Division of Laboratory Sciences Laboratory Protocol

Analyte: Nicotine, Cotinine, *Trans*-3'-hydroxycotinine, Nornicotine, Nicotine N'-oxide, Cotinine N-oxide, 4-Hydroxy-4-(3-pyridyl)-butanoic acid, Anatabine, Anabasine

Matrix: **Urine**

Method: **LC/MS/MS**

Method No: **2021.04**

Revised: **July 31, 2020**

as performed by:

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

contact:

Dr. June Feng
Phone: 770-488-7980
Fax: 770-488-7518
Email: czf2@cdc.gov

Dr. James Pirkle, MD, PhD
Director, Division of Laboratory Sciences

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

Public Release Data Set Information

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

Data File Name	Variable Name	SAS Label
UCOT_J	URXANBT	Anabasine, urine (ng/mL)
	URXANTT	Anatabine, urine (ng/mL)
	URXCOXT	Cotinine-n-oxide, urine (ng/mL)
	URXHPBT	4-Hydroxy-4-(3-pyridyl) C4H802 (ng/mL)
	URXNICT	Nicotine, urine (ng/mL)
	URXNNCT	Nornicotine, urine (ng/mL)
	URXNOXT	Nicotine 1’ -Oxide, urine (ng/mL)
	URDTNE2	TNE - 2 (nmol/mL)
	URDTNE3	TNE - 3 (nmol/mL)
	URDTNE6	TNE - 6 (nmol/mL)
	URDTNE7	TNE - 7 (nmol/mL)

1. Clinical Relevance and Summary of Test Principle

List of analytes in this method:

4-Hydroxy-4-(3-pyridyl)-butanoic acid;
CAS#:15569-97-8; $C_9H_{11}NO_3$; Mol. Wt: 181.1885; m.p. 78-88°C.

(S)-Cotinine N-oxide;
CAS#: 36508-80-2; $C_{10}H_{12}N_2O_2$; Mol. Wt: 192.2145; m.p.: 116-117°C.

(1'S,2'S)-Nicotine N'-oxide;
CAS#: 51095-86-4; $C_{10}H_{14}N_2O$; Mol. Wt: 178.2310; M.P.: 168-170°C.

(-)-*Trans*-3'-Hydroxycotinine;
CAS#: 34834-67-8; $C_{10}H_{12}N_2O_2$; Mol. Wt: 192.2145; m.p. 107-109°C.

(-)-Cotinine;
CAS#: 486-56-6; $C_{10}H_{12}N_2O$; Mol. Wt.: 176.2151; m.p.: 35-37°C; b.p.: 145-150°C/3 mm.

(R,S)-Nornicotine;
CAS#: 5746-86-1; $C_9H_{12}N_2$; Mol. Wt.: 148.2050; b.p.: 108-110°C/0.5mm.

(R,S)-Anatabine;
CAS#: 2743-90-0; $C_{10}H_{12}N_2$; Mol. Wt.: 160.2157; b.p.: 136°C.

(R,S)-Anabasine;
CAS#: 13078-04-1; $C_{10}H_{14}N_2$; Mol. Wt.: 162.2316; b.p.: 270-272°C

(-)-Nicotine;
CAS#: 54-11-5; $C_{10}H_{14}N_2$; Mol. Wt.: 162.2316; 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). *Trans*-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 4-Hydroxy-4-(3-pyridyl)-butanoic acid), is of interest when elucidating differences in metabolic profiles of various ethnic, age, and gender groups {James E.

McGuffey, 2013 #52; Rangiah, 2011 #1422}. 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 nitrosoamines, 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, (4-Hydroxy-4-(3-pyridyl)-butanoic acid (HPBT), cotinine N-oxide (COXT), nicotine N'-oxide (NOXT), *trans*-3'-hydroxycotinine (HCTT), 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 HPBT-D3, methyl-D3-COXT, methyl-D3-NOXT, methyl-D3-HCTT, 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, Indigo Ascent data

processing, and Microsoft Access data review is required prior to being 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 store 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

For the sample preparation step of the analysis, proficiency in iLink and Maestro (current version 6.0) automation software package is required for all users. Proficiency in the analytical software package of the HPLC and mass spectrometer used in the analysis is required for all analytical instrument users. AB Sciex Analyst software (current version 1.6.2) is used for data acquisition on the AB Sciex triple quadrupole 6500 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 equivalent statistical software package.

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 factor, 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. Raw data and processed data file are automatically backed-up on the CDC network on a routine basis via ISLE(e.g. weekly).

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, access authorization, and physical location access. 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 (or equivalent vials) 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	LC/MS	Fisher Scientific (Suwanee, GA, USA)	A955-4
methanol	LC/MS	Fisher Scientific (Suwanee, GA, USA)	A456-4
water	LC/MS	Fisher Scientific (Suwanee, GA, USA)	W6-4
isopropanol	LC/MS	Fisher Scientific (Suwanee, GA, USA)	A461-4
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 LC/MS-grade 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 LC/MS-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, 10 mL of 650mM stock ammonium acetate solution is added to 990 mL of LC/MS-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% NH_3). 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 LC/MS-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) Needle wash solution

Approximately 2 L of a mixture of 2-propanol, acetonitrile, and water in 4:3:3 volume ratio with 0.3% ammonium hydroxide 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 #
(+/-)-4-hydroxy-4-(3-pyridyl)butanoic acid	98%	^{1}H NMR	P992550
(+/-)-4-hydroxy-4-(3-pyridyl)butanoic acid-D3	98%	^{1}H NMR	H952972
(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 N'-oxide	98%	^{1}H NMR	N427500
(+/-)-*trans*-nicotine N'-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
(-)-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 gravimetric determination with consideration of the compound purity on the analyte label provided by the manufacturer. The final concentration of each analyte prepared in our latest preparation is listed in Table 6.3.2.1. (+/-)-4-hydroxy-4-(3-pyridyl)butanoic acid and its labeled internal standards are obtained as salt form. When the following stocks are prepared, the concentrations listed reflect that of the pure analyte of interest.

Table 6.3.2.1. Analyte concentration in original stock

Analyte name	Analyte code	Amout weighed (mg)	Purity	Conc. (µg/mL)
(+/-)-4-hydroxy-4-(3-pyridyl)butanoic acid	HPBT	27.28	0.98	244.372
(S)-cotinine N-oxide	COXT	24.81	0.98	243.1707
(1'*S*,2'*S*)-nicotine N'-oxide	NOXT	99.87	0.98	978.7378
trans-3'-hydroxy-cotinine	HCTT	75.38	0.98	738.695
(-)-cotinine	COTT	118.55	0.98	1161.757
R,S-nornicotine	NNCT	53.83	0.98	527.534
RS-anatabine	ANTT	8.29	0.98	81.2028
(*R*,*S*)-anabasine	ANBT	11.09	0.98	108.633
(-)-nicotine	NICT	102.88	0.99	1018.545

(2) Isotopically-labeled Internal Standard Stock Solutions

Each original isotopically-labeled stock solutions are prepared by gravimetric determination with consideration of chemical purity of the analyte. Exact amount of chemical was purchased and used based on the quantity indicated by the manufacturer.

The individually prepared internal standard stock solutions are combined in appropriate volumes and diluted with LC/MS-grade water into a solution mixture of working concentrations. An example for the final concentration for each labeled analyte in the mixture is listed in Table 6.3.2.3.

Table 6.3.2.3. Concentration of individual labeled analyte in the working internal standard solution mixture

ISTD	Analyte Code	Original Stock of individual internal standard solution, µg/mL	Working concentration of internal standard mixture, ng/mL
HPBT-D3	HPBT-ISTD	189.391	1326
COXT-D3	COXT-ISTD	142.619	214
NOXT-D3	NOXT-ISTD	95.080	95
HCTT-D3	HCTT-ISTD	92.169	461
COTT-^{13}CD3	COTT-ISTD	95.656	96
NNCT-D4	NNCT-ISTD	47.540	238
ANTT-D4	ANTT-ISTD	91.728	92
ANBT-D4	ANBT-ISTD	46.085	115
NICT-^{13}CD3	NICT-ISTD	187.96	1880

6.3.3. Preparation of Calibration Standard Solutions

(1) Blank Urine Pool

Collect urine samples from non-users of tobacco product in 120 mL sterile specimen containers and store them at or below -20°C (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 4°C overnight.

(2) Concentration of standard solutions

Thirteen standard solutions (SL01–SL13) are prepared in pooled blank urine by diluting a desired amount of the native stock solution, to a final volume of 500 mL (100 mL for SL13) in a volumetric flask. The analyte concentrations at each level are listed in Table 6.3.3.1.

Twelve standards were used to build a calibration curve when “total” form of the analytes were measured (excluding SL12). When “free” form of these analytes were measured, ten standards (excluding SL05, SL09, and SL12) were used to build a calibration curve.

Table 6.3.3.1. Calibration standard levels and analyte concentrations at each level (ng/mL)

Levels	HPBT	COXT	NOXT	HCTT	COTT	NNCT	ANTT	ANBT	NICT
SL01	1.00	1.50	2.00	3.00	1.50	0.00	0.25	0.20	0
SL02	1.98	2.97	3.96	5.94	2.97	1.49	0.50	0.40	3.03
SL03	3.94	5.91	7.87	11.81	5.91	2.36	0.98	0.79	8.52
SL04	14.00	12.00	14.00	80.00	50.00	2.40	1.00	0.80	40.00
SL05	35.00	30.00	35.00	200.00	125.01	6.00	2.50	2.00	100.00
SL06	70.00	60.00	70.00	400.01	250.01	12.00	5.00	4.00	200.01
SL07	140.01	120.01	140.00	800.01	500.02	24.00	10.00	8.00	400.02
SL08	350.02	300.01	350.01	2000.03	1250.05	60.01	25.01	20.01	1000.05
SL09	875.05	750.04	875.03	5000.08	3125.13	150.03	62.53	50.02	2500.12
SL10	1750.09	1500.07	1750.06	10000.16	6250.25	300.06	125.05	100.04	5000.24
SL11	3500.19	3000.14	3500.12	20000.32	12500.51	600.12	250.10	200.08	10000.48
SL12	6000.31	5000.075	6001.608	55000.29	25001.02	1200.245	800.0506	800.0386	20000.15
SL13	2400.12	2000.03	2400.64	22000.12	10000.4	480.98	320.02	320.02	8000.06

*Note: SL01-SL12 were prepared during the original development of the assay. Later it was realized that the high concentration of SL12 reached instrument saturation of the mass spectrometer, thus SL13 was subsequently prepared via dilution of SL12 in blank urine.

6.4. Quality-Control (QC) Materials

6.4.1. Preparation of Blank Urine Pool

Preparation of blank urine pool is described in Section 6.3.3.

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 tobacco 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 pool 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. Concentration of these two pools are described in Table 6.3.3.1.

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.

- HPLC: Shimadzu modular system, consisting of a DGU-20A5R degasser, two LC-30AD pumps, one SIL-30ACMP autosampler, one CTO-20AC column oven, and a CBM20A controller (Shimadzu Corp, Columbia, MD).
- Mass spectrometer: AB Sciex 6500 triple quadrupole mass spectrometer with a TurboIonSpray source (ABSciex, Foster City, CA).
- Nitrogen gas generator: MS-Table-2N 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 turn table, a 1D barcode reader, and a 2D barcode reader (FluidX).
- 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.
- Repeat Manager 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)
- Tissue Culture Rotator (Glas –Col, Terre Haute, IN)
- Repeater E3x repeat pipette (Pipettes.com)

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)
- 1, 2.5, 5, 10, 20 mL pipette tips for Repeater E3x pipette

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, prepared in a 96-well plate. Sample plates naming nomenclature and preparation steps are described further in subsequent sections 8.1 and 8.2.,

8.1. Assay Run Nomenclature

Each sample plate prepared for an analytical run is assigned a run ID, in the following format: AAAABBB_(X_Rx)_CmmddyyyyD, where:

- **AAAA** is the four digits plate number (e.g. 1251)
- **BBB** is the dataset ID, it can be one to three letters or numbers (e.g. P4A). When samples from two dataset were included on the same plate, they were separated by an underscore "_" (e.g. P3B_P3C)
- **X** denotes repeat preparation of the same plate, **and Rx** denotes reinjection of the same plate ID, these two items are optional to a plate
- **C** denotes the one letter instrument ID (e.g. Letter "A" for SashaJr, "C" for Galle, "D" for Zeus)
- **mmddyyyy** reflects instrument analysis date (e.g. 10222018)
- **D** denotes the one letter analyst ID (e.g. P for Patrick)

In the above example listed in brackets, the run ID of the plate would be "1251P4A_2_R1_D10222018P". This indicates a plate number 1251 for PATH W4 G2 (P4A) sample set. The plate is a re-preparation of the original 1251 plate (The original 1251 plate would have identical sample/standards layout as this re-preparation). It is a reinjection of the plate on Zeus. The plate was run on October 22, 2018 by Analyst "P".

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.

8.2. Sample Preparation

8.2.1. Sample Tracking

Prior to aliquotting, each sample is placed on the automation rack to be read by a scanner. Any samples missed by the initial scan will require manual input of the sample barcode into the system. As the samples are being aliquoted to the 96-well plate, the list of sample barcodes, their rack location, and the specific wells they were aliquoted into on the 96-well plate are being automatically generated. The generated list is then renamed after the specific run ID and stored in the directory specified by the user at the start of the method for later use.

8.2.2. Sample Organization and Preparation

Samples, including unknown urine samples, QCs, control blanks and standards are prepared in the same manner using the automated sample preparation system. These samples are either aliquoted on the Caliper Staccato System or the Hamilton Star System, depending on the dimensions of the vials they are originally shipped from (Either 2-mL Nalgene cryo vials or FluidX vials). Initially, all samples are set on bench in order to equilibrate to room temperature from their original storage conditions. If necessary, thawing can be sped up for samples stored in freezers via incubation in water bath at 37 °C for 1 hour. After complete thawing to liquid form, all samples are placed on an end-over-end rotator for 30 min to ensure thorough mixing of solutions prior to aliquoting.
Subsequently, the mixed samples are moved to racks for the automation system, where 50 µL of internal standard solution (ISTD) and 100 µL of each of the mixed samples are placed on individual wells of a 96-well plate. Solution in each well is aspirated and dispensed repeatedly by the system, then 60 µL of enzyme solution (1000 units of beta glucuronidase, *Helix pomatia*. See Section 6.2.5) is added to each well. **For the "free" analysis, 60 µL of water is added instead.** The well plate is then sealed and incubated for 12 hours at 45 °C with continuous agitation. **For the "free" analysis, this step is skipped.**

After the incubation step, the plate is centrifuged for 2 min at 1651 rpm to spin samples down to the bottom of the wells. Next, the foil seal of the well plate is pierced, and 450 µL of cold acetone (-20 °C) is added to each well. The acetone is mixed into each well via continuous aspiration/dispensation with pipette tips by the system, afterwards the plate is re-sealed again and incubated in the centrifuge at -20 °C for 30 min.

For the final steps, after the low temperature incubation, the plate seal is pierced and 180 µL of supernatant from each well is transferred to a brand new 96-well injection plate. This

injection plate is then placed inside a Turbovap for 12 min (30 °C, N_2 flow rate 35 Fh). Finally, 250 µL of water is added to each well and the injection plate is sealed and ready to be placed in the LC/MS instrument autosampler for analysis.

8.2.3. Preparation run time

Total sample preparation run time is approximately 14.5 hours for one plate. Finished plates are sealed and placed in Rack 3. After samples are finished, pass the sample plate along with the plate layout to the 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.

8.2.4. Maintenance and calibration of Caliper and Hamilton Star System

(1) Daily maintenance
   a. Daily maintenance includes confirming each component to be functional, checking available supplies, and cleaning up after the run is finished.
   b. Main array on Sciclone deck: the O rings on the main array should be lubricated with a grease plate (provided by Caliper) daily.

(2) Monthly maintenance and calibration
   a. Volume verification should be conducted on a monthly basis for both Caliper and Hamilton Star systems.
   b. 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 6500 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, 110 Å, 2.0 x 100 mm, 3.0-µm particle size from Phenomenex (# 00D-4453-B0), or Gemini-NX, C18, 3.0 x 100 mm, 3.0-µm particle size from Phenomenex (# 00D-4453-YO), or equivalent
(2) Two pre-column filters: 1st an A-100 (2.0 µm) SS frit, and the 2nd an A-103 (0.5 µm) SS frit (IDEX Health & Science, or their equivalents).

(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	0.30 min
Rinse Sequence	R2→R0
Rinsing Volume R0,R1,R2	300 µL
Injection Port Rinsing	R2
Equilibration Start Time	6.40 min
Equilibration Hold Time	0.60 min
R0,R1,R2 Purge Time	10.0 min

(5) Running gradient on the Shimadzu HPLC system

Time	Module	Event	Percentage of buffer B, (%)
0.50	Pumps	%B	0
2.20	Pumps	%B	4
2.30	Pumps	%B	6
2.90	Pumps	%B	6
3.00	Pumps	%B	15
3.50	Pumps	%B	15
4.70	Pumps	%B	18
4.80	Pumps	%B	22
5.40	Pumps	%B	22
5.50	Pumps	%B	60
6.00	Pumps	%B	60
6.05	Pumps	%B	0
7.00	Controller	Stop	

8.3.2. Mass Spectrometry Conditions and Settings

(1) Typical AB-Sciex Triple quadruple 6500 Mass Spectrometer Settings:

Instrument C (Galle SL)

Ion source	Turbo spray
SC type	Scheduled MRM
Polarity	Positive
Curtain Gas (CUR)	40
Collision Gas (CAD)	8
IonSpray Voltage (IS)	2500
Temperature (TEM)	650
Ion Source Gas 1 (GS1)	70
Ion Source Gas 2 (GS2)	70

Instrument D (Zeus)

Ion source	Turbo spray
SC type	Scheduled MRM
Polarity	Positive
Curtain Gas (CUR)	40
Collision Gas (CAD)	9
IonSpray Voltage (IS)	3500
Temperature (TEM)	600
Ion Source Gas 1 (GS1)	45
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.

Instrument C (Galle SL)

	Precursor Ion	Daughter Ion (quant/confirm)	CXP (V)	EP (V)	DP (V)	CE (V) (quant/confirm)
HPBT	182.0	108.1/80.0	14	10	50	60/64
HPBT-D3	185.0	109.0	14	10	50	43
COXT	193.1	96.0/79.0	15	10	49	30/50
COXT-D3	196.2	96.0	11	10	49	30
NOXT	179.2	130.0/117.0	17	10	35	45/38
NOXT-D3	182.1	130.0	17	10	33	30
HCTT	193.1	80.0/134.0	4	10	55	72/25
HCTT-D3	196.1	80.0	4	10	55	34
COTT	177.1	80.0/98.0	10	10	65	68/65
COTT-^{13}CD3	181.2	80.0	10	10	65	32
NNCT	149.2	80.0/130.0	10	10	26	29/24
NNCT-D4	153.2	84.0	10	10	26	29
ANTT	161.1	144.0/80.0	18	10	23	20/40
ANTT-D4	165.1	148.0	18	10	23	20
ANBT	163.1	80.0/120.0	14	10	30	30/22
ANBT-D4	167.2	84.0	14	10	30	30
NICT	163.1	130.1/117.1	12	10	35	27/33
NICT-^{13}CD3	167.1	130.0	12	10	35	27

Instrument D (Zeus)

	Precursor Ion	Daughter Ion (quant/confirm)	CXP (V)	EP (V)	DP (V)	CE (V) (quant/confirm)
HPBT	182.1	108.0/80.0	5	10	50	65/66
HPBT-D3	185.1	109.0	9	10	50	47
COXT	193.3	96.0/79.0	6	10	43	29/54
COXT-D3	196.1	96.0	11	10	43	31
NOXT	179.1	130.2/117.1	7	10	40	30/40
NOXT-D3	182.1	130.1	8	10	40	36
HCTT	193.2	80.0/134.1	9	10	60	37/27
HCTT-D3	196.2	79.9	9	10	60	40
COTT	177.0	80.0/98.0	13	10	60	53/28
COTT-^{13}CD3	181.0	79.9	9	10	60	35
NNCT	149.5	80.1/130.0	10	10	40	27/25
NNCT-D4	153.5	84.0	9	10	40	29
ANTT	161.3	144.1/80.0	7	10	30	21/39
ANTT-D4	165.3	148.2	8	10	30	21
ANBT	163.3	80.0/120.0	9	9	40	30/23
ANBT-D4	167.2	84.1	10	9	40	33
NICT	163.3	130.0/117.2	11	10	40	28/35
NICT-^{13}CD3	167.3	130.1	10	10	40	28

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

8.3.3. LC/MS/MS Maintenance

(4) 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. Preventive maintenance (PM) on Liquid Chromatography (LC) system is performed according to manufacturer's guidance. Normally, the PM is conducted 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 6500 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 shifts 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 swab. 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.

(4) This procedure should be done once every month.

c) Procedure for changing HPLC column and pre-column filters

(1) The HPLC column is replaced after every 800 injections, or earlier as necessary when analyst observes broadening peaks, high back pressure, low response, or distorted peak shapes. The pre-column filters are replaced every 400 injections and 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.

(2) Prior to changing filters and column, deactivate the system profile from the Analyst software, then turn off controller (CBM) and oven of the HPLC system. Make sure the oven is completely cooled to room temperature before replacing the column and pre-column filters housings.

(3) Use appropriate sized wrenches to dissemble the column and pre-column filters. Pay attention to the flow direction (examine the arrow indicators on column and the filter housings) to ensure correct flow direction. Also replace any metal capillaries that appears to be clogged or kinked.

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 latter, 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 600 mL of buffer and 200 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:

- Vacuum readings after starting gas flow. Be sure the readings have stabilized before recording them.
- The three pressure gauges on the Peak gas generator.
- HPLC Pump pressures readings after equilibrium (normally from 2500 to 3500 psi).
- 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 method will allow the instrument to warm up, reach a stable back pressure, and equilibrate the column from the 50/50 Acetonitrile/Water 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_04 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_04 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 15000 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).

Overall, a typical HPLC-MS/MS running sequence is displayed as follows:

Blank_01
Standards 01-13 (no 12)
Blank_02
Blank_03
QC-LOW
1st half unknown Samples (36 samples)
Blank_04
2nd half (remaining) unknown Samples
Blank_05
QC-HIGH

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

(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 are automatically backed up on the CDC network.

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” button and notify data certifier 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 a designated folder on the CDC network. 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 Section 6.3.4.

(2) Blank correction for unknown samples for NNCT and NICT 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 77 total unknowns on any repeat plate.

For example, if 500 sample-analyte combinations require repeating, stage 72 (3 racks of samples) 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 72 unknowns.

Likewise, the analyst can stage an additional 77 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.

(5) 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.

(6) 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.

(7) 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 results to “Send.” Repeat this process until the Review Repeats tab is empty.

(8) 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. Reportable Range of Results

9.1. 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 9.2). 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 $2x10^8$ cps require a rerun for that analyte, following the addition of 0.5 mL LC/MS-grade water into the pre-treated solutions.

9.2. 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 concentration (Taylor, 1987; ICH, 1994; DNR, 1996). Estimated LODs are as follows:

Analyte	Estimated LOQ (ng/mL)
HPBT	1.38
COXT	2.02
NOXT	2.50
HCTT	3.00
COTT	1.50
NNCT	2.50
ANTT	0.39
ANBT	0.51
NICT	10.5

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 12 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 15000 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 $2x10^8$ cps, a suitable volume of LC/MS-grade 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. QC limits can be updated periodically or as needed, however they should not deviate from the original values significantly (±15%).

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

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 $1.3x10^5$ 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 occur 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 peak selection and integration, resulting in variations of measured 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 $2x10^8$, 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 peak broadening.

(3) 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. No known interferences have yet been reported for analytes listed in 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
4-hydroxy-4-(3-pyridyl)-butanoic acid	HPBT	ng/mL	21-2500	10
trans-3'-Hydroxycotinine	HCTT	ng/mL	50-32700	6, 9
Nicotine	NICT	ng/mL	16-10100	5, 6, 9
Nornicotine	NNCT	ng/mL	4.4 -616	5
Nicotine N'-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 -60°C 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. 4°C). 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 -20°C for short-term use (within one week), and at or below -60°C 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 -20°C 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 -60°C 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. Once the StarLIMS extract file is generated after data review and evaluation, the laboratory QC officer evaluates the results based on the division QA/AC requirements. 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. Method Performance Documentation

19.1. Accuracy

Accuracy of the method was determined by two ways: Analyses of certified reference materials by the test method, and recovery analysis of the test method.

19.1.1. Accuracy Assessment using Certified Reference Materials

Certified reference materials of each analyte were used in this analysis. Three concentrations of each analyte in the reportable range of the method were measured. For each concentration, two measurements were made in five different analytical runs for a total of ten measurements for each of the concentrations. The five runs were done on five different days. The deviation of the mean from the nominal value was used to assess accuracy. As shown in Appendix 3 A3a, the mean value of the measurements from all analytes were determined to be within ±15% of the nominal value.

19.1.2. Recovery Analysis of the Test Method

For this analysis, two urine pools (Sample 1 and Sample 2) were selected. Aliquots of these pools were left unspiked (zero concentration) and spiked with three concentration levels of each analyte within the reportable range of the method. For each of these concentrations, spiking is done in triplicates, resulting in a total of 12 samples for each urine pool. The 12 samples were analyzed in two analytical runs on two separate days resulting in a total of 24 results for each of Samples 1 and 2 urine pool, as shown in the tables listed in Appendix 3 A3b.

19.2. Precision

Precision of the method was evaluated by using two concentrations of QC reference materials (QCH and QCL). For each QC material, 20 measurements were made in ten different analytical runs (Duplicate analyses per run) in order to calculate within-run, between-run, and total precision. As shown in the tables listed in Appendix 4, all analytes tested in the method have high precision that deviates well within the ±15% relative standard deviation limit set by the division.

19.3. 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 data from all standards above three times the detection limit of each analyte. This ion ratio was examined for results from 50 persons' urine sample to establish the appropriate range and ensure absence of any interfering substance. When

testing unknown samples with this method, the sample is repeated when it does not meet the confirmation ratio limits. The result is evaluated for its validity on a case by case basis.

19.4. Stability

Stability tests were performed as listed in Appendix 5. Two spiked urine pools, quality material 1 (QCL) and quality material 2 (QCH) were analyzed in triplicates to obtain initial measurements. Bench-top stability was performed by storing samples at room temperature for 1 day prior to analysis. Processed samples stability was conducted by treating samples through the sample preparation steps (i.e. samples processed and placed in 96-well plates ready for instrument analysis) and stored at 4 °C (mimicking autosampler storage temperature) for 1 day prior to analysis. For long-term stability, the samples were stored at -80 °C for 2 years prior to analysis. As listed in the results tables, all final measurements deviate within 15% from the initial measurements.

19.5. 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. 19.1).

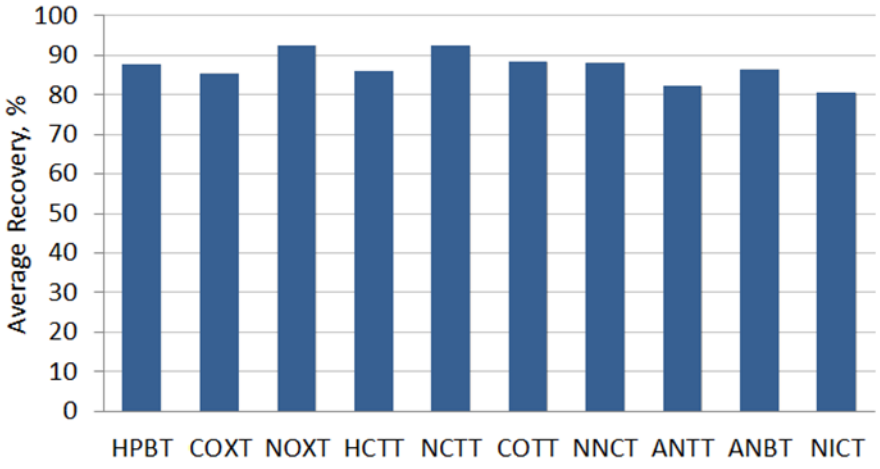


Figure 19.1. Sample preparation recovery

19.6. Carryover

To investigate the carry-over, a water blank was injected before and after a high calibration standard. Data from two LC/API6500 MS systems were compiled for each analyte totaling

50 values for each analyte. The carry-over for each run was determined by comparing the peak area difference between the two water blanks (Water blank after standard injection minus that before) with the peak area of the standard. The carry-over for each analyte on each instrument is summarized in Table 19.1 below. The “Max” column below listed the higher level of carryover and were used for instrument carryover threshold setting in Indigo data processing software.

Table 19.1. Carryover by instrument and used for method (max carryover)

Compound	Zeus	Galle	Max
HPBT	3.97E-06	1.64E-06	3.97E-06
COXT	0.00E+00	9.98E-07	9.98E-07
NOXT	4.51E-07	1.67E-06	1.67E-06
HCTT	0.00E+00	2.39E-06	2.39E-06
COTT	6.59E-05	9.27E-05	9.27E-05
NNCT	2.21E-03	2.10E-03	2.10E-03
ANTT	7.54E-04	6.48E-04	7.54E-04
ANBT	1.86E-03	1.21E-03	1.86E-03
NICT	3.43E-04	3.09E-04	3.43E-04

19.7. 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.

19.7.1. 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. 19.7.1a).

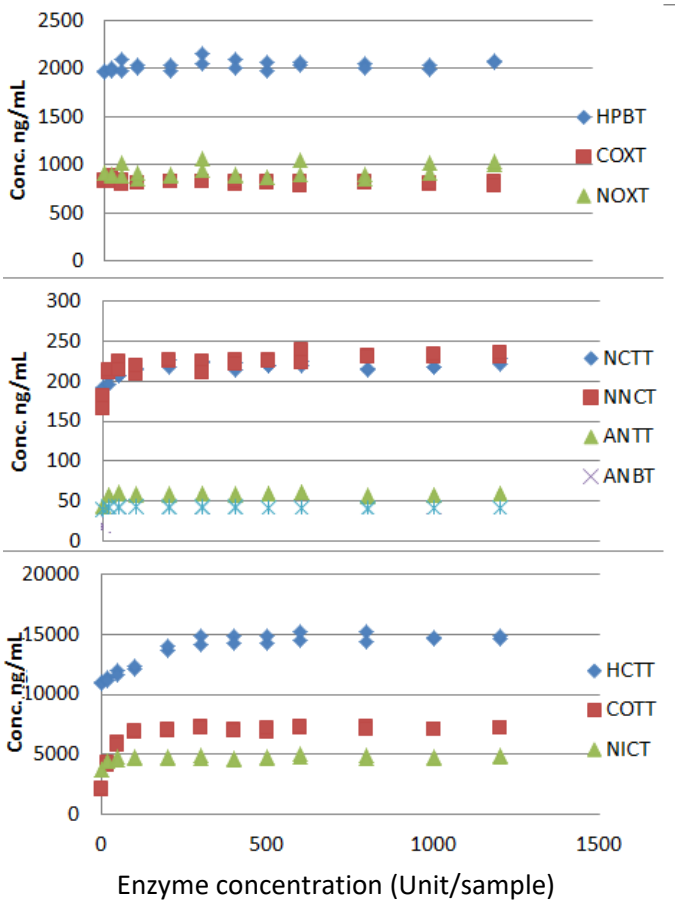


Figure 19.7.1a. Glucuronide forms in a pooled smoker urine sample

The glucuronide forms for other analytes, including cotinine N-oxide, nicotine N'-oxide, nornicotine, anatabine, and anabasine are very low and are not affected significantly by enzyme concentration used in the method. This is shown in Fig. 19.7.2a-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 19.7.1b).

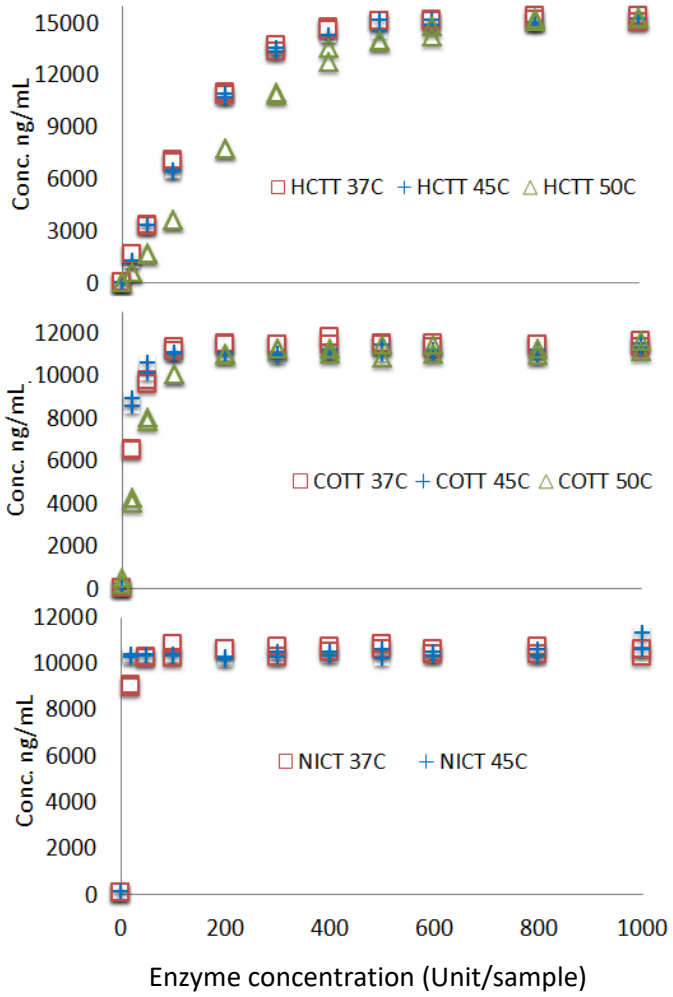


Figure 19.7.1b. Performance of *H. pomatia* by varying amount per sample at different temperatures

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 (45°C) for *H. pomatia* is relatively 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.

19.7.2. 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. 19.7.2a-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.

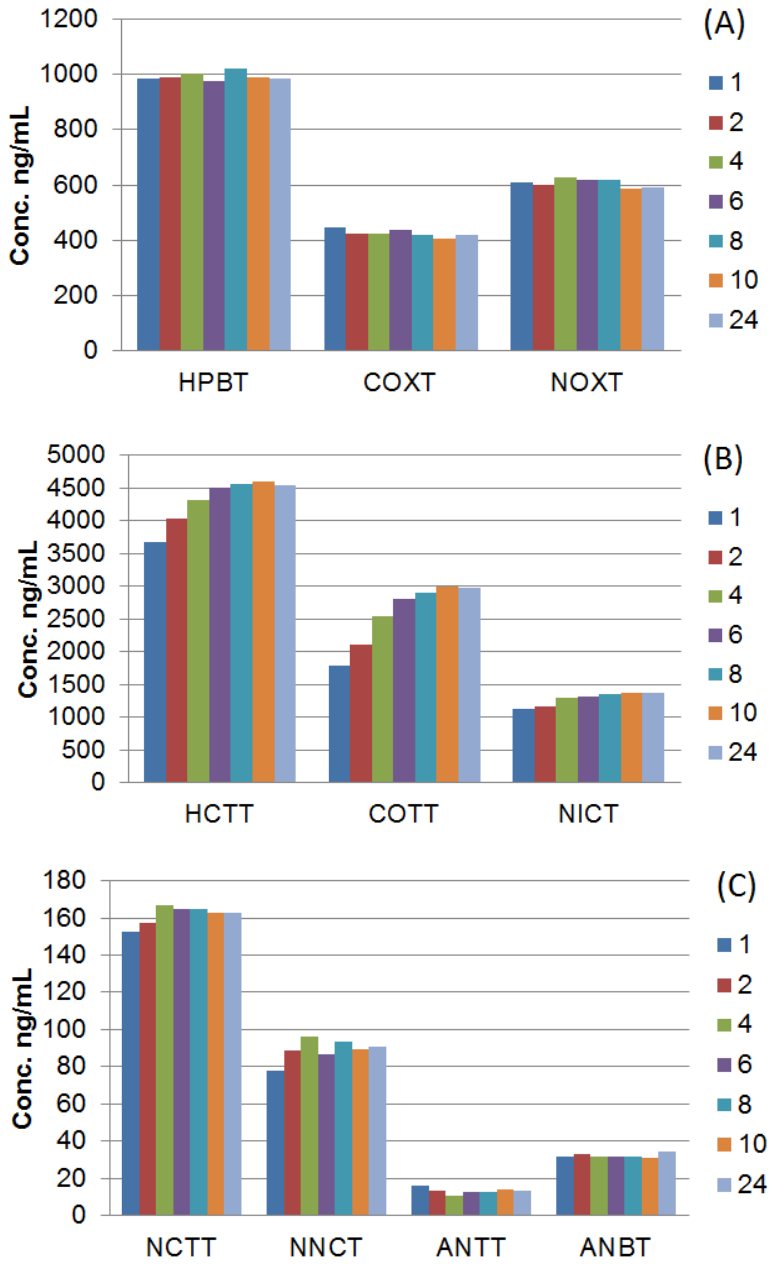


Figure 19.7.2a-c. The effect of hydrolysis time on analyte concentration

19.7.3. 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×2.0mm, 3.0um) was selected, and the gradient was adjusted to obtain a reasonable run time (7.0min 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 19.7.3.

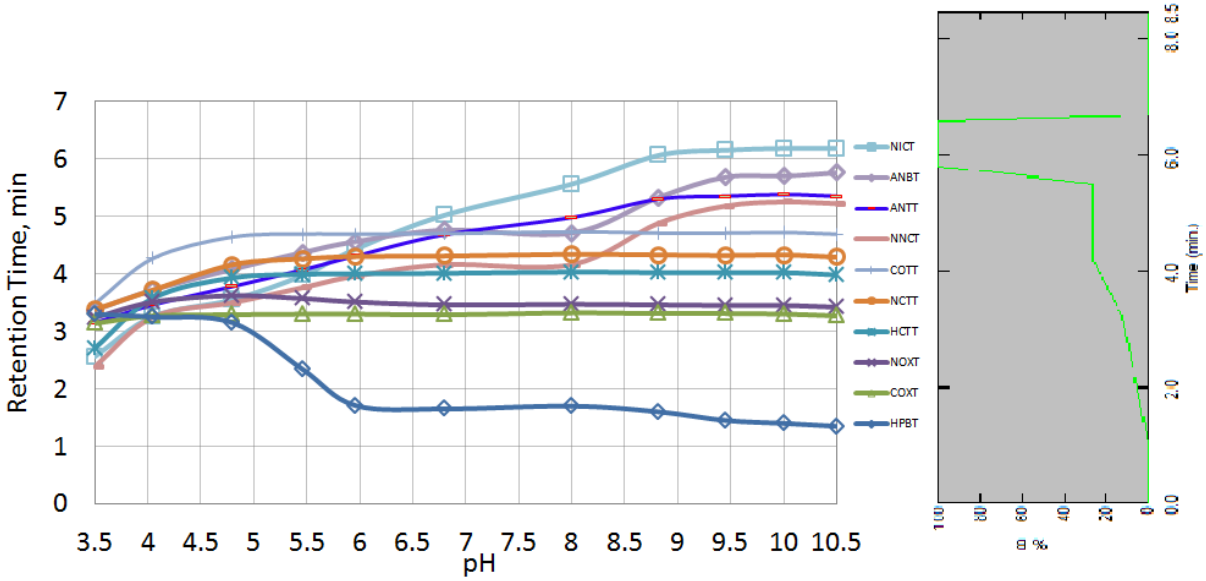


Figure 19.7.3. The effect of buffer pH on retention time.

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 ammonium hydroxide, was utilized in this method.

19.7.4. 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 6). No significant differences were observed for the analyte concentrations among these three groups.

19.7.5. Freeze-Thaw Cycle

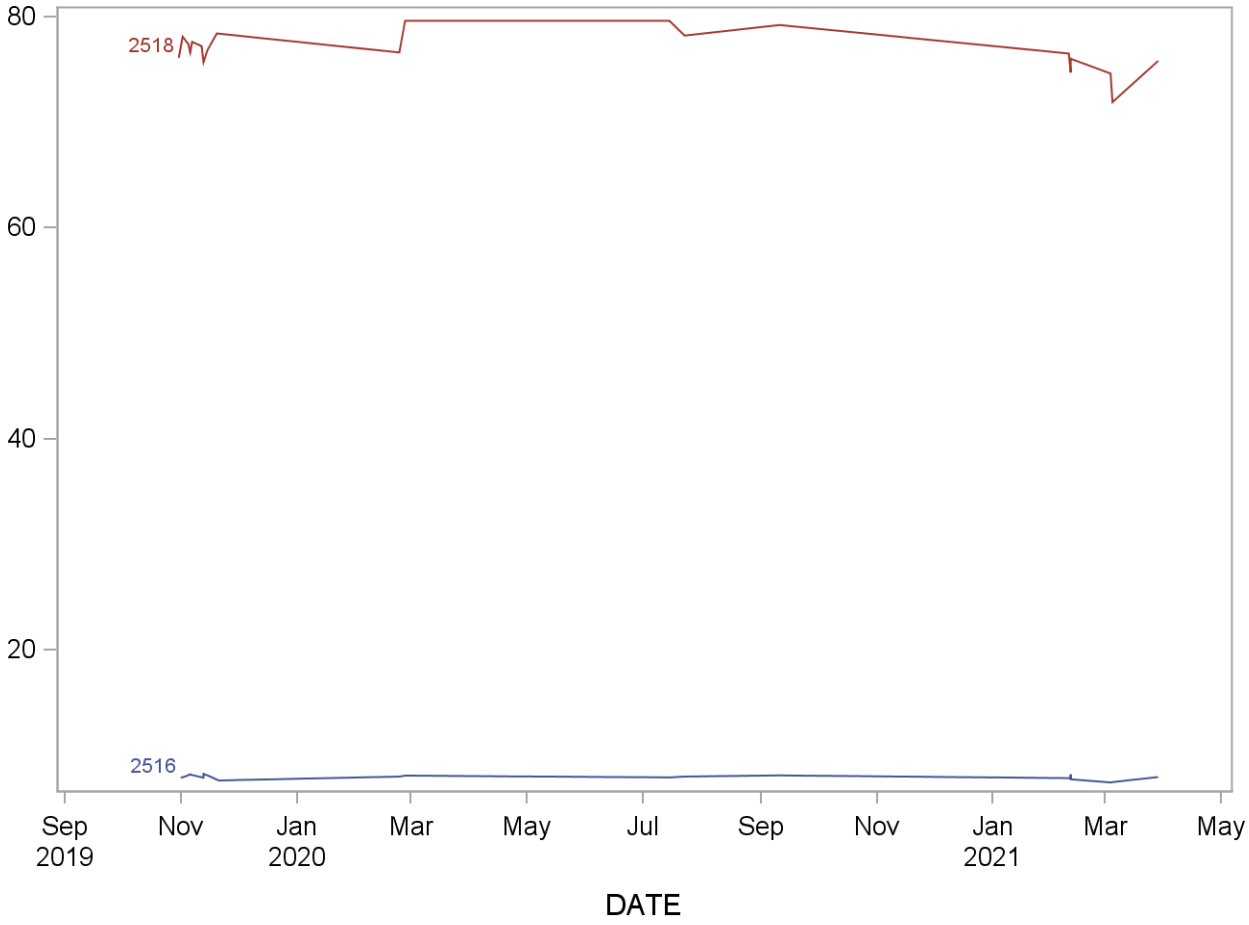
A Freeze-Thaw test was performed to test whether this process will cause any potential variations on analyte concentrations using two prepared pools over 25 cycles. The results indicated no significant effect on the calculated concentration values was detected (Appendix 7).

20. Summary Statistics and QC Graphs

Please see follow page.

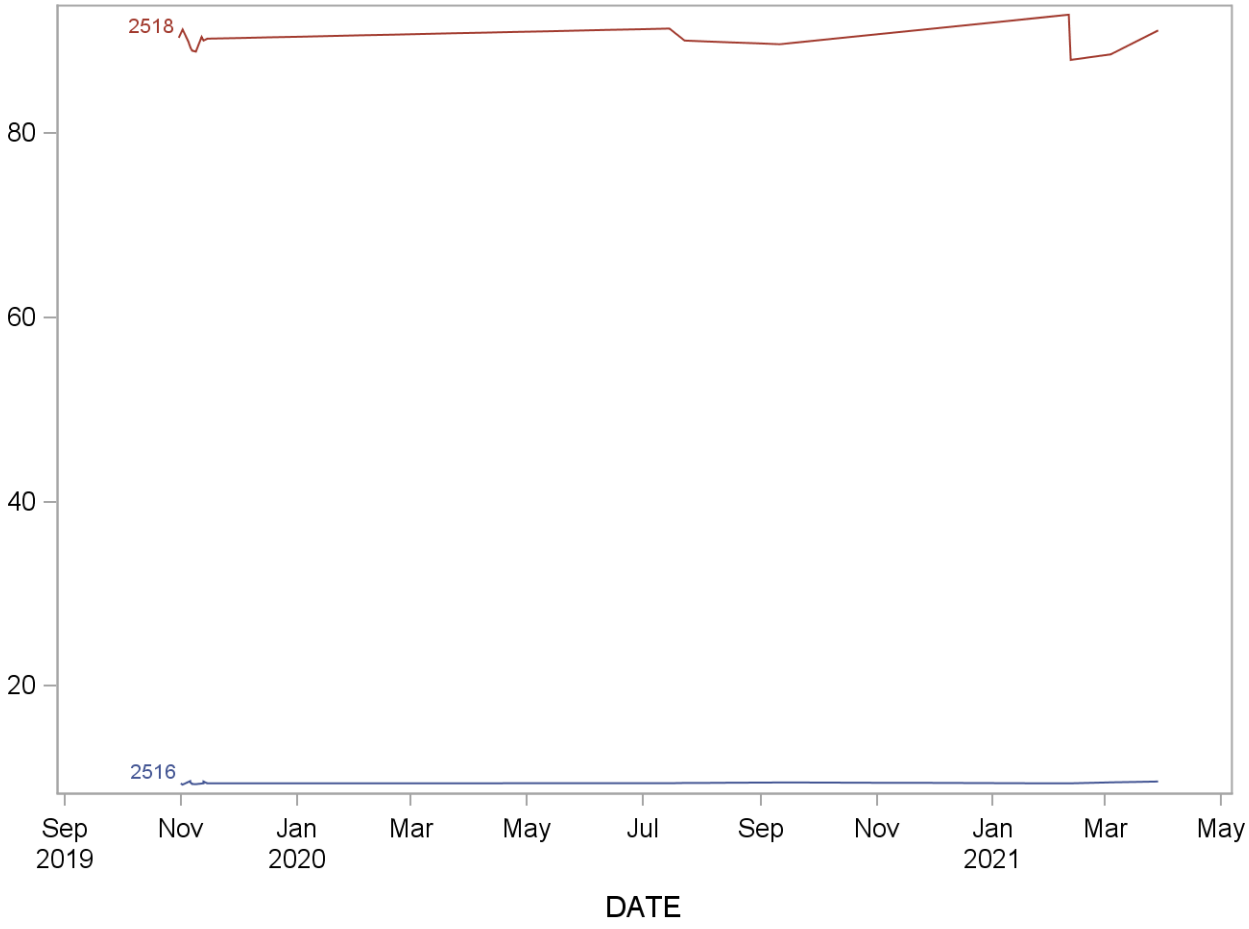
2017-2018 Summary Statistics and QC Chart
URXANBT (Anabasine, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	20	31OCT19	29MAR21	76.830	1.858	2.4
2516	20	01NOV19	29MAR21	7.955	0.234	2.9



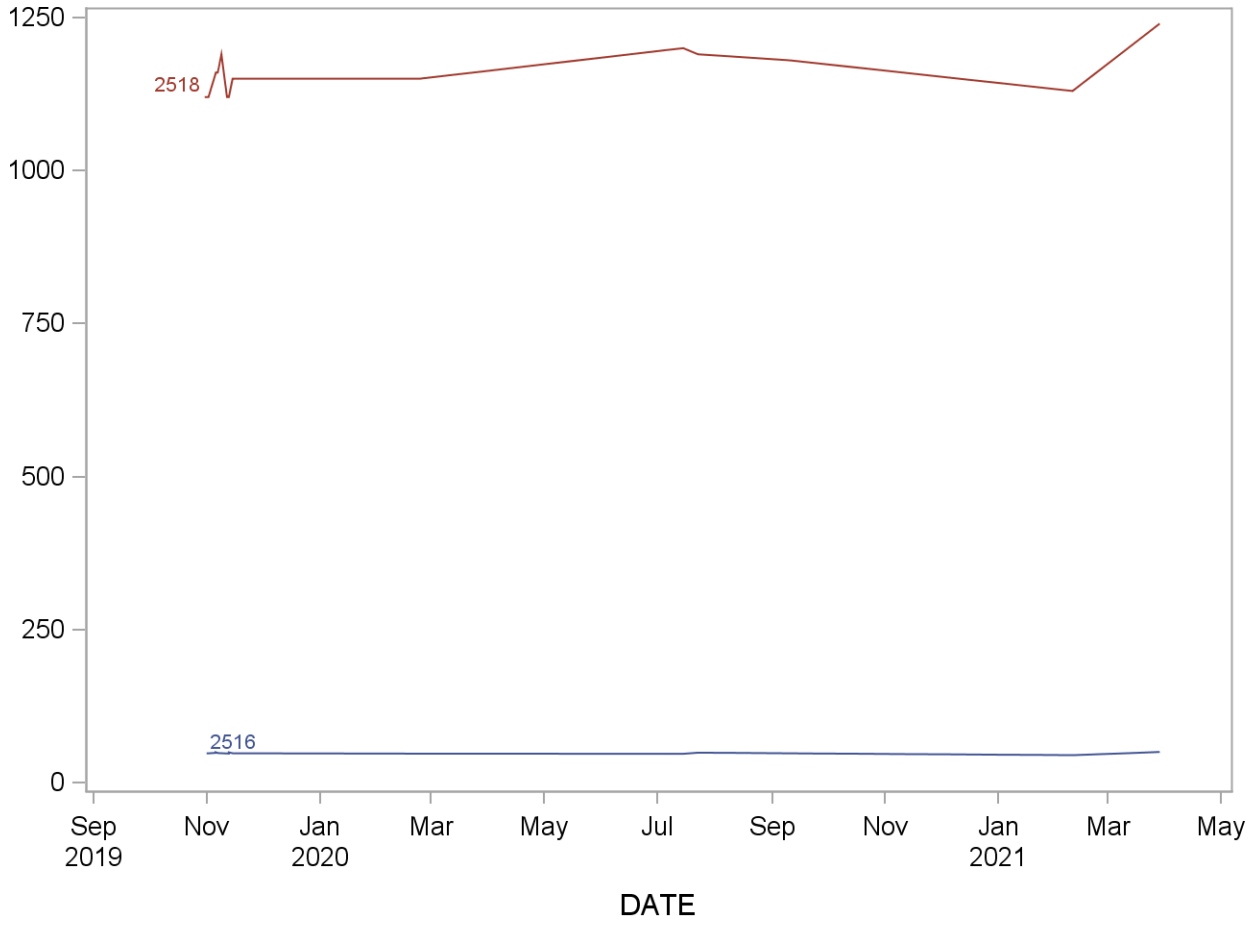
2017-2018 Summary Statistics and QC Chart
URXANTT (Anatabine, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	16	31OCT19	29MAR21	90.013	1.223	1.4
2516	16	01NOV19	29MAR21	9.477	0.109	1.2



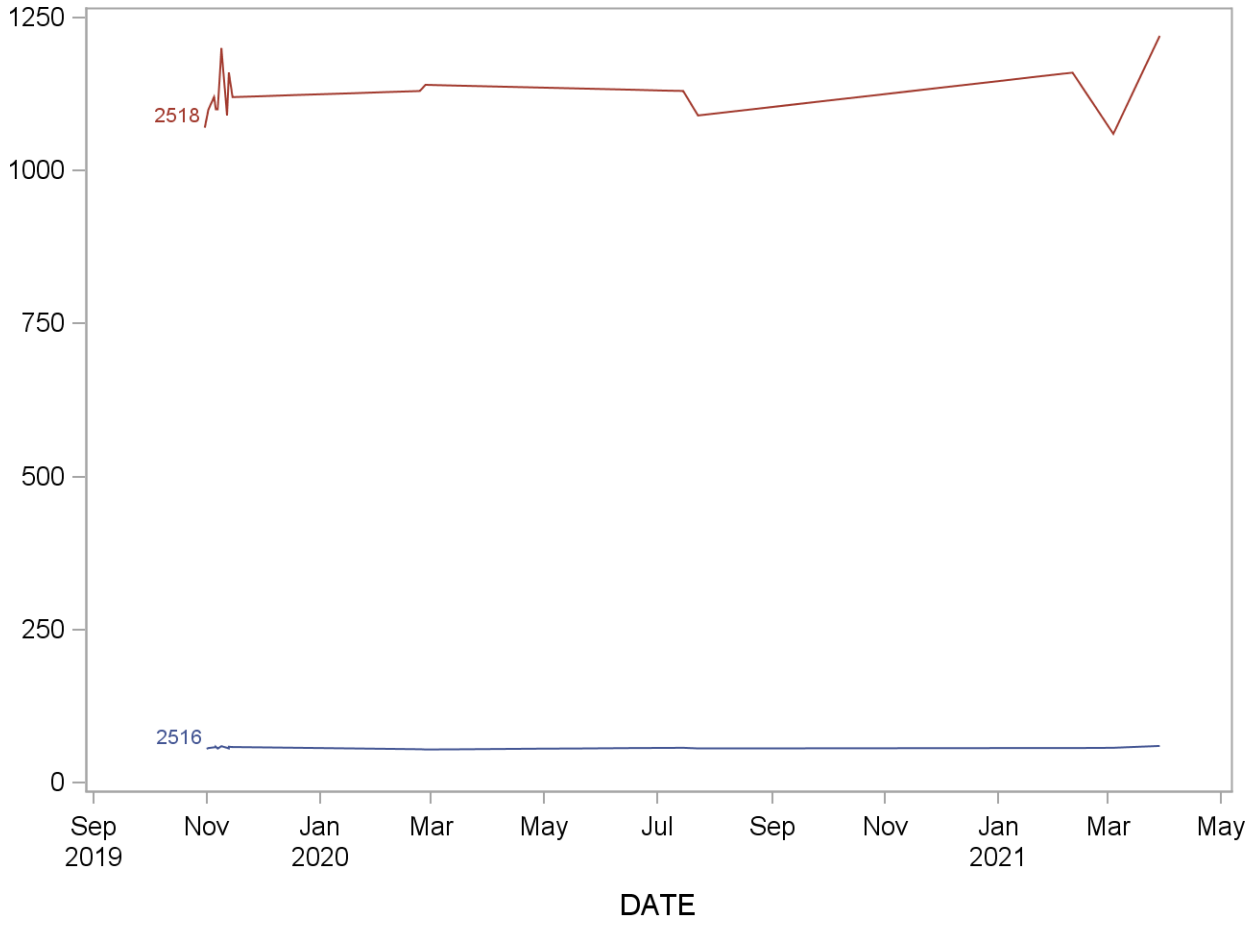
2017-2018 Summary Statistics and QC Chart
URXCOXT (Cotinine-n-oxide, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	15	31OCT19	29MAR21	1158.667	35.630	3.1
2516	15	01NOV19	29MAR21	48.153	1.253	2.6



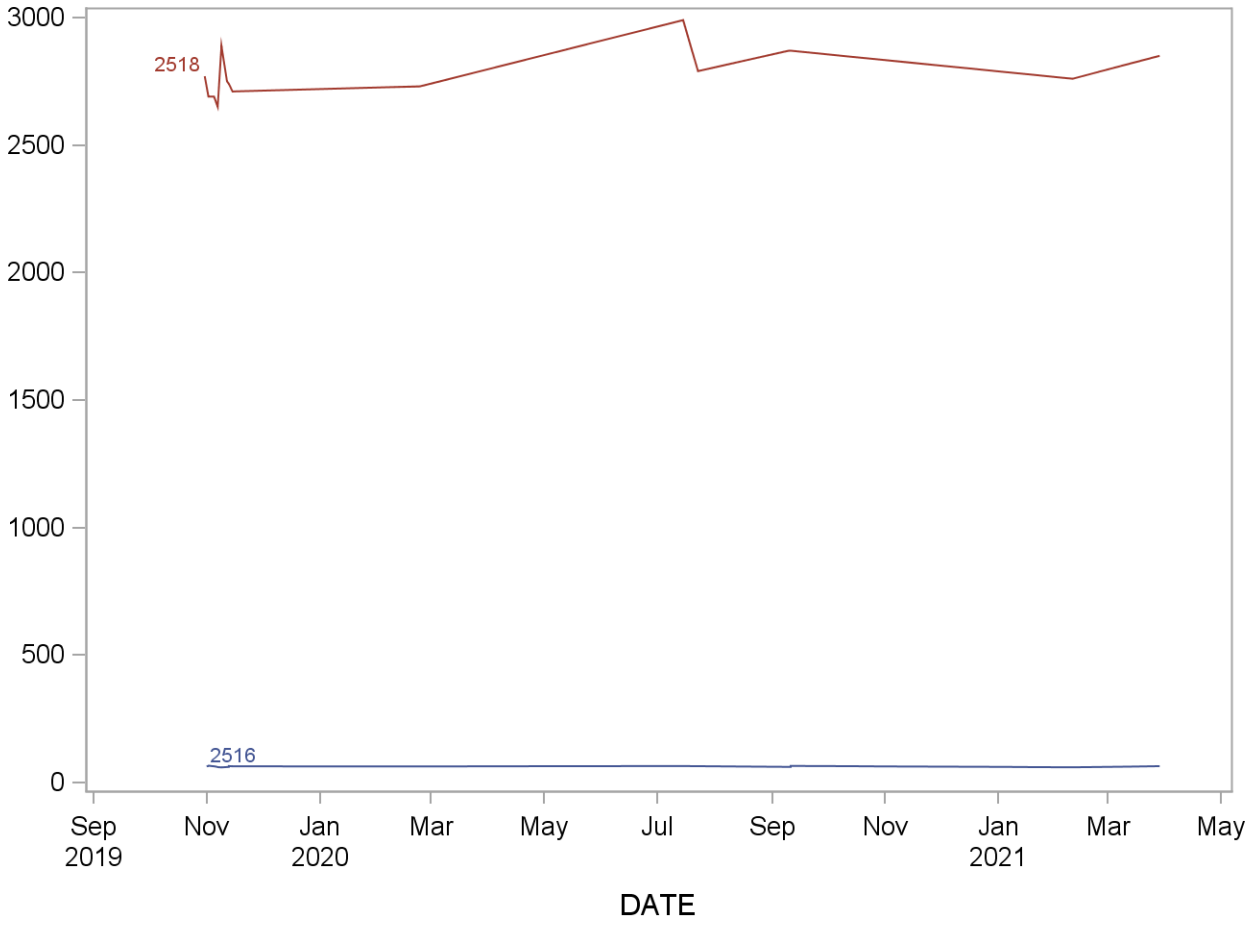
2017-2018 Summary Statistics and QC Chart
URXHPBT (4-Hydroxy-4-(3-pyridyl) butanoic acid (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	16	31OCT19	29MAR21	1124.375	43.965	3.9
2516	16	01NOV19	29MAR21	57.013	1.714	3.0



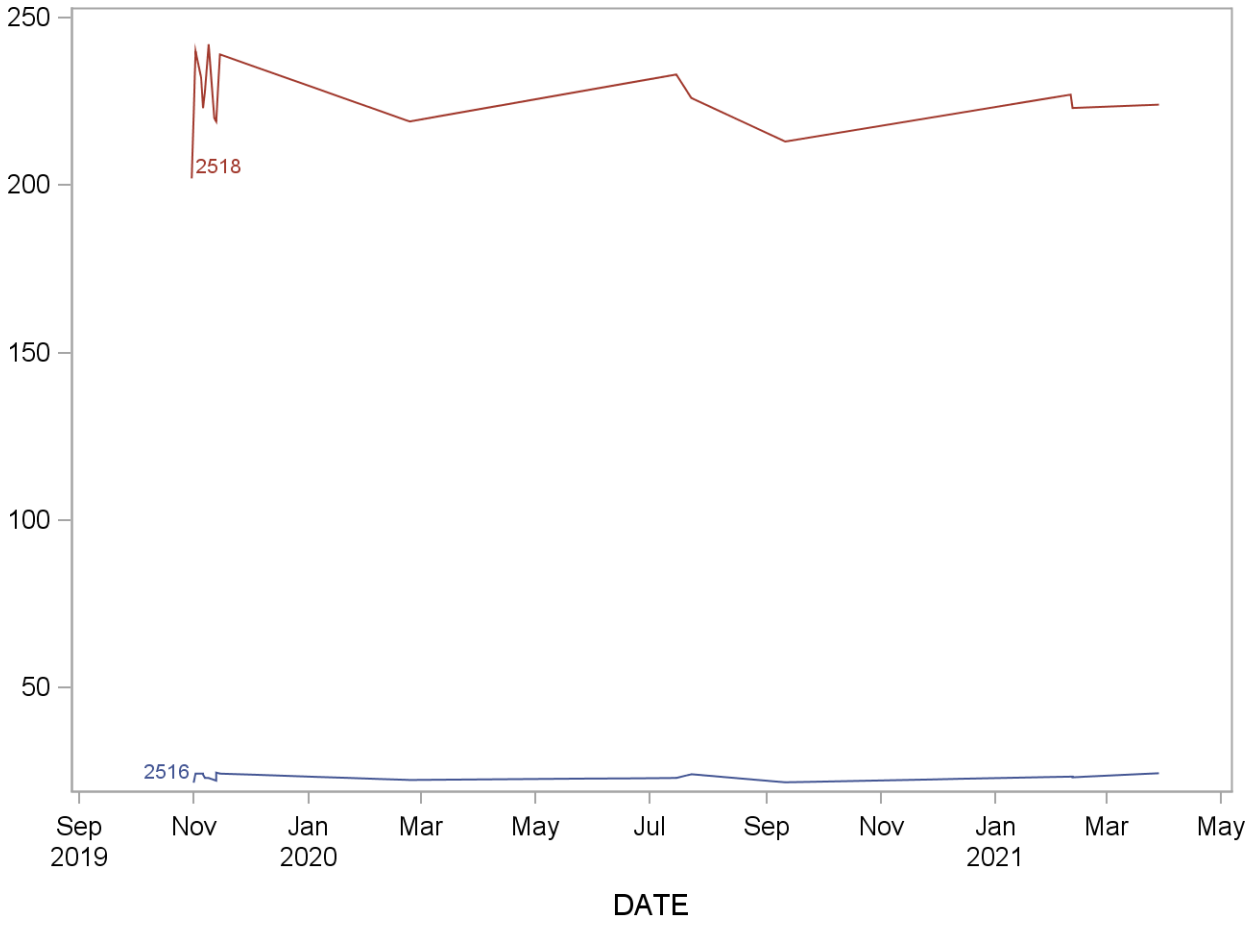
2017-2018 Summary Statistics and QC Chart
URXNICT (Nicotine, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	15	31OCT19	29MAR21	2783.333	92.864	3.3
2516	15	01NOV19	29MAR21	63.353	2.011	3.2



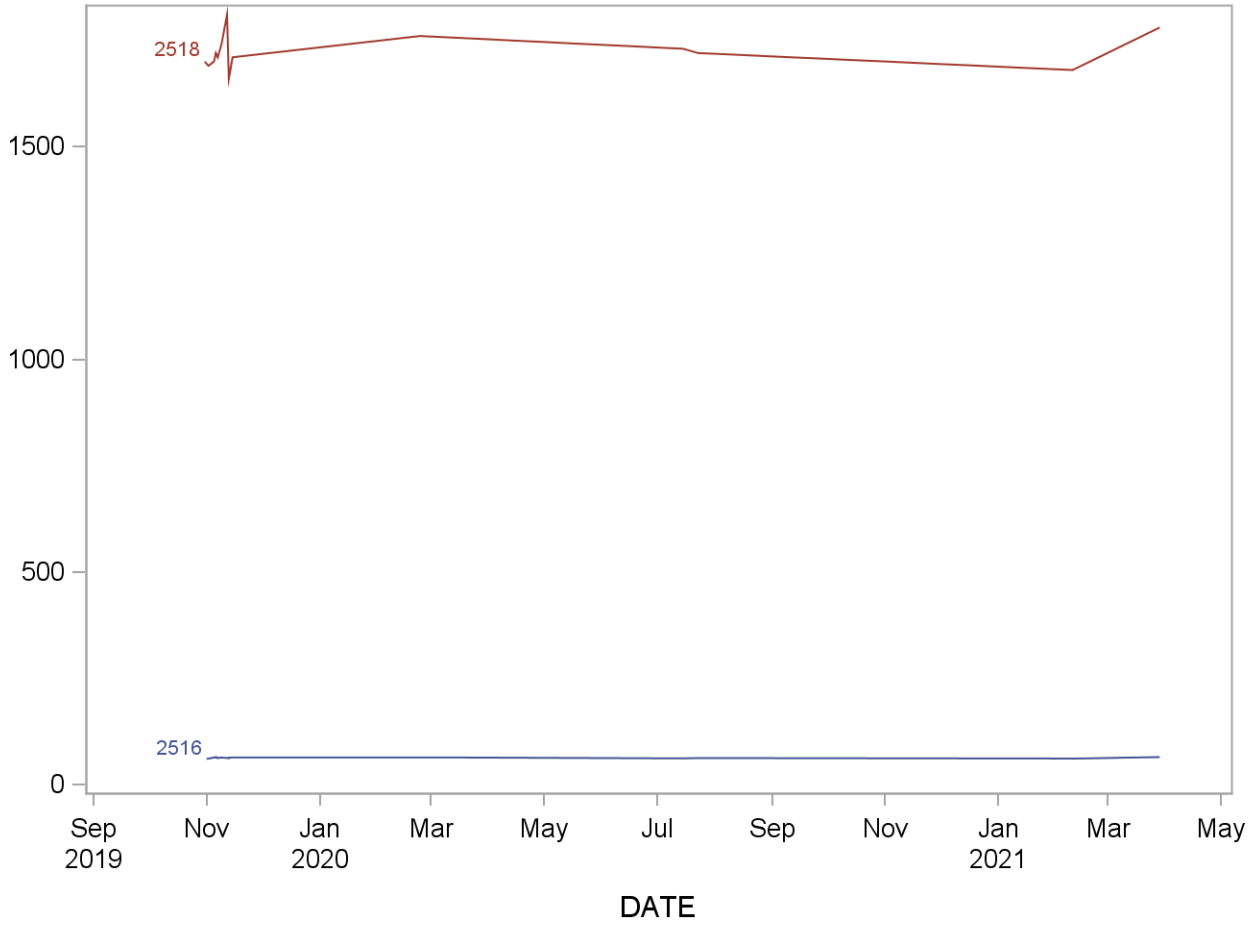
2017-2018 Summary Statistics and QC Chart
URXNNCT (Nornicotine, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	16	31OCT19	29MAR21	225.625	10.359	4.6
2516	16	01NOV19	29MAR21	23.444	0.992	4.2



2017-2018 Summary Statistics and QC Chart
URXNOXT (Nicotine-1 N-oxide, urine (ng/mL))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2518	14	31OCT19	29MAR21	1722.143	40.034	2.3
2516	14	01NOV19	29MAR21	62.979	1.346	2.1



21. References

1) DNR, 1996. Analytical detection limit guidance & laboratory guide for determining method detection limits. Wisconsin Department of Natural Resources: Laboratory Certification Program, http://dnr.wi.gov/regulations/labcert/documents/guidance/-LODguide.pdf.
2) ICH, 1994. ICH harmonised tripartite guideline. Validation of analytical procedures: Text and methodology. Available from http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf [Accessed October 25th, 2013].
3) McGuffey JE, Wei B, Bernert JT, Morrow JC, Xia B, et al. (2014) Validation of a LC-MS/MS Method for Quantifying Urinary Nicotine, Six Nicotine Metabolites and the Minor Tobacco Alkaloids—Anatabine and Anabasine—in Smokers' Urine. PLoS ONE 9(7): e101816. doi:10.1371/journal.pone.0101816
4) Taylor, J.K., 1987. Quality assurance of chemical measurements. CRC Press.
5) Jacob III, P., Hatsukami, D., Severson, H., et al. Anabasine and anatabine as biomarkers for tobacco use during nicotine replacement therapy. Cancer Epidemiology, Biomarkers Prevention 11(12), 1668-1673 (2002).
6) Rangiah, K., Hwang, W-T., Mesaros, C., Vachani, A., and Blair, I., Nicotine exposure and metabolizer phenotypes from analysis of urinary nicotine and its 15 metabolites by LC-MS, Bioanalysis 3(7), 745-761 (2011).
7) Xu, X., M. M. Iba, et al. (2004). Simultaneous and sensitive measurement of anabasine, nicotine, and nicotine metabolites in human urine by liquid chromatography-tandem mass spectrometry. Clin Chem **50**(12): 2323-2330.
8) Zhang, Y., P. Jacob, 3rd, et al. (1990). Determination of nornicotine in smokers' urine by gas chromatography following reductive alkylation to N'-propylnornicotine. J Chromatogr **525**(2): 349-357.
9) Nagano, T., M. Shimizu, et al. (2010). Biomonitoring of urinary cotinine concentrations associated with plasma levels of nicotine metabolites after daily cigarette smoking in a male Japanese population. Int J Environ Res Public Health **7**(7): 2953-2964.
10) Moyer, T. P., J. R. Charlson, et al. (2002). Simultaneous analysis of nicotine, nicotine metabolites, and tobacco alkaloids in serum or urine by tandem mass spectrometry, with clinically relevant metabolic profiles. Clin Chem **48**(9): 1460-1471.

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

For all tables:

Units:	ng/mL
Analyte:	anatabine, anabasine, nicotine, cotinine N-oxide, nicotine N'-oxide, 4-hydroxy-4-(3-pyridyl)-butanoic acid, *trans*-3'-hydroxycotinine, cotinine, nornicotine

A3a. Accuracy compared to Reference Material

4-hydroxy-4-(3-pyridyl)-butanoic acid (HPBT)											
			Measured concentration								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	45.50399	40.78	37.04	43.96	46.18	44.59	42.49	3.22	7.58	-6.6
	2		40.83	37.86	44.56	43.29	45.87				
Level 2	1	364.0319	322.8	310.3	351.5	350.7	354.4	336.7	20.43	6.07	-7.5
	2		310.6	311.5	347.7	361.7	345.5				
Level 3	1	819.0718	809.4	697.3	815.4	770.6	792.6	768.0	51.03	6.65	-6.2
	2		717.3	681.9	825.5	783.1	786.9				

Cotinine-N-oxide (COXT)											
			Measured concentration								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	18.347	19.10	16.91	16.17	18.67	17.77	17.33	1.04	6.02	-5.6
	2		17.22	17.80	15.72	16.98	16.92				
Level 2	1	89.439	85.1	81.6	80.2	88.6	88.3	85.6	4.57	5.34	-4.3
	2		84.7	87.8	77.9	92.6	89.0				
Level 3	1	758.506	734.4	685.9	627.4	760.7	742.3	708.9	46.16	6.51	-6.5
	2		725.3	721.0	632.1	743.2	716.6				

Nicotine-N-oxide (NOXT)											
Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	100.499	98.65	91.36	94.61	97.87	100.13	95.42	3.06	3.20	-5.1
	2		92.59	92.30	93.04	96.13	97.50				
Level 2	1	401.996	383.7	352.9	385.9	380.7	399.8	377.5	15.94	4.22	-6.1
	2		372.7	350.2	386.3	371.6	391.2				
Level 3	1	803.992	852.2	735.4	748.5	748.2	801.3	775.1	37.04	4.78	-3.6
	2		760.3	734.5	792.1	780.8	798.0				

Trans-3'-hydroxycotinine (HCTT)											
Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	121.475	119.66	112.70	131.42	124.72	125.91	122.39	6.36	5.20	0.8
	2		119.92	112.43	128.50	121.73	126.95				
Level 2	1	592.171	583.8	577.4	641.4	624.4	612.1	609.9	22.10	3.62	3.0
	2		583.3	607.7	634.9	617.2	617.2				
Level 3	1	5022.051	4639.7	4765.2	5125.4	4974.0	4933.6	4916.1	180.91	3.68	-2.1
	2		4718.8	4974.4	5237.6	4869.3	4923.2				

Cotinine (COTT)											
			Measured concentration								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	76.413	77.01	77.74	92.52	81.35	81.09	81.55	4.75	5.83	6.7
	2		78.60	78.10	86.62	80.45	82.04				
Level 2	1	372.501	391.7	383.2	432.9	410.5	412.0	402.5	24.91	6.19	8.0
	2		370.8	370.4	445.9	396.6	410.8				
Level 3	1	3159.086	3122.3	3201.8	3560.9	3324.6	3292.9	3309.2	152.00	4.59	4.8
	2		3277.4	3216.9	3592.2	3225.8	3277.6				

Nornicotine (NNCT)											
			Measured concentration								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	9.234	7.69	7.83	8.98	7.04	8.67	8.18	0.94	11.45	-11.4
	2		8.54	8.17	9.74	6.55	8.58				
Level 2	1	17.998	15.8	16.3	17.2	15.6	16.6	16.7	0.72	4.33	-7.4
	2		17.7	16.9	17.6	16.2	16.7				
Level 3	1	152.635	135.5	140.3	150.2	148.6	137.8	144.6	6.43	4.45	-5.3
	2		144.5	144.0	153.6	152.6	138.9				

Anatabine (ANTT)											
			Measured concentration								
Reference	Replicate	Nominal	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from
Level 1	1	2.052306	2.10	1.93	1.82	2.10	2.18	2.08	0.19	9.36	1.2
	2		2.21	1.92	2.51	2.00	2.02				
Level 2	1	5.130765	5.3	4.9	4.5	5.5	5.1	5.1	0.31	6.02	-0.7
	2		5.2	4.7	5.2	5.3	5.2				
Level 3	1	20.52306	23.8	19.3	20.3	20.9	21.9	20.9	1.48	7.07	1.9
	2		20.2	18.5	20.9	21.4	21.8				

Anabasine (ANBT)											
			Measured concentration								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	6.144	6.02	5.84	6.09	6.28	5.92	6.15	0.27	4.33	0.0
	2		6.35	6.66	5.89	6.00	6.41				
Level 2	1	11.975	11.4	12.1	11.8	11.7	12.8	11.9	0.52	4.40	-0.7
	2		11.2	11.8	11.4	12.1	12.7				
Level 3	1	101.561	95.5	102.3	98.2	102.9	102.8	101.5	3.07	3.02	-0.1
	2		100.2	105.8	99.6	103.8	103.6				

Nicotine (NICT)											
			Measured concentration								
Reference material	Replicate	Nominal value	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	CV (%)	Difference from nominal value (%)
Level 1	1	153.76	140.37	140.40	160.45	152.06	167.31	152.65	9.41	6.17	-0.7
	2		149.24	145.86	161.16	148.50	161.19				
Level 2	1	299.688	286.2	287.4	304.5	276.5	309.7	296.9	12.64	4.26	-0.9
	2		291.0	288.5	300.9	312.1	312.4				
Level 3	1	2541.573	2459.3	2444.1	2439.6	2471.0	2563.8	2481.0	49.42	1.99	-2.4
	2		2449.4	2450.9	2490.8	2464.5	2577.0				

A3b. Accuracy using Spike Recovery

4-hydroxy-4-(3-pyridyl)-butanoic acid (HPBT)

		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	-0.399	-0.496	-0.5		0	-0.567	-0.56	-0.6		97.3	2.0
	2		-0.475	-0.505				-0.586	-0.572				
	3		-0.591	-0.5				-0.501	-0.5				
Sample + Spike 1	1	182.0159605	182.77	184.232	181.8	100.2	182.0159605	173.747	165.1	172.7	95.2		
	2		171.467	185.511				177.639	175.273				
	3		181.406	185.568				175.522	168.86				
Sample + Spike 2	1	364.0319211	343.368	353.9	347.2	95.5	364.0319211	364.457	343.2	349.5	96.1		
	2		345.112	348.809				355.981	346.838				
	3		338.25	353.995				353.827	332.518				
Sample + Spike 3	1	728.0638421	708.347	756.779	717	98.5	728.0638421	720.23	714.482	713	98.1		
	2		691.832	708.863				714.434	720.872				
	3		704.848	730.1				702.302	708.306				

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				

Cotinine (COTT)

		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	0.417	-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)

		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	0.41	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 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	0.024	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)

		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	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				

Appendix 4: Precision

For all tables:

Units:	ng/mL
Analyte:	anatabine, anabasine, nicotine, cotinine N-oxide, nicotine N'-oxide, 4-hydroxy-4-(3-pyridyl)-butanoic acid, *trans*-3'-hydroxycotinine, cotinine, nornicotine

4-hydroxy-4-(3-pyridyl)-butanoic acid (HPBT)						
Quality material 1 (QCL)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	59.27	60.03	59.65	0.145924	0.145924	7116.722208
2	58.823	58.653	58.74	0.007225	0.007225	6900.305288
3	61.135	60.493	60.81	0.103041	0.103041	7396.685192
4	54.387	59.557	56.97	6.682225	6.682225	6491.617568
5	59.222	57.303	58.26	0.92064025	0.92064025	6789.037813
6	61.165	55.791	58.48	7.219969	7.219969	6839.352968
7	58.597	57.705	58.15	0.198916	0.198916	6763.077602
8	61.301	63.196	62.25	0.89775625	0.89775625	7749.751505
9	57.23	60.77	59.00	3.12405625	3.12405625	6961.646005
10	57.686	57.503	57.59	0.00837225	0.00837225	6634.252861
Grand sum	1179.818	**Grand mean**	58.9909			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	38.61625	3.861625	1.965101779	3.33		
Between Run	43.9233518	4.880372422	0.713704218	1.21		
Total	82.5396018		2.090693357	**3.54**		
Quality material 2 (QCH)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	1177.52	1119.58	1148.55	839.492676	839.492676	2638329.611
2	1183.30	1160.81	1172.05	126.3938063	126.3938063	2747423.502
3	1174.31	1186.52	1180.41	37.25271225	37.25271225	2786756.784
4	1028.19	1095.91	1062.05	1146.702769	1146.702769	2255900.405
5	1061.13	1091.60	1076.37	232.0899902	232.0899903	2317125.379
6	1075.88	1090.39	1083.14	52.58425225	52.58425225	2346365.023
7	1185.52	1206.12	1195.82	106.048804	106.048804	2859970.945
8	1233.44	1239.97	1236.70	10.666756	10.666756	3058858.727
9	1085.58	1069.68	1077.63	63.154809	63.154809	2322577.144
10	1098.75	1126.62	1112.69	194.1702903	194.1702903	2476146.945
Grand sum	22690.817	**Grand mean**	1134.54085			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	5617.113731	561.7113731	23.7004509	2.09		
Between Run	65795.65813	7310.628681	58.09009084	5.12		
Total	71412.77186		62.73890362	**5.53**		

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	1.41729025	1.41729025	4856.938241
2	45.008	43.204	44.11	0.813604	0.813604	3890.678472
3	49.727	49.377	49.55	0.030625	0.030625	4910.801408
4	47.36	48.412	47.89	0.276676	0.276676	4586.137992
5	47.695	48.138	47.92	0.04906225	0.04906225	4591.981945
6	49.448	50.413	49.93	0.23280625	0.23280625	4986.109661
7	50.66	43.034	46.85	14.538969	14.538969	4389.282818
8	47.751	52.123	49.94	4.778596	4.778596	4987.407938
9	46.63	52.14	49.38	7.57625625	7.57625625	4877.262613
10	51.819	45.518	48.67	9.92565025	9.92565025	4737.245785
Grand sum	967.011	**Grand mean**	48.35055			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	79.2790705	7.92790705	2.815653929	5.82		
Between Run	58.33316445	6.481462717	0	0.00		
Total	137.6122349		2.815653929	**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	0.05546025	0.05546025	2888812.331
2	1173.52	1152.08	1162.80	114.9291202	114.9291202	2704223.959
3	1190.43	1249.64	1220.04	876.456025	876.456025	2976985.443
4	1132.55	1193.75	1163.15	936.3294003	936.3294003	2705819.561
5	1128.16	1143.01	1135.58	55.18261225	55.18261225	2579099.771
6	1176.39	1077.35	1126.87	2452.081842	2452.081842	2539683.263
7	1197.39	1137.38	1167.39	900.2700203	900.2700203	2725587.15
8	1290.24	1170.78	1230.51	3567.6729	3567.6729	3028304.798
9	1128.41	1213.11	1170.76	1793.607201	1793.607201	2741372.004
10	1214.71	1174.44	1194.57	405.5189063	405.5189063	2854016.472
Grand sum	23547.025	**Grand mean**	1177.35125			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	22204.20698	2220.420698	47.12134015	4.00		
Between Run	20785.43466	2309.49274	6.673531375	0.57		
Total	42989.64163		47.59156142	**4.04**		

Nicotine-N-oxide (NOXT)						
Quality material 1 (QCL)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	60.33	63.57	61.95	2.621161	2.621161	7675.109408
2	62.278	60.937	61.61	0.44957025	0.44957025	7590.968113
3	61.789	61.869	61.83	0.0016	0.0016	7645.650482
4	57.553	56.664	57.11	0.19758025	0.19758025	6522.761545
5	59.051	64.135	61.59	6.461764	6.461764	7587.395298
6	57.27	57.494	57.38	0.012544	0.012544	6585.387848
7	59.817	62.327	61.07	1.575025	1.575025	7459.578368
8	56.693	63.243	59.97	10.725625	10.725625	7192.322048
9	62.54	65.19	63.86	1.76757025	1.76757025	8157.348721
10	65.178	59.623	62.40	7.71450625	7.71450625	7787.644801
Grand sum	1217.546	**Grand mean**	60.8773			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	63.053892	6.3053892	2.511053404	4.12		
Between Run	83.2535242	9.250391578	1.2134666	1.99		
Total	146.3074162		2.788886944	**4.58**		
Quality material 1 (QCH)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	1758.13	1672.93	1715.53	1814.7174	1814.7174	5886069.207
2	1759.32	1606.42	1682.87	5844.220256	5844.220256	5664092.777
3	1666.05	1756.81	1711.43	2059.75284	2059.75284	5857981.867
4	1597.41	1603.78	1600.59	10.13467225	10.13467225	5123799.104
5	1858.02	1787.34	1822.68	1249.162992	1249.162992	6644321.119
6	1672.44	1639.26	1655.85	275.294464	275.294464	5483678.445
7	1767.77	1704.52	1736.14	1000.140625	1000.140625	6028391.977
8	1779.95	1824.96	1802.45	506.3625063	506.3625063	6497684.449
9	1709.84	1774.56	1742.20	1047.20196	1047.20196	6070525.164
10	1733.00	1780.90	1756.95	573.6743523	573.6743522	6173743.091
range.						
Grand sum	34453.394	**Grand mean**	1722.6697			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	28761.32414	2876.132414	53.62958525	3.11		
Between Run	78469.29511	8718.810568	54.04941329	3.14		
Total	107230.6193		76.14112877	**4.42**		

Trans-3'-hydroxycotinine (HCTT)						
Quality material 1 (QCL)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	284.26	268.04	276.15	65.74777225	65.74777225	152514.8835
2	285.74	293.342	289.54	14.447601	14.447601	167667.9814
3	296.64	288.324	292.48	17.288964	17.288964	171091.4406
4	265.814	281.263	273.54	59.66790025	59.66790025	149646.622
5	272.217	275.748	273.98	3.11699025	3.11699025	150132.8206
6	283.867	271.728	277.80	36.83883025	36.83883025	154342.902
7	285.691	284.948	285.32	0.13801225	0.13801225	162814.4342
8	290.322	272.545	281.43	79.00543225	79.00543225	158409.6298
9	277.00	304.08	290.54	183.3722223	183.3722223	168822.9157
10	301.154	292.159	296.66	20.22750625	20.22750625	176010.158
Grand sum	5674.87	**Grand mean**	283.7435			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	959.702462	95.9702462	9.796440486	3.45		
Between Run	1246.311921	138.4791023	4.610252495	1.62		
Total	2206.014383		10.82703442	**3.82**		
Quality material 2 (QCH)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	10199.86	10037.38	10118.62	6600.262564	6600.262564	204772900.9
2	10827.30	10404.66	10615.98	44655.08581	44655.08581	225398126.4
3	10439.48	10590.99	10515.24	5738.820025	5738.820025	221140334.2
4	9574.00	9716.65	9645.32	5086.970329	5086.970329	186064550.1
5	10487.88	10118.28	10303.08	34151.04	34151.04	212307079.8
6	9864.30	10215.97	10040.13	30917.77139	30917.77139	201608561.4
7	10330.15	10166.62	10248.38	6685.106406	6685.106406	210058728.7
8	10283.52	10120.58	10202.05	6637.523841	6637.523841	208163607.6
9	9773.31	9519.73	9646.52	16076.08447	16076.08447	186110599.8
10	10547.88	10317.89	10432.88	13223.96502	13223.96502	217689991.1
Grand sum	203536.415	**Grand mean**	10176.82075			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	339545.2597	33954.52597	184.2675391	1.81		
Between Run	1960868.49	217874.2766	303.248867	2.98		
Total	2300413.749		354.844193	**3.49**		

Cotinine (COTT)						
Quality material 1 (QCL)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	126.49	125.65	126.07	0.173889	0.173889	31786.78552
2	128.338	129.86	129.10	0.579121	0.579121	33333.1036
3	136.62	132.671	134.65	3.89865025	3.89865025	36258.82134
4	137.394	140.809	139.10	2.91555625	2.91555625	38698.4546
5	127.378	125.594	126.49	0.795664	0.795664	31997.41639
6	132.297	125.431	128.86	11.785489	11.785489	33211.86099
7	137.356	134.241	135.80	2.42580625	2.42580625	36882.4652
8	134.473	135.611	135.04	0.323761	0.323761	36472.68353
9	128.58	134.27	131.43	8.09687025	8.09687025	34545.3241
10	130.359	132.121	131.24	0.776161	0.776161	34447.8752
Grand sum	2635.542	**Grand mean**	131.7771			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	63.541936	6.3541936	2.520752586	1.91		
Between Run	330.7087978	36.74542198	3.898155229	2.96		
Total	394.2507338		4.642177053	**3.52**		
Quality material 2 (QCH)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	4839.52	4887.75	4863.64	581.677924	581.677924	47309890.83
2	4762.26	4768.16	4765.21	8.71135225	8.71135225	45414481.28
3	4951.49	5218.42	5084.96	17813.84049	17813.84049	51713544.87
4	4826.46	4793.15	4809.81	277.355716	277.355716	46268448.28
5	4817.17	4872.84	4845.01	774.7037222	774.7037223	46948156.59
6	4812.79	4975.09	4893.94	6585.24135	6585.24135	47901248.51
7	5048.06	5087.66	5067.86	391.8618203	391.8618202	51366399.82
8	5091.46	5300.22	5195.84	10894.87126	10894.87126	53993496.22
9	4870.75	5015.51	4943.13	5239.153924	5239.153924	48869107.94
10	4924.47	5021.17	4972.82	2337.67415	2337.67415	49457827.78
Grand sum	98884.397	**Grand mean**	4944.21985			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	89810.18343	8981.018343	94.76823488	1.92		
Between Run	336403.6096	37378.17884	119.1577956	2.41		
Total	426213.793		152.2484765	**3.08**		

Nornicotine (NNCT)						
Quality material 1 (QCL)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	23.66	23.75	23.70	0.00225625	0.00225625	1123.617013
2	22.935	22.675	22.81	0.0169	0.0169	1040.13605
3	23.224	23.379	23.30	0.00600625	0.00600625	1085.919805
4	19.531	21.526	20.53	0.99500625	0.99500625	842.8386245
5	24.036	23.763	23.90	0.01863225	0.01863225	1142.372201
6	22.902	20.922	21.91	0.9801	0.9801	960.271488
7	24.304	24.348	24.33	0.000484	0.000484	1183.508552
8	22.7705	22.845	22.81	0.001387563	0.001387563	1040.38692
9	23.48	25.11	24.29	0.659344	0.659344	1180.396872
10	24.865	23.727	24.30	0.323761	0.323761	1180.591232
Grand sum	463.7455	**Grand mean**	23.187275			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	6.007755125	0.600775513	0.775097099	3.34		
Between Run	27.04431761	3.004924179	1.096391506	4.73		
Total	33.05207274		1.342702441	**5.79**		
Quality material 2 (QCH)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	234.22	241.60	237.91	13.601344	13.601344	113201.3846
2	238.24	233.73	235.99	5.089536	5.089536	111378.7844
3	242.50	237.18	239.84	7.07294025	7.07294025	115048.8496
4	218.38	215.60	216.99	1.93627225	1.93627225	94168.88622
5	271.63	267.73	269.68	3.798601	3.798601	145454.6048
6	236.49	241.75	239.12	6.895876	6.895876	114355.7923
7	239.89	234.79	237.34	6.52547025	6.52547025	112660.0765
8	238.70	239.62	239.16	0.213444	0.213444	114394.0546
9	221.27	240.80	231.04	95.374756	95.374756	106756.1907
10	243.50	243.26	243.38	0.013924	0.013924	118466.6753
Grand sum	4780.881	**Grand mean**	239.04405			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	281.0443275	28.10443275	5.301361405	2.22		
Between Run	3044.142203	338.2380226	12.45258186	5.21		
Total	3325.186531		13.53407654	**5.66**		

Anatabine (ANTT)

Quality material 1 (QCL)						
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
2	9.186	9.253	9.22	0.00112225	0.00112225	169.9983605
3	9.721	9.353	9.54	0.033856	0.033856	181.908738
4	8.777	9.094	8.94	0.02512225	0.02512225	159.6863205
5	9.373	9.141	9.26	0.013456	0.013456	171.384098
6	9.617	9.021	9.32	0.088804	0.088804	173.687522
7	9.563	9.83	9.70	0.01782225	0.01782225	188.0442245
8	9.418	9.866	9.64	0.050176	0.050176	185.936328
9	9.53	10.00	9.77	0.054289	0.054289	190.749512
10	9.473	9.12	9.30	0.03115225	0.03115225	172.8498245
Grand sum	187.628	**Grand mean**	9.3814			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.70005	0.070005	0.26458458	2.82		
Between Run	1.2936588	0.143739867	0.192008941	2.05		
Total	1.9937088		0.326913495	**3.48**		

Quality material 2 (QCH)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	83.02	90.27	86.65	13.126129	13.126129	15014.71205
2	88.05	85.86	86.95	1.18919025	1.18919025	15122.17014
3	81.37	87.35	84.36	8.952064	8.952064	14232.54433
4	85.15	87.75	86.45	1.69650625	1.69650625	14946.6863
5	82.18	86.53	84.36	4.721929	4.721929	14232.2069
6	88.66	89.37	89.01	0.12852225	0.12852225	15847.16242
7	92.37	91.12	91.74	0.391876	0.391876	16833.5561
8	91.24	95.47	93.35	4.477456	4.477456	17429.19181
9	84.77	91.64	88.20	11.799225	11.799225	15559.18561
10	88.43	89.82	89.13	0.48233025	0.48233025	15886.7095
Grand sum	1760.4	**Grand mean**	88.02			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	93.930456	9.3930456	3.064807596	3.48		
Between Run	153.717156	17.079684	1.960438522	2.23		
Total	247.647612		3.638181524	**4.13**		

Anatabine (ANTT)						
Quality material 1 (QCL)						
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
2	9.186	9.253	9.22	0.00112225	0.00112225	169.9983605
3	9.721	9.353	9.54	0.033856	0.033856	181.908738
4	8.777	9.094	8.94	0.02512225	0.02512225	159.6863205
5	9.373	9.141	9.26	0.013456	0.013456	171.384098
6	9.617	9.021	9.32	0.088804	0.088804	173.687522
7	9.563	9.83	9.70	0.01782225	0.01782225	188.0442245
8	9.418	9.866	9.64	0.050176	0.050176	185.936328
9	9.53	10.00	9.77	0.054289	0.054289	190.749512
10	9.473	9.12	9.30	0.03115225	0.03115225	172.8498245
Grand sum	187.628	**Grand mean**	9.3814			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	0.70005	0.070005	0.26458458	2.82		
Between Run	1.2936588	0.143739867	0.192008941	2.05		
Total	1.9937088		0.326913495	**3.48**		
Quality material 2 (QCH)						
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	83.02	90.27	86.65	13.126129	13.126129	15014.71205
2	88.05	85.86	86.95	1.18919025	1.18919025	15122.17014
3	81.37	87.35	84.36	8.952064	8.952064	14232.54433
4	85.15	87.75	86.45	1.69650625	1.69650625	14946.6863
5	82.18	86.53	84.36	4.721929	4.721929	14232.2069
6	88.66	89.37	89.01	0.12852225	0.12852225	15847.16242
7	92.37	91.12	91.74	0.391876	0.391876	16833.5561
8	91.24	95.47	93.35	4.477456	4.477456	17429.19181
9	84.77	91.64	88.20	11.799225	11.799225	15559.18561
10	88.43	89.82	89.13	0.48233025	0.48233025	15886.7095
Grand sum	1760.4	**Grand mean**	88.02			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	93.930456	9.3930456	3.064807596	3.48		
Between Run	153.717156	17.079684	1.960438522	2.23		
Total	247.647612		3.638181524	**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	0.09394225	137.0009045
2	8.594	7.427	8.01	0.34047225	0.34047225	128.3362205
3	7.311	7.95	7.63	0.10208025	0.10208025	116.4490605
4	8.717	9.006	8.86	0.02088025	0.02088025	157.0523645
5	7.798	7.647	7.72	0.00570025	0.00570025	119.2740125
6	7.731	7.511	7.62	0.0121	0.0121	116.159282
7	7.668	7.516	7.59	0.005776	0.005776	115.276928
8	7.357	7.932	7.64	0.08265625	0.08265625	116.8767605
9	7.90	8.53	8.22	0.099856	0.099856	134.97245
10	7.953	7.664	7.81	0.02088025	0.02088025	121.9453445
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	10290.52926
2	73.51	73.34	73.42	0.00714025	0.00714025	10781.13964
3	69.28	75.10	72.19	8.485569	8.485569	10422.21469
4	71.31	75.26	73.28	3.912484	3.912484	10740.79618
5	79.87	79.18	79.52	0.11730625	0.11730625	12647.65601
6	70.10	71.34	70.72	0.38750625	0.38750625	10002.77824
7	72.42	71.74	72.08	0.11526025	0.11526025	10391.19696
8	72.23	72.22	72.23	1.225E-05	1.225E-05	10433.62351
9	76.73	77.23	76.98	0.0625	0.0625	11851.22497
10	80.41	79.72	80.06	0.117649	0.117649	12819.84769
Grand sum	1484.426	**Grand mean**	74.2213			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**		
Within Run	40.281499	4.0281499	2.007025137	2.70		
Between Run	204.9796752	22.77551947	3.061647397	4.13		
Total	245.2611742		3.660851634	**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	8136.797312
2	68.57	66.118	67.34	1.503076	1.503076	9070.428672
3	62.246	70.413	66.33	16.67497225	16.67497225	8799.205141
4	63.641	75.859	69.75	37.319881	37.319881	9730.125
5	57.546	64.483	61.01	12.03049225	12.03049225	7445.538421
6	61.434	60.84	61.14	0.088209	0.088209	7475.465538
7	66.821	74.155	70.49	13.446889	13.446889	9937.116288
8	68.845	70.091	69.47	0.388129	0.388129	9651.606048
9	62.50	74.23	68.37	34.398225	34.398225	9348.093378
10	66.116	70.4	68.26	4.588164	4.588164	9318.309128
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	151.0072322	151.0072323	16727009.88
2	2913.54	2852.34	2882.94	936.115216	936.115216	16622686.09
3	2876.47	2935.48	2905.97	870.5155203	870.5155203	16889352.34
4	2682.74	2812.11	2747.42	4183.631761	4183.631761	15096677.27
5	3008.40	2884.59	2946.49	3832.105216	3832.105216	17363630.21
6	2926.53	2962.26	2944.40	319.1403603	319.1403602	17338965.05
7	3083.27	3052.06	3067.67	243.516025	243.516025	18821137.1
8	3051.86	3022.56	3037.21	214.710409	214.710409	18449289.17
9	2905.91	3064.12	2985.01	6258.233881	6258.233881	17820617.16
10	2930.92	2951.95	2941.44	110.544196	110.544196	17304115.02
Grand sum	58701.053	**Grand mean**	2935.05265			
	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**		
Within Run	34239.03963	3423.903963	58.51413473	1.99		
Between Run	142798.1308	15866.45898	78.87507532	2.69		
Total	177037.1704		98.20988479	**3.35**		

Appendix 5. 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

4-hydroxy-4-(3-pyridyl)-butanoic acid (HPBT)									
Quality material 1 (QCL)									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	54.387	56.007	54.387	54.34	59.222	56.312	58.688	57.24	54.387
Replicate 2	59.557	54.056	59.557	54.045	56.605	55.301	52.612	58.249	59.557
Replicate 3	63.104	54.57	63.104	54.405	50.311	55.337	50.881	57.923	63.104
Mean	59.016	54.87766667	59.016	54.3	55.37933333	55.65		57.804	59.0
% difference from initial measurement	--	**-7.0**	--	**-8.1**	--	**0.5**		--	**2.1**
Quality material 2 (QCH)									
	Initial measurement	**Three freeze-thaw cycles**	**Initial measurement**	**Bench-top stability**	**Initial measurement**	**Processed sample stability**		**Initial measurement**	**Long-term stability**
Replicate 1	1095.913	1095.775	1095.913	1038.858	1034.386	1133.85	1068	1103.454	1095.913
Replicate 2	1056.315	995.075	1056.315	1091.936	964.524	1066.301	1071	1227.513	1056.315
Replicate 3	1028.187	1017.562	1028.187	1057.818	1061.131	1106.33	1123	1161.296	1028.187
Mean	1060.138333	1036.1	1060.138333	1062.9	1020.013667	1102.160333		1164.087667	1060.1
% difference from initial measurement	--	**-2.3**	--	**0.3**	--	**8.1**		--	**-8.9**

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**

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	131.598	127.648	137.394
Replicate 2	140.809	127.22	140.809	134.784	130.212	127.732	129.728	140.809
Replicate 3	139.984	129.994	139.984	128.366	122.355	125.807	131.567	139.984
Mean	139.3956667	132.2543333	139.3956667	128.9	126.6483333	128.379	129.6476667	139.4
% difference from initial measurement	--	**-5.1**	--	**-7.5**	--	**1.4**	--	**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	4939.695	4895.164	4793.151
Replicate 2	5036.044	4672.813	5036.044	5024.275	4571.985	4822.489	5177.211	5036.044
Replicate 3	4826.459	4704.874	4826.459	4888.005	4817.172	5035.941	4995.004	4826.459
Mean	4885.218	4871.0	4885.218	4891.7	4653.636667	4932.708333	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**

Appendix 6. Ruggedness test results for the volume of water added to each sample prior to injection (analyte concentrations displayed in ng/mL)

			HPBT	COXT	NOXT	HCTT	COTT	NNCT	ANTT	ANBT	NICT
-30% volume	POOL01	Mean	59.98	50.70	65.86	288.75	137.49	24.24	10.04	8.42	69.42
		STD	1.03	1.39	1.84	4.99	3.49	0.85	0.22	0.18	1.37
	POOL02	Mean	1192.31	1250.50	1802.89	11024.70	5172.72	229.72	92.61	80.74	3078.90
		STD	18.88	16.65	24.53	78.12	76.52	8.47	1.66	3.87	28.32
Optimized volume	POOL01	Mean	59.60	50.76	64.64	282.35	135.88	24.30	9.90	8.65	68.20
		STD	0.63	1.25	1.53	8.15	2.30	1.27	0.36	0.63	0.74
	POOL02	Mean	1184.89	1268.95	1806.48	10985.86	5097.80	240.98	91.19	87.38	3025.32
		STD	19.32	18.66	37.70	190.60	68.14	9.24	1.91	1.66	34.76
+ 30% volume	POOL01	Mean	60.17	48.99	65.85	278.39	122.40	24.32	9.63	8.24	68.48
		STD	1.35	1.03	2.46	5.85	4.58	0.69	0.21	0.26	1.03
	POOL02	Mean	1200.36	1233.15	1794.42	10727.22	4707.08	240.74	89.79	82.63	3074.71
		STD	26.17	21.86	36.46	182.87	62.98	7.61	1.31	1.51	33.66

Appendix 7. Freeze-thaw test results. (Analyte concentrations displayed in ng/mL)

			HPBT	COXT	NOXT	HCTT	COTT	NNCT	ANTT	ANBT	NICT
POOL01	1 Cycle	Mean	58.8	47.9	63.2	280.9	130.5	23.7	9.6	8.1	67.4
		STD	0.9	1.3	1.1	8.7	4.4	0.5	0.3	0.3	1.4

	5 cycles	Mean	59.9	49.8	65.1	283.5	132.0	24.6	9.9	8.6	69.0
		STD	0.9	1.3	1.1	10.1	7.2	0.7	0.3	0.4	1.4
	10 Cycles	Mean	59.6	49.1	64.7	279.4	131.1	24.6	9.7	8.5	68.7
		STD	0.7	1.3	1.6	4.8	8.9	1.0	0.2	0.3	1.1
	25 Cycles	Mean	59.5	49.9	64.2	283.0	131.6	23.7	9.9	8.1	68.4
		STD	1.0	1.0	1.2	6.1	7.5	0.9	0.4	0.5	1.0
POOL02	1 Cycle	Mean	1157.0	1214.6	1743.7	10769.0	4912.5	230.0	90.3	81.0	2987.4
		STD	24.3	39.1	38.9	311.1	156.9	9.0	2.8	3.0	16.4
	5 cycles	Mean	1209.0	1252.8	1819.1	10921.8	5055.9	241.1	91.7	84.7	3085.7
		STD	24.3	39.1	38.9	311.1	156.9	9.0	2.8	3.0	16.4
	10 Cycles	Mean	1197.2	1253.6	1784.5	10929.9	4986.9	236.8	90.5	84.3	3050.2
		STD	14.7	19.2	17.7	199.7	226.3	6.2	2.0	3.3	34.9
	25 Cycles	Mean	1171.4	1246.3	1800.2	10886.2	4934.8	233.6	91.3	81.8	3043.1
		STD	16.8	17.9	35.7	232.1	209.3	11.2	1.9	4.8	44.4

Appendix 8. Total Nicotine Equivalent (TNE) Calculations

Total Nicotine Equivalent (TNE) is calculated by dividing the concentration in ng/mL by the MW for each analyte. NIST's molecular weights, which includes 4 digits after the decimal point, are used in TNE calculations. If any of the individual analyte values used in the respective formulas are missing, the TNE variable for that sample is set as missing.

TNE-2 = (Total Cotinine /176.2151) + (Total *Trans*-3'-Hydroxycotinine/192.2145) nmol/mL

TNE-3 = (Total Nicotine/162.2316) + (Total Cotinine /176.2151) + (Total *Trans*-3'-Hydroxycotinine/192.2145) nmol/mL

TNE-6 = (Total Nicotine/162.2316) + (Total Cotinine /176.2151) + (Total *Trans*-3'-Hydroxycotinine/192.2145) + (Total (S)-Cotinine N-oxide/ 192.2145) + ((1'S,2'S)-Nicotine N'-oxide/ 178.231) + ((R,S)-Nornicotine/ 148.2050) nmol/mL

TNE-7 = (Total Nicotine/162.2316) + (Total Cotinine /176.2151) + (Total *Trans*-3'-Hydroxycotinine/192.2145) + (Total (S)-Cotinine N-oxide/ 192.2145) + ((1'S,2'S)-Nicotine N'-oxide/ 178.231) + ((R,S)-Nornicotine/ 148.2050) + (4-Hydroxy-4-(3-pyridyl)-butanoic acid/ 181.1885) nmol/mL