# Laboratory Procedure Manual

*Analyte:* **Tobacco-Specific Nitrosamines**

*Matrix:* **Urine**

*Method:* **HPLC API Tandem Mass Spectrometry**

*Method No:* **2014**

*Revised:*

*As performed by:*

Tobacco and Volatiles Branch
Division of Laboratory Sciences
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**Important Information for Users**

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

## Public Release Data Set Information

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

| Data File Name | Variable Name | SAS Label |
|---|---|---|
| **TSNA_H** | URXNAL | Urinary Total NNAL (ng/mL) |
| | URXNAB | N'-Nitrosanabasine, urine (ng/mL) |
| | URXNAT | N'-Nitrosanatabine, urine (ng/mL) |
| | URXNNN | N'-Nitrosonornicotine, urine (ng/mL) |

# 1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

a. ***Analyte.***

*4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N' - nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT) and N'-nitrosoanabasine (NAB)*

b. ***Clinical Relevance.***

Tobacco-specific nitrosamines (TSNAs) are formed during tobacco smoking and tobacco curing. TSNAs are a leading class of carcinogens in tobacco products and thus become an important class of biomarkers for tobacco carcinogen uptake (1-3). There are seven major TSNAs identified from tobacco smoke, which includes 4- (Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), *N'* -nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT) and *N'*-nitrosoanabasine (NAB). Due to their specificity to tobacco, the study of TSNA uptake will provide very useful insight into the mechanistic and epidemiologic role of these compounds in human cancer.

c. ***Assay Principle.***

NNAL, NNN, NAT and NAB in urine are measured by an isotope-dilution high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (ID HPLC-ESI MS/MS). For "total" TSNA assays, the urine sample is fortified with internal standards, and then hydrolyzed using β-glucuronidase in incubations for at least 24 hours. The samples are then extracted with liquid-liquid extraction followed by solid phase extraction, after which the analyte is eluted and analyzed by LC/MS/MS. The m/z 210/180, 210/93 and 210/186 are monitored for NNAL quantitation, confirmation and ISTD respectively. The m/z 178/148, 178/105 and 182/152 are monitored for NNN quantitation, confirmation and ISTD respectively. The m/z 190/160, 190/106 and 194/164 are monitored for NAT quantitation, confirmation and ISTD respectively. The m/z 192/162, 192/106 and 196/166 are monitored for NAB quantitation, confirmation and ISTD respectively. The concentrations are derived from their respective ratios of native to isotope-labeled in the sample by comparing to their standard curves. Free TSNA measurements are conducted in a similar manner, but with the omission of prior enzymatic hydrolysis.

d. ***Special Precaution.***

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

**Safety glasses, gloves and clothing must be worn during the extraction and processing of samples by this method**

# 2 SAFETY PRECAUTIONS

a. ***Reagent Toxicity / Carcinogenicity.***

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

b. ***Radioactive Hazards.***

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

c. ***Biological Hazards.***

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

d. ***Chemical Hazards.***

Reagents and solvents are used in this method including those listed below in Section 6.a. MSDSs for these chemicals are readily accessible on the internet (e.g., http://www.msdssearch.net/MSDSSearch.asp, or http://msds.ehs.cornell.edu/msdssrch.asp). Hardcopies are maintained on file.

e. ***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 and centrifuge covers, etc. should not be defeated during normal operations.

f. ***Protective equipment.***

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

g. ***Training.***

Method specific training in the use of tandem mass spectrometry is required. All analysts must be CLIA-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.

h. ***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 it 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 appropriate covered container until autoclaved. Unshielded needles, pipette tips 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) each day. 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

a. ***Software and knowledge requirements.***

This method has been validated using multiple steps of liquid/liquid extraction and solid-phase extraction (SPE) sample preparation procedure on automation systems, followed by liquid chromatography ESI-tandem mass spectrometry utilizing Sciex API6500 with Analyst software (current version 1.6.2). The acquired the data is uploaded to Indigo Biosystem' 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. The results with additional information (retention times, area counts, etc) are exported to local laboratory information management system STARLIMS. The final results and QC evaluation are performed in STARLIMS. Knowledge of, and experience with these software packages is required in performing these functions.

The sample master database is maintained in STARLIMS. Sample sequence is generated on automation systems, such as Hamilton liquid handler and Caliper Staccato systems. The analyst should upload the sequence file to STARLIMS where it is merged with the analytical results data. Contact the supervisor for emergency assistance with any custom files and databases used in this method; contact the DLS LAN manager for assistance with any DLS network problems.

b. ***Sample information.***

All samples are analyzed in runs of, typically, 96 samples including 3 blank, 6 QCs and unknowns. Each run is recorded in STARLIMS that contains such information as Run and sample ID, date of analysis, analyst, sample weight, internal standard, cleanup cartridge plate information, and special notes and observations for each run. STARLIMS containing this information has been developed by this DLS and is maintained on the intranet.

c. ***Data maintenance.***

Following the analytical (LC/MS/MS) analysis, the standards and samples are processed

online using Indigo Ascent™ software, and information for each run is individually documented in Indigo Ascent™ files, which includes sample file number, sample I.D., date and time assayed, integrated peak area counts, retention times, quantitated results, etc. These files are transferred and uploaded to STARLIMS where data are checked and QCs are evaluated. The final data are stored in STRALIMS for reporting.

d. ***Information security.***

Information security is provided at multiple levels. The data systems (such as Indigo Ascent and STARLIMS) used in this work are accessed via computers that require individual login and passwords and that default to locked conditions during extended periods of nonuse. In addition, all systems and equipment are located on the Chamblee campus of CDC which has restricted access with security personnel approving all entry. Furthermore, the individual laboratory building has multiple levels of controlled access including the requirement for card keys to access the building itself, and also the individual floors where the equipment is located. Confidentiality of the results is protected by use of blind coded ID numbers only (no personal identifiers are ever used).

## 4 COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. ***Special requirements.***

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

b. ***Sample collection.***

The specimen for these analyses is human urine. Samples are collected in standard urine collection cups or vials (note: cups and all other materials contacting samples should be pre-screened and approved by this laboratory before they are used to avoid background contamination issues). Samples should be refrigerated as soon as possible after collection, and frozen for longer term storage. The sample should be well-mixed, and placed in an appropriate vial (e.g. Fluidx 10 mL vial with 2-D barcode), and the tubes capped securely. Be careful not to overfill the tube (maximum volume of approximately 8 mL for 10 mL vials) to allow for expansion in the freezer.

c. ***Sample handling.***

Specimen handling conditions for urine samples including general collection and transport requirements are outlined in the DLS protocol for urine collection and handling (available from this laboratory or the DLS specimen handling activity). In general, urine samples should be frozen at –20°C and shipped with dry ice by overnight air. A packing list must be included with the samples, and the laboratory (or the specimen handling group) should be notified before shipment. Unless special arrangements are made, shipment schedules should avoid having samples arrive at CDC on the weekends or holidays since sample handling at those times may not be appropriate. After receipt, samples are stored frozen at –20°C, or in some cases at –70°C for long-term storage.

d. ***Sample quantity.***

A minimum of 4-8 ml of urine is needed depending on the nature of the analysis (e.g. whether both free and total measurements will be made). The optimum volume is approximately 5–10 ml of urine.

e. ***Unacceptable specimens.***

Criteria for defining a sample as unacceptable include (1) use of improper collection materials or techniques leading to elevated background contamination; (2) sample volumes less than the required minimum; or (3) improper shipment or storage of samples leading to thawing, leaking, sleeve cracking or similar problems. All samples are logged in at receipt and problems with storage or shipment are identified at that point. Inadequate volumes will generally be identified when the samples are thawed for analysis. If a sample must be rejected as unacceptable, a description of the problem must be entered into the database and associated with that sample.

f. ***Long-term stability.***

Long-term stability results are currently under investigation for these analytes. Until more information is available, samples should be stored at $\leq$ –50°C and protected from light for long term storage.

## 5 PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6 PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

Note: Class A glassware such as pipets and volumetric flasks are used unless otherwise stated. The accuracy of balances, and automated pipettes should be confirmed at least annually.

a. ***Solvents and reagents. Identification and handling.***

(1) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)- this is a suspected lung carcinogen, and suitable protective clothing, gloves and eye/face protection must be utilized. It can be harmful if inhaled, swallowed or absorbed through the skin, and should only be used in a chemical safety hood. If contact occurs, flush area immediately with copious amounts of water.

(2) *N'* - nitrosonornicotine (NNN)- this is a carcinogen, and suitable protective clothing, gloves and eye/face protection must be utilized. It can be harmful if inhaled, swallowed or absorbed through the skin, and should only be used in a chemical safety hood. If contact occurs, flush area immediately with copious amounts of water.

(3) *N'*-nitrosoanatabine (NAT) – this may be harmful by inhalation, ingestion, or skin absorption. Suitable protective clothing, gloves and eye/face protection must be utilized. If contact occurs, flush area immediately with copious amounts of water.

(4) *N*'-nitrosoanabasine (NAB)- this may be harmful by inhalation, ingestion, or skin absorption. It is toxic when swallowed. Suitable protective clothing, gloves and eye/face protection must be utilized. If contact occurs, flush area immediately with copious amounts of water.

(5) Potassium Hydroxide – this is a very caustic base, corrosive to all tissues. It is used to adjust the pH of phosphate buffer. It generates considerable heat when mixed with water or an acid. It is nonflammable but would be harmful if inhaled or swallowed. Safety glasses and gloves must be worn while working with this reagent.

(6) Ammonium Acetate – this is used to make the LC mobile phase. It may be harmful by inhalation, ingestion or skin absorption. Inhalation may cause irritation to mucous membranes and the upper respiratory tract. Safety glasses and gloves must be worn while working with this reagent.

(7) Formic Acid - this is used to adjust the pH of the LC mobile phase. It will bum skin tissue and is harmful if inhaled or swallowed. If exposure occurs, flush the area with copious amounts of water. Always wear protective clothing and safety glasses when working with this reagent.

(8) Toluene - this is a flammable liquid, and also may form explosive vapors. Remember that the vapor is heavier than air and may travel some distance to an ignition source. As a liquid it is a skin irritant, and may be absorbed through the skin. Large volumes of toluene should be handled with gloves in a chemical fume hood.

(9) Methylene chloride - this solvent is chemically stable and relatively unreactive. It poses a relatively low hazard. It is not flammable, but the vapor can be irritating to the eyes, nose and throat, and skin or eye contact with the liquid should be avoided. Flush copiously with water if any contact should occur. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator, or in a chemical fume hood.

(10) Methanol - this solvent is used to pre-condition SPE columns. It is toxic by ingestion, inhalation and skin absorption. It may cause acidosis, blindness and death. It is also flammable. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator or in a chemical fume hood. Safety glasses and gloves must be worn when handling this solvent.

(11) Acetonitrile - used as a mobile phase for LC. It is toxic by ingestion, inhalation and skin absorption, and can be a source of cyanide toxicity. It is also flammable. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator, or in a chemical fume hood. Wear appropriate protective clothing to prevent skin exposure.

(12) Sodium Hydroxide – this is a very caustic base, corrosive to all tissues. It is used to adjust the pH of phosphate buffer. It generates considerable heat when mixed with water or an

acid. It is nonflammable but would be harmful if inhaled or swallowed. Safety glasses and gloves must be worn while working with this reagent.

(13) Ammonium Hydroxide - this is a caustic base, corrosive to all tissues. It is used to make the 10% ammonium hydroxide in methanol solution for sample cleanup.

(14) Hydrochloric Acid - this is a strong inorganic acid which is very corrosive to metal. May be fatal if inhaled or swallowed. Skin irritant. Open the bottle in a chemical fume hood. Wear appropriate protective clothing to prevent skin exposure.

(15) Biotage Evolute CX 96-well SPE plate, 60 mg

(16) Biotage Isolute 96-well SLE plate, 400 µL

(17) Supelco SupelMIP NNAL SPE 96-well plate, 25 mg

b. ***Stock reagent preparation***

See appendix A

c. ***Calibration materials***

See appendix B

d. ***Controls.***

***1. Quality control materials.*** **(Q1 and Q2)** There are two quality control pools for the urinary TSNA assay. Pools Q1 and Q2 represent the low and high TSNA quality control pools for total TSNA analysis. Pools Q1 and Q2 were prepared in house by spiking blank urine with TSNA standards. Pools were pooled, mixed well and 8.0 ml aliquots were dispensed into appropriate sample tubes and frozen at -70°C.

***2. Proficiency testing materials.*** **(A, B and C)** These materials are prepared from nonsmokers urine spiked at known levels with TSNAs. Three pools are prepared at known concentration levels with target amounts of approximately 25, 50 and 250 pg/ml. The pools are stored at –70ºC labeled as Pool A, B and C. Five aliquots of all 3 pools are analyzed by the standard procedure at least twice a year. The coded results are reviewed by DLS personnel not involved in the analysis to confirm acceptable method performance.

e. ***Major Instrumentation and Other Equipment.***

Liquid Handler. Hamilton MicroLab STAR, 8-channel, deepwell plate carrier.

Automation system. Caliper Staccato: Sciclone, 2D Fluidex barcode reader, Fluidex decapper, Hettich centrifuge, Inheco IVD, plate sealer, Biotage Turbovap.

HPLC. Shimadzu LC-30AD module which consists of degasser, binary pump, SIL-30AC autosampler, LC-20AD pump and CTO-30A column oven.

Mass Spectrometer. Sciex API 6500 Triple Quad Tandem Mass Spectrometer with electrospray interface.

Data System. Dell Optiplex 960 or the equivalent using Windows XP and ABSciex Analyst software v. 1.6 or higher version.

Data integration and review system. Indigo Ascent Automated Data Analysis and Review software, Indigo Biosystems, Indianapolis, IN.

Data Management System, Microsoft Access. Microsoft Corporation, Redmond, WA.

## 7 CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. *Cleaning and instrument tune procedure.*

For automation systems (Hamilton STAR and Caliper Staccato), calibration is performed once per week at 3 concentrations levels (low, medium, and high) following manufacturer's procedure. For LC/MS/MS instrument, the inlet skimmer plate is removed and the system front end is cleaned at the sign of the buildup of stain. When it is needed, the system calibration is confirmed by infusing a polypropylene glycol tune solution provided by Sciex. Overall performance and complete mass calibrations with PPGs are conducted at each preventive maintenance (1 to 2/year), following significant repairs or other changes to the instrument, and on other occasions as indicated.

### b. *Calibration Curve*

A calibration curve for this assay is based on the analysis of the standard set described in Section 6.c. A set of 14 standards ranging in value from zero to 20 ng/mL of TSNAs is analyzed in duplicate in the forward and the reverse direction prior to the start of each sample run. After adjusting for dilution, the calibration range for this method corresponds to a range of values from the limit of detection (LOD) to concentrations at the highest levels in calibration curves.

### c. *Verification*

Initial. The initial accuracy of this method was established by analyzing a series of pure standards prepared as described above. The ratio of native and labeled TSNA area counts was regressed on concentration using 1/X weighting using both Sciex system software Analyst and Indigo Ascent. The resulting calibration curves were linear to a concentration level of 20 ng/ml, and R-squared values were typically > 0.98.

Daily. Prior to assaying each run of unknowns, the results from standard analyses are reviewed for acceptable accuracy, precision and instrument sensitivity. The results from the 28 calibration standards analyzed prior to each run are reviewed daily. Acceptable back-calculated values for standards above the detection limit are typically in the range of nominal concentration ± 15% for high standards and ± 30% for low standards.

## 8 PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

When the run is prepared, each sample or control is assigned an alphanumeric laboratory ID with a five-character prefix, and a three-digit suffix in the format XXXXX-nnn, where XXXXX is the run designation (e.g., PA001) and -nnn is the sample's position in the run (e.g., 096). These working ID numbers are linked to the original IDs (sample barcodes) in the sample database. The first run in the analysis has been designated 001, with each subsequent run to increment by 1. Each run is set up in the following format: sample #001, #011 and # 53 are urine blank, #027, #033 and #77 are the low bench QC, #007, #049 and #083 are the high bench QC, and the rest of the samples are unknowns. Standards run with the samples will be named corresponding to the study. Standards IDs are TSNA_STD_XX (where XX ranges from 01 to 14) in STARLIMS. The standards run in ascending order will have a different run number than the standards run in descending order. Each run should have two different standards sets associated with it. Both the sample run number and standards run numbers should be recorded on the daily instrument log.

### a. *Sample Preparation*

See appendix C

### b. *LC/MS/MS Analysis*

1. The LC mobile phase A contains 0.08% ammonium hydroxide in water (pH = 10.50). The mobile phase B is 100% acetonitrile. Prepare the mobile phase A stock as needed and discard old stock after one week. Equilibrate the pump at least 15 minutes prior to starting the run for the day to ensure the pH is stable throughout the sample path.

2. Operate the ESI source of the API 6500 mass spectrometer at a source temperature of $600^0$C with a spray voltage of 1800 volts. Record all LC/MS/MS data in MRM (multiple reaction monitoring) mode. Data of m/z 210/180 (NNAL quantitation), m/z 210/93 (NNAL confirmation) and m/z 216/186 (NNAL ISTD) transitions are recorded for NNAL. Data of m/z 178/148 (NNN quantitation), m/z 178/105 (NNN confirmation), and m/z 182/152 (NNN ISTD) are collected for NNN. Data of m/z 190/160 (NAT quantitation), m/z 190/106 (NAT confirmation) and m/z 194/164 (NAT ISTD) transitions are recorded for NAT. Data of m/z 192/162 (NAB quantitation), m/z 192/106 (NAB confirmation) and m/z 196/166 (NAB ISTD) transitions are recorded for NAB. The total run time is 15 min. Optimize the compound-related mass spectrometric parameters for each individual ion transition. Optimize the collision gas and curtain gas for the m/z 210/180 transition. Set the analytical quadrupole to unit resolution. The typical MS parameters are as follows:

CAD: 8
CUR: 35
GS1: 80
GS2: 90
IS: 1800
TEM: 600
DP: 25
EP: 10

| Analyte | Precursor ions(Q1) | product ions(Q3) | DP[a] | CE[b] | CXP[c] |
|---|---|---|---|---|---|
| NNAL Quan[d] | 210.2 | 180.1 | 31 | 15 | 8 |
| NNAL Conf[e] | 210.2 | 93.0 | 31 | 27 | 14 |
| NNAL ISTD[f] | 216.1 | 186.1 | 31 | 15 | 10 |
| NNN Quan | 178.1 | 148.1 | 21 | 15 | 18 |
| NNN Conf | 178.1 | 105.0 | 21 | 39 | 8 |
| NNN ISTD | 182.1 | 152.1 | 21 | 15 | 12 |
| NAT Quan | 190.0 | 160.0 | 16 | 15 | 18 |
| NAT Conf | 190.0 | 106.1 | 16 | 23 | 14 |
| NAT ISTD | 194.2 | 164.1 | 16 | 15 | 18 |
| NAB Quan | 192.1 | 162.1 | 31 | 17 | 20 |
| NAB Conf | 192.1 | 106.1 | 31 | 45 | 18 |
| NAB ISTD | 196.1 | 166.1 | 31 | 17 | 18 |

[a] Declustering Potential
[b] Collision energy
[c] Collision cell exit potential
[d] Quantitation ion
[e] Confirmation ion
[f] Internal standard ion

3. HPLC separation is achieved using a Gemini-NX C18 column (3 µm, 2.0x150 mm) or the equivalent, purchased from Phenomenex (Torrance, CA). Maintain the column temperature at ca. $50^0$C and the eluant flow rate at 0.600 ml/min. The third pump is 100% acetonitrile with flow rate 0.40ml/min. Injection volume is 5 uL for standards and unknowns.

4. A typical LC gradient program is as follows:

| Time (min) | Mobile phase A | Mobile phase B | Flow rate (ml/min) |
|---|---|---|---|
| 0.00 | 97% | 3% | 0.6 |
| 9.00 | 70% | 30% | 0.6 |
| 10.50 | 70% | 30% | 0.6 |
| 10.51 | 0% | 100% | 0.6 |
| 12.00 | 0% | 100% | 0.6 |
| 12.01 | 97% | 3% | 0.6 |
| 14.98 | 97% | 3% | 0.6 |

5. Prior to performing a run, inject the 1st TSNA standard to determine accurate system operation and suitable sensitivity.

6. Immediately after the run, wash the LC column using 100 % B for 60 minutes and place the system in standby (see appendix D).

c. ***Data Processing***

1. The peak integration is automatically performed by Indigo Ascent™. Quality control rules are established in Assay Configuration. A complete list of QC rules can be found in Appendix F.

2. After the sample is quantitated by Indigo Ascent™ software, each peak integration result is checked manually and re-integrated as needed. Peaks with low quality scores are flagged, and the analyst confirms automatically generated flags or adds flags as necessary. A second analyst reviews the data again and updates the Indigo Ascent™ review status to Certified. The quantitation result file is downloaded from Indigo Ascent™

3. After the quantitation result file is generated, it is uploaded to the Microsoft Access database that serves as the TSNA assay repeat manager. Results with flags are marked as “Pending” in the database. Results with no flags are marked “Send.” Results that are repeat analyses for which there is already a result marked “Send” are automatically marked “Don’t Send.” Blanks are evaluated by the user. A sample run fails blank evaluation if the average calculated concentration of the three water blanks exceeds LOD. If a sample run fails blank evaluation, unknown samples with calculated concentrations below 20x the mean blank concentration are marked “Don’t Send.”

   A blank subtraction is performed by subtracting the average calculated concentration of the three water blanks from each Unknown and QC calculated concentration. Individual data flags are evaluated by the user. Only data that is marked “Send” is exported to a file that is compatible with STARLIMS and is uploaded to STARLIMS. QCs are evaluated in STARLIMS. If any QCs fail STARLIMS evaluation, the user updates affected results to “Don’t Send” in the repeat manager database and uses the database to generate a list of sample IDs that must be staged for repeat analysis. After repeats are analyzed and the data are processed through Indigo Ascent, they are uploaded into repeat manager. These results are compared with previous results, and the process is repeated until there is a result marked “Send” for every sample-analyte result.

d. ***Calculations***

The results are reported in ng/mL urine or it can be expressed relative to the urine creatinine value when available: TSNA (ng/ml)/Creatinine (mg/ml) = TSNA ng/mg creatinine.

## 9 REPORTABLE RANGE OF RESULTS

a. ***Linearity Limits***

Samples are obtained from both smokers and non-smokers. Therefore, a broad range of urine TSNA levels can be expected. If the value of a sample exceeds the highest point on the

standard curve, the sample is repeated using a smaller sample aliquot and re-analyzed.

b. ***Limit of Detection***

We followed DLS Policy and Procedure Manual Section 22 limit of Detection (LOD) to determine the LODs for 4 TSNAs. TSNA pools were spiked at 4 levels: 0, 1, 2 and 5 pg/mL. These pools were analyzed in 60 different runs over a few months. The instructions in Policy and Procedure manual were followed to calculate the
LODs for all 4 TSNAs.

The calculated LODs are listed below and Appendix E:

| ANALYTE | LOD (ng/mL) |
|---|---|
| NNAL | 0.0006 |
| NNN | 0.0028 |
| NAB | 0.0016 |
| NAT | 0.0042 |

c. ***Precision***

The precision of the method is reflected in the variance of quality control samples analyzed over time. The coefficient of variation (CV) of the method was determined based on 20 independent analyses of the QC samples. The precision study was performed for total TSNA analysis. (Appendix E)

d. ***Accuracy***

The accuracy of the assay is established by blind analysis of Proficiency Testing (PT) samples. The PT samples were spiked in blank urine at three different concentration levels. The accuracy study was performed for total TSNA analysis. (Appendix E)

e. ***Analytical Specificity***

Analytical specificity in this method is mainly conferred through the use of tandem mass spectrometry which is a very specific technique. Further assurance of the identity of the analyte in unknowns is provided by comparison of retention times during HPLC of the unknowns with that observed with standards; by coelution of the analyte with the labeled internal standard; and by the calculation of appropriate confirmation ion ratios.

f. ***Carryover***

Carryover effects were investigated in urine samples at TSNA concentrations up to ULOQ. No carryover was observed between samples in these evaluations. Samples above 20 ng/mL are subject to be repeated in dilution. The following sample will be repeated too.

g. ***Freeze-Thaw, Bench-Top Stability and Storage Stability***

Freeze-thaw stability: The stability of TSNA was analyzed for 3 freeze-thaw cycles using 2 spiked urine pools. Three replicates were subjected to 0 to 3 freeze thaw cycles with the samples removed from the -70°C freezer and allowed to stand at room temperature for 1 hour before refreezing. Bench-top stability: The three samples of 2 spiked urine pools (not yet prepared for instrument analysis) stored at room temperature for 1 day and stored at -70°C freezer. All the samples were then analyzed and data were calculated and summarized in Appendix E. For the long term storage stability: we monitor our spiked urine pools (QCL and QCH) from the fresh prepared time, 1 years and 2 years storage at -70°C freezer. The data are summarized in Appendix E.

h. ***Ruggedness validation***

The following four factors that may influence the accuracy of the method were tested for ruggedness validation:

1. The centrifuge duration before SPE by CX. The duration of 5, 10, and 15 min was evaluated and the final method uses 10 min.
2. The time course for TSNA hydrolysis. The time course of 0-48 hours was examined and the final method uses 24 hours (see Appendix G).
3. % of ammonium hydroxide in LC mobile phase. The % of ammonium hydroxide was examined at 0.03%, 0.05%, 0.08%, 0.1%, and was optimized at 0.08% (see Appendix G).
4. The pH of phosphate buffer. The pH of phosphate was examined from 5 to 10, and was validated at 6.4 in the final method (see Appendix G).

## 10 Quality Assessment and Proficiency Testing

There are several human urine pools that are used for QC in this analysis. All pools were subjected to an initial characterization run series with at least 20 replicates over a 2-week period. The preparation of these pools was described previously in section 6.d. In addition, water blanks are included in all sample runs.

a. ***QUALITY CONTROL (QC) PROCEDURES***

1. The water blanks for TSNAs are typically negative or below LOD for the method (non-detectable). For the lower concentration sample, the average blank value is not higher than the LOD. In the higher concentration samples plates, the sample concentration is less than 20 times of average of the blank, these samples need to repeat. If a calculated concentration of any amount is obtained, the blank value will be subtracted from the sample value.

2. For the bench QC, the run is rejected if any pool is outside of the 3-sigma limits of the mean, or if any two pools are outside of 2-sigma limits in the same direction. All data are periodically batched and analyzed by using the Division of Laboratory Science SAS-QC program incorporating standard criteria for analysis. Any run failing the DLS SAS QC analysis is repeated if sufficient sample volumes exist; otherwise, no quantitative results for the samples analyzed in that run are reported.

3. TSNA concentrations are checked to make certain the values are within the range of the method. The actual measured concentration must be no greater than the highest standard value. If above that limit, the sample must be diluted and reanalyzed if sufficient volume exists. In addition, expected ion ratios for confirmation and quantitation ions, the expected retention times, etc. are checked for each sample.

b. ***Proficiency Testing***

1. Proficiency testing is performed at least semi-annually. Currently, no external source of PT materials is available. Therefore, PT assays are conducted using nonsmokers' urine spiked with known amounts of TSNA. In addition, split sample comparisons with an external reference laboratory are conducted periodically to help confirm our results.

2 Analytical PT results are reviewed by the analyst and the supervisor. Acceptable results require that > 80% of the results agree with the target value ± 25%. If the assay fails PT, all analyses are stopped and the source of 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 SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. ***Calibration.***

System calibration and general readiness is assessed on a daily basis from a review of the instrument's operating conditions, the values for the water blanks, and the results of the pre-run standard (e.g. Internal standard area counts and calculated concentration). When corrective actions are indicated, they are performed and the system is re-evaluated with additional standards until acceptable results are obtained before any unknowns are analyzed.

b. ***Quality Control.***

If the results from analysis of QC samples are outside the acceptable limits and a reason is identified for the apparent problem, it is indicated and the run is scheduled for repeat sample preparation and analysis for samples that have sufficient quantity. If the problem is not identified, sample preparation and analysis is suspended until the problem or problems are discovered and corrected for each analyte. Any questionable sample identified by QC or individual sample evaluation that cannot be confirmed by repeat analysis is not included for the affected analyte in the reportable database of results.

## 12 LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Some plastic materials, solvents, air and water may provide trace amounts of TSNAs (especially NNAL and NNN), which could contribute as a contaminant to the level measured in the urine sample. Besides, some interfering substances do exist in some urine samples. However, this issue can be resolved by monitoring the confirmation ratio. In addition, the specificity of LC/MS/MS detection helps to avoid background chemical interferences with the quantitation ion.

## 13 REFERENCE RANGES (NORMAL VALUES)

Study populations typically include both smokers and non-smokers, therefore, a large range of TSNA levels in urine are expected. The geometric mean concentration of NNAL in nonsmokers is below LOD, with 75$^{th}$ and 95$^{th}$ percentile to be 0.0027 and 0.0244 ng/mL respectively (4). The reference range of NNAL in smokers is 0.333 ng/mL at 50$^{th}$ and 1.86 ng/mL at 95$^{th}$ percentile, with a mean concentration of 0.299 ng/mL (5). No defined reference range currently exists for NNN, NAB or NAT. For total NNN, NAT and NAB, the average levels for smokers ranged from 4 to 47 pg/mL, 50 to 160 pg/mL and 10 to 47 pg/mL, respectively (6-7). For our method, expected levels for both free and total TSNA levels are currently under investigation.

## 14 CRITICAL CALL RESULTS (“PANIC VALUES”)

Not applicable for this procedure.

## 15 SPECIMEN STORAGE AND HANDLING DURING TESTING

Samples are received frozen and typically stored frozen at or below –20 °C until analysis. After samples are aliquoted, the remainder of the samples is returned to freezer at -70°C until duplicate analysis is completed or for repeat analysis if required.

## 16 ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If a problem with the method exists, samples are held in the freezer until the problem can be resolved. If necessary, filtered and extracted samples ready for analysis can be stored, well-sealed, at –70 °C for at least 1 month before they are assayed.

## 17 TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable at this time.

## 18 TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND ATRACKING

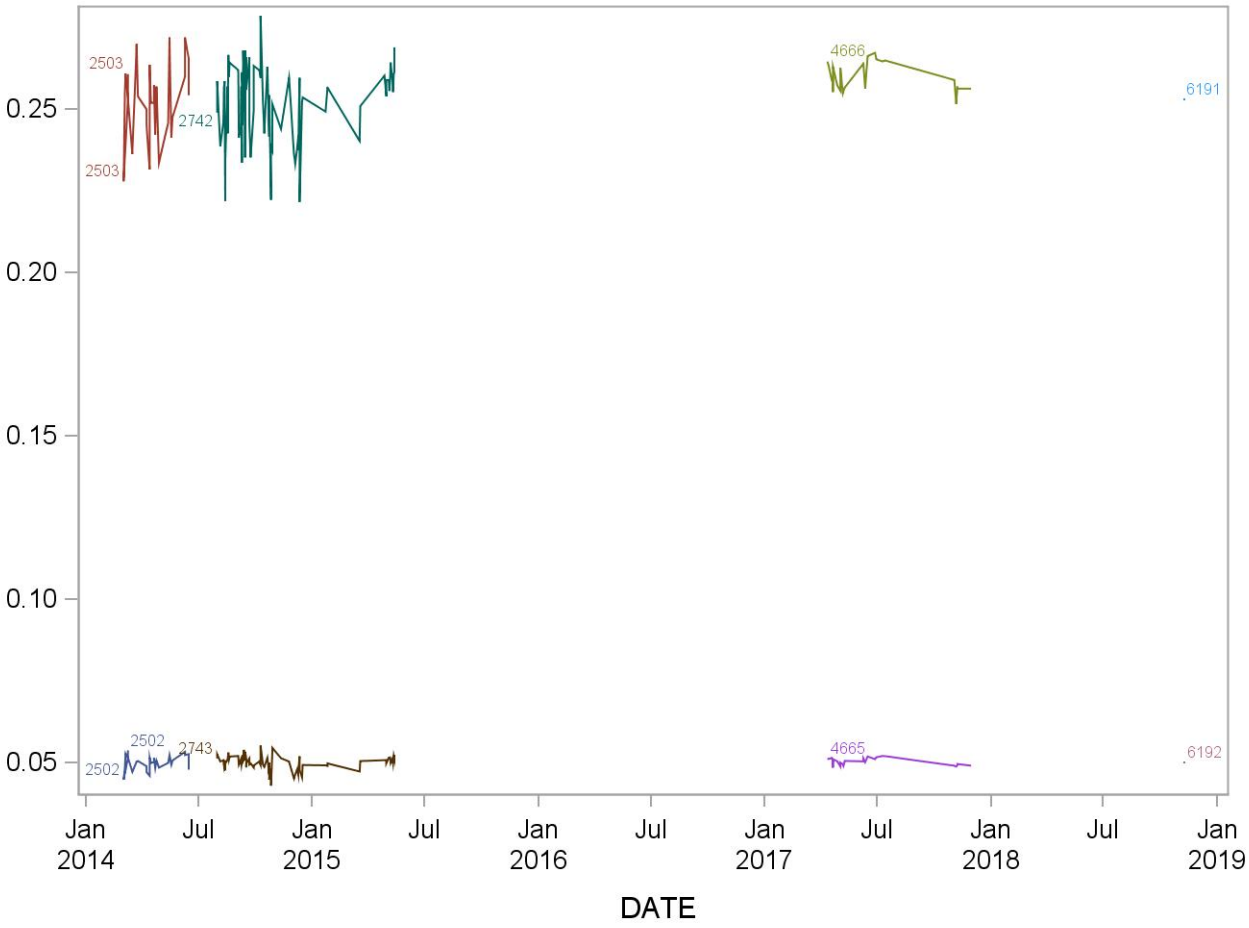
Following analysis, residual samples, if available, are held in storage at approximately -70°C in Chamblee buildings.

## 19 Summary Statistics and QC graphs

See following pages.

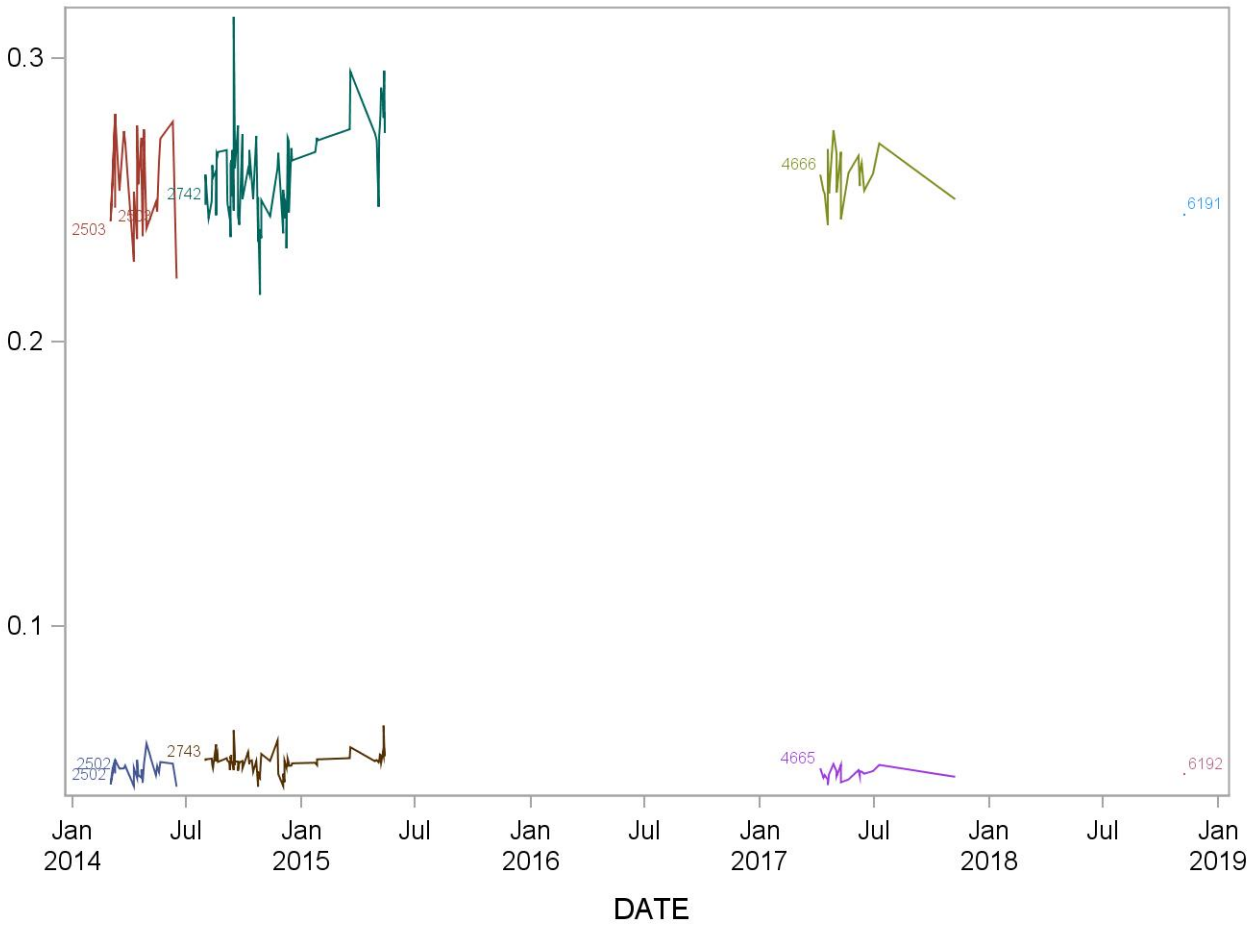
## 2013-2014 Summary Statistics and QC Chart for N'-Nitrosanabasine, urine (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 2503 | 25 | 03MAR14 | 16JUN14 | 0.25205 | 0.01215 | 4.8 |
| 2502 | 25 | 03MAR14 | 16JUN14 | 0.04992 | 0.00223 | 4.5 |
| 2503 | 2 | 10MAR14 | 10MAR14 | 0.25702 | 0.00535 | 2.1 |
| 2502 | 2 | 10MAR14 | 10MAR14 | 0.05273 | 0.00160 | 3.0 |
| 2742 | 79 | 01AUG14 | 14MAY15 | 0.25238 | 0.01184 | 4.7 |
| 2743 | 79 | 01AUG14 | 14MAY15 | 0.05021 | 0.00219 | 4.4 |
| 4666 | 25 | 12APR17 | 30NOV17 | 0.26005 | 0.00446 | 1.7 |
| 4665 | 25 | 12APR17 | 30NOV17 | 0.05042 | 0.00112 | 2.2 |



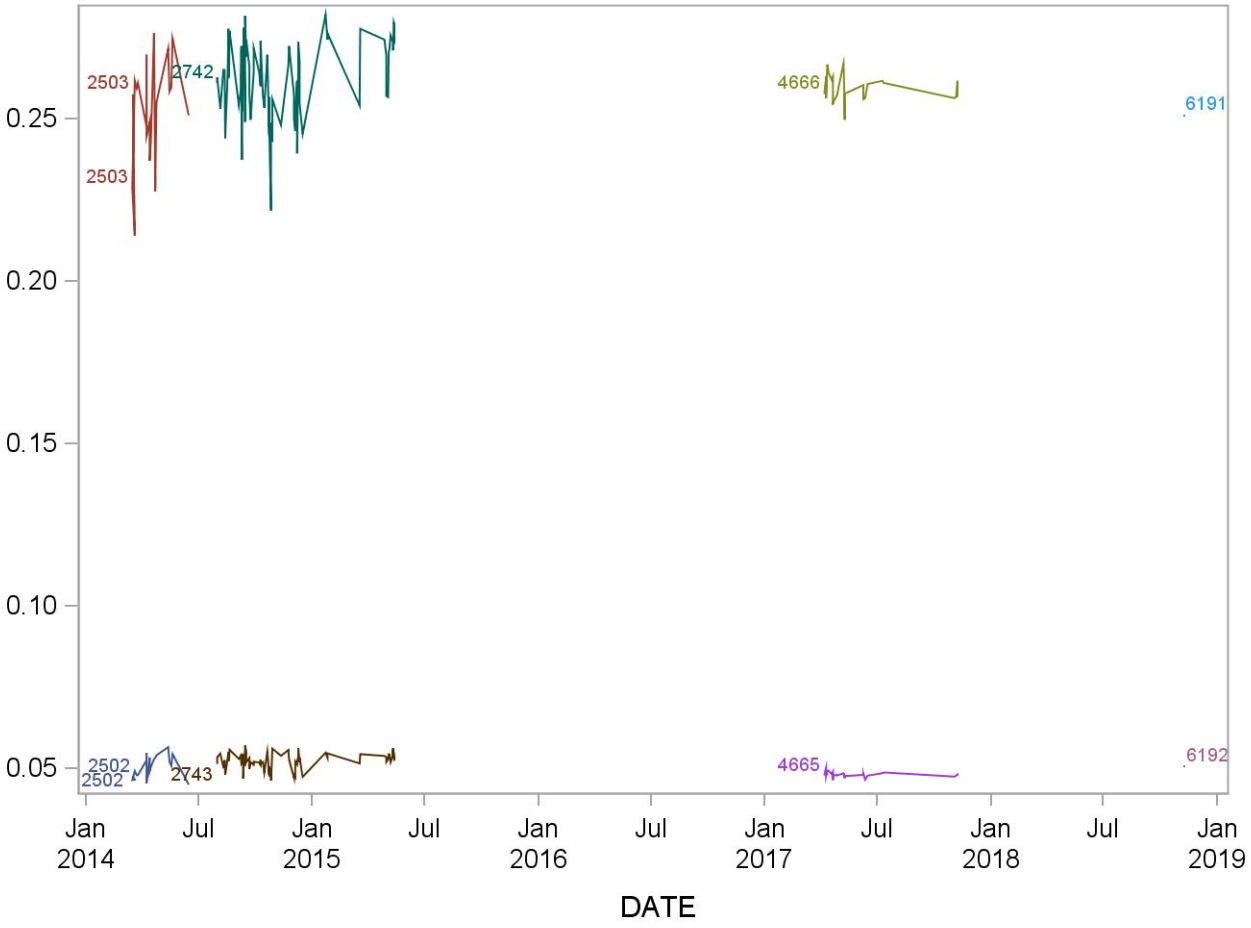
## 2013-2014 Summary Statistics and QC Chart for N'-Nitrosanatabine, urine (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 2503 | 23 | 03MAR14 | 16JUN14 | 0.25636 | 0.01681 | 6.6 |
| 2502 | 23 | 03MAR14 | 16JUN14 | 0.04897 | 0.00351 | 7.2 |
| 2503 | 2 | 10MAR14 | 10MAR14 | 0.26388 | 0.02341 | 8.9 |
| 2502 | 2 | 10MAR14 | 10MAR14 | 0.05027 | 0.00335 | 6.7 |
| 2742 | 80 | 01AUG14 | 14MAY15 | 0.26074 | 0.01626 | 6.2 |
| 2743 | 80 | 01AUG14 | 14MAY15 | 0.05218 | 0.00346 | 6.6 |
| 4666 | 20 | 07APR17 | 08NOV17 | 0.25830 | 0.00876 | 3.4 |
| 4665 | 20 | 07APR17 | 08NOV17 | 0.04781 | 0.00202 | 4.2 |



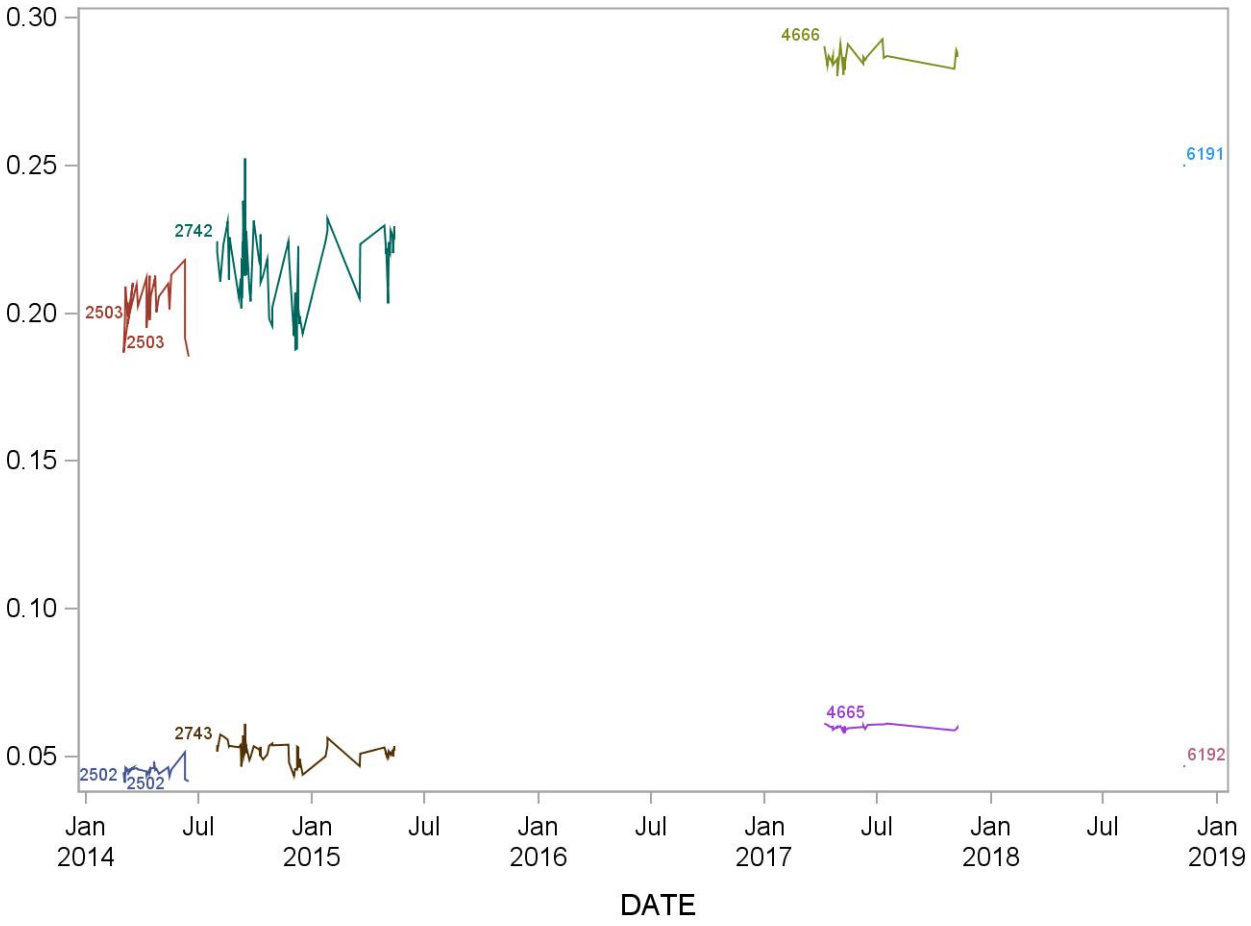
## 2013-2014 Summary Statistics and QC Chart for N'-Nitrosonornicotine, urine (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 2503 | 18 | 17MAR14 | 16JUN14 | 0.25424 | 0.01449 | 5.7 |
| 2502 | 18 | 17MAR14 | 16JUN14 | 0.05085 | 0.00340 | 6.7 |
| 2503 | 2 | 18MAR14 | 21MAR14 | 0.23570 | 0.03078 | 13.1 |
| 2502 | 2 | 18MAR14 | 21MAR14 | 0.04700 | 0.00071 | 1.5 |
| 2742 | 82 | 01AUG14 | 14MAY15 | 0.26252 | 0.01197 | 4.6 |
| 2743 | 82 | 01AUG14 | 14MAY15 | 0.05231 | 0.00248 | 4.7 |
| 4666 | 25 | 07APR17 | 08NOV17 | 0.25916 | 0.00402 | 1.5 |
| 4665 | 25 | 07APR17 | 08NOV17 | 0.04804 | 0.00084 | 1.7 |



## 2013-2014 Summary Statistics and QC Chart for Urinary Total NNAL (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| 2503 | 22 | 03MAR14 | 16JUN14 | 0.20377 | 0.00897 | 4.4 |
| 2502 | 22 | 03MAR14 | 16JUN14 | 0.04522 | 0.00220 | 4.9 |
| 2503 | 3 | 10MAR14 | 18MAR14 | 0.20347 | 0.00694 | 3.4 |
| 2502 | 3 | 10MAR14 | 18MAR14 | 0.04476 | 0.00130 | 2.9 |
| 2742 | 67 | 01AUG14 | 14MAY15 | 0.21659 | 0.01298 | 6.0 |
| 2743 | 67 | 01AUG14 | 14MAY15 | 0.05172 | 0.00336 | 6.5 |
| 4666 | 29 | 07APR17 | 08NOV17 | 0.28594 | 0.00298 | 1.0 |
| 4665 | 29 | 07APR17 | 08NOV17 | 0.05991 | 0.00078 | 1.3 |



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# Appendix A

## Stock reagent preparation

### 1. TSNA solutions preparation

β-Glucuronidase Enzyme –
For a set of 96 samples, weigh approximately 0.2169g of 1,660,000 units/g enzyme into a scintillation vial. Store in -20°C freezer until needed. On preparation date of used, add 18mL of phosphate buffer solution into vial.
*If run is smaller than 96 samples, ask for special preparation instructions.

5% Ammonia (28.8%, 14.8N) in Methanol –
In a 1L flask, combine 50mL of ammonium hydroxide (28.8%, 14.8N) with 950mL of methanol. Store in fume hood.

2% Formic Acid in Water –
In a 1L flask, combine 20mL formic acid with 980mL of water. Store in fume hood.

10N Sodium Hydroxide (NaOH) –
Weight 48g of sodium hydroxide pellets for 120mL of water; you can used 6g of sodium hydroxide pellets for each addition 12mL of water desired. First add pellets slowly to flask. Then, fill to respective water level. Make sure cap is on tight, prior to inverting. Invert as needed until all pellets are dissolved (CAUTION: Flask gets very hot!). Store in fume hood.

0.1N Hydrochloric Acid (HCl) –
For preparation in a 2L flask, use 16.6mL of concentrated hydrochloric acid. For preparations of 1L, use 8.3mL of concentrated hydrochloric acid. Pour water into flask (about half full), add designated amount of concentrated hydrochloric acid, then fill to capacity with water. Place top on flask and invert to mix. Store in fume hood.
*Make sure to neutralize glassware with water when finished.
**Place used volumetric pipets into pipet washer.

0.5N ($PO_4$) Phosphate Buffer (pH 6.4) –
For 2L Solution – Weigh 136.09g of potassium phosphate, monobasic into flask. Bring up to 2L with graduated cylinder. Mix solution until pellets dissolve completely. Calibrate your pH meter (using the instructions on the fume hood), then place probe into mixed solution. Adjust pH to 6.4 using potassium hydroxide (KOH) pellets. Mix and store in refrigerator.

90:10 Toluene: Dichloromethane –
Using a graduated cylinder, prepare 500mL of solution. For 450mL of toluene into cylinder, then add 50mL of dichloromethane. To prepare 250mL, use 225mL of toluene and 25mL of dichloromethane. Store in fume hood.

Calibration Solution (Low Volume- 3:100) – Add 15ml of dye solution into 500ml volumetric flask and dilute to volume with water. Prepare as needed and store in fume hood.

Calibration Solution (High Volume 1:200) - Add 2.5 ml of dye solution into a 500ml volumetric flask and dilute to volume with water. Prepare as needed and store in fume hood.

## 2. SLE plate solutions preparation

0.5N Potassium Hydroxide (KOH) –

Using a 1L flask, pour in 100mL of 5N KOH and then fill to volume with water.

*To make 5N KOH, add 159.4g of KOH pellets into a 500mL flask, then fill to volume with water.

# Appendix B

## Calibration materials

The stock solutions were stored in freezer in Building103/3318. The internal standard spike solutions and working standards are prepared as needed. A total of 14 standards were prepared ranging from 0 to 20 ng/ml. The internal standard spiking solution was prepared as a 4 ng/ml solution in water, and is stored in freezer in 103/3318.

| Standard name | Purity | Method | Catalog # |
|---|---|---|---|
| 4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanol | 99% | $^{1}$H NMR/MS | M325740 |
| 4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanol-1,2',3',4',5',6'-13C6 | 99% | $^{1}$H NMR/MS | M325741 |
| Rac-N'-Nitroso Nornictine | 99% | $^{1}$H NMR/MS | N535000 |
| Rac-N'-Nitroso Nornictine-$D_4$ | 99% | $^{1}$H NMR/MS | N535002 |
| (R,S)-Nitroso Anatabine | 99% | $^{1}$H NMR/MS | N524750 |
| (R,S)-Nitroso Anatabine-2,4,5,6-d4 | 99% | $^{1}$H NMR/MS | N524752 |
| (R,S)-Nitroso Anabasine | 99% | $^{1}$H NMR/MS | N524250 |
| (R,S)-Nitroso Anabasine-d4 | 99% | $^{1}$H NMR/MS | N524252 |
| 4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanone | 99% | $^{1}$H NMR/MS | M325750 |
| 4-(Methylnitrosoamino)-1-(3-pyridyl-d4)-1-butanone | 99% | $^{1}$H NMR/MS | M325751 |

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Toronto Research Chemicals M-325740, $C_{10}H_{15}N_3O_2$, Mol Wt 209.12.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol - 1,2',3',4',5',6'-$^{13}C_6$ (NNAL-$^{13}C_6$). Cambridge Isotope Laboratories, Mol Wt 215.2.

*N'* -nitrosonornicotine (NNN). Toronto Research Chemicals N-535000, $C_9H_{11}N_3O$, Mol Wt 177.20.

*N'* –nitrosonornicotine-d4 (NNN-d4), Toronto Research Chemicals N-535002, Mol Wt 181.23.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Toronto Research Chemicals M-325750, $C_{10}H_{13}N_3O_2$, Mol Wt 207.23.

4-(Methylnitrosamino)-1-(3-pyridyl-d4)-1-butanone (NNK-d4), Toronto Research Chemicals M-325751, Mol Wt 211.25.

*N'*-nitrosoanatabine (NAT), Toronto Research Chemicals N-524750, $C_{10}H_{11}N_3O$, Mol Wt 189.21.

*N'*-nitrosoanatabine-d4 (NAT-d4), Toronto Research Chemicals N-524752, Mol Wt 193.24.

*N*'-nitrosoanabasine (NAB), Toronto Research Chemicals N-524252, Mol Wt 191.23.

*N*'-nitrosoanabasine-d4 (NAB-d4), Toronto Research Chemicals N-524250, $C_{10}H_{13}N_3O$, Mol Wt 195.25.

Original stocks:

Stock NNAL O: dissolve 13.05 mg of (99.9+%) NNAL in 50 ml water. 261,000 ng/ml

Stock NNN O: dissolve 14.02 mg of (99.9+%) NNN in 50 ml water. 280,000 ng/ml

Stock NNK O: dissolve 13.43 mg of (99.9+%) NNK in 50 ml water. 268,600 ng/ml

Stock NAT O: dissolve 13.44 mg of (99.9+%) NAT in 50 ml water. 268,800 ng/ml

Stock NAB O: dissolve 15.39 mg of (99.9+%) NAB in 50 ml water. 307,800 ng/ml

Stock NNAL-$^{13}C_6$ O: dissolve 1 mg of (99.9+%) NNAL-$^{13}C_6$ in 100 ml water. 10,000 ng/ml

Stock NNN-D4 O: dissolve 1 mg of (99.9+%) NNN-D4 in 100 ml water. 10,000 ng/ml

Stock NNK-D4 O: dissolve 1 mg of (99.9+%) NNK-D4 in 100 ml water. 10,000 ng/ml

Stock NAT-D4 O: dissolve 1 mg of (99.9+%) NAT-D4 in 100 ml water. 10,000 ng/ml

Stock NAB-D4 O: dissolve 1 mg of (99.9+%) NAB-D4 in 100 ml water. 10,000 ng/ml

Working Stocks:

Level NNAL A: dilute Stock NNAL O with water (19.16 ml of Stock Native O q.s. to 50 ml); 100,000 ng/ml.

Level NNN A: dilute Stock NNN O with water (17.83 ml of Stock Native O q.s. to 50 ml); 100,000 ng/ml.

Level NNK A: dilute Stock NNK O with water (18.62 ml of Stock Native O q.s. to 50 ml); 100,000 ng/ml.

Level NAT A: dilute Stock NAT O with water (18.60 ml of Stock Native O q.s. to 50 ml); 100,000 ng/ml.

Level NAB A: dilute Stock NAB O with water (16.24 ml of Stock Native O q.s. to 50 ml); 100,000 ng/ml.

Mixed Level B: Dilute from NNAL A, NNN A, NNK A, NAT A and NAB A. The final concentration contains 10,000 ng/mL of NNAL, NNN, NNK, NAT and NAB.

Mixed Level C: Dilute from mixed level B. The final concentration contains 1,000 ng/mL of NNAL, NNN, NNK, NAT and NAB.

Mixed Level D: Dilute from mixed level C. The final concentration contains 100 ng/mL of each NNAL, NNN, NNK, NAT and NAB.

Mixed Level E: Dilute from mixed level D. The final concentration contains 10 ng/mL of NNAL, NNN, NNK, NAT and NAB.

Mixed Level F: Dilute from mixed level E. The final concentration contains 1 ng/mL of NNAL, NNN, NNK, NAT and NAB.

Internal Standard Level A: dilute from the original stocks of the internal standards. The final internal standard A contains 1000 ng/mL of NNAL-$^{13}C_6$, NNN-D4, NNK-D4, NAT-D4 and NAB-D4.

Internal Standard Level B: dilute from Internal Standard Level A. The final Internal Standard Level B contains 100 ng/mL of NNAL-$^{13}C_6$, NNN-D4, NNK-D4, NAT-D4 and NAB-D4.

Internal Standard Level C (ISC): dilute from Internal Standard Level A. The final ISC contains 40 ng/mL of NNAL-$^{13}C_6$, NNN-D4, NNK-D4, NAT-D4 and NAB-D4.

Internal Standard Spike Solution: Dilute from Internal Standard Level B. The final internal standard spike solution contains 4 ng/mL of NNAL-$^{13}C_6$, NNN-D4, NNK-D4, NAT-D4 and NAB-D4.

Below is a complete list of the TSNA standards:

| STD | ng/ml | Final volume<br>ml | Total ng<br>Stock | Stock | Volume of stock<br>ml | Total ng<br>ISTD | Volume of ISTD A<br>ml | ISTD<br>ng/ml |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| 1 | 0 | 100 | 0 | NA | 0 | 40 | 0.04 | 0.4 |
| 2 | 0.0025 | 100 | 0.25 | Level F (1 ng/ml) | 0.25 | 40 | 0.04 | 0.4 |
| 3 | 0.005 | 100 | 0.5 | Level F (1 ng/ml) | 0.5 | 40 | 0.04 | 0.4 |
| 4 | 0.01 | 100 | 1 | Level F (1 ng/ml) | 1 | 40 | 0.04 | 0.4 |
| 5 | 0.02 | 100 | 2 | Level E (10 ng/ml) | 0.2 | 40 | 0.04 | 0.4 |
| 6 | 0.04 | 100 | 4 | Level E (10 ng/ml) | 0.4 | 40 | 0.04 | 0.4 |
| 7 | 0.1 | 100 | 10 | Level E (10 ng/ml) | 1 | 40 | 0.04 | 0.4 |
| 8 | 0.2 | 100 | 20 | Level D (100 ng/ml) | 0.2 | 40 | 0.04 | 0.4 |
| 9 | 0.4 | 100 | 40 | Level D (100 ng/ml) | 0.4 | 40 | 0.04 | 0.4 |
| 10 | 0.8 | 100 | 80 | Level D (100 ng/ml) | 0.8 | 40 | 0.04 | 0.4 |
| 11 | 2 | 100 | 200 | Level C (1000 ng/ml) | 0.2 | 40 | 0.04 | 0.4 |
| 12 | 5 | 100 | 500 | Level C (1000 ng/ml) | 0.5 | 40 | 0.04 | 0.4 |
| 13 | 10 | 100 | 1000 | Level C (1000 ng/ml) | 1 | 40 | 0.04 | 0.4 |
| 14 | 20 | 100 | 2000 | Level B (10000 ng/ml) | 0.2 | 40 | 0.04 | 0.4 |

# Appendix C

### Sample Preparation

## TSNA Sample Clean-up Procedure for Total TSNA

1. **Sample Hydrolysis**

a. Remove samples from freezer and allow them to thaw completely. Let ISTD come to room temperature.
b. Pipet 50 µl ISTD into each well on a deep 96-well collection plate (CP#1).
c. Pipet 1.7 mL urine sample into each well.
d. Pipet 170 µl of β-glucuronidase solution (20,000 u/ml) into each well.
e. Mix well (aspirate and dispense 3 x).
f. Place CP#1 in water bath that is set to 37°C for 24 hours to hydrolyze. Leave the shaking function on (at settting = 40 rpm) during the incubation.

2. **Solid Phase Extraction by CX**

a. Condition the CX 96-well plate with 1.5 ml of 5% ammonia in methanol, followed by 1.5ml mL of H2O and 1.5 mL of 2% formic acid in H2O at 2 ml/min.
b. If the plate has been refrigerated, place the plate in 37°C water bath for 30 min with shaking function on.
c. Centrifuge CP#1 at 6000 rpm for 10 min.
d. Transfer 1800 µl of supernatant in each well in CP#1 to CX 96-well plate (avoid disturbing silt at bottom of the well).
e. Add 40 µL formic acid into each well in CX plate.
f. Mix well (aspirate and dispense 3 x).
g. Apply pressure to CX plate so that the sample is drawn through the plate at a rate of about 0.5 ml/min.
h. Once all of the samples have loaded, allow the columns to dry under nitrogen at 10 psi for 1 min.
i. After drying, the plate is washed at 10 psi with 1.5 ml of 2% formic acid in H2O, followed by 1.5 mL of methanol.
j. Dry the plate under nitrogen at 10 psi for 5 min.
k. The analyte is eluted at 0.5 ml/min with 0.6 ml of 5% ammonia in methanol into a new 96-well collection plate (CP#2).
l. Dry down CP#2 at 38 psi and 38 $^0$C for 30 min.
m. Eluted again at 0.5 ml/min with 0.6 ml of 5% ammonia in methanol into the 96-well collection plate (CP#2).
n. Dry down CP#2 at 38 psi and 38 $^0$C for 30 min.
o. Eluted again at 0.5 ml/min with 0.6 ml of 5% ammonia in methanol into the 96-well collection plate (CP#2).
p. Dry down CP#2 at 38 psi and 38 $^0$C for 68 min.
q. The residue is reconstituted in 300 µL water.

3. **Liquid-liquid Extraction by SLE (96-well Isolute)**

a. Pipet 30 μl 10N NaOH (freshly made daily, 2 grams in 5 ml water) to each well in CP#2.
b. Mix well (aspirate and dispense 3 x).
c. Apply the entire sample to a pre-washed SLE plate.
d. Allow to stay on the column for 4 min.
e. Add 3 x 0.65 ml of methylene chloride to the column. Allow to elute by positive pressure. Collect methylene chloride fraction a new deep 96-well plate (CP#3).
f. Dry down CP#3. The residue is reconstituted in 800 µL methylene chloride.

**1. Liquid-liquid extraction by HCl**

a. Add 0.8 ml of 0.1 N HCl into each well in CP#3.
b. Mix for 10 min (aspirate and dispense 10x).
c. Centrifuge the plate for 10 min at 3000 rpm.
d. Transfer HCl layer into a new 96-well collection plate CP#4.
e. Add 10 μl 10N NaOH into each well in CP#4 to neutralize HCl layer.
f. Add 1 ml PO4 buffer into each well in CP#4. Mix well (aspirate and dispense 3 x).

**6. MIP SPE procedure**

a. Condition the MIP 96-well plate with 1 ml methylene chloride, followed by 1 ml methanol and 1 ml water at 2 ml/min.
b. Vortex the sample and load sample on MIP plate. Adjust the pressure so that the sample is drawn through the plate at a rate of about 0.5 ml/min.
c. Once all of the samples have loaded, allow the columns to dry under nitrogen at 10 psi for 1 min.
d. After drying, the plate is washed at 10 psi with 2 x 1 ml water, followed by vacuum drying for 10 min.
e. The plate is then washed by 1 ml toluene and 1 ml toluene/methylene chloride (9:1).
f. Dry the plate at 30 psi for 10 min to remove toluene.
g. The analyte is eluted at 0.5 ml/min with 1.5 ml methylene chloride into a new 96-well collection plate (CP#5), and dry down methylene chloride.
h. Elute the analyte with a second aliquot of 1.5 ml methylene chloride into the same 96-well collection plate (CP#5).
i. The elutent is dried down in the plate CP#5. The residue is reconstituted in 40 µl water. 5 µl is injected into the LC for analysis.

## TSNA Sample Clean-up Procedure for Free TSNA

1. **Solid Phase Extraction by CX**

a. Pipet 1700 μl of sample into each well in a deep 96-well collection plate (CP#1).
b. Pipet 50 μl ISTD into each well in CP#1.
c. If the plate has been refrigerated, place the plate in 37°C water bath for 30 min with shaking

function on.
d. Centrifuge CP#1 at 6000 rpm for 10 min.
e. Condition the CX 96-well plate with 1.5 ml of 5% ammonia in methanol, followed by 1.5 mL of H2O and 1.5 mL of 2% formic acid in H2O at 2 ml/min.
f. Transfer 1700 µl of supernatant in each well in CP#1 to CX 96-well plate (avoid disturbing silt at bottom of the well).
g. Add 40 µL formic acid into each well in CX plate.
h. Mix well (aspirate and dispense 3 x).
i. Apply pressure to CX plate so that the sample is drawn through the plate at a rate of about 0.5 ml/min.
j. Once all of the samples have loaded, allow the columns to dry under nitrogen at 10 psi for 1 min.
k. After drying, the plate is washed at 10 psi with 1 ml of 2% formic acid in H2O, followed by 1 mL of methanol.
l. Dry the plate under nitrogen at 10 psi for 2 min.
m. The analyte is eluted at 0.5 ml/min with 0.6 ml of 5% ammonia in methanol into a new 96-well collection plate (CP#2).
n. Dry down CP#2 at 38 psi and 38 $^0$C for 30 min.
o. Eluted again at 0.5 ml/min with 0.6 ml of 5% ammonia in methanol into the 96-well collection plate (CP#2).
p. Dry down CP#2 at 38 psi and 38 $^0$C for 30 min.
q. Eluted again at 0.5 ml/min with 0.6 ml of 5% ammonia in methanol into the 96-well collection plate (CP#2).
r. Dry down CP#2 at 38 psi and 38 $^0$C for 68 min.
s. The residue is reconstituted in 300 µL water.

2. **Liquid-liquid Extraction by SLE (96-well Isolute)**

a. Pipet 30 µl 10N NaOH (freshly made daily, 2 grams in 5 ml water) to each well in CP#2.
b. Mix well (aspirate and dispense 3 x).
c. Apply the entire sample to a pre-washed SLE plate.
d. Allow to stay on the column for 4 min.
e. Add 3 x 0.65 ml of methylene chloride to the column. Allow to elute by positive pressure. Collect methylene chloride fraction a new deep 96-well plate (CP#3).
f. Dry down methylene chloride to 800 µL in CP#3.

**3. Liquid-liquid extraction by HCl**

a. Add 0.8 ml of 0.1 N HCl into each well in CP#3.
b. Mix for 10 min (aspirate and dispense 10x).
c. Centrifuge the plate for 10 min at 3000 rpm.
d. Transfer HCl layer into a new 96-well collection plate CP#4.
e. Add 10 µl 10N NaOH into each well in CP#4 to neutralize HCl layer.
f. Add 1 ml PO4 buffer into each well in CP#4. Mix well (aspirate and dispense 3 x).

**5. MIP SPE procedure**

a. Condition the MIP 96-well plate with 1 ml methylene chloride, followed by 1 ml methanol and 1 ml water at 2 ml/min.
b. Vortex the sample and load sample on MIP plate. Adjust the pressure so that the sample is drawn through the plate at a rate of about 0.5 ml/min.
c. Once all of the samples have loaded, allow the columns to dry under nitrogen at 10 psi for 1 min.
d. After drying, the plate is washed at 10 psi with 2 x 1 ml water, followed by vacuum drying for 10 min.
e. The plate is then washed by 1 ml toluene and 1 ml toluene/methylene chloride (9:1).
f. Dry the plate at 10 psi for 2 min to remove toluene.
g. The analyte is eluted at 0.5 ml/min with 1.5 ml methylene chloride into a new 96-well collection plate (CP#5), and dry down methylene chloride.
h. Elute the analyte with a second aliquot of 1.5 ml methylene chloride into the same 96-well collection plate (CP#5).
i. The elutent is dried down in the plate CP#5. The residue is reconstituted in 40 µl water. 5 µl is injected into the LC for analysis.

## Sample preparation check sheet

HYDROLYSIS - BEFORE STARTING HAMILTON 1 (Bonnie)
START TIME _____DATE _____ END TIME_____

| CALIPER(CALIPER _____1-Capone _____2-Gotti) | HAMILTON |
|---|---|
| | |
| ☐ Set up deck layout | ☐ Sample Carriers 1-4 is at rows 14-17 |
| ☐ Make sure enzyme and ISTD are in a NEW 12-way reservoir | ☐ Invert then load samples onto sample carriers 1-4 from rows 14-17 (positions 1 to 24) facing scanner |
| ☐ Tip Box is empty at C3 and CP1 is at C4 | |
| ☐ Tip Reformattor is screwed in place and empty at B2 | ☐ Tip Carrier has two full 1000µl tip trays in the first two positions of the tip carrier and a 50ul tip tray in the fourth position |
| ☐ Tip waste chute (A2) box is empty (or has room for 4 extra sets of tips) | |
| ☐ With lids capped tightly, invert tube carrier racks a few times to mix urine | ☐ On row 25, load ISTD in positions (1-8) of carrier, skip space 9, then place 8 tubes of enzyme filled (position 10-17) |
| ☐ Urine Sample Carriers are on Rack 4 (right-most rack) in positions 1-4 | ☐ 96 well plate is in fixed position at 36 row |
| ☐ Hyperstack 1 has 4+ sets of tips alternating with empty tip racks; Hyperstack 2 has 1 set of tips. Hyperstack 1 must press Input then Output setting to view 1st tip rack slightly above red light, Hyperstack 2 must press Output then Input for red light to show above first empty stack | ☐ Load "NHANES hydrolysis v 1.3" from run editor on Hamilton if NHANES study for complete run |
| | ☐ Load "PATH hydrolysis v1.2" if this is a PATH study run on Hamilton for complete run |
| ☐ Make sure decapper isn't storing any caps (if so, throw them away) | ☐ SECTION FOR SPLIT PREP:<br>TIME AND DATE OF HYDROLYSIS INCUBATION:<br>_____ |
| ☐ INITIALS_____ or N/A | ☐ If preparing ISTD and SAMPLE VOLUME ONLY (Friday run), load method for NHANES – "nhaneshydISTDSAMPVOLONLYfri" |
| ☐ SAMPLE ALIQUOTTING VERIFICATION<br>☐ 96 SAMPLES COUNTED PRIOR TO ALIQUOTTING<br>ANALYST/DATE _____<br>VERIFIED BY/DATE_____<br>☐ 96 SAMPLES COUNTED AFTER ALIQUOTTING<br>ANALYST/DATE _____<br>VERIFIED BY/DATE_____<br>Rotate samples for 10 minutes prior to aliquotting – INITIALS_____ | ☐ For continuation of NHANES Friday run, to add enzyme, load method – "nhaneshydENZONLYmond" |
| | ☐ If preparing ISTD and SAMPLE VOLUME ONLY (Friday run), load method for PATH --- "pathhydISTDSAMPVOLONLYfri" |
| | ☐ For continuation of PATH Friday run, to add enzyme, load method – "pathhydENZONLYmond" |
| | INITIALS_____<br>Removed plate after 24 hours from date/initials _____ |

☐ CENTRIFUGE AFTER STARTING: Centrifuge after hydrolysis at _____ rpm for _____ minutes (not refrigerated)

- ☐ If refrigerated, warm and agitate 30 minutes in water bath at 37deg C, then centrifuge at _____ rpm for _____ minutes
- ☐ Make sure there is a 1920ul balance plate in Bucket2

CX TO ISOLUTE - BEFORE STARTING Caliper 1 – Capone
START TIME_____DATE_____ END TIME_____

- ☐ Set up deck layout
- ☐ Tip Box is empty at C3
- ☐ Put a TipBox2 at C2 – remove after run
- ☐ 5% Ammonia in Methanol Reservoirs have lids at B3 and B2
- ☐ Collection Plate 1 (from Hydrolysis) has the seal taken off and is at D4
- ☐ Bulk Dispenses and Reservoirs: DCM bottle has at least 1000ml, Water bottle has at least 800ml, and reservoir volumes are at least 220ml
- ☐ Waste bottle is less than half full and tubing for waste chute is tightly attached to the bottom of B4
- ☐ Tip waste chute (A2) box is empty and autoclave is positioned to catch table waste
- ☐ Collection Plates 2&3 are labeled and on rack 2 (middle rack) positions 2 and 3
- ☐ Hyperstack 1 has 10+ sets of tips alternating with empty tip racks; Hyperstack 2 has 1 set of tips. Hyperstack 1 must press Input then Output setting to view 1st tip rack slightly above red light, Hyperstack 2 must press Output then Input for red light to show above first empty stack
- ☐ First position of Turbovap has a flow rate of 38/Plate temp 38deg C INITIALS _____

LLE TO COMPLETION_LATEST_12-05-16 - BEFORE STARTING Caliper 2 – Gotti
START TIME_____DATE_____END TIME_____

- ☐ Set up deck layout
- ☐ Tip Box is empty at C3
- ☐ Collection Plate 3 (from Caliper 1) has the seal taken off and is at D4
- ☐ Bulk Dispenses and Reservoirs: DCM bottle has at least 1000ml, Water bottle has at least 800ml, and reservoir volumes are at least 220ml
- ☐ Waste bottle is less than half full and tubing for waste chute is tightly attached to the bottom of B4
- ☐ Tip waste chute (A2) box is empty and empty autoclave bin is at waste chute
- ☐ Collection Plates 4-5 are labeled and on rack 1 (right rack) positions 4-5
- ☐ First position of Turbovap has a flow rate of 38/Plate temp 38deg C
- ☐ Hyperstack 1 has 10+ sets of tips alternating with empty tip racks; Hyperstack 1 must press Input then Output setting to view 1st tip rack slightly above red light and empty tip holder waste container
- ☐ Make sure Centrifuge has 1600ul balance plate in Bucket 2 and Regrip Station basket biased towards robotic arm
- ☐ Centrifuge Parameters set to: _____ RPM for _____ minutes INITIALS _____

Additional Comments:

Note sheet

TOTAL/FREE TSNA OBSERVATIONS DURING RUN

RUN NUMBER _____ INITIALS_____

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | | | | | | | | | | | | |
| **B** | | | | | | | | | | | | |
| **C** | | | | | | | | | | | | |
| **D** | | | | | | | | | | | | |
| **E** | | | | | | | | | | | | |
| **F** | | | | | | | | | | | | |
| **G** | | | | | | | | | | | | |
| **H** | | | | | | | | | | | | |

# Appendix D

## TSNA Assay LC/MS/MS Analysis Checklist

Run Start Date/Time_____ STD Curve Name _____

Instrument: T1 □ T2 □ T3 □ T5 □ T6 □ Instrument Analyst_____

LC Column S/No._____ Run # on column _____

STD Curve Lot _____ STD Aliquot # _____

**Sample run type** (circle) original reinjection repeat INITIAL:_____

**Prepare the instrument to run the TSNA method** INITIAL:

◯ Fill A1 mobile phase reservoir to at least 1500 mL 0.08% Ammonium Hydroxide in JT Baker Water.

*To make 0.08% Ammonium Hydroxide in Water, fill a clean A1 reservoir with 1000 mL JT Baker Water using a graduated cylinder. Spike with 800 µL Ammonium Hydroxide using a 1000 µL pipette.*

◯ Fill B1 mobile phase reservoir to at least 1000 mL 100% Honeywell Acetonitrile.

◯ Fill C1 mobile phase reservoir to at least 1500 mL 100% Honeywell Acetonitrile.

◯ Note the mobile phase lot numbers:

Acetonitrile _____ Water _____ Ammonium Hydroxide _____

◯ Purge A, B, and C pumps and preheat the column oven.

◯ Initialize/re-initialize the LC Controller.

◯ Equilibrate the system on the 2014_TSNA_PATH acquisition method for approximately 10 minutes.

◯ At approx. 10 minutes record the A, B, and C pump pressures.

Pump A, B _____psi Pump C _____psi

◯ Run ISTD check injections and manually integrate the ISTD peaks. Record acceptable ISTD Area counts before proceeding.

ISTD Aliquot Date _____ ISTD Aliquot # _____

NNAL _____counts NNN _____counts NNK _____counts

NAT _____counts NAB _____counts

**Sample plate** INITIAL:

◯ Retrieve Sample Prep Checklist in LC/MS/MS folder on side of refrigerator in Room 3308, attach Analysis Checklist.

◯ Retrieve sample plate from the LC/MS/MS bin in -20C freezer in room 3318, thaw, hold to vortexer for ~30 seconds, and check for air bubbles.

◯ Place the plate in the correct sample rack position: _____.

**Start sample acquisition** INITIAL:

Create the sample acquisition batch from Sample Prep batch file.

◯ Submit the sample acquisition batch to the instrument queue.

◯ Submit a column flush sample or sample set to the queue.

◯ Start the queue.

◯ Add any relevant notes:

circle if NONE

**Maintain the instrument during the sample run** INITIAL:

◯ Refill mobile phase reservoirs as needed 4-24 hours after the start of the sample run.

◯ Integrate the STD_03_01 Native STD peaks. Record Native STD Area counts.

NNAL _____counts NNN _____counts NNK _____counts

NAT _____counts NAB _____counts

◯ Integrate the STD_03_01 ISTD peaks. Record ISTD Area counts.

NNAL _____counts NNN _____counts NNK _____counts

NAT _____counts NAB _____counts

**Put finished plate in long term storage** INITIAL:

◯ Add 20µL JT Baker water to each well and seal.

◯ Put in -20°C freezer in room 3318.

**Integrate the sample data** INITIAL:

◯ Upload the sample data to Indigo ASCENT.

# Appendix E

## **Table E1**. Accuracy using spike recovery

**Accuracy using Spike Recovery** - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: TSNA in urine
Method #: 2014
Matrix: Urine
Units: pg/mL
Analyte: **NNAL**

| | | 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 | 0.0 | -0.3 | 0.0 | | 0 | -0.1 | -0.2 | -0.1 | | 102.3 | 1.0 |
| | 2 | | 0.2 | -0.2 | | | | -0.1 | -0.2 | | | | |
| | 3 | | 0.4 | 0.0 | | | | 0.0 | -0.1 | | | | |
| Sample + Spike 1 | 1 | 25 | 25.9 | 25.8 | 25.7 | 102.7 | 25 | 25.6 | 26.0 | 25.8 | 103.6 | | |
| | 2 | | 26.2 | 25.5 | | | | 25.8 | 25.9 | | | | |
| | 3 | | 25.8 | 24.9 | | | | 25.7 | 25.7 | | | | |
| Sample + Spike 2 | 1 | 50 | 51.6 | 50.5 | 51.1 | 102.2 | 50 | 51.0 | 52.0 | 51.3 | 102.8 | | |
| | 2 | | 52.0 | 51.1 | | | | 51.0 | 50.5 | | | | |
| | 3 | | 51.8 | 49.8 | | | | 51.5 | 51.8 | | | | |
| Sample + Spike 3 | 1 | 250 | 255.3 | 244.7 | 252 | 100.8 | 250 | 256.7 | 252.4 | 254 | 101.5 | | |
| | 2 | | 255.5 | 252.6 | | | | 258.9 | 250.4 | | | | |
| | 3 | | 251.3 | 253.4 | | | | 248.9 | 253.9 | | | | |

**Accuracy using Spike Recovery** - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: TSNA in urine
Method #: 2014
Matrix: Urine
Units: pg/mL
Analyte: **NNN**

| | | 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 | 0.8 | 0.1 | 0.3 | | 0 | 1.1 | 0.0 | 0.2 | | 103.0 | 2.0 |
| | 2 | | 0.6 | 0.0 | | | | 0.0 | 0.0 | | | | |
| | 3 | | -0.1 | 0.3 | | | | 0.0 | 0.3 | | | | |
| Sample + Spike 1 | 1 | 25 | 26.3 | 25.8 | 26.0 | 102.8 | 25 | 26.7 | 26.6 | 26.9 | 106.5 | | |
| | 2 | | 26.2 | 25.3 | | | | 27.8 | 26.2 | | | | |
| | 3 | | 26.2 | 26.1 | | | | 28.3 | 25.6 | | | | |
| Sample + Spike 2 | 1 | 50 | 53.4 | 52.5 | 52.2 | 103.9 | 50 | 51.3 | 51.8 | 51.4 | 102.4 | | |
| | 2 | | 52.1 | 51.1 | | | | 51.2 | 50.4 | | | | |
| | 3 | | 53.7 | 50.6 | | | | 51.2 | 52.6 | | | | |
| Sample + Spike 3 | 1 | 250 | 254.2 | 249.9 | 252 | 100.6 | 250 | 251.0 | 253.8 | 255 | 101.9 | | |
| | 2 | | 256.5 | 250.3 | | | | 259.0 | 253.7 | | | | |
| | 3 | | 252.0 | 248.4 | | | | 260.3 | 252.0 | | | | |

**Accuracy using Spike Recovery** - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: TSNA in urine
Method #: 2014
Matrix: Urine
Units: pg/mL
Analyte: **NAT**

| | | Sample 1 | | | | | Sample 2 | | | | | Mean recovery (%) | SD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Replicate | Spike concentration | Measured concentration Day 1 | Day 2 | Mean | Recovery (%) | Spike concentration | Measured concentration Day 1 | Day 2 | Mean | Recovery (%) | | |
| Sample | 1 | 0 | 2.5 | 0.0 | | | 0 | 1.1 | 0.6 | | | 103.5 | 1.6 |
| | 2 | | 2.7 | -0.1 | 1.1 | | | 0.3 | 0.2 | 0.6 | | | |
| | 3 | | 1.0 | 0.3 | | | | 1.0 | 0.2 | | | | |
| Sample + Spike 1 | 1 | 25 | 28.7 | 26.5 | | | 25 | 27.3 | 26.7 | | | | |
| | 2 | | 27.6 | 25.7 | 27.3 | 104.8 | | 28.0 | 26.0 | 27.1 | 106.0 | | |
| | 3 | | 28.8 | 26.3 | | | | 27.9 | 26.5 | | | | |
| Sample + Spike 2 | 1 | 50 | 52.8 | 52.7 | | | 50 | 51.0 | 51.7 | | | | |
| | 2 | | 54.3 | 50.7 | 52.8 | 103.4 | | 52.1 | 51.1 | 51.6 | 102.0 | | |
| | 3 | | 53.3 | 52.9 | | | | 51.9 | 51.5 | | | | |
| Sample + Spike 3 | 1 | 250 | 256.4 | 260.2 | | | 250 | 257.9 | 258.6 | | | | |
| | 2 | | 255.3 | 257.2 | 256 | 101.9 | | 257.4 | 255.6 | 258 | 103.1 | | |
| | 3 | | 252.9 | 253.6 | | | | 258.6 | 261.7 | | | | |

**Accuracy using Spike Recovery** - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: TSNA in urine
Method #: 2014
Matrix: Urine
Units: pg/mL
Analyte: **NAB**

| | | Sample 1 | | | | | Sample 2 | | | | | Mean recovery (%) | SD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Replicate | Spike concentration | Measured concentration Day 1 | Day 2 | Mean | Recovery (%) | Spike concentration | Measured concentration Day 1 | Day 2 | Mean | Recovery (%) | | |
| Sample | 1 | 0 | 1.0 | 0.0 | | | 0 | 1.0 | 1.0 | | | 104.8 | 1.8 |
| | 2 | | 0.8 | 0.0 | 0.4 | | | 0.0 | 0.0 | 0.4 | | | |
| | 3 | | 0.0 | 0.5 | | | | 0.6 | 0.0 | | | | |
| Sample + Spike 1 | 1 | 25 | 26.2 | 26.8 | | | 25 | 27.4 | 27.2 | | | | |
| | 2 | | 27.0 | 27.5 | 26.7 | 105.3 | | 28.1 | 26.6 | 27.4 | 107.9 | | |
| | 3 | | 26.4 | 26.3 | | | | 28.1 | 27.0 | | | | |
| Sample + Spike 2 | 1 | 50 | 52.9 | 53.9 | | | 50 | 52.9 | 53.3 | | | | |
| | 2 | | 54.1 | 52.0 | 53.0 | 105.3 | | 51.9 | 51.9 | 52.6 | 104.4 | | |
| | 3 | | 52.6 | 52.7 | | | | 52.7 | 53.0 | | | | |
| Sample + Spike 3 | 1 | 250 | 255.8 | 260.0 | | | 250 | 258.1 | 260.8 | | | | |
| | 2 | | 255.9 | 256.8 | 257 | 102.7 | | 260.6 | 260.9 | 259 | 103.5 | | |
| | 3 | | 254.3 | 260.2 | | | | 259.5 | 255.7 | | | | |

## **Table E2**. Precision

**Precision** - fill in yellow shaded cells

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

| | |
|---|---|
| Method name: | TSNAs in urine |
| Method #: | 2014 |
| Matrix: | Urine |
| Units: | pg/mL |
| Analyte: | **NNAL** |

**Quality material 1**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 58.2 | 57.9 | 58.07 | 0.03 | 0.03 | 6743.09 |
| 2 | 58.3 | 58.0 | 58.12 | 0.02 | 0.02 | 6755.87 |
| 3 | 58.9 | 58.2 | 58.57 | 0.14 | 0.14 | 6859.72 |
| 4 | 59.6 | 58.2 | 58.91 | 0.46 | 0.46 | 6940.78 |
| 5 | 59.9 | 58.9 | 59.39 | 0.22 | 0.22 | 7054.34 |
| 6 | 59.9 | 59.3 | 59.58 | 0.10 | 0.10 | 7099.55 |
| 7 | 60.4 | 59.7 | 60.03 | 0.12 | 0.12 | 7207.20 |
| 8 | 60.8 | 59.8 | 60.25 | 0.25 | 0.25 | 7260.13 |
| 9 | 61.1 | 63.1 | 62.12 | 1.00 | 1.00 | 7717.79 |
| 10 | 62.0 | 63.7 | 62.89 | 0.71 | 0.71 | 7909.05 |

| | | | |
|---|---|---|---|
| **Grand sum** | 1195.83 | **Grand mean** | 59.7915 |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) |
|---|---|---|---|---|
| **Within Run** | 6.11 | 0.61 | 0.78 | 1.31 |
| **Between Run** | 47.04 | 5.23 | 1.52 | 2.54 |
| **Total** | 53.15 | | 1.71 | **2.86** |

**Quality material 2**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 281.25 | 280.55 | 280.90 | 0.12 | 0.12 | 157809.62 |
| 2 | 281.89 | 281.18 | 281.54 | 0.13 | 0.13 | 158523.91 |
| 3 | 285.70 | 282.69 | 284.20 | 2.27 | 2.27 | 161533.60 |
| 4 | 287.46 | 282.77 | 285.12 | 5.50 | 5.50 | 162581.13 |
| 5 | 288.11 | 283.22 | 285.67 | 5.98 | 5.98 | 163208.98 |
| 6 | 288.40 | 285.38 | 286.89 | 2.28 | 2.28 | 164611.74 |
| 7 | 290.40 | 286.29 | 288.35 | 4.22 | 4.22 | 166285.68 |
| 8 | 291.39 | 287.64 | 289.52 | 3.52 | 3.52 | 167637.87 |
| 9 | 296.48 | 303.33 | 299.91 | 11.73 | 11.73 | 179886.02 |
| 10 | 306.27 | 304.64 | 305.46 | 0.66 | 0.66 | 186605.51 |

| | | | |
|---|---|---|---|
| **Grand sum** | 5775.04 | **Grand mean** | 288.75 |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev |
|---|---|---|---|---|
| **Within Run** | 72.8 | 7.3 | 2.7 | 0.93 |
| **Between Run** | 1129.7 | 125.5 | 7.7 | 2.66 |
| **Total** | 1202.5 | | 8.1 | **2.82** |

## **Precision** - fill in yellow shaded cells

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

| | |
|---|---|
| Method name: | TSNAs in urine |
| Method #: | 2014 |
| Matrix: | Urine |
| Units: | pg/mL |
| Analyte: | **NNN** |

**Quality material 1**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 46.9 | 47.0 | 46.94 | 0.01 | 0.01 | 4406.73 |
| 2 | 47.1 | 47.5 | 47.31 | 0.03 | 0.03 | 4475.53 |
| 3 | 48.1 | 47.8 | 47.98 | 0.03 | 0.03 | 4603.20 |
| 4 | 48.2 | 48.5 | 48.33 | 0.02 | 0.02 | 4670.61 |
| 5 | 48.4 | 48.6 | 48.49 | 0.02 | 0.02 | 4701.59 |
| 6 | 48.6 | 48.9 | 48.79 | 0.02 | 0.02 | 4759.95 |
| 7 | 48.7 | 49.3 | 48.98 | 0.08 | 0.08 | 4798.08 |
| 8 | 48.8 | 49.3 | 49.07 | 0.06 | 0.06 | 4815.73 |
| 9 | 49.5 | 50.5 | 49.99 | 0.25 | 0.25 | 4997.00 |
| 10 | 50.1 | 51.3 | 50.67 | 0.38 | 0.38 | 5133.88 |
| **Grand sum** | 973.03 | **Grand mean** | 48.65 | | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) |
|---|---|---|---|---|
| **Within Run** | 1.78 | 0.18 | 0.42 | 0.87 |
| **Between Run** | 22.94 | 2.55 | 1.09 | 2.24 |
| **Total** | 24.72 | | 1.17 | **2.40** |

**Quality material 2**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 255.50 | 256.66 | 256.08 | 0.3 | 0.3 | 131153.9 |
| 2 | 256.83 | 257.77 | 257.30 | 0.2 | 0.2 | 132406.6 |
| 3 | 257.18 | 259.75 | 258.47 | 1.7 | 1.7 | 133608.3 |
| 4 | 257.42 | 260.99 | 259.21 | 3.2 | 3.2 | 134374.5 |
| 5 | 257.81 | 263.75 | 260.78 | 8.8 | 8.8 | 136012.4 |
| 6 | 258.03 | 264.73 | 261.38 | 11.2 | 11.2 | 136639.0 |
| 7 | 261.14 | 266.00 | 263.57 | 5.9 | 5.9 | 138938.3 |
| 8 | 261.33 | 266.77 | 264.05 | 7.4 | 7.4 | 139444.8 |
| 9 | 265.02 | 273.62 | 269.32 | 18.5 | 18.5 | 145066.5 |
| 10 | 271.51 | 273.85 | 272.68 | 1.4 | 1.4 | 148708.8 |
| **Grand sum** | 5245.66 | **Grand mean** | 262.28 | | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev |
|---|---|---|---|---|
| **Within Run** | 117.20 | 11.72 | 3.42 | 1.31 |
| **Between Run** | 505.66 | 56.18 | 4.72 | 1.80 |
| **Total** | 622.86 | | 5.83 | **2.22** |

## **Precision** - fill in yellow shaded cells

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

| | |
|---|---|
| Method name: | TSNAs in urine |
| Method #: | 2014 |
| Matrix: | Urine |
| Units: | pg/mL |
| Analyte: | **NAT** |

**Quality material 1**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 44.1 | 46.0 | 45.07 | 0.87 | 0.87 | 4061.71 |
| 2 | 44.8 | 46.6 | 45.68 | 0.86 | 0.86 | 4172.41 |
| 3 | 45.7 | 48.1 | 46.90 | 1.36 | 1.36 | 4398.28 |
| 4 | 47.1 | 48.1 | 47.59 | 0.28 | 0.28 | 4529.62 |
| 5 | 47.7 | 50.1 | 48.94 | 1.44 | 1.44 | 4790.25 |
| 6 | 47.9 | 50.3 | 49.08 | 1.44 | 1.44 | 4817.69 |
| 7 | 48.7 | 50.6 | 49.68 | 0.88 | 0.88 | 4936.20 |
| 8 | 48.8 | 51.2 | 50.00 | 1.39 | 1.39 | 5000.00 |
| 9 | 51.1 | 51.5 | 51.34 | 0.04 | 0.04 | 5271.59 |
| 10 | 51.2 | 52.2 | 51.71 | 0.23 | 0.23 | 5347.85 |
| **Grand sum** | 971.95 | **Grand mean** | 48.60 | | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) |
|---|---|---|---|---|
| **Within Run** | 17.59 | 1.76 | 1.33 | 2.73 |
| **Between Run** | 91.26 | 10.14 | 2.05 | 4.21 |
| **Total** | 108.85 | | 2.44 | **5.02** |

**Quality material 2**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 241.25 | 244.78 | 243.02 | 3.1 | 3.1 | 118112.6 |
| 2 | 243.77 | 251.14 | 247.46 | 13.6 | 13.6 | 122468.0 |
| 3 | 247.79 | 252.75 | 250.27 | 6.2 | 6.2 | 125270.1 |
| 4 | 248.15 | 253.24 | 250.70 | 6.5 | 6.5 | 125696.0 |
| 5 | 249.56 | 257.70 | 253.63 | 16.6 | 16.6 | 128656.4 |
| 6 | 251.02 | 264.10 | 257.56 | 42.8 | 42.8 | 132674.3 |
| 7 | 252.62 | 264.50 | 258.56 | 35.3 | 35.3 | 133706.5 |
| 8 | 253.26 | 264.63 | 258.95 | 32.3 | 32.3 | 134105.0 |
| 9 | 259.71 | 269.70 | 264.71 | 25.0 | 25.0 | 140137.5 |
| 10 | 261.56 | 280.40 | 270.98 | 88.7 | 88.7 | 146860.3 |
| **Grand sum** | 5111.63 | **Grand mean** | 255.58 | | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev |
|---|---|---|---|---|
| **Within Run** | 539.90 | 53.99 | 7.35 | 2.87 |
| **Between Run** | 1248.61 | 138.73 | 6.51 | 2.55 |
| **Total** | 1788.51 | | 9.82 | **3.84** |

## **Precision** - fill in yellow shaded cells

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

| | |
|---|---|
| Method name: | TSNAs in urine |
| Method #: | 2014 |
| Matrix: | Urine |
| Units: | pg/mL |
| Analyte: | **NAB** |

**Quality material 1**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 46.8 | 48.7 | 47.72 | 0.94 | 0.94 | 4554.40 |
| 2 | 47.7 | 49.3 | 48.48 | 0.59 | 0.59 | 4700.62 |
| 3 | 48.3 | 49.5 | 48.88 | 0.35 | 0.35 | 4777.53 |
| 4 | 48.4 | 49.8 | 49.09 | 0.44 | 0.44 | 4818.67 |
| 5 | 48.8 | 50.1 | 49.44 | 0.40 | 0.40 | 4888.63 |
| 6 | 49.0 | 50.1 | 49.55 | 0.28 | 0.28 | 4910.41 |
| 7 | 49.6 | 50.3 | 49.93 | 0.13 | 0.13 | 4986.01 |
| 8 | 50.2 | 50.5 | 50.33 | 0.02 | 0.02 | 5065.21 |
| 9 | 51.5 | 50.7 | 51.08 | 0.15 | 0.15 | 5217.31 |
| 10 | 52.1 | 51.0 | 51.55 | 0.28 | 0.28 | 5314.81 |
| **Grand sum** | 992.06 | **Grand mean** | 49.60 | | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) |
|---|---|---|---|---|
| **Within Run** | 7.17 | 0.72 | 0.85 | 1.71 |
| **Between Run** | 24.44 | 2.72 | 1.00 | 2.02 |
| **Total** | 31.61 | | 1.31 | **2.64** |

**Quality material 2**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 | 2*mean^2 |
|---|---|---|---|---|---|---|
| 1 | 239.98 | 249.78 | 244.88 | 24.0 | 24.0 | 119932.4 |
| 2 | 249.62 | 253.74 | 251.68 | 4.2 | 4.2 | 126685.6 |
| 3 | 255.32 | 256.94 | 256.13 | 0.7 | 0.7 | 131205.2 |
| 4 | 256.08 | 257.16 | 256.62 | 0.3 | 0.3 | 131707.6 |
| 5 | 257.52 | 257.88 | 257.70 | 0.0 | 0.0 | 132818.6 |
| 6 | 257.88 | 259.11 | 258.50 | 0.4 | 0.4 | 133639.3 |
| 7 | 258.53 | 261.10 | 259.82 | 1.7 | 1.7 | 135007.7 |
| 8 | 258.97 | 261.19 | 260.08 | 1.2 | 1.2 | 135283.2 |
| 9 | 264.30 | 265.26 | 264.78 | 0.2 | 0.2 | 140216.9 |
| 10 | 269.00 | 267.10 | 268.05 | 0.9 | 0.9 | 143701.6 |
| **Grand sum** | 5156.46 | **Grand mean** | 257.82 | | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev |
|---|---|---|---|---|
| **Within Run** | 67.26 | 6.73 | 2.59 | 1.01 |
| **Between Run** | 744.18 | 82.69 | 6.16 | 2.39 |
| **Total** | 811.44 | | 6.69 | **2.59** |

**Table E3**. Stability

**Stability** - fill in yellow shaded cells

**The initial measurement can be from the same day for all stability experiments.**

**Freeze and thaw stability** = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: example: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
**Bench-top stability** = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: example: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day
**Processed sample stability** = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: example: processed samples (ready for instrument analysis) stored at room temperature for 1 day
**Long-term stability** = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

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

Method name: TSNAs in urine
Method #: 2014
Matrix: Urine
Units: pg/mL
Analyte: **NNAL**

| **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 | 53.9 | 52.5 | 53.9 | 53.8 | 53.9 | 53.2 | 55.8 | 57.9 |
| Replicate 2 | 54.0 | 52.4 | 54.0 | 52.4 | 54.0 | 53.2 | 60.7 | 58.7 |
| Replicate 3 | 52.9 | 52.6 | 52.9 | 53.8 | 52.9 | 52.9 | 61.9 | 59.2 |
| Mean | 53.6 | 52.5 | 53.6 | 53.3 | 53.6 | 53.1 | 59.5 | 58.6 |
| **% difference from initial measurement** | -- | **-2.1** | -- | **-0.5** | -- | **-0.9** | -- | **-1.5** |

| **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 | 269.6 | 263.1 | 269.6 | 271.9 | 269.6 | 265.8 | 304.4 | 277.9 |
| Replicate 2 | 267.5 | 255.9 | 267.5 | 263.4 | 267.5 | 263.4 | 285.8 | 280.5 |
| Replicate 3 | 270.9 | 266.0 | 270.9 | 268.3 | 270.9 | 264.8 | 284.3 | 286.9 |
| Mean | 269.3 | 261.7 | 269.3 | 267.9 | 269.3 | 264.7 | 291.5 | 281.8 |
| **% difference from initial measurement** | -- | **-2.9** | -- | **-0.5** | -- | **-1.7** | -- | **-3.3** |

**Stability** - fill in yellow shaded cells

**The initial measurement can be from the same day for all stability experiments.**

**Freeze and thaw stability** = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: example: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
**Bench-top stability** = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: example: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day
**Processed sample stability** = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: example: processed samples (ready for instrument analysis) stored at room temperature for 1 day
**Long-term stability** = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

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

| | |
|---|---|
| Method name: | TSNAs in urine |
| Method #: | 2014 |
| Matrix: | Urine |
| Units: | pg/mL |
| Analyte: | **NNN** |

| **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 | 51.8 | 50.5 | 51.8 | 51.2 | 51.8 | 49.7 | 48.5 | 47.6 |
| Replicate 2 | 50.5 | 49.8 | 50.5 | 49.6 | 50.5 | 50.2 | 46.2 | 47.7 |
| Replicate 3 | 50.6 | 49.8 | 50.6 | 52.0 | 50.6 | 50.7 | 47.2 | 48.0 |
| Mean | 51.0 | 50.0 | 51.0 | 50.9 | 51.0 | 50.2 | 47.3 | 47.7 |
| **% difference from initial measurement** | -- | **-1.8** | -- | **-0.1** | -- | **-1.5** | -- | **0.9** |

| **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 | 253.2 | 248.5 | 253.2 | 266.6 | 253.2 | 257.2 | 255.7 | 258.1 |
| Replicate 2 | 261.7 | 251.0 | 261.7 | 249.2 | 261.7 | 254.1 | 256.4 | 259.3 |
| Replicate 3 | 255.1 | 265.0 | 255.1 | 257.5 | 255.1 | 258.2 | 268.0 | 262.1 |
| Mean | 256.6 | 254.8 | 256.6 | 257.8 | 256.6 | 256.5 | 260.0 | 259.8 |
| **% difference from initial measurement** | -- | **-0.7** | -- | **0.4** | -- | **-0.1** | -- | **-0.1** |

**Stability** - fill in yellow shaded cells

**The initial measurement can be from the same day for all stability experiments.**

**Freeze and thaw stability** = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: example: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
**Bench-top stability** = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: example: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day
**Processed sample stability** = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: example: processed samples (ready for instrument analysis) stored at room temperature for 1 day
**Long-term stability** = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

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

| | |
|---|---|
| Method name: | TSNAs in urine |
| Method #: | 2014 |
| Matrix: | Urine |
| Units: | pg/mL |
| Analyte: | **NAT** |

| **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 | 52.8 | 51.4 | 52.8 | 53.7 | 52.8 | 51.7 | 47.1 | 47.3 |
| Replicate 2 | 52.6 | 52.3 | 52.6 | 52.0 | 52.6 | 52.7 | 46.5 | 47.7 |
| Replicate 3 | 51.8 | 52.5 | 51.8 | 52.8 | 51.8 | 52.8 | 48.5 | 48.0 |
| Mean | 52.4 | 52.1 | 52.4 | 52.9 | 52.4 | 52.4 | 47.4 | 47.6 |
| **% difference from initial measurement** | -- | **-0.6** | -- | **0.9** | -- | **0.0** | -- | **0.5** |

| **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 | 262.2 | 264.2 | 262.2 | 269.0 | 262.2 | 254.2 | 254.6 | 248.1 |
| Replicate 2 | 266.7 | 256.1 | 266.7 | 256.7 | 266.7 | 267.5 | 251.2 | 251.2 |
| Replicate 3 | 268.3 | 266.4 | 268.3 | 262.6 | 268.3 | 269.0 | 270.8 | 257.6 |
| Mean | 265.7 | 262.2 | 265.7 | 262.8 | 265.7 | 263.5 | 258.9 | 252.3 |
| **% difference from initial measurement** | -- | **-1.3** | -- | **-1.1** | -- | **-0.8** | -- | **-2.5** |

**Stability** - fill in yellow shaded cells

**The initial measurement can be from the same day for all stability experiments.**

**Freeze and thaw stability** = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: example: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
**Bench-top stability** = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: example: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day
**Processed sample stability** = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: example: processed samples (ready for instrument analysis) stored at room temperature for 1 day
**Long-term stability** = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

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

Method name: TSNAs in urine
Method #: 2014
Matrix: Urine
Units: pg/mL
Analyte: **NAB**

| **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 | 51.5 | 49.9 | 51.5 | 50.4 | 51.5 | 50.7 | 50.6 | 48.6 |
| Replicate 2 | 51.2 | 50.3 | 51.2 | 50.3 | 51.2 | 50.3 | 49.5 | 49.1 |
| Replicate 3 | 51.4 | 49.7 | 51.4 | 51.4 | 51.4 | 50.0 | 47.7 | 49.8 |
| Mean | 51.4 | 50.0 | 51.4 | 50.7 | 51.4 | 50.3 | 49.3 | 49.2 |
| **% difference from initial measurement** | -- | **-2.7** | -- | **-1.3** | -- | **-2.0** | -- | **-0.2** |

| **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 | 255.7 | 251.1 | 255.7 | 262.7 | 255.7 | 252.9 | 257.1 | 248.6 |
| Replicate 2 | 255.5 | 250.5 | 255.5 | 254.3 | 255.5 | 262.0 | 257.1 | 250.2 |
| Replicate 3 | 259.3 | 262.2 | 259.3 | 261.1 | 259.3 | 259.1 | 253.7 | 252.5 |
| Mean | 256.8 | 254.6 | 256.8 | 259.4 | 256.8 | 258.0 | 256.0 | 250.4 |
| **% difference from initial measurement** | -- | **-0.9** | -- | **1.0** | -- | **0.5** | -- | **-2.2** |

## Table E4. LOD, Specificity, Fit for intended use

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

| | |
|---|---|
| Method name: | TSNAs in urine |
| Method #: | 2014 |
| Matrix: | Urine |
| Units: | ng/mL |

| Analytes | Limit of Detection (LOD) | Interferences successfully checked in at least 50 human samples | Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use |
|---|---|---|---|
| NNAL | 0.0006 | yes | yes |
| NNN | 0.0028 | yes | yes |
| NAT | 0.0042 | yes | yes |
| NAB | 0.0016 | yes | yes |

# Appendix F

## TSNA Assay Rules for Indigo ASCENT

This is a list of the QA rules that are currently available in ASCENT. All are enabled except for two labeled "Not enabled." Rules can be switched from 'enabled' to 'not enabled' in Assay Configuration, and most can be enabled or not enabled on a compound-by-compound basis.

*available but not requested
**requested but not available

**CALIBRATION**

CDC Concentration Deviation*

'Concentration Deviation (%)'

*Flag standards with level ≥ High Calibrator Low Level (b)*
*AND conc deviation ≥ High Calibrator deviation threshold(c%)*
*OR with level ≥ Low Calibrator Low Level (d)*
*AND level ≤ Low Calibrator High Level (e)*
*AND conc deviation ≥Low Calibrator deviation threshold (f%)*

| | | | | | |
|---|---|---|---|---|---|
| NNAL_1 | c = 20 | d = 5 (0.02ng/mL) | e = 9 (0.4ng/mL) | f = 50 | b = 10 (0.8ng/mL) |
| NNAL_2 | - | - | - | - | - |
| NNN | c = 20 | d = 5 | e = 9 | f = 50 | b = 10 |
| NAT | c = 20 | d = 5 | e = 9 | f = 50 | b = 10 |
| NAB | c = 20 | d = 5 | e = 9 | f = 50 | b = 10 |
| NNK_1 | c = 20 | d = 5 | e = 9 | f = 50 | b = 10 |
| NNK_2 | - | - | - | - | - |

Calibration Standards Excluded

'>x Standards excluded'

*Flag ALL Standard determinations if > x number of standards are excluded from calibration curve.*

| | |
|---|---|
| NNAL_1 | x = 5 |
| NNAL_2 | - |
| NNN | x = 5 |
| NAT | x = 5 |
| NAB | x = 5 |
| NNK_1 | x = 5 |
| NNK_2 | - |

Calibration Regression Failed

'Calibration Failed'

*Flag ALL Standard determinations for an analyte if calibration could not be performed.*

**Calibration cont'd**

Calibration Concentration Deviation
'Concentration Deviation (%)'
*For standards, flag determination if absolute value of concentration deviation ≥ x% from nominal concentration.*

| | | | |
|---|---|---|---|
| NNAL_1 | - | NNAL_1 ISTD | - |
| NNAL_2 | - | NNAL_2 ISTD | - |
| NNN | - | NNN ISTD | - |
| NAT | - | NAT ISTD | - |
| NAB | - | NAB ISTD | - |
| NNK_1 | - | NNK_1 ISTD | - |
| NNK_2 | - | NNK_2 ISTD | - |

Not enabled. Redundant with CDC Concentration Deviation.

Calibration No Intercept
*Only for determinations with cubic or quadratic regression equations.*
Not enabled

**INTERNAL STANDARD**
CDC2014 Internal Standard Recovery
'IS recovery (%)'
*Flag determination if the deviation of the peak area as a percentage of the mean of QC areas exceeds the Upper (b) or Lower(c) QC Area Deviation Threshold*

| | | |
|---|---|---|
| NNAL_1 | b = 10 | c = -10 |
| NNAL_2 | b = 10 | c = -10 |
| NNN | b = 10 | c = -10 |
| NAT | b = 10 | c = -10 |
| NAB | b = 10 | c = -10 |
| NNK_1 | b = 10 | c = -10 |
| NNK_2 | b = 10 | c = -10 |

CDC2014 Instrument Sensitivity
'Low sensitivity"
*Flag a determination if the internal standard area (x) is below a threshold.*

| | |
|---|---|
| NNAL_1 | x = 6666.67 |
| NNAL_2 | x = 6666.67 |
| NNN | x = 6666.67 |
| NAT | x = 6666.67 |
| NAB | x = 6666.67 |
| NNK_1 | x = 6666.67 |
| NNK_2 | x = 6666.67 |

CDC Internal Standard Recovery
'IS recovery (%)'
*Flag determination if the mean area of the standards falls between the Upper (b) and Lower (c) IS Area Deviation Thresholds*

| | | |
|---|---|---|
| NNAL_1 | - | - |
| NNAL_2 | - | - |
| NNN | - | - |
| NAT | - | - |

| | | |
|---|---|---|
| NAB | - | - |
| NNK_1 | - | - |
| NNK_2 | - | - |

Not enabled.

Internal Standard Area Deviation
'IS Area Deviation (%)'
*Flag the internal standard in a sample if the absolute value of the internal standard area deviation from the mean IS area of standards > x%.*

| | |
|---|---|
| NNAL_1 | - |
| NNAL_2 | - |
| NNN | - |
| NAT | - |
| NAB | - |
| NNK_1 | - |
| NNK_2 | - |

Not enabled. Redundant with CDC Internal Standard Recovery.

**ION RATIO**

Ion Ratio Peak Missing
'Ion Ratio (No Quant)'
*Flag a sample if a Qual peak is integrated but no Quant peak is integrated.*

CDC Ion Ratio
'Ion Ratio (%)'
*Flag a sample if the concentration > b AND the absolute value of the ion ratio deviation > x% from the deviation in standards above level c and below level d.*

| | | | | |
|---|---|---|---|---|
| NNAL_1 | b = 0.01 (ng/mL) | x = 6 | c = 4 (0.01ng/mL) | d = 14 (20ng/mL) |
| NNAL_2 | - | - | - | - |
| NNN | b = 0.01 | x = 11 | c = 4 | d = 14 |
| NAT | b = 0.01 | x = 6 | c = 4 | d = 14 |
| NAB | b = 0.01 | x = 35 | c = 4 | d = 14 |
| NNK_1 | b = 0.01 | x = 11 | c = 4 | d = 14 |
| NNK_2 | - | - | - | - |

Ion Ratio Deviation
'Ion Ratio (%)'
*Flag a sample if the absolute value of the ion ratio deviation from mean ion ratio in the standards > x% and the calculated concentration > LLOQ.*
See LLOQ below, under "Concentration Over Diluted."

| | |
|---|---|
| NNAL_1 | - |
| NNAL_2 | - |
| NNN | - |
| NAT | - |
| NAB | - |
| NNK_1 | - |
| NNK_2 | - |

Not enabled. Redundant with CDC Ion Ratio.

**RETENTION TIME**

CDC Quant Qual Retention Time Difference

'Quant/Qual RT difference > Absolute Difference Quant Qual Retention Times min'

*Flag sample if the absolute value of Quant RT – Qual RT > x (minutes).*

| | |
|---|---|
| NNAL_1 | x = 0.016 |
| NNAL_2 | x = 0.016 |
| NNN | x = 0.016 |
| NAT | x = 0.016 |
| NAB | x = 0.016 |
| NNK_1 | x = 0.016 |
| NNK_2 | x = 0.016 |

RT Shift from Standards

'RT Shift'

*Flag a sample if the Quant RT – mean standard Quant RT > x (minutes).*
*OR if the Qual RT – mean standard Qual RT > x (minutes).*
*OR if the ISTD RT – mean standard ISTD RT > x (minutes).*

| | | | | | |
|---|---|---|---|---|---|
| NNAL_1 | x = 0.083 | NNAL_1 CONF | x = 0.083 | NNAL_1 ISTD | x = 0.083 |
| NNAL_2 | x = 0.083 | NNAL_2 CONF | x = 0.083 | NNAL_2 ISTD | x = 0.083 |
| NNN | x = 0.083 | NNN CONF | x = 0.083 | NNN ISTD | x = 0.083 |
| NAT | x = 0.083 | NAT CONF | x = 0.083 | NAT ISTD | x = 0.083 |
| NAB | x = 0.083 | NAB CONF | x = 0.083 | NAB ISTD | x = 0.083 |
| NNK_1 | x = 0.083 | NNK_1 CONF | x = 0.083 | NNK_1 ISTD | x = 0.083 |
| NNK_2 | x = 0.083 | NNK_2 CONF | x = 0.083 | NNK_2 ISTD | x = 0.083 |

CDC Quant IS Retention Time Difference

'Quant/IS RT difference > x min'

*Flag sample if the absolute value of Quant RT – IS RT > x (minutes).*

| | |
|---|---|
| NNAL_1 | x = 0.0083 |
| NNAL_2 | x = 0.0083 |
| NNN | x = 0.066 |
| NAT | x = 0.066 |
| NAB | x = 0.058 |
| NNK_1 | x = 0.066 |
| NNK_2 | x = 0.066 |

RT Quant Qual Difference

'Quant-Qual RT > x'

*Flag a sample if the absolute value of the Quant RT – Qual RT> x (minutes).*

| | |
|---|---|
| NNAL_1 | - |
| NNAL_2 | - |
| NNN | - |
| NAT | - |
| NAB | - |
| NNK_1 | - |
| NNK_2 | - |

Not enabled. Redundant with CDC Quant/Qual Retention Time Difference.

**RETENTION TIME cont'd**

RT Relative RT Deviation

'Relative RT (%)'

*Flag a sample if the absolute value of the Relative RT deviation from mean Relative RT of standards > x%. (Relative RT = Quant RT / IS RT)*

| | |
|---|---|
| NNAL_1 | - |
| NNAL_2 | - |
| NNN | - |
| NAT | - |
| NAB | - |
| NNK_1 | - |
| NNK_2 | - |

Not enabled.

CDC Retention Time Shift**

*Flag sample if the absolute value of IS – mean standard IS > x (minutes).*

Not available (Would be redundant with RT Shift from Standards)

**CARRYOVER**

CDC Carryover

'Possible carryover'

*Flag sample (except standards) and next N samples if response > mean response of standards with possible carryover above standard level x.*

| | | |
|---|---|---|
| NNAL_1 | x = 8 | N = 1 |
| NNAL_2 | - | - |
| NNN | x = 8 | N = 1 |
| NAT | x = 8 | N = 1 |
| NAB | x = 8 | N = 1 |
| NNK_1 | x = 8 | N = 1 |
| NNK_2 | - | - |

Carryover Above ULOQ

'> ULOQ'

*Flag determination if the analyzed concentration is > ULOQ.*

| | |
|---|---|
| NNAL_1 | ULOQ = 20 ng/mL |
| NNAL_2 | ULOQ = 20 |
| NNN | ULOQ = 20 |
| NAT | ULOQ = 20 |
| NAB | ULOQ = 20 |
| NNK_1 | ULOQ = 20 |
| NNK_2 | ULOQ = 20 |

Not enabled. Redundant with CDC Carryover.

Carryover Flag Subsequent Samples

'Possible Carryover'

*Flag next x samples if analyzed concentration > ULOQ.*

| | |
|---|---|
| NNAL_1 | - |
| NNAL_2 | - |
| NNN | - |

| | |
|---|---|
| NAT | - |
| NAB | - |
| NNK_1 | - |
| NNK_2 | - |

Not enabled. Redundant with CDC Carryover.

**SENSITIVITY**

CDC Instrument Sensitivity

'Low Sensitivity'

*Flag standards if mean area of ISTD < x.*

| | |
|---|---|
| NNAL_1 | 6666.67 |
| NNAL_2 | 6666.67 |
| NNN | 6666.67 |
| NAT | 6666.67 |
| NAB | 6666.67 |
| NNK_1 | 6666.67 |
| NNK_2 | 6666.67 |

CDC Max Peak Height

'Above Detector Limit'

*Flag a determination in a sample if the peak height > x.*

| | | | | | |
|---|---|---|---|---|---|
| NNAL_1 | x = 2.0e8 | NNAL_1 CONF | x = 2.0e8 | NNAL_1 ISTD | x = 2.0e8 |
| NNAL_2 | x = 2.0e8 | NNAL_2 CONF | x = 2.0e8 | NNAL_2 ISTD | x = 2.0e8 |
| NNN | x = 2.0e8 | NNN CONF | x = 2.0e8 | NNN ISTD | x = 2.0e8 |
| NAT | x = 2.0e8 | NAT CONF | x = 2.0e8 | NAT ISTD | x = 2.0e8 |
| NAB | x = 2.0e8 | NAB CONF | x = 2.0e8 | NAB ISTD | x = 2.0e8 |
| NNK_1 | x = 2.0e8 | NNK_1 CONF | x = 2.0e8 | NNK_1 ISTD | x = 2.0e8 |
| NNK_2 | x = 2.0e8 | NNK_2 CONF | x = 2.0e8 | NNK_2 ISTD | x = 2.0e8 |

**DILUTION**

Concentration Over Diluted

'Over Diluted'

*Flag a determination in a sample if 0 < analyzed concentration < LLOQ AND the sample dilution factor >1.*

| | |
|---|---|
| NNAL_1 | LLOQ = 0.0006 ng/mL |
| NNAL_2 | LLOQ = 0.0006 |
| NNN | LLOQ = 0.0028 |
| NAT | LLOQ = 0.0042 |
| NAB | LLOQ = 0.0016 |
| NNK_1 | LLOQ = 0.01 |
| NNK_2 | LLOQ = 0.01 |

Concentration Dilution Required

'Needs Dilution'

*Flag a determination in a sample if the analyzed concentration > ULOQ.*

See ULOQ above, under "Carryover Above ULOQ."

Concentration Present But Below LLOQ

'Present < LLOQ'

*Flag a determination in a sample if a peak is integrated but calc conc < LLOQ.*

See LLOQ above, under "Concentration Over Diluted."

**QCs**

QC Concentration Deviation

'Concentration Deviation (%)'

*Flag a QC sample if the absolute value of the calculated concentration deviation from the nominal concentration ≥ x%.*

| | |
|---|---|
| NNAL_1 | x = 10 |
| NNAL_2 | x = 10 |
| NNN | x = 10 |
| NAT | x = 10 |
| NAB | x = 10 |
| NNK_1 | x = 10 |
| NNK_2 | x = 10 |

QC Exceeds Standard Deviation

'Concentration outside xSD'

*Flag a QC sample if the absolute value of the calculated concentration ≥ x number of standard deviations from the nominal concentration.*

| | |
|---|---|
| NNAL_1 | x = 3 |
| NNAL_2 | x = 3 |
| NNN | x = 3 |
| NAT | x = 3 |
| NAB | x = 3 |
| NNK_1 | x = 3 |
| NNK_2 | x = 3 |

QC Negative Control

'Negative QC > x% LLOQ (calculated concentration)'

*Flag a determination in a negative QC if the calculated concentration > x% of LLOQ.*

See LLOQ above, under "Concentration Over Diluted."

| | |
|---|---|
| NNAL_1 | x = 50 |
| NNAL_2 | x = 50 |
| NNN | x = 50 |
| NAT | x = 50 |
| NAB | x = 50 |
| NNK_1 | x = 50 |
| NNK_2 | x = 50 |

Contamination of Blank Sample

'Blank >= x% LLOQ'

*Flag a determination in a blank if the calculated con of blank ≥ x% of LLOQ.*

See LLOQ above, under "Concentration Over Diluted."

| | |
|---|---|
| NNAL_1 | x = 100 |
| NNAL_2 | x = 100 |
| NNN | x = 100 |
| NAT | x = 100 |
| NAB | x = 100 |
| NNK_1 | x = 100 |

| | |
|---|---|
| NNK_2 | x = 100 |

**QCs cont'd**

QC Flag Positive Unknowns
'QC Flagged'
*Flag a determination in an unknown sample if a QC was flagged AND the calculated concentration if the analyte ≥ LLOQ.*
See LLOQ above, under "Concentration Over Diluted."

**PEAK QUALITY**

Peak Quality Fit Quality

'Fit quality (%)'

*Flag a determination in a sample if the peak quality < x%.*

| | | | | | |
|---|---|---|---|---|---|
| NNAL_1 | x = 60 | NNAL_1 CONF | x = 40 | NNAL_1 ISTD | x = 70 |
| NNAL_2 | x = 60 | NNAL_2 CONF | x = 40 | NNAL_2 ISTD | x = 70 |
| NNN | x = 90 | NNN CONF | x = 80 | NNN ISTD | x = 90 |
| NAT | x = 90 | NAT CONF | x = 80 | NAT ISTD | x = 90 |
| NAB | x = 90 | NAB CONF | x = 80 | NAB ISTD | x = 90 |
| NNK_1 | x = 60 | NNK_1 CONF | x = 60 | NNK_1 ISTD | x = 60 |
| NNK_2 | x = 65 | NNK_2 CONF | x = 65 | NNK_2 ISTD | x = 65 |

Peak Quality High Signal No Peak

'High Signal, No Peak'

*Flag a determination in a sample if no peak is integrated and the max signal in the retention time window > x.*

| | | | | | |
|---|---|---|---|---|---|
| NNAL_1 | x = 10,000 | NNAL_1 CONF | x = 10,000 | NNAL_1 ISTD | x = 10,000 |
| NNAL_2 | x = 10,000 | NNAL_2 CONF | x = 10,000 | NNAL_2 ISTD | x = 10,000 |
| NNN | x = 10,000 | NNN CONF | x = 10,000 | NNN ISTD | x = 10,000 |
| NAT | x = 10,000 | NAT CONF | x = 10,000 | NAT ISTD | x = 10,000 |
| NAB | x = 10,000 | NAB CONF | x = 10,000 | NAB ISTD | x = 10,000 |
| NNK_1 | x = 10,000 | NNK_1 CONF | x = 10,000 | NNK_1 ISTD | x = 10,000 |
| NNK_2 | x = 10,000 | NNK_2 CONF | x = 10,000 | NNK_2 ISTD | x = 10,000 |

CDC Base Peak Width*

'Base peak width outlier'

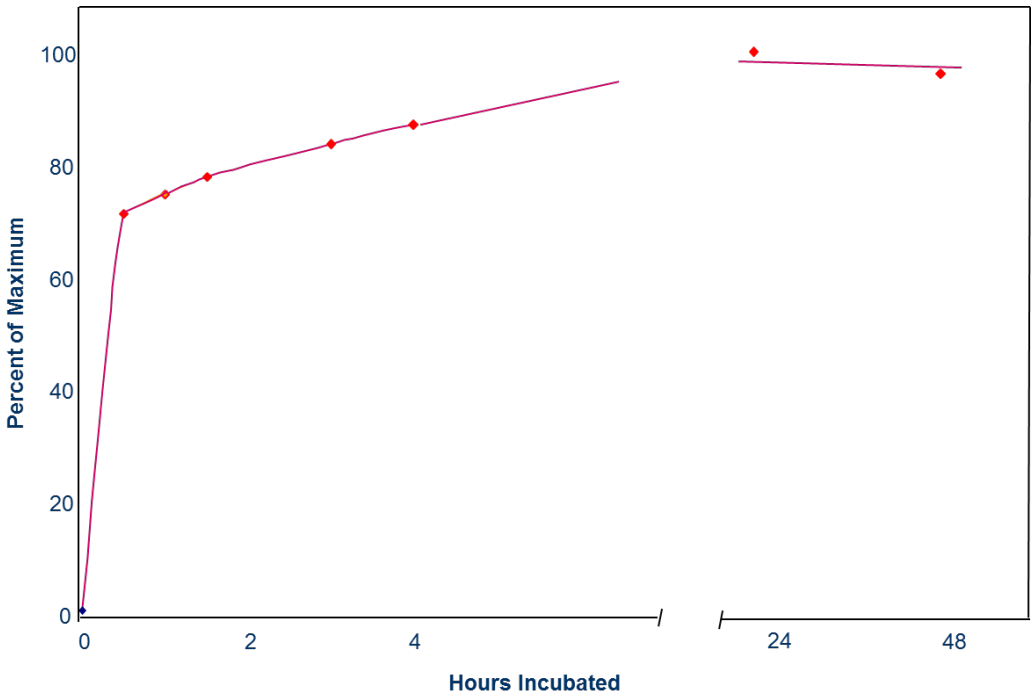
*Flag unknown samples if the calculated concentration > b and the base peak width is between c and d.*

| | | | | |
|---|---|---|---|---|
| NNAL_1 | b = 0.04 | QUANT | d = 10 | c = 3 |
| | | QUAL | d = 10 | c = 3 |
| NNAL_2 | - | QUANT | - | - |
| | | QUAL | - | - |
| NNN | b = 0.04 | QUANT | d = 30 | c = 5 |
| | | QUAL | d = 30 | c = 5 |
| NAT | b = 0.04 | QUANT | d = 30 | c = 5 |
| | | QUAL | d = 30 | c = 5 |
| NAB | b = 0.04 | QUANT | d = 20 | c = 5 |
| | | QUAL | d = 20 | c = 5 |
| NNK_1 | b = 0.04 | QUANT | d = 10 | c = 3 |
| | | QUAL | d = 10 | c = 3 |
| NNK_2 | b = 0.04 | QUANT | d = 10 | c = 3 |
| | | QUAL | d = 10 | c = 3 |

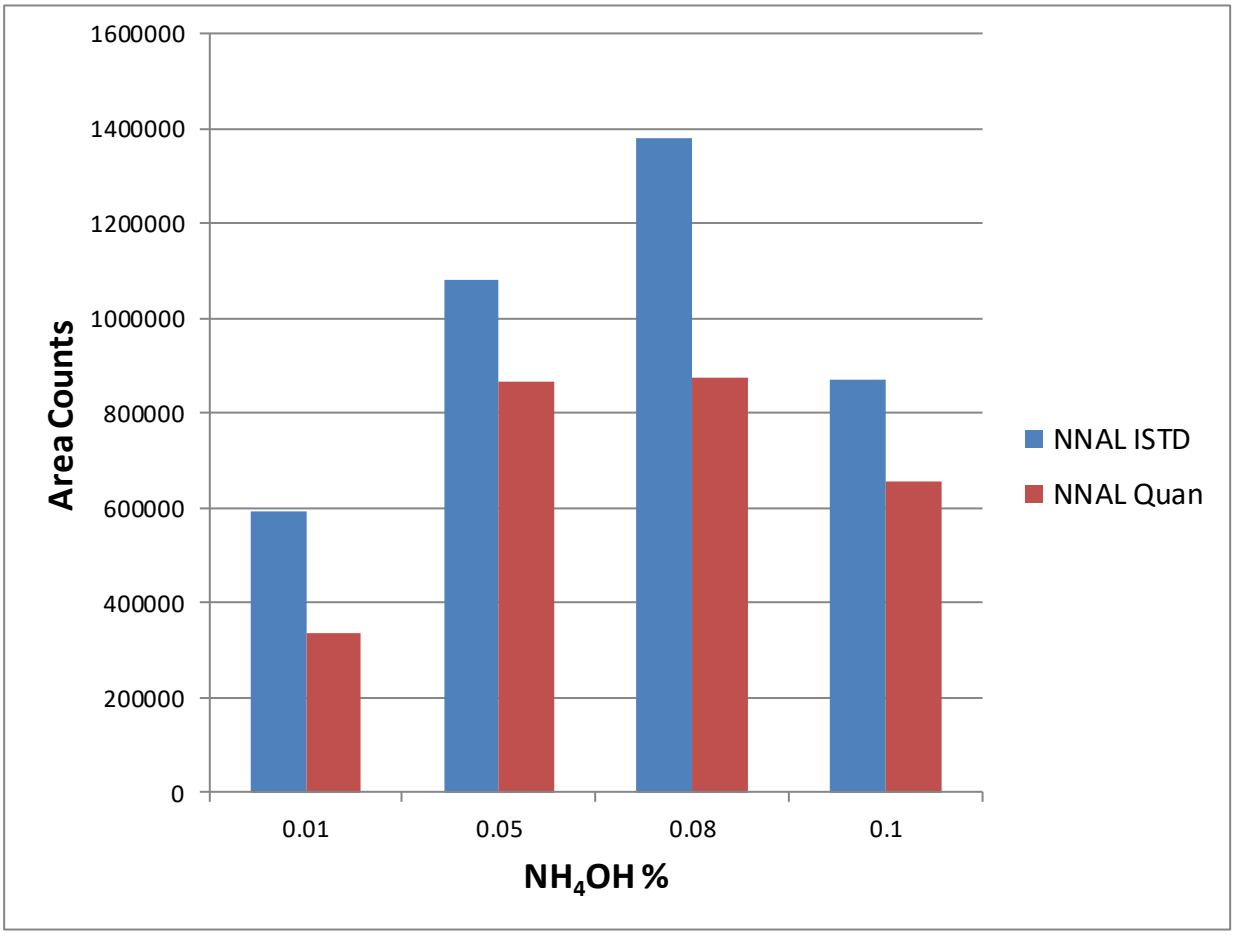
# Appendix G

## Ruggedness Tests

1. The time course for TSNA hydrolysis.



2. % of ammonium hydroxide in LC mobile phase.



3. The pH of phosphate buffer.