Division of Laboratory Sciences
Laboratory Protocol

Analytes: **Volatile N-Nitrosamines:**
N-Nitrosomethylethylamine (NMEA), N-Nitrosodiethylamine (NDEA), N-Nitrosopiperidine (NPIP), N-Nitrosopyrrolidine (NPYR), N-nitrosomorpholine (NMOR)

Matrix: **Urine**

Method: **GC/MS/MS**

Method No: **2016**

Revised:

As performed by:

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

Contact:

Dr. Tiffany Seyler
Phone: 770-488-4527
Fax: 770-488-0181
Email: tvh2@cdc.gov

James Pirkle, M.D., Ph.D.
Director, Division of Laboratory Sciences

Important Information for Users

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

Public Release Data Set Information

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

Data File Name	Variable Name	SAS Label
VNA_H & VNAS_H	URXNDEA	N-Nitrosodiethylamine (NDEA) (ng/L)
	URXNMEA	N-Nitrosoethylmethylamine (NMEA) (ng/L)
	URXNMOR	N-Nitrosomorpholine (NMOR) (ng/L)
	URXNPIP	N-Nitrosopiperidine (NPIP) (ng/L)
	URXNPYR	N-Nitrosopyrrolidine (NPYR) (ng/L)

1. Clinical Relevance and Summary of Test Principle.

a. Clinical Relevance

Volatile nitrosamines (VNAs) are harmful constituents in tobacco smoke. High levels of exposure have been reported during the consumption of smokeless or burned tobacco. Nitrate content in tobacco has significant influence on the tobacco smoke composition, cancer risk, and other disease risks relative to cigarette smoking. Nitrates are reduced to nitrites and can react with amines to form N-nitrosamine compounds. VNAs are well-established teratogens and carcinogens in animals and are classified as probable (group 2A) or possible (group 2B) carcinogens in humans. These compounds have also been shown to have genotoxic effects. It has been reported that VNAs exposure may play an important part in the etiology of esophageal and schistosome-associated bladder cancer. Studies have also shown correlations between VNA exposure and lipid peroxidation and oxidative stress (insulin-resistance and inflammation), chronic diseases (diabetes), and neurodegenerative diseases such as Alzheimer's.

b. Test Principle

VNAs in human urine are measured using an isotope dilution gas chromatography tandem mass spectrometric (GC/MS-MS) method. Urine samples are collected and stored at -60-70ºC. Urine sample is spiked with a mixture of isotopically labeled internal standards, and VNAs are extracted from urine with dichloromethane. Isotopically labelled internal standards are used for accurate quantification. Standard solutions with known VNA concentrations are analyzed, and the reconstructed ion chromatogram peak area ratios of native analytes to labeled internal standards are used to determine VNAs levels in samples.

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

Many VNAs are carcinogenic. Care should be taken to avoid inhalation or dermal exposure. Use a chemical fume hood when working with VNAs. Appropriate use

of personal protection including lab coat, gloves, and safety goggles are required when preparing or handling neat materials, standard solutions, extraction solutions, or collected samples.

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 any direct contact with the samples. Lab coats, gloves, and protective eyewear (as required) should be worn while handling the specimens.

d. 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.

e. Protective Equipment

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

f. Training

Training for sample preparation, sample handling, and equipment operation is required

g. Personal Hygiene

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

h. Disposal of Wastes

Dispose all waste materials in compliance with laboratory, federal, state, and local regulations. Solvents and reagents should always be disposed of in an appropriate container that has been clearly marked for waste products and temporarily stored in a chemical fume hood. Place disposable laboratory supplies such as vials, pipette tips, syringe, etc. that directly contact VNAs or samples in a biohazard autoclave bag or similar approved storage container.

3. Computerization and Data-System Management

a. Software and Knowledge Requirements

Proficiency is required for the analytical software package of the GC and mass spectrometer used in the analysis. For the Agilent GC/MS triple quadrupole mass spectrometer, this package is MassHunter™. Instrument raw data is uploaded to Indigo, a web based integration software, for evaluating the quality control and calculating analyte concentrations Further, statistical analysis of results requires proficiency in a standard statistical analysis software package. The Statistical Analysis System (SAS Institute, Cary, NC) is one such package.

b. Sample Information

Information pertaining to particular specimens entered into the database is either manually or electronically transferred. Data entered include the sample identification number, the sample type, standard number, and any other information not associated with the GC/MS/MS analysis. The GC/MS-MS relative response data for each sample and associated calibrators, QC, and blank data are transferred electronically into the database.

c. Data Maintenance

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

d. Information Security

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

4. Procedures for Collecting, Storing, and Handling Samples; Criteria for Sample Rejection

a. Special Instructions

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

b. Sample Collection

The specimen for these analyses is human urine. Based on the relatively short physiological half-lives of these analytes, urine samples integrating over longer time periods are preferred over spot urine samples. Urine can be collected by using standard equipment; mix the sample well before aliquoting, and freeze the urine aliquot in polypropylene cryogenic, screw-cap vials.

c. Sample Handling

Specimen handling and transport should be frozen prior to shipment, must be sent and received frozen where they will be stored at -60°C to -70°C until analysis. All samples are vortexed thoroughly prior to preparation (more details in sample preparation section).

d. Sample Quantity

The sample size is 1.9 ml of urine. This sample volume is required to quantify the analyte concentrations listed for the Limits of Detection in Section 9b. However, detectable concentrations can likely be measured in a reduced volume sample (1 ml).

e. Unacceptable Specimens

Criteria for defining a sample as unacceptable include (1) use of improper collection materials or techniques leading to possible background contamination; and (2) sample volume below 1 ml.

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

Not applicable for this procedure.

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

a. Reagents, Preparation Materials and Sources

Reagents and sources used in this method are listed below.

Solvents and sources.

Reagent	Grade	Source*
Dichloromethane	HPLC	B&J, VWR, Suwanee, GA
Methanol	HPLC	B&J, VWR, Suwanee, GA
Acetonitrile	HPLC	B&J, VWR, Suwanee, GA

* Or equivalent

All chemicals and solvents are used without further purification.

b. Reagent Preparation

There is none for this assay.

c. Standard Materials

1) Native Standards

   Purchase native VNA standards from a commercial vendor. One such vendor is Supelco (Belafonte, PA). Standard stock solution is in dichloromethane and stored in a -20ºC freezer. Prepare standard solutions in ACN.

2) Isotopically Labeled Internal Standards

   Purchase ^{2}H-labeled VNAs internal standards from a commercial vendor. One such vendor is Cambridge Isotope Laboratories (CIL) (Andover, MA) or Toronto Research Chemical (TRC).

3) Quality Control Materials

   There are three quality control pools for the urinary volatile nitrosamine assay. Pools QC low and QC high contain relatively low and high levels of VNAs, respectively. A QC dilute pool is prepared at a level that exceeds the highest calibrator of the calibration curve. The QC pools were prepared in house from urine collected from non-smokers spiked with standard stock solution. Each of these pools was mixed well, dispensed in 2.00 ml aliquots into 2 ml cryovials with screw cap, and frozen at -60°C to -70°C.

d. Other Material and Supplies

Materials, supplies and sources used during the development and validation of this method are listed below. Materials and supplies for use with this method should be equivalent to those listed if obtained from other sources.

- Pipettes and disposable tips capable of accurately dispensing the following volumes: 0.5µl to 200µl, 1ml, 2ml (Hamilton, Fisher Scientific)
- Disposable siliconized test tubes, 13x100 mm
- Disposable 48-well plate (Axygen)
- 300 µl insert amber vials (Fisher Scientific)
- Assorted glassware

e. Equipment

- Commercial Caliper Staccato automation system
- Commercial Hamilton STAR automation system
- Commercial tandem mass spectrometer such as the Agilent triple quad G7001C (or comparable)
- Commercial gas chromatography system (GC) such as the Agilent 7968 GC system (or comparable).
- Commercial autosampler system (AS) such as the Agilent G4514A system (or comparable)
- Commercial ThermoSavant SpeedVac SPD 2010 (vacuum evaporator)

f. Instrumentation

1) Gas chromatograph

Instrument operating parameters (specific to Agilent GC 7968)

Parameter	Setting
Injection port	PTV solvent vent
Injection port temperature	5°C for 0.85 min.
Temperature gradient	600°C/min
Final temperature	280°C
Temperature hold time	11.5 min
Pressure	13.662 psi
Pressure hold time	0 min
Vent flow	200 mL/min, 0 psi, 0.7min.
Vent pressure	5 psi, 0.7 min.
Injection volume	5 µl
Purge flow to split vent	20 ml/min
Purge flow to split vent time	2 min
Cryo (liquid nitrogen)	ON
Cryo use temperature	200°C
Back flush flow to inlet	25 ml/min
Back flush time	4 min
Column 1, J & W 122-733DB-WAXetr 30m x 250 µm x 0.5 µm	Constant flow 1.2 ml/min.
Column 2, deactivate fuse silica 1m x 150 µm x 0.0 µm	Constant pressure 1 psi, post run at 25 psi

GC oven temperature gradient for VNAs separation and post-run back flush.

	Rate (°C /min)	Final temp °C	Hold time (min.)
Initial		35	1
Ramp 1	20	245	0
Post run		250	4 min

2). Mass Spectrometer Source/Gas Parameters

The MS/MS configuration is described below.

Instrument Operating Parameters (specific to Agilent 7000C)

Parameter	**Setting**
Mass spectrometer mode	Positive ion detection
Ionization source	Chemical ionization (CI)
CI gas	Ammonia
CI gas flow	25%
Source temperature	250°C
Quad temperature	150°C
Collision gas	Nitrogen at 1.5 ml/min
Collision pressure	35 psi
Quench gas	Helium at 2.25 ml/min
Detector setting	Gain at 50

3). Selected Reaction Monitoring Parameters

The ion pairs and compound dependent parameters are listed below.

Quantification (Quan) and conformation (Conf) ions.

Analyte	**Precursor (amu)***	**Quan**	**Conf**	**CE Quan/Conf**	**DT (msec)**
NMEA	106	89	61	2/21	30
NDEA	120	103	75	2/16	30
NPIP	132	115	69	2/33	30
NPYR	118	55	41	26/40	30
NMOR	134	117	87	5/18	40
NDEA-$^{2}H_{10}$	130	113	--	5	30
NMEA-$^{2}H_{3}$	109	92	--	10	30
NPIP-$^{2}H_{10}$	142	125	--	2	30
NPYR-$^{2}H_{8}$	126	109	--	4	30
NMOR-$^{2}H_{8}$	142	125	--	5	40

**Precursor ions are [M+18] or [M+NH_3] adducts*

The Agilent Triple Quad specific operational variables CE and DT refer to collision energy and dwell time.

4) GC-MS/MS Instrument Control Program

An instrument control program for the Agilent GC/MS Triple Quad created using the MassHunter™ software that incorporates the above parameters is used for data acquisition.

7. Calibration and Calibration Verification

a. Creation of Calibration Curve

1) Data Collection

A calibration curve is constructed at the beginning of each study using a ten-point curve of response factors (i.e., peak area ratio of analyte to labeled internal standard) versus calibration standard amount (pg). All 10 standard solutions are injected 3 times (5 µL/each) and subsequently analyzed on the GC/MS-MS to evaluate the linearity response for each analyte. A calibration curve is obtained with each analytical run and after a major service on the instrument (such as oil change, filament replacement, source cleaning, etc.). A consistent smoothing factor may be used to facilitate auto-integration of all peaks (samples, standards, blanks, and QCs). The smoothing variables used in the Agilent MassHunter™ instrument software are referred to as smoothing factor and bunching factor. The smoothing factor ranges from 0 to 10, but a value of 1 is typical. The bunching factor can be set between 1 and 100; a value of 1 is typical.

2) Calculation and Evaluation of Curve Statistics

The slope and intercept of the calibration curve are generated using linear regression with 1/x weighting. This analysis can be performed using the instrument's data analysis software or other suitable data analysis software. The resulting plot should be visually examined for linearity over the entire

calibration range. Determine the slope and intercept of the calibration curve by linear least squares fit. Any deviations from this procedure (e.g., using a quadratic fit) must have a valid scientific justification and be approved by a supervisor.

b. Usage of Curve

The calibration range was chosen based on VNA levels from previously measured urine samples from smokers and nonsmokers. Quantification can only be reported for values that fall within the calibration range (between highest and lowest calibrator points).

For sample results that are higher than the highest calibrator, the analysis can be repeated with a smaller amount of sample to bring the result within the calibration range. For these repeated samples, a QC dilute sample will be used in place of the QC high pool sample.

c. Calibration Verification

The accuracy of the calibration curve is verified by using testing calibrators. The testing calibrators were prepared using native standards purchased from a different vendor. If a different vendor is not available, a different lot of native standards from the same vendor can be used. Three levels (low, medium, and high) of testing calibrators were prepared. The back calculated values of the testing calibrators must be within 5-7% of the expected values. This accuracy test is performed each time a new standard calibration set is prepared and used for analyte quantitation.

Calibrations are further confirmed semi-annually through the use of PT pools with previously characterized concentrations.

8. Procedure Operation Instructions; Calculations; Interpretation of Results

An analytical run consists of 10 calibration standards, 2 blanks, 1 QC low, 1 QC high, and unknown urine samples.

a. Hamilton- volume verification

Volume verification is done weekly using methanol and water. Temperature is measured. Set volumes are dispensed in pre-weighted vials, and the pre- and post-weight difference is calculated. Density of methanol and water are used to calculate the actual volume dispensed by the Hamilton. If the difference between the set and actual volume delivered is less than 5%, then the volume delivered is accurate. If the difference is more than 5%, then a service call will be placed with Hamilton. No aliquot can be performed until a service engineer from Hamilton services and certifies the volume verification.

b. Sample Preparation

Before preparing any samples, check for background contamination from the Caliper. Dispense 1 mL DCM from the Caliper's bulk dispenser into a 1.5 mL amber GC vial. Analyze 5 µL on the GC/MS-MS under the same conditions as the samples to check for any background contamination for all six analytes. If the DCM is clean, proceed to sample preparation as below. If the DCM is not clean, flush the Caliper tubing liner with methanol, and repeat the testing until there is no background contamination.

i. Thaw samples at room temperature if they are frozen
ii. Decap samples and load onto two Hamilton sample carriers. On carrier one: a DCM blank in position 1, QC low in position 2, unknown samples from position 3-24. On carrier 2: unknown samples from 1-22, QC high in position 23, and another DCM blank in position 24. This will result in one run/batch with a total of 2 blanks, 1 QC low, 1 QC high, and 44 unknown samples.
iii. Place a labeled 48-well collection plate C1 on the Hamilton deck.
iv. Place 4 1.5 mL amber GC vials of ISTD spiking solution (10 pg/µL) in position 25-28 of Hamilton carrier 1.

v. Scan in the barcodes from the original sample vials and mixing plate to generate a Hamilton output file that is used to make a sequence file and run sheet.
vi. Aliquot 1.9 mL of each sample to a well on 48-well sample plate (Hamilton). If a QC dilute sample is use, then deliver only 1 mL.
vii. Add 50 µL of 10 pg/µL ISTD to each well (Hamilton)
viii. Transfer the 48-well sample plate from Hamilton to Caliper
ix. Dispense 2.5 mL DCM to each well, pipette mix for approximately 40 minutes (Caliper)
x. Seal plate and centrifuge for 5 minutes (1500rpm, room temperature) (Caliper)
xi. Pierce seal and transfer 1.5 mL of DCM layer to new 48-well collection plate C2(Caliper)
xii. Dry down C2 plate in TurboVap for 9.75 minutes, seal plate (Caliper)
xiii. Remove seal and manually transfer residue samples in C2 plate to 48 labeled GC vials
xiv. Dry down GC vials to approximately 75 µL in Savant (approximately 9-10 minutes)
xv. Add 50 µL acetonitrile as a keeper solvent to each GC vial, and evaporate the rest of DCM in Savant (approximately 7 minutes)
xvi. Cap vials with aluminum crimp caps
xvii. Run samples on GC-QQQ or store at -70°C

c. Sample Analysis

This method uses GC coupled with a triple quadrupole mass spectrometer to quantitate VNAs in human urine. The Hamilton output file that was generated during the sample preparation process is converted to a MassHunter sequence file. This sequence file is used to run the prepared batch on an Agilent GC triple quad 7000C.

The analytes are first resolved from other potential interferences on an Agilent WaxETR™ column (comparable column could be used).

Further selectivity is accomplished using a triple quadrupole mass spectrometer operated under positive chemical ionization and multiple reaction monitoring (MRM) mode. Comparison of the area ratio (native analyte area/isotope labeled analyte area) with previously generated calibration curve yields individual analyte concentrations.

Before the run:

1) Analyze an ACN solvent blank to check for contamination in the GC/MS-MS system.
2) Inject a check standard under the same conditions as the samples to check the performance of the GC/MS-MS system.
3) Check retention times and responses (area counts) for the internal standards. If the retention times are similar (± 0.3 min) to the retention times from previous runs (unless the analytical column is replaced or trimmed), and the responses are within ±20% of the typical responses from the previous runs ($1x10^5$ counts) the system is ready to perform the analysis. The acceptable calculated values for each analyte concentration are the nominal concentration ± 10%.
4) The run order should be an ACN blank, ten calibrators (0.0-400.0 pg/µL), an ACN blank, a QC blank, a QC low, unknown samples, a QC high, and a QC blank.

d. Processing of Data

Process all the raw data files using instrument's quantification software (or comparable software package).

1) Analyzing and Storing the GC/MS-MS Data
   a) Upon completion of an analytical run, load the raw data files and sequence files onto the Indigo ASCENT™ web server. Quantitation will

be done automatically via Indigo ASCENT™ software: selection and integration of quantification and confirmation peaks for all native analytes and the internal standards; sample QC verification such as retention time, internal standard counts, carry over, etc.

b) Review the automated integrations of peaks to ensure correct integration. Manually re-integrate if the limits were chosen incorrectly.
c) Verify and certify the quantitation results.
d) Download the result .csv file and upload it to the DLS STARLIMS server.
e) Download the .pdf report and save it in the TEB share drive folder.
f) If Indigo ASCENT server is not available, Agilent MassHunter™ Quant can be used to quantify the results:
    i. Verify the quantitation results
    ii. Generate a report using a STARLIMS template report and upload it to the DLS STARLIMS server.
g) Store all Hamilton output files, raw data files, and Indigo reports in the TEB share drive folder: \\cdc\project\CCEHIP_NCEH_DLS_TVB_TEBL\VNA, organized according to run sheet name.
h) Backup all data files weekly.

2) Evaluation of Calibration Curves

The y-intercept of each calibration curve should not be significantly different from zero ($p > 0.05$); if it is, the source of bias should be identified. An R^2 of > 0.98 is acceptable. Through visual inspection, check to see if any single standard is an outlier. If removal of a point changes the slope or intercept by more than 10% it should be considered an outlier. No more than one standard point may be discarded. If either the high or low standard is removed, the reporting limits must be adjusted to reflect the new reporting range.

3) Evaluation of Quality Control Material

After the completion of a run, the calculated results from the analysis of quality control samples are compared to the established quality control limits to determine if the run is “in control”. Quality control procedures implemented in this method are defined by the Division’s Policies and Procedures Manual (for more information see: Caudill SP, Schleicher RL, and Pirkle JL (2008) Multi-rule quality control for the age-related eye disease study, Stat Med, 27: 4094-4106.). QC samples are subjected to the complete analytical process. The data from these materials are then used to estimate method precision and to assess the magnitude of any time-associated trends. The concentrations of these materials should cover the expected concentration range of the analytes for the method.

a) If both the low and the high QC results are within the 2σ limits, then accept the run.

b) If one of two QC results is outside the 2σ limits, then apply the rules below and reject the run if any condition is met.

i. Extreme Outlier – Run result is beyond the characterization mean +/- 4 S_i
ii. 3S Rule - Run result is outside a $3S_i$ limit
iii. 2S Rule - Both run results are outside the same 2Si limit
iv. 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
v. R 4S Rule – Two consecutive standardized run results differ by more than $4S_i$. Note: Since runs have a single result per pool for 2 pools, comparison of results for the R 4S rule will be with the previous result within run or the last result of the previous run. Standardized results are used because different pools have different means.

9. Reportable Range of Results

a. Limit of Detection

The method detection limits for VNAs in human samples are determined according to the guideline for determination of limits of detection by the Clinical and Laboratory Standard Institute (CLSI. Protocols for Determination of Limits of Detection and Limits of Quantitation: Approved Guideline. CLSI document EP17-A. Wayne, PA: Clinical and Laboratory Standards Institute, 2004).

b. Accuracy

Neat reference and internal standards are obtained from commercial sources. Stock solution concentrations are based on stated purity using gravimetric analysis. The source, purity, and manufacture's purity confirmation procedures are listed below.

Standard name	Manufacturer	Purity	Method for determining purity	Method for determining concentration	Cat #
NMEA	Supelco	99.8%	GC-FID	^{1}H NMR	502138
NDEA	Supelco	99.9%	GC-FID	^{1}H NMR	502138
NPIP	Supelco	99.9%	GC-FID	^{1}H NMR	502138
NPYR	Supelco	99.9%	GC-FID	^{1}H NMR	502138
NMOR	Supelco	99.9%	GC-FID	^{1}H NMR	502138
NMEA-$^{2}H_{3}$	TRC	>96%	GC-MS	^{1}H NMR	N525952
NDEA-$^{2}H_{10}$	Cambridge Isotope lab.	>98%	NEAT	GC-FID	DLM-7982-S-0
NPIP-$^{2}H_{10}$	TRC	>98%	GC-MS	^{1}H NMR	N545372
NPYR-$^{2}H_{8}$	Cambridge Isotope lab.	>98%	NEAT	GC-FID	DLM-8252-S
NMOR-$^{2}H_{8}$	Cambridge Isotope lab.	>96%	NEAT	GC-FID	DLM-8254-S

Accuracy was determined by spiking known amounts of VNA standard solution into ACN (accuracy in solution) and urine (accuracy in matrix). The spiked urine samples were processed the same as for unknown samples. The accuracy was calculated by the following formula.

%bias =100 (observed VNA level-expected VNA level)/expected VNA level*

The acceptable range for accuracy is ±5-7% for accuracy in solution and ±20% for accuracy in matrix.

c. Precision

The relative %RSD values calculated over 20 runs spanning at least 5 days include both within-day and between day error. Acceptable RSD values should consistently fall below 20% for all the analytes. If higher RSD values are obtained, the origin should be investigated and corrective action discussed with supervisor.

d. Analytical Specificity

A high degree of analytical specificity is achieved with this approach. Correct retention times, correct ion mass-to-charge ratios, and correct precursor/product ion transitions help ensure a very high degree of specificity and minimize the influence from any potential interference.

An established range of ratios of the response of quantitation ion to that of confirmation ion of QC samples is used to determine if an unknown sample tests positive for a given analyte.

e. Recovery

Sample matrix effects for each analyte are evaluated. Spiking same amounts of isotope labeled VNA internal standards in urine samples. Prepare these spiked samples according to sample preparation procedures. % recovery is calculated as the ratio of the responses of labeled internal standards (area count) in the urine samples to the responses of internal standard in the standards. The average recovery is 35% for NMEA and NDEA, and 70% for NPIP, NPYR, and NMOR.

f. Linearity Limits

The VNA calibration curves established are linear over the concentration ranges from the low and high standard with R^2 values greater than or equal to 0.98. The

lower reportable limit is either the LOD or the lowest standard concentration, whichever is higher. The upper reportable limit is the highest standard concentration. A residual plot of the calibrators is checked to confirm linearity.

g. Ruggedness test

Ruggedness testing was performed to assess the potential of important analytical variables to affect results. Each of these variables was systematically varied to examine their influence, if any, on the analytical results and was optimized to achieve sensitivity and high throughput.

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

Quality assessment procedures follow standard practices. Daily experimental checks are made on the stability of the analytical system.

First, a check standard is analyzed under the same instrument conditions as that for samples to check the performance of the GC/MS-MS system. If the retention times and peak intensities of the analytes are within the acceptable ranges described in section 8.b., then add ten standards, blanks, QCs and unknown samples to the run sequence.

Examine the blank prior to running the samples to check for possible contamination in the system or extraction solution or reagents. Compare the QC results obtained from the run with the acceptance criteria to assure the proper operation of the analysis. If a QC result is “out of control”, the cause of the failure should be determined. No results from the associated batch may be reported.

b. Establishing QC Limits

As per division policy, acceptable QC concentration limits must be calculated from the concentration results observed in at least 20 characterization runs. During the 20 characterization runs, previously characterized QCs or pools with

target values assigned by outside laboratories should be included to evaluate the analysis. The process of limits calculation is performed using the laboratory database and the SAS division QC characterization program (for more information see: Caudill SP, Schleicher RL, and Pirkle JL (2008) Multi-rule quality control for the age-related eye disease study, Stat Med, 27: 4094-4106.).

c. Proficiency Testing

1. PT is conducted twice a year, usually in March and September each year. At this time there are no external PT programs or certified reference materials for volatile nitrosamines in human urine. Therefore, we developed an in-house PT program administered by an independent quality control officer. We purchased pure standards of all six VNAs from different vendor or different lots from the same vendor than the corresponding standard material used to prepare calibrators. PT solutions are prepared from these standard materials using dilution schemes as outlined in Section 6. PT samples are prepared at 2-3 different levels covering the calibration range and blind-coded by the branch statistician. Five blind-coded PT samples are prepared as described in Section 8a for urine samples.
2. PT is done by analyzing a series of 5 “blinded” (the analyst is unaware of the target concentrations for “blinded” samples) VNA spiked urine samples once every six months. A correct determination, within 20%, on at least 4 of the 5 samples must be achieved to be considered proficient.
3. Performance in the PT program along with documentation of remedial action taken for unacceptable performance is to be documented in a QC Manual in the laboratory that is available for review.

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

If the calibration or QC fails, all operations are suspended until the source or cause of failure is identified and corrected. Analytical results are not reported.

After calibration and /or quality control have been reestablished, analytical runs may be resumed.

a. Internal Standard Response

If the area counts of the internal standards of the check standard fall below 30% of the median of the previously obtained values, this indicates that the instrumental sensitivity has fallen below acceptable limits. The following steps should be taken, and the instrument sensitivity rechecked after each is performed. Once sensitivity has been reestablished, further steps are not necessary.

1) Replace the syringe wash vials and rinse or replace the syringe
2) Replace injection liner, o-ring, and septum
3) Clean the mass spectrometer ion source
4) Clean the mass spectrometer quads.
5) If the sensitivity is lowered due to band broadening, inspect all GC connections and consider changing the analytical column.

b. Calibration Regression

If the linearity of the calibration curve criterion 0.98 is not met, check if the standards are prepared correctly or if an instrument malfunction has occurred. If no standard preparation is found in error, check if the detector is saturated. Also check GC delivery pressure for deviation from normal. Other instrument specific factors that could cause calibrations problems, such as leaks, should be checked and corrective action taken as needed.

c. Analyte in Standards or QC Materials

If an unexpectedly large amount of analyte is measured in one of the calibration standards or QC materials but is not seen in the remainder of the samples, this indicates a contamination of this particular sample. The source of this incident

should be investigated to prevent repeat occurrences, but no further action is required.

d. Analyte in All Samples

If an unexpectedly large amount of analyte is present in all measurements for a particular day, it is likely that one or more of the spiking solutions are contaminated. If necessary, prepare new solutions.

e. QC Sample Outside of Control Limits

Verify the integrity of the QC material if the result of QC sample falls outside the control limits. Check if the proper amount of internal standard was added to that sample. Also confirm that the integration was performed correctly. No analytical results can be reported for runs for which the QC is outside of the control limits.

12. Limitation of Method; Interfering Substances and Conditions

This method is an isotope dilution mass spectrometry method and provides a high degree of specificity. However, occasional interferences from unknown substances could be encountered and any spurious result should be investigated. Samples with concentrations outside the calibration range are not reportable.

13. Reference Range (Normal Values)

The study population typically includes both smokers and non-smokers; therefore, a large range of urinary volatile nitrosamine levels are expected. There are no current literature reported mean levels for nonsmokers and smokers. We plan to apply the method described in this document to characterize population-based ranges for U.S. smokers and non-smokers.

14. Critical-Call Results (“Panic” Values)

Not applicable to this procedure.

15. Sample Storage and Handling during Testing

Human urine samples selected for analysis are stored in a freezer -70°C±10°C.

VNAs are light sensitive, and samples should be protected from light and stored in the dark.

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

If the instrument measurement is delayed, samples should be stored in a freezer -70°C±10°C. Prepared samples can be stored in a freezer -70°C±10°C for up to 60 days prior to analysis.

17. Test-Result Reporting System; Protocol for Reporting Critical Calls (if applicable)

Analytical results are reportable once the validity of the data is established by the division's QC/QA policies and procedures and verified by a DLS statistician. One hardcopy and one electronic copy (ASCII format) of the data will be generated. This data, a cover letter, and a table of method specifications will be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director). After approval, the report will be sent to the contact person who requested the analyses.

18. Procedures for Specimen Accountability and Tracking

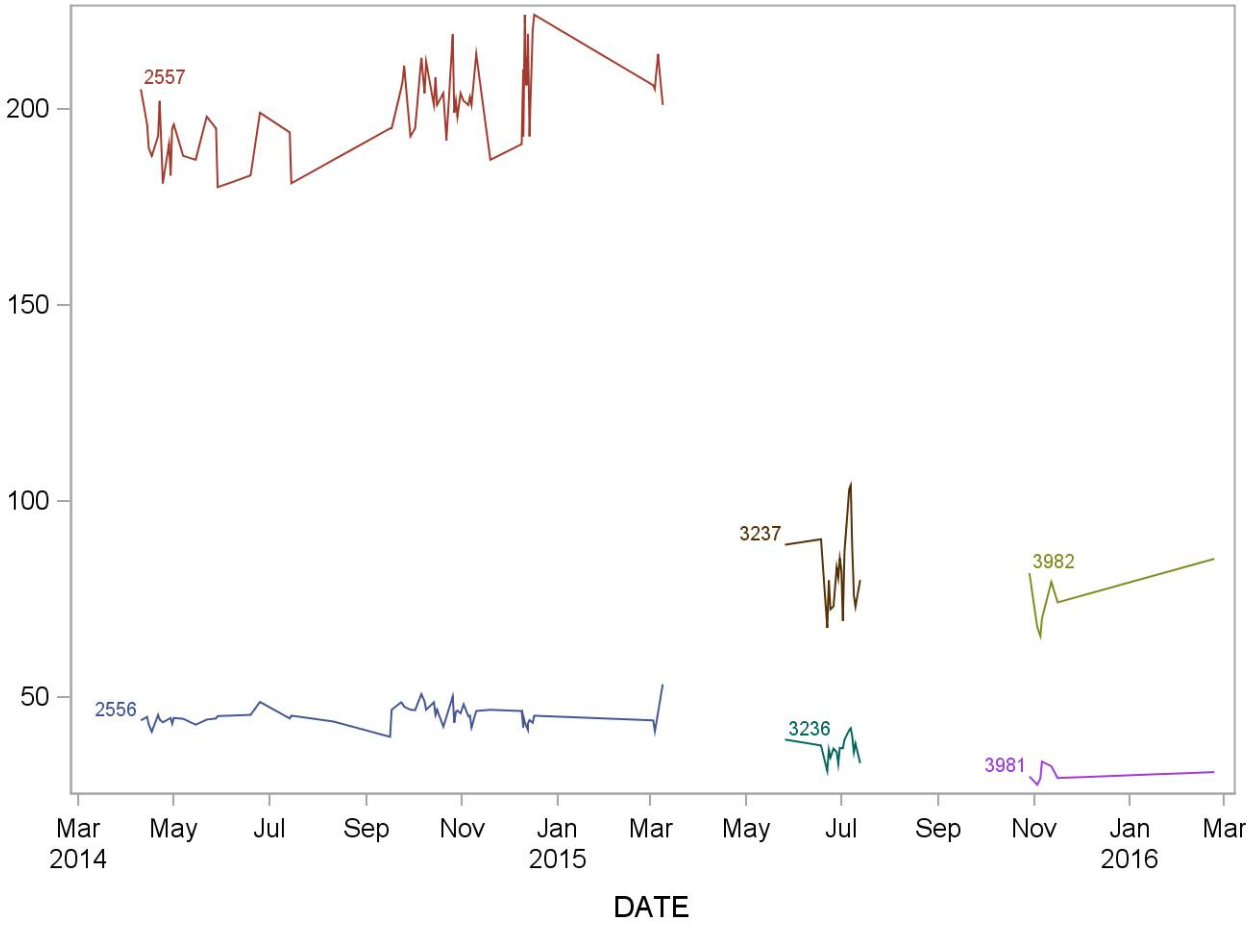
Standard record keeping (e.g., sample ID, notebooks, data files, database, etc.) is used for sample tracking. All records are maintained in accordance with the HHS Records Management guidance. (See http://www.hhs.gov/open/records/index.html).

19. Summary Statistics

See following pages.

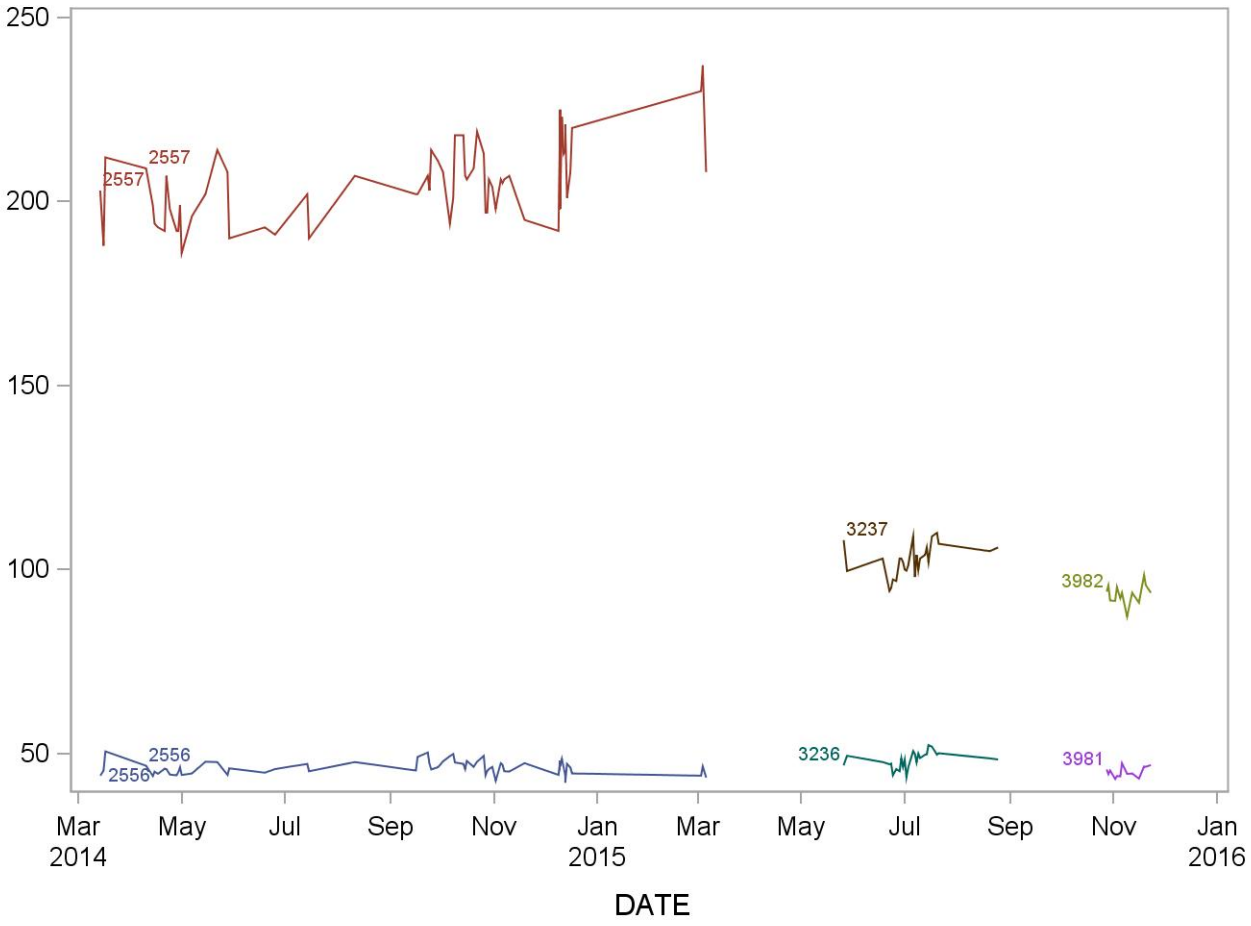
2013-2014 Summary Statistics and QC Chart for N-Nitrosodiethylamine (NDEA) (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2557	61	10APR14	09MAR15	199.9672	10.7625	5.4
2556	61	10APR14	09MAR15	45.1951	2.4501	5.4
3237	18	26MAY15	13JUL15	82.3444	10.2156	12.4
3236	18	26MAY15	13JUL15	36.9278	2.8615	7.7
3982	7	29OCT15	24FEB16	74.7857	7.4573	10.0
3981	7	29OCT15	24FEB16	30.3429	2.0140	6.6



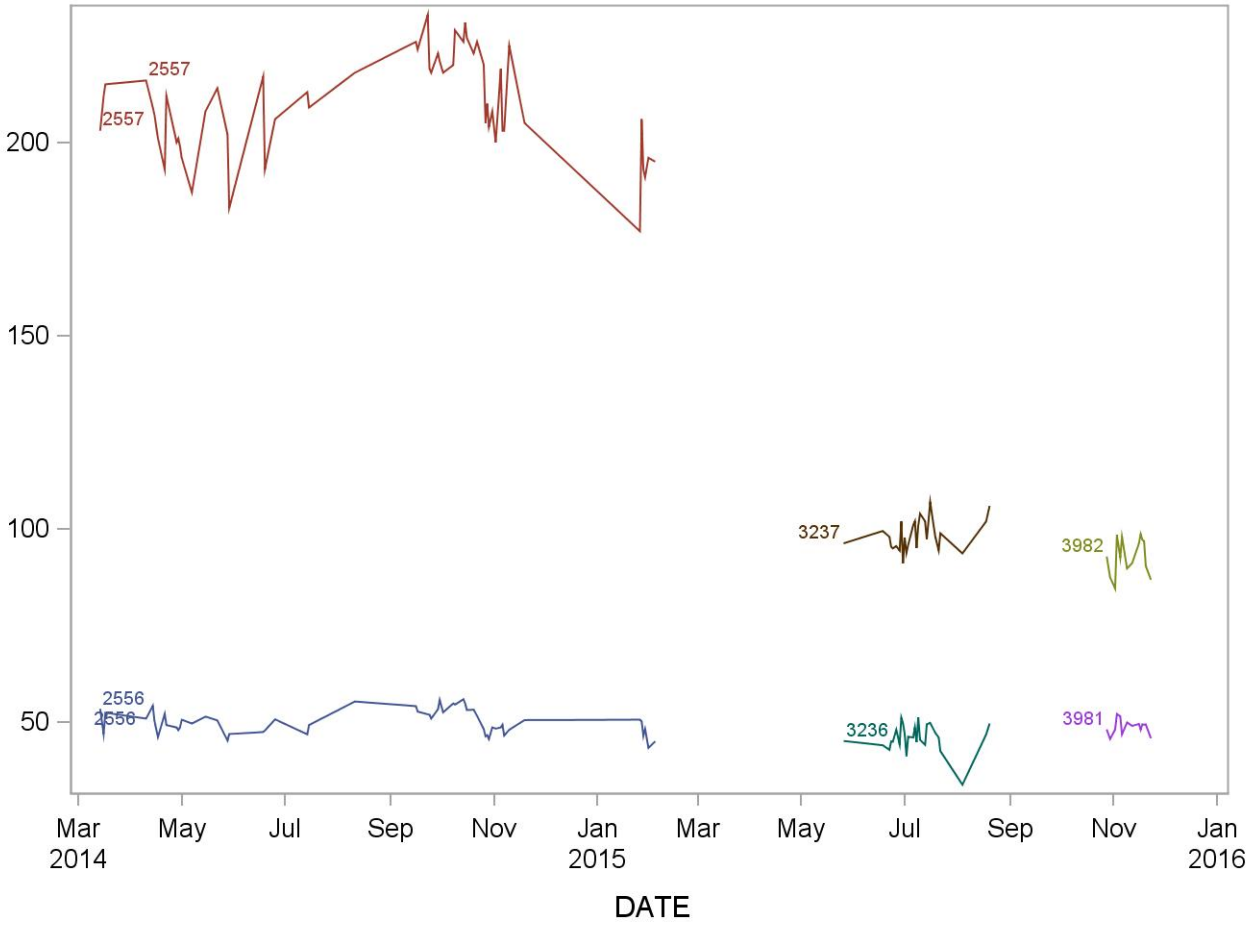
2013-2014 Summary Statistics and QC Chart for N-Nitrosoethylmethylamine (NMEA) (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2557	3	14MAR14	17MAR14	201.0000	12.1244	6.0
2556	3	14MAR14	17MAR14	46.6667	3.4775	7.5
2557	60	10APR14	06MAR15	204.9500	10.6571	5.2
2556	60	10APR14	06MAR15	46.1400	1.7879	3.9
3237	26	26MAY15	25AUG15	102.5192	4.3073	4.2
3236	26	26MAY15	25AUG15	48.2346	2.2073	4.6
3982	13	28OCT15	23NOV15	93.3769	2.7956	3.0
3981	13	28OCT15	23NOV15	45.0615	1.4274	3.2



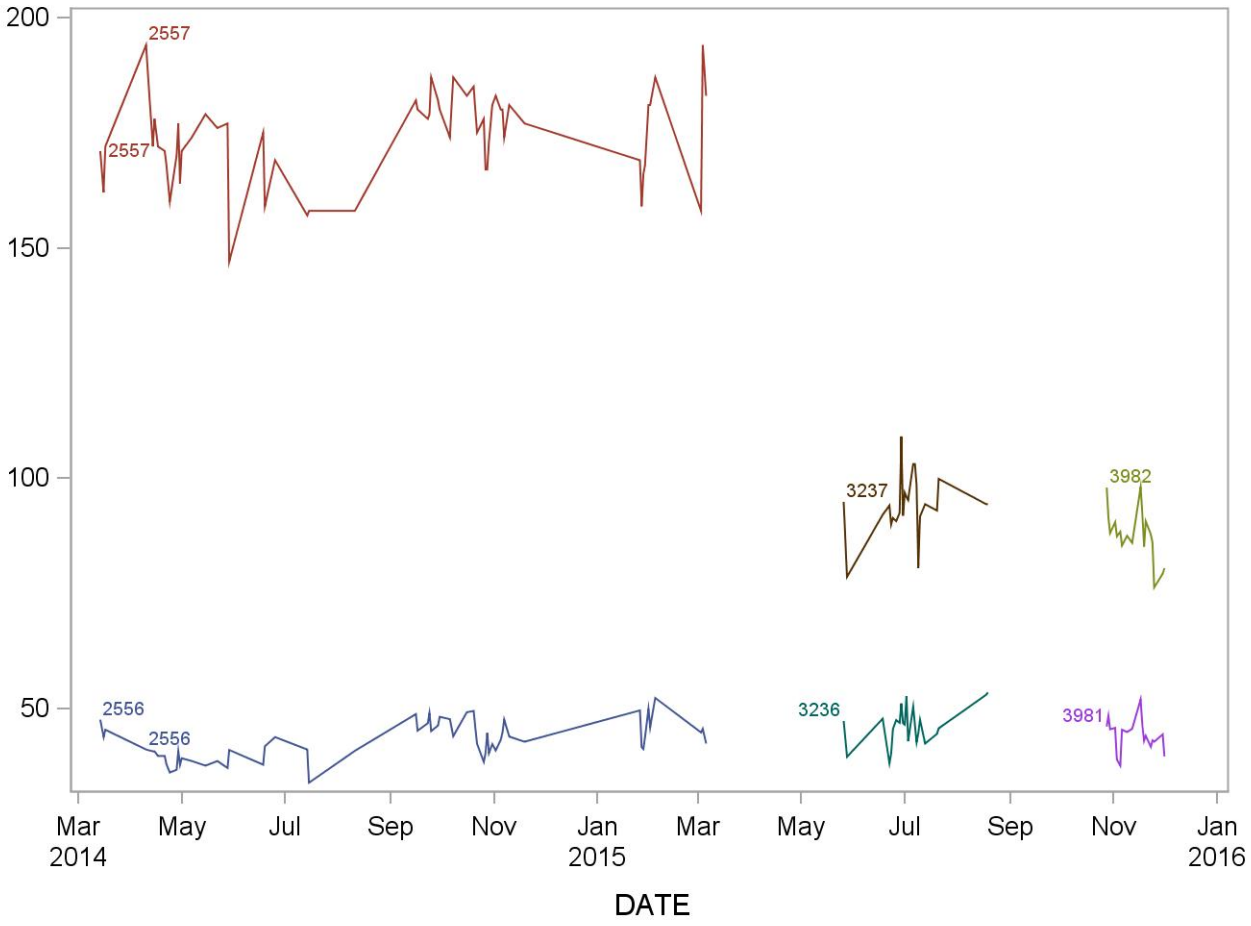
2013-2014 Summary Statistics and QC Chart for N-Nitrosopiperidine (NPIP) (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2557	3	14MAR14	17MAR14	210.0000	6.2450	3.0
2556	3	14MAR14	17MAR14	51.0000	3.5000	6.9
2557	53	10APR14	04FEB15	209.5849	13.0832	6.2
2556	53	10APR14	04FEB15	50.1038	3.0935	6.2
3237	26	26MAY15	20AUG15	98.3577	4.0484	4.1
3236	26	26MAY15	20AUG15	46.0308	3.6253	7.9
3982	14	28OCT15	23NOV15	92.9714	4.7181	5.1
3981	14	28OCT15	23NOV15	48.8500	1.8752	3.8



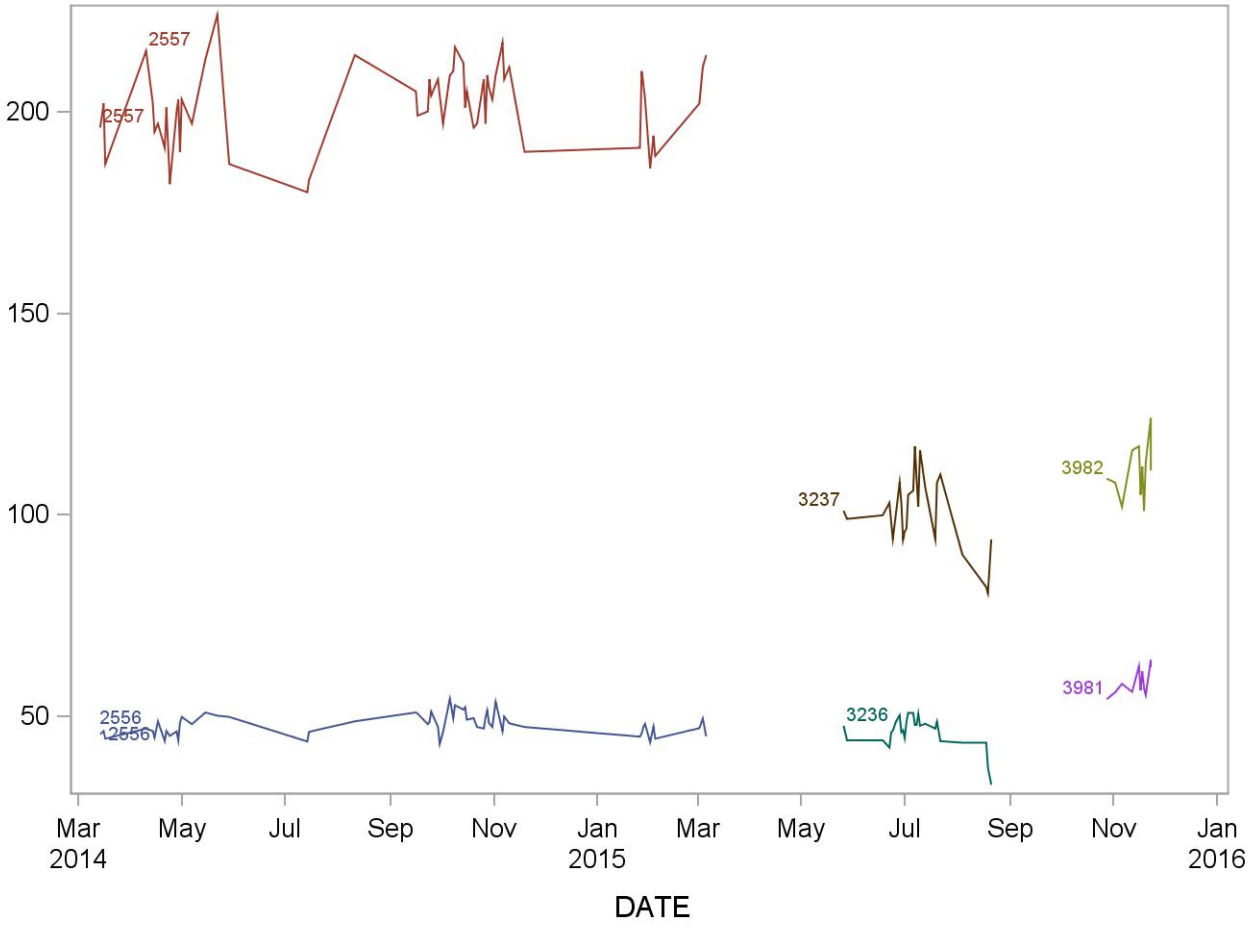
2013-2014 Summary Statistics and QC Chart for N-Nitrosopyrrolidine (NPYR) (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2557	3	14MAR14	17MAR14	168.3333	5.5076	3.3
2556	3	14MAR14	17MAR14	45.5000	1.9079	4.2
2557	55	10APR14	06MAR15	174.2727	9.7420	5.6
2556	55	10APR14	06MAR15	42.6455	4.1415	9.7
3237	23	26MAY15	19AUG15	94.0957	6.5116	6.9
3236	23	26MAY15	19AUG15	46.1478	4.1675	9.0
3982	18	28OCT15	01DEC15	87.6167	5.6164	6.4
3981	18	28OCT15	01DEC15	44.0833	3.4195	7.8



2013-2014 Summary Statistics and QC Chart for N-Nitrosomorpholine (NMOR) (pg/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2557	3	14MAR14	17MAR14	195.0000	7.5498	3.9
2556	3	14MAR14	17MAR14	45.5000	0.9539	2.1
2557	54	10APR14	06MAR15	202.0926	9.6666	4.8
2556	54	10APR14	06MAR15	48.0778	2.6488	5.5
3237	26	26MAY15	21AUG15	100.5577	8.8380	8.8
3236	26	26MAY15	21AUG15	46.0500	4.0553	8.8
3982	11	28OCT15	23NOV15	110.7273	6.8131	6.2
3981	11	28OCT15	23NOV15	58.4727	3.3850	5.8



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Appendix A

Standard Materials

1) Native Standards

VNA standards 2000 µg/mL were purchased from Supelco (Belafonte, PA).

Standard name	Manufacturer	Purity	Method for determining purity	Method for determining identity	Cat #	Lot number
NMEA	Supelco	99.8%	GC-FID	^{1}H NMR	502138	LB88399
NDEA	Supelco	99.9%	GC-FID	^{1}H NMR	502138	LB88399
NPIP	Supelco	99.9%	GC-FID	^{1}H NMR	502138	LB88399
NPYR	Supelco	99.9%	GC-FID	^{1}H NMR	502138	LB88399
NMOR	Supelco	99.9%	GC-FID	^{1}H NMR	502138	LB88399

Standard stock solution is in dichloromethane and stored in a -20ºC freezer. Prepare combined standard solutions in ACN from the above standard stock 2000µg/ml (Stock I) as described below.

Stock	Standard stock vol .	Final volume	Final conc.
A	50 µL stock I	50 mL	2000 pg/µL
B	2.5 mL stock A	50 mL	100 pg/µL
C	125 µL stock A	25 mL	10 pg/µL

2) Isotopically Labeled Internal Standards

Purchase ^{2}H-labeled VNAs internal standards from a commercial vendor. One such vendor is Cambridge Isotope Laboratories (CIL) Inc.(Andover, MA) or Toronto Research Chemical (TRC).

Standard name	Manufacturer	Purity	Method for determining purity	Method for determining identity	Cat #	Lot #
NMEA-$^{2}H_{3}$	TRC	>96%	GC-MS	^{1}H NMR	N525952	5-RFS-73-3
NDEA-$^{2}H_{10}$	Cambridge Isotope lab.	>98%	NEAT	GC-FID	DLM-7982-S-0	SCJD-008
NPIP-$^{2}H_{10}$	TRC	>98%	GC-MS	^{1}H NMR	N545372	3-JTN-154-1
NPYR-$^{2}H_{8}$	Cambridge Isotope lab.	>98%	NEAT	GC-FID	DLM-8252-S	SDCC-013
NMOR-$^{2}H_{8}$	Cambridge Isotope lab.	>96%	NEAT	GC-FID	DLM-8254-S	SDCG-017

i) Individual VNA Internal Standard Stocks (1 mg/ml)

The individual internal standard stocks purchased from CIL are in dichloromethane: NDEA-$^{2}H_{10}$, NPYR-$^{2}H_{8}$, NMOR-$^{2}H_{8}$. Internal standards purchased from TRC are: NPIP-$^{2}H_{10}$ and NMEA-$^{2}H_{3}$.

ii) Mixed VNAs Internal Standard Sample Spiking Solution (10 pg/µl)

Pipette 100 µl of each internal standard stock (1 mg/ml) to a 100 ml volumetric flask, dilute to mark with methanol, and shake well. This 1 ng/µl stock (stock X) is used to prepare the subsequence 10 pg/µl spiking solutions.

Pipette 2000 µl of the mixed internal standard 1 ng/µl (stock X) to a 200 ml volumetric flask, dilute to mark with methanol and shake well. This mixed internal standard solution, stock Y (10 pg/µl), is used in unknown sample preparation.

iii) Mixed VNAs Internal Standard Calibrator Spiking Solution (1250 pg/µL)

Pipette 125 µl of each of the internal standard stocks (1 mg/mL) to a 100ml volumetric flask, dilute to mark with ACN, and shake well. This mixed internal standard solution, stock Z (1250 ng/ml), is used to prepare calibrator solutions.

Stock	Std Stock Volume	Final Volume	Final Concentration	Solvent
X	100 µL each indiv stock (1 mg/mL)	100 mL	1 ng/µL	MeOH
Y	2000 µL stock X	200 mL	10 pg/µL	MeOH
Z	125 µL each indiv stock (1 mg/mL)	100 mL	1250 pg/µL	ACN

3) VNAs calibration standards

Prepare VNA calibrator solutions according to the table below. Spike variable volumes of the VNA mixed standard stocks (A-C) to a 25 ml volumetric flask; add 1000 µl of stock Z (1250 pg/µl) and bring to final aliquot using ACN. The internal standard concentration in every calibrator is 50 ng/mL. These calibrator solutions are aliquoted to 1 ml amber vials and stored at -20°C.

Standard	Stock Volume Added	Volume VNA ISTD (stock Z) added	Concentration in standard (ng/mL)	Concentration in sample (ng/mL)*	Concentration in sample (pg/mL)
0	0 mL stock	1000 µL	0	0	0
1	125 µL stock C	1000 µL	0.05	0.0005	0.5
2	250 µL stock C	1000 µL	0.1	0.001	1.0
3	125 µL stock B	1000 µL	0.5	0.005	5.0
4	1.25 mL stock B	1000 µL	5	0.05	5.0

5	125 µL stock A	1000 µL	10	0.1	100
6	625 µL stock A	1000 µL	50	0.5	500
7	1.25 mL stock A	1000 µL	100	1.0	1000
8	2.5 mL stock A	1000 µL	200	2.0	2000
9	5.0 mL stock A	1000 µL	400	4.0	4000

** In each unknown sample, we spike 50 µL of 10 pg/µL ISTD solution, so there is a total of 500 pg or 0.5 ng of ITSD in each sample. The difference in the amount of ISTD in standards vs. in sample is 100 time.*
This calculation is based on 1 mL sample volume. If your sample volume is 2 mL, then you need to divide the concentration by 2. In case of diluted samples, if your sample volume is 0.5 mL, then you divide the concentration by 0.5.

4) Quality Control Materials

There are three quality control pools for the urinary volatile nitrosamine assay. Pools “QC low” and “QC high” contain relatively low and high levels of VNAs, respectively. QC dilute pools contain levels for VNAs that exceed the highest calibrator of the calibration curve. The QC pools were prepared in-house from urine collected from non-smokers spiked with standard stock solution. Each of these pools was mixed well, dispensed in 2.00 ml aliquots into 2 ml cryovials with screw cap, and frozen at -60°C to -70°C.

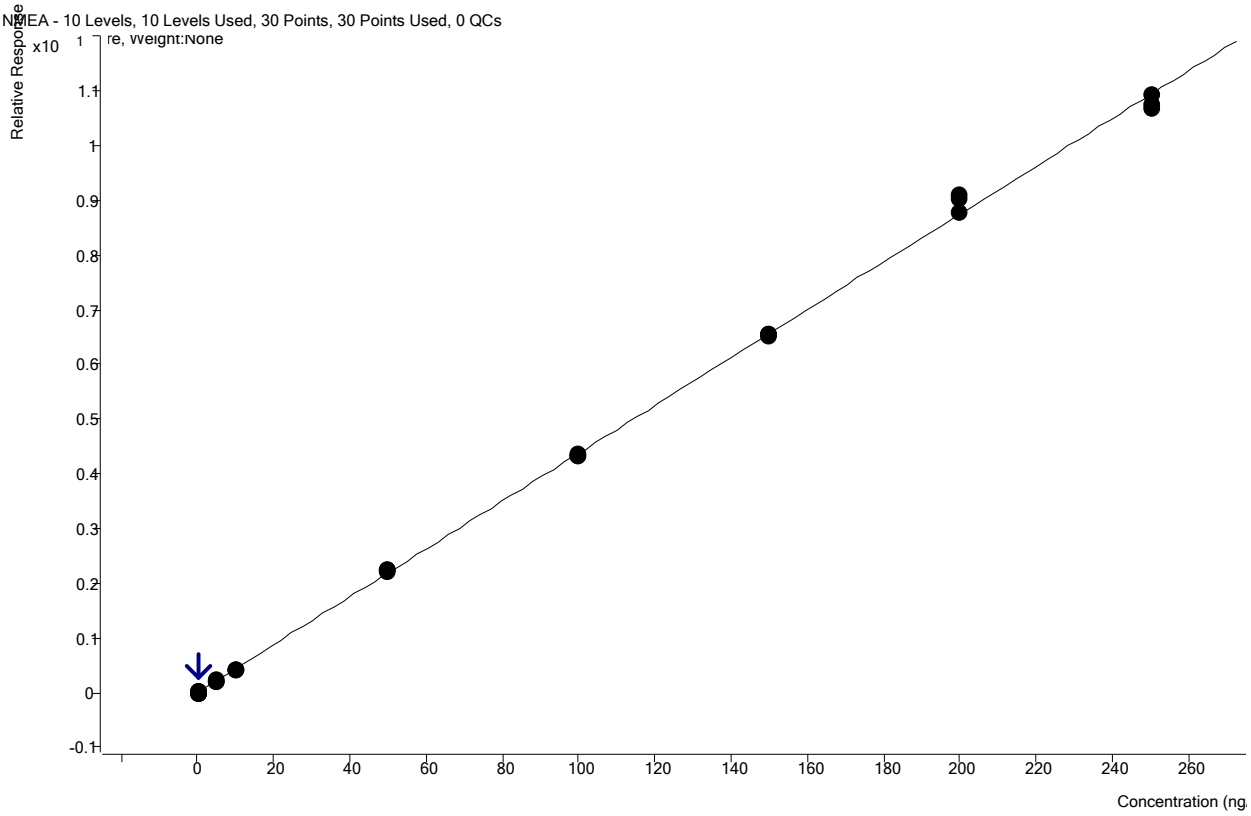
Pool	Stock solution (in MeOH)	Amount spiked (µL)	Final vol. (urine)	Final conc.
Low	100 pg/µL	500	1000 mL	50 pg/ml
High	100 pg/µL	2000	1000 mL	200 pg/mL
Dilute	2000 pg/µL	375	1500 mL	500 pg/mL

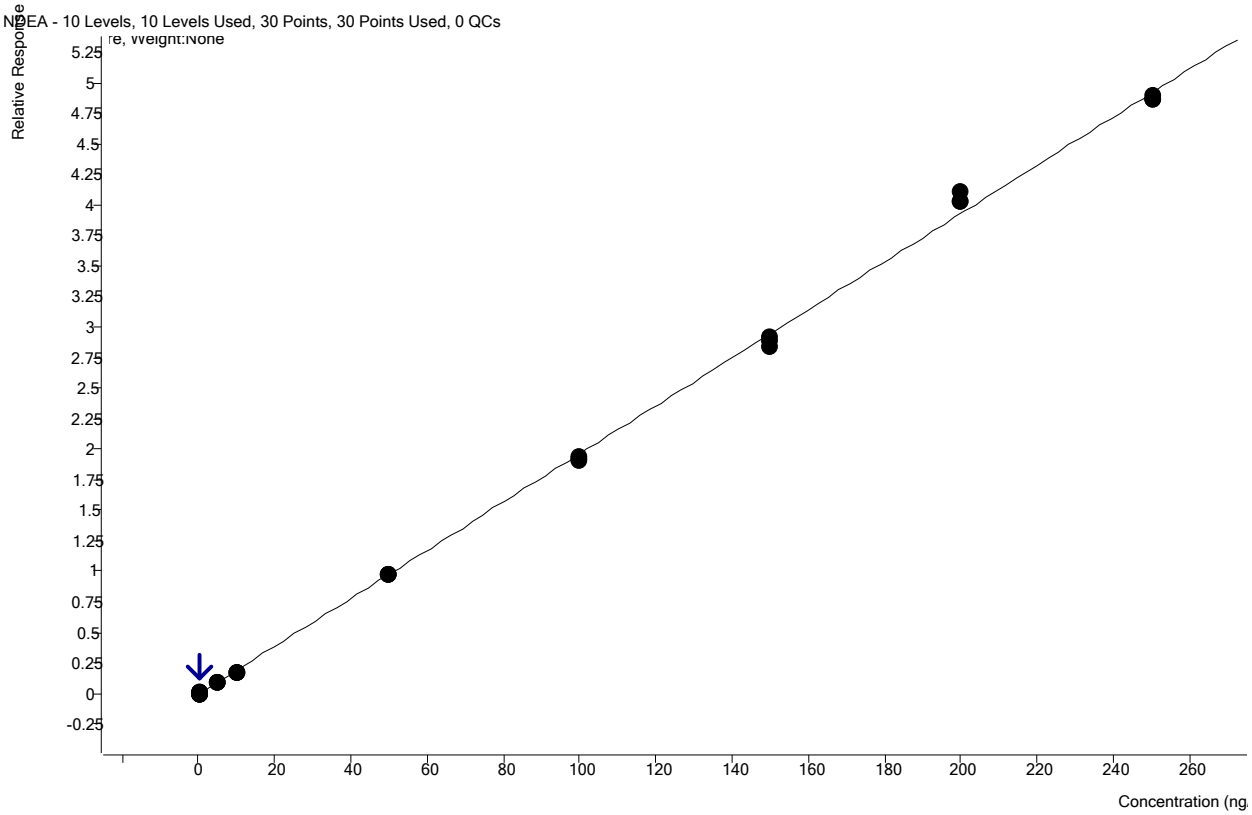
Appendix B

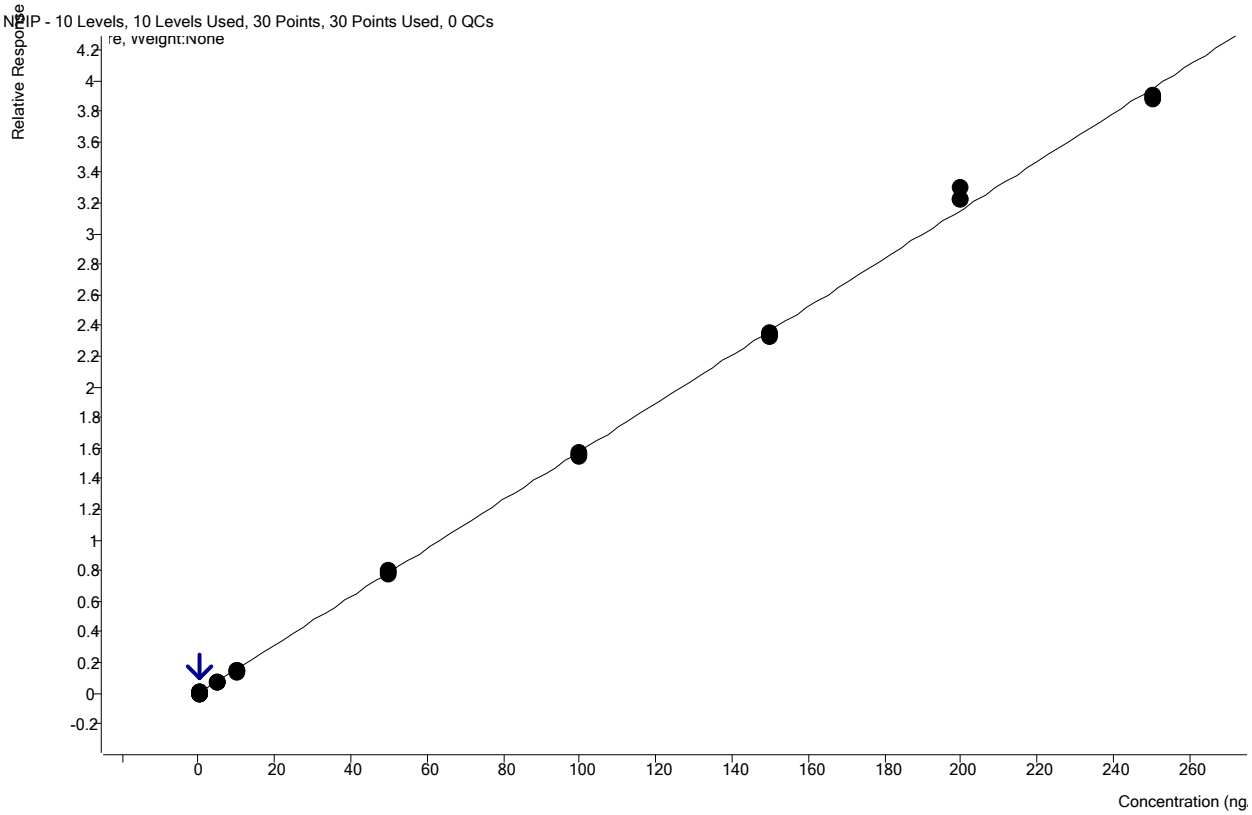
Method Validation Data

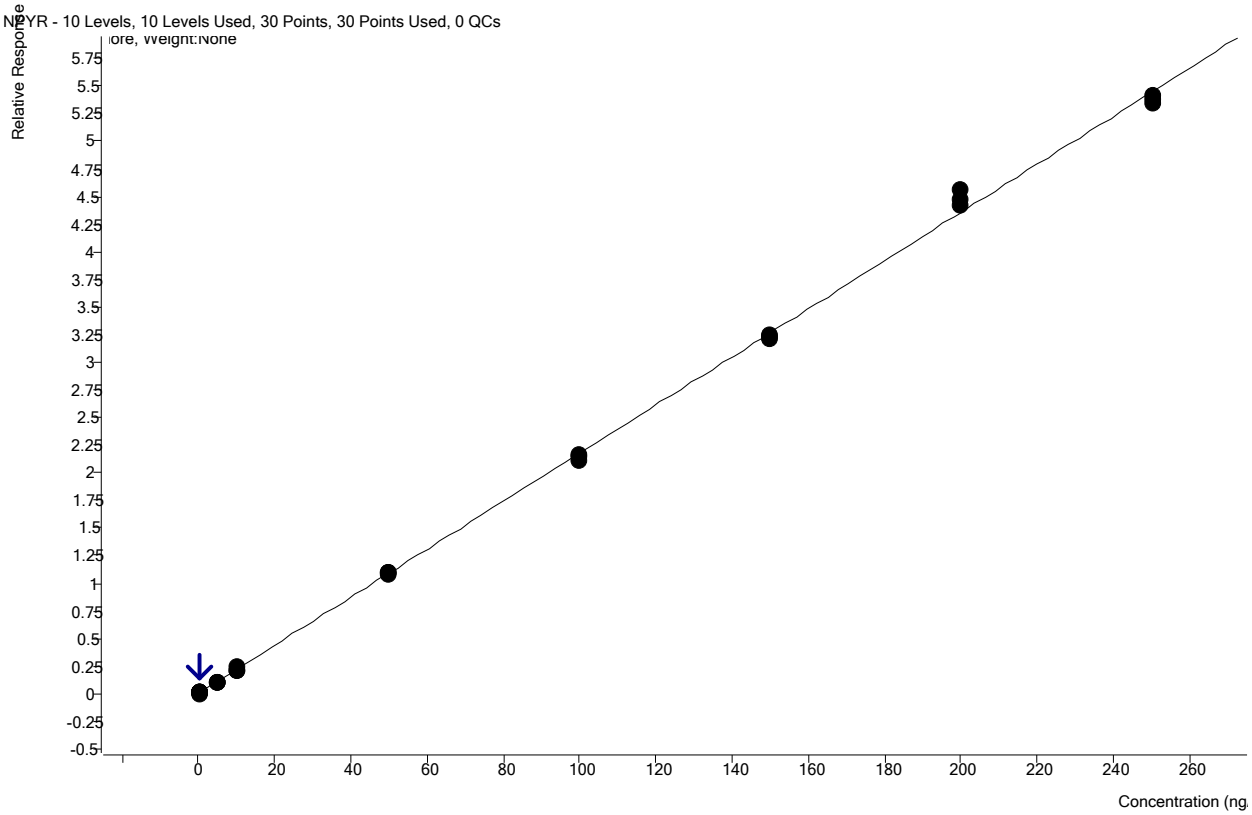
a. Linearity and weighting factor evaluation

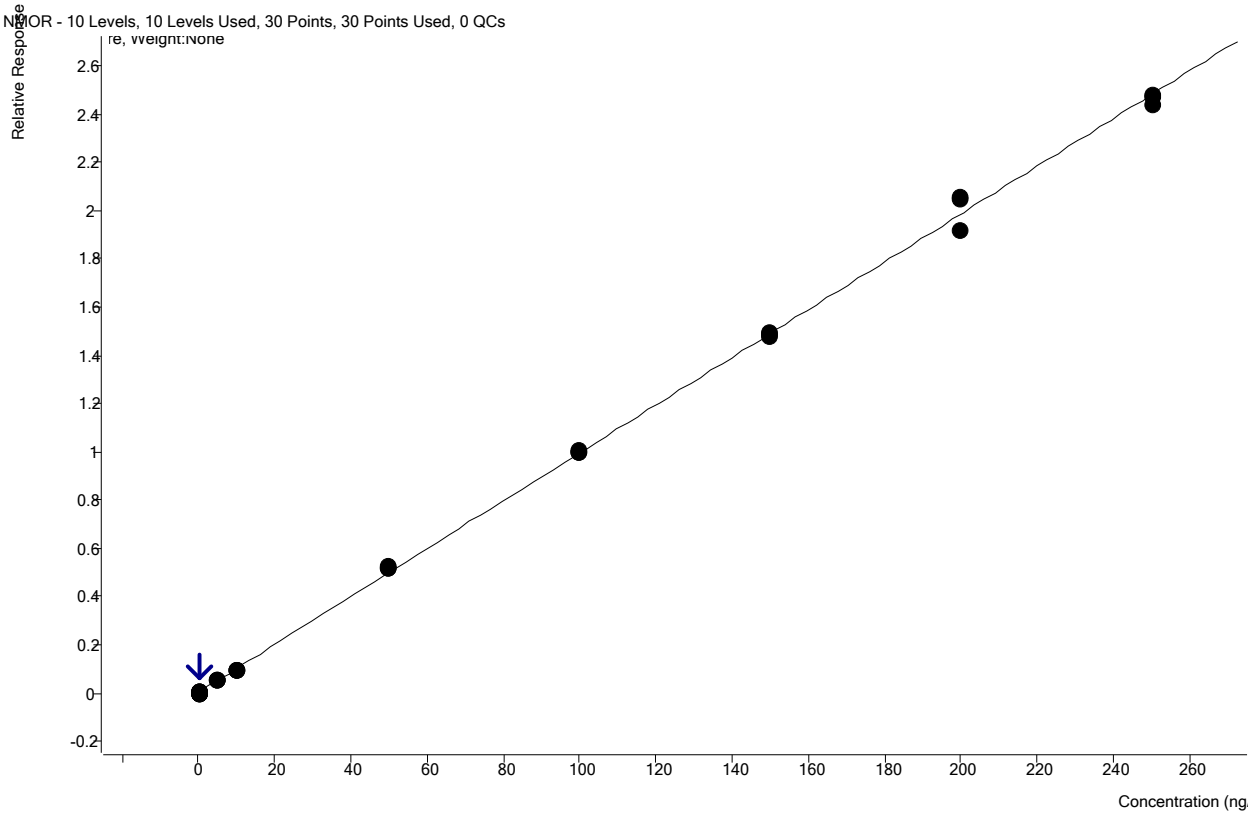
We have confirmed linear responses for all analytes across a broad range of 10 analyte concentrations relevant to urinary levels of volatile nitrosamine ($R^2 \geq 0.99$). The linear range of the analytical method extends from 0.0 to highest calibrator. Shown below are calibration curves of 6 analytes (n = 3).



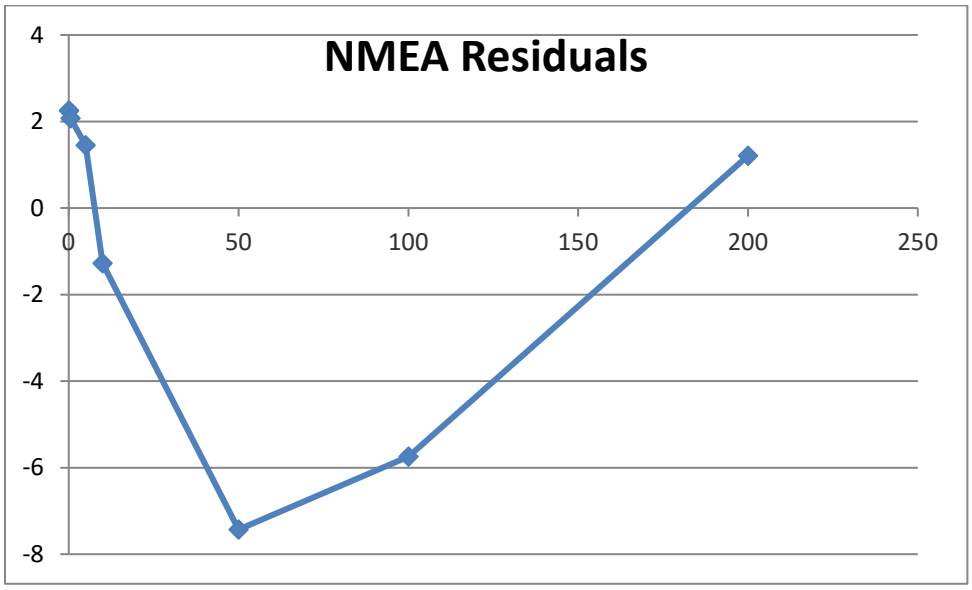

NDEA - 10 Levels, 10 Levels Used, 30 Points, 30 Points Used, 0 QCs
re, Weight:None
Relative Response
5.25
5
4.75
4.5
4.25
4
3.75
3.5
3.25
3
2.75
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
-0.25
0
20
40
60
80
100
120
140
160
180
200
220
240
260
Concentration (ng

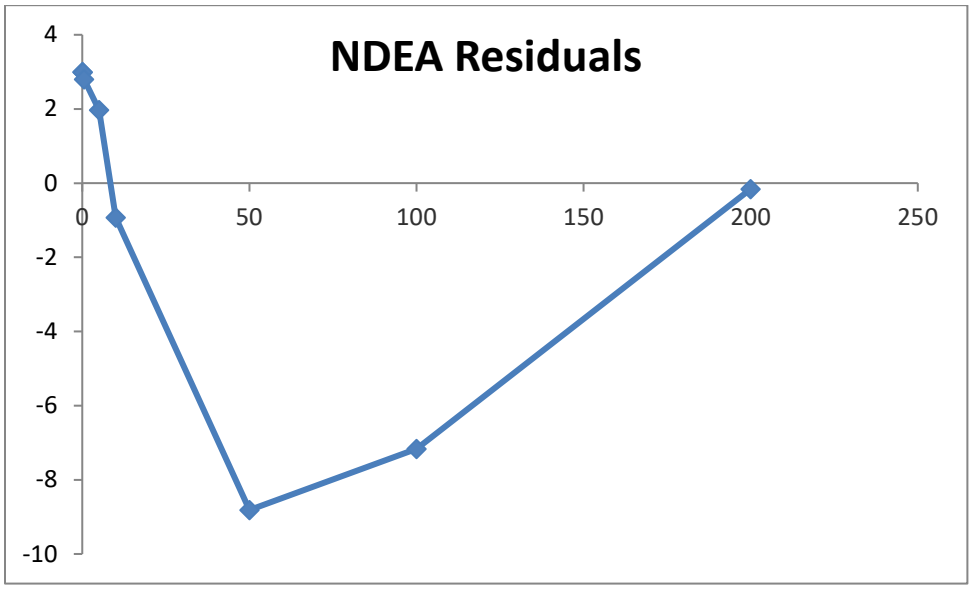

NDIP - 10 Levels, 10 Levels Used, 30 Points, 30 Points Used, 0 QCs
re, Weight:None
Relative Response
4.2
4
3.8
3.6
3.4
3.2
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
20
40
60
80
100
120
140
160
180
200
220
240
260
Concentration (ng

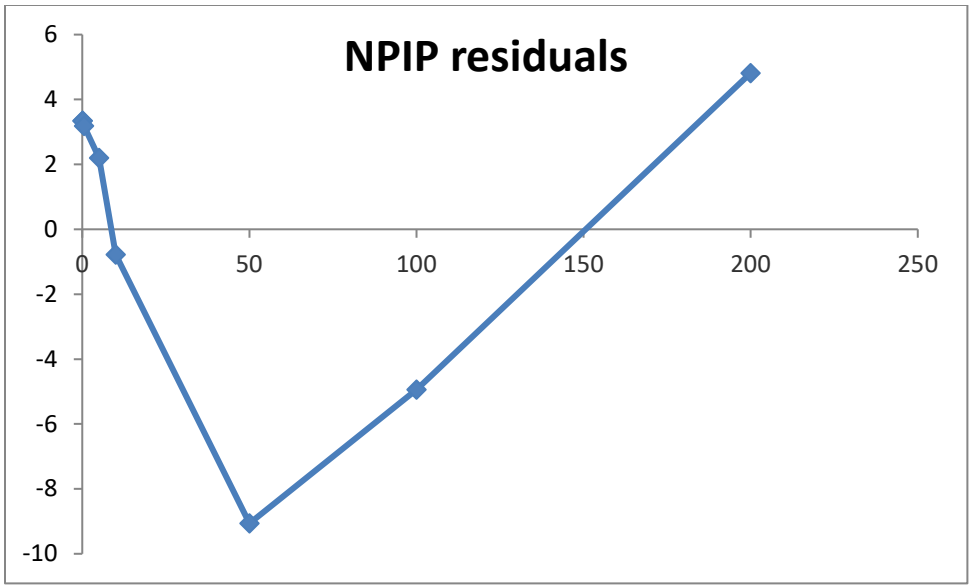

NPYR - 10 Levels, 10 Levels Used, 30 Points, 30 Points Used, 0 QCs
Relative Response
Concentration (ng

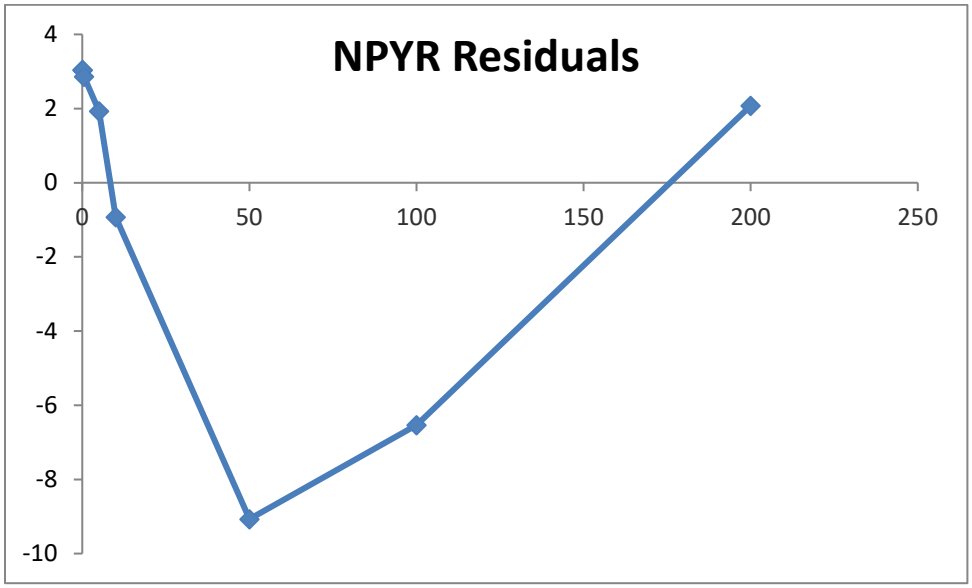

NMOR - 10 Levels, 10 Levels Used, 30 Points, 30 Points Used, 0 QCs
Relative Response
Concentration (ng

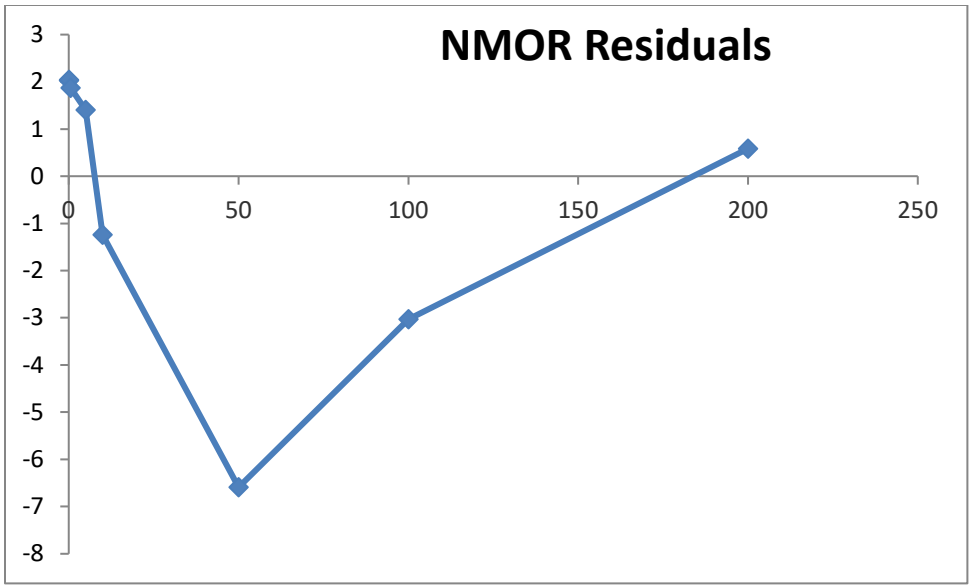
A weighting factor of 1/x is used for all analytes base on the residual plot of each analyte show below.






NPIP residuals
6
4
2
0
-2
-4
-6
-8
-10
0
50
100
150
200
250


NPYR Residuals
4
2
0
-2
-4
-6
-8
-10
0
50
100
150
200
250



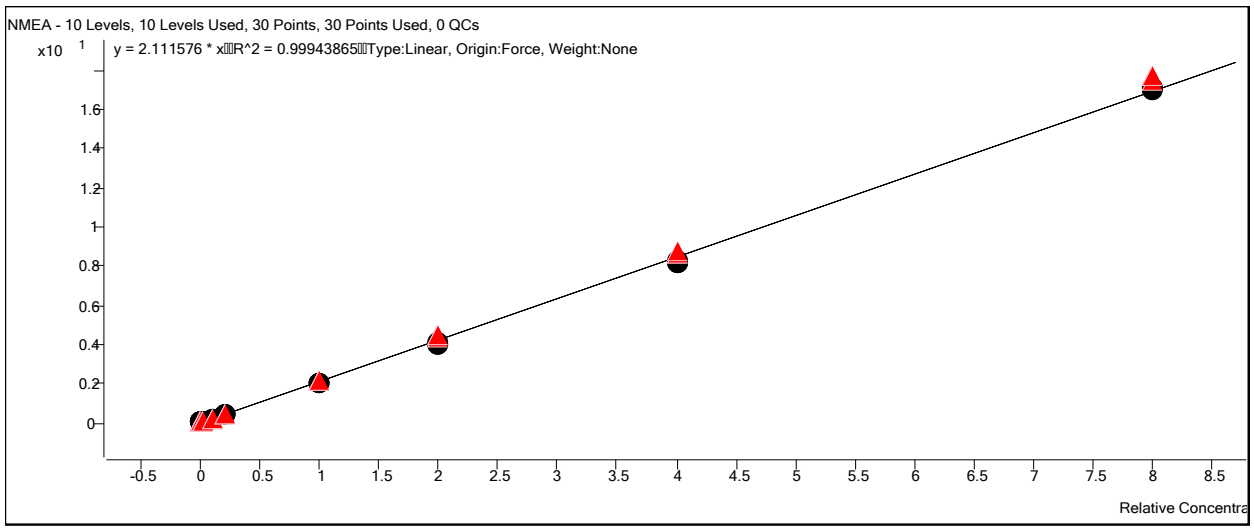
b. Calibration curve- Matrix vs. Non-matrix based calibrators

To verify matrix equivalency, 10 standard solutions were prepared in ACN (non-matrix) as specified in Appendix A, Section 3. Another 10 standard solutions were prepared in urine (matrix), and carried through sample preparation as described in Section 8a for urine samples. Each calibration set was run in triplicate. Shown below are calibrations curves of all six analytes prepared in ACN and in urine. The average of three slopes obtained from ACN and urine were calculated, and the difference of the two average slopes was obtained. Equivalency of slopes is within acceptance limit of DLS policy and procedure manual (%difference < 5%).

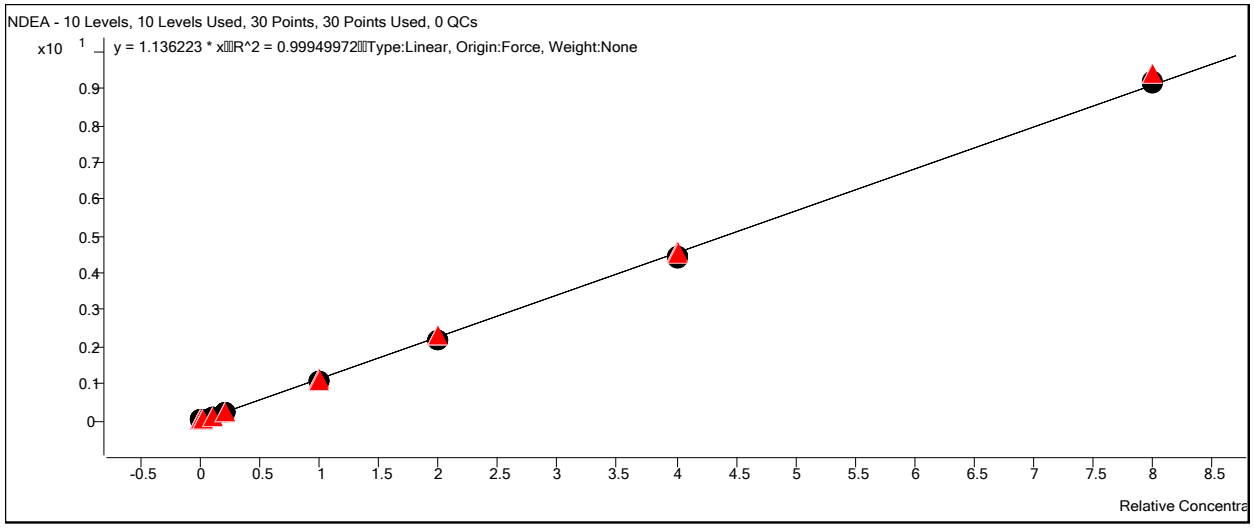
	ACN Slope (N=3)	R^2 Value	Urine Slope (N=3)	R^2 Value	% Difference
NMEA	2.1116	0.9994	2.1968	0.9998	4.0366
NDEA	1.1362	0.9995	1.1703	0.9998	2.9998
NPIP	0.8901	0.9994	0.8475	0.9999	4.7881
NPYR	1.2872	0.9993	1.2934	0.9996	0.4775
NMOR	1.1633	0.9996	1.1296	0.9999	2.8925

Below are calibration curves prepared in ACN (black circle) and urine (red triangle).

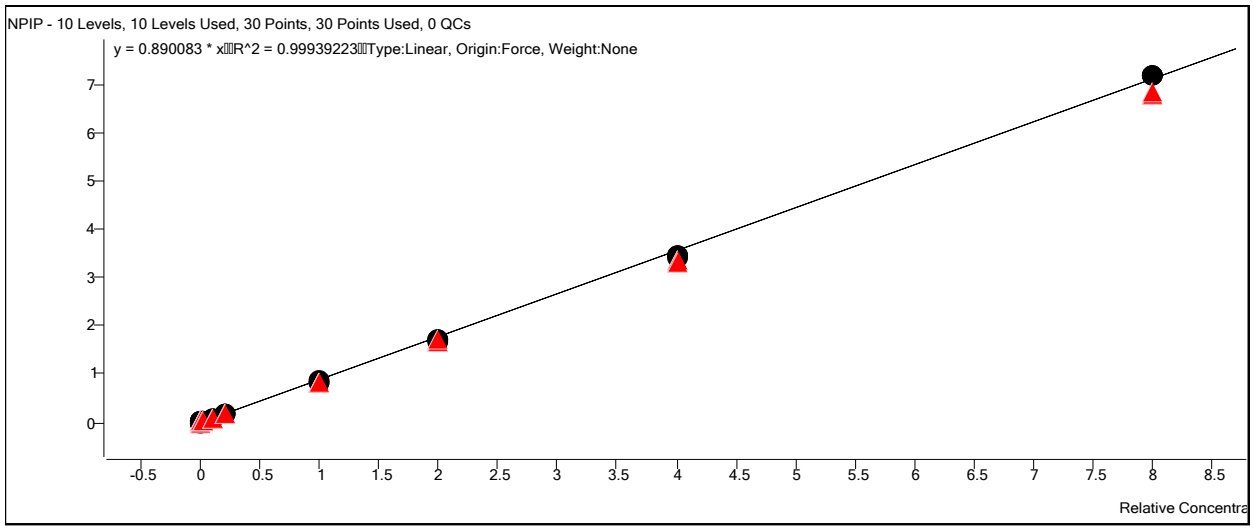
NMEA



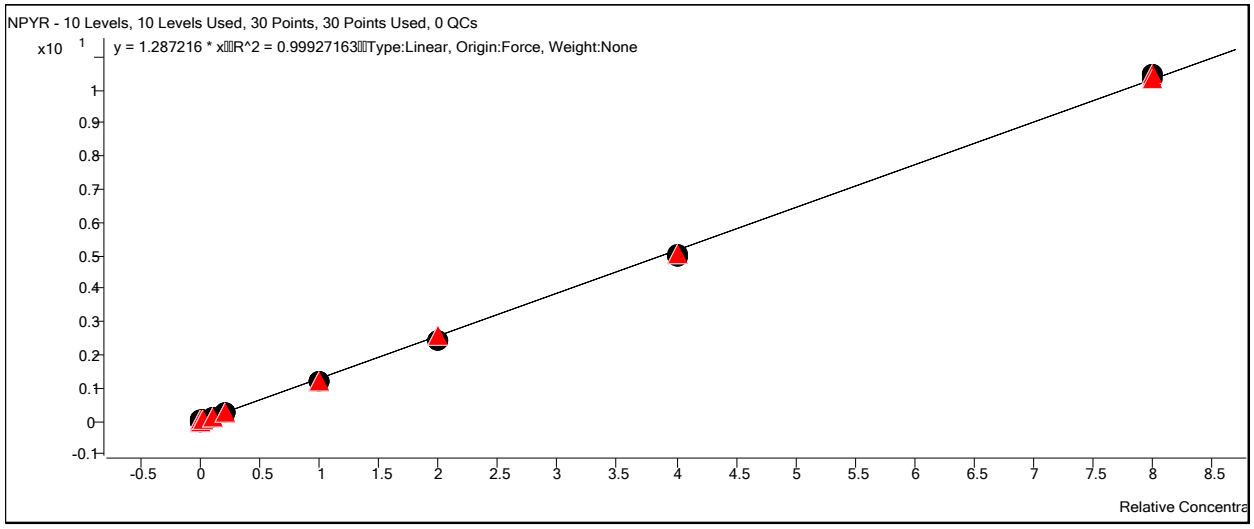
NDEA



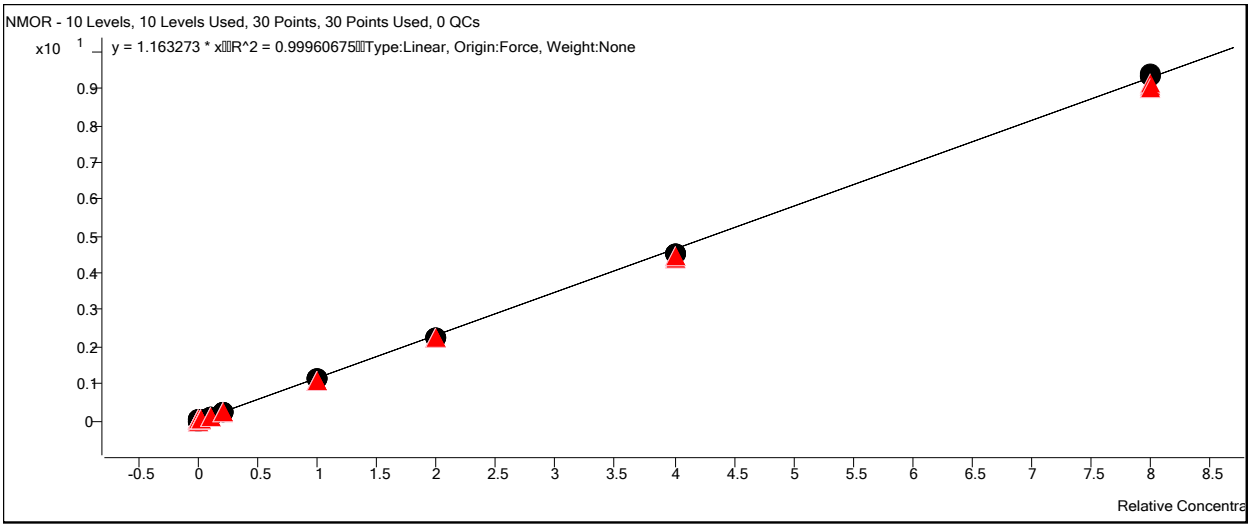
NPIP


NPIP - 10 Levels, 10 Levels Used, 30 Points, 30 Points Used, 0 QCs
y = 0.890083 * x R^2 = 0.99939223 Type:Linear, Origin:Force, Weight:None
7
6
5
4
3
2
1
0
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
Relative Concentra

NPYR


NPYR - 10 Levels, 10 Levels Used, 30 Points, 30 Points Used, 0 QCs
x10 1
y = 1.287216 * x R^2 = 0.99927163 Type:Linear, Origin:Force, Weight:None
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
Relative Concentra

NMOR



c. Limit of Detection

The limit of detection is determined according to the guideline for determination of limits of detection by the Clinical and Laboratory Standard Institute (CLSI. Protocols for Determination of Limits of Detection and Limits of Quantitation: Approved Guideline. CLSI document EP17-A. Wayne, PA: Clinical and Laboratory Standards Institute, 2004). Four urine pools: one non-smoker blank pool, non-smoker blank spiked at 2.5, 5.0, and 7.5 pg/mL for all 6 analytes. These four pools were used to estimate the LoD of NPYR. Due to detectable background levels of NMEA, NDEA, NPIP, and NMOR in sample blanks, the detection limits for these five analytes were estimated as $3S_0$, where S_0 is the standard deviation of samples blanks. LoDs are obtained from 18 independent runs. Additional 46 runs will be conducted, and the LoDs for all six VNAs will be re-evaluated.

Analyte	LOD (pg/ml)
NMEA	0.8
NDEA	1.2
NPIP	0.7
NPYR	5.0
NMOR	1.6

b. Accuracy

All native compounds were purchased from Supelco and used to prepare calibration curve. Native standards purchased from a different vendor were used to prepare accuracy testing solutions.
Accuracy was determined by spiking known amounts of VNA standard solution into ACN (accuracy in solution) and urine (accuracy in matrix). The spiked urine standards were prepared using the same procedure as for unknown samples.

%bias =100 (observed VNA level-expected VNA level)/expected VNA level*

Accuracy in solution- NDEA, NPYR, and NMOR were purchased from Cambridge Isotope Laboratory. NMEA and NPIP were not available from a different vendor, and a different lot from Supelco was used.

Standard name	**Manufacturer**	**Purity**	**Method for determining purity**	**Method for determining concentration**	**Cat #**	**Lot #**
NMEA	Supelco	99.9%	GC-FID	^{1}H NMR	502138	LB96504
NDEA	Cambridge Isotope Lab	>98%	GC-FID	Gravimetric	UML-7984-S	SCGH-002
NPIP	Supelco	99.9%	GC-FID	^{1}H NMR	502138	LB96504
NPYR	Cambridge Isotope Lab	>98%	NEAT	Gravimetric	ULM-8253-S	SDDE-019
NMOR	Cambridge Isotope Lab	>96%	NEAT	Gravimetric	ULM-8255-S	SDCG-017

NDEA, NPYR, and NMOR results are highlighted in orange. NMEA and NPIP results are highlight in green. All %bias is less than 5-7%, and thus accuracy tests were passed.

Test solution	NMEA	NDEA	NPIP	NPYR	NMOR
Mixed - 2 pg/µl	-4.92	-5.35	-5.64	-4.20	-5.31
Mixed - 100 pg/µl	0.05	0.89	1.80	-1.01	1.35
Mixed - 200 pg/µl	2.63	-0.02	1.77	-1.47	0.96
Mixed - 200 pg/µl	3.32	0.79	-0.01	-0.09	1.68
Mixed - 100 pg/µl	1.39	-0.48	-0.62	-1.10	2.04
Mixed - 2 pg/µl	-5.11	-5.00	-4.50	-4.00	-6.34

Mixed - 2 pg/ul	-3.41	-3.37	-5.43	-3.22	-5.74
Mixed - 100 pg/µl	2.56	0.84	1.24	-1.84	2.71
Mixed - 200 pg/µl	2.28	1.93	-1.34	-2.30	2.03
Indiv - 2 pg/µl		-4.71		-2.38	-2.87
Indiv - 100 pg/µl		-0.37		-3.63	3.56
Indiv - 200 pg/µl		2.08		-1.74	3.86
Indiv - 200 pg/µl		-0.77		-1.47	3.76
Indiv - 100 pg/µl		-0.91		-4.22	3.62
Indiv - 2 pg/µl		-5.73		-3.62	-0.67
Indiv - 2 pg/µl		-5.60		-3.78	-2.40
Indiv - 100 pg/µl		-1.57		-1.80	4.98
Indiv - 200 pg/µl		0.64		-0.38	4.75

Accuracy in matrix was tested with three separate runs in three days, at three levels, and in triplicate at each level. All %bias except one are less than 20%, and accuracy in matrix tests are acceptable.

Run	NMEA	NDEA	NPIP	NPYR	NMOR
VA179	2.43	-5.37	-8.09	15.43	-7.70
	-5.97	-8.87	-11.91	18.27	-13.87
	-2.36	-10.06	-15.57	23.84	-13.50
	8.63	0.39	-2.91	11.95	-0.38
	5.91	-2.54	-3.45	12.74	-4.35
	4.53	-4.55	-6.81	7.78	-6.61
	-2.55	-6.87	-11.96	-4.55	-10.34
	0.20	-4.66	-7.97	3.68	-6.12
	-1.39	-4.73	-11.04	-1.43	-6.37
VA180	-5.57	-11.43	-12.41	0.85	-10.50
	-10.71	-11.82	-19.14	1.08	-13.96
	-8.22	-15.88	-18.09	8.58	-10.51
	-0.51	-5.52	-10.96	4.14	-4.66
	2.09	-4.66	-9.68	6.03	-7.95
	-7.55	-12.64	-16.15	-5.39	-12.79
	4.27	-0.90	-7.70	-0.44	-1.75
	1.30	-4.06	-8.59	1.19	-4.91
	-6.50	-9.85	-14.52	-8.58	-10.30
VA181	-3.15	-7.29	-13.59	12.75	-8.07
	-3.42	-9.52	-8.59	10.03	-5.71
	-3.10	-9.77	-12.82	8.27	-7.85
	6.14	2.13	-4.44	5.37	-2.05
	10.35	4.54	-2.46	9.10	0.90
	6.13	-0.98	-8.07	4.74	-2.00
	5.93	0.95	-6.08	0.14	-2.17
	8.42	2.54	-2.56	4.44	1.32
	10.20	3.61	-2.83	5.86	1.58

c. Precision

Two pools: non-smoker urine spiked at 50 and 200 pg/mL were used in precision testing. Six independent runs were performed each day for 5 days. The relative %RSD values are calculated and listed in the table below. All RSD values except one are less than 10% and thus acceptable.

Low pool, 50 pg/mL in urine

Intra/Inter	Stat.	NMEA	NDEA	NPIP	NPYR	NMOR
Intra-run	STDEV	2.28	2.03	0.78	1.78	1.86
(N= 6 runs)	MEAN	46.78	43.80	40.81	38.26	39.73
	CV	4.87	4.63	1.90	4.65	4.69
Intra-run	STDEV	0.96	1.65	1.94	2.71	0.99
(N= 6 runs)	MEAN	47.44	45.80	42.97	42.14	40.71
	CV	2.03	3.60	4.51	6.43	2.43
Intra-run	STDEV	1.17	1.23	0.43	2.39	0.85
(N= 6 runs)	MEAN	48.45	45.71	41.04	36.62	39.14
	CV	2.42	2.68	1.05	6.54	2.18
Intra-run	STDEV	1.24	0.84	0.88	1.16	1.29
(N= 6 runs)	MEAN	46.24	43.66	40.03	36.22	40.80
	CV	2.68	1.93	2.19	3.19	3.16
Intra-run	STDEV	1.61	2.49	2.26	1.82	1.12
(N= 6 runs)	MEAN	53.40	48.25	44.93	33.37	44.10
	CV	3.02	5.16	5.04	5.44	2.54
Inter-run	STDEV	2.98	2.35	2.25	3.49	2.11
(N= 5 days)	MEAN	48.46	45.44	41.96	37.32	40.89
	CV	6.14	5.17	5.36	9.34	5.16

High pool, 200 pg/mL

Intra/Inter	Stat.	NMEA	NDEA	NPIP	NPYR	NMOR
Intra-run	STDEV	4.28	4.80	3.42	4.59	9.07
(N= 6 runs)	MEAN	190.77	178.81	173.68	171.59	178.97
	CV	2.24	2.68	1.97	2.68	5.07
Intra-run	STDEV	2.47	3.09	3.86	3.59	2.21
(N= 6 runs)	MEAN	195.45	182.91	175.94	172.75	178.71
	CV	1.26	1.69	2.19	2.08	1.24
Intra-run	STDEV	5.77	4.36	4.68	4.80	4.45
(N= 6 runs)	MEAN	198.43	187.28	175.49	174.62	178.59
	CV	2.91	2.33	2.67	2.75	2.49
Intra-run	STDEV	4.22	5.11	5.20	3.89	3.97
(N= 6 runs)	MEAN	195.40	185.27	174.62	170.15	180.00
	CV	2.16	2.76	2.98	2.29	2.20
Intra-run	STDEV	7.50	7.59	6.63	8.10	6.79
(N= 6 runs)	MEAN	229.42	213.60	205.30	195.01	204.75
	CV	3.27	3.55	3.23	4.16	3.32
Inter-run	STDEV	14.24	12.80	12.52	10.05	11.24
(N= 5 days)	MEAN	200.95	188.74	180.17	176.20	183.49
	CV	7.09	6.78	6.95	5.70	6.12

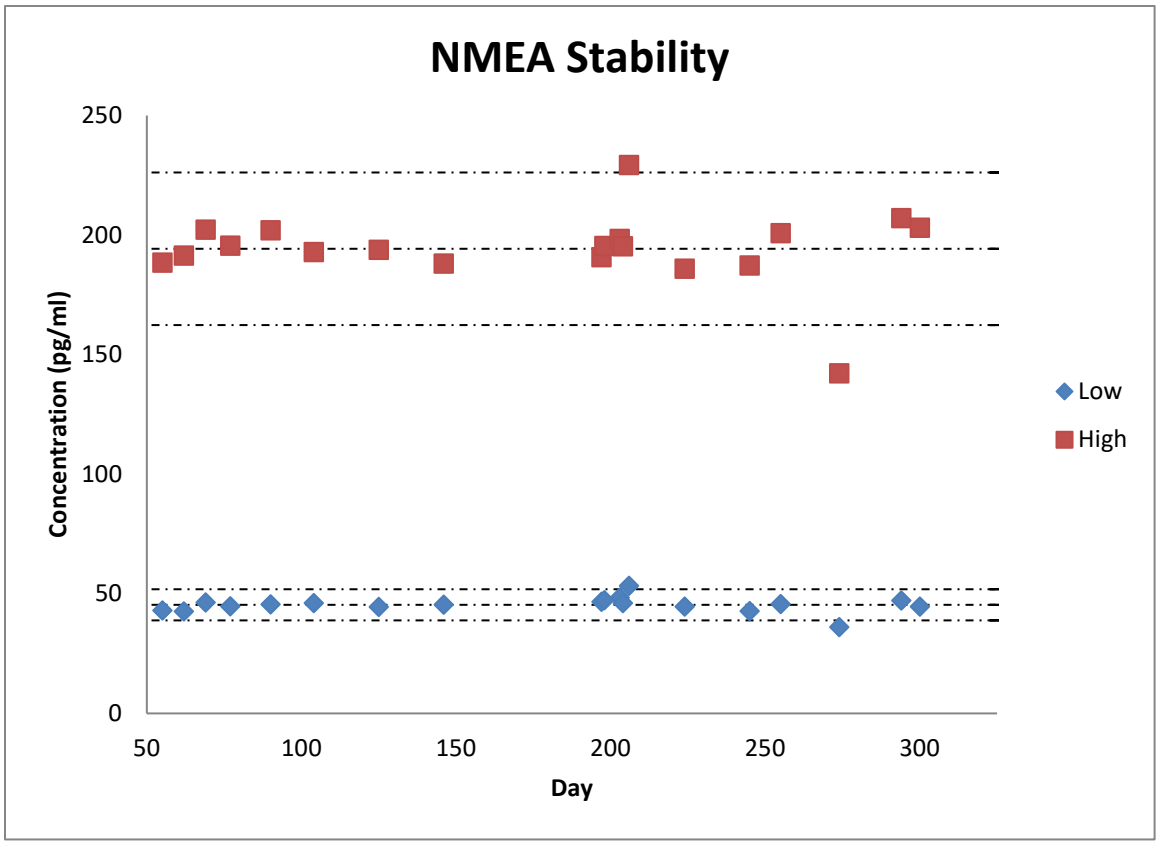
d. Carry over

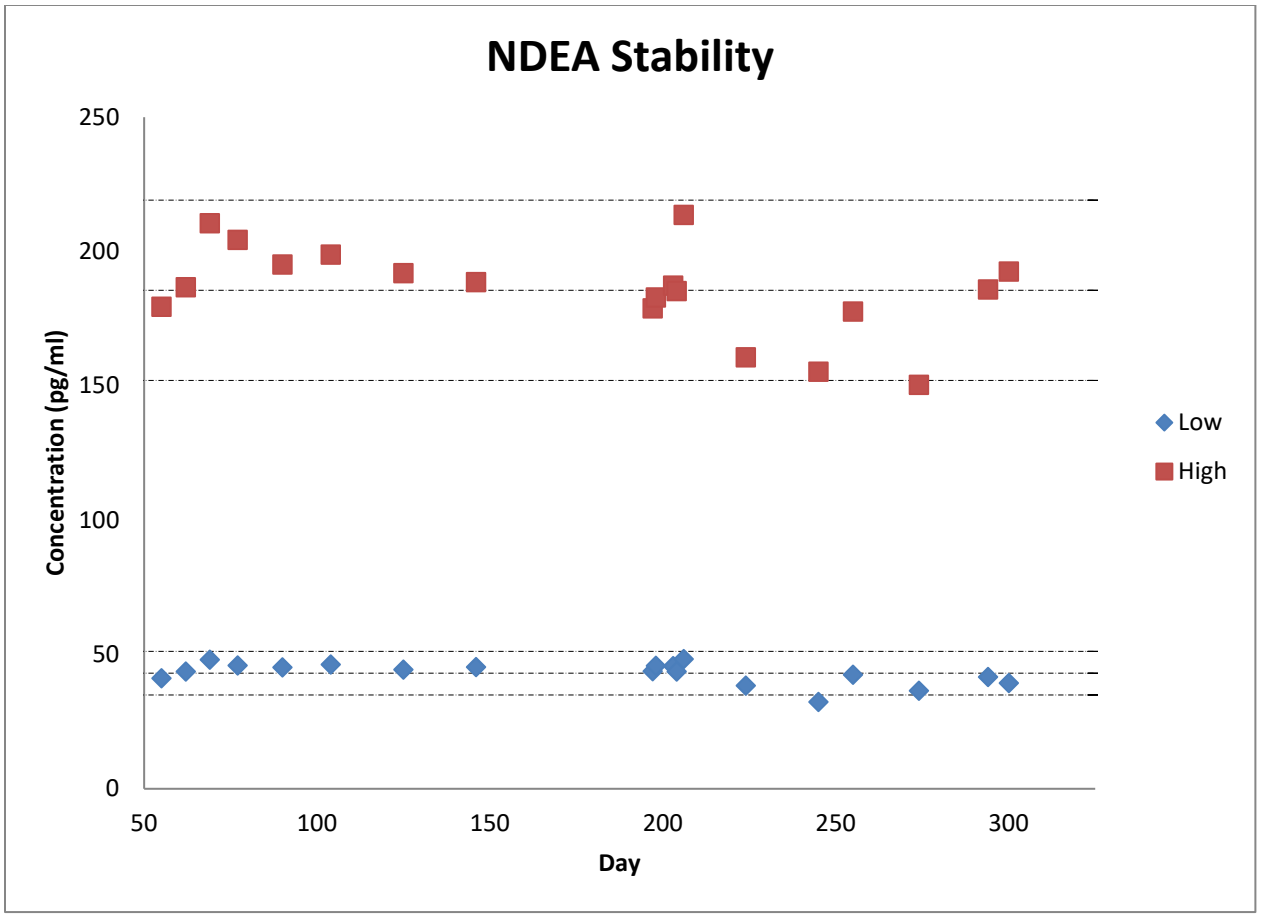
Carryover was examined by comparing successive pairs of injections of the high calibrator or high QC samples followed by ACN blank. No carryover was detected. Therefore the carryover limit was defined as the analyte concentration of the highest standard 400 pg/µl (see VNA standard calibration table in Appendix A).

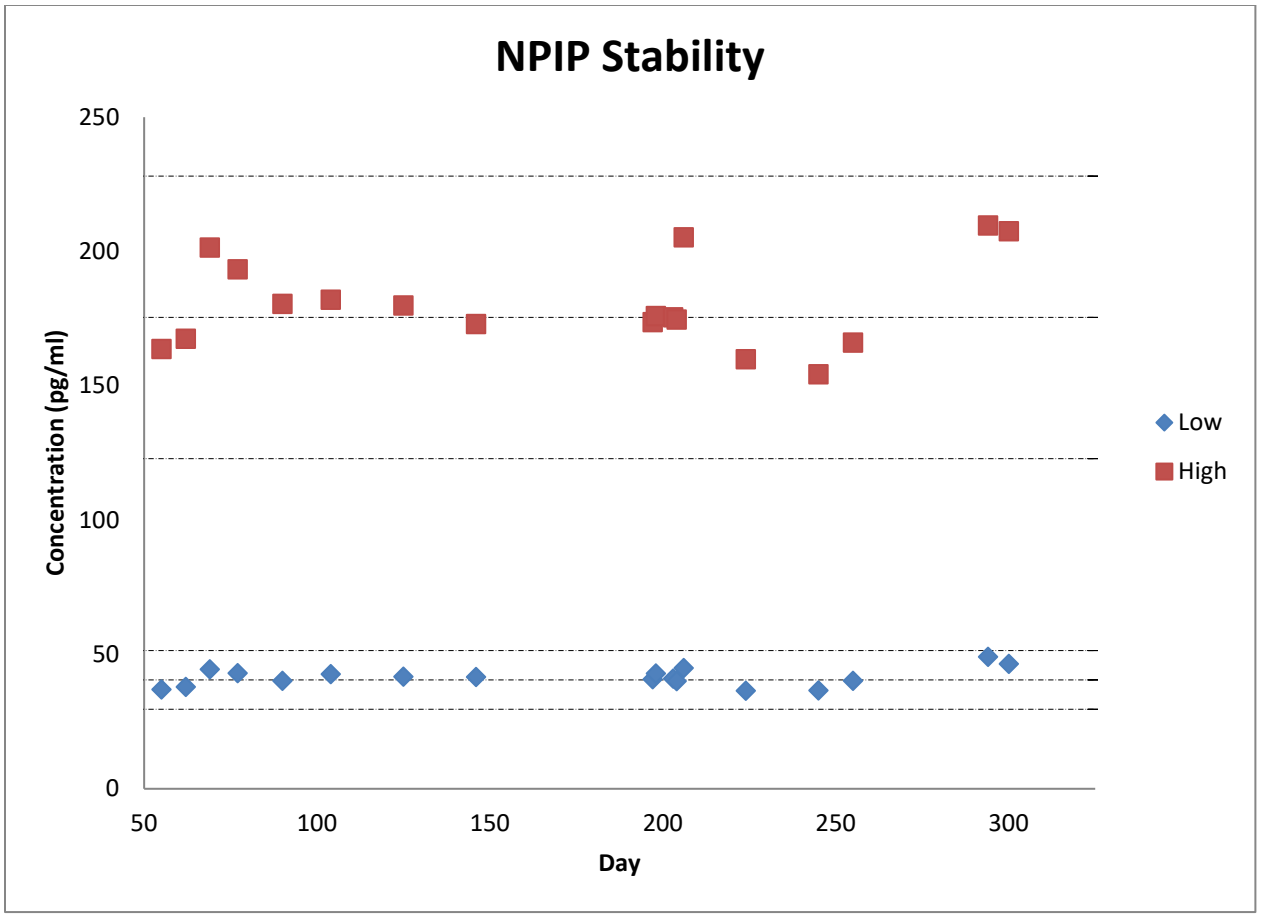
e. Thaw-Refreeze and storage stability

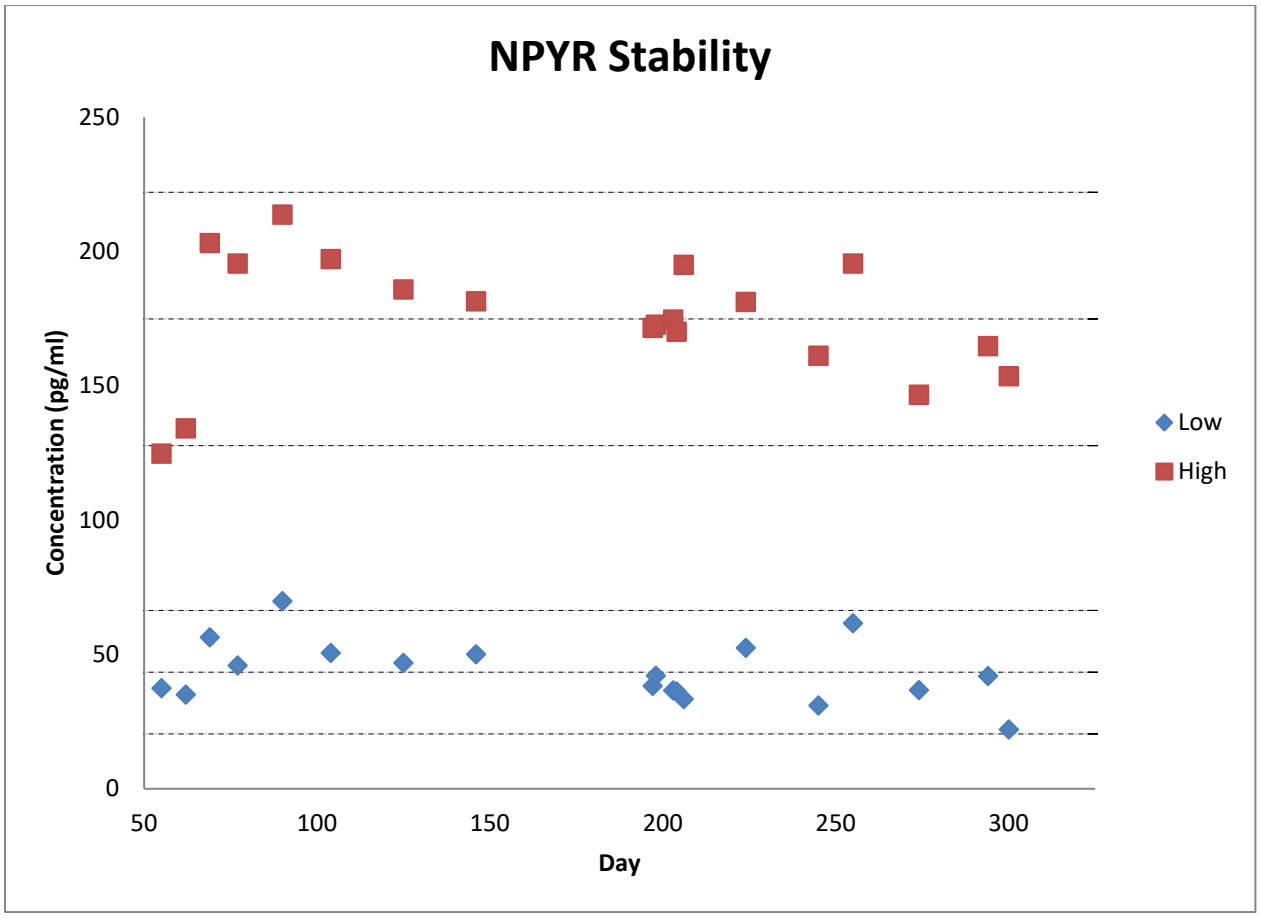
Normal and accelerated stability studies of the analytes in urine matrix were conducted. QC pools low and high, 50 pg/mL and 200 pg/mL respectively, were used. Repeated analysis of these QC samples that were frozen at -70°C

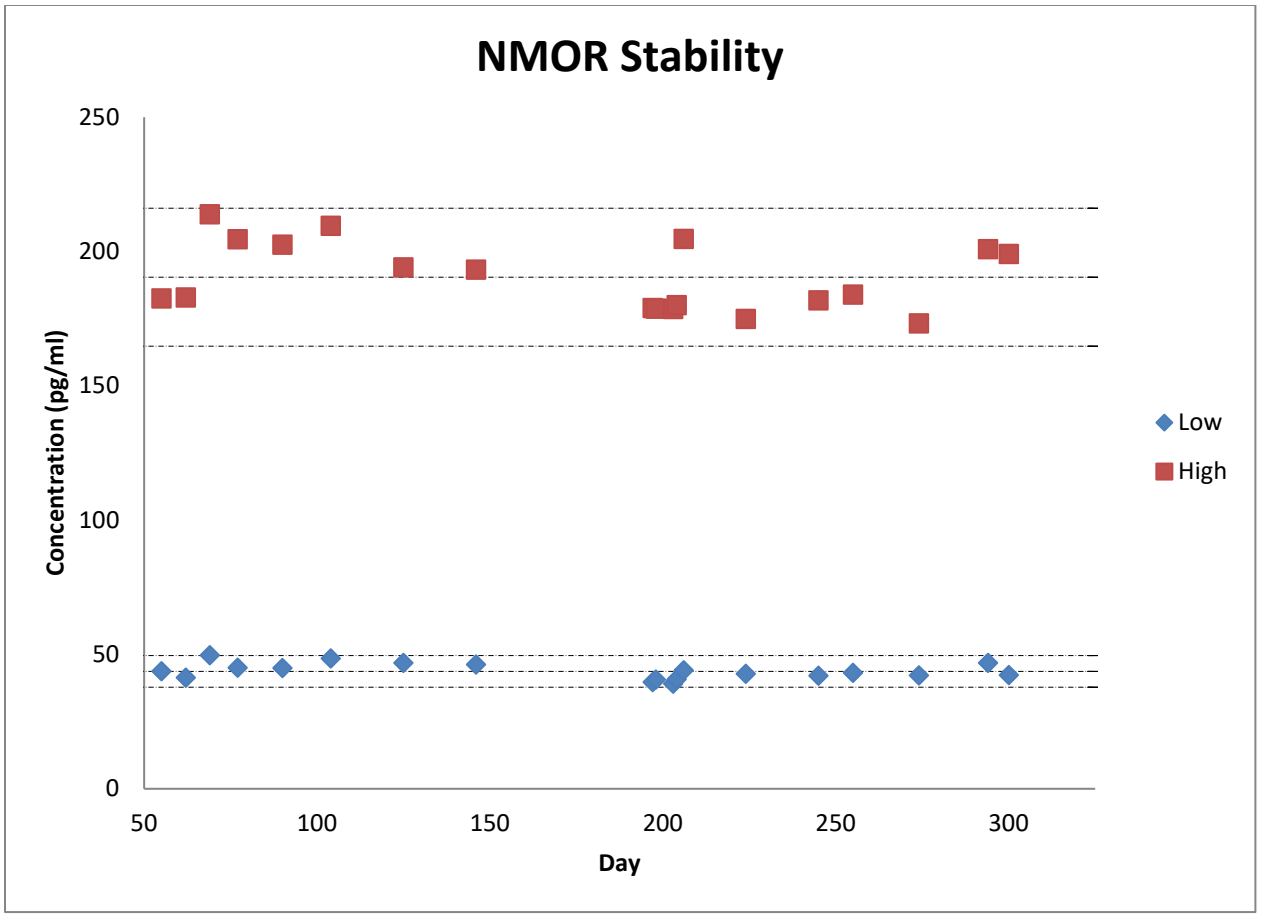
indicated that all six VNAs are stable at -70°C for at least 10 months. Urine samples are routinely stored in the dark at approximately -70 °C and analyte levels should not be impacted by long term storage at these conditions. Additional experiments indicate that NMEA, NDEA, and NPIP were stable following at least 5 thaw-refreeze cycles (T/RF) (sample loss less than 5%)




NDEA Stability
250
200
150
100
50
0
Concentration (pg/ml)
50
100
150
200
250
300
Day
Low
High


NPIP Stability
250
200
150
100
50
0
Concentration (pg/ml)
50
100
150
200
250
300
Day
Low
High


NPYR Stability
Concentration (pg/ml)
250
200
150
100
50
0
50
100
150
200
250
300
Day
Low
High



f. Ruggedness test

A non-smoker sample pool was spiked with all six analytes at 50 pg/mL. 1.9 mL aliquots of this pool were analyzed for ruggedness by varying the following parameters:

1) Number of pipet extraction loops: 8, 10 (protocol level), and 12 loops.
2) Amount of DCM use for analyte extraction: 2 mL, 2.5 mL (protocol level), and 3 mL. Maximum working volume of each well plate is 5 mL.
3) Source temperature: 225°C, 250°C (protocol level), and 275°C.
4) Chemical ionization gas flow: 20%, 25% (protocol level), and 30%.
5) Volume injection: 4, 5 (protocol level), and 6 µL.

No significant difference was observed in all six analytes under 5 testing parameters.

Parameter testing	NMEA	NDEA	NPIP	NPYR	NMOR
Ruggedness - PipExtr 8 loops	46.5	44.2	40.8	64.5	52.9
Ruggedness - PipExtr 10 loops	44.3	43.8	40.6	61.5	51.5
Ruggedness - PipExtr 12 loops	46.8	47.0	43.6	61.9	52.7
Ruggedness - DCMvol 2 mL	46.0	46.5	41.4	62.9	57.9
Ruggedness - DCMvol 2.5 mL	47.0	46.8	43.4	63.4	54.9
Ruggedness - DCMvol 3 mL	38.4	42.0	35.7	56.3	44.0
Ruggedness - SourceTemp 225°C	46.7	50.2	42.4	62.5	52.2
Ruggedness - SourceTemp 250°C	45.9	51.3	44.1	64.2	52.8
Ruggedness - SourceTemp 275°C	47.9	51.9	46.6	63.8	53.8
Ruggedness - CIgas 20%	40.7	43.5	37.5	58.2	48.4
Ruggedness - CIgas 25%	39.4	44.1	37.0	56.9	47.6
Ruggedness - CIgas 30%	40.7	43.2	35.8	57.5	45.9
Ruggedness - InjVol 4 µL	46.6	51.3	44.1	60.6	53.8
Ruggedness - InjVol 5 µL	45.7	50.5	43.5	64.2	54.9
Ruggedness - InjVol 6 µL	46.4	50.8	44.1	63.6	53.1

**yellow highlight are protocol levels.*

g. Quantitation via MassHunter™ and Indigo ASCENT™

Accuracy and precision results were obtained by either MassHunter or Indigo ASCENT, and the difference was less than 5%. Quantitation is done via Indigo ASCENT software. It can be performed via MassHunter if necessary.

Accuracy (%)
Run VA179

	100pg MassHunter			Indigo		
	VA179_016	VA179_017	VA179_018	VA179_016	VA179_017	VA179_018
NMEA	102.43	94.03	97.64	103.48	94.82	98.71
NDEA	94.63	91.13	89.94	89.94	87.53	89.20
NPIP	91.91	88.09	84.43	94.37	91.29	91.04
NPYR	115.43	118.27	123.84	115.66	113.05	123.84
NMOR	92.30	86.13	86.50	91.88	86.01	85.56

Run VA180

	100pg MassHunter			Indigo		
	VA180_016	VA180_017	VA180_018	VA180_016	VA180_017	VA180_018
NMEA	94.43	89.29	91.78	96.47	90.54	93.20
NDEA	88.57	88.18	84.12	87.34	85.90	83.59
NPIP	87.59	80.86	81.91	87.55	80.80	84.16
NPYR	100.85	101.08	108.58	100.35	99.65	106.35
NMOR	89.50	86.04	89.49	87.16	84.21	86.31

Run VA181

	100pg MassHunter			Indigo		
	VA181_016	VA181_017	VA181_018	VA181_016	VA181_017	VA181_018
NMEA	96.85	96.58	96.90	98.33	97.55	97.83
NDEA	92.71	90.48	90.23	89.75	89.78	89.08
NPIP	86.41	91.41	87.18	88.33	91.75	89.89
NPYR	112.75	110.03	108.27	115.32	112.10	108.75
NMOR	91.93	94.29	92.15	91.20	92.13	92.32

Run VA179

	200pg MassHunter			Indigo		
	VA179_019	VA179_020	VA179_021	VA179_019	VA179_020	VA179_021
NMEA	108.63	105.91	104.53	109.15	108.24	105.80
NDEA	100.39	97.46	95.45	97.66	95.85	94.53
NPIP	97.09	96.55	93.19	99.40	99.11	94.39
NPYR	111.95	112.74	107.78	109.84	113.49	107.37
NMOR	99.62	95.65	93.39	99.21	96.03	92.62

Run
VA180

	200pg MassHunter			Indigo		
	VA180_019	VA180_020	VA180_021	VA180_019	VA180_020	VA180_021
NMEA	99.49	102.09	92.45	101.40	103.42	94.20
NDEA	94.48	95.34	87.36	94.26	95.41	86.52
NPIP	89.04	90.32	83.85	91.55	91.44	85.13
NPYR	104.14	106.03	94.61	103.40	105.30	93.49
NMOR	95.34	92.05	87.21	92.19	93.09	86.31

Run
VA181

	200pg MassHunter			Indigo		
	VA181_019	VA181_020	VA181_021	VA181_019	VA181_020	VA181_021
NMEA	106.14	110.35	106.13	107.44	112.15	107.41
NDEA	102.13	104.54	99.02	101.54	104.05	99.42
NPIP	95.56	97.54	91.93	98.19	99.53	94.38
NPYR	105.37	109.10	104.74	106.57	110.49	104.99
NMOR	97.95	100.90	98.00	97.36	101.51	97.75

Precision
(CV)

	MassHunter 50 pg QC				
Intra-Run	NMEA	NDEA	NPIP	NPYR	NMOR
VA180	4.87	4.63	1.90	4.65	4.69
VA181	2.03	3.60	4.51	6.43	2.43
VA183	2.42	2.68	1.05	6.54	2.18
VA184	2.68	1.93	2.19	3.19	3.16
VA185	3.02	5.16	5.04	5.44	2.54
Inter-Run	6.14	5.17	5.36	9.34	5.16

Indigo

Intra-Run	NMEA	NDEA	NPIP	NPYR	NMOR
VA180	4.72	3.93	1.91	3.03	3.00
VA181	2.79	4.00	3.17	5.48	1.41
VA183	2.55	3.39	2.24	4.26	1.73
VA184	2.78	1.56	2.34	2.74	2.68
VA185	2.84	2.74	3.72	3.85	2.25
Inter-Run	5.49	5.53	3.56	5.86	3.63

MassHunter

200 pg QC

Intra-Run	NMEA	NDEA	NPIP	NPYR	NMOR
VA180	2.24	2.68	1.97	2.68	5.07
VA181	1.26	1.69	2.19	2.08	1.24
VA183	2.91	2.33	2.67	2.75	2.49
VA184	2.16	2.76	2.98	2.29	2.20
VA185	3.27	3.55	3.23	4.16	3.32
Inter-Run	7.09	6.78	6.95	5.70	6.12

Indigo

Intra-Run	NMEA	NDEA	NPIP	NPYR	NMOR
VA180	1.95	2.00	1.43	2.47	1.23
VA181	1.29	1.90	1.37	1.50	1.16
VA183	2.49	1.94	2.68	2.33	2.22
VA184	2.52	2.52	2.35	2.27	1.90
VA185	2.49	5.56	3.53	3.37	2.94
Inter-Run	7.95	8.37	6.08	5.61	6.18

h. Hamilton- volume verification

Volume verification is done weekly using methanol and water. Temperature is measured. Set volumes are dispensed in pre-weighted vials, and the pre- and post-weight difference is calculated. Density of methanol and water are used to calculate the actual volume dispensed by the Hamilton. If the difference between the set and actual volume delivered is less than 5%, then the volume delivered is accurate. If the

difference is more than 5%, then a service call will be placed with Hamilton. No aliquot can be performed until a service engineer from Hamilton services and certifies the volume verification.

Volume verification for ISTD addition: ISTD sample spiking solution is prepared in methanol, and 50 µL of this spiking solution is added to each QC and unknown sample using 50 µL tips.

Solvent: methanol (MeOH)

Volume delivered: 50 µL

Temperature: 21ºC

Density of methanol at 20°C: 0.7913

Tube	Channel	Target	Tip	Initial weight (mg)	Final weight (mg)	Weight difference	Vol MeOH delivered
Tube 1	1	50	50uL	1803.55	1842.43	38.88	49.13
Tube 2	2	50	50uL	1812.53	1852.28	39.75	50.23
Tube 3	3	50	50uL	1811.89	1851.18	39.29	49.65
Tube 4	4	50	50uL	1804.28	1842.51	38.23	48.31
Tube 5	1	50	50uL	1822.2	1862.51	40.31	50.94
Tube 6	2	50	50uL	1812.33	1852.55	40.22	50.83
Tube 7	3	50	50uL	1819.14	1858.82	39.68	50.15
Tube 8	4	50	50uL	1804.53	1844.26	39.73	50.21
Tube 9	1	50	50uL	1802.41	1842.81	40.4	51.06
Tube 10	2	50	50uL	1819.21	1859.26	40.05	50.61
Tube 11	3	50	50uL	1799.66	1838.57	38.91	49.17
Tube 12	4	50	50uL	1797.95	1837.84	39.89	50.41

Channel	Average	StDev	RSD
Channel 1	50.38	1.08	2.14
Channel 2	50.56	0.30	0.59
Channel 3	49.66	0.49	0.98
Channel 4	49.64	1.16	2.33
All Channels	50.06	0.84	1.67

Volume verification for sample aliquot: QC and unknown samples are aliquoted via 1x1000 µL and 1x900µL using 1000 µL tips.

Solvent: water

Volume delivered: 1000 µL

Temperature: 23ºC

Density of water at 25°C: 0.997

Tube	Channel	Target	Tip	Initial weight (mg)	Final weight (mg)	Weight difference	Vol H_2O delivered
Tube 1	1	1000	1mL	1217.5	2236.6	1019.1	1021.612
Tube 2	2	1000	1mL	1218.1	2235.8	1017.7	1020.208
Tube 3	3	1000	1mL	1211.3	2228.6	1017.3	1019.807
Tube 4	4	1000	1mL	1210.1	2227.9	1017.8	1020.308
Tube 5	1	1000	1mL	1217.3	2235.6	1018.3	1020.81
Tube 6	2	1000	1mL	1216.8	2233.5	1016.7	1019.206
Tube 7	3	1000	1mL	1222.7	2238.8	1016.1	1018.604
Tube 8	4	1000	1mL	1220	2236.1	1016.1	1018.604

Channel	Average	StDev	RSD
Channel 1	1021.21	0.57	0.06
Channel 2	1019.71	0.71	0.07
Channel 3	1019.21	0.85	0.08
Channel 4	1019.46	1.21	0.12
All Channels	1019.89	1.06	0.10

Appendix C

Method Validation Data

Limit of Detection

The LoD of all six analytes were updated after 60 independent runs were obtained:VA224 to V141029.

Analyte	LoD
NMEA	3.64
NDEA	5.02
NPIP	5.08
NPYR	8.15
NMOR	7.84

Appendix D

Method Validation Data

Limit of Detection

The LoD of NDMA was due to high background observed from blank plates. Previously, the average of NDMA blank was 2.1 ng/L. Currently, the average of NDMA blank has increased to 11.1 ng/L.

Analyte	LoD (ng/L)
NDMA (not reported)	30.8
NMEA	3.64
NDEA	5.02
NPIP	5.08
NPYR	8.15
NMOR	7.84

Appendix E

NDMA Repeatability

Due to high %CV observed in NDMA repeatability, final results for NDMA collected for the NHANES 2013-14 1/3 subset were not reported.

Relevant records are kept in our QC manual.