Laboratory Procedure Manual

Analyte: **Cotinine and Hydroxycotinine**
Matrix: **Serum and Saliva**
Method: **HPLC - APCI Tandem Mass Spectrometry**
Method No: **2017**
Revised: **June 13, 2016**

As performed by:

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

Contact:

Connie Sosnoff
Phone: 770-488-7910
Email: CSS3@cdc.gov

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

COT and HC 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 HC concentrations tend to be two to four times lower than COT concentrations (**9-10**).

The ratio of HC to COT is called the nicotine metabolite ratio (NMR). It is highly correlated with the rate of nicotine metabolism in smokers (**11-12**). 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 (**13**). The conversion of nicotine to COT, as well as the conversion of COT to HC is largely mediated by the liver enzyme cytochrome P450 2A6 (CYP2A6**) (14-15**). 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 (**16-20**). 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 HC are measured by an isotope-dilution high-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometric (ID HPLC-APCI MS/MS) method (**21-23**). Briefly, the serum sample is spiked with methyl-D_3-COT and methyl-D_3-HC 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 concentrated, and the residue 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 HC. 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.

Special Precaution

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

Note: This same method is used to measure COT and HC in saliva by substituting suitable saliva QC pools. All other aspects including calibration, cleanup and analysis are identical to serum procedures (**22**).

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

e. Protective Equipment

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

f. Training

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

g. Personal Hygiene

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

h. Disposal of Wastes

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

3. Computerization; Data-System Management

a. Software and Knowledge Requirements

Proficiency is required in the analytical software package of the HPLC and mass spectrometer used in the analysis. For the AB Sciex API 6500 mass spectrometer this package is Analyst. Statistical analysis of results requires proficiency in a standard statistical analysis software package. The Statistical Analysis System (SAS Institute, Cary, NC) is one such package. Sample cleanup is accomplished using an automated sample preparation system and a liquid handler; these require knowledge of the operating software (currently i-Link, Maestro, and Microlab STAR RUN). Proficiency is required in the software that automatically integrates the sample chromatograms (currently ASCENT from Indigo BioSystems, Indianapolis, IN).

b. Sample Information

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

The spreadsheet information is uploaded 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 need to be backed up monthly onto an external hard drive or the laboratory's 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 or saliva. 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.

Collection of saliva is most conveniently accomplished using a Salivette® or similar commercial device (**22**). Salivettes may be frozen directly after sample collection for subsequent transfer to the laboratory without any further processing required.

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 low-temperature freezers at or below -60°C.

d. Sample Quantity

A minimum of 1.0 mL of serum or saliva 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 HC. However, record other unusual sample characteristics on the runsheet for tracking purposes.

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

Not applicable for this procedure.

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

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

a. Reagents, Materials and Sources

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

Reagent	Grade	Source	Catalog #
acetic acid, glacial	HPLC	Tedia, Fairfield, OH	AS-1102
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	85-90% reagent	Fisher Scientific, Pittsburgh, PA	P-250
water	Optima LC/MS	Fisher Scientific, Pittsburgh, PA	W6
Isolute SLE+ 400 mg extraction plate	n/a	Biotage, Charlotte, NC	820-0400-P01

b. Reagent Preparation

Prepare the following solutions on an as-needed basis.

(1) 1N and 0.2N Potassium Hydroxide (KOH) solutions
Fisher P-250, 85-90% reagent, FW = 56.11, stored at room temperature. To prepare 100 mL of 1N KOH 0.1 mols or 5.611 g is required. Using the percentage purity listed on the bottle, calculate the required weight by dividing 5.611 by the (decimal) purity. For example, if the reagent is 85%, then the required weight of KOH is 5.611/0.85 = 6.601 grams. Weigh out the indicated amount of KOH, dissolve in 50-60 mL HPLC-grade water, transfer to a 100-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 1N KOH solution 1:5 with HPLC-grade water.

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

950 mL HPLC-grade water and 50 mL methanol. Store in a labeled, capped, glass bottle.

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. Samples will be analyzed as smoker or nonsmoker samples if smoking status is known. If smoking status is not known, samples will be analyzed as nonsmoker samples, and if analytes are found to have concentrations above the highest standard in the low serum standard set, the samples will be repeated as smoker samples and re-analyzed with the high serum standards using a smaller sample volume.

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

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

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

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

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 HCD3_A. Dissolve 25 mg of D_3-HC in water, q.s. to 100 mL. Nominal final concentration = 250 µg/mL D_3-HC.

- Stock HCD3_B. Dilute Stock D3HC_A 1:50 with water by taking 2 mL of Stock HCD3_A and q.s. to 100 mL with water. Nominal final concentration = 5 µg/mL (5000 ng/mL) D_3-HC.

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-HC 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 HC_D (mL)	Stock HC_C (mL)	Stock HCD3_B (mL)	Conc D3_COT and D3_HC[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_HC are actual concentrations in the standard solution.
[d]Conc of in diluted sample is the calculated concentration of analyte if sample vol = 200 uL.

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 HC_C (mL)	Stock HC_B (mL)	Stock HCD3_B (mL)	Conc D3_COT and D3_HC[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

[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_HC are actual concentrations in the standard solution.
[d]Conc of in diluted sample is the calculated concentration of analyte if sample vol = 50 uL.

Analyze the standards for two weeks to confirm their suitability (see section 7 below for standards acceptance criteria). Seal the standards in approximately 3mL aliquots in pre-cleaned (rinsed 3 times with methylene chloride), 5mL amber ampules. Store them at approximately 4°C.

6) Internal Standard Spiking Solution

Place 6 mL COTD3_B and 6 mL HCD3_B into a 3 L volumetric flask, q.s. to 3 L with water. Seal this solution in approximately 3mL aliquots in pre-cleaned (rinsed 3 times with methylene chloride), 5mL amber ampules. Store the ampules at approximately 4°C. Nominal final concentration = 10 ng/mL D_3-COT and 10 ng/mL D_3-HC. Add 50 µL of the spiking solution to each sample. The amount of ISTD per sample is 0.5 ng for both ISTDs.

This ISTD spiking solution must exactly match the concentration of the ISTDs in the standards.

7) Standards Acceptance Criteria

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

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

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

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

d. Quality-Control (QC) Materials

Prepare four QC 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)	HC (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 at approximately 4°C. The next day, mix the pools at room temperature for about 5 hours, then with continuous stirring, dispense into labeled 2mL cryovials. Store vials at or below -60°C.

e. Major Instrumentation and Other Equipment

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

Liquid Handler. Hamilton STARlet automated liquid handler.

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

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

7. Calibration and Calibration Verification

a. Creation of Calibration Curve

Base the calibration curves for this assay on the analysis of the standards described above in Section **6c**. If the samples are from nonsmokers or the smoking status is unknown use the low serum standard set (N=12 standards). If the samples are from smokers use the high serum standard set (N=10 standards). Each day analyze the standards in order from Standard 1 to Standard 10 (or 12). Repeat the analysis in reverse order, from Standard 10 (or 12) to Standard 1. Use both standard sets, 20 (or 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 do not use Standard #1, the standard with ISTD only, in the calibration curve.

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%*
(B) R-squared value > 0.9990

* 30% for standards less than 0.05 ng/mL

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

c. Calibration Verification

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

8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of a rack of 24 vials that contains the following: one blank in position 1, two QC samples, one in position 2 and one in a random position in the rack, and 21 unknown samples. Smoker samples are analyzed with high QC samples, nonsmoker samples are analyzed with low QC samples. A batch consists of four runs worked up together on one 96-well SLE plate. All four runs are analyzed with one set of 20 calibration standards for high runs or 24 calibration standards for low runs. Sometimes 2 plates are analyzed at the same time, if so the 2 plates and the standards set comprise a batch.

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

(1) Remove samples from the freezer and let thaw at room temperature. The sample racks may be placed in a few inches of cool water in the sink to facilitate thawing. During the week, the next day's samples are generally placed in the refrigerator the night before to thaw.

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

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

Use the Hamilton Starlet for Steps 4-11:

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

(5) Remove ISTD spiking solution ampules (as many ampules as needed for the batch) from the refrigerator, let warm to room temperature. Open the ampules and pour the ISTD spiking solution from all ampules into column 1 of a 12-column reservoir.

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

(7) Scan in the barcodes from the vials and mixing plate to make the sequence file and fill out the runsheet.

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

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

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

(11) Enter the SLE Plate lot #, cleanup analyst initials (if different from LC/MS operator), and any pertinent notes on the run sheet.

Use the PerkinElmer Staccato System robot for Steps 12-24:

(12) Place the mixing plate containing the samples and ISTD onto the Caliper deck of the PerkinElmer Staccato System robot.

(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 5 times.

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

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

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

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

(20) Evaporate the solvents under nitrogen at 15 psi for 60 min at 40°C using the Turbovap.

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

(22) Seal the collection plate.

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

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

Assay (this will determine whether a high or low run and thus the volume/dilution factor to use)
Initials of LC/MS operator
Number of samples
Run IDs

b. LC/MS/MS Analysis

Listed below are the conditions and settings for the Shimadzu Nexera HPLC and AB Sciex API 6500 mass spectrometer. If you have different instrumentation, then you will need to optimize the conditions and settings for your equipment. Several analytical columns have been validated for this method and are listed below.

1) <u>HPLC Conditions and Settings</u>

Analytical columns: Gemini NX C18, 4.6 x 50 mm, 5 µm particle size from Phenomenex (catalog # 00B-4454-E0)

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

Acquity UPLC BEH C18, 2.1 x 100 mm, 1.7 µm particle size from Waters (catalog# 186002352)

Pre-column frits: A-100X (2 µm pore) followed by A-103X (0.5 µm pore) from IDEX Health and Science

Injection volume: 5 µL for UPLC column, 10 µL for other columns

HPLC Settings	
Pump A	Buffer A
Pump B	Methanol
Flow rate	1 mL/min (0.6 mL/min for UPLC column)
Pressure limits	Min = 0, Max = varies depends on column
Column Oven Settings	
Temperature	50°C
Maximum temp	52°C
Injection Settings	
Sampling speed	5.0 µL/sec
Cooler temp	4°C
Measuring line purge vol	600 µL
Air gap vol	0.5 µL
Rinse type	External/ Internal
Rinse Settings	
Rinsing speed	35.0 µL/sec
Rinse port liquid	R2
Rinsing vol	500 µL
Rinse mode	Before and After
Rinse dip time	4 sec
Rinse method	Rinse port only
Rinse time	4 sec
Purge settings	
R1	5.0 min
R2	0.5 min
R3	5.0 min

Solvent Program		
Time (min)	A%	B%
0.01	100	0
1.80	68	32
2.20	68	32
2.21	0	100
3.20	0	100
3.21	100	0
3.98	100	0
4.00		

2) Mass Spectrometry Conditions and Settings

Mass Spec Settings	
Exp	1
SC type	MRM
Polarity	Positive
Duration	4.005
Delay	0
MCA	no
Optimized Gas, Temp & Voltage Settings*	
CAD	10
CUR	50
GS1	35
NC	3.0
TEM	625
Res Q1	Unit
Res Q3	Unit
Pause between mass range	5.007
Dwell	22-30, depends on column
DP	60
EP	9.0
CE (COT)	31
CE (HC)	45
CXP (COT)	10
CXP (HC)	9.0

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

different values for these settings.

3) Tuning Procedure

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

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

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

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

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

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

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

(7) After completing the tunes, close the configuration for MS only and remove the IS probe. Clean the interface skimmer plate and the surrounding area with methanol. Change the two pre-column frits, wipe off the injector needle with a

cotton swab and methanol. Clean the IS probe with methanol and put the APCI probe back in. Clean out the syringe with methanol, and wash the buffer bottle and replace with fresh buffer. Return the configuration to LM LCMS and the project to the latest current project setting. Analyze a set of standards up and down (see Daily Procedure, below) to verify the instrument is running correctly.

4) Daily Procedure

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

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

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

(3) Check to make sure the mobile phase bottles are full enough to complete the runs planned for the day. It takes approximately 400 mL of Buffer A and 300 mL of 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) 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. The three pressure gauges on the Peak gas generator.
c. HPLC Pump A pressure readings after approximately 10 min 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.

Hard copies of the daily sample log are kept in a maintenance log binder. Any repairs which are made to the instrument are also recorded in an electronic file that is kept on the desktop of the instrument computer for quick reference.

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

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

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

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

c. Uploading Data to Indigo Ascent

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

(1) Obtain data from your instrument computer

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

(2) On your network computer, open citgo by going to citgo.cdc.gov/

a. Use your smartcard or username and password to access citgo
b. Click on “CITGO Virtual Desktop”
c. Use your smartcard to access the virtual desktop
d. If you are using a flash drive citgo will ask permission to access it. If so, click “Permit/Use”
e. The “CITGO Virtual Desktop – Desktop Viewer” should now be open

(3) Open the Firefox Web browser

a. Click on the Microsoft icon on the bottom left of the screen
b. In the search bar type “Firefox”
c. Click on the program “Mozilla Firefox”

(4) Access Indigo Ascent’s webpage at https://cdc.poweredbyascent.net/

a. Type in your username and password

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

a. From Indigo's home page, click on "Accessioning" in the top left corner
b. Choose your assay from the drop down list: "2017-SCOH-high" (for high runs) or "2017-SCOH-low" (for low runs)
c. Locate your sequence file and drag/drop it into the box containing the words "Currently Viewing [filename]"

   If your sequence file is on your flash drive, click on the folder icon, then click on the "Removable Disk" that represents your flash drive. Citgo will ask again to access your local files. Click on "Read/write access"

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

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

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

a. Click on the folder icon on the bottom left of your screen to open a new window
b. In the new window, locate the folder labeled "+NCEH_DLS_Indigo_Dropbox-FC" and click on it so that it is highlighted.
c. Right-click and select "New → Folder". A blank folder will appear. Inside the folder, type the name of the batch you are uploading.
d. Once your folder is created, you will need to put the .csv file for this run into the folder. To do this, click on the windows icon on the bottom left of your screen. Then type the name of the "Batch" that you converted.
   This is the name you should remember from step 5d
c. Your search should open a new window with the .csv file in it. Drag/drop this file into the folder you created in the Indigo Dropbox.
d. Now, locate your wiff and wiff.scan files

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

e. Select all of the wiff and wiff.scan files associated with the run you are uploading and drag/drop them into the folder you created in the Indigo Dropbox.
This is the same folder where you just put the .csv file

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

d. Quality Assurance (QA) Review in Indigo Ascent

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

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

(1) Check the calibration curves for both analytes.
- R2 needs to be >0.9990
- All concentration deviations need to be < 15% except standards ≤ 0.05 ng/mL need to have concentration deviations < 30%

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

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

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

(4) Set batch status to "Reviewed".

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

e. Calculations

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

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

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

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

9. Reportable Range of Results

a. Limit of Detection

The method detection limits are defined as 3 times S_0, where S_0 is the estimate of the standard deviation at zero analyte concentration. The value of S_0 is taken as the y-intercept of a linear regression of standard deviation versus concentration as specified by Taylor (**25**). See Appendix A for LOD calculation.

b. Accuracy

Accuracy was tested on at least three 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 HC; these were then subtracted from the spiked sample results.

Acceptable results were obtained at all concentration levels for both analytes. The mean value was within ±9% of the theoretical value at all levels except at the LLOQ where it was within ±14% of the theoretical values. See Appendix A for accuracy results.

c. Precision

Within-day precision was measured using the accuracy samples described above. Acceptable results were obtained at all concentration levels for both analytes. The coefficient of variation (CV) did not exceed 5% for either analyte at any concentration level except at the LLOQ where it did not exceed 16%.

Between-day precision was also measured using the accuracy samples described above. Acceptable results were obtained at all concentration levels for both analytes.

The CV did not exceed 4% for either analyte at any concentration level except at the LLOQ where it did not exceed 10%. See Appendix A for precision results.

d. Analytical Specificity

A high degree of analytical specificity is achieved with this HPLC/MS/MS method; however there is always a possibility that a sample will have an unknown interferent. 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.

e. Matrix Effects and Recovery

Matrix effects (ME) and extraction recoveries (RE) were measured according to the method of Matuszewski (**26**). 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 six 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 104% for these 6 serum samples and HC had an average ME of 101%. Average extraction recoveries were 50% and 24% respectively. See Appendix A for matrix effects results.

f. Linearity Limits

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. Ruggedness test

Method ruggedness for the serum assay was tested by varying the following parameters: KOH concentration, volume of KOH solution, volume of water in blank, N2 evaporation pressure, N2 evaporation temp, N2 evaporation time, and volume of sample. Each parameter was tested at the method level and at a lower and higher 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 HC. 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 HC QC and blank samples are in control, then the HC results for the samples in the run will be acceptable, however the samples will need to be reanalyzed in a repeat run for COT.

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

1) QC results. Confirm all QC results for the mean and range values using the current DLS QC rules based on the division SAS QC program (**27**).

2) Blanks. For a low run, reject run if COT blank > 0.015 ng/mL or if HC blank > 0.015 ng/mL. Also reject run if the blank batch average > 0.006 ng/mL for either analyte. For a high run, reject high run if COT blank > 0.050 ng/mL or if HC blank > 0.050 ng/mL.

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

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

COT Confirmation ion = m/z 177 → 98
COT Quantitation ion = m/z 177 → 80

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

The confirmation ion ratio range is determined from the mean of the standards for that day with concentrations ≥ 0.02 ng/mL. Because of low ion counts for the confirmation ion, these evaluations are limited to samples with a calculated concentration ≥ 0.1 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 (**27**).

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 at or below approximately -60^{o}C. Characterize the pools in at least 20 analytical runs over a period of at least two weeks.

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

assays will resume until the problem has been resolved and a repeat PT assay has been passed.

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

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

a. Internal Standard Response

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

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

b. Calibration Regression

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

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

c. Analyte in Standards or QC Materials

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

d. Analyte in All Samples

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

Note: We have seen contamination when the bottle containing the needle rinse solvent becomes dry. The fix is to monitor the solvent level. Contamination has also occurred when the needle rinse program was not working properly. This was caused by a miscommunication between the software running the LC (Shimadzu) and the software running the MS (Analyst). This problem was solved by updating Analyst software to 1.6.2. To avoid contamination and carryover confirm that the rinse program is working properly by monitoring the solvent levels in the rinse solvent bottles.

e. QC Sample Outside of Control Limits

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

12. Limitations of Method; Interfering Substances and Conditions

In some studies other nicotine metabolites (e.g., HC) and physiological substances (e.g., caffeine) have interfered with immunoassay or chromatographic assays of COT. However, we are aware of no known interferents for this tandem mass spectrometric method for either COT or HC. The presence of other interfering substances in a particular sample should be indicated by a deviation in the expected confirmation ratio for that sample.

13. Reference Ranges (Normal Values)

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 (**28**). Subsequent evaluations have indicated a decline in the median level of serum COT among nonsmokers (**23**). The most recent NHANES survey, for which serum cotinine data have been published (survey years 2009-2010), found the 50^{th} and 95^{th} percentile level in nonsmokers to be 0.03 and 1.29 ng/mL, respectively (**29-30**).

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.

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

Not applicable for this procedure.

15. Specimen Storage and Handling During Testing

Store samples at or below approximately -60^{o} C 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 re-freeze residual samples at or below approximately -60^{o}C.

16. 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 until the problem is resolved. Samples that have been extracted and reconstituted can be analyzed after one day at room temperature or after one week at -20^{o} C.

b. Proper Storage Procedures

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

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

Analytical results are reported as ng/mL for each sample. Final results that meet all QC/QA criteria are reviewed by a DLS statistician 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.

18. Procedures for Specimen Accountability and Tracking

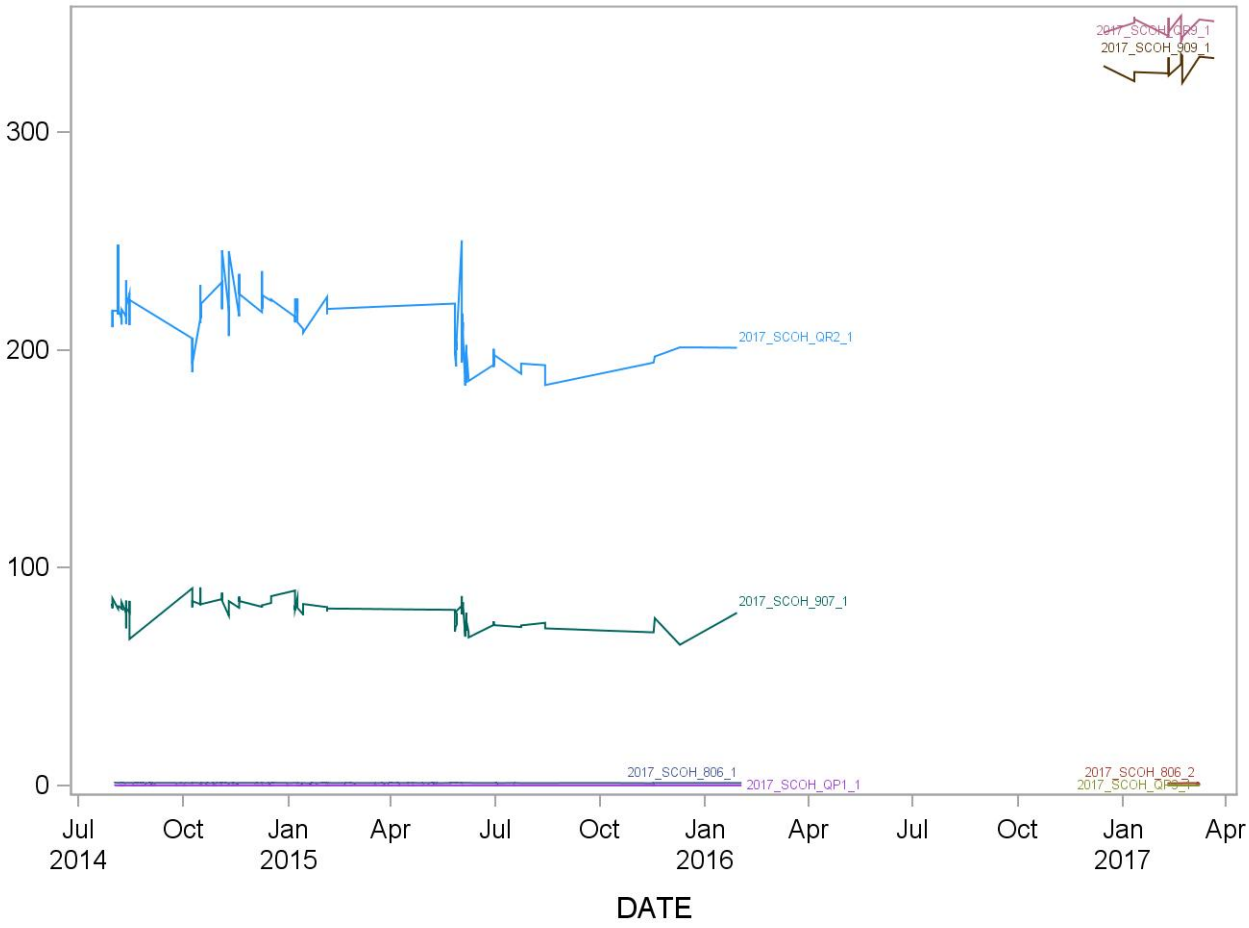
Standard record keeping (e.g., sample ID, notebooks, data files, databases, etc.) is used for sample tracking. All records are maintained in accordance with the HHS Records Management guidance. (See: http://www.hhs.gov/open/records/index.html)

19. Summary Statistics and QC Graphs

See following pages.

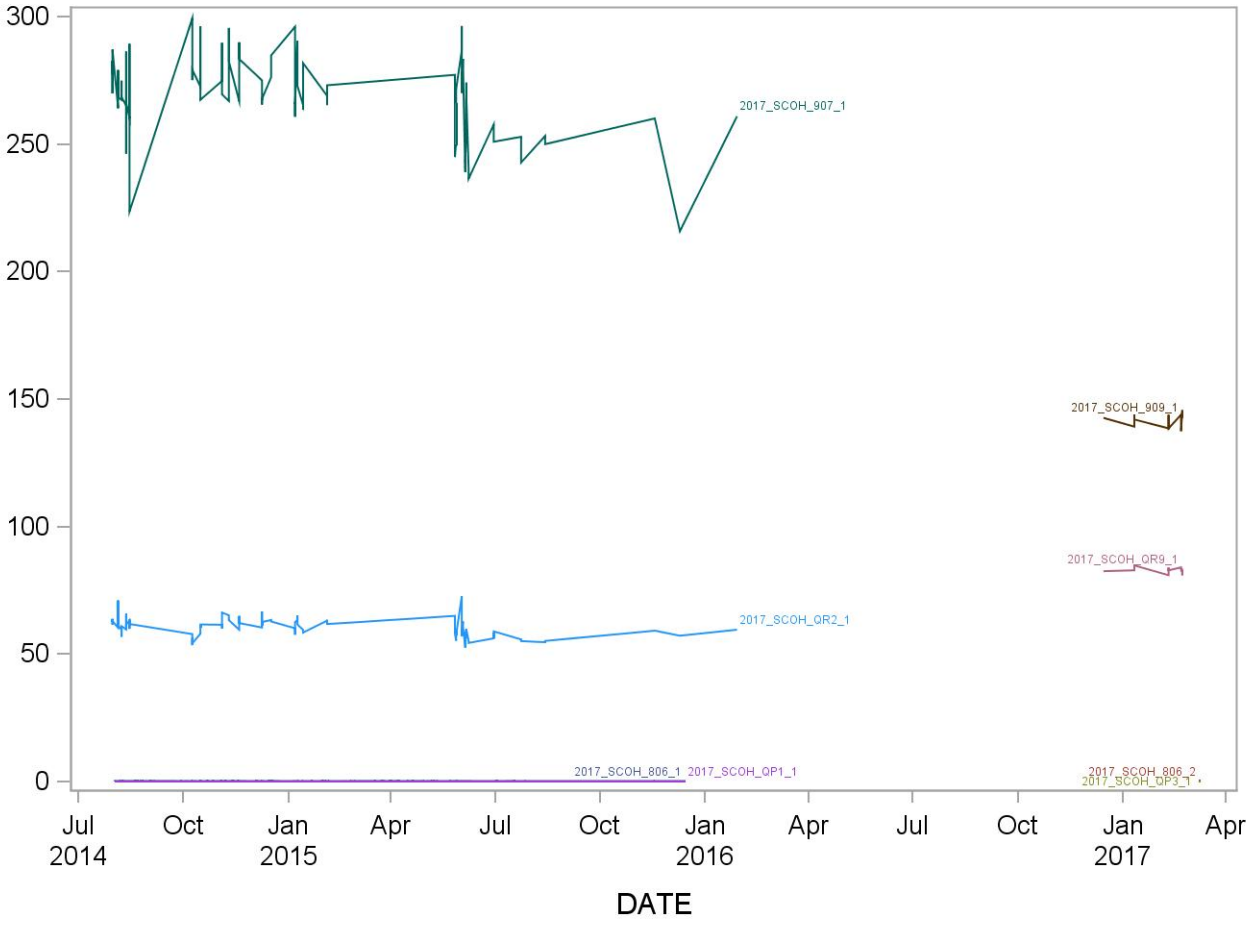
2013-2014 Summary Statistics and QC Chart for Cotinine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2017_SCOH_QR2_1	95	31JUL14	29JAN16	210.8931	14.9227	7.1
2017_SCOH_907_1	95	31JUL14	29JAN16	79.8888	5.5270	6.9
2017_SCOH_806_1	364	02AUG14	02FEB16	1.2525	0.0463	3.7
2017_SCOH_QP1_1	364	02AUG14	02FEB16	0.1163	0.0070	6.0
2017_SCOH_909_1	15	15DEC16	22MAR17	329.9896	4.4743	1.4
2017_SCOH_QR9_1	15	15DEC16	22MAR17	347.9685	4.2812	1.2
2017_SCOH_806_2	8	09FEB17	09MAR17	1.1469	0.0248	2.2
2017_SCOH_QP3_1	8	09FEB17	09MAR17	0.1271	0.0175	13.8



2013-2014 Summary Statistics and QC Chart for Hydroxycotinine, Serum (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2017_SCOH_907_1	95	31JUL14	29JAN16	267.5847	15.8327	5.9
2017_SCOH_QR2_1	95	31JUL14	29JAN16	60.2513	3.6467	6.1
2017_SCOH_806_1	363	02AUG14	15DEC15	0.2713	0.0116	4.3
2017_SCOH_QP1_1	363	02AUG14	15DEC15	0.0573	0.0030	5.3
2017_SCOH_909_1	13	15DEC16	22FEB17	141.7464	2.6007	1.8
2017_SCOH_QR9_1	13	15DEC16	22FEB17	82.9056	1.1187	1.3



References

1. Watts RR, Langone JJ, Knight GJ, Lewtas J. Cotinine analytical workshop report: consideration of analytical methods for determining cotinine in human body fluids as a measure of passive exposure to tobacco smoke. Environ Health Perspec 1990;84:173-82.

2. Benowitz NL. Cotinine as a biomarker of environmental tobacco smoke exposure. Epidemiol Rev 1996;18:188-204.

3. Jarvis MJ, Russell MAH, Benowitz NL, Feyerabend C. Elimination of cotinine from body fluids: implications for noninvasive measurement of tobacco smoke exposure. Am J Public Health 1988;78:696-8.

4. Benowitz NL, Kuyt F, Jacob P, Jones RT, Osman AL. Cotinine disposition and effects. Clin Pharmacol Ther 1983;34:604-11.

5. Kyerematen GA, Morgan ML, Chattopadhyay B, deBethizy JD, Vesell ES. Disposition of nicotine and eight metabolites in smokers and nonsmokers: identification of two metabolites that are longer lived than cotinine. Clin Pharmacol Ther 1990;48:641-51.

6. Jacob P, Yu L, Wilson M, Benowitz NL. Selected ion monitoring method for determination of nicotine, cotinine and deuterium-labeled analogs: absence of an isotope effect in the clearance of (S)-nicotine-3',3'-d_2 in humans. Biol Mass Spec 1991;20:247-52.

7. Armitage AK, Dollery CT, George CF, Houseman TH, Lewis PJ, Turner DM. Absorption and metabolism of nicotine from cigarettes. Br Med J 1975;4:313-16.

8. Scherer G, Jarczyk L, Heller WD, Biber A, Neurath GB, Adlkofer F. Pharmacokinetics of nicotine, cotinine, and 3'-hydroxycotinine in cigarette smokers. Klin Wochenschr 1988;66 Suppl 11:5-11.

9. Benowitz NL, Jacob P. Trans-3'-hydroxycotinine: disposition kinetics, effects and plasma levels during cigarette smoking. Br J Clin Pharmacol 2001;51(1):53-9.

10. Bentley MC, Abrar M, Kelk M, Cook J, Phillips K. Validation of an assay for the determination of cotinine and 3-hydroxycotinine in human saliva using automated solid-phase extraction and liquid chromatography with tandem mass spectrometric detection. J Chromatogr B Biomed Sci Appl. 1999 Feb 19;723(1-2):185-94.

11. Dempsey D, Tutka P, Jacob P, Allen F, Schoedel K, Tyndale RF, Benowitz NL. Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. Clin Pharmacol Ther. 2004 Jul;76(1):64-72.

12. Johnstone E, Benowitz N, Cargill A, Jacob R, Hinks L, Day I, Murphy M, Walton R. Determinants of the rate of nicotine metabolism and effects on smoking behavior. Clin Pharmacol Ther. 2006 Oct;80(4):319-30.

13. Benowitz NL. Nicotine addiction. N Engl J Med. 2010 Jun 17;362(24):2295-303.

14. Nakajima M, Yamamoto T, Nunoya K, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N, Kamataki T, Kuroiwa Y. Characterization of CYP2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. J Pharmacol Exp Ther. 1996 May;277(2):1010-5.

15. Hukkanen J, Jacob P, Benowitz NL. Metabolism and disposition kinetics of nicotine. Pharmacol Rev. 2005 Mar;57(1):79-115.

16. Yamazaki H, Inui Y, Yun CH, Guengerich FP, Shimada T. Cytochrome P450 2E1 and 2A6 enzymes as major catalysts for metabolic activation of N-nitrosodialkylamines and tobacco-related nitrosamines in human liver microsomes. Carcinogenesis. 1992 Oct;13(10):1789-94.

17. Smith TJ, Liao AM, Liu Y, Jones AB, Anderson LM, Yang CS. Enzymes involved in the bioactivation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in patas monkey lung and liver microsomes. Carcinogenesis. 1997 Aug;18(8):1577-84.

18. Patten CJ, Smith TJ, Friesen MJ, Tynes RE, Yang CS, Murphy SE. Evidence for cytochrome P450 2A6 and 3A4 as major catalysts for N'-nitrosonornicotine alpha-hydroxylation by human liver microsomes. Carcinogenesis. 1997 Aug;18(8):1623-30.

19. Loriot MA, Rebuissou S, Oscarson M, Cenee S, Miyamoto M, Ariyoshi N, Kamataki T, Hemon D, Beaune P, Stucker I. Genetic polymorphisms of cytochrome P450 2A6 in a case-control study on lung cancer in a French population. Pharmacogenetics. 2001 Feb;11(1):39-44.

20. Xu C, Goodz S, Sellers EM, Tyndale RF. CYP2A6 genetic variation and potential consequences. Adv Drug Deliv Rev. 2002 Nov 18;54(10):1245-56.

21. Bernert JT, Turner WE, Pirkle JL, Sosnoff CS, Akins JR, Waldrep MK, Ann Q, Covey TR, Whitfield WE, Gunter EW, Miller MM, Patterson DG, Needham LL, Hannon WH, Sampson EJ. Development and validation of a sensitive method for determination of serum cotinine in smokers and nonsmokers by liquid chromatography / atmospheric pressure ionization tandem mass spectrometry. Clin Chem 1997;43:2281-91.

22. Bernert JT, McGuffey JE, Morrison, Pirkle JL. Comparison of serum and salivary cotinine measurements by a sensitive high-performance liquid chromatography – tandem mass spectrometry method as an indicator of exposure to tobacco smoke among smokers and nonsmokers. J Anal Toxicol 2000;24:333-9.

23. Pirkle JL, Bernert JT, Caudill SP, Sosnoff CS, Pechacek TF. Trends in the exposure of nonsmokers in the U.S. population to secondhand smoke: 1988 through 2002. Env Health Perspectives. 2006;114:853-8.

24. Bernert JT, Jacob P, Holiday DB, Benowitz NL, Sosnoff CS, Doig MV, Feyerabend C,Aldous KM, Sharifi M, Kellogg MD, Langman LJ. Interlaboratory comparability of serum cotinine measurements at smoker and nonsmoker concentration levels: A round robin study. Nicotine Tob Res. 2009;11(12):1458-66.

25. J.K. Taylor, Quality Assurance of Chemical Measurements, Lewis Publishers, Chelsea, MI, 1987.

26. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS. Anal Chem. 2003;75:3019-30.

27. Caudill SP, Schleicher RL, and Pirkle JL (2008) Multi-rule quality control for the age-related eye disease study, Stat Med, 27: 4094-4106.

28. Pirkle JL, Flegal KM, Bernert JT, Brody DJ, Etzel RA, Maurer KR. Exposure of the US population to environmental tobacco smoke. The Third National Health and Nutrition Examination Survey, 1988 to 1991. J Am Med Assoc 1996;275:1233-40.

29. Fourth National Report on Human Exposure to Environmental Chemicals. 2009. http://www.cdc.gov/exposurereport/.

30. http://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Sep2013.pdf

31. Jarvis MJ, Tunstall-Pedoe H, Feyerabend C, Vesey C, Saloojee Y. Comparison of tests used to distinguish smokers from nonsmokers. Am J Public Health 1987;77:1435-8.

32. Benowitz NL, Bernert JT, Caraballo RS, Holiday DB, Wang J. Optimal serum cotinine levels for distinguishing cigarette smokers and nonsmokers within different racial/ethnic groups in the United States between 1999 and 2004. Am J Epidemiol 2009;169:236-48.

33. Official Journal of the European Communities L 221, 17.8.2002, p. 16.

Appendix A: Validation Results

1. Ruggedness Testing Results

Method ruggedness was tested by varying the following parameters:

KOH concentration (N=4, 4 and 2)
N2 evaporation pressure (all N=3)
N2 evaporation temp (all N=3)
N2 evaporation time (all N=3)
Volume of sample (all N=24)
% IPA in DCM (N=16, 16 and 15)

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

The parameter means were tested using a one-way anova (by Proc GLM in SAS) and found to be the same for all parameters ($p > 0.05$) except sample volume and %IPA. The means for those parameters were within 7% of each other.

Parameter	Method Level	Lower level	Upper Level	COT Result at Method level (ng/mL)	COT Result at Lower level (ng/mL)	COT Result at Upper Level (ng/mL)	HC Result at Method level (ng/mL)	HC Result at Lower level (ng/mL)	HC Result at Upper Level (ng/mL)
KOH conc	0.2N	0.1N	0.5N	1.07	1.05	1.09	0.235	0.230	0.246
sample vol	200 uL	150 uL	250 uL	1.38	1.27	1.37	0.370	0.351	0.374
N2 time*	40 min	35 min	45 min	1.16	1.15	1.14	0.254	0.247	0.255
N2 pressure	15 psi	10 psi	20 psi	1.16	1.17	1.15	0.254	0.252	0.251
N2 temp*	60°C	45°C	75°C	1.16	1.17	1.21	0.254	0.259	0.263
IPA in DCM	5%	3%	8%	1.30	1.28	1.33	0.394	0.394	0.377

*N2 evaporation temp was changed to 35°C on 6/1/2014.
**N2 evaporation time was changed to 30 min after each elution on 6/1/2014.

Note: we changed back to one 60 min evaporation at 15 psi and 40 °C after we started using the re-grip station on the Caliper instead of the turn-table.

2. Accuracy and Precision Results

Accuracy and precision were tested on at least three 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 HC; these were then subtracted from the spiked sample results. All results are in the table below.

Acceptable results: accuracy with 15% of theoretical except at the LLOQ where it should be within 20%, precision less than 15% coefficient of variation (CV) except at the LLOQ where it should be less than 20%.

Accuracy and Precision Results

Sample ID	Analyte	Mean conc (ng/mL)	Std Dev (ng/mL)	Conc exp	Accuracy (%)	CV within day	CV between day	N	Note
LL1	OH	0.010	0.0036	0.011	89	35.5	21.2	23	CV ok, at LOD*
LL2	OH	0.114	0.0052	0.117	97	4.6	1.2	14	
LL3	OH	0.381	0.0117	0.393	97	3.1	1.4	15	
LL4	OH	6.1	0.1133	6.28	97	1.9	1.1	14	
LL5	OH	15.4	0.2196	15.7	98	1.4	1.0	15	
LLOQ	OH	0.033	0.0052	0.034	97	15.8	9.7	25	
HL1	OH	6.59	0.081	6.28	105	1.2	0.6	14	
HL2	OH	16.3	0.503	15.7	104	3.1	2.8	14	
HL3	OH	81.1	2.729	78.3	104	3.4	3.0	14	
HL4	OH	163	4.267	157	104	2.6	1.2	14	
LL1	COT	0.007	0.0055	0.015	49	78.7	57.9	23	Acc & CV ok, at LOD*
LL2	COT	0.142	0.0054	0.15	95	3.8	4.0	14	
LL3	COT	0.506	0.0125	0.5	101	2.5	1.3	15	
LL4	COT	8.25	0.1470	8	103	1.8	0.8	14	
LL5	COT	20.9	0.7969	20	105	3.8	2.4	15	
LLOQ	COT	0.039	0.0045	0.045	86	11.6	8.1	25	
HL1	COT	8.75	0.125	8	109	1.4	1.1	14	
HL2	COT	21.6	0.382	20	108	1.8	0.8	14	
HL3	COT	107	2.755	100	107	2.6	1.3	14	
HL4	COT	216	6.118	200	108	2.8	0.9	14	

*The precision and accuracy of Pool LL1 is acceptable given that the spiked levels are at or below our LODs.

Acceptable accuracy was obtained at all concentration levels for both analytes. The mean value was within ±9% of the theoretical value at all levels except at the LLOQ where it was within ±14% of the theoretical values.

Within-day and between-day precision were measured using the accuracy samples described above. Acceptable precision was obtained at all concentration levels for both analytes. The CV for within-day precision did not exceed 5% for either analyte at any concentration level except at the LLOQ where it did not exceed 16%. The CV for between-day precision did not exceed 4% for either analyte at any concentration level except at the LLOQ where it did not exceed 10%.

3. 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 each concentration level of the accuracy and precision samples. Recoveries were reproducible at each concentration (3*SD error bars overlap for all levels of each analyte).

Recovery Results

Analyte	POOL	Target Conc (ng/mL)	MEAN recov (%)	Std Dev recov (%)	N
HC	LL1	0.01145	30.0	5.7	23
HC	LLOQ	0.03435	31.9	6.0	25
HC	LL2	0.117	38.9	15.2	14
HC	LL3	0.393	35.9	16.5	15
HC	LL4	6.28	37.1	16.2	14
HC	LL5	15.6624	37.9	15.1	15
HC	HL1	6.28	37.0	15.8	14
HC	HL2	15.6624	35.1	16.8	14
HC	HL3	78.312	32.7	11.1	14
HC	HL4	156.624	32.7	10.6	14
HC	**Average**		**34.9**		

Analyte	POOL	Target Conc (ng/mL)	MEAN recov (%)	Std Dev recov (%)	N
COT	LL1	0.015	51.4	9.5	23
COT	LLOQ	0.045	54.1	9.0	25
COT	LL2	0.15	63.2	13.5	14
COT	LL3	0.5	59.5	14.0	15
COT	LL4	8	61.7	12.2	14
COT	LL5	20	61.1	10.2	15
COT	HL1	8	63.7	13.5	14
COT	HL2	20	60.0	16.9	14
COT	HL3	100	55.2	9.4	14
COT	HL4	199.784	56.0	10.2	14
COT	**Average**		**58.6**		

Note: 6/1/2014 we changed our collection plate and found our recoveries greatly improved: COT recovery changed to about 90%, HC recovery changed to about 70%.

4. 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 HC in them. Therefore

we have chosen to make our calibration curve in water. In order to test the equivalency of our water calibration curve to a calibration curve in the matrix, we created two calibration curves: one in serum and one in water. Then we compared the slopes of the two calibration curves. The division policy is if the slopes differ by no more than 5% then the calibration curves can be considered equivalent and the alternate matrix (in this case water) can be used for the assay calibration curve.

We followed the procedure outlined below:

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

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

3. Calibration curves were constructed by averaging the four instrument responses (Quant area/ISTD area) for each calibrator and plotting those averages against the expected calibrator concentrations. Three sets of calibration curves were compared: curves using all 13 standards (“all”), curves using the lowest 10 standards (“low”), and curves using the highest 10 standards (“high”). 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.

Calibrator Concentrations

Calibrator #	COT Conc (ng/mL)	HC Conc (ng/mL)
1	0.0128	0.0100
2	0.0638	0.0500
3	0.0956	0.0750
4	0.1275	0.1000
5	0.6375	0.5000
6	0.9563	0.7500
7	1.275	1.000
8	6.375	5.000
9	9.563	7.500
10	12.75	10.00
11	40	31.3
12	100	78.3
13	300	235

Matrix Calibration Curve Comparison

Analyte	Curve	Weight	Slope	Intercept	R2	slope diff (%)	Standards
HC	water	1/x	0.47812	0.00611	0.9999	-1.1	all
HC	serum	1/x	0.48364	0.01605	0.9995		all
COT	water	1/x	0.60577	0.01983	0.9978	4.6	all
COT	serum	1/x	0.57846	0.04189	0.9995		all
HC	water	1/x	0.4856	0.00561	0.9994	0.0	low
HC	serum	1/x	0.48538	0.01586	0.999		low
COT	water	1/x	0.58779	0.0215	0.997	0.3	low
COT	serum	1/x	0.58632	0.04117	0.9997		low
HC	water	1/x	0.47805	0.00911	0.9999	-1.2	high
HC	serum	1/x	0.48358	0.0181	0.9995		high
COT	water	1/x	0.60632	-0.00613	0.9979	4.7	high
COT	serum	1/x	0.57834	0.04757	0.9995		high

5. Matrix Effects

We measured the effect of the matrix according to the method of Matuszewski (26). We compared the instrumental response for the following three cases:

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

The Case A is just the ISTD injected with the standards. Result A is the average of all the standards analyzed the same day. Cases B and C were measured in five different nonsmoker 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 104% for these 6 serum samples and HC had an average ME of 101%. Average extraction recoveries were 50% and 24% respectively.

Matrix Effects Results

Sample	HC ISTD Case B	COTISTD Case B	HC ISTD Case C	Cot ISTD Case C	ME(%) COT (B/A)	ME(%) HC (B/A)	RE(%) COT (C/B)	RE(%) HC (C/B)
R229008	12067	30815	3392	15965	105	101	52	28
R228988	12026	30211	2374	14801	102	101	49	20
BB	12023	30327	2980	13841	103	101	46	25
CS	11959	30727	2713	15417	104	101	50	23
CW	12021	30814	3207	16444	105	101	53	27
LQ	12125	30747	2986	14535	104	102	47	25
Averages					**104**	**101**	**49**	**24**

	HC ISTD Case A	Cot ISTD Case A
Standards	11898	29482

Note: 6/1/2014 we changed our collection plate and found our recoveries greatly improved: COT recovery changed to about 90%, HC recovery changed to about 70%.

6. 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 and two QC pools in saliva 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
SA04	saliva	1.60	0.034	COT	1

SA04	saliva	1.58	0.017	COT	4
SA04	saliva	0.279	0.006	HC	1
SA04	saliva	0.269	0.016	HC	4
SA05	saliva	213	1.94	COT	1
SA05	saliva	215	3.55	COT	4
SA05	saliva	64.0	1.37	HC	1
SA05	saliva	65.3	1.25	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 $p > 0.05$).

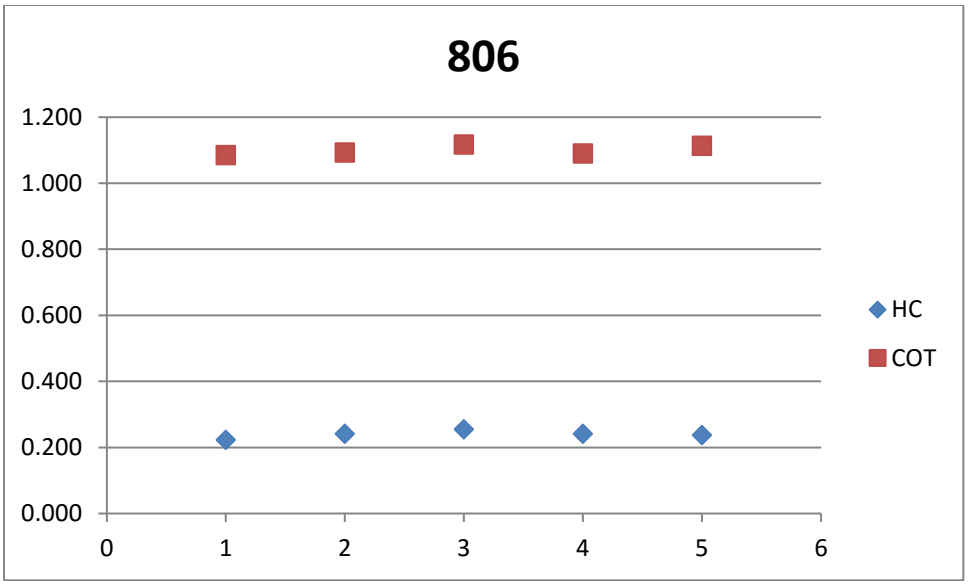
Stability of Extracted Samples

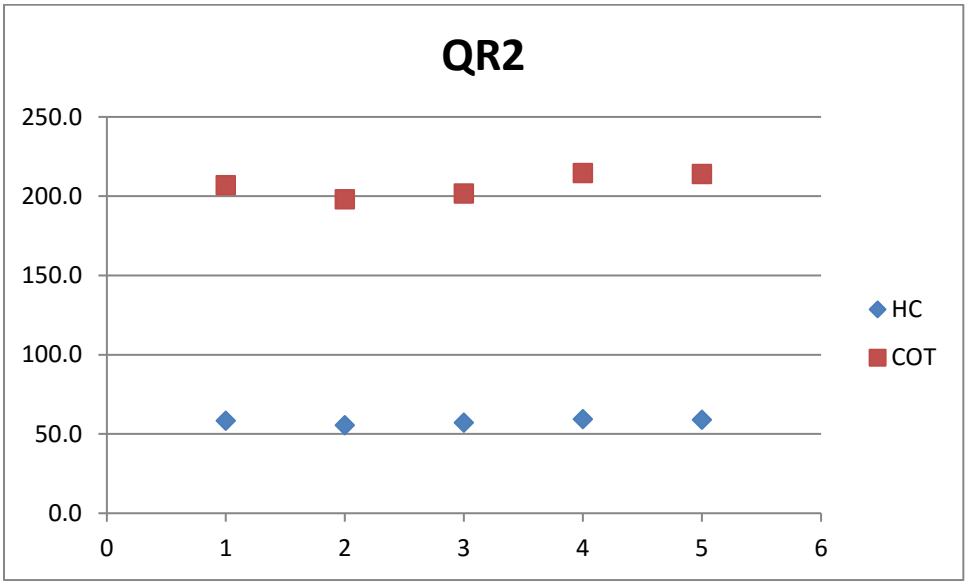
Pool	Matrix	Mean (SD) (ng/mL)	Std Dev (ng/mL)	Analyte	Storage
806	serum	1.18	0.047	COT	1 day
806	serum	1.20	0.055	COT	1 week
806	serum	1.16	0.051	COT	none
806	serum	0.251	0.014	HC	1 day
806	serum	0.261	0.027	HC	1 week
806	serum	0.258	0.009	HC	none
QR2	serum	202	1.90	COT	1 day
QR2	serum	207	5.24	COT	1 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
SA04	saliva	1.83	0.045	COT	1 day
SA04	saliva	1.89	0.037	COT	1 week
SA04	saliva	1.83	0.024	COT	none
SA04	saliva	0.315	0.007	HC	1 day
SA04	saliva	0.328	0.011	HC	1 week
SA04	saliva	0.330	0.011	HC	none
SA05	saliva	223	2.88	COT	1 day
SA05	saliva	223	8.70	COT	1 week
SA05	saliva	218	0.767	COT	none
SA05	saliva	65.4	0.640	HC	1 day
SA05	saliva	67.0	1.73	HC	1 week
SA05	saliva	66.3	2.08	HC	none

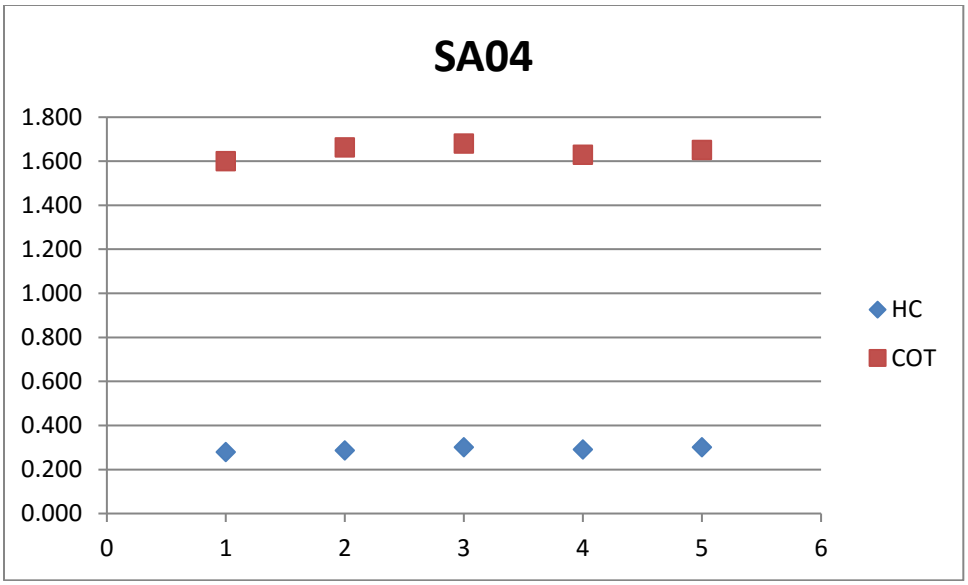
(c) An accelerated stability study was also performed. Samples were held at room temp and in a water bath at 37°C for up to two weeks then analyzed in triplicate along with samples that had remained in the -70°C freezer for the two week period. Results are in the table and charts below. The data show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

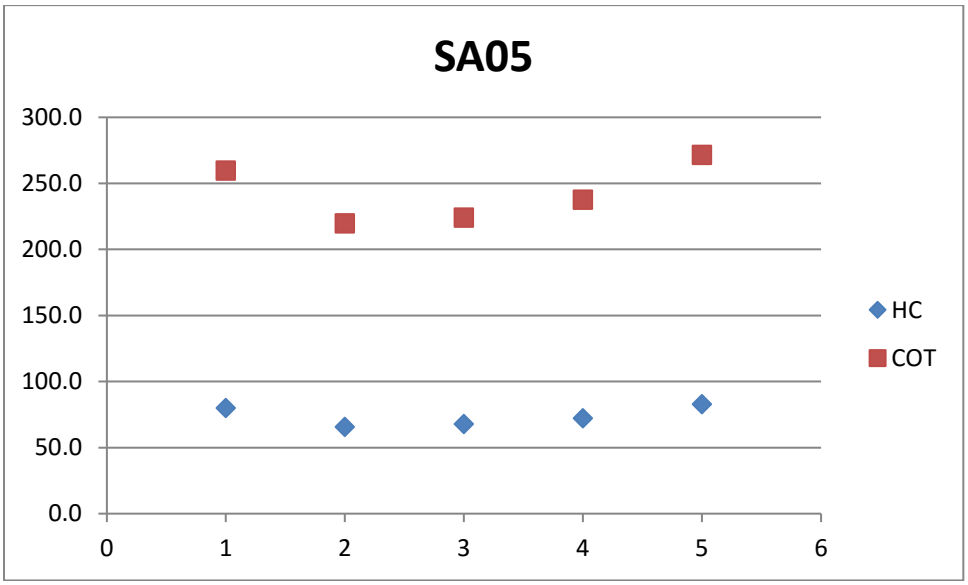
Accelerated Stability Study

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
SA04	low saliva	control	0.279	1.601
SA04	low saliva	RT1	0.286	1.66
SA04	low saliva	RT2	0.301	1.68
SA04	low saliva	37_1	0.291	1.63
SA04	low saliva	37_2	0.301	1.65
SA05	high saliva	Control	79.9	260
SA05	high saliva	RT1	65.7	220
SA05	high saliva	RT2	67.9	224
SA05	high saliva	37_1	72.3	238
SA05	high saliva	37_2	82.9	272


806
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
3
4
5
6
HC
COT


QR2
250.0
200.0
150.0
100.0
50.0
0.0
0
1
2
3
4
5
6
HC
COT





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

7. Specificity

The specificity of the assay was established by analyzing 84 serum samples from nonsmokers. No interferences were seen in the Quant chromatograms for either analyte in any of the samples.

8. Confirmation Ion Ratio Calculation

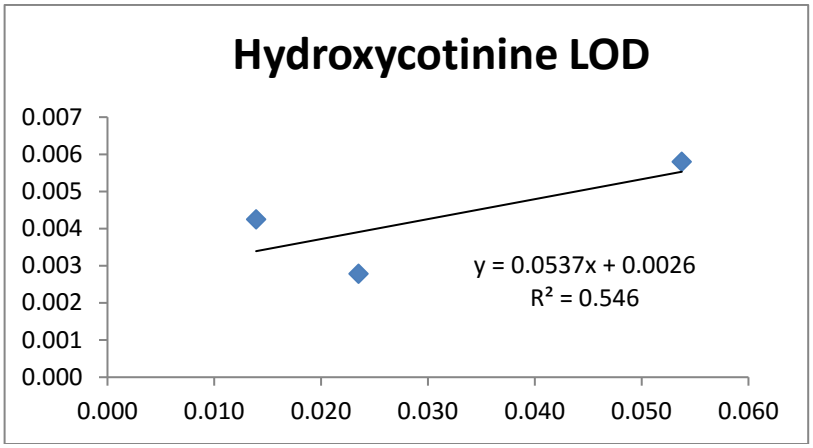
On a daily basis, specificity is monitored by checking confirmation ion ratios. The confirmation ion ratio is calculated for each analyte by dividing the confirmation ion area by the quantitation ion area. The ion transitions are given below.

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

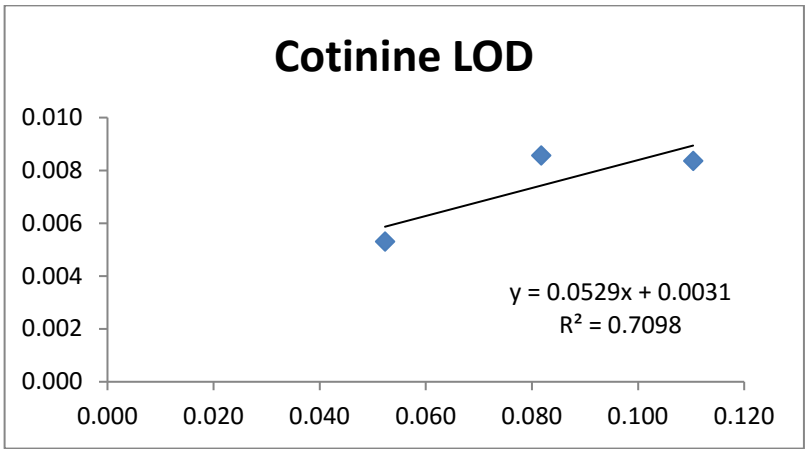
The confirmation ion ratio range is determined for each instrument from the mean of the standards for that day with concentrations ≥ 0.02 ng/mL. Because of low ion counts for the confirmation ion, these evaluations are limited to samples with a calculated concentration ≥ 0.1 ng/mL. Samples are repeated if they have a calculated concentration ≥ 0.1 ng/mL and a confirmation ratio greater than 25% from the mean (**33**).

9. LOD by Taylor's Method (**25**)

Pool	Analyte	Mean	Std Dev	N
PT1	COT	0.052	0.005	9
R-008	COT	0.082	0.009	13
QP1	COT	0.110	0.008	15
R-008	OH	0.014	0.004	13
PT1	OH	0.023	0.003	9
QP1	OH	0.054	0.006	15



LOD = 3*S_0 = 3*(0.0026) = 0.0078 for Hydroxycotinine



LOD = 3*S_0 = 3*(0.0031) = 0.0093 for Cotinine

We are going to keep the old LOD of 0.015 ng/mL for both COT and HC until we reassess the LOD using the DLS method.

10. Blank Carryover

To investigate potential carryover that might occur in a low sample immediately following a high sample, we compared the results for water blanks that were analyzed as the first sample in the run to water blanks that were analyzed immediately following a high QC. All results were below the LOD (0.015 ng/mL) for both analytes. We conclude that there is no carryover problem.

Analyte	Position in run	Mean conc (ng/mL)	Std Dev (ng/mL)	N
COT	first in run	0.0033	0.0014	18
COT	following high QC	0.0066	0.0041	15
HC	first in run	0.0006	0.0008	18
HC	following high QC	0.0005	0.0011	15

11. Blank Cutoff

The blank cutoff is set at the mean + 3*SD of the analyte concentrations found in the water blank samples (N=106 for HC, N=102 for COT).

	HC	COT
Mean	0.0007	0.0042

Std Dev	0.0016	0.0033
Minimum	0	0.0008
Maximum	0.01	0.0139
Count	106	102
3*SD	0.0049	0.0100
M+3*SD	0.0056	0.0142

COT calculated blank cutoff = 0.015 ng/mL
HCT calculated blank cutoff = 0.006 ng/mL, we will use a blank cutoff of 0.015 for HCT.

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.

12. Comparison of Method on two Instruments

In order to compare the responses of the four instruments that are used in this assay, multiple analytical runs were analyzed on pairs of instruments. The concentration of the analytes in the samples spanned the calibration curve.

The paired results were plotted and linear regression was performed. The regression line statistics are listed below.

Comparison	Samples	N	Instruments	Run Type	Analyte	Slope	Intercept	R2
BB856…BB861	serum pools	189	GG vs OP	Low	COT	0.9928	0.0104	0.9981
BB856…BB861	serum pools	189	GG vs OP	Low	HCT	1.0014	0.0034	0.9996
AD127…AD158	Hanes	113	GG vs OP	Low	COT	0.9925	0.0089	0.9992
AD127…AD158	Hanes	113	GG vs OP	Low	HCT	0.9963	0.0045	0.9995
AD171-174	Hanes	64	GG vs DR	Low	COT	0.9936	-0.0018	0.9998
AD171-174	Hanes	64	GG vs DR	Low	HCT	1.008	-0.0009	0.9995
BC121-128	PATH	144	MC vs DR	high	COT	0.9535	8.0259	0.9983
BC121-128	PATH	144	MC vs DR	high	HCT	1.0104	-1.3479	0.9992

The conclusion from these tests is that there is no difference between the instrument responses, so all four instruments can be used interchangeably.