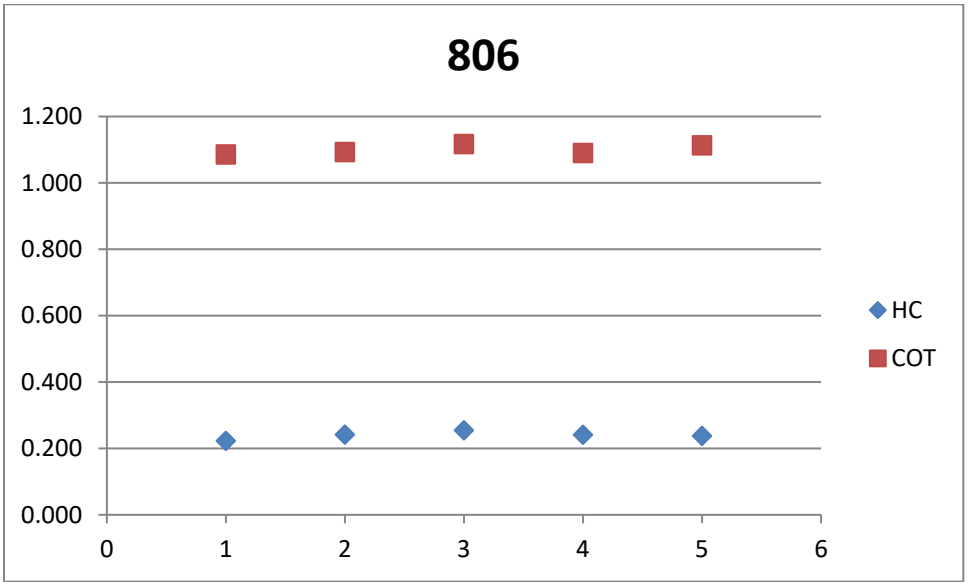
Pool	Matrix	Mean (SD) (ng/mL)	Std Dev (ng/mL)	Analyte	Storage
806	serum	1.18	0.047	COT	1 day
806	serum	1.20	0.055	COT	1 week
806	serum	1.16	0.051	COT	none
806	serum	0.251	0.014	HC	1 day
806	serum	0.261	0.027	HC	1 week
806	serum	0.258	0.009	HC	none
QR2	serum	202	1.90	COT	1 day
QR2	serum	207	5.24	COT	1 week
QR2	serum	201	1.42	COT	none
QR2	serum	56.2	1.08	HC	1 day

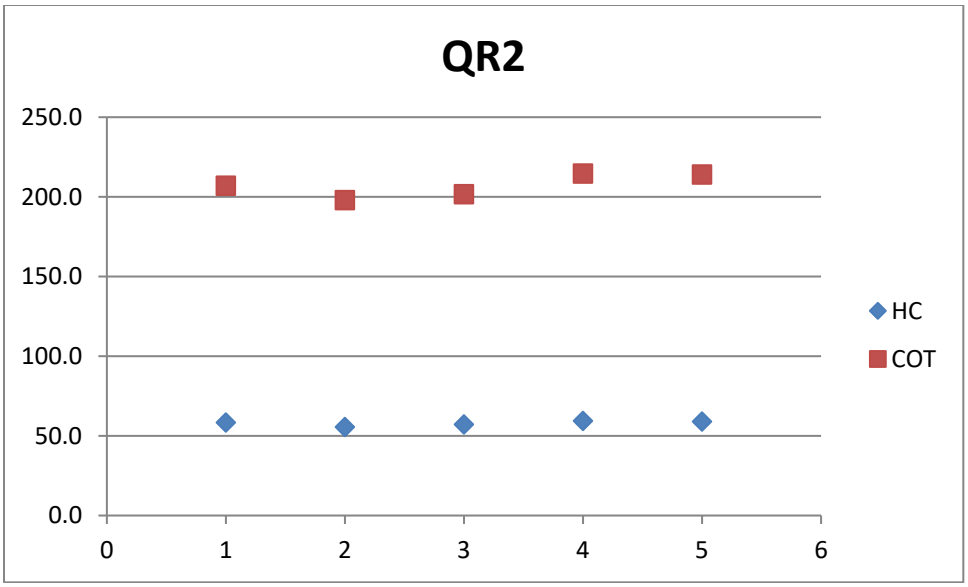
QR2	serum	56.1	1.01	HC	1 week
QR2	serum	56.8	0.837	HC	none

(c) 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 charts below. The data show no systematic decrease in analyte values.

Accelerated Stability Study

POOL	Pool Type	EXPERIMENT	MEAN HC conc (ng/mL)	MEAN COT conc (ng/mL)
806	low serum	control	0.223	1.086
806	low serum	RT1	0.242	1.09
806	low serum	RT2	0.255	1.12
806	low serum	37_1	0.241	1.09
806	low serum	37_2	0.238	1.11
QR2	high serum	Control	58.4	207
QR2	high serum	RT1	55.6	198
QR2	high serum	RT2	57.2	202
QR2	high serum	37_1	59.4	215
QR2	high serum	37_2	59.0	214





Note: For all the above charts the following define the points:

X-Axis point	EXPERIMENT
1	control
2	Room Temp 1 week
3	Room Temp 2 weeks
4	37°C 1 week
5	37°C 2 weeks

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: four times frozen at ≤ -70°C and then thawed (4 freeze-thaw cycles) (RunID=BB850, 1/8/14)
Bench-top stability1 and 2 = 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, 2 weeks (RunID=BB851, 1/8/14)
Bench-top stability3 and 4 = Accelerated stability study to assess extreme conditions
Describe condition: original samples (not yet prepared for instrument analysis) stored at 37°C in a water bath for 1 week, 2 weeks (RunID=BB851, 1/8/14)
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 (RunID=BB853, 1/8/14)
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for >5 years (806) and >2 years (QR2) (AF189-191, 7/5/19, 806; BC712-714, 4/27/16, QR2)

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

Method name: Cotinine and Hydroxycotinine in Serum and Saliva
Method #: 2017
Matrix: serum
Units: µg/L
Analyte: **cotinine**

Quality material 1	**806**							
	Initial measurement	**Four freeze-thaw cycles**	**Bench-top stability1**	**Bench-top stability2**	**Bench-top stability3**	**Bench-top stability4**	**Processed sample stability**	**Long-term stability**
Replicate 1	1.09	1.10	1.11	1.12	1.12	1.12	1.20	1.17
Replicate 2	1.07	1.02	1.11	1.15	1.05	1.11	1.12	1.13
Replicate 3	1.10	1.05	1.06	1.08	1.10	1.11	1.21	1.12
Mean	1.09	1.06	1.09	1.12	1.09	1.11	1.18	1.14
% difference from initial measurement	--	**-2.8**	**0.7**	**3.0**	**0.5**	**2.6**	**8.4**	**4.9**

Quality material 2	**QR2**							
	Initial measurement	**Four freeze-thaw cycles**	**Bench-top stability1**	**Bench-top stability2**	**Bench-top stability3**	**Bench-top stability4**	**Processed sample stability**	**Long-term stability**
Replicate 1	195	194	204	213	215	218	203	195
Replicate 2	196	195	198	195	221	215	204	214
Replicate 3	194	197	192	197	208	209	200	199
Mean	195	195	198	202	215	214	202	203
% difference from initial measurement	--	**0.1**	**1.5**	**3.4**	**10.1**	**9.7**	**3.5**	**3.8**

Stability

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: four times frozen at ≤ -70°C and then thawed (4 freeze-thaw cycles) (RunID=BB850, 1/8/14)
Bench-top stability1 and 2 = 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, 2 weeks (RunID=BB851, 1/8/14)
Bench-top stability3 and 4 = Accelerated stability study to assess extreme conditions
Describe condition: original samples (not yet prepared for instrument analysis) stored at 37°C in a water bath for 1 week, 2 weeks (RunID=BB851, 1/8/14)
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 (RunID=BB853, 1/8/14)
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for >5 years (806) and >2 years (QR2) (AF189-191, 7/5/19, 806, BC712-714, 4/27/16, QR2)

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

Method name: Cotinine and Hydroxycotinine in Serum and S
Method #: 2017
Matrix: serum
Units: µg/L
Analyte: **trans-3'-hydroxycotinine**

Quality material 1	**806**							
	Initial measurement	**Four freeze-thaw cycles**	**Bench-top stability1**	**Bench-top stability2**	**Bench-top stability3**	**Bench-top stability4**	**Processed sample stability**	**Long-term stability**
Replicate 1	0.226	0.234	0.236	0.285	0.252	0.242	0.242	0.261
Replicate 2	0.225	0.221	0.252	0.240	0.234	0.235	0.243	0.248
Replicate 3	0.218	0.215	0.237	0.239	0.237	0.236	0.267	0.252
Mean	0.223	0.223	0.242	0.255	0.241	0.237	0.251	0.254
% difference from initial measurement	--	**0.1**	**8.3**	**14.2**	**7.9**	**6.5**	**12.4**	**13.7**

Quality material 2	**QR2**							
	Initial measurement	**Four freeze-thaw cycles**	**Bench-top stability1**	**Bench-top stability2**	**Bench-top stability3**	**Bench-top stability4**	**Processed sample stability**	**Long-term stability**
Replicate 1	55.2	54.5	58.2	60.4	59.3	61.6	57.4	55.1
Replicate 2	55.2	55	55.2	55.5	61.6	58.4	55.4	61.6
Replicate 3	54.7	56	53.3	55.6	57.3	57.0	55.6	52.4
Mean	55.0	55.2	55.6	57.2	59.4	59.0	56.2	56.4
% difference from initial measurement	--	**0.2**	**1.0**	**3.9**	**7.9**	**7.2**	**2.0**	**2.4**

d. LOD, Specificity and fit for intended use

Method name:	Cotinine and Hydroxycotinine in Serum
Method #:	2017
Matrix:	serum
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	serum	0.010	yes	yes
Hydroxycotinine	serum	0.004	yes	yes

Specificity

COT and HCT specificity in serum

BE520-523 on 03/09/2022
no interferences for any of the 96 samples in either analyte quant chromatogram
COT range: 0.001 to 4.43 ng/mL (not blank subtracted)
HCT range: 0.000 to 4.94 ng/mL (not blank subtracted)

e. Ruggedness

Method ruggedness was tested by varying the following parameters:

%IPA in DCM (N=5)
DCM Elution Pressure (N=5)
N2 Temperature (N=5)
N2 Pressure (N=5)
N2 Dry Time (N=5)

Each parameter was tested at the method level and at a lower and higher level, unless otherwise specified, using a low QC pool.

Parameter	Method Level	Lower level	Upper Level	COT (ng/mL)			HCT (ng/mL)		
				Result at Method level	Result at Lower level	Result at Upper Level	Result at Method level	Result at Lower level	Result at Upper Level
%IPA in DMC	5%	0%	10%	1.11	1.11	1.12	0.248	0.256	0.251
DCM Elution pressure*	0.5 PSI	1 PSI	2 PSI	1.11	1.12	1.13	0.248	0.248	0.256
N2 Temp	40°C	35°C	45°C	1.11	1.14	1.16	0.248	0.254	0.259
N2 pressure	50 PSI	40 PSI	55 PSI	1.11	1.18	1.17	0.248	0.264	0.262
N2 Dry Time*	35 min	40min	45min	1.11	1.16	1.17	0.248	0.258	0.266

All mean values were within 7% of method level values.

f. Recovery

Recoveries were estimated from the raw area counts observed for the ISTD relative to the mean observed for all of the standards assayed that day. Recoveries were tested separately for a range of concentrations using NHANES samples. Recoveries were reproducible at each concentration (3*SD error bars overlap for all levels of each analyte).

%IS recovery averages

	BE504-507			BE516-519			BE520-523			BE524-527		
	average	CV	N	average	CV	N	average	CV	N	average	CV	N
COT	-1.47%	7.70%	84	11.2%	7.02%	82	1.46%	5.72%	84	4.87%	10.6%	84
HCT	-14.7%	12.4%	84	-9.49%	7.89%	82	-10.6%	7.76%	84	-1.08%	13.4%	84

g. Matrix Calibration Curve Comparison

Calibrators should be in the same matrix as unknown samples to be analyzed. However, there are no sources of serum that do not have residual COT and HCT in them. Therefore, we made our calibration curve in water and validated the equivalency of calibration curve in water versus the curve in the matrix by comparing 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. A 12 calibrator matrix low curve and a 10-calibrator matrix high curve, see table below, were prepared as unknowns. A second set of calibrators was prepared by spiking the same amounts of COT and HCT into water. See the table below for the calibrator concentrations.

Low Matrix Curve

Calibrator #	COT and HCT Conc (ng/mL)
1	0
2	0.01
3	0.025
4	0.05
5	0.1
6	0.25
7	0.5
8	1
9	2.5
10	5
11	10
12	25

High Matrix Curve

Calibrator #	COT and HCT Conc (ng/mL)
1	0
2	2
3	4
4	10
5	20
6	40
7	100
8	200
9	300
10	400

2. The serum 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. The two sets of calibrators were analyzed on the instrument three times.

3. The three sets of calibration curves were compared. The slopes 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. All slope comparisons were within 5% so the slopes are equivalent. See table below for all data.

Low Curves

	Slopes								
	Non-matrix 1 CL0157	Matrix 1 BE636-1	Non-matrix 2 CL158	Matrix 2 BE636-2	Non-matrix 3 CL159	Matrix 3 BE636-3	Non-matrix avg.	Matrix avg.	%diff
COT	2.9258	2.8738	2.9445	2.8539	2.8450	2.8740	2.9051	2.8672	-1.30
HCT	2.4845	2.5153	2.4255	2.5528	2.5198	2.4984	2.4766	2.5222	1.84

High Curves

	Slopes								
	Non-matrix 1 GH593	Matrix 1 BE637-1	Non-matrix 2 GH594	Matrix 2 BE637-2	Non-matrix 3 GH595	Matrix 3 BE637-3	Non-matrix avg.	Matrix avg.	%diff
COT	2.7100	2.7269	2.6738	2.6667	2.6895	2.6842	2.6911	2.6926	0.06
HCT	2.2186	2.1972	2.2471	2.2196	2.1986	2.2350	2.2214	2.2173	-0.19

h. Matrix Effects

We measured the effect of the matrix according to the method of Matuszewski [34]. 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 eighteen different nonsmoker sera in duplicate 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 99.1% for these 18 serum samples and HCT had an average ME of 100%. Average extraction recoveries were 79.4% and 74.4% respectively. The average peak area of the internal standard spiking solution (case A, n=21) for HCT and COT was 23,806 and 46,784, respectively.

Results

Sample ID	HCT-ISTD peak area (case B)	COT-ISTD peak area (case B)	HCT-ISTD peak area (case C)	COT-ISTD peak area (case C)	ME% HCT (B/A)	ME% COT (B/A)	RE% HCT (C/B)	RE% COT (C/B)
3.33E+08	27,092	47,105	20,777	36,024	101.1	100.7	76.7	76.5
3.58E+08	26,252	45,308	20,165	37,739	97.9	96.8	76.8	83.3
1.82E+08	26,567	46,731	18,987	33,440	99.1	99.9	71.5	71.6
7.49E+08	25,660	44,655	20,004	37,722	95.7	95.4	78.0	84.5
2.21E+08	27,048	47,705	20,670	39,375	100.9	102.0	76.4	82.5
2.55E+08	24,758	43,300	20,362	33,221	92.4	92.6	82.2	76.7
5.14E+08	28,492	48,300	18,997	36,631	106.3	103.2	66.7	75.8
6.36E+08	26,912	45,645	18,361	34,965	100.4	97.6	68.2	76.6
8.94E+08	25,818	46,243	19,916	38,510	96.3	98.8	77.1	83.3
9.01E+08	26,239	44,738	21,529	37,335	97.9	95.6	82.1	83.5
2.15E+08	27,445	46,740	21,008	37,486	102.4	99.9	76.5	80.2
6.22E+08	28,143	47,682	19,546	37,412	105.0	101.9	69.5	78.5
9.49E+08	26,403	45,774	19,548	38,855	98.5	97.8	74.0	84.9
8.08E+08	26,514	46,847	18,905	33,437	98.9	100.1	71.3	71.4
9.7E+08	27,432	47,012	19,206	37,683	102.3	100.5	70.0	80.2
7.51E+08	27,863	47,096	19,008	37,877	103.9	100.7	68.2	80.4
3.01E+08	27,487	47,131	20,331	37,921	102.5	100.7	74.0	80.5
8.57E+08	26,480	46,420	21,071	36,556	98.8	99.2	79.6	78.8
Averages					100.0	99.1	74.4	79.4

i. Confirmation Ion Ratio Calculation

On a daily basis, specificity is monitored by checking confirmation ion ratios. The confirmation ion ratio is calculated 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 → 98
COT Quantitation ion = m/z 177 → 80
HCT Confirmation ion = m/z 193 → 134
HCT Quantitation ion = m/z 193 → 80

The confirmation ion ratio range is determined for each instrument from the mean of the standards for that day with concentrations > 0.04 ng/mL. See full ion ratio concentration plots in tables 1 and 3 below. Tables 2 and 4 show low concentration range to visualize how the cutoff was estimated. A final determination will be established upon completion of more analyses. Because of low ion counts for the confirmation ion, these evaluations are limited to samples with a calculated concentration > 0.1 ng/mL. Samples are repeated if they have a calculated concentration > 0.1 ng/mL and a confirmation ratio greater than 25% from the mean [36].

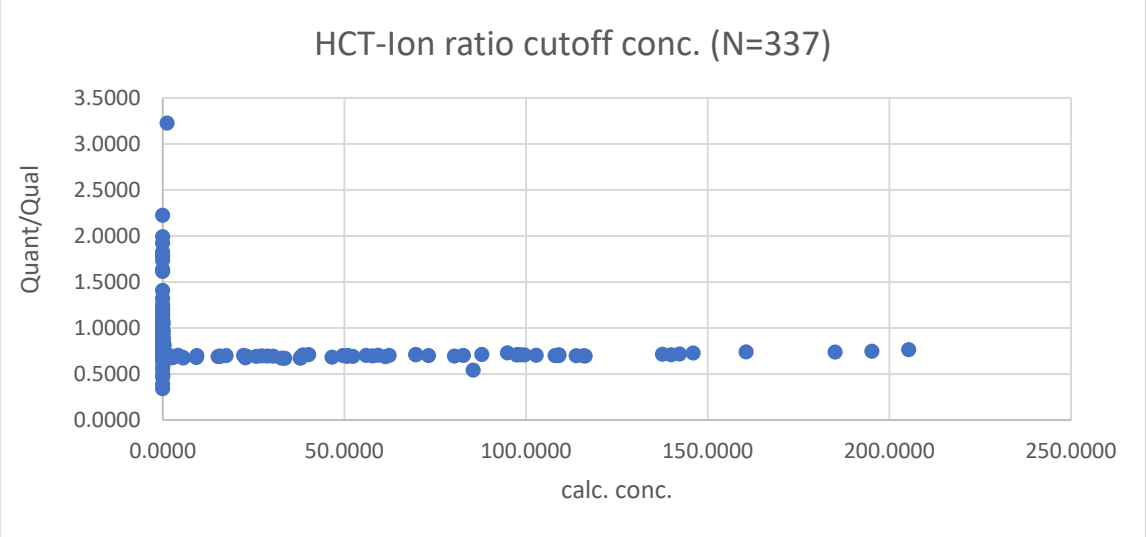


Figure 1: HCT ion ratio full concentration range

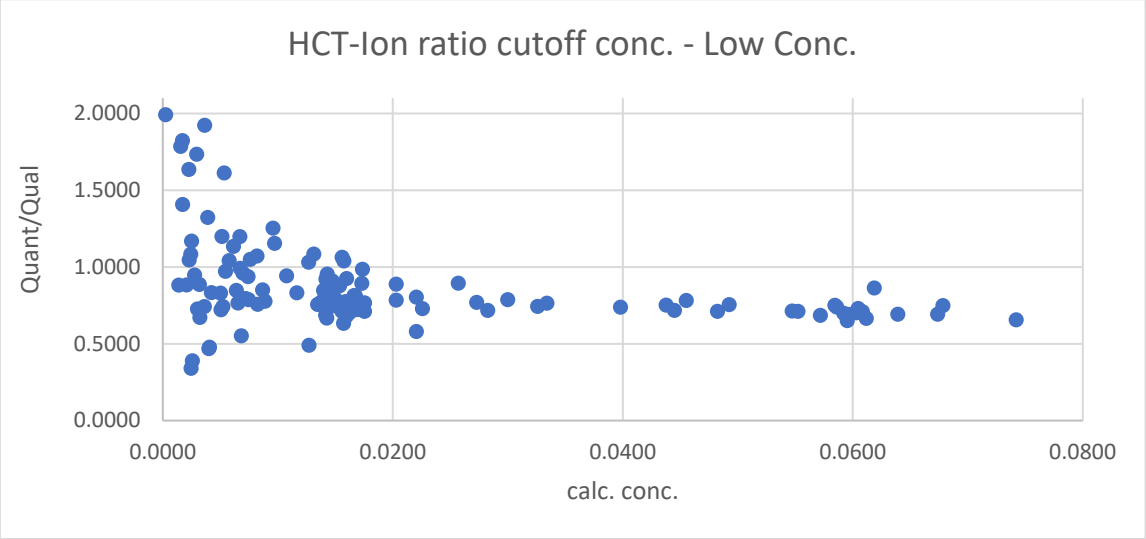


Figure 2: HCT ion ratio low concentration range

Table 1: COT Ion Ration Full Concentration Range

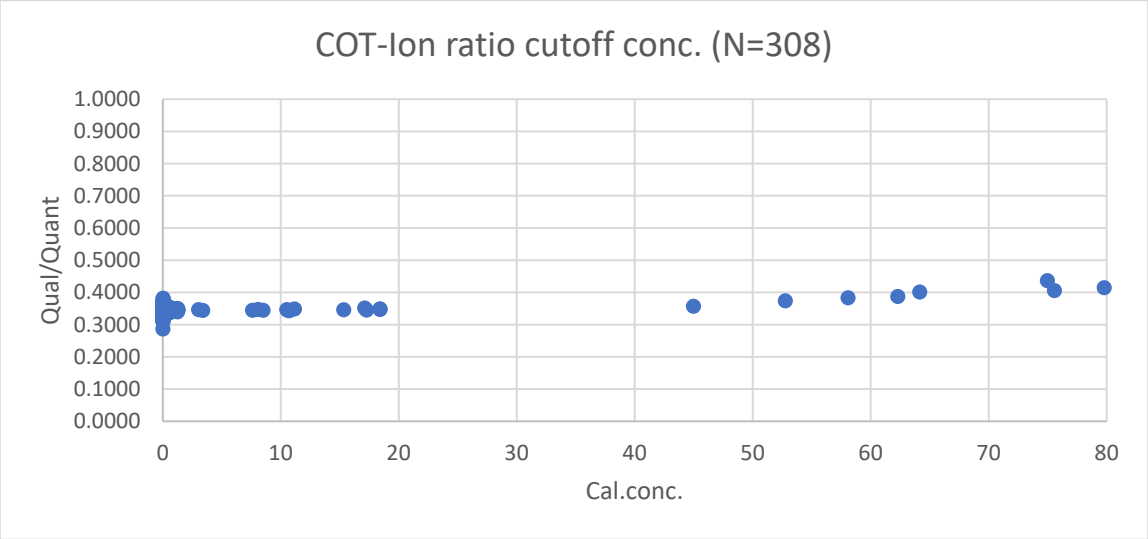


Figure 3: COT ion ratio full concentration range

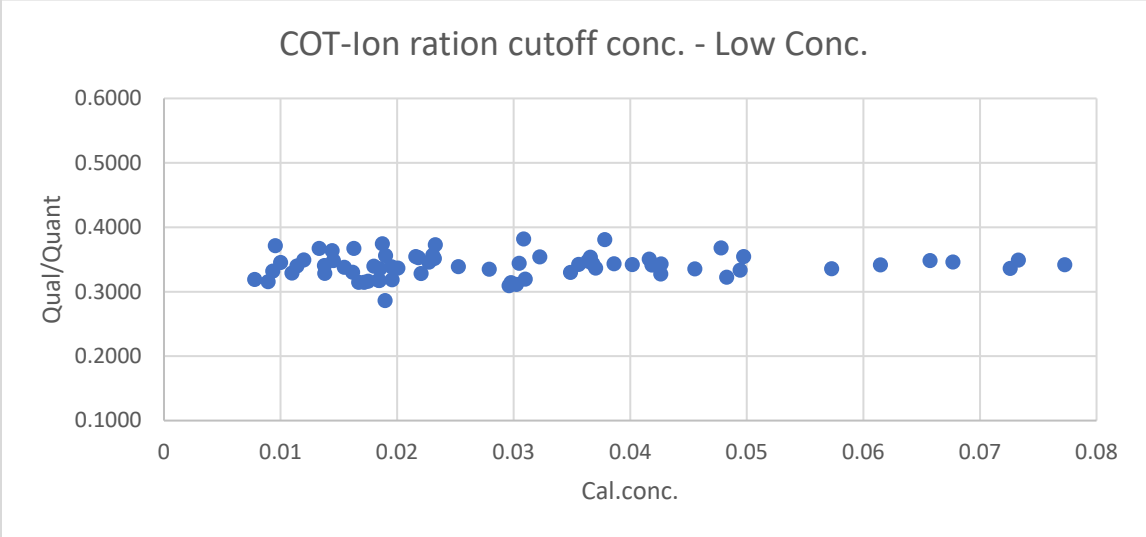


Figure 4: COT ion ratio low concentration range

j. Blank Carryover

To investigate potential carryover that might occur in a low sample immediately following a high sample, we compared the results of prepared water blank samples that were injected with vs. without a high sample injection preceding these blanks. There were 9 high samples with COT concentration ranging from 25 to 300 ng/mL, and each high sample injection was followed by 8 individual water blanks samples. At 73 ng/mL for COT concentration, we observed elevated baselines in one water blank sample following the high sample. The average difference of blank concentration with vs. without high sample injection was 0.0020 to 0.0066 ng/mL, which were below the estimated LOD and blank limits. We conclude that there is no significant carryover for serum COT. As a precaution, we will reinject one sample if its calculated concentration

is between 0.0086-0.0160 ng/mL, and if it is injected after a high COT sample concentration at 73 ng/mL or above.

k. Blank Cutoff

The blank cutoff is set at the mean + 3*SD of the analyte concentrations found in the water blank samples (N=64 for HCT, N=64 for COT). Blank cutoff will be recalculated as more blank samples are obtained in next analysis set.

	COT	HCT
Mean	0.0021	0.0001
Std Dev	0.0019	0.0002
Min	0.0008	0
Max	0.0203	0.0014
N	64	64
3*SD	0.00575	0.00072
M+3*SD	0.00788	0.00083

COT blank cutoff set at LOD= 0.010 ng/mL
HCT blank cutoff set at LOD= 0.005 ng/ml

For high runs the blank cutoffs can be higher without affecting the sample results. We will use a blank cutoff of 0.050 for both analytes in high runs.

l. Comparison of Method on two Instruments

In 2022, the UPLC systems were upgraded from Shimadzu Nexera systems to Waters I-Class systems. To compare the responses and compare across instruments, multiple analytical runs were analyzed before and after upgrade and across two low concentration instruments (7500 MS) and one high concentration instrument (6500 MS) The concentration of the analytes in the samples spanned the low and high calibration curves.

The paired results were plotted and linear regression was performed for concentrations 0-25 ng/mL on the two 7500 MS systems.
Method A: Instrument Cyberman, Shiamdzu Nexera UPLC System
Method B: Instrument Alexander, Waters I-Class UPLC System

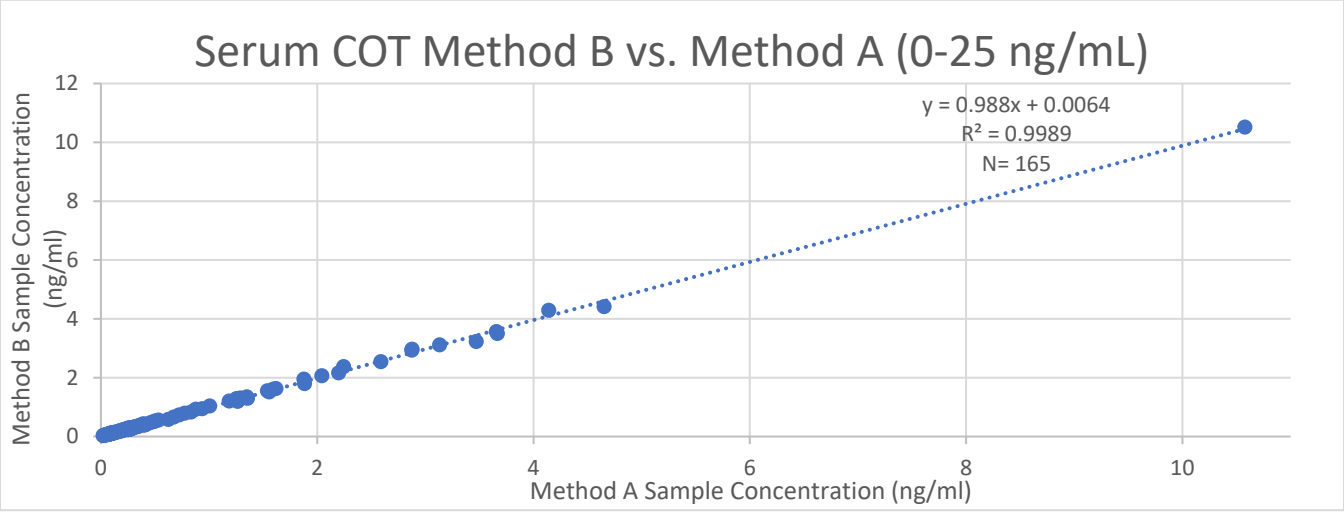


Figure 5: Comparison plot of the cotinine concentrations of all samples run with Method B vs. the same samples run with Method A for concentrations 0-25 ng/mL

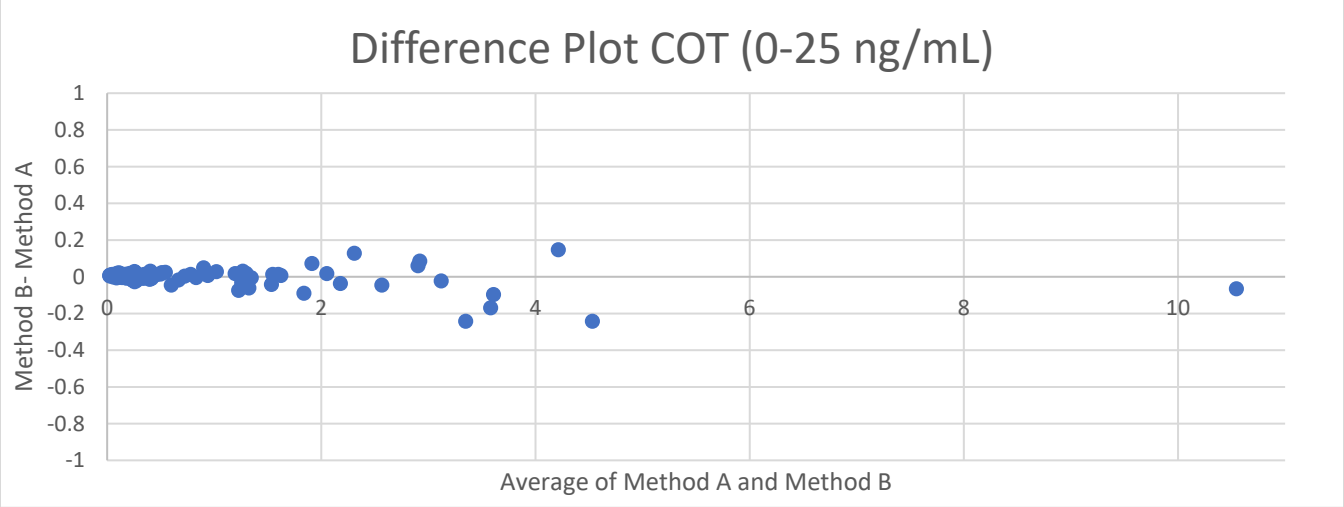


Figure 6: Method A-Method B vs. average of Method A and Method B plot for cotinine for concentrations 0-25 ng/mL

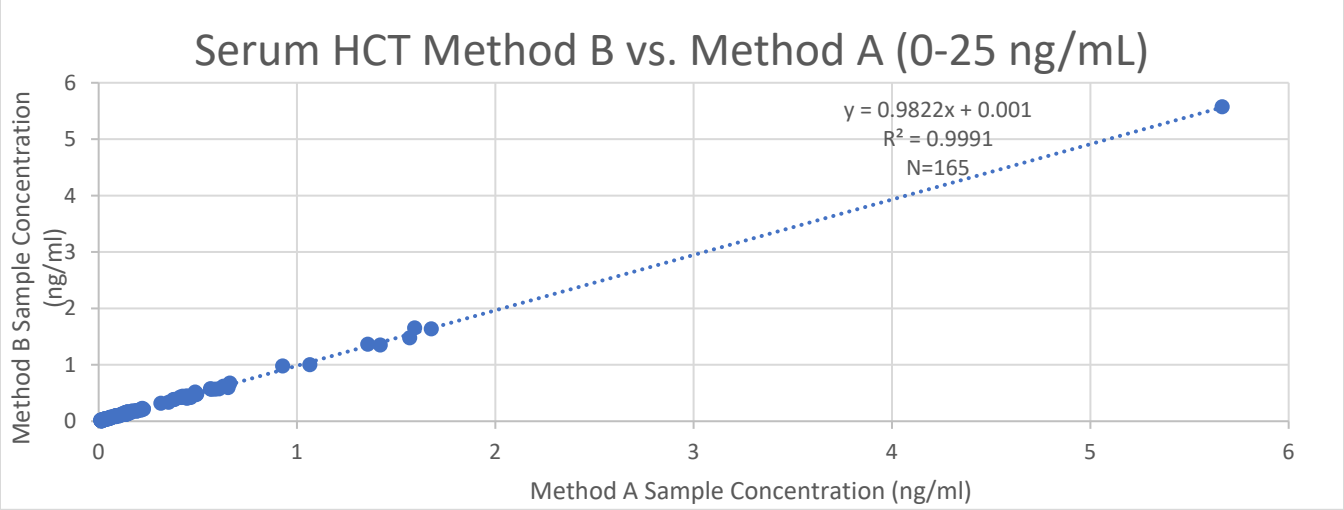


Figure 7: Comparison plot of the trans-3-hydroxycotinine concentrations of all samples run with Method B vs. the same samples run with Method A for concentrations 0-25 ng/mL

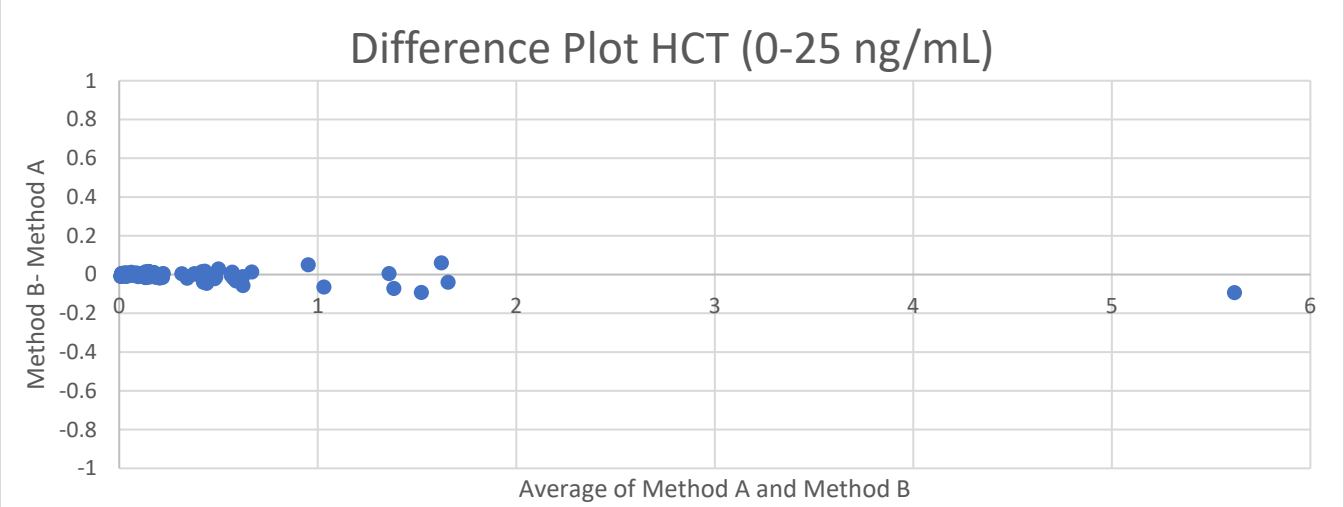


Figure 8: Method A-Method B vs. average of Method A and Method B plot for trans-3-hydroxycotinine for concentrations 0-25 ng/mL

Also, paired results were plotted and linear regression was performed for concentrations 25-400 ng/mL on the 6500 MS.

Method A: Instrument K9, Shimadzu Nexera UPLC System
Method B: Instrument K9, Waters I-Class UPLC System

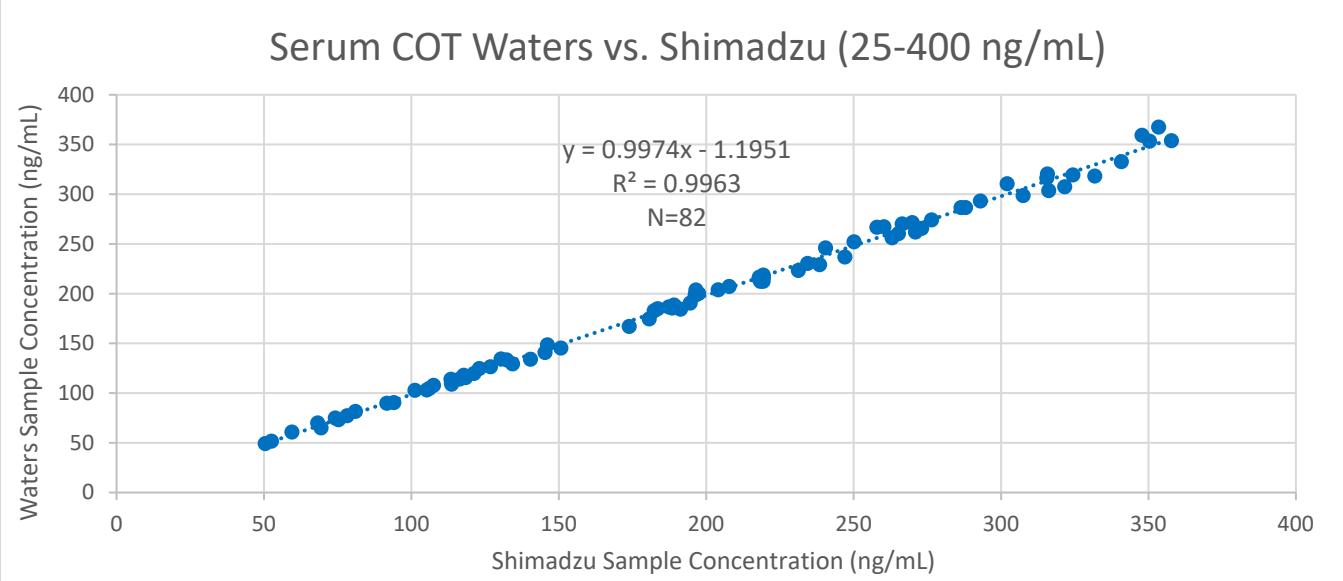


Figure 9: Comparison plot of the cotinine concentrations of all samples run with Method B vs. the same samples run with Method A for concentrations 25-400 ng/mL

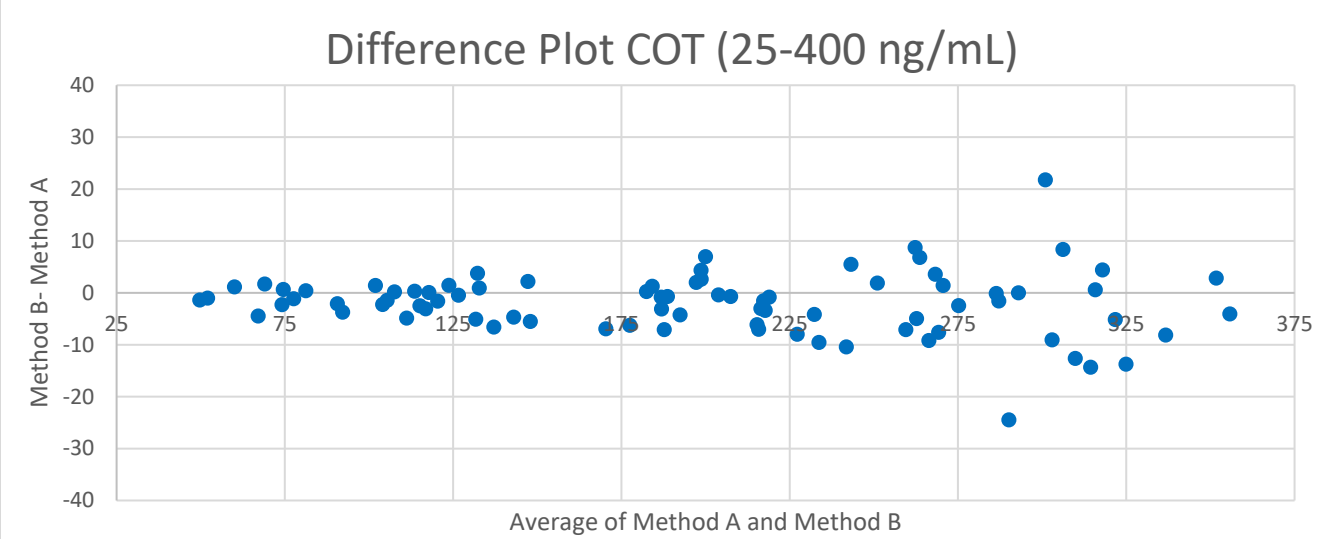


Figure 10: Method A-Method B vs. average of Method A and Method B plot for cotinine for concentrations 25-400 ng/mL

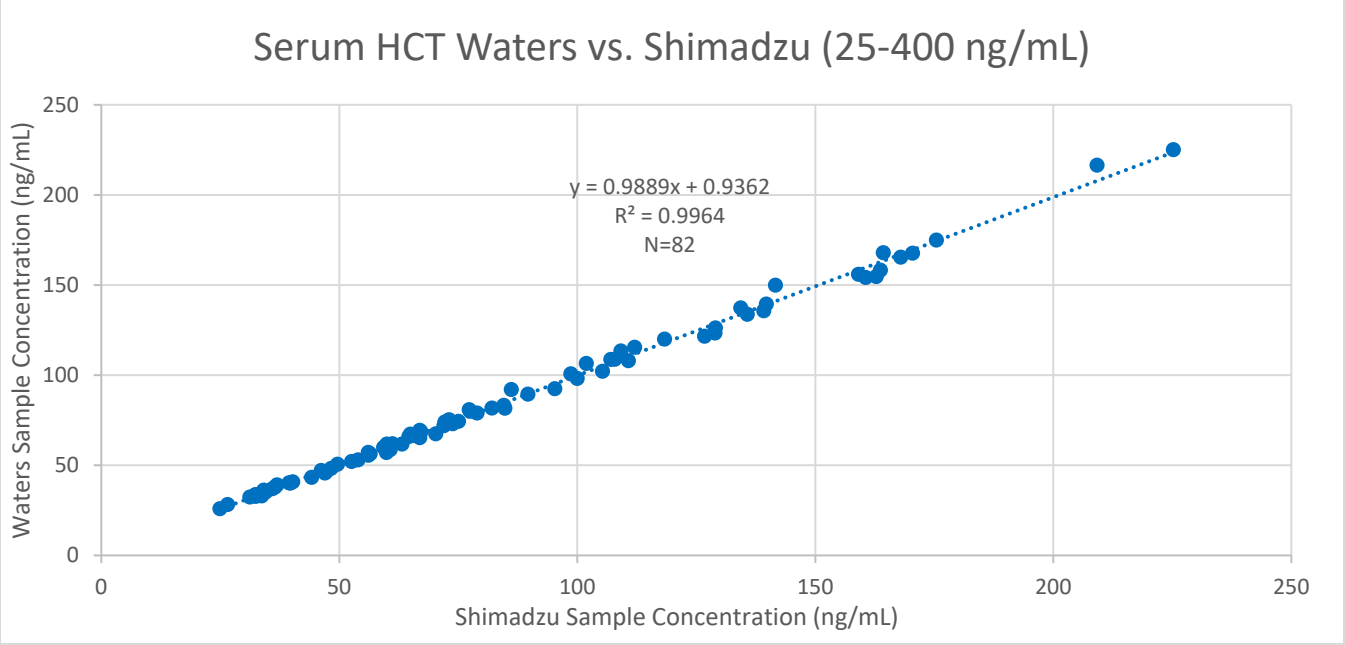


Figure 11: Comparison plot of the trans-3-hydroxycotinine concentrations of all samples run with Method B vs. the same samples run with Method A for concentrations 25-400 ng/mL

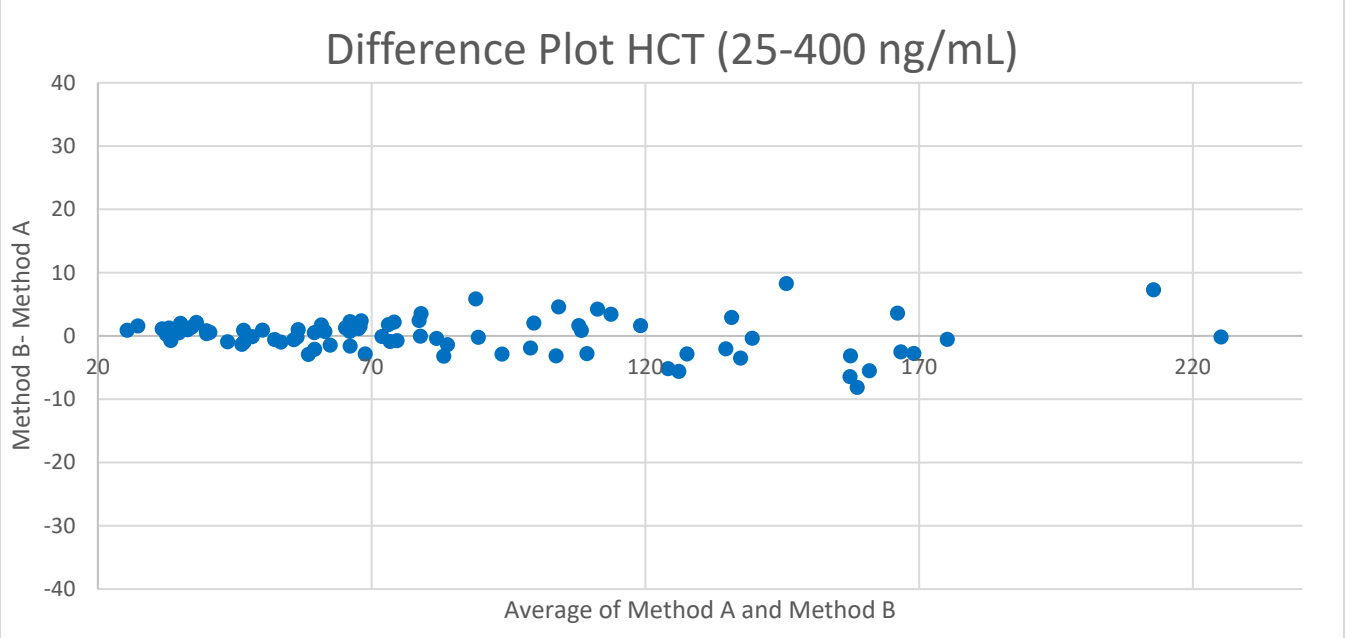


Figure 12: Method A-Method B vs. average of Method A and Method B plot for trans-3-hydroxycotinine for concentrations 25-400 ng/mL

The conclusion from these tests is that there is no significant difference between the Shimadzu and Waters UPLC systems and thus the instruments can be used interchangeably. In addition, there is no difference between the two low 7500 MS instruments used in this assay.

m. 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 three low concentration urine pools (LOD Pool 1, LOD Pool 2, LOD Pool 3 and QP1) were analyzed in runs over a period > 2 years. Analysis date range: 04/21/2022-07/09/2024.
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.

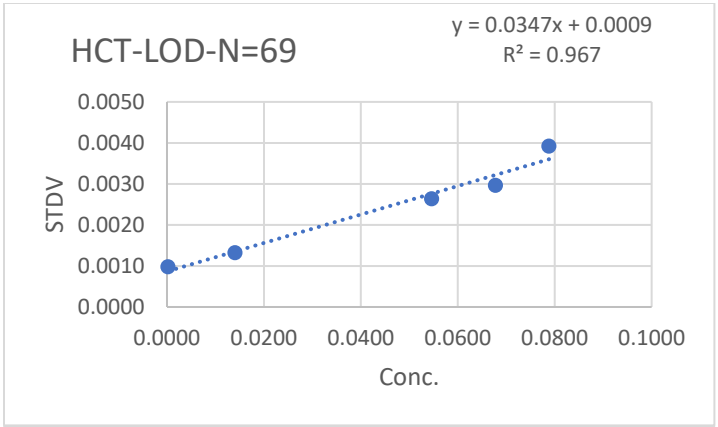
$$\text{Equation (1):}\quad Conc_{LOD} = [Mean_b + 1.645*(S_b + B)]/(1-1.645*A)$$

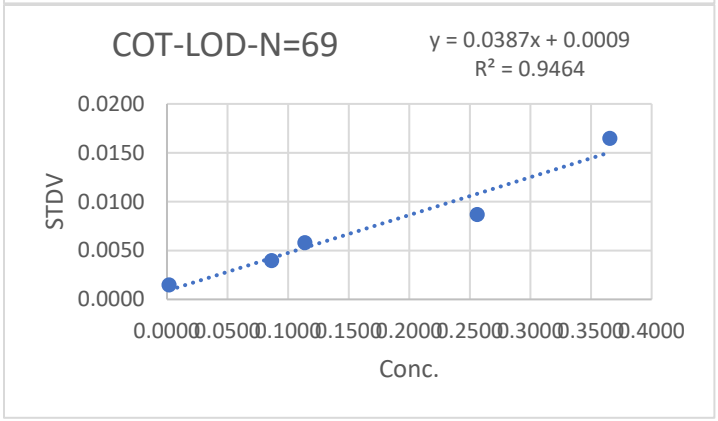
Where $Mean_b$ = mean of blank and S_b = std dev of blank

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

Pool	Analyte	Mean	Std Dev	N
LOD Pool 1	HCT	0.06781	0.00296	72
LOD Pool 2	HCT	0.01406	0.00132	71
LOD Pool 3	HCT	0.07883	0.00392	70
QP1	HCT	0.05463	0.00263	69
Blank	HCT	0.00021	0.00098	321
LOD Pool 1	COT	0.25613	0.00866	72
LOD Pool 2	COT	0.08651	0.00396	71
LOD Pool 3	COT	0.36567	0.01645	70
QP1	COT	0.11397	0.00579	69
Blank	COT	0.00185	0.00146	321

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


HCT-LOD-N=69
y = 0.0347x + 0.0009
R² = 0.967
STDV
0.0050
0.0040
0.0030
0.0020
0.0010
0.0000
0.0000
0.0200
0.0400
0.0600
0.0800
0.1000
Conc.


COT-LOD-N=69
y = 0.0387x + 0.0009
R² = 0.9464
STDV
0.0200
0.0150
0.0100
0.0050
0.0000
0.0000
0.0500
0.1000
0.1500
0.2000
0.2500
0.3000
0.3500
0.4000
Conc.

ConcLOD was obtained using the regression parameters and Equation (1).

Analyte	A=slope	B=inter	$Conc_{LOD}$	#pts
COT	0.0387	0.0009	0.006124	5
HCT	0.0347	0.00090	0.003501	5

LOD for HCT: estimated at 0.0035 ng/ml, will be set at 0.004 ng/mL
LOD for COT: 0.0061 ng/ml, will be set at 0.010 ng/mL

n. Strip Test Specificity, Sensitivity and Accuracy

481 Samples with a self-report nonsmoking status or no status were strip tested to determine cotinine concentration. These samples were then run in the corresponding run type determined by the strip test and specificity, sensitivity and accuracy were calculated.

Strip Test Lot	Date	Sample ID	Result	COT Concentration (ng/mL)
COT23060024	11/14/2023	SCOH_910	Positive	268
COT23060024	11/14/2023	SCOH_QR10	Positive	217
COT23060024	11/14/2023	SCOH_806	Negative	1.16
COT23060024	11/14/2023	SCOH_QP3	Negative	0.1299

	LC/MS/MS Positive (>25 ng/mL)	LC/MS/MS Negative (<25 ng/mL)
Strip Test Positive	210	34*
Strip Test Negative	0	399

*False positive concentrations range from 3-24 ng/mL

Specificity	92.1%
Sensitivity	100%
Classification Accuracy	94.7%