Division of Laboratory Sciences Laboratory Protocol

Analyte: Nicotine, Cotinine, *Trans*-3'-hydroxycotinine, Nornicotine, Nicotine-N-oxide, Cotinine-N-oxide, Norcotinine, Anatabine
Anabasine

Matrix: **Urine**

Method: **LC/MS/MS**

Method No: **2021.02**

Revised: **March 08, 2018**

as performed by:

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

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Important Information for Users

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

Division of Laboratory Sciences

Laboratory Protocol

Analytes: **Nicotine Metabolites and Analogs**

Matrix: **Urine**

Method: **Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS)**

Method code: 2021.02

Branch: **Tobacco and Volatile Branch (TVB)**

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Date current version of method first used in lab: March 15, 2018
Date

Director's Signature Block:

Reviewed:	_____ Signature	_____ Date

DIVISION OF ENVIRONMENTAL HEALTH LABORATORY SCIENCES

Procedure Change Log

Procedure: Nicotine Metabolites and Analogs in urine

DLS Method Code: 2021

Date	Changes Made	By	Rev'd By (Initials)	Date Rev'd
10/3/2014	Added steps for aliquoting on Hamilton Star system and made other minor updates on method	JF		10/3/2014
5/13/2016	Added Indigo data processing information	SM		9/8/2016
5/13/2016	Added Repeat Manager data review information	SM		9/8/2016
5/13/2016	Added modified procedure for pre-instrument water blank analysis	SM		9/8/2016
5/13/2016	Added more details on the rules for quality assurance	SM		9/8/2016
5/13/2016	Updated with more details on remedial action when QC fails	SM		9/8/2016
9/6/2016	Added reference range for this method	JF		9/12/2016
9/6/2016	Updated minor details and formatted the documents	JF		9/12/2016
03/2018	Added method update tables (Appendix 6) to meet DLS requirement	WS		3/6/2018
03/2018	Added method description for "free" analysis (1.2, 6.4, 8.2)	JF		3/6/2018

Public Release Data Set Information

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

Data File Name	Variable Name	SAS Label
UCOT_H & UCOTS_H	URXANBT	Anabasine, urine (ng/mL)
	URXANTT	Anatabine, urine (ng/mL)
	URXCOXT	Cotinine-n-oxide, urine (ng/mL)
	URXNICT	Nicotine, urine (ng/mL)
	URXNNCT	Nornicotine, urine (ng/mL)
	URXNOXT	Nicotine-1 N-oxide, urine (ng/mL)

1. Clinical Relevance and Summary of Test Principle

List of analytes in this method:

(S)-Cotinine N-oxide; $C_{10}H_{12}N_2O_2$; Mol. Wt: 192.21; m.p.: 116-117°C.

(1'S,2'S)-Nicotine 1'-oxide; $C_{10}H_{14}N_2O$; Mol. Wt: 178.23; M.P.: 168-170°C.

(-)-*Trans*-3'-Hydroxycotinine; $C_{10}H_{12}N_2O_2$; Mol. Wt: 192.21; m.p. 107-109°C.

(R,S)-Norcotinine; $C_9H_{10}N_2O$; Mol. Wt.: 162.19; m.p.: 94-96°C.

(-)-Cotinine; $C_{10}H_{12}N_2O$; Mol. Wt.: 176.22; m.p.: 35-37°C; b.p.: 145-150°C/3 mm.

(RS)-Nornicotine; $C_9H_{12}N_2$; Mol. Wt.: 148.2; b.p.: 108-110°C/0.5mm.

(R,S)-Anatabine; $C_{10}H_{12}N_2$; Mol. Wt.: 160.21; b.p.: 136°C.

(R,S)-Anabasine; $C_{10}H_{14}N_2$; Mol. Wt.: 162.23; b.p.: 270-272°C

(-)-Nicotine; $C_{10}H_{14}N_2$; Mol. Wt.: 162.23; m.p.: -79°C. b.p.: 247°C.

1.1. Clinical Relevance

Nicotine is the primary tobacco-specific alkaloid in tobacco plants and tobacco smoke. Although, nicotine is not a direct cause of most diseases associated with tobacco use, it is highly addictive, which can lead to tobacco product dependence and chronic exposure to the carcinogens and bioactive compounds in tobacco. The presence of nicotine in biological specimens indicates exposure to tobacco, either through the active use of tobacco, or from passive exposure to secondhand smoke (SHS). T*rans*-3'-hydroxycotinine and cotinine are the two predominant nicotine metabolites in urine. Because their concentrations are greater and their elimination half-lives are longer, these metabolites are generally preferred over nicotine itself as exposure biomarkers. The concentration ratio of hydroxycotinine to cotinine has been used as an index of cytochrome P-450 A6 activity. The relative concentration of nicotine to its six major metabolites (hydroxycotinine, cotinine, cotinine-N-oxide, nicotine-N-oxide, nornicotine, and norcotinine), is of interest when elucidating differences in metabolic profiles of various ethnic, age, and gender groups (James E. McGuffey, 2013). Furthermore, the sum of these metabolites may more accurately describe the exposure of individuals and groups to tobacco products compared to the most commonly used biomarker, urinary cotinine. Lastly, anatabine and anabasine are nicotine analogs in tobacco product, and are precursors for nitrosamines, which are known carcinogens. Therefore levels of these two compounds have been used in monitoring compliance of smoking cessation programs as well as in biomonitoring for nitrosamine-related studies.

1.2. Test Principle

"Total" urinary anatabine (ANTT), anabasine (ANBT), nicotine (NICT) and its six major metabolites (cotinine-N-oxide (COXT), nicotine-N-oxide (NOXT), trans-3'-hydroxycotinine (HCTT), norcotinine (NCTT), cotinine (COTT) and nornicotine (NNCT)), including the unconjugated and glucuronide conjugated forms, are measured by an isotope-dilution high performance liquid chromatography/electrospray ionization tandem mass spectrometric (HPLC-ESI-MS/MS) method. Beta-glucuronidase is used for the hydrolysis of the conjugated forms prior to analysis. Briefly, a urine sample is spiked with an internal standard mixture of methyl-D3-COXT, methyl-D3-NOXT, methyl-D3-HCTT, pyridyl-D4-NCTT, methyl-13CD3-COTT, pyridyl-D4-NNCT, ANTT-D4, ANBT-D4, and methyl-13CD3-NICT and incubated with beta-glucuronidase to hydrolyze the conjugated analytes. Following the incubation period, proteins and salts in the sample are precipitated with cold acetone. The sample is centrifuged and part of the supernatant is transferred and evaporated to remove acetone. The sample is further diluted 5-10 fold prior to analysis on a HPLC-ESI-MS/MS system. Chromatography is performed using a C18 analytical column; mass spectrometric analysis is carried out under positive mode using scheduled multiple reaction monitoring (MRM). One quantitation transition, one confirmation transition, and one corresponding internal standard transition are monitored for each analyte. Sample concentration is derived from the ratio of the native transition ions to the labeled transition ions in the sample by comparing to a standard curve.

"Free" form of all the urinary nicotine metabolites and minor tobacco alkaloids mentioned in the above paragraph can be measured by the same isotope-dilution high performance liquid chromatography/electrospray ionization tandem mass spectrometric (HPLC-ESI-MS/MS) method. In the sample preparation of this measurement, the step of beta-glucuronidase hydrolysis prior to analysis is eliminated, while all other steps are kept the same.

Special Precautions

Because of the nature of these assays, all analysts involved in this study must be non-users of tobacco product, and measurements must be performed in a smoke-free environment.

2. Safety Precautions

Eye protection and suitable protective equipment should be worn at all times during the sample pre-treatment and analysis procedures.

2.1. Reagent Toxicity/Carcinogenicity

Some of the analytes used in this procedure are toxic. Universal safety precautions must be taken to avoid inhalation or dermal exposure to samples and analytes.

2.2. Radioactive Hazards

This procedure does not use radioactive materials and there are no radioactive hazards associated with it.

2.3. Biological Hazards

This assay involves human urine samples. Universal precautions must be followed. Analysts working directly with the specimens must use proper techniques and avoid any direct contact with the sample. Lab coats, gloves and protective eyewear (as required) should be worn while handling the specimens.

2.4. Mechanical Hazards

There are no unusual mechanical hazards associated with this method. Analysts should know and follow the manufacturer's recommendations concerning the safe handling of instruments and other equipment. High voltages are found within certain areas of the mass spectrometer and care must be taken when working in those areas. Safety interlocks on instruments, such as the mass spectrometer, LC autosampler, centrifuge, etc. should not be disabled during normal operations.

2.5. Protective Equipment

Standard safety precautions should be followed when performing this procedure including the use of lab coats or disposable gowns, safety glasses, appropriate gloves, and the use of biological safety cabinets and chemical fume hoods. Refer to the laboratory Chemical Hygiene Plan and standard CDC/DLS safety policies and procedures guidelines for details of specific activities or reagents.

2.6. Training

Training is required for carrying out the analytical procedure. Training in the use of automated sample preparation system, tandem mass spectrometry, and Indigo Accent data processing, Microsoft Access data review, is required prior to be certified to perform the job. All analysts must be certified and demonstrate proficiency in the analysis before handling samples. Educational and specific training information is maintained for all analysts certified to work on this method.

2.7. Personal Hygiene

Follow standard precaution and comply with all established laboratory safety practices. Care should be taken when handling chemicals to avoid inhalation or dermal exposure. Lab coats, gloves, and safety glasses should be worn at all times when handling standards or samples.

2.8. Disposal of Wastes

All waste disposals must be in compliance with DLS policy. Discard solvents and other waste reagents into an appropriate container marked for waste handling and stored in a chemical fume hood. Place all disposable items that come in contact with biological specimens in a biohazard autoclave bag, which is maintained in an appropriately covered container until autoclaved. Unshielded needles and disposable syringes with attached needles must be placed in a sharps container and autoclaved when the container is full. Wipe down all surfaces potentially exposed to biological samples with a freshly prepared bleach solution (10% dilution of commercial sodium hypochlorite or the equivalent). Non-disposable glassware or other equipment that comes into contact with biological samples must be rinsed with bleach before cleaning and reuse.

3. Computerization; Data-System Management

3.1. Software and Knowledge Requirements

Proficiency in the analytical software package of the HPLC and mass spectrometer used in the analysis is required for all users. AB Sciex Analyst software (current version 1.6.2) is used for data acquisition on the AB Sciex triple quadrupole 5500 mass spectrometer. Analyzed data is uploaded to Indigo Biosystems' Ascent, a web based integration software, for evaluating the quality control and calculating analyte concentrations. Quality control evaluation is continued throughout sample results tracking and repeat run staging, in Microsoft Access. Statistical analysis of results requires proficiency in a standard statistical analysis software package, e.g. Statistical Analysis System (SAS Institute, Cary, NC) or SPSS (IBM Corporation, NY).

3.2. Sample Information

Typically, a batch of 96 samples, including calibration standards, blanks, QCs and unknowns, are analyzed in a sample run. Individual sample information is manually or electronically entered into a database, and each run sheet is prepared using a Microsoft Excel worksheet that contains the run number, sample ID, dilution, date of analysis, and volume of enzyme solution added to each sample.

3.3. Data Maintenance

Check data entered into the database for transcription or transmission errors. Routinely back-up the database on a weekly basis.

3.4. Information Security

The information management systems, including the instrument workstation and database server containing the raw data and final reportable results, are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the campus, buildings, and individual laboratories.

4. Procedures for Collecting, Storing, and handling Specimens; Criteria for Specimen Rejection

4.1. Special Requirements

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

4.2. Sample Collection

Urine can be collected using standard equipment. Mix the sample well before aliquoting, and freeze the urine aliquot in polypropylene cryogenic, screw-cap vial at or below -20°C before preparation is initiated. Long-term storage of samples requires freezer temperature at or below -60°C.

4.3. Sample Handling

Sample processing does not require special preservatives, or unusual sterility procedures. Urine should be handled according to standard equipment and protocols. Ensure that samples remain frozen during shipment and subsequent storage. The laboratory should 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 cause significant contamination, and only equipment that has been prescreened and/or found to be acceptable by this laboratory should be used for collecting samples.

4.4. Sample Quantity

A minimum of 0.5 mL of urine is needed for this assay to provide sufficient volume for repeat analyses if indicated/necessary.

4.5. Long-Term Stability and Storage

Analytes in the method are stable during the analysis duration under the conditions described in this assay. Samples should be stored at or below -60°C for long-term

storage.

4.6. Unacceptable Specimens

Currently, there is no evidence that atypical specimen characteristics influence the HPLC/MS/MS analysis. However, record unusual sample characteristics and maintain this information in database file for tracking purposes.

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

Not applicable to this procedure.

6. Preparation of Reagents, Calibration (standards), Controls and Other Materials, Equipment, and Instrumentation

6.1. Reagents, Materials and Sources

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

Chemical	Grade	Source	Catalog #
acetone	HPLC	Sigma-Aldrich (St. Loius, MO, USA)	650501-4X4L
acetonitrile	HPLC	Honeywell (Muskegon, MI, USA)	AH015-4PC
methanol	HPLC	Honeywell (Muskegon, MI, USA)	230-4
water	HPLC	J.T.Baker	4218-03
ammonium acetate	99.999%	Sigma-Aldrich (St. Loius, MO, USA)	73594-100G-F
ammonium hydroxide	28-30% NH_3	Sigma-Aldrich (St. Loius, MO, USA)	21228-100ML-A
helix Pomatia (type H-1)	>200,000 units/g	Sigma-Aldrich (St. Loius, MO, USA)	G0751-2MU

6.2. Stock Reagents Preparation

The following solutions are prepared on an as-needed basis. Buffer containers and all glassware used for buffer preparation should be rinsed a minimum of three times with 20-50 ml HPLC water.

(1) 650 mM Ammonium Acetate Stock Solution

Weigh 50.61 g of the solid ammonium acetate (Sigma-Aldrich, Item No: 73594-100G-F, or equivalent), and dissolved it HPLC-grade water to a total volume of 1.0 L. Store the labeled buffer in the refrigerator.

(2) HPLC Mobile Phase Buffer A

Fresh mobile buffer "A" (6.5 mM ammonium acetate, pH 10.0) is prepared based on the volume needed for the total batch samples. For a total volume of 1.0 liter buffer, 10mL of 650mM stock ammonium acetate solution is added to 990mL of HPLC-grade water yielding a running buffer of 6.5 mM ammonium acetate. The initial pH should be between 6.7 and 7.0. A significantly higher or lower value indicates a significant amount of residue buffer from a previous preparation, which is not desired. A new buffer solution should be prepared when this happens. The pH of the solution is adjusted to 10.00±0.05 with concentrated ammonium hydroxide solution (Sigma-Aldrich, 28-30% NH3). The buffer solution is degassed for 5 min using an ultrasonic water bath.

(3) HPLC Mobile Phase Buffer B

Mobile phase "B" is 100% acetonitrile. As with the buffer A, it is degassed for 5 min using an ultrasonic water bath.

(4) 0.5 M Ammonium Acetate (pH 5.1)

Weigh 38.5 g of solid ammonium acetate (Sigma-Aldrich, Item No: 73594-100G-F, or equivalent), and dissolve it in HPLC-grade water to a total volume of 1.0 L. The pH is adjusted to 5.1 with glacial acetic acid. Store the labeled buffer in the refrigerator.

(5) β-Glucuronidase Solution, type H-1 from Helix Pomatia

A total unit of 1000 for Helix Pomatia in 60 µL solution (pH 5.1, 16.7 Units/µL) is needed for each urine sample. The enzyme product may be different from lot to lot or source to source. Thus, for a different lot or equivalent source, the amount of the enzyme should be calculated correspondingly. For example, using the Sigma Aldrich product with the specific activity of 3,854,000 units/g, weigh 28.5 mg of β-glucuronidase in 6.5 mL of 0.5 M ammonium acetate (pH 5.1, stored at 4^{o}C). Mix gently by inversion and shaking gently for at least 30 minutes before using to ensure that all enzyme powder has dissolved.

(6) 1:1 Methanol: Water Solution

Approximately 1L of a 1:1 methanol (Honeywell, CAT #: 650501-4X4L, or equivalent)/ HPLC-grade water solution is prepared daily for internal and external auto sampler syringe rinsing. Ensure solution is degassed before connecting to LC instrument, by placing it in the ultra-sonic water bath for 5 min.

6.3. Standards

6.3.1. Standard Commercial Sources

Standard materials are obtained from Toronto Research Chemicals (TRC, Ontario, Canada), but equivalent sources may be used. Stock solution concentrations are calculated based on the stated purity of the compounds and their mass as determined by an analytical balance. The purity, manufacture's purity confirmation procedures, and catalog numbers are given in the table below.

Standard name	Purity	Method	Catalog #
(S)-cotinine-N-oxide	98%	^{1}H NMR	C725200
(*R,S*)-cotinine-N-oxide-methyl-D3	98%	^{1}H NMR	C725203
(1'*S*,2'*S*)-nicotine-1'-oxide	98%	^{1}H NMR	N427500
(+/-)-*trans*-nicotine-1'-oxide-methyl-D3	98%	^{1}H NMR/MS	N427492
trans-3´-hydroxycotinine	98%	^{1}H NMR	H924500
trans-3'-hydroxycotinine-D3	98%	^{1}H NMR	H924510
(*R*,*S*)-norcotinine	98%	^{1}H NMR	N662000
(*R*,*S*)-norcotinine-pyridyl-D4	98%	^{1}H NMR	N662002
(-)-cotinine	98%	^{1}H NMR	C725000
(+/-)-cotinine-^{13}CD3	98%	^{1}H NMR/MS	C725007
(*R*,*S*)-nornicotine	98%	^{1}H NMR	N757000
(*R*,*S*)-nornicotine-D4	98%	^{1}H NMR	N757010
(*R*,*S*)-anatabine	98%	^{1}H NMR	A637500
(*R*,*S*)-anatabine-2,4,5,6-D4	97.5%	^{1}H NMR	A637505
(*R*,*S*)-anabasine	98%	^{1}H NMR/MS	A637175
(*R*,*S*)- anabasine-2,4,5,6-D4	98%	^{1}H NMR/MS	A637180
(-)-nicotine	98%	^{1}H NMR/MS	N412450
(+/-)-nicotine-^{13}CD3	98%	^{1}H NMR	N412424

6.3.2. Stock Solution Preparation

(1) Native Stock Solutions

Initial native stock solutions are prepared by dissolving the accurately weighed compound in a desired volume (100 or 250 mL) of HPLC water in volumetric flasks (Table 6.3.2.1).

Table 6.3.2.1. Analyte concentration in original stock

Analyte name	Analyte code	Amout weighed (mg)	Purity	Final volume (mL)	Conc. (µg/mL)
(S)-cotinine-N-oxide	COXT	24.81	0.98	100	243.1707

(1'*S*,2'*S*)-nicotine-1'-oxide	NOXT	99.87	0.98	100	978.7378
trans-3'-OH-cotinine	HCTT	75.38	0.98	250	738.695
R,S-norcotinine	NCTT	9.94	0.98	100	97.412
(-)-Cotinine	COTT	118.55	0.98	100	1161.757
R,S-Nornicotine	NNCT	53.83	0.98	100	527.534
RS-Anatabine	ANTT	8.29	0.98	100	81.2028
(*R*,*S*)-Anabasine	ANBT	11.09	0.98	100	108.633
(-)-Nicotine	NICT	102.88	0.99	100	1018.545

Sub-stock 1 of the native stock solutions are prepared by diluting original stock concentrations as listed in Table 6.3.2.2.

Table 6.3.2.2. Analyte concentration in sub-stock 1 solution

Analyte	Original Stock Concentration (µg/mL)	Volume transferred (mL)	Sub-stock 1 Concentration (µg/mL)	Final Volume (mL)
COXT	243.171	10.00	24.317	100
NOXT	978.736	10.00	97.874	100
HCTT	738.695	10.00	73.870	100
NCTT	97.412	10.00	9.741	100
COTT	1161.757	5.00	58.088	100
NNCT	527.534	14.22	75.015	100
ANTT	81.203	35.00	28.421	100
ANBT	108.633	23.00	24.986	100
NICT	1018.545	10.00	101.855	100

(2) Isotopically-labeled Internal Standard Stock Solutions

Original isotopically-labeled stock solutions are prepared in 100 mL of HPLC water, as with the native solutions (Table 6.3.2.3). Exact amount of chemical was purchased and used based on the quantity indicated by the manufacturer.

Table 6.3.2.3. Concentration of internal standard stock solution

	Analyte code	Amount used (mg)	Chemical Purity	Isotopic purity	Recovery	Conc (µg/mL)
(*R,S*)-cotinine-N-oxide-methyl-D3	COXT-ISTD	15.0	0.98	0.99	0.98	142.62
(+/-)-*trans*-nicotine-1'-oxide-methyl-D3	NOXT-ISTD	10.0	0.98	0.99	0.98	95.08

trans-3'-hydroxycotinine-D3	HCTT-ISTD	10.0	0.95	0.99	0.98	92.17
(*R*,*S*)-norcotinine-pyridyl-D4	NCTT-ISTD	10.0	0.98	0.995	0.98	95.56
(+/-)-cotinine-^{13}CD3	COTT-ISTD	10.0	0.98	0.996	0.98	95.66
(*R*,*S*)-nornicotine-D4	NNCT-ISTD	5.0	0.98	0.99	0.98	47.54
(*R*,*S*)-anatabine-2,4,5,6-D4	ANTT-ISTD	10.0	0.96	0.975	0.98	91.73
(*R*,*S*)-anabasine-2,4,5,6-D4	ANBT-ISTD	5.0	0.95	0.99	0.98	46.08
(+/-)-nicotine-^{13}CD3	NICT-ISTD	10.0	0.95	0.98	0.98	91.24

6.3.3. Spiking Internal Standard Solution

500 mL internal standard solution is prepared by adding and mixing adequate internal standard stock solutions and diluting with HPLC water, to a final volume of 500 mL in a volumetric flask. An example for the volume transferred from each original stock solution and the concentration for each labeled standard is also listed in Table 6.3.3.1.

Table 6.3.3.1. Internal standard stock solutions and spiking concentration

ISTD	Analyte Code	Original Stock µg/mL	Volume transferred mL	Concentration in 500 mL spiking solution, ng/mL
COXT-D3	COXT-ISTD	142.619	1.60	456.38
NOXT-D3	NOXT-ISTD	95.080	1.60	304.25
HCTT-D3	NCTT-ISTD	92.169	7.00	1290.37
NCTT-D4	NCTT-ISTD	95.560	3.80	726.25
COTT-^{13}CD3	COTT-ISTD	95.656	1.80	344.36
NNCT-D4	NNCT-ISTD	47.540	6.00	570.48
ANTT-D4	ANTT-ISTC	91.728	2.00	366.91
ANBT-D4	ANBT-ISTD	46.085	5.50	506.93
NICT-^{13}CD3	NICT-ISTD	91.238	30.00	5474.28

6.3.4. Preparation of Calibration Standard Solutions

(1) Blank Urine Pool

Collect urine samples from non-cigarette users in 120 mL sterile specimen containers and

store them at or below -20ºC (e.g. in this study n=200). Chromatographically, screen the urine samples collected to identify the samples with potentially detectable levels of the analytes in this assay. Combine and thoroughly mix the urine supernatant from these background-clean urine samples, and store the pooled urine at 4ºC overnight.

(2) Preparation of Standard Solutions

Eleven standard solutions (SL01 – SL11) are prepared in pooled blank urine by diluting a desired amount of the native stock solution, to a final volume of 500 mL in a volumetric flask. The analyte concentrations at each level are listed in Table 6.3.4.1.

All eleven standards were used to build a calibration curve when “total” form of the analytes were measured. When “free” form of these analytes were measured, nine standards (excluding SL05 and SL09) were used to build a calibration curve.

Table 6.3.4.1. Calibration standard levels and analyte concentrations at each level (ng/mL)

Levels	COXT	NOXT	HCTT	NCTT	COTT	NNCT	ANTT	ANBT	NICT
SL01	1.50	2.00	3.00	0.75	1.50	0.00	0.25	0.20	0
SL02	2.97	3.96	5.94	1.49	2.97	1.49	0.50	0.40	3.03
SL03	5.91	7.87	11.81	2.95	5.91	2.36	0.98	0.79	8.52
SL04	12.00	14.00	80.00	3.40	50.00	2.40	1.00	0.80	40.00
SL05	30.00	35.00	200.00	8.50	125.01	6.00	2.50	2.00	100.00
SL06	60.00	70.00	400.01	17.00	250.01	12.00	5.00	4.00	200.01
SL07	120.01	140.00	800.01	34.00	500.02	24.00	10.00	8.00	400.02
SL08	300.01	350.01	2000.03	85.01	1250.05	60.01	25.01	20.01	1000.05
SL09	750.04	875.03	5000.08	212.51	3125.13	150.03	62.53	50.02	2500.12
SL10	1500.07	1750.06	10000.16	425.03	6250.25	300.06	125.05	100.04	5000.24
SL11	3000.14	3500.12	20000.32	850.06	12500.51	600.12	250.10	200.08	10000.48

The following tables present the details on preparation of each calibration standard solution (500mL volumetric flask).

Prepare a high concentration working solution A (WS-A) in pooled blank urine using the native stock solutions, to a volume of 250 mL in volumetric flasks. The analyte concentrations in WS-A are listed in Table 6.3.4.3.

- **Table 6.3.4.3. Working Solution A**

Analyte	Stock solution used µg/mL	Volume Transferred mL	WS-A µg/mL

COXT	243.171	15.422	15.001
NOXT	978.736	4.470	17.501
HCTT	738.695	33.844	100.001
NCTT	97.412	10.908	4.250
COTT	1161.757	13.450	62.503
NNCT	75.015	10.000	3.001
ANTT	28.421	11.000	1.251
ANBT	24.986	10.010	1.000
NICT	1018.545	12.273	50.002

Calibration standard levels 8-11 are prepared by diluting 10, 25, 50 and 100 mL of WS-A respectively, to 500 mL with the blank urine pool in volumetric flasks. The analyte concentrations at each level are listed in Table 6.3.4.4.

- **Table 6.3.4.4. Standard Levels 8-11**

Analyte	WS-A µg/mL	SL08 ng/mL	SL09 ng/mL	SL10 ng/mL	SL11 ng/mL
COXT	15.001	300.01	750.04	1500.07	3000.14
NOXT	17.501	350.01	875.03	1750.06	3500.12
HCTT	100.001	2000.03	5000.08	10000.16	20000.32
NCTT	4.250	85.01	212.51	425.03	850.06
COTT	62.503	1250.05	3125.13	6250.25	12500.51
NNCT	3.001	60.01	150.03	300.06	600.13
ANTT	1.251	25.01	62.53	125.05	250.10
ANBT	1.000	20.01	50.02	100.04	200.08
NICT	50.002	1000.05	2500.12	5000.24	10000.48

Transfer 20.0 mL SL08 into a clean 500 mL volumetric flask, and fill to the line with the blank urine pool. Analyte concentrations at each level are listed in Table 6.3.4.5.

- **Table 6.3.4.5. Standard Level 4 (SL04)**

Analyte	SL08 ng/mL	Transfer volume, mL	SL04 ng/mL
COXT	300.01	20	12.00
NOXT	350.01		14.00
HCTT	2000.03		80.00
NCTT	85.01		3.40
COTT	1250.05		50.00
NNCT	60.01		2.40

ANTT	25.01		1.00
ANBT	20.01		0.80
NICT	1000.05		40.00

Transfer 20.0 mL SL09 into a clean 500 mL volumetric flask, and fill to the line with the blank urine pool. Analyte concentrations at each level are listed in Table 6.3.4.6.

- **Table 6.3.4.6. Standard Level 5 (SL05)**

Analyte	SL09 ng/mL	Transfer volume, mL	SL05 ng/mL
COXT	750.04	20	30.00
NOXT	875.03		35.00
HCTT	5000.08		200.00
NCTT	212.51		8.50
COTT	3125.13		125.01
NNCT	150.03		6.00
ANTT	62.53		2.50
ANBT	50.02		2.00
NICT	2500.12		100.00

Transfer 20.0 mL SL10 into a clean 500 mL volumetric flask, and dilute to 500 mL using blank urine pool. Analyte concentrations at each level are listed in Table 6.3.4.7.

- **Table 6.3.4.7. Standard Level 6 (SL06)**

Analyte	SL10 ng/mL	Transfer volume, mL	SL06 ng/mL
COXT	1500.07	20	60.00
NOXT	1750.06		70.00
HCTT	10000.16		400.01
NCTT	425.03		17.00
COTT	6250.25		250.01
NNCT	300.06		12.00
ANTT	125.05		5.00
ANBT	100.04		4.00
NICT	5000.24		200.01

Transfer 20.0 mL SL11 into a clean 500 mL volumetric flask, and fill to the line with the blank urine pool. Analyte concentrations at each level are listed in Table 6.3.4.8.

- **Table 6.3.4.8. Standard Level 7 (SL07)**

Analyte	SL11 ng/mL	Transfer volume, mL	SL07 ng/mL
COXT	3000.14	20	120.01
NOXT	3500.12		140.00
HCTT	20000.32		800.01
NCTT	850.06		34.00
COTT	12500.51		500.02
NNCT	600.13		24.00
ANTT	250.10		10.00
ANBT	200.08		8.00
NICT	10000.48		400.02

Prepare a low concentration working solution B (WS-B) in pooled blank urine using sub-stock 1 solution, in a 100 mL volumetric flask. Working solution C (WS-C) is prepared by transferring 10.0 ml WS-B to a clean volumetric flask, filling with 100 mL of pooled blank urine. These two stocks contain all the analyte except NNCT and NICT. Analyte concentrations are listed in Table 6.3.4.9.

Table 6.3.4.9. Working Solution B &C

Analyte	Sub-stock 1 (µg/mL)	Volume transferred (mL)	WS-B (ng/mL)	WS-C (ng/mL)
COXT	24.317	12.338	3000.24	300.02
NOXT	97.874	4.087	4000.09	400.01
HCTT	73.870	8.123	6000.42	600.04
NCTT	9.741	15.400	1500.15	150.01
COTT	58.088	5.165	3000.24	300.02
ANTT	28.421	1.760	500.21	50.02
ANBT	24.986	1.601	400.02	40.00

Prepare two low concentration stocks for NICT and NNCT in a similar way to WS-B and WS-C. Prepare a low concentration working solution B (WS-B) for each analyte in pooled blank urine using sub-stock 1 solution, in a 100 mL volumetric flask. Corresponding working solution C (WS-C) is prepared by transferring 10.0 ml WS-B to a clean volumetric flask, filling with 100 mL of pooled blank urine. The names of these are stocks and concentrations of the analyte are listed in Table 6.3.4.10.

Table 6.3.4.10. Low concentration stocks for NICT and NNCT

Analyte	Sub-stock 1 (µg/mL)	Volume transferred to make WS-B (mL)	Stock name	Analyte concentration (ng/mL)
NNCT	75.015	2.000	NNCT-WS-B	1500.31

NNCT			NNCT-WS-C	150.031
NICT	101.855	5.000	NICT-WS-B	5092.73
NICT			NICT-WS-C	509.27

To prepare calibration standard levels 01-03, dilute designated amount of low concentration stocks prepared above, which is shown in table 6.3.4.11, using blank urine pool, each to designated volume. Analyte concentrations for each level are listed in Table 6.3.4.12.

Table 6.3.4.11. Volume of the stocks added to each standard level

Standard	WS-C Volume (mL)	NICT-WS-C Volume (mL)	NNCT-WS-C Volume (mL)	Final volume (mL)
SL 01	2.5	0	0	500
SL 02	5	3	5	505
SL 03	10	8.5	8	508

- **Table 6.3.4.12. Standard Levels 01-03**

Analyte	WS-C (ng/mL)	SL01 ng/mL	SL02 ng/mL	SL03 ng/mL
COXT	300.02	1.499	2.971	5.906
NOXT	400.01	1.998	3.960	7.874
HCTT	600.04	2.997	5.941	11.811
NCTT	150.01	0.749	1.485	2.953
COTT	300.02	1.499	2.971	5.906
ANTT	50.02	0.250	0.495	0.985
ANBT	40.00	0.200	0.396	0.787
NNCT	150.03	0.000	1.485	2.363
NICT	509.27	0.000	3.025	8.521

(3) Standards Acceptance Criteria

Analyze the standards in the order from the lowest concentration to highest concentration 20 times. In order to accept the prepared standard solutions, the following must be true:

- Correlation coefficient $R^2 \geqslant 0.99$. No more than two out of the 20 calibration curves can have an $R^2 < 0.99$.
- Back-calculated standard value should be within15% of the nominal concentration for standards with their concentrations greater than 3x LOD, within 30% for standards with their concentrations between 1-3x LOD, and within 50% for standards with their

concentrations less than LOD. Standard data points that do fall outside of this limit are excluded from calibration.

- No more than two standards per calibration curve can fall outside this limit. For “total” analysis, nine out of eleven standards should meet the requirement; for “free” analysis, seven out of nine standards should meet the requirement.

6.4. Quality-Control (QC) Materials

6.4.1. Preparation of Blank Urine Pool

Preparation of blank urine pool is described in Section 6.3.4.

6.4.2. Preparation of QC Pools

QC pools for analyzing “total” form of analytes of interest

Two QC urine pools, QC-Low (QCL) and QC-High (QCH), are used in each analytical run of the “total” analysis. Each of the QC pools is prepared by combining desired amount of the following urinary and standard stock sources: 50 urine samples from cigarettes users, standard cocktail solution, and blank urine pool collected from ~100 non-tobacco users.

First, each of the users’ urine samples is screened and the level of each analyte included in the method is quantified. Then, make the low QC pool by transferring a calculated volume of each urine sample to a large volume of the low concentration stock pool. Finally, make the high QC pools by combining a large amount of the high-concentration samples and diluting with low concentration urine or blank urine pool.

The high QC pools that resulted from the mixing process will often require spiking with an additional amount of each native analyte from stock solutions to achieve the desired final concentrations. This should be done when the concentration of a given analyte is not high enough in the users’ urine samples.

Mix the pools well and store them overnight at approximately 4^{o}C. The next day, mix the pools at room temperature, and aliquot the pools into labeled 2mL Fluidx vials or cryovials. All QC samples are store at or below -60^{o}C.

QC pools for analyzing “free” form of analytes of interests

Two QC urine pools, QC-Low (SL05) and QC-High (SL09), are used in each analytical run of the “free” analysis. Preparation of these two pools are described in 6.3.4.

6.5. Instrumentation and Equipment

Instruments, supplies, and sources used during the development and validation of this method are listed below. Materials and supplies for use with this method should be equivalent to those listed if obtained from other alternative sources. The following is a list of equipment used in this method.

- HPLC: Shimadzu modular system, consisting of a DGU-20A5R degasser, two LC-20ADXR pumps, one SIL-30AC autosampler, one CTO-20AC column oven, and a CBM20A controller (Shimadzu Corp, Columbia, MD).
- Mass spectrometer: AB Sciex 5500 triple quadrupole mass spectrometer with a TurboIonSpray source (ABSciex, Foster City, CA).
- Nitrogen gas generator: NM20ZA Peak Generator (Peak Scientific Instruments Ltd.)
- Automated sample preparation system: Caliper Staccato System, built in an enclosure with the depth of 1.5 m, width of 2.3 m and height of 2.2 m, containing a Mitsubishi robot, a Sciclone G3 automated liquid handling workstation (Perkin Elmer, Waltham, MA, USA), a Rotanda 460 auto-centrifuge (Hettich), a thermal sealer (Thermo Scientific), a TurboVap 96 (Biotage), a capper/de-capper (FluidX), four incubators/shakers (Inheco), a 1D barcode reader, and a 2D barcode reader (FluidX).
- Liquid Handler: Hamilton Star automated liquid handler
- Data acquisition system: Analyst software (current version 1.6.2), Applied Biosystems International (ABI).
- Data integration and review system: Indigo Ascent Automated Data Analysis and Review software, Indigo Biosystems, Indianapolis, IN.
- Data Management System, Microsoft Access, (Microsoft Corporation, Redmond, WA)
- Branson 5510 ultrasonic cleaner, Branson Ultrasonics, Danbury, CT.
- Eon Microplate Spectrometer, Biotek, Winooski, VT.
- Plate vortexer, Scilogex, Berlin, CT.
- UB-10 Ultrabasic pH meter (Denver Instrument, NY, USA)
- SevenCompact™ pH meter (Mettler Toledo, Columbus, OH)
- Tissue Culture Rotator (Glas –Col, Terre Haute, IN)

6.6. Other Supplies

Other supplies used in this method are listed below. Equivalent sources may be used:

- Assay plate: Nunc 96 deepwell-1 mL natural polypropylene (Fisher: #12-565-394)
- Injection plate: Supelco SPE 96 deep square well collection plate (Sigma: #575653-U)
- 12-column Reservoir: Seahorse Bioscience Res Seahorse 12 CL 21 ML (Fisher: #50-995-865)
- Nalgene cryogenic vials: 2 mL and 5 mL (Fisher Scientific, NNI # 5000-0020 and 5000-0050)
- 2D Coded Jacket Cryo Tubes: 1.8ml (FluidX Part #: 65-7532)
- 0.2 mL pipet tips (Perkin Elmer)
- Aluminum sealing foil (Fisher Scientific)
- 50 µL CORE tips and 1 mL high volume tips (Hamilton, Part # 235948 and 235905, respectively)

7. Calibration and Calibration-Verification Procedures

7.1. Calibration Curve for LC-MS/MS Assay

The calibration curve for this assay is created using the standards described above in Section 6. Calibration standards are prepared and run in the same manner as QCs, blanks, and unknown urine samples in every analytical batch. Calibration curves are established as described in Section 8.

7.2. Usage of Calibration Curve

Quantification can only be performed for values that fall within the calibration range, between the lowest and the highest calibration point. Calculated concentrations that are outside the calibration range, low or high end, may have high variations. Unknowns that yield calculated concentrations exceeding the highest level calibrator included in calibration will be diluted and re-prepared for analysis.

7.3. Calibration Verification

QCs (low and high) are analyzed in every analytical batch to validate that the calibration and analysis in general are within acceptable limits.

Calibrations are further confirmed semi-annually through proficiency testing (PT) pools with previously characterized concentrations.

8. Procedure Operation Instructions, Calculations, Interpretation of Results

An analytical run consists of up to five water blanks, 11 calibration standards, two quality control (QC) samples, and a maximum of 78 unknown samples.

8.1. Run Sheet

Each run consists of up to 96 samples prepared in a 96-well plate. When the running batch is prepared, each plate is assigned a run ID in the following format: Ammddyyyy_NNNXY, where:

- **A**09122013_0123P1A:
  - A denotes Sasha Jr HPLC-MS/MS instrument
  - B denotes NIC1 HPLCS-MS/MS instrument
- A**09122013**_0123P1A:
  - reflects the mmddyyyy of plate injection (not plate preparation)
- A09122013_**0123**P1A:
  - reflects the plate number
- A09122013_0123**P1A**:
  - reflects the study name and the study set

For example, a sample with a run ID of "A09122013_0123P1A" represents a plate ran on Sasha Jr, on September 12, 2013. The plate number is 123, and the study set in P1A.

For every sample within a given run, a sample name, sample id, and output file name is assigned. The sample name reflects the sample barcode. The sample id, which is the same as the instrument data file name, reflect the specific run information and positioning on the plate.

A typical HPLC-MS/MS running sequence is displayed as follows:

Blank_01
Standards 01-11
Blank_02
Blank_03
QC-LOW
1st half unknown Samples
Blank_04
2nd half unknown Samples
Blank_05
QC-HIGH

Note: Blank_04 can be omitted if less than 36 unknowns are run on a plate.

8.2. Sample Preparation

Samples, including unknown urine samples, QCs, control blanks and standards are prepared in the same manner using the automated sample preparation system.

The sample preparation setup and procedures listed here are examples used to illustrate the method. Minor changes in the set up (such as position changes on the deck or plate) should not affect analytical results. When minor changes are made, results should be compared to results from before the change; however, a full validation of the method is not necessary. If samples arrive in a format different than the one mentioned in this document, analytical results should be valid as long as the chemistry is the same and accuracy and precision of all steps are not compromised.

To be compatible with the automation system, samples to be tested with this method should be in either 2-mL Nalgene cryo vials (or of the vials with same diameter and similar height) or 2-mL FluidX vials. If they are in 2-mL FluidX vials, they will be aliquoted on Caliper staccato system. If the samples are in Nalgene vials or equivalent, they will be aliquoted on the Hamilton Star System (or equivalent). After the addition of the internal standard solution and samples onto a 96-well plate, the rest of the preparation is carried out on the Caliper Staccato System to completion.

When the quantity of sample volume is insufficient to complete a single run, an aliquot of that sample will be obtained, if available. Sample aliquots transferred from other labs will be ran (on instrument and in data processing software) according to the specified barcode associated with the aliquot, and not the barcode associated with the original identification. When reporting, the aliquot identification will be changed after completion of QA/QC assessment for the entire sample.

The same sample preparation steps were applied to both the “total” and the “free” analyses except the hydrolysis step. The following are a description of the “total” analysis where this method applied most often. Special steps for the “free” analysis is listed as needed.

8.2.1. Sample Organization and Initial preparation for the Caliper system

(1) Remove the samples from the freezer and thaw at room temperature. If needed, samples may be thawed in incubator or water bath at 37°C for 1 hour. All stds/QCs/Samples are mixed on an end-over-end rotator for 30 minutes prior to aliquoting.

(2) Check each sample cap to verify it is well-sealed. Record any samples where spilling, leaking, or any other abnormality of the sample is observed.

(3) Transfer unknown urine sample vials to a 24-vial rack, which is designed for barcoded FluidX tubes and sanded-down to fit on the Caliper system.

(4) Transfer standards/QCs/blanks to rack 4 in the following layout:

	1	2	3	4	5	6
A	Standard 1	Standard 5	Standard 9	Blank	Blank	
B	Standard 2	Standard 6	Standard10	Blank	QCL	
C	Standard 3	Standard 7	Standard11	Blank	QCH	
D	Standard 4	Standard 8		Blank		

The remaining open positions can be used for unknown urine samples.

(5) Repeat steps 1 through 2 for as many 96-well plates as needed, up to four plates per day. A typical final sample layout on the 96-well plate is as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	US	US	US	US	US	US	US	US	US	US	US	US
B	US	S1	US	S5	US	S9	US	BLK	US	BLK	US	
C	US	US	US	US	US	US	US	US	US	US	US	US
D	US	S2	US	S6	US	S10	US	BLK	US	QCL	US	
E	US	US	US	US	US	US	US	US	US	US	US	US
F	US	S3	US	S7	US	S11	US	BLK	US	QCH	US	
G	US	US	US	US	US	US	US	US	US	US	US	US
H	US	S4	US	S8	US		US	BLK	US		US	

US = Unknown sample
S(X) = Standard
BLK = Water Blank
QCL = Quality Control Low
QCH= Quality Control High

Rack 1 = Red
Rack 2 = Green
Rack 3 = Blue
Rack 4 (QC/Standards) = Orange

8.2.2. Deck/Consumable Setup

(1) For each 96 well plate, consumables, racks, and reservoirs are placed on the Caliper as follows:

Rack 1: Urine sample racks for positions 1 through 4 with the standard/QC/blank rack placed in the 4th position. Place 96-well Nunc plates in position 9.

Rack 3: As many 96-well deep-well Supelco plates as needed, starting from position 1 (four maximum).

- Ensure that there are at least three boxes of tips in the hyperstack per plate that will be prepared. Remove lid before use and place lid back after run is finished.
- Rinse out the bulk dispenser reservoirs (the acetone bottle in the circulating bath and the water bottle on top of the Sciclone) and replace with fresh acetone or water daily (at least 150 mL per plate). Acetone bottles should be rinsed daily three times with 50 mL acetone each time, and water bottles should be rinsed daily three times with 50 mL water each time.
- Make sure the first position of the TurboVap (left position) is turned on and has a flow rate of around 35.
- All plates should be taken out of their package immediately before use to reduce the possibility of environmental nicotine contamination, as nicotine readily deposits on surfaces.

(2) Set the Sciclone deck up as follows:

A scheme for the Sciclone deck is displayed in table below. The 12-column reservoir should contain sufficient enzyme solution in column 1 (6.5 mL per 96-sample plate) and internal standard solution (ISTD) in column 12 (6 mL per plate).

The tip box at B2 should contain at least two columns of tips per plate.

	1	2	3	4	5
A		Tip Waste	Shaker	Tip formatter	Positive Pressure SPE
B		**Partial tip box**		Liquid waste	Piercing array
C				**12-column reservoir**	
D		Clamp station	Tip box (empty)		

Items highlighted in blue need to be manually placed on deck before running the method.

8.2.3. Sample Preparation Steps on Caliper

Parameters used here are tested to obtain accurate and reproducible results. If any parameters are changed, the method accuracy and precision should be compared with previous results. However, a full validation is not necessary.

1) Bring assay plate (1 mL Nunc deep well) from rack to Sciclone deck
2) Add 50 µL of IS solution to assay plate

- 40.5 µL/sec aspirate with 25 µL leading air gap (LAG) and 25 µL trailing air gap (TAG)
- 30 µL/sec dispense

3) Decap and scan 24 vial sample rack with 2D barcode reader before it is brought to the Sciclone deck

4) Mix sample and add 100 µL of sample/standard/QC/blank (procedure is same for all)

- Ten mix cycles (160 µL, tip touch at end)
- 3.333 µL/sec aspirate with 75 µL LAG
- 20.5 µL/sec dispense

5) Repeat steps 3 and 4 for each 24-sample rack for one full 96-well plate

6) Add 60 µL of enzyme solution (1000 units of beta glucuronidase, Helix. Pomatia. See Section 6.2.5). **For the "free" analysis, 60 µL of water is added.**

- 40.5 µL/sec aspirate with 100 µL LAG
- 100 µL/sec dispense

7) Seal assay plate

- Sealed at 169°C, 7 seconds

8) Incubate the sealed sample plate for 12 hours at 45°C. **For the "free" analysis, this step is skipped and step 9 below is performed.**

- Shake at 660 RPM, 3 mm amplitude in x and y directions

9) Centrifuge the assay plate at -20°C for 2 minutes (1651 RPM)

10) Return assay plate to Sciclone deck, pierce foil seal

11) Add 450 µL of cold acetone (-20°C) using bulk dispenser

- Dispenser primed for three cycles of 2.5 mL before use
- Dispensed at 200 µL/sec

12) Mix the acetone using nine mix cycles of aspirating/dispensing with 200 µL tips

13) Seal the assay plate(Same parameters as in step 6)

14) Incubate the assay plate in centrifuge at -20°C for 30 minutes

15) Centrifuge the assay plate at -20°C for 30 minutes (4716 RPM)

16) Return the assay plate to Sciclone deck, pierce foil seal

17) Bring an injection plate (Supelco 2 mL deep well plate) to the deck

18) Transfer 180 µL of supernatant from assay plate to the injection plate

- 40.5 µL/sec aspirate
- 50 µL/sec dispense with tip touch at end

19) Take the injection plate and place it inside TurboVap for 12 minutes
    - Evaporate at 30°C, nitrogen flow rate: 35 Fh
20) Transfer the injection plate to the deck, and add 250 µL of water to each well using the bulk dispenser.
    - Dispenser primed for three cycles of 2.5 mL before use
    - Dispensed at 533.33 µL/sec
21) Seal the injection plate (same parameters in step 6) and store it on Caliper rack

8.2.4. Automation Method Running on Caliper

(1) Open iLink Pro. Allow all the ICPs to start up. If there is an error loading the Sciclone, power it down for a few seconds, turn it back on, and click “Cancel” to retry. This usually solves the problem. However, if the problem persists, restart the computer and try again.

(2) Open the method “preincubation – automatic”. Click “Initalize”. If any initialization errors occur, restart the computer and try again. Note: If running samples that are low volume (less than 300 µL in sample tube), run the method “preincubation – low volume samples – automatic” instead, which will have a lower aspiration depth to reliably accommodate the lower sample volume.

(3) Click on the first instruction and click the “Run” button. A prompt will pop up allowing one to specify the number of tips in the partial tip box, the directory where the barcode results are saved in, and the run number of the plate. User intervention will be required during the recap steps due to an iLink error. The error can be cleared by clicking “Skip Instruction” icon, allowing the recapping to function normally. Finishing this method will result in a sealed plate containing sample, internal standard, and enzyme in each well.

(4) Take sealed plate and place it in the first available IVD incubator, starting from the left. Have the bottom of the plate (the side with notches for Nunc brand plates) face to the right. Open the ICP for the incubator and click “start” to start the shaker.

(5) Repeat steps 3 and 4 for each additional 96 well plate being run.

(6) When finished preparing each assay plate, open the iLink method set file “Incubate and Remove (acetone mix).mst.” Initialize as before and click on the first instruction. Unless there is an error, no user intervention is required after this point.

8.2.5. Sample Organization and Initial preparation on Hamilton Star system

(1) Remove the samples from the freezer and thaw at room temperature. If needed, samples may be thawed in incubator or water bath at 37°C for 1 hour. All stds/QCs/Samples are mixed on an end-over-end rotator for 30 minutes prior to aliquoting.

(2) Decap all standards/QCs/samples and discard caps to avoid cross contamination. Note any samples that have less than 200 µL, these samples need to be manually transferred onto the plate with a pipette. Record any unusual characteristics about the samples (unusual color, high turbidity, leaking, etc).

(3) Place standards, blanks, and QCs in first 24 vial rack in the following order, making sure the barcodes of unknown samples are facing outward: Water blank 1, Standard 1 to 11 (in order), Water blank 2 and 3, QC-low, Water blank 4 and 5, QC-high

If blanks/QCs/standards are in pre-barcoded vials, do not let the barcode face the scanner. This will let you manually input what these samples are so you do not have to change the output file later. This can also apply to samples that are not in their original vial (diluted samples, samples from other vials being placed in a fresh fluid x vial for any reason).

(4) Place unknown samples starting from the second 24 vial rack, barcodes facing outward. Make sure the correct barcode is displayed if multiple ones are on the sample tubes.

(5) Place 1 mL tips in the first tip rack position from the top if aliquoting into a plate, another rack of 1mL tips in the second position if aliquoting into new vials, and 50 µL tips in the third position. Barcodes for the tip rack should be facing to the right.

(6) Place vial racks in the positions adjacent to the tip racks. If less than four are used, leave spaces empty for the racks that would be there normally.

(7) Place 1 mL 96 well Nunc plate in the third position on the plate holder.

(8) Invert bottle of internal standard ~10 times and place appropriate amount into four tubes (either FluidX or Nalgene will work) and place tubes in positions 25-28 on rack 1

Aliquoting the samples:

(1) Open the aliquoting method for nicotine metabolites

(2) Make sure simulation mode is not checked.

(3) Click the start button on the toolbar at the top of the screen. Wait for the system to initialize.

(4) Answer the prompts that appear. This will let you specify the number of samples, set mix volume for both standards/blanks/QCs and unknowns, and determine whether you want to aliquot into a plate, tubes, or both. Ensure that your mix volume is less than the volume in the vial containing the least amount of sample.

(5) The samples and plates will now be scanned. Water blanks and standards that lack a barcode will have to be input manually at this point. Manually input the barcode of any samples that are missed by the scanner exactly as they appear on the vials.

(6) When prompted, enter the desired ID number of the plate. This will also serve as the file name your output file will be saved as.

(7) The standards/blanks/QCs will be aliquoted first in fixed positions, followed by samples. Low volume samples will result in an error message. If you are certain there is enough volume in the tube, choose the option to take from the bottom rather than the top. If the error persists, the volume is too low to be aliquoted by Hamilton Star.

(8) Once the samples are aliquoted, it will ask if you are ready to aliquot internal standard. Place the internal standard tubes on rack 1 if you haven't already done so.

(9) After the internal standard is aliquoted and the method finishes, an output file will be generated in C:\Output Files and the racks will be ejected. Make sure there isn't anything on the deck that will be pushed or knocked over when the racks eject.

(10) Seal the plate, recap the samples with new caps and return to storage, retrieve your output file from C:\Output Files.

8.2.6. Completing preparation of samples aliquoted on the Hamilton Starlet (section 8.2.1)

(1) Place the sealed plate containing internal standard, samples, and standards/QCs/blanks in the clamp position on the Sciclone deck (D2). Carefully remove the foil seal. Place the 12 column reservoir on the Sciclone deck containing enzyme solution as mentioned in section 8.2.3.

(2) Open iLink Pro. Maestro should open automatically with it.

(3) Open the application "Nic metabolites".

(4) Run the method "OnInitilization" and wait for it to complete.

(5) Run the method "UNM – Add enzyme w/ parameters". Upon running the method, a menu will open that will allow the operator to specify the number of available tips at B2 as well as the column number containing the enzyme solution on the reservoir.

(6) Reseal the plate and place in the first available IVD incubator as specified in 8.2.5

(7) Repeat steps 1 through 6 for any additional plates, up to four plates per day. With a full load of 78 samples, the final sample layout on the 96-well plate is as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S8	US	US	US	US	US	US	US	US	US	US
B	S1	S9	US	US	US	US	US	US	US	US	US	US
C	S2	S10	US	US	US	US	US	US	US	US	US	US
D	S3	S11	US	US	US	US	US	US	US	US	US	US
E	S4	BLK	US	US	US	US	BLK	US	US	US	US	US
F	S5	BLK	US	US	US	US	US	US	US	US	US	US
G	S6	QCL	US	US	US	US	US	US	US	US	US	BLK
H	S7	US	US	US	US	US	US	US	US	US	US	QCH

US = Unknown sample, S(X) = Standard, BLK = Blank, QC = Quality control

(8) When finished preparing each assay plate, open the iLink method set file “Incubate and Remove (acetone mix).mst”. Initialize as before and click on the first instruction. Unless there is an error, no user intervention is required after this point.

8.2.7. Method run time

Total method run time is approximately 14.5 hours for one plate. Finished plates are sealed and stored in Rack 3. After samples are finished, pass the sample plate along with the plate layout to analytical instrument operator, or store plate in refrigerator. For each additional plate prepared on the same day, an additional 2.5 hours is required. A maximum of four plates can be prepared each day.

8.2.8. Sample Tracking (Caliper)

(1) Each sample rack is read by a 2D scanner before it is moved to the Sciclone deck and transferred to the assay plate. After being read, the barcodes are stored in “tubes.csv”.

(2) Microsoft Excel is opened in the background and tubes.csv is opened and saved as a new file so that the barcodes within the file are not overwritten by the next scanned plate.

(3) Steps 1 and 2 are repeated for each of the four plates that are transferred to a specific assay plate.

(4) The four new files generated from each scan are opened by Microsoft Excel and compiled into a template file that indicates the location of each barcoded sample on the assay plate.

(5) The template file is then saved as a new file named after the specified run and is stored in the directory specified by the user at the start of the method for later use.

8.2.9. Sample Tracking (Starlet)

Each sample rack is read by a 1D scanner before aliquoting. Any samples that are missed by the initial scan will result in a prompt by the system that requires the user to manually input the barcode to continue. Upon completion of the method, the software will automatically generate a list of sample barcodes, their rack location, and the specific well they were aliquoted into on the 96-well plate. If aliquoted into empty barcoded tubes, a second list containing the barcode of the original sample and the corresponding aliquot tube will be generated. The generated lists are named after the run ID (which is obtained by user input at the beginning of the method) and the date/time.

8.2.10. Maintenance and calibration of Caliper and Hamilton Star System

(1) Daily maintenance

Daily maintenance includes confirming each component to be functional, checking available supplies, and clean up after the run is finished.

(2) Monthly maintenance and calibration
   a. Main array on Sciclone deck: the O rings on the main array should be lubricated with a grease plate (provided by Caliper) after every 50 tip loads or monthly.
   b. Volume verification should be conducted on a monthly basis for both Caliper and Hamilton Star systems.
   c. Ethylene glycol/water bath should be checked to ensure proper liquid level for operation.

(3) Preventive maintenance (PM) on Caliper and Hamilton Star is performed according to manufacturer’s guidance. Normally, the PM is conducted annually.

8.3. HPLC-MS/MS Analysis

Conditions and settings for the Shimadzu HPLC and AB Sciex 5500 triple quadrupole

mass spectrometer are listed below. If different instrumentation is used, the conditions and settings need to be optimized for that instrument.

8.3.1. HPLC Conditions and Settings

(1) Analytical column: Gemini-NX, C18, 3.0 x 100 mm, 3.0-µm particle size from Phenomenex (# 00D-4453-YO). The analytical column is replaced as necessary when analyst observes broadening peaks, high back pressure, low response, or distorted peak shapes.

(2) Two pre-column filters: 1st an A-103X SS frit, and the 2nd an A-100X SS frit (Upchurch Scientific, or their equivalents). Frits are replaced every time the column is changed. Increasing back pressure is one of the indications of impending blockage. When impending blockage is observed, the frits are also replaced.

(3) HPLC Pump Settings:

Pump A	Buffer A (Section 6.2)
Pump B	Acetonitrile (Section 6.3)
Pumping mode	Binary flow
Total flow rate	0.65 mL/min
Column temp	40°C
Initial Pump B Conc.	0.0%
Pressure range (Pump A/B)	Min = 0, Max = 6000 psi

(4) Auto sampler Settings:

Model	SIL-30AC
Rinsing volume	500 µL
Rinsing speed	35 µL/sec.
Sampling speed	5.0 µL/sec.
Rinse mode	Before and after aspiration
Cooler enabled	Yes
Cooler temperature	4ºC
Rinse method	Rinse port Then Pump
Rinse time	1 sec
Measuring line purge volume	600 µL

Discharge speed	1.0 µL/sec
Injection Volume	2 µL
Rinse Dip Time	0 sec
Rinse Start Time	0.5 min
Rinse Sequence	R2→R0
Rinsing Volume R0,R1,R2	300 µL
Injection Port Rinsing	R2
Equilibration Start Time	7.0 min
Equilibration Hold Time	0.9 min
R0,R1,R2 Purge Time	10.0 min

(5) Running gradient on the Shimadzu HPLC system

Time	Module	Event	Percentage of buffer B, (%)
1.00	Pumps	%B	0
3.20	Pumps	%B	12
4.20	Pumps	%B	26
5.40	Pumps	%B	26
5.50	Pumps	%B	60
6.60	Pumps	%B	60
6.65	Pumps	%B	0
8.50	Controller	Stop	

8.3.2. Mass Spectrometry Conditions and Settings

(1) Typical AB-Sciex Triple quadruple 5500 Mass Spectrometer Settings:

a. Instrument A (Sasha Jr.)

Ion source	Turbo spray
SC type	Scheduled MRM
Polarity	Positive
Curtain Gas (CUR)	45
Collision Gas (CAD)	7
IonSpray Voltage (IS)	2300
Temperature (TEM)	650
Ion Source Gas 1 (GS1)	65

Ion Source Gas 2 (GS2)	60

b. Instrument B (NIC1)

Ion source	Turbo spray
SC type	Scheduled MRM
Polarity	Positive
Curtain Gas (CUR)	45
Collision Gas (CAD)	7
IonSpray Voltage (IS)	2800
Temperature (TEM)	650
Ion Source Gas 1 (GS1)	65
Ion Source Gas 2 (GS2)	60

(2) Typical MRM transitions and voltage settings.

MS parameter voltage settings (DP, EP, CE, CXP) are optimized using infusion. A syringe pump is used to introduce a solution containing the native or labeled analyte. The optimal voltage settings are determined for each analyte and these optimal settings are used for the initial MS analysis. However, concentration of most analytes, except for anabasine, in smoker's urine may exceed the linear limits of the detector, if voltage settings are optimal. Thus, the voltage settings (DP and CE) are detuned to avoid saturation on the detector as displayed in the following table. A dummy scan is introduced at the beginning to ensure desired way of displaying chromatogram in Ascent. The scan data for the dummy scan is not processed.

a. Instrument A (Sasha Jr.)

	Precursor Ion	Daughter Ion (quant/confirm)	CXP (V)	EP (V)	DP (V)	CE (V) (quant/confirm)
Dummy	200.00	195.00	12	10	130	5
COXT	193.1	96/79	12	10	70	60/88
COXT-D3	196.2	96	12	10	65	29
NOXT	179.2	130.1/117	12	10	75	66/70
NOXT-D3	182.1	130.1	15	10	55	31
HCTT	193.1	80/134	12	10	75	100/45
HCTT-D3	196.2	80.1	12	10	60	36
NCTT	163.1	80/118	12	10	70	55/27
NCTT-D4	167.1	84	13	10	70	34
COTT	177.1	80/98	12	10	75	97/60
COTT-^{13}CD3	181.2	80.1	8	10	65	35
NNCT	149.1	80/130	11	6	55	45/30
NNCT-D4	153.2	84.1	12	10	55	31

ANTT	161.1	144/80	12	8	55	20/36
ANTT-D4	165.1	148.1	12	10	55	20
ANBT	163.1	80/120	12	8	60	29/22
ANBT-D4	167.2	84.1	11	10	60	30
NICT	163.1	130.1/117.1	15	10	75	48/53
NICT-^{13}CD3	167.1	130.1	8	10	65	28

Abbreviations: DP: declustering potential; EP: entrance potential; CXP: collision cell exit potential; CE: collision offset energy.

b. Instrument B (NIC1)

	Precursor Ion	Daughter Ion (quant/confirm)	CXP (V)	EP (V)	DP (V)	CE (V) (quant/confirm)
Dummy	200.00	195.00	12	10	130	5
COXT	193.1	96/79	12	10	70	60/88
COXT-D3	196.2	96	12	10	65	29
NOXT	179.2	130.1/117	12	10	75	66/70
NOXT-D3	182.1	130.1	15	10	55	31
HCTT	193.1	80/134	12	10	75	100/45
HCTT-D3	196.2	80.1	12	10	60	36
NCTT	163.1	80/118	12	10	70	55/27
NCTT-D4	167.1	84	13	10	70	34
COTT	177.1	80/98	12	10	75	97/60
COTT-^{13}CD3	181.2	80.1	8	10	65	35
NNCT	149.1	80/130	11	6	55	45/30
NNCT-D4	153.2	84.1	12	10	55	31
ANTT	161.1	144/80	12	8	55	20/36
ANTT-D4	165.1	148.1	12	10	55	20
ANBT	163.1	80/120	12	8	60	29/22
ANBT-D4	167.2	84.1	11	10	60	30
NICT	163.1	130.1/117.1	15	10	75	48/53
NICT-^{13}CD3	167.1	130.1	8	10	65	28

8.3.3. LC/MS/MS Maintenance

The mass spectrometer is cleaned quarterly, or as needed to rectify high background or low sensitivity. Mass calibration and resolution tuning are conducted semi-annually. Additional maintenance is also done on an as-needed basis or after an unusually low response for any of the analyte is noted. A professional maintenance service by AB Sciex Company is performed semi-annually. If a different instrument is used, follow the manufacturer's procedure for tuning and maintenance.

a) Procedure for tuning mass spectrometer

(1) Place the ESI (Electrospray Ionization) probe in the source housing for the Triple Quadruple 5500 MS. Fill a 1.0 ml glass syringe with appropriate polypropylene glycol (PPG) solution. Flow the solution into the ESI needle at a rate of 10 µL/min with the instrument syringe pump.

(2) Set the mass spec configuration to MS only mode. Select the instrument project “API instrument.” Choose the tune icon on the tool bar to activate the TUNE menu and load the Q1 tuning file from the methods folder. Once the flow is stable, start the tuning method by choosing the start key.

(3) Perform PPG scan.

(4) Click the calculate button at the top and on the next screen; verify that the correct peaks are selected for calibration. Choose “Calibrate”. A screen will appear showing both the peak shift (from assigned calibration mass) and peak width (at half height). Peak shift must be less than 0.1. Peak width must be between 0.6 to 0.8 amu. If the shift is off, it is necessary to update or replace the calibration. Continue this process until all calibration peak shits are less than 0.1 mass units. Once the calibration is assigned, adjust the peak widths by altering the offsets. To obtain wider peaks, go lower on the offsets and vice versa for narrower peaks. When all the calibration parameters appear to be satisfied, print the results and place the printouts in the “Calibration Log” binder. Repeat the same procedure for Q3.

(5) After completing the calibration, clean the curtain plate and the surrounding area with methanol and a lint-free paper towel. Clean the IS probe with methanol.

b) Procedure for cleaning the source assembly and curtain plate

(1) Deactivate the system from Analyst software. Then, vent the instrument by pressing the side vent button for 3 seconds. After the system has vented, (approximately 20 minutes) turn off MS power source. Wait until the source cools to room temperature.

(2) Remove the source assembly and the curtain plate from the front of the MS. Using a Lint-free swab (to avoid blocking the orifice with fibers) and methanol, clean the source chamber, the curtain plate, and the orifice thoroughly and carefully. Rinse the probe needle with methanol.

(3) Carefully, install the curtain plate and the source back on the mass spectrometer, turn it on, and allow at least 40 min for it to pump down and stabilize.

8.4. Daily Assay Procedure

a) Sample Inventory

(1) After receiving samples from logistics, add new samples into Microsoft Access sample inventory system.

(2) Include received date, study ID, associated DLS barcode, assay ID, box #, position, and any additional comments that indicate sample issues such as low volume, no volume, unusual physical appearance, loose vial caps, etc.

(3) Update the sample inventory after each plate is prepared, to reflect the new box and respective location. If an alternate aliquot of a sample is ran, identify the original sample barcode under the alternate # column.

(4) If samples get transferred to other internal labs for analysis, update the lab location to reflect the lab change. Maintain an electronic log of all incoming and outgoing samples for each study, in addition to, recording samples in Microsoft Access inventory.

(5) To identify the sample disposition at any time, create an inventory report by inputting a sample barcode. The inventory output file will generate the box # and location of that sample. To identify all runs associated with a sample, review the inventory, locate the sample barcode, and select the “+” button. This will display a tracking table that shows all box #, locations, and associated dates, since receiving the sample.

b) Sample Preparation

(1) Maintain a daily sample log either as a hard copy or in electronic format. For the later, log an MS Excel file in the network team folder on a CDC network computer. Hard copies of this file are kept in the “Sample Log” binder.

(2) An example of daily sample prep log sheet is listed in Appendix 1.

(3) Record the following in the daily sample log:

- Caliper settings: i.e. nitrogen flow rate, centrifuge temperature, etc.
- The run standards, QC’s, and samples for that day.
- Whether the acetone bottle was washed and had new solvent added
- Whether the water bottle was rinsed and had new water added.
- Any repairs, additions, or other changes made to the sample prep system.

(4) Details on sample preparation are described in Section 8.2.

c) HPLC-MS/MS analysis

(1) Fresh buffer, “Mobile Phase A”, and “Mobile Phase B” are prepared daily. It takes approximately 200 mL of buffer and 100 mL of acetonitrile to analyze one analytical run of an entire 96-well plate. Details on how to prepare buffer is given in Section 6.

(2) A daily sample run log sheet is listed in Appendix 2.

(3) Purge both LC pumps, the autosampler, and all internal rinse lines used (R0, R1, and R2). System purging helps to ensure that there are no air bubbles in the lines and the degasser, which might affect the buffer pH and system pressure.

(4) Record the following in the daily run log:

- o Vacuum readings before and after starting gas flow. Be sure the readings have stabilized before recording them.
- o The three pressure gauges on the Peak gas generator.
- o HPLC Pump pressures readings after equilibrium (normally from 1500 to 2500 psi).
- o The run ID numbers of the standards and samples for that day, the instrument analyst, and notes on cleaning or repairs made to the instrument.

(5) Prepare a batch file for the current standards and samples according to the description in Section 8.1.

(6) Start the sample run method, and submit the condition method using a water blank injection. Submitting this 10min method will allow the instrument to warm up, reach a stable back pressure, and equilibrate the column from the 100% Acetonitrile storage condition to the 100% buffer in run starting condition.

(7) If run is following the install of a new column, please submit the “New Col Install” method which will gradually increase the system flow until reaching UNICM method flow of 0.65mL/min. This method will prepare the column for system running conditions, and remove any potential hydrophobic and hydrophilic contaminating residuals caused by the column packing process.

(8) After the condition run is complete, inject the following pre-screening sequence of water runs using the normal analytical method.

Sample Name	Sample Description	Reason for Injecting
Pre-Blank A	Water blank containing no internal standard	Stabilize system pressure and establish equilibrium
Pre-Blank B	Water blank containing no internal standard	Monitor system background to evaluate potential instrument contamination
Pre-Blank C	Water blank_03 from plate	Observe internal standard recovery and native response to ensure proper instrument sensitivity

		and no contamination caused by sample preparation
Pre-Blank D	Water blank with internal standard	Ensure proper instrument sensitivity and monitor nicotine peak height independent of sample preparation
Pre-Blank E	Water_blank_03 from plate	Assess system stability and document nicotine peak height

(9) Confirm a stable retention time and verify that the peak height for nicotine in the blank water sample is lower than 3000 cps (counts per second); otherwise, corrective actions must be taken to reduce the background. This is typically done by a thorough cleaning or replacing the frits and column.

(10) Submit the first water blank, WBK_01, of the run, which contains only analyte internal standards (ISTD's). Document and record the NICT-ISTD peak height (typically ~$1x10^6$ cps), peak width, and retention time before submitting the run. Additionally, document the NICT native peak height in the HPLC run log for blank monitoring. If poor sensitivity and peak shape occurs, replace the column. Column replacement usually occurs after approximately 1000 runs on a column.

(11) Submit the standards and samples from the batch file, and then submit the Wash/Shutdown batch to wash the column and to shut the instrument down after the run. (The column should be washed with acetonitrile for 30 min before shutting down to maintain ideal storage conditions and maximize column durability).

(12) After the sample run is complete, move the data into a designated batch file folder on instrument computer. Upload the acquired data to Indigo Ascent for data processing and quality control evaluation.

(13) All data files should be backed up on a 2nd external hard drive weekly.

8.5. Data Processing

The data is processed using Ascent (Indigo Biosystems, Indianapolis, IN), a web based peak integration software. The following is a description of how to transfer and process data using Ascent. Modifications are needed if different instrumentation and software are used.

(1) After each sample run is finished, data is retrieved from the instrument computer and uploaded to the Indigo host server on the internet.

(2) Verify the data for calibration and QC in the batch is as expected. This is done by checking percent concentration deviation of the standards and QC values against the established nominal concentrations and QC limits. Refer to section 6.3 and 7.3 on specific acceptance criteria and standard/QC evaluation.
(3) Extensively review all unknowns within the batch. Check the peak shapes and retention times to ensure the correct peaks are chosen for quantitation, especially for those samples flagged by the Ascent QA program for all blanks, standards, and QCs. Review the reasons for flagging and manually correct any integration, as necessary.
(4) Double-check analyte calibration curves following integration. Exclude data points, where necessary. If >2 points for any analyte calibration curve are excluded, the run fails for that analyte.
(5) Identify any analyte run QC fail based on the DLS PPM regulations. For all analytes whose run QC passed, process all unknown sample data.
(6) Following data processing completion, click “Review complete” key and notify data reviewer for certification of the batch.
(7) Data certifier will evaluate all flagged samples to ensure and monitor correct integration and peak selection. At-random samples without QA flags will be checked for additional review. Any unusual sample-analyte circumstances, such as peak interference or poor chromatography, will be documented by selecting the repeat drop down tab, and inserting a comment within the Indigo quantitation table. This will enable the Data Analyst to do a third review on the sample-analyte data, before deciding to repeat or report.
(8) Data certifier will certify the batch after data review is complete. Once the batch is certified, the results can’t be modified, and a .pdf result file will be generated within the hour to be printed, as needed.
(9) Following project completion, copy the project onto an external hard drive. This should include all of the raw data, results sets, and all relevant instrument parameters used for each sample during that quarter. After the project is copied all project information can be deleted from the data system hard drive to clear up space.

8.6. Calculations

(1) The Ascent program produces a regression chart for each analyte based on 1/X weighting. Standard acceptance criteria can be reviewed in the Section 6.3.4.

(2) Blank correction for unknown samples is done by subtracting the average of water blank 01 and water blank 03 calculated concentrations for the run from the sample results for that analyte. For example, if the NICT blank is 0.68 ng/mL and the sample is calculated to be 56.88 ng/mL, then the blank-subtracted result is: 56.88-0.68 = 56.20 ng/mL. Among samples containing analyte concentrations at or near the LOD, blank subtraction may result in a negative number for the concentration. Replace all negative numbers with a zero as the blank-corrected result.

(3) The concentration of each unknown sample is calculated using the blank-corrected standard curves generated by the Ascent program. The analyte concentrations are calculated from the quantitation ratios of the analytes (quant ion area/ISTD ion area) compared to the standard curve.

8.7. Data Evaluation

(1) Following batch certification from Indigo Ascent data processing software, the batch results file is uploaded into Repeat Manager Microsoft Access program; the two programs are synchronous with UNICM configured QA/QC rules.

(2) Access the review repeats table and select the appropriate study-set name from the drop down tab. One-by-one, review each sample-analyte within the study-set to observe the QA rule violated. If reviewed sample-analyte is deemed a valid result, in compliance with all configured QA/QC, then manually change the StarLIMS status from “Pending” to “Send.” After all sample-analytes have completed initial evaluation, proceed with sample repeat staging.

(3) Pending the total number of sample-analytes requiring repeat results, stage samples accordingly to minimize time spent on sample preparation and data processing. Analyst may choose to stage repeats individually (sample-analyte), by compound (any of the 9 analytes), or by internal status (QA rule violated), with no more than 78 total unknowns on any repeat plate.

For example, if 500 sample-analyte combinations require repeating, stage 78 sample-analyte combinations for “dilution required” internal status, so that all unknowns on repeat plate will undergo dilution. This will reduce time spent preparing the repeat run, since the analyst will perform a dilution on all 78 unknowns.

Likewise, the analyst can stage an additional 78 samples on another repeat plate for specific analyte, so that the repeat plate only gets processed for that analyte. This will reduce time spent on data processing, since the analyst will not have to process for all 9 analytes.

Note, if a sample is staged based on an individual compound, and another compound for that sample violated a QA rule, both analytes will appear in the stage samples table, even though the sample was only selected to stage for one compound. This is designed to avoid repeating samples multiple times.

(4) Assign a repeat run name to staged samples, and access the repeat run pull sheet based on the selected set and repeat run name. Extract the repeat run pull sheet into excel, and sort by sample “current box #” and “current location.” This will allow the sample preparation analyst to easily retrieve samples from multiple locations in a

logical and time-efficient manner. Notify sample preparation analyst that repeat run is ready for preparation.

(5) Continue staging samples until no additional samples require repeating. After repeat batch is certified, import repeat run results file, by selecting processed-only analytes. Review the side-by-side comparison of initial and repeat run results. View the calculated concentration % difference cells to ensure that all runs results are ≤25% difference. If results are outside of this criteria, repeat for confirmation, until two valid results satisfy %difference criteria. Report the first-valid result accordingly.

(6) After deciding which result to report, change the StarLIMS status of all non-reporting results to “Don’t Send.” Change the StarLIMS status of reporting result to “Send.” Repeat this process until the Review Repeats tab is empty.

(7) Identify any missing results, i.e. samples that do not have StarLIMS status as “Send” for all 9 analytes, by accessing the missing results table. Identify any potential duplicated results, i.e. sample-analyte combinations that had more than one result with StarLIMS send status, by selecting the “look for duplicates.”

If no missing or duplicate results were identified, create a StarLIMS-compatible extract file.

9. Method Reliability

9.1. Accuracy

Native and internal standards are obtained from commercial sources. Stock solution concentrations are based on stated purity and gravimetric measurement of the compounds. The source, purity, and manufacture’s purity confirmation procedure are listed in Section 6.

The initial accuracy was evaluated using five non-smoker urine pools spiked with known amounts of analyte. Each pool was analyzed five times for three weeks. The results are summarized in Appendix 3.

9.2. Precision

Intra-day and inter-day accuracy, expressed as a percent of the target concentration, and imprecision, expressed as the relative standard deviation (RSD), for each analyte were evaluated with five different concentrations. The concentrations were distributed across the entire calibration curves of measured analytes and were prepared by spiking known amounts of analytes in the pooled blank urine (Appendix 3). Specifically, inter-day accuracy and imprecision were evaluated with 16 replicates for each concentration level

within an analytical batch each day. The inter-day data was assessed with three separate analytical batches over three consecutive days. The total accuracy and imprecision were calculated with all data sets (n=48 for each analyte). The intra-day imprecision was generally 5%, except at concentrations near the LOQ, which had coefficient of variation (CV) of 10% or, in a few cases, 12%.

9.3. Analytical Specificity

A high degree of analytical specificity is achieved with this approach; however, there is always a possibility that a sample will have an unknown interference. Correct retention times and correct precursor/product ion transitions help ensure a very high degree of specificity and minimize the influence from any potential interference.

Also, an established range of ratios of the response of the confirmation ion to that of the quantitation ion of all samples is used to determine if an unknown sample tests positive for a given analyte. Section 10 describes how to establish the limits. The confirmation ion ratio ranges are determined using standards data from all standards above the detection limit of each analyte. If a sample does not meet the confirmation ratio limits, then it is repeated. If it fails again, the result is not reported as there is likely a contaminant in the sample.

9.4. Limit of Detection

The limit of detection (LOD) is 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 (Taylor, 1987; ICH, 1994; DNR, 1996). Estimated LODs are as follows:

Analyte	Estimated LOQ (ng/mL)
COXT	2.00
NOXT	2.50
*HCTT	9.50
NCTT	1.10
*COTT	4.60
NNCT	2.50
ANTT	0.40
ANBT	0.50
NICT	10.50

Limits of detection for HCTT and COTT can be found in the Urinary Cotinine and Hydroxycotinine Laboratory Procedure Manual

9.5. Sample Recovery

Two sets of samples, including three replicates from each of a low, medium and high concentration pools, were prepared. Deuterated internal standard solution was added to one set of samples at the beginning of the sample preparation, and to another set just before the injection. Sample preparation recovery (%) was calculated by comparing the average peak area of processed internal standards with the average peak area of unprocessed internal standards. The average recoveries for all analytes are above 70% (Fig. 9.5).

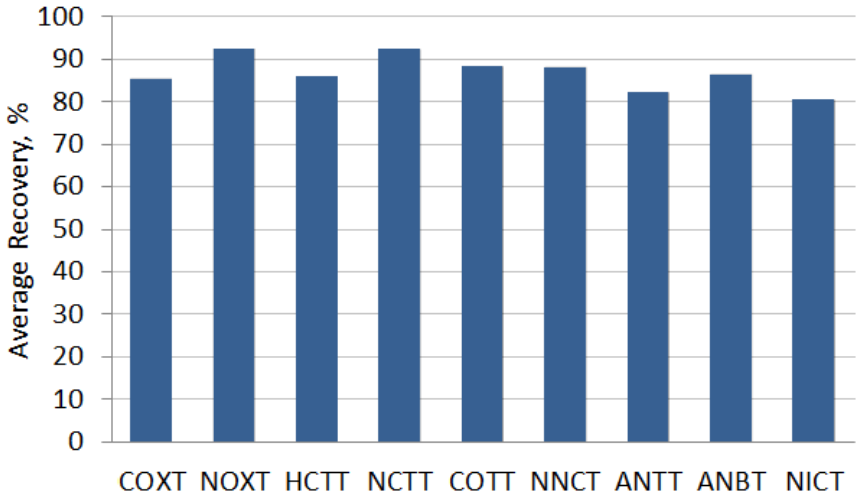


Figure 9.5. Sample preparation recovery

9.6. Carryover

To investigate the carry-over, each processed sample with ISTD from the low, medium, and high pools was injected, followed by three blank urine samples. The carry-over was determined by comparing the average peak area in the first blank urine sample with the average peak area of the processed sample. The overall carry-over was negligible.

9.7. Linearity Limits

This method can be applied to urine samples from both smokers and exposed nonsmokers. Consequently, a broad range of urinary concentrations may be encountered, ranging from less than 0.5 ng/mL to greater than 50,000 ng/mL depending on the analyte.

The lower reportable limit for each analyte is the LOD (described in Section 9.1). The upper reportable limit for each analyte is its highest standard concentration that is listed in Section 6.3.4. Dilute, re-prepare, and reanalyze any sample from a run with an analyte concentration greater than upper limit of the standard curve for that analyte. Samples with an analyte peak height greater than $2x10^6$ cps require a rerun for that analyte, following the addition of 0.5 mL HPLC water into the pre-treated solutions.

9.8. Ruggedness Test

Method ruggedness was tested by varying the following parameters: enzyme amount, hydrolysis temperature, hydrolysis time, buffer pH, and volume of added water. Details are listed in the following sections.

9.8.1. Enzyme Amount and Hydrolysis Temperature

This method has been developed to measure the “total” concentration for each analyte. Thus, hydrolysis is a critical step to “free” the analyte from its conjugated forms, e.g. glucuronides. Experiment results shown in the figure below demonstrate that deconjugation of hydroxycotinine is the rate limiting step that determines the amount of enzyme needed for complete hydrolysis during sample preparation of this method. Nicotine-glucuronide and cotinine-glucuronide are two other major conjugates with relatively high concentrations (Fig. 9.8.1a).

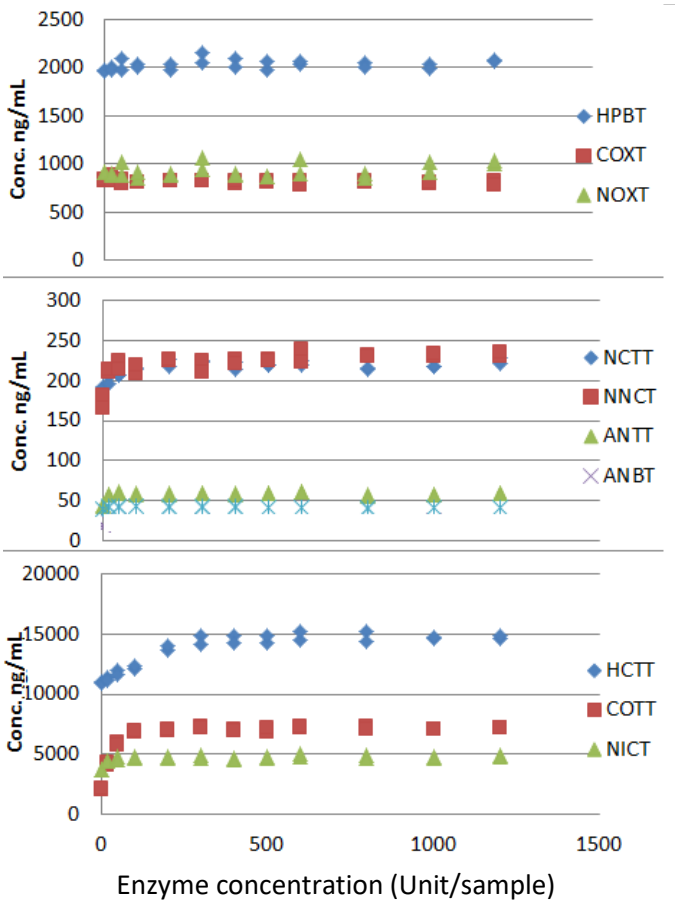


Figure 9.8.1.a. Glucuronide forms in a pooled smoker urine sample

The glucuronide forms for other analytes, including cotinine-N-oxide, nicotine-N-oxide,

norcotinine, nornicotine, anatabine, and anabasine are very low and are not affected significantly by enzyme concentration used in the method. This is shown in Fig. 9.8.2a-c.

Thus, a mixed solution including *trans*-3´-hydroxy-cotinine-O-β-D-glucuronide, cotinine-N-β-D-glucuronide and nicotine-N-β-D-glucuronide, was prepared in blank urine pools, and then the hydrolysis performance of Helix. Pomatia was evaluated by changing the amount added per sample and the hydrolysis incubation temperatures (37, 45 and 50^{0}C) (Figure 9.8.1b).

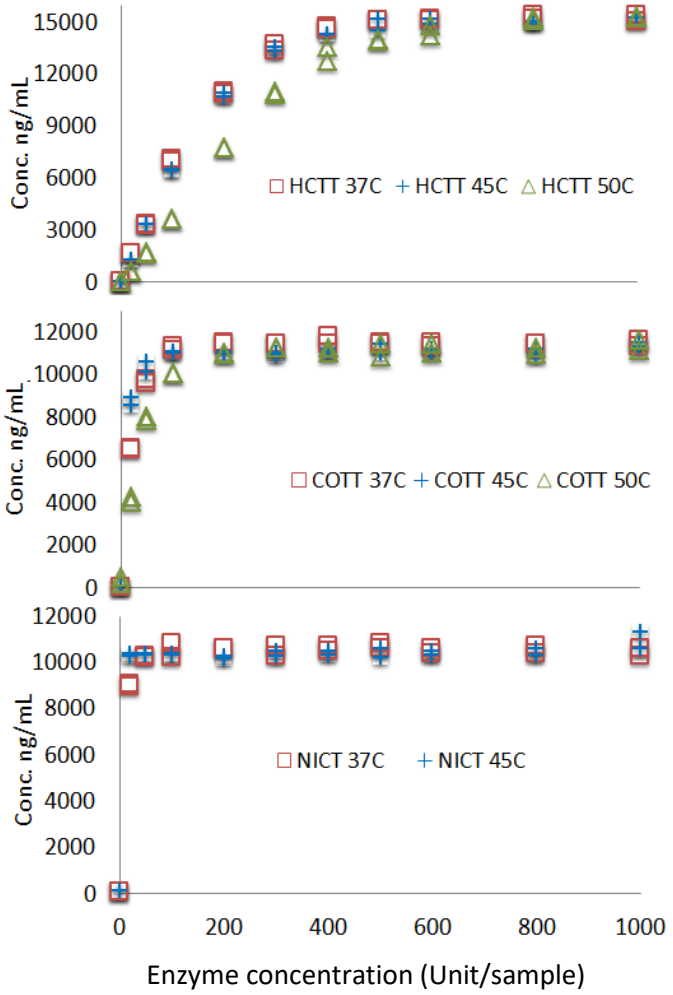


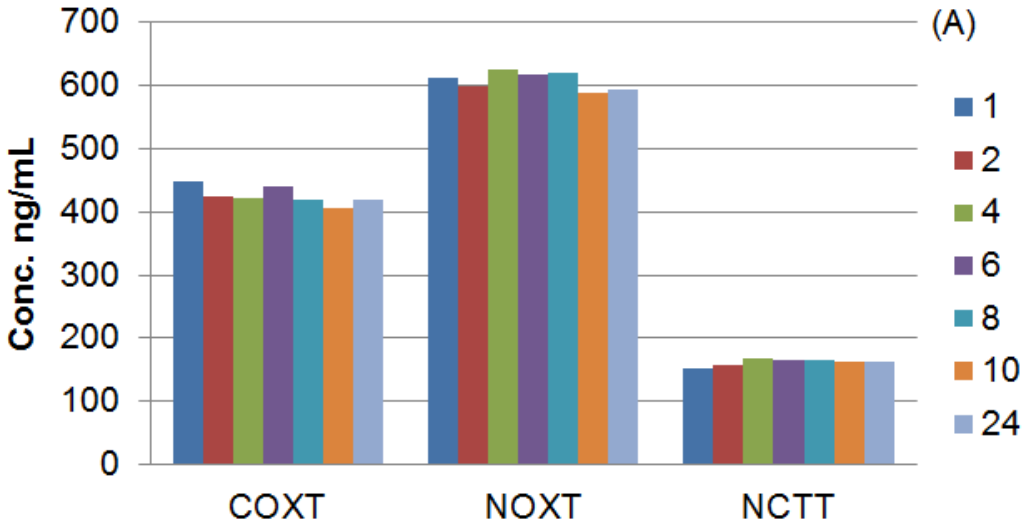
Figure 9.8.1b. Performance of H. Pomatia by varying amount per sample at different temperatures

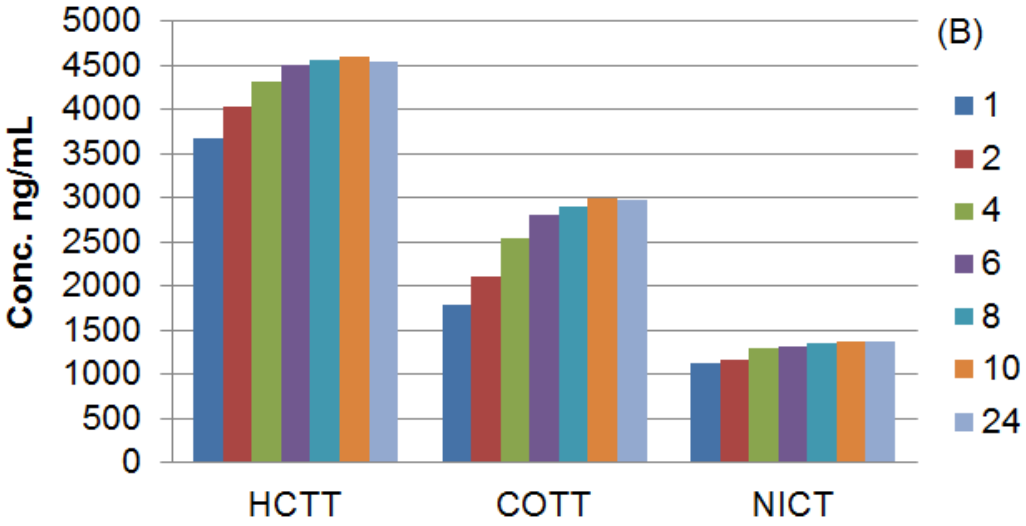
For the three predominant analytes, increasing the enzyme temperature from 45 to 50°C will slowly increase the activity of H. Pomatia. Thus, the working incubation temperature (45ºC) for H. Pomatia is relative broad, which contributes to the robustness of the method. Usually, it takes a longer time to complete the hydrolysis at 37°C than at 45°C using the same conditions for the other parameters. 45°C was selected as the

incubation temperature so that the enzymatic hydrolysis can be completed within 12 hours.

9.8.2. Hydrolysis Incubation Duration

A pooled urine sample prepared by mixing samples from smokers was used to determine the hydrolysis incubation duration. A total of 21 samples were initially treated equally, and were put in the incubator. Triplicate aliquots were collected at 1 h, 2 h, 4 h, 6 h, 8 h, 10 h and 24 h, and were treated according to the method preparation steps. The results are shown in Fig. 9.8.2a-c, indicating that a minimum of 10 hours, using H. Pomatia, is needed for complete hydrolysis. Based on the time scale of the automated preparation, a 12-hour incubation was selected. The extra two hours ensures complete hydrolysis.





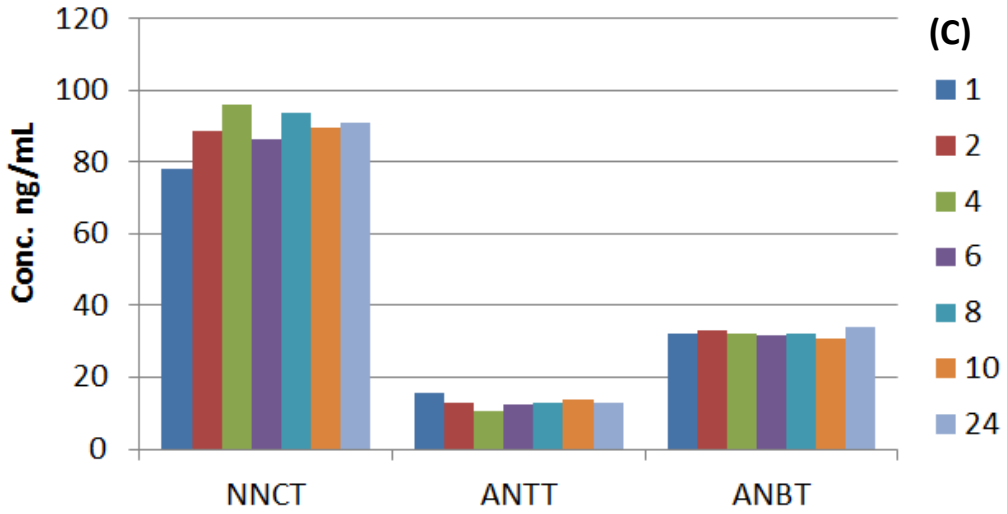


Figure 9.8.2a-c. The effect of hydrolysis time on analyte concentration

9.8.3. Buffer pH and Retention Time

Several major factors influencing the chromatography, e.g. buffer pH and gradient, were tested to achieve the best resolution and reduce the run time. These factors are not independent of one another. Baseline separation of all analyte peaks is highly desired to achieve high analytical specificity. A short run time is also highly desired to increase the sample throughput. To optimize these factors: a Gemini-NX column with moderate dimensions (100mm×3.0mm, 3.0um) was selected, and the gradient was adjusted to obtain a reasonable run time (8.5min per sample) (Table 2). Additionally, the injection volume was reduced to 2 µl to avoid overload and detector saturation, and the flow rate was set to 0.65 mL/min. The responses of all analytes on the detector and their resolutions were compared at different buffer pH values from 3.5 to 10.5 as presented in Figure 9.8.3.

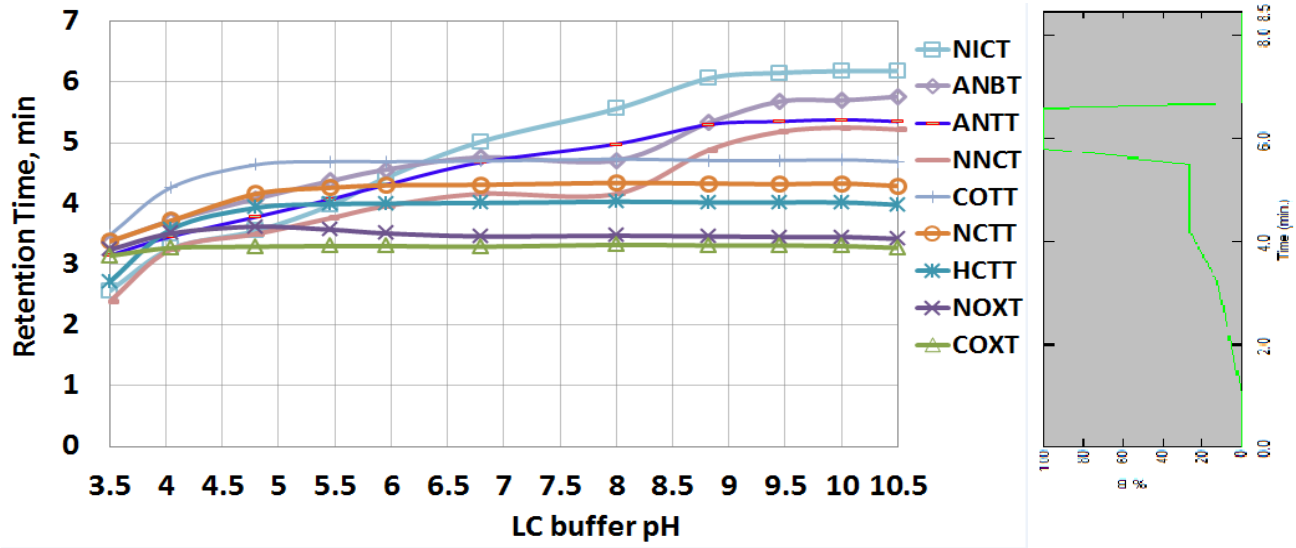


Figure 9.8.3. The effect of buffer pH on retention time

Results indicate that basic buffer (pH > 9.6) produced not only a higher response, but also improved resolution. Thus, a buffer pH of 10.0, adjusted using concentrated ammonia hydroxide, was utilized in this method.

9.8.4. Volume of Water Added for Injection Solutions

The volume of water is determined based on following factors: (a) less water volume, i.e. less dilution, will increase the peak response and cause instrumental saturation, resulting in lower calibration linearity; (2) more water volume, i.e. higher dilution, will reduce the peak response, resulting in lower sensitivity. In this study, an optimized water volume of 250 µL is added to each sample prior to injection. For the ruggedness test, two tests including two pools were done: one test with 30% less volume of water, and the second one with 30% more volume of water prior to injection (Appendix 4). No significant differences were observed for the analyte concentrations among these three groups.

9.8.5. Freeze-Thaw Cycle

A Freeze-Thaw test was performed to test whether this process will cause any potential variations on analyte concentrations using two prepared pools over 25 cycles. The results indicated no significant effect on the calculated concentration values was detected (Appendix 5).

10. Quality Assessment and Proficiency Testing

10.1. Quality Assessment

Quality assessment procedures follow standard practices. Daily experimental checks are made on the stability of the analytical system. Quality control (QC) measures employed to ensure the reliability of the data include:

(1) For long-term storage, calibration standards (STD) and QC samples are all kept at or below −60°C.

(2) Samples are calibrated using 11 point curves, and both calibration standards and QC samples are prepared in pooled urine samples to account for potential matrix effects. The preparation of the QC materials is described in Section 6.

(3) STDs, QCs, and laboratory control blanks are simultaneously prepared and analyzed in the same manner as the urine unknown samples in each analytical batch.

(4) The measured concentration for each sample is assessed by the accuracy and precision specifications of the quality control/quality assurance program of the Division of Laboratory Sciences, National Center for Environmental Health, CDC. Specifically, the following criteria are applied:

a. **QC results:** Confirm all QC results for the mean and range values using the current DLS QC rules. The QC evaluation considers each of the 9 analytes independent of the other; therefore, a run may be out of control for one analyte and in control for the other analyte (+/- 2SD). For example, if NICT is found to be out of QC limits, but all COTT QCs are in control, then the COTT results for the samples in the run will be acceptable; however, the samples will need to be re-prepared and processed for NICT.

b. **Relative retention times:** If the retention time difference between the quantitation and ISTD ions is more than 5 sec, inspect the chromatogram carefully for any possible interference. If the identity of the peak cannot be confirmed, then the sample is re-prepared for confirmation. If interference is still present in repeat run, and calculated concentrations are not within 25% difference between the initial and repeat run, then the sample-analyte result is invalid.

c. **Confirmation ratios:** Calculate the confirmation ratio for each analyte by dividing the confirmation ion area by the quantitation ion area. The confirmation ion ratio range is determined from the mean of the standards above Standard 5 within a run in the Indigo Ascent program. Samples should be further evaluated if the confirmation ratio deviation is greater than 25%.

d. **Control blanks:** Examine the control blanks prior to running the samples to check for possible contamination in the analytical instruments, extraction solution, or reagents. Check the background level of nicotine. If its peak height is above 3000 cps, stop running samples and take measures to inspect any potential contamination sources, e.g. buffer, dilution water etc.

e. **Linear range:** Check all measured concentrations to make certain that the values are within the linear range of the method (described in Section 6). Actual measured values must not exceed the highest standard for that analyte. Any samples with uncorrected values greater than the corresponding highest acceptable standard levels require a further suitable dilution before a repeat of pre-treatment and analysis. If any analyte in a sample has peak height higher than $2x10^6$ cps, a suitable volume of HPLC water should be added to the pre-treated sample, followed by reinjection of that sample on the LC/MS/MS system.

f. **Recoveries:** For all samples, the IS area of an unknown sample chromatogram is set at 200% for upper and 20% for lower limits. Reanalyze any sample with

sample recovery less than 20%, if sufficient residual sample is available. However, low recovery alone is not grounds for rejecting a sample result.

g. Other checks:

- Ascent has the ability to flag samples in many ways desired by analyst. Comprehensive rules are set up to flag samples for further evaluation on possible problems.
- Some configured Indigo Ascent QA rules are set more stringent than actual rule thresholds. This is to enable additional sample-analyte review by the analyst, certifier, and data analyst.
- Double check run log files to make sure correct dilution factor is entered for each sample.
- Compare results between initial and repeat analyses to ensure that no reported result it outside of 25% calculated difference threshold (when both results are considered valid quantitatively).

(5) This method prepares the standards, QCs, and blanks in the same manner to the unknown urine samples. Calculated concentrations of unknown samples are non-blank-corrected raw values. If blank-corrected values need to be reported, blank concentrations should be subtracted from the raw values. Calculations are described in Section 8.5.

(6) Instruments are regularly evaluated to maintain the highest sensitivity, e.g. a preventative maintenance (PM) is done semi-annually. Source assembly cleaning should be performed routinely to prevent front end residue build-up and maintain the required sensitivity. Responses of ISTDs in each batch run should be checked (as described in 10.1.4.f). If their responses are significantly low compared to normal average values, the instrument needs to be inspected, and a PM might need to be conducted.

(7) Data quantitation is performed on certified third-party software, Indigo Ascent, customized for this method. Specific QA rules are configured to ensure data quality. QA rules and QC limits are carried throughout data evaluation, using Microsoft Access.

10.2. Establishing QC Limits

Acceptable QC concentration limits are calculated initially from at least 20 analyses of the QC pools over a period of seven weeks. During the 20 characterization runs, previously characterized QCs or pools with target values assigned by outside laboratories, or second source products should be included to evaluate the analysis. The process of limits calculation is performed using the laboratory database and the division SAS QC characterization program.

10.3. Proficiency Testing

Three urine proficiency testing (PT) pools were prepared for this assay by spiking different levels of standard solutions with known concentrations into blank urine pools. The pools are aliquoted into 2 mL FludiX vials, labeled, and frozen at or below -60^{o}C. All pools are characterized in at least 20 analytical batch runs over a period of several weeks.

Proficiency testing is performed at least semi-annually. PT assays are conducted using the characterized PT pools. Analytical 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 the characterized mean ± 25%. If the assay fails PT, all analyses are stopped and the source of the error is investigated. No assays will resume until the problem has been resolved and a repeat PT assay has been passed.

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

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

11.1. Internal Standard Response

If the average peak height of the NICT ISTD in the standards fall below 2x10^{5} cps, this indicates that either the instrumental sensitivity has fallen below acceptable limits, or the LC column needs to be replaced. As the column ages, the retention time for early eluting analytes gradually shifts towards the void time, which increase the ion suppression. If this occurs, the following steps may need to be taken:

a. Clean the mass spectrometer front end including curtain gas plate, orifice, and ESI needle.

b. Replace the old column and old column frits with new ones and check the response. Ensure appropriate column conditioning and equilibrium prior to submitting batch sequence. Extensively clean all mobile phase bottles and LC system lines. Replace tubing, when necessary if pressure fluctuates or is not maintained.

c. Break vacuum and clean Q0 of the mass spectrometer.

d. Submit a service call with mass spectrometer manufacturer.

11.2. Peak Tailing

Tailing issues often occurs when column ages, usually after 800 runs. Since there can be slight variations observed from column to column, tailing issue may occur earlier between different batches of the same column. Heavy tailing might affect peaking picking and integration, resulting in variations on measure concentrations. If this occurs, check mobile phases followed by column replacement.

11.3. Calibration

Assess system calibration and general readiness on a daily basis by reviewing instrument operating conditions (temperature, pressure, etc.). If the calibration curve becomes non-linear, first determine if the problem is with the LC, the MS, or the standards.

a. Saturation check: check the peak height for each native analyte. If the height is above $2x10^6$, check the evaporation step during sample preparation. Evaporation flow rate, temperature, and duration need to be set accurately according to the sample preparation procedure.

b. Integration check: make sure all peaks are correctly integrated relative to sample baseline. Any errors need to be manually corrected.

c. Standards checks: analyze the standards on another instrument. If the standards have become unsuitable or experience degradation, prepare new standards.

d. HPLC checks: check for leaks or high back pressure, make sure the pumps are delivering the correct volumes. Prepare fresh mobile phase daily, and check the auto-sampler and injection needle for correct positioning and potential clogging.

e. MS checks: clean the front end, recalibrate instrument, and conduct a PM. Record source, exhaust, and curtain gas responses on instrument run log for each batch.

11.4. 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 it is not seen in the remainder of the samples, this indicates a contamination of that particular sample. The source of the contamination should be investigated to prevent repeat occurrences, but no further action is required unless analyte QC fails for that run. If analyte QC fails for run, all samples must be re-prepared and re-analyzed for that analyte.

11.5. Analyte in Samples

If an unexpectedly large amount of analyte is present in all measurements (including the blanks) for a particular day, it is likely that the source of contamination is in the reagents/buffers, the tubes, the vials, and/or the instrument. All of these should be tested to identify the source of the contamination. Reagents can be replaced. Tubes, vials, columns can be re-washed or replaced, and the instrument can be cleaned or parts replaced. Depending if contamination source is identified as sample preparation and/or instrument, samples will be re-prepped or re-analyzed and processed for contaminating analyte. All other analyte results, if in compliance with QA rules and QC limits, will be acceptable in initial run.

Note: Nicotine is usually a ubiquitous pollutant in the environment. A background level could be often detected in control blanks using the HPLC-MS/MS method. Thus, sample preparation and instrument operation should strictly follow the assay monitoring steps.

11.6. 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. Additionally, the following steps should be taken:

(1) Repeat the run. If several runs in a row are found to be out of control, analyses should be suspended while the source of the problem is investigated.

(2) Other possible sources of error: automation liquid handling parts, i.e. needles that could be out of calibration, and potential contamination.

(3) Test the entire system, i.e. reagents, tubes, plates, and mobile phases for contamination.

(4) Wipe down the lab bench area and Caliper enclosure where samples are prepared. Clean any waste tips and liquids in the Caliper enclosure.

11.7. HPLC Column Replacement

There is no constant parameter to tell when to replace the HPLC column. However, when the following conditions are observed, it typically indicates a need for column replacement:

(1) Heavy tailing occurs (usually for NNCT, ANBT, ANTT and NICT) as described in Section 11.2.

(2) Significant drop for average peak height for NCTT-ISTD, as described in Section 8.3.4.

(3) Significant peak broadening.

(4) Retention time shift across all analytes.

After replacing a column, it must be conditioned using low flow (0.1 mL/min), 100% Acetonitrile, 50:50 Acetonitrile:water, 50:50 Buffer: Acetonitrile, and ending with the initial starting conditions of the run (% Acetonitrile) and 0.65 mL/min flow. Gradually increase the gradient and flow rate with respect to time to remove any silica particles, dust, or air gaps retained in the column.

12. Limitation of Method: Interfering Substances and Conditions

In some studies, other physiological substances (e.g. caffeine) have interfered with immunoassays or chromatographic assays of cotinine. However, no known interferences have yet been reported for cotinine for this tandem mass spectrometric method. However, there are several other limitations associated this method:

(a) NOXT typically has an interfering peak eluted before NOXT in smoker's urine sample. The relative difference for retention time for both NOXT/ISTD and the interfering peak is about 3-4 s. Our investigation shows that this peak is from other isomers of NOXT.

(b) NICT exists ubiquitously in the environment. All the commercially available labeled nicotine IS also contains trace amount of NNCT which affects the LOD of this method. Measuring NNCT and NICT using HPLC-MS/MS method is challenging due to their detectable background levels regardless of the matrix used or how the calibration standards are prepared. It should be pointed out that nicotine background levels are not constant, varying from day to day. Thus, a cut-off blank value for these two analytes are set high enough to accommodate the variation. Due to this blank issue, higher variations at low concentrations may be seen.

13. Reference Ranges (Normal Values)

Since the population includes both smokers and nonsmokers, levels of analytes in this method in urine are distributed in broad ranges. This method focuses on measurements on tobacco users, and the following ranges are expected.

Analyte (total)	Analyte Code	Units of measure	Reference range (smokers)	Reference range literature reference
Anabasine	ANBT	ng/mL	0.15-208	5
Anatabine	ANTT	ng/mL	0.06-456	5
Cotinine	COTT	ng/mL	17-9470	5, 6
Cotinine N-oxide	COXT	ng/mL	9-2520	7, 8
trans-3'-Hydroxycotinine	HCTT	ng/mL	50-32700	6, 9
Norcotinine	NCTT	ng/mL	6-1010	7, 8, 10
Nicotine	NICT	ng/mL	16-10100	5, 6, 9
Nornicotine	NNCT	ng/mL	4.4 -616	5
Nicotine 1'-Oxide	NOXT	ng/mL	29-2280	7, 8

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

Not applicable to this procedure.

15. Specimen Storage and Handling during Testing

Samples are stored frozen at or below -60ºC until they are prepared and analyzed. Remove the rack of frozen samples from the freezer and allow them to thaw overnight in a refrigerator (e.g. 4ºC). Bring the samples to room temperature on the morning of the analysis, and mix well prior to preparation. After analysis, re-freeze the samples at or below approximately -20ºC for short-term use (within one week), and at or below -60ºC for long-term storage.

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

Generally, if a problem exists with the method, store samples in the freezer until the problem can be solved. Samples that have been pre-treated can be stored at or below -20ºC for at least one week before analysis.

Currently, two LC-MS/MS instruments have been validated for this method. If one system needs maintenance, then samples can be run on the second instrument. There is no validated method other than this HPLC-MS/MS method for these analytes in CDC.

If any problem occurs on the Caliper staccato system, samples can still be prepared manually step by step using individual modules incorporated in the Caliper staccato system. If unexpected problems occur to individual modules, samples can be prepared on available equivalent instruments in the lab. Otherwise, store all urine samples at or below -60ºC until the instruments are fixed.

17. Test-Result Reporting System: Protocol for Reporting Critical Calls

Analytical results are reported as ng/mL for each sample. Final results that meet all QA/QC criteria are then reviewed by a DLS statistician. The data, a cover letter, and a table of method specifications will be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director). Formally, the data will be 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

Residual samples from specific studies will remain in the same freezer, organized in identifiable storage racks. Sample disposition is imported, updated, and maintained in Microsoft Access inventory system. To identify the current location of a given sample, simply input the sample barcode to create an inventory report. Reviewing the complete inventory will provide complete sample disposition- after receiving from sample logistics and following and subsequent runs.

Following the completion and reporting of each study, residual samples may be saved in long term storage, returned, or discarded as determined by the study administrators.

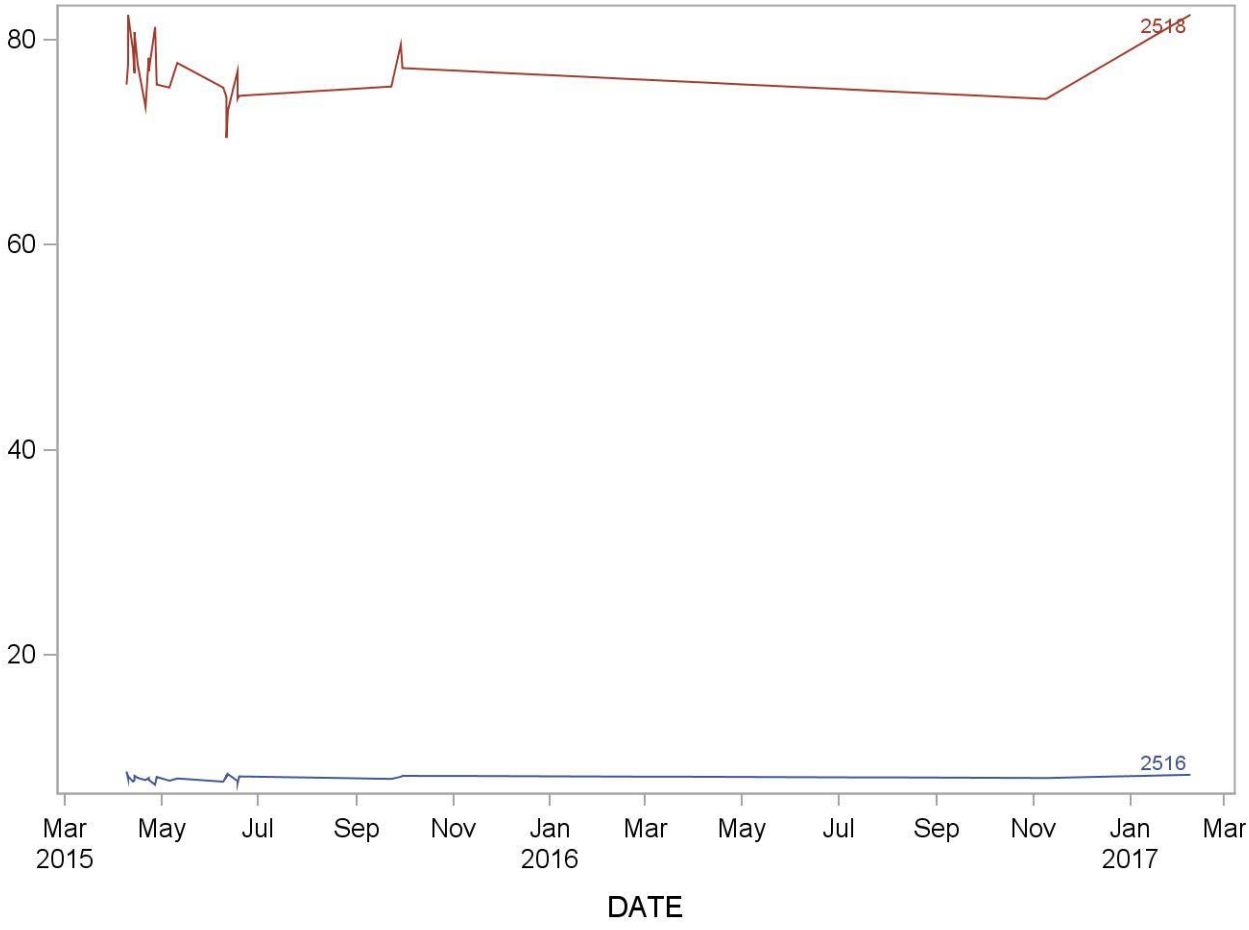
Standard record keeping (e.g., sample ID, notebooks, data files, database, etc.) is used for additional sample tracking. All records are maintained in accordance with the HHS Records Management guidance. (See: http://www.hhs.gov/open/records/index.html).

19. Summary Statistics and QC Graphs

See the following pages.

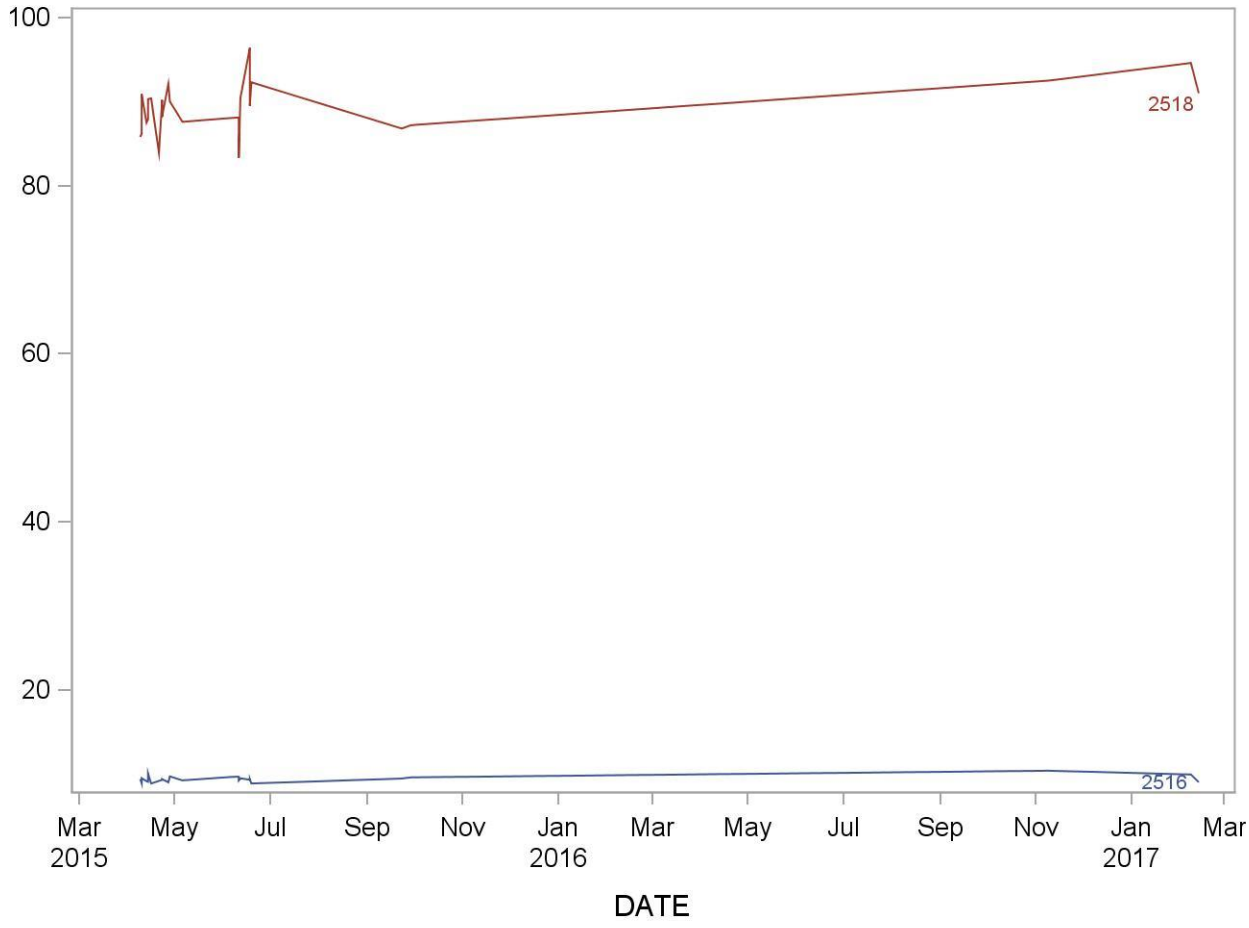
2013-2014 Summary Statistics and QC Chart for Anabasine, urine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2516	26	09APR15	08FEB17	8.015	0.291	3.6
2518	26	09APR15	08FEB17	76.746	2.924	3.8



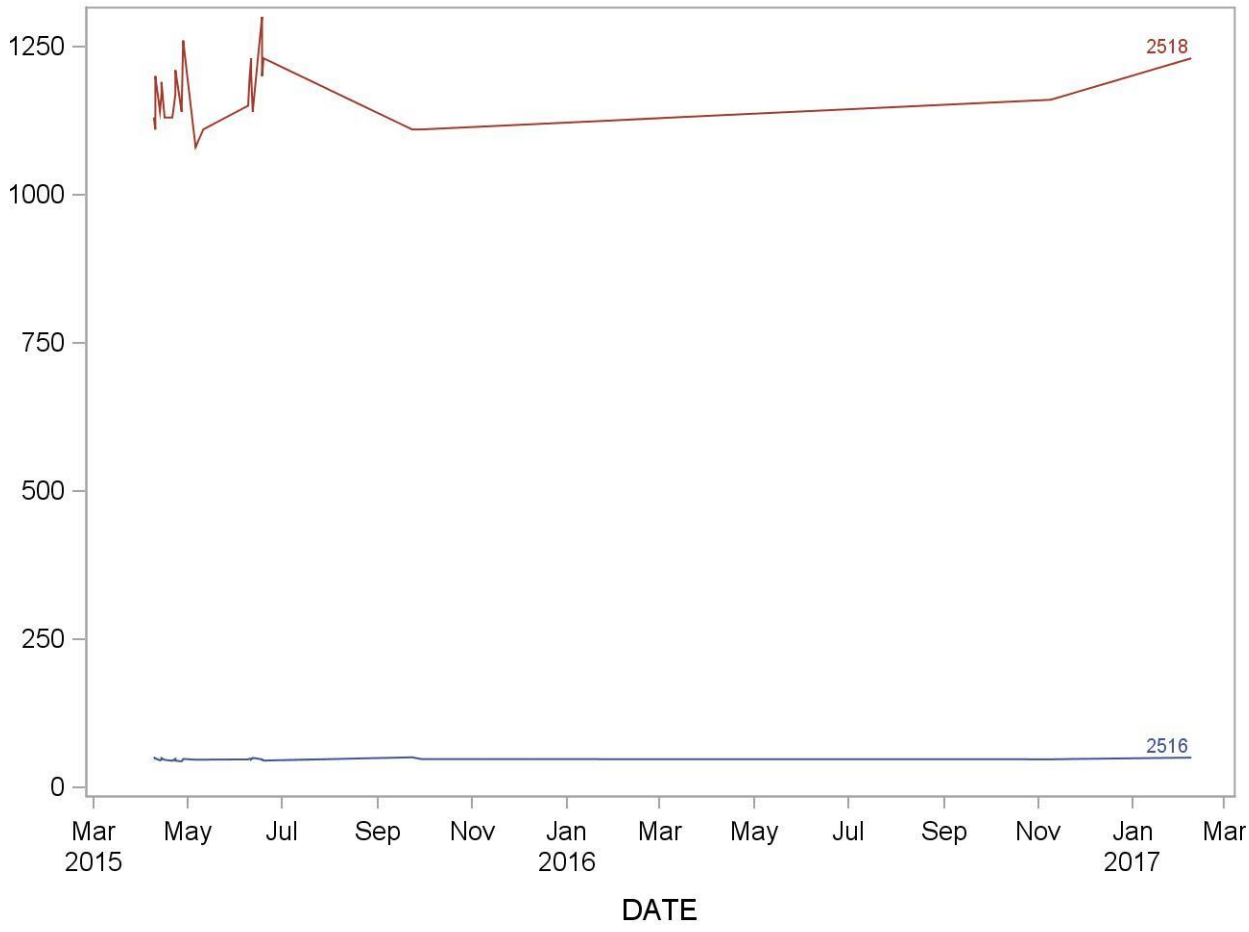
2013-2014 Summary Statistics and QC Chart for Anatabine, urine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2516	25	09APR15	13FEB17	9.404	0.380	4.0
2518	25	09APR15	13FEB17	89.248	3.066	3.4



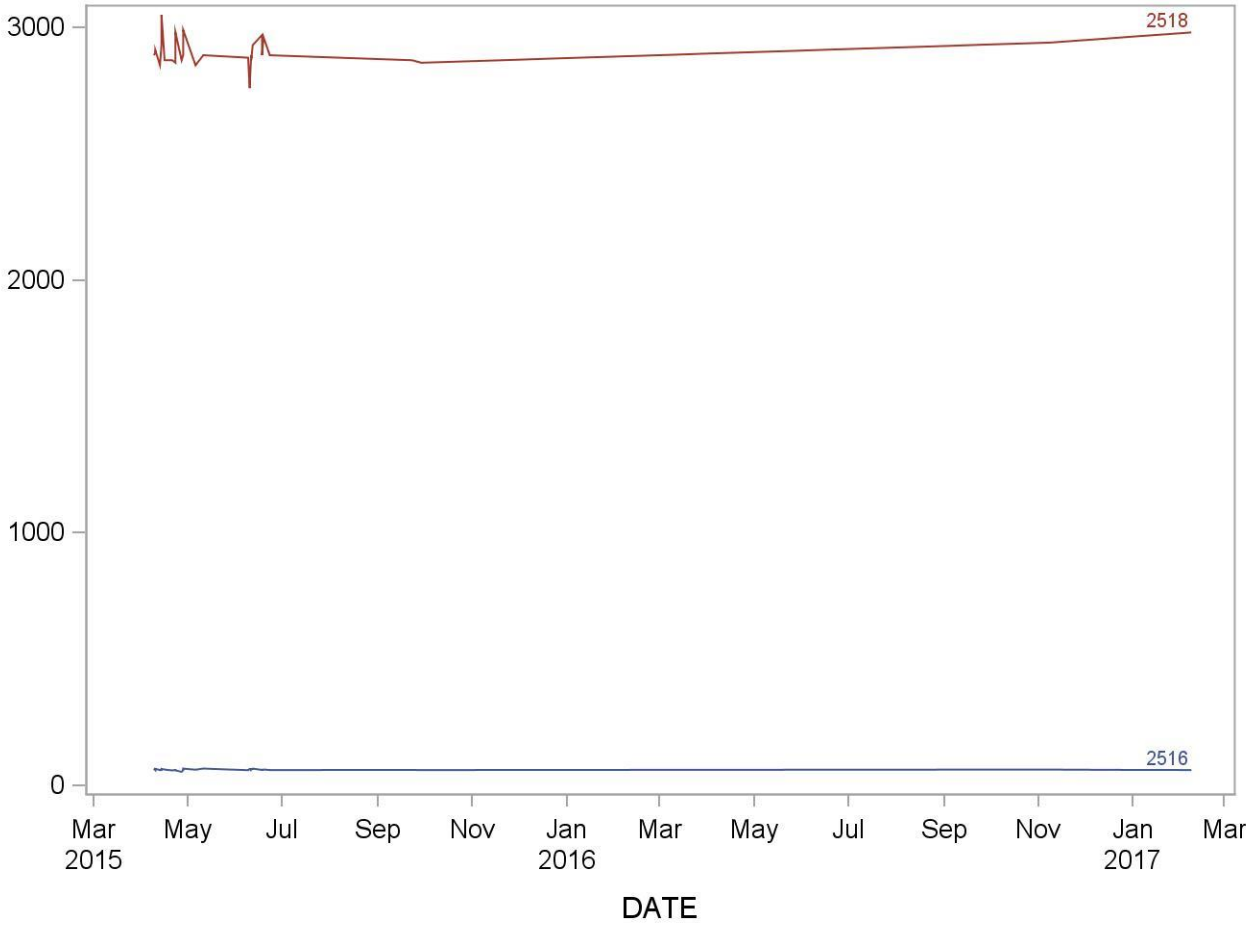
2013-2014 Summary Statistics and QC Chart for Cotinine-n-oxide, urine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2516	26	09APR15	08FEB17	47.712	1.828	3.8
2518	26	09APR15	08FEB17	1170.385	53.627	4.6



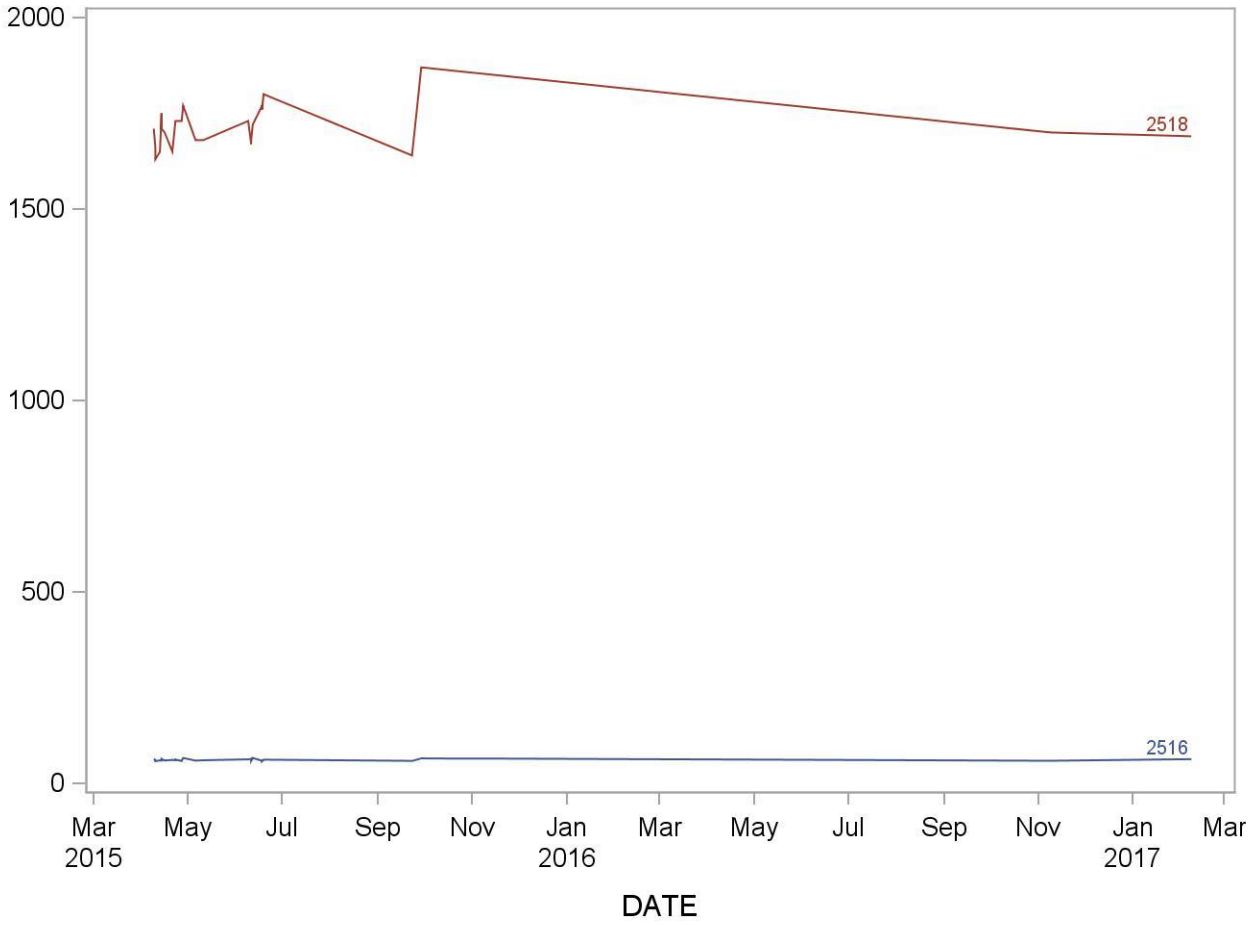
2013-2014 Summary Statistics and QC Chart for Nicotine, urine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2516	28	09APR15	08FEB17	62.604	2.955	4.7
2518	28	09APR15	08FEB17	2903.214	57.223	2.0



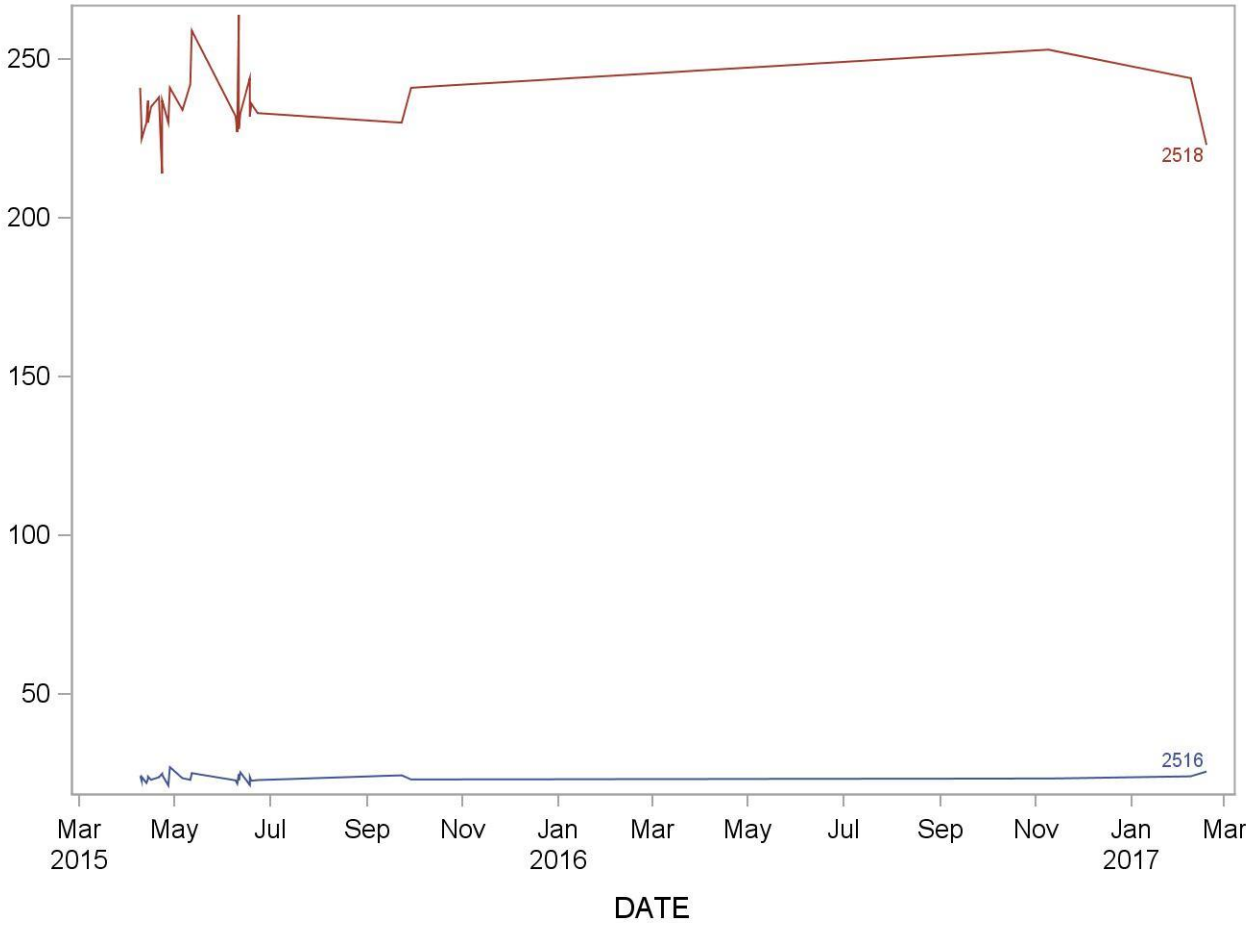
2013-2014 Summary Statistics and QC Chart for Nicotine-1 N-oxide, urine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2516	25	09APR15	08FEB17	61.584	2.621	4.3
2518	25	09APR15	08FEB17	1712.400	54.945	3.2



2013-2014 Summary Statistics and QC Chart for Norcotinine, urine (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2516	29	09APR15	18FEB17	23.517	1.301	5.5
2518	29	09APR15	18FEB17	235.862	10.429	4.4



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Appendix

Appendix 1: Example of Sample Preparation Log Sheet

	Sample Prep Log Sheet
Operator:	
Date:	
Run Plate label:	
Study Name:	
Number of unknowns:	
Total number of samples	
# of blanks included:	
# of times standards have been used (0-4):	
# of times QCs have been used (0-4):	
IS stock batch:	
Enzyme lot number	
Enzyme specific activity (U/g)	
Enzyme weighed (mg)	
Incubation time (12-24 hrs):	
Acetone last rinsed:	
H2O bottle last rinsed:	
Nitrogen flow rate:	
Centrifuge temperature:	
Caliper/Hamilton hardware and software issues:	
Comments: (maintenance, errors, layout, etc.)	

Appendix 2: Example of Sample Run Log Sheet.

LC/MS analysis Log Sheet				
Operator:				
Date:				
Run Plate label:				
Instrument Name:				
Total # of samples				
Preparation for LC				
Mobile Phase A: Ammonium Acetate Buffer A volume prepared (mL)				
Initial pH				
final pH (10 ± 0.05 temp. corrected)				
Mobile Phase B: Acetonitrile	**New solvent**	previously left		
Details on preparation:				
Peak height for NICT in blank water runs (< 3 x 10e3)	**water_01**	**water_02**	**water_03**	
LC pre-column stable pressure (1550-1750 psi)	**Pump A (psi)**	**Pump B (psi)**		
Preparation for MS				
Nitrogen generator readings				
Source (psi) (80-120)				
Curtain (psi) (50-80)				
Exhaust (psi) (40-80)				

Source assembly is clean:	**Yes**	No
Retention time (min) (5.85-6.05):		
NICT IS height (cps) (>2.0 x 10^5):		
Peak width (min) (0.1-0.25):		
Vacuum gauge (10^{-5} torr) (1.5 - 3.0):		
Pre-column filter last changed:		
Any instrument hardware and software issues:	N/A	
Comments: (maintenance, errors, layout, etc.)		
Column replaced:		
Column Lot #:		
# Injections on Column (max 1300):		

Appendix 3：Accuracy and Precision (target and measured concentrations displayed in ng/mL)

			Day-1 (n=16)				Day-2 (n=16)				Day-3 (n=16)				Overall (n=16)			
		TARGET	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%
COXT	LEVEL-1	8.00	8.22	0.61	7.44	2.74	8.30	0.38	4.55	3.75	8.05	0.48	5.91	0.61	8.19	0.49	6.10	2.37
	LEVEL-2	40.00	39.35	1.77	4.51	-1.63	40.19	1.28	3.19	0.48	39.79	1.23	3.10	-0.53	39.78	1.43	3.57	-0.56
	LEVEL-3	320.00	319.06	10.66	3.34	-0.29	321.25	10.28	3.20	0.39	322.88	4.83	1.50	0.90	321.06	8.59	2.68	0.33
	LEVEL-4	1600.00	1605.00	27.57	1.72	0.31	1572.50	55.80	3.55	-1.72	1617.50	27.93	1.73	1.09	1598.33	37.10	2.32	-0.10
	LEVEL-5	4000.00	4064.38	122.53	3.01	1.61	4001.25	141.51	3.54	0.03	3983.75	71.36	1.79	-0.41	4016.46	111.80	2.79	0.41
NOXT	LEVEL-1	8.00	7.97	0.35	4.38	-0.39	8.14	0.36	4.38	1.77	8.30	0.33	3.96	3.72	8.14	0.34	4.31	1.70
	LEVEL-2	40.00	39.71	0.99	2.49	-0.73	39.81	1.27	3.19	-0.48	40.48	2.02	4.98	1.19	40.00	1.42	3.56	-0.01
	LEVEL-3	320.00	315.38	10.58	3.35	-1.45	321.63	10.29	3.20	0.51	322.94	7.95	2.46	0.92	319.98	9.61	3.00	-0.01
	LEVEL-4	1600.00	1615.63	19.31	1.20	0.98	1593.75	66.72	4.19	-0.39	1644.38	24.49	1.49	2.77	1617.92	36.84	2.30	1.12
	LEVEL-5	4000.00	4065.63	90.70	2.23	1.64	4095.63	152.36	3.72	2.39	4094.38	86.48	2.11	2.36	4085.21	109.85	2.75	2.13
HCTT	LEVEL-1	80.00	80.64	2.22	2.75	0.80	78.50	2.05	2.62	-1.87	83.21	2.49	2.99	4.01	80.78	2.25	2.82	0.98
	LEVEL-2	400.00	396.75	11.95	3.01	-0.81	391.44	13.56	3.46	-2.14	403.00	10.30	2.56	0.75	397.06	11.94	2.98	-0.73
	LEVEL-3	3200.00	3123.13	74.45	2.38	-2.40	3143.75	99.92	3.18	-1.76	3179.38	80.79	2.54	-0.64	3148.75	85.05	2.66	-1.60
	LEVEL-4	16000.00	15693.75	184.28	1.17	-1.91	15812.50	326.34	2.06	-1.17	15825.00	264.58	1.67	-1.09	15777.08	258.40	1.61	-1.39
	LEVEL-5	40000.00	40012.50	1009.21	2.52	0.03	39262.50	1237.13	3.15	-1.84	38768.75	737.31	1.90	-3.08	39347.92	994.55	2.49	-1.63
NCTT	LEVEL-1	2.20	2.26	0.13	5.74	2.56	2.02	0.09	4.22	1.16	2.03	0.12	5.69	1.37	2.10	0.11	5.01	-4.44
	LEVEL-2	11.00	10.61	0.37	3.49	-3.58	9.92	0.44	4.45	-0.77	10.25	0.39	3.82	2.51	10.26	0.40	3.65	-6.73
	LEVEL-3	88.00	88.33	4.24	4.80	0.37	77.79	3.07	3.95	-2.76	83.73	1.55	1.85	4.66	83.28	2.95	3.35	-5.36

Continued Appendix 3：Accuracy and Precision

			Day-1 (n=16)				Day-2 (n=16)				Day-3 (n=16)				Overall (n=16)			
		TARGET	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%
	LEVEL-4	440.00	452.69	7.37	1.63	2.88	378.75	22.06	5.82	-5.31	418.00	6.80	1.63	4.50	416.48	12.08	2.75	-5.35
	LEVEL-5	1100.00	1133.75	35.00	3.09	3.07	953.50	48.33	5.07	-4.65	1043.75	22.47	2.15	4.38	1043.67	35.27	3.21	-5.12
COTT	LEVEL-1	40.00	40.59	1.87	4.61	1.48	40.54	1.53	3.77	1.36	40.98	1.97	4.80	2.44	40.70	1.79	4.47	1.76
	LEVEL-2	200.00	204.06	7.45	3.65	2.03	202.81	7.97	3.93	1.41	208.00	5.13	2.46	4.00	204.96	6.85	3.42	2.48
	LEVEL-3	1600.00	1665.00	43.05	2.59	4.06	1606.88	50.69	3.15	0.43	1638.75	32.84	2.00	2.42	1636.88	42.19	2.64	2.30
	LEVEL-4	8000.00	8273.13	154.82	1.87	3.41	8067.50	409.32	5.07	0.84	8145.00	264.27	3.24	1.81	8161.88	276.14	3.45	2.02
	LEVEL-5	20000.00	20825.00	444.97	2.14	4.13	20393.75	895.52	4.39	1.97	20362.50	686.90	3.37	1.81	20527.08	675.80	3.38	2.64
NNCT	LEVEL-1	2.00	2.18	0.33	14.89	9.17	1.91	0.07	3.74	-4.44	2.08	0.10	4.96	3.94	2.06	0.17	8.33	2.89
	LEVEL-2	10.00	10.46	0.96	9.16	4.59	10.29	0.48	4.62	2.93	10.01	0.59	5.86	0.06	10.25	0.67	6.73	2.53
	LEVEL-3	80.00	85.84	3.18	3.70	7.30	81.71	1.94	2.37	2.13	80.76	2.84	3.51	0.95	82.77	2.65	3.31	3.46
	LEVEL-4	400.00	415.81	13.70	3.29	3.95	415.13	18.38	4.43	3.78	403.31	11.16	2.77	0.83	411.42	14.41	3.60	2.85
	LEVEL-5	1000.00	1080.00	30.55	2.83	8.00	1038.94	43.37	4.17	3.89	1035.44	68.99	6.66	3.54	1051.46	47.63	4.76	5.15
ANTT	LEVEL-1	1.20	1.24	0.06	4.93	3.23	1.23	0.05	3.82	2.19	1.20	0.08	6.30	0.26	1.22	0.06	5.10	1.89
	LEVEL-2	6.00	6.00	0.27	4.57	0.07	6.09	0.17	2.87	1.49	6.10	0.27	4.41	1.70	6.07	0.24	3.99	1.09
	LEVEL-3	48.00	51.27	1.23	2.39	6.81	49.63	1.81	3.64	3.40	49.71	1.80	3.62	3.55	50.20	1.61	3.36	4.59
	LEVEL-4	240.00	250.44	4.59	1.83	4.35	253.69	15.27	6.02	5.70	248.06	7.43	3.00	3.36	250.73	9.10	3.79	4.47
	LEVEL-5	600.00	599.81	14.76	2.46	-0.03	629.13	28.15	4.48	4.85	618.50	11.97	1.94	3.08	615.81	18.30	3.05	2.64

Continued Appendix 3：Accuracy and Precision

			Day-1 (n=16)				Day-2 (n=16)				Day-3 (n=16)				Overall (n=16)			
		TARGET	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%	MEASURED	STD	RSD%	ERROR%
ANBT	LEVEL-1	1.00	1.04	0.09	9.00	4.41	1.08	0.11	11.00	7.64	0.98	0.10	9.78	-1.91	1.03	0.13	13.00	3.38
	LEVEL-2	5.00	4.92	0.23	4.72	-1.55	4.93	0.15	3.05	-1.45	5.05	0.30	5.88	1.08	4.97	0.23	4.53	-0.64
	LEVEL-3	40.00	39.85	3.39	8.51	-0.37	38.61	2.12	5.48	-3.48	40.55	1.80	4.45	1.37	39.67	2.44	6.09	-0.83
	LEVEL-4	200.00	192.56	6.79	3.53	-3.72	188.38	9.92	5.27	-5.81	194.38	6.59	3.39	-2.81	191.77	7.77	3.88	-4.11
	LEVEL-5	500.00	485.38	11.48	2.36	-2.93	490.06	15.20	3.10	-1.99	484.75	21.78	4.49	-3.05	486.73	16.15	3.23	-2.65
NICT	LEVEL-1	30.00	30.09	1.56	5.20	0.29	30.58	1.26	4.11	1.94	30.84	1.32	4.27	2.81	30.50	1.38	4.60	1.68
	LEVEL-2	150.00	149.69	4.50	3.00	-0.21	151.50	3.08	2.03	1.00	152.06	5.23	3.44	1.38	151.08	4.27	2.85	0.72
	LEVEL-3	1200.00	1207.50	26.46	2.19	0.63	1198.75	48.97	4.09	-0.10	1215.63	36.69	3.02	1.30	1207.29	37.37	3.11	0.61
	LEVEL-4	6000.00	6163.75	61.31	0.99	2.73	5968.13	173.94	2.91	-0.53	6125.63	170.25	2.78	2.09	6085.83	135.17	2.25	1.43
	LEVEL-5	15000.00	15450.00	203.31	1.32	3.00	15412.50	351.90	2.28	2.75	15100.00	136.63	0.90	0.67	15320.83	230.61	1.54	2.14

Appendix 4. Ruggedness test results for the volume of water added to each sample prior to injection (analyte concentrations displayed in ng/mL)

			COXT	NOXT	HCTT	NCTT	COTT	NNCT	ANTT	ANBT	NICT
-30% volume	POOL01	Mean	50.70	65.86	288.75	16.53	137.49	24.24	10.04	8.42	69.42
		STD	1.39	1.84	4.99	0.80	3.49	0.85	0.22	0.18	1.37
	POOL02	Mean	1250.50	1802.89	11024.70	302.14	5172.72	229.72	92.61	80.74	3078.90
		STD	16.65	24.53	78.12	6.62	76.52	8.47	1.66	3.87	28.32
Optimized volume	POOL01	Mean	50.76	64.64	282.35	17.29	135.88	24.30	9.90	8.65	68.20
		STD	1.25	1.53	8.15	0.56	2.30	1.27	0.36	0.63	0.74
	POOL02	Mean	1268.95	1806.48	10985.86	299.44	5097.80	240.98	91.19	87.38	3025.32
		STD	18.66	37.70	190.60	14.79	68.14	9.24	1.91	1.66	34.76
+ 30% volume	POOL01	Mean	48.99	65.85	278.39	17.36	122.40	24.32	9.63	8.24	68.48
		STD	1.03	2.46	5.85	0.43	4.58	0.69	0.21	0.26	1.03
	POOL02	Mean	1233.15	1794.42	10727.22	308.15	4707.08	240.74	89.79	82.63	3074.71
		STD	21.86	36.46	182.87	9.15	62.98	7.61	1.31	1.51	33.66

Appendix 5. Freeze-thaw test results. (Analyte concentrations displayed in ng/mL)

			COXT	NOXT	HCTT	NCTT	COTT	NNCT	ANTT	ANBT	NICT
POOL01	1 Cycle	Mean	47.9	63.2	280.9	16.6	130.5	23.7	9.6	8.1	67.4
		STD	1.3	1.1	8.7	0.5	4.4	0.5	0.3	0.3	1.4
	5 cycles	Mean	49.8	65.1	283.5	17.3	132.0	24.6	9.9	8.6	69.0
		STD	1.3	1.1	10.1	0.4	7.2	0.7	0.3	0.4	1.4
	10 Cycles	Mean	49.1	64.7	279.4	16.9	131.1	24.6	9.7	8.5	68.7
		STD	1.3	1.6	4.8	0.5	8.9	1.0	0.2	0.3	1.1
	25 Cycles	Mean	49.9	64.2	283.0	16.6	131.6	23.7	9.9	8.1	68.4
		STD	1.0	1.2	6.1	0.8	7.5	0.9	0.4	0.5	1.0
POOL02	1 Cycle	Mean	1214.6	1743.7	10769.0	304.6	4912.5	230.0	90.3	81.0	2987.4

		STD	39.1	38.9	311.1	9.6	156.9	9.0	2.8	3.0	16.4
	5 cycles	Mean	1252.8	1819.1	10921.8	303.2	5055.9	241.1	91.7	84.7	3085.7
		STD	39.1	38.9	311.1	9.6	156.9	9.0	2.8	3.0	16.4
	10 Cycles	Mean	1253.6	1784.5	10929.9	303.5	4986.9	236.8	90.5	84.3	3050.2
		STD	19.2	17.7	199.7	11.9	226.3	6.2	2.0	3.3	34.9
	25 Cycles	Mean	1246.3	1800.2	10886.2	303.0	4934.8	233.6	91.3	81.8	3043.1
		STD	17.9	35.7	232.1	3.9	209.3	11.2	1.9	4.8	44.4

Appendix 6. Method performance tests

For all tables:

Units:	ng/mL
Analyte:	anatabine, anabasine, nicotine, cotinine-N-oxide, nicotine-N-oxide, *trans*-3'-hydroxycotinine, norcotinine, cotinine, nornicotine

1. Accuracy using Spike Recovery

Cotinine-N-oxide (COXT)

		Sample 1					Sample 2						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	-0.224	0.24	0.1		0	-0.293	0.3	0.0		91.3	1.7
	2		-0.335	0.583				-0.159	0.317				
	3		-0.243	0.4				-0.325	0.2				
Sample + Spike 1	1	151.7016	142.519	137.419	139.4	91.8	151.7016	129.366	131.9	133.6	88.1		
	2		136.235	146.08				141.886	129.434				
	3		138.583	135.354				135.545	133.777				
Sample + Spike 2	1	295.9828	282.634	253.3	271.6	91.7	295.9828	286.814	271.9	275.1	92.9		
	2		293.988	247.301				277.091	266.413				
	3		289.941	262.258				277.414	270.792				
Sample + Spike 3	1	591.5866	549.677	528.414	547	92.5	591.5866	561.32	540.62	537	90.8		
	2		576.368	540.289				533.687	532.691				
	3		567.709	521.697				551.861	503.524				

Nicotine-N-oxide (NOXT)

		Sample 1					Sample 2						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.594	0.621	0.5		0	0.55	0.277	0.4		96.5	4.4
	2		0.517	0.293				0.486	0.333				
	3		0.442	0.4				0.468	0.3				
Sample + Spike 1	1	200.998	179.271	179.03	178.7	88.6	200.998	189.068	194.8	191.9	95.3		
	2		176.976	179.971				196.743	190.116				
	3		177.818	178.904				194.668	185.98				
Sample + Spike 2	1	401.996	380.429	386.5	388.2	96.4	401.996	392.069	393.4	392.7	97.6		
	2		382.763	400.674				402.009	397.155				
	3		376.431	402.296				389.849	381.931				
Sample + Spike 3	1	803.992	797.283	841.768	807	100.3	803.992	795.634	841.357	810	100.6		
	2		762.833	819.635				781.059	824.364				
	3		790.365	830.879				798.922	815.793				

Trans-3'-hydroxycotinine (HCTT)

		Sample 1					Sample 2						
			Measured concentration					Measured concentration					
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	-1.206	-0.689	-1.1		0	-1.414	-0.652	-1.2		96.1	1.7
	2		-1.371	-0.616				-1.463	-0.917				
	3		-1.58	-1.1				-1.487	-1.1				
Sample + Spike 1	1	1004.41	960.237	1001.45	993.5	99.0	1004.41	936.748	935.0	945.1	94.2		
	2		964.068	1023.05				961.852	935.217				
	3		987.796	1024.52				948.32	953.285				
Sample + Spike 2	1	1959.689	1908.53	1841.6	1867.8	95.4	1959.689	1930.37	1863.8	1900.4	97.0		
	2		1865.1	1904.53				1889.1	1898.13				
	3		1862.13	1825.19				1903.97	1916.85				
Sample + Spike 3	1	3916.871	3772.92	3694.65	3754	95.9	3916.871	3776.62	3700.36	3714	94.8		
	2		3722.83	3857.59				3692.72	3729.8				
	3		3703.68	3774.12				3706.38	3677.78				

Norcotinine (NCTT)

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1	Sample 1 Measured concentration Day 2	Sample 1 Measured concentration Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1	Sample 2 Measured concentration Day 2	Sample 2 Measured concentration Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	-0.092	0.081	0.0		0	-0.219	-0.077	-0.1		107.0	2.2
	2		-0.148	-0.058				-0.161	-0.106				
	3		-0.092	0.3				-0.174	0.0				
Sample + Spike 1	1	50.5874	54.389	57.186	55.6	109.8	50.5874	52.518	53.1	52.3	103.5		
	2		54.567	58.679				51.479	53.15				
	3		52.439	56.088				51.652	51.6				
Sample + Spike 2	1	98.7004	104.281	103.9	104.5	105.9	98.7004	104.799	108.1	107.0	108.6		
	2		104.611	103.872				104.19	108.496				
	3		107.775	102.696				105.362	111.166				
Sample + Spike 3	1	197.2746	212.709	209.573	211	107.2	197.2746	205.952	214.859	211	107.0		
	2		213.479	216.397				212.651	210.198				
	3		209.256	207.012				207.085	214.752				

Cotinine (COTT)

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1	Sample 1 Measured concentration Day 2	Sample 1 Measured concentration Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1	Sample 2 Measured concentration Day 2	Sample 2 Measured concentration Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.417	-0.697	-0.3		0	-0.689	-0.655	-0.5		101.0	1.7
	2		-0.732	-0.232				-0.263	-0.393				
	3		0.119	-0.9				-0.145	-0.6				
Sample + Spike 1	1	631.8172	631.051	677.779	654.5	103.6	631.8172	619.045	646.8	622.8	98.6		
	2		630.604	676.087				630.753	618.139				
	3		631.058	680.333				603.403	618.676				
Sample + Spike 2	1	1232.729	1248.2	1249.4	1237.9	100.4	1232.729	1264.62	1281.4	1256.5	102.0		
	2		1241.42	1232.13				1256.06	1224.32				
	3		1228.35	1227.72				1238.45	1274.14				
Sample + Spike 3	1	2463.88	2457.2	2471.3	2492	101.2	2463.88	2458.09	2492.48	2474	100.4		
	2		2455.44	2615.89				2449.55	2516.88				
	3		2502.33	2449.57				2400.29	2524.72				

Nornicotine (NNCT) *

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1	Sample 1 Measured concentration Day 2	Sample 1 Measured concentration Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1	Sample 2 Measured concentration Day 2	Sample 2 Measured concentration Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.41	0.25	0.2		0	1.951	1.738	2.1		94.3	7.4
	2		0.208	0.327				2.257	2.532				
	3		0.013	0.2				2.231	2.0				
Sample + Spike 1	1	30.527	37.642	33.953	32.7	106.5	30.527	30.435	29.9	29.7	90.3		
	2		31.054	31.464				29.838	29.283				
	3		31.316	31.03				29.665	28.9				
Sample + Spike 2	1	59.5606	55.74	51.9	53.2	89.0	59.5606	53.589	52.9	54.7	88.2		
	2		54.804	54.921				59.963	55.561				
	3		51.937	50.113				54.329	51.622				
Sample + Spike 3	1	119.045	121.468	133.345	120	100.4	119.045	109.166	107.289	111	91.4		
	2		117.985	117.032				108.303	104.335				
	3		115.32	113.63				124.545	111.518				

Anatabine (ANTT)

		Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1	Sample 1 Measured concentration Day 2	Sample 1 Measured concentration Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1	Sample 2 Measured concentration Day 2	Sample 2 Measured concentration Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample		1	0	0.034	0.036	0.0		0	0.103	0.041	0.1		106.2	9.9
		2		0.023	0.008				0.08	0.014				
		3		0.025	0.0				0.03	0.1				
Sample + Spike 1	*	1	14.9396	13.128	13.655	13.2	87.9	10.26153084	10.515	12.0	11.5	111.4		
		2		11.929	13.464				10.77	11.972				
		3		12.596	14.179				11.003	12.629				
Sample + Spike 2		1	20.52306167	20.767	21.2	21.7	105.7	20.52306167	21.624	24.5	23.2	112.7		
		2		22.192	22.835				22.333	24.625				
		3		21.641	21.601				21.374	24.747				
Sample + Spike 3		1	41.04612334	43.299	43.076	43	104.3	41.04612334	43.087	50.159	47	115.0		
		2		41.627	43.946				43.671	52.53				
		3		41.442	43.726				43.593	50.464				

Anabasine (ANBT)

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1	Sample 1 Measured concentration Day 2	Sample 1 Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1	Sample 2 Measured concentration Day 2	Sample 2 Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	0.024	0.063	0.1		0	0.765	0.139	0.3		97.1	1.5
	2		0.054	0.105				0.607	0.116				
	3		0.032	0.1				0.311	0.0				
Sample + Spike 1	1	20.3122	19.432	20.118	20.2	99.0	20.3122	18.998	19.3	19.6	94.9		
	2		19.524	20.831				19.798	19.794				
	3		19.781	21.272				19.403	20.304				
Sample + Spike 2	1	39.6308	39.468	37.4	38.3	96.4	39.6308	39.306	39.8	38.8	97.1		
	2		37.986	37.95				37.325	39.213				
	3		38.359	38.454				38.195	39.105				
Sample + Spike 3	1	79.2108	78.196	78.152	77	96.6	79.2108	78.758	78.553	78	98.6		
	2		75.016	78.729				77.644	80.056				
	3		72.719	76.426				76.672	78.887				

Nicotine (NICT)

	Replicate	Sample 1 Spike concentration	Sample 1 Measured concentration Day 1	Sample 1 Measured concentration Day 2	Sample 1 Mean	Sample 1 Recovery (%)	Sample 2 Spike concentration	Sample 2 Measured concentration Day 1	Sample 2 Measured concentration Day 2	Sample 2 Mean	Sample 2 Recovery (%)	Mean recovery (%)	SD (%)
Sample	1	0	2.007	10.585	4.0		0	5.056	7.127	4.9		94.7	2.1
	2		0.264	3.959				2.068	6.336				
	3		5.46	1.6				1.129	7.8				
Sample + Spike 1	1	508.3146	486.682	515.905	500.9	97.8	508.3146	466.041	457.9	470.5	91.6		
	2		500.09	510.046				476.62	473.329				
	3		487.484	505.251				475.276	473.994				
Sample + Spike 2	1	991.7648	925.051	909.4	931.0	93.5	991.7648	933.858	953.7	951.3	95.4		
	2		935.014	946.517				954.706	957.313				
	3		934.112	935.868				953.947	954.341				
Sample + Spike 3	1	1982.26	1906.57	1863.2	1886	94.9	1982.26	1889.37	1901.43	1886	94.9		
	2		1867.97	1928.52				1881.35	1893.51				
	3		1899.59	1848.81				1874.71	1876.78				

2. Precision

Cotinine-N-oxide (COXT)

Quality material 1 (QCL)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	50.47	48.09	49.28	1.417	1.417	4857
2	45.008	43.204	44.11	0.814	0.814	3891
3	49.727	49.377	49.55	0.031	0.031	4911
4	47.36	48.412	47.89	0.277	0.277	4586
5	47.695	48.138	47.92	0.049	0.049	4592
6	49.448	50.413	49.93	0.233	0.233	4986
7	50.66	43.034	46.85	14.539	14.539	4389
8	47.751	52.123	49.94	4.779	4.779	4987
9	46.63	52.14	49.38	7.576	7.576	4877
10	51.819	45.518	48.67	9.926	9.926	4737
Grand sum	967.011	Grand mean	48.35055			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	79.279	7.928	2.816	5.82
Between Run	58.333	6.481	0	0.00
Total	137.612		2.816	**5.82**

Quality material 2 (QCH)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	1202.07	1201.60	1201.83	0.06	0.06	2888812
2	1173.52	1152.08	1162.80	114.93	114.93	2704224
3	1190.43	1249.64	1220.04	876.46	876.46	2976985
4	1132.55	1193.75	1163.15	936.33	936.33	2705820
5	1128.16	1143.01	1135.58	55.18	55.18	2579100
6	1176.39	1077.35	1126.87	2452.08	2452.08	2539683
7	1197.39	1137.38	1167.39	900.27	900.27	2725587
8	1290.24	1170.78	1230.51	3567.67	3567.67	3028305
9	1128.41	1213.11	1170.76	1793.61	1793.61	2741372
10	1214.71	1174.44	1194.57	405.52	405.52	2854016
Grand sum	23547.025	Grand mean	1177.351			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	22204	2220	47.121	4.00
Between Run	20785	2309	6.674	0.57
Total	42990		47.592	**4.04**

Nicotine-N-oxide (NOXT)

Quality material 1 (QCL)							Quality material 1 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2	Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	60.33	63.57	61.95	2.621161	2.621161	7675.1094	1	1758.13	1672.93	1715.53	1814.7174	1814.7174	5886069
2	62.278	60.937	61.61	0.44957025	0.44957	7590.9681	2	1759.32	1606.42	1682.87	5844.22026	5844.220256	5664093
3	61.789	61.869	61.83	0.0016	0.0016	7645.6505	3	1666.05	1756.81	1711.43	2059.75284	2059.75284	5857982
4	57.553	56.664	57.11	0.19758025	0.19758	6522.7615	4	1597.41	1603.78	1600.59	10.1346722	10.13467225	5123799
5	59.051	64.135	61.59	6.461764	6.461764	7587.3953	5	1858.02	1787.34	1822.68	1249.16299	1249.162992	6644321
6	57.27	57.494	57.38	0.012544	0.012544	6585.3878	6	1672.44	1639.26	1655.85	275.294464	275.294464	5483678
7	59.817	62.327	61.07	1.575025	1.575025	7459.5784	7	1767.77	1704.52	1736.14	1000.14063	1000.140625	6028392
8	56.693	63.243	59.97	10.725625	10.72563	7192.322	8	1779.95	1824.96	1802.45	506.362506	506.3625063	6497684
9	62.54	65.19	63.86	1.76757025	1.76757	8157.3487	9	1709.84	1774.56	1742.20	1047.20196	1047.20196	6070525
10	65.178	59.623	62.40	7.71450625	7.714506	7787.6448	10	1733.00	1780.90	1756.95	573.674352	573.6743522	6173743
Grand sum	1217.546	Grand mean	60.8773				Grand sum	34453.394	Grand mean	1722.67			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)				Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	63.053892	6.3053892	2.511053404	4.12			Within Run	28761.324	2876.132414	53.62959	3.11		
Between Run	83.2535242	9.250391578	1.2134666	1.99			Between Run	78469.295	8718.810568	54.04941	3.14		
Total	146.3074162		2.788886944	**4.58**			Total	107230.62		76.14113	**4.42**		

Trans-3'-hydroxycotinine (HCTT)

Quality material 1 (QCL)							Quality material 2 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2	Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	284.26	268.04	276.15	65.7477722	65.74777	152514.88	1	10199.86	10037.38	10118.62	6600.26256	6600.262564	2.05E+08
2	285.74	293.342	289.54	14.447601	14.4476	167667.98	2	10827.30	10404.66	10615.98	44655.0858	44655.08581	2.25E+08
3	296.64	288.324	292.48	17.288964	17.28896	171091.44	3	10439.48	10590.99	10515.24	5738.82003	5738.820025	2.21E+08
4	265.814	281.263	273.54	59.6679002	59.6679	149646.62	4	9574.00	9716.65	9645.32	5086.97033	5086.970329	1.86E+08
5	272.217	275.748	273.98	3.11699025	3.11699	150132.82	5	10487.88	10118.28	10303.08	34151.04	34151.04	2.12E+08
6	283.867	271.728	277.80	36.8388303	36.83883	154342.9	6	9864.30	10215.97	10040.13	30917.7714	30917.77139	2.02E+08
7	285.691	284.948	285.32	0.13801225	0.138012	162814.43	7	10330.15	10166.62	10248.38	6685.10641	6685.106406	2.1E+08
8	290.322	272.545	281.43	79.0054323	79.00543	158409.63	8	10283.52	10120.58	10202.05	6637.52384	6637.523841	2.08E+08
9	277.00	304.08	290.54	183.372222	183.3722	168822.92	9	9773.31	9519.73	9646.52	16076.0845	16076.08447	1.86E+08
10	301.154	292.159	296.66	20.2275063	20.22751	176010.16	10	10547.88	10317.89	10432.88	13223.965	13223.96502	2.18E+08
Grand sum	5674.87	Grand mean	283.7435				Grand sum	203536.42	Grand mean	10176.82			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)				Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	959.702462	95.9702462	9.796440486	3.45			Within Run	339545.26	33954.52597	184.2675	1.81		
Between Run	1246.311921	138.4791023	4.610252495	1.62			Between Run	1960868.5	217874.2766	303.2489	2.98		
Total	2206.014383		10.82703442	**3.82**			Total	2300413.7		354.8442	**3.49**		

Norcotinine (NCTT)

Quality material 1 (QCL)							Quality material 2 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2	Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	16.14	16.54	16.34	0.038809	0.038809	533.9912	1	273.42	275.48	274.45	1.054729	1.054729	150646.7
2	17.095	15.958	16.53	0.32319225	0.323192	546.2504	2	303.63	285.34	294.48	83.6035923	83.60359225	173438.7
3	16.385	16.65	16.52	0.01755625	0.017556	545.65561	3	285.17	299.64	292.40	52.388644	52.388644	170999
4	16.721	16.834	16.78	0.00319225	0.003192	562.96901	4	274.08	276.94	275.51	2.056356	2.056356	151811.5
5	16.56	16.302	16.43	0.016641	0.016641	539.95552	5	276.85	280.34	278.59	3.038049	3.038049	155229.2
6	18.003	17.023	17.51	0.2401	0.2401	613.41034	6	284.06	299.60	291.83	60.3340562	60.33405625	170328.9
7	17.553	17.318	17.44	0.01380625	0.013806	607.99332	7	303.43	286.04	294.73	75.6117202	75.61172025	173736.9
8	17.263	17.115	17.19	0.005476	0.005476	590.92344	8	297.77	290.19	293.98	14.356521	14.356521	172848.5
9	16.66	18.23	17.45	0.61858225	0.618582	608.83051	9	292.66	302.57	297.62	24.532209	24.532209	177151.8
10	16.003	16.937	16.47	0.218089	0.218089	542.5218	10	288.53	295.86	292.20	13.402921	13.402921	170755.8
Grand sum	337.295	Grand mean	16.86475				Grand sum	5771.591	Grand mean	288.5796			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)				Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	2.9908885	0.29908885	0.546890163	3.24			Within Run	660.7576	66.07575955	8.1287	2.82		
Between Run	4.10531325	0.456145917	0.28022943	1.66			Between Run	1383.899	153.7665513	6.621586	2.29		
Total	7.09620175		0.614505804	**3.64**			Total	2044.6566		10.48433	**3.63**		

Cotinine (COTT)

Quality material 1 (QCL)							Quality material 2 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2	Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	126.49	125.65	126.07	0.173889	0.173889	31786.786	1	4839.52	4887.75	4863.64	581.677924	581.677924	47309891
2	128.338	129.86	129.10	0.579121	0.579121	33333.104	2	4762.26	4768.16	4765.21	8.71135225	8.71135225	45414481
3	136.62	132.671	134.65	3.89865025	3.89865	36258.821	3	4951.49	5218.42	5084.96	17813.8405	17813.84049	51713545
4	137.394	140.809	139.10	2.91555625	2.915556	38698.455	4	4826.46	4793.15	4809.81	277.355716	277.355716	46268448
5	127.378	125.594	126.49	0.795664	0.795664	31997.416	5	4817.17	4872.84	4845.01	774.703722	774.7037223	46948157
6	132.297	125.431	128.86	11.785489	11.78549	33211.861	6	4812.79	4975.09	4893.94	6585.24135	6585.24135	47901249
7	137.356	134.241	135.80	2.42580625	2.425806	36882.465	7	5048.06	5087.66	5067.86	391.86182	391.8618202	51366400
8	134.473	135.611	135.04	0.323761	0.323761	36472.684	8	5091.46	5300.22	5195.84	10894.8713	10894.87126	53993496
9	128.58	134.27	131.43	8.09687025	8.09687	34545.324	9	4870.75	5015.51	4943.13	5239.15392	5239.153924	48869108
10	130.359	132.121	131.24	0.776161	0.776161	34447.875	10	4924.47	5021.17	4972.82	2337.67415	2337.67415	49457828
Grand sum	2635.542	Grand mean	131.7771				Grand sum	98884.397	Grand mean	4944.22			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)				Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	63.541936	6.3541936	2.520752586	1.91			Within Run	89810.183	8981.018343	94.76823	1.92		
Between Run	330.7087978	36.74542198	3.898155229	2.96			Between Run	336403.61	37378.17884	119.1578	2.41		
Total	394.2507338		4.642177053	**3.52**			Total	426213.79		152.2485	**3.08**		

Nornicotine (NNCT)

Quality material 1 (QCL)							Quality material 2 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2	Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	23.66	23.75	23.70	0.00225625	0.002256	1123.617	1	234.22	241.60	237.91	13.601344	13.601344	113201.4
2	22.935	22.675	22.81	0.0169	0.0169	1040.1361	2	238.24	233.73	235.99	5.089536	5.089536	111378.8
3	23.224	23.379	23.30	0.00600625	0.006006	1085.9198	3	242.50	237.18	239.84	7.07294025	7.07294025	115048.8
4	19.531	21.526	20.53	0.99500625	0.995006	842.83862	4	218.38	215.60	216.99	1.93627225	1.93627225	94168.89
5	24.036	23.763	23.90	0.01863225	0.018632	1142.3722	5	271.63	267.73	269.68	3.798601	3.798601	145454.6
6	22.902	20.922	21.91	0.9801	0.9801	960.27149	6	236.49	241.75	239.12	6.895876	6.895876	114355.8
7	24.304	24.348	24.33	0.000484	0.000484	1183.5086	7	239.89	234.79	237.34	6.52547025	6.52547025	112660.1
8	22.7705	22.845	22.81	0.00138756	0.001388	1040.3869	8	238.70	239.62	239.16	0.213444	0.213444	114394.1
9	23.48	25.11	24.29	0.659344	0.659344	1180.3969	9	221.27	240.80	231.04	95.374756	95.374756	106756.2
10	24.865	23.727	24.30	0.323761	0.323761	1180.5912	10	243.50	243.26	243.38	0.013924	0.013924	118466.7
Grand sum	463.7455	Grand mean	23.187275				Grand sum	4780.881	Grand mean	239.0441			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)				Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	6.007755125	0.600775513	0.775097099	3.34			Within Run	281.04433	28.10443275	5.301361	2.22		
Between Run	27.04431761	3.004924179	1.096391506	4.73			Between Run	3044.1422	338.2380226	12.45258	5.21		
Total	33.05207274		1.342702441	**5.79**			Total	3325.1865		13.53408	**5.66**		

Anatabine (ANTT)

Quality material 1 (QCL)							Quality material 2 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2	Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	9.33	8.96	9.15	0.034225	0.034225	167.26205	1	83.02	90.27	86.65	13.126129	13.126129	15014.71
2	9.186	9.253	9.22	0.00112225	0.001122	169.99836	2	88.05	85.86	86.95	1.18919025	1.18919025	15122.17
3	9.721	9.353	9.54	0.033856	0.033856	181.90874	3	81.37	87.35	84.36	8.952064	8.952064	14232.54
4	8.777	9.094	8.94	0.02512225	0.025122	159.68632	4	85.15	87.75	86.45	1.69650625	1.69650625	14946.69
5	9.373	9.141	9.26	0.013456	0.013456	171.3841	5	82.18	86.53	84.36	4.721929	4.721929	14232.21
6	9.617	9.021	9.32	0.088804	0.088804	173.68752	6	88.66	89.37	89.01	0.12852225	0.12852225	15847.16
7	9.563	9.83	9.70	0.01782225	0.017822	188.04422	7	92.37	91.12	91.74	0.391876	0.391876	16833.56
8	9.418	9.866	9.64	0.050176	0.050176	185.93633	8	91.24	95.47	93.35	4.477456	4.477456	17429.19
9	9.53	10.00	9.77	0.054289	0.054289	190.74951	9	84.77	91.64	88.20	11.799225	11.799225	15559.19
10	9.473	9.12	9.30	0.03115225	0.031152	172.84982	10	88.43	89.82	89.13	0.48233025	0.48233025	15886.71
Grand sum	187.628	Grand mean	9.3814				Grand sum	1760.4	Grand mean	88.02			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)				Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)		
Within Run	0.70005	0.070005	0.26458458	2.82			Within Run	93.930456	9.3930456	3.064808	3.48		
Between Run	1.2936588	0.143739867	0.192008941	2.05			Between Run	153.71716	17.079684	1.960439	2.23		
Total	1.9937088		0.326913495	**3.48**			Total	247.64761		3.638182	**4.13**		

Anabasine (ANBT)

Quality material 1 (QCL)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	7.97	8.58	8.28	0.09394225	0.093942	137.0009
2	8.594	7.427	8.01	0.34047225	0.340472	128.33622
3	7.311	7.95	7.63	0.10208025	0.10208	116.44906
4	8.717	9.006	8.86	0.02088025	0.02088	157.05236
5	7.798	7.647	7.72	0.00570025	0.0057	119.27401
6	7.731	7.511	7.62	0.0121	0.0121	116.15928
7	7.668	7.516	7.59	0.005776	0.005776	115.27693
8	7.357	7.932	7.64	0.08265625	0.082656	116.87676
9	7.90	8.53	8.22	0.099856	0.099856	134.97245
10	7.953	7.664	7.81	0.02088025	0.02088	121.94534
Grand sum	158.765	Grand mean	7.93825			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	1.5686875	0.15686875	0.396066598	4.99
Between Run	3.02706625	0.336340694	0.29955963	3.77
Total	4.59575375		0.496593115	**6.26**

Quality material 2 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	69.10	74.36	71.73	6.93532225	6.93532225	10290.53
2	73.51	73.34	73.42	0.00714025	0.00714025	10781.14
3	69.28	75.10	72.19	8.485569	8.485569	10422.21
4	71.31	75.26	73.28	3.912484	3.912484	10740.8
5	79.87	79.18	79.52	0.11730625	0.11730625	12647.66
6	70.10	71.34	70.72	0.38750625	0.38750625	10002.78
7	72.42	71.74	72.08	0.11526025	0.11526025	10391.2
8	72.23	72.22	72.23	1.225E-05	1.225E-05	10433.62
9	76.73	77.23	76.98	0.0625	0.0625	11851.22
10	80.41	79.72	80.06	0.117649	0.117649	12819.85
Grand sum	1484.426	Grand mean	74.2213			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	40.281499	4.0281499	2.007025	2.70
Between Run	204.97968	22.77551947	3.061647	4.13
Total	245.26117		3.660852	**4.93**

Nicotine (NICT)

Quality material 1 (QCL)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	62.50	65.07	63.78	1.653796	1.653796	8136.7973
2	68.57	66.118	67.34	1.503076	1.503076	9070.4287
3	62.246	70.413	66.33	16.6749722	16.67497	8799.2051
4	63.641	75.859	69.75	37.319881	37.31988	9730.125
5	57.546	64.483	61.01	12.0304923	12.03049	7445.5384
6	61.434	60.84	61.14	0.088209	0.088209	7475.4655
7	66.821	74.155	70.49	13.446889	13.44689	9937.1163
8	68.845	70.091	69.47	0.388129	0.388129	9651.606
9	62.50	74.23	68.37	34.398225	34.39822	9348.0934
10	66.116	70.4	68.26	4.588164	4.588164	9318.3091
Grand sum	1331.88	Grand mean	66.594			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	244.183667	24.4183667	4.941494379	7.42
Between Run	217.468205	24.16313389	0	0.00
Total	461.651872		4.941494379	**7.42**

Quality material 2 (QCH)						
Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	2879.68	2904.26	2891.97	151.007232	151.0072323	16727010
2	2913.54	2852.34	2882.94	936.115216	936.115216	16622686
3	2876.47	2935.48	2905.97	870.51552	870.5155203	16889352
4	2682.74	2812.11	2747.42	4183.63176	4183.631761	15096677
5	3008.40	2884.59	2946.49	3832.10522	3832.105216	17363630
6	2926.53	2962.26	2944.40	319.14036	319.1403602	17338965
7	3083.27	3052.06	3067.67	243.516025	243.516025	18821137
8	3051.86	3022.56	3037.21	214.710409	214.710409	18449289
9	2905.91	3064.12	2985.01	6258.23388	6258.233881	17820617
10	2930.92	2951.95	2941.44	110.544196	110.544196	17304115
Grand sum	58701.053	Grand mean	2935.053			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	34239.04	3423.903963	58.51413	1.99
Between Run	142798.13	15866.45898	78.87508	2.69
Total	177037.17		98.20988	**3.35**

3. LOD, specificity and fit for intended use

Analytes	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-N-oxide (COXT)	2	yes	yes
Nicotine-N-oxide (NOXT)	2.5	yes	yes
Trans-3'-hydroxycotinine (HCTT)	9.5	yes	yes
Norcotinine (NCTT)	1.1	yes	yes
Cotinine (COTT)	4.6	yes	yes
Nornicotine (NNCT)	2.5	yes	yes
Anabasine (ANTT)	0.4	yes	yes
Anabasine (ANBT)	0.5	yes	yes
Nicotine (NICT)	10.5	yes	yes

4. Stability

Freeze and thaw stability	Three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability	original samples stored at room temperature for 1 day
Processed sample stability	processed samples stored at 4°C for 1 day
Long-term stability	samples stored at -80°C for 2 years

Cotinine-N-oxide (COXT)									
Quality material 1									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	47.36	47.578	47.36	46.974	47.695	47.242	48.136	46.19	47.36
Replicate 2	48.412	47.02	48.412	50.71	41.02	42.658	45.633	48.389	48.412
Replicate 3	47.977	46.731	47.977	46.918	40.74	46.562	46.993	51.826	47.977
Mean	47.91633333	47.10966667	47.91633333	48.2	43.15166667	45.48733333		48.80166667	47.9
% difference from initial measurement	--	**-1.7**	--	**0.6**	--	**5.4**		--	**-1.8**
Quality material 2									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	1193.746	1203.422	1193.746	1135.96	1087.584	1183.014	1339.5	1138.052	1193.746
Replicate 2	1143.075	1121.956	1143.075	1221.026	1011.923	1116.707	1176.9	1220.016	1143.075
Replicate 3	1132.547	1071.479	1132.547	1139.234	1128.155	1230.369	1221.3	1182.337	1132.547
Mean	1156.456	1132.3	1156.456	1165.4	1075.887333	1176.696667		1180.135	1156.5
% difference from initial measurement	--	**-2.1**	--	**0.8**	--	**9.4**		--	**-2.0**

Nicotine-N-oxide (NOXT)

Quality material 1

	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	57.553	62.756		57.553	54.846		59.051	57.494	58.177	65.63	57.553
Replicate 2	56.664	56.014		56.664	59.75		61.192	52.418	60.574	62.3	56.664
Replicate 3	57.552	56.197		57.552	55.195		60.277	54.451	59.784	59.599	57.552
Mean	57.25633333	58.32233333		57.25633333	56.6		60.17333333	54.78766667		62.50966667	57.3
% difference from initial measurement	--	**1.9**		--	**-1.2**		--	**-9.0**		--	**-8.4**

Quality material 2

	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	1603.777	1521.734		1603.777	1510.877		1785.294	1578.183	1692.1	1755.556	1603.777
Replicate 2	1574.677	1462.138		1574.677	1628.134		1716.973	1503.485	1692.3	1898.483	1574.677
Replicate 3	1597.41	1498.777		1597.41	1630.574		1858.023	1659.418	1685.8	1788.927	1597.41
Mean	1591.954667	1494.2		1591.954667	1589.9		1786.763333	1580.362		1814.322	1592.0
% difference from initial measurement	--	**-6.1**		--	**-0.1**		--	**-11.6**		--	**-12.3**

Trans-3'-hydroxycotinine (HCTT)

Quality material 1

	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	265.814	274.528		265.814	260.527		272.217	297.03	278.21	279.243	265.814
Replicate 2	281.263	253.417		281.263	261.772		265.432	282.606	272.41	276.211	281.263
Replicate 3	281.182	257.867		281.182	261.989		269.473	289.451	292.08	269.606	281.182
Mean	276.0863333	261.9373333		276.0863333	261.4		269.0406667	289.6956667		275.02	276.1
% difference from initial measurement	--	**-5.1**		--	**-5.3**		--	**7.7**		--	**0.4**

Quality material 2

	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	9716.647	10012.496		9716.647	9560.213		9874.098	10213.795	10317	10253.879	9716.647
Replicate 2	9782.693	9314.649		9782.693	10363.701		9326.009	10293.601	10209	10086.728	9782.693
Replicate 3	9574.001	9689.675		9574.001	9704.242		10487.884	10961.999	10257	10366.643	9574.001
Mean	9691.113667	9672.3		9691.113667	9876.1		9895.997	10489.79833		10235.75	9691.1
% difference from initial measurement	--	**-0.2**		--	**1.9**		--	**6.0**		--	**-5.3**

Norcotinine (NCTT)

Quality material 1

	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	16.25	16.398		16.25	16.09		16.56	18.359	16.662	13.966	16.25
Replicate 2	16.834	16.693		16.834	16.671		16.016	15.606	16.24	15.498	16.834
Replicate 3	18.012	14.548		18.012	15.669		16.521	15.416	15.153	16.088	18.012
Mean	17.032	15.87966667		17.032	16.1		16.36566667	16.46033333		15.184	17.0
% difference from initial measurement	--	**-6.8**		--	**-5.2**		--	**0.6**		--	**12.2**

Quality material 2									
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	276.944	286.655	276.944	278.763	268.63	285.446	290.71	259.434	276.944
Replicate 2	277.941	271.037	277.941	283.695	258.417	274.148	268.87	249.365	277.941
Replicate 3	274.076	271.737	274.076	271.188	276.851	294.097	282.94	276.692	274.076
Mean	276.3203333	276.5	276.3203333	277.9	267.966	284.5636667		261.8303333	276.3
% difference from initial measurement	--	**0.1**	--	**0.6**	--	**6.2**		--	**5.5**

Cotinine (COTT)

Quality material 1									
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	137.394	139.549	137.394	123.591	127.378	131.88		127.648	137.394
Replicate 2	140.809	127.22	140.809	134.784	130.212	128.016		129.728	140.809
Replicate 3	139.984	129.994	139.984	128.366	122.355	126.091		131.567	139.984
Mean	139.3956667	132.2543333	139.3956667	128.9	126.6483333	128.6623333		129.6476667	139.4
% difference from initial measurement	--	**-5.1**	--	**-7.5**	--	**1.6**		--	**7.5**

Quality material 2									
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	4793.151	5235.213	4793.151	4762.736	4571.753	4939.511		4895.164	4793.151
Replicate 2	5036.044	4672.813	5036.044	5024.275	4571.985	4822.317		5177.211	5036.044
Replicate 3	4826.459	4704.874	4826.459	4888.005	4817.172	5035.747		4995.004	4826.459
Mean	4885.218	4871.0	4885.218	4891.7	4653.636667	4932.525		5022.459667	4885.2
% difference from initial measurement	--	**-0.3**	--	**0.1**	--	**6.0**		--	**-2.7**

Nornicotine (NNCT)

Quality material 1									
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	19.531	21.747	19.531	19.406	24.036	25.089	24.176	24.287	19.531
Replicate 2	21.526	18.85	21.526	19.548	24.084	21.011	24.012	23.914	21.526
Replicate 3	22.784	19.904	22.784	18.478	24.71	22.304	23.559	23.668	22.784
Mean	21.28033333	20.167	21.28033333	19.1	24.27666667	22.80133333		23.95633333	21.3
% difference from initial measurement	--	**-5.2**	--	**-10.0**	--	**-6.1**		--	**-11.2**

Quality material 2									
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	215.598	209.713	215.598	203.206	258.852	240.783	249.16	233.409	215.598
Replicate 2	218.907	209.512	218.907	212.413	253.591	234.953	226.92	232.863	218.907
Replicate 3	218.381	206.985	218.381	212.944	271.629	240.005	249.19	234.334	218.381
Mean	217.6286667	208.7	217.6286667	209.5	261.3573333	238.5803333		233.5353333	217.6
% difference from initial measurement	--	**-4.1**	--	**-3.7**	--	**-8.7**		--	**-6.8**

Anatabine (ANTT)									
Quality material 1									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	8.777	9.157	8.777	8.433	9.373	8.813	9.213	9.212	8.777
Replicate 2	9.094	8.418	9.094	8.713	8.23	8.646	9.298	9..391	9.094
Replicate 3	9.965	8.438	9.965	8.385	8.371	8.332	8.583	8.933	9.965
Mean	9.278666667	8.671	9.278666667	8.5	8.658	8.597		9.0725	9.3
% difference from initial measurement	--	**-6.5**	--	**-8.3**	--	**-0.7**		--	**2.3**
Quality material 2									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	87.751	84.337	87.751	79.186	79.468	85.322	77.975	84.509	87.751
Replicate 2	83.78	76.906	83.78	83.179	76.992	79.301	80.997	91.087	83.78
Replicate 3	85.146	81.222	85.146	81.341	82.184	84.077	81.778	89.753	85.146
Mean	85.559	80.8	85.559	81.2	79.548	82.9		88.44966667	85.6
% difference from initial measurement	--	**-5.5**	--	**-5.1**	--	**4.2**		--	**-3.3**

Anabasine (ANBT)									
Quality material 1									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	8.717	7.973	8.717	8.101	7.798	7.934	7.34	7.919	8.717
Replicate 2	9.006	7.906	9.006	7.562	7.264	7.205	7.013	8.037	9.006
Replicate 3	8.876	7.779	8.876	7.692	7.572	6.929	7.408	8.1	8.876
Mean	8.866333333	7.886	8.866333333	7.8	7.544666667	7.356		8.018666667	8.9
% difference from initial measurement	--	**-11.1**	--	**-12.2**	--	**-2.5**		--	**10.6**
Quality material 2									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	75.261	69.956	75.261	67.734	74.388	72.259	72.242	74.977	75.261
Replicate 2	70.178	67.077	70.178	72.925	70.957	72.014	69.811	79.823	70.178
Replicate 3	71.305	72.137	71.305	70.713	79.865	78.496	74.781	71.507	71.305
Mean	72.248	69.7	72.248	70.5	75.07	74.25633333		75.43566667	72.2
% difference from initial measurement	--	**-3.5**	--	**-2.5**	--	**-1.1**		--	**-4.2**

Nicotine (NICT)									
Quality material 1									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	63.641	71.842	63.641	68.523	57.546	64.054	64.106	64.268	63.641
Replicate 2	75.859	68.757	75.859	74.558	58.202	62.723	61.228	63.826	75.859
Replicate 3	78.345	63.003	78.345	70.653	72.449	63.5	60.885	67.131	78.345
Mean	72.615	67.86733333	72.615	71.2	62.73233333	63.42566667		65.075	72.6
% difference from initial measurement	--	**-6.5**	--	**-1.9**	--	**1.1**		--	**11.6**

Quality material 2										
	Initial measurement	Three freeze-thaw cycles		Initial measurement	Bench-top stability		Initial measurement	Processed sample stability		Initial measurement	Long-term stability
Replicate 1	2812.105	2851.806		2812.105	2709.061		2886.625	2891.977	2902.1	2932.093	2812.105
Replicate 2	2700.772	2606.684		2700.772	2866.437		2820.1	2752.996	2768.8	2928.577	2700.772
Replicate 3	2682.743	2633.323		2682.743	2732.311		3008.396	2917.733	3052.3	2891.672	2682.743
Mean	2731.873333	2697.3		2731.873333	2769.3		2905.040333	2854.235333		2917.447333	2731.9
% difference from initial measurement	--	**-1.3**		--	**1.4**		--	**-1.7**		--	**-6.4**