CONFIDENTIAL

UK SMOKE CONSTITUENTS STUDY

Part 8: Determination of Tobacco Specific Nitrosamines Yields in Cigarette Smoke

AnnexA - method

Commissioned by:

Tobacco Manufacturers Association

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March 2003

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DEFINITIONS

The acronyms used in this procedure are listed and defined below.

(v/v)	Volume/Volume
1R4F	Industry Reference Cigarette produced by the University of Kentucky
TSNAs	Tobacco specific N-nitrosamines
NNN	N-nitrosonornicotine
NNK	4-(N-nitrosomethylamino)- I -(3-pyridyl)- 1 -butanone
NAT	N-nitrosoanatabine
NAB	N-nitrosoanabasine
Cal	Calibration
Cigt.	Cigarette
Conc	Concentration
D	Difference
ISO	International Standards Organization
ID	Identification
LOD	Limits of Detection
LOQ	Limits of Quantitation
MS	Mainstream Smoke
MSDS	Material Safety Data Sheet
N	Number of Replicates
PTFE	Polytetrafluoroethylene (Teflon)
QC	Quality Control
RE	Relative Error
RSD	Relative Standard Deviation
SD	Standard Deviation
ACN	Acetonitrile
MeOH	Methanol

DETERMINATION OF TOBACCO SPECIFIC N-NITROSAMINES IN MAINSTREAM SMOKE

I. PURPOSE/SCOPE

Tobacco specific N-nitrosamines (TSNAs) are a known component of mainstream cigarette smoke. This method describes the procedure for the determination of TSNAs in mainstream cigarette smoke. Note that the term TSNAs in this SOP refers to the following compounds: N-nitrosonornicotine (NNN), 4-(N-nitrosomethylamino)- I -(3-pyridyl)- 1 -butanone (NNK), N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB).

II. PRINCIPLE OF METHOD

The TSNAs are quantitated from mainstream smoke from five cigarettes smoked under ISO conditions using an analytical smoking machine and a 44 mm Cambridge filter as the collection media.

LC-MS/MS (liquid chromatography coupled to a triple quadrupole mass spectrometer) allows for the determination of the targeted nitrosamines with minimum interference from other components in mainstream smoke. The LC-MS/MS method uses a high performance liquid chromatograph (HPLC) equipped with a C18 reversed phase column to resolve TSNAs from potential interference. Further selectivity is accomplished using a triple quadrupole mass spectrometer with electrospray ionization (ESI) and selective reaction monitoring of characteristic daughter ions. Deuterated internal standards (d-NNN, and d-NNK) are added to the buffer extraction solution and are used to correct for recovery of nitrosamines.

The concentrations of the TSNAs determined by this method are reported in units of mass-to-volume (i.e., ng/mL). The measured concentration, the number of cigarettes smoked, and the sample solution volume(s) are also used to calculate the total analyte mass on a per cigarette basis.

III. APPARATUS, CHEMICALS, AND LABORATORY SUPPLIES

A. Required Chemicals

Chemical	Supplier	Catalog Number	Certified Purity or Concentration
N-Nitrosonornicotine (NNN)	**Midwest Research Institute**	**E0151**	**Specify**
4-(N-Methyl-N-Nitrosamino)-1-(3-Pyrridyl)-1-Butanone (NNK)	**Midwest Research Institute**	**E0698**	**Specify**
N-Nitrosoanabasine (NAB)	**Midwest Research Institute**	**E0840**	**Specify**
N-Nitrosoanatabine (NAT)	**Midwest Research Institute**	**E0831**	**Specify**
Deuterated N-Nitrosonornicotine (d_4-NNN)	**CDN Isotopes**	**D-4141**	**98+%**
Deuterated 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyrridyl)-1-Butanone (d_4-NNK)	**CDN Isotopes**	**D-4152**	**99+%**
Acetic Acid	**Mallinckrodt**	**MK250144**	**99.5%**
Methanol	**EM Science**	**EM-MX0475**	**HPLC Grade**
Ammonia Acetate	**Aldrich**	**15-852-6**	**98%**
Acetonitrile	**EM Science**	**AX0142-1**	**HPLC Grade**

B. Laboratory Apparatus and Supplies

- Automated liquid sampler (ALS) vials, with screw PTFE-lined lids.
- Volumetric glass pipettes, calibrated “to deliver” specified volumes, Class A
- 44-mm Cambridge filter, part number 8 0 20 2851, Borgwaldt Technik (Hamburg, Germany)
- Laboratory balance with 0.1mg accuracy
- 50mL Erlenmeyer Flasks
- 100 – 1000μL mechanical micropipette
- 1 – 100μL mechanical micropipette
- Mechanical Dispenser (10 – 50 mL), Brand Tech model Dispensette III (Wertheim Germany)
- HPLC grade water or water system capable of delivering 18 MΩ-cm water
- Orbital Shaker, VWR scientific
- Aluminum Foil

C. Preparation of mobile phase and extraction solutions

See Appendix A

IV. PREPARATION OF ANALYTICAL SOLUTIONS

Precaution: TSNAs are light sensitive. In the preparation of standards and samples, unnecessary prolonged exposure to light should be avoided.

A. TSNAs Stock Solution

See Appendix B

B. TSNAs Intermediate Solutions

See Appendix B

C. Working Standards

See Appendix B

D. Quality Control Stock Solutions

See Appendix B

E. Internal Standard Solutions

See Appendix B

V. SAMPLE COLLECTION

- Each mainstream sample will consist of the smoke collected from five cigarettes smoked under ISO conditions. The sample quantity for product cigarettes is defined in the study-specific Cigarette Product Testing Protocol. Mainstream samples are collected using a linear smoking machine as specified in T-003, Operation of Analytical Smoking Machines. Before smoke collection, the smoking laboratory must conform to the environmental conditions specified in SOP T-002, Smoke Laboratory Environmental Control.

Authors Comment – conditions for smoke generation and collection are described elsewhere – a summary is reproduced below.

- Cigarettes are conditioned[1] at a temperature of 22 ± 1°C and 60 ± 3 % relative humidity for a minimum of 48 hours but not exceeding 10 days.
- Butt marking will be ISO butt length specifications[2]. Filtered cigarettes will be smoked to a measured butt length equal to either the tipping paper + 3 mm or filter length + 8 mm whichever is longer. The minimum butt length will be 23 mm and this will also be used for non filter brands. All smoking shall be conducted in an environment of temperature 22 ± 2°C and 60 ± 5 % relative humidity[1].
- ISO conditions[3] for smoking cigarettes will apply. The smoking machine puffing parameters will be 35 ± 0.2 cm^3 puff volume with 2.0 ± 0.05 second puff duration once every 60.0 ± 0.5 seconds.
- As a check that cigarettes have been smoked in accordance with ISO standard conditions, TPM yields were determined and compared with that normally achieved. Results for cigarettes that give significantly low or high TPM yields (± 3 × standard deviation) will be discarded.
- A minimum of five determinations will be performed for each brand. The smoking of the cigarette brands is randomised so that samples from the same brand are smoked on different days.
- With each batch of samples a 2R4F cigarette is smoked.
- Five cigarettes are smoked using ISO specifications[2] - and the mainstream smoke is collected onto a pad.

A flow diagram of the sample collection and workup procedure is given in Appendix C.

VI. PROCEDURE

A. Extraction of Filter Pads:

- Remove the mainstream pad from its holder, folding it into quarters and wiping the inside of the holder with the clean side of the pad.
- Transfer the pad to a suitable flask or amber bottle.
- Add 200 μL of Internal Standard to the pad using a mechanical micropipette.
- Add 20 mL of 100mM Ammonium Acetate to the Erlenmeyer flask using the dispenser.
- Protect the sample from light if necessary.
- Shake the sample on an orbital shaker for 60 minutes at 200rpm or wrist-action shaker for 30 min.
- Filter Sample with a 0.45 μM PTFE Filter
- Pipette ~1.5 mL of sample into a properly labeled ALS Vial and cap using PTFE lined caps.

NOTE: Samples are stable in the refrigerator for a minimum of 5 days at ~5°C.

VI. INSTRUMENT ANALYSIS

A. Sample Run Order

Sampling analysis order is as follows:

1) Internal Standard Blank
2) Calibration standards = Calibration Curve Standards (e.g. S1 to S5)

[1] ISO 3402: 2000 - Tobacco and tobacco products – atmosphere for conditioning and testing
[2] ISO :4387: 2000 - Methods for chemical analysis of tobacco and tobacco products – Determination of total and nicotine- free dry particulate matter using a routine analytical smoking machine
[3] ISO 3308:2000 – Routine analytical cigarette smoking machine – 1: Definitions and standard conditions

3) QC Low = Low calibration check standard
4) QC High = High calibration check standard
5) Samples in sets of ten or less
6) Alternate QC-Low and QC-High

B. HPLC Apparatus and Operation Parameters

Equilibrate the column with the initial mobile phase conditions for 15-30 minutes or until obtaining a stable baseline before use.

Example Chromatograms are depicted in Figures IX.1 - IX.3.

TABLE IX.1
HPLC Parameters

Equipment / Parameter	Make	Model	
HPLC System Identification #	Shimadzu	10VP Series	
Detector	Micromass	Ultima	
Autosampler	HTC-PAL	Leap Autosampler	
Data Acquisition Hardware	Mass Lynx	Version 3.5	
Operating System Software	Windows NT		
Analytical Columns:	Water Xterra Stainless Steel Column 5cm x 2.1mm i.d., 2.5µm particle size	Part Nos. 186000595	
Guard Column	Keystone Science	Part Nos. 842015-715-P	
Mobile Phase	Gradient (see below)		
Flow Rate	0.2 mL/min		
Column Temperature	65°C		
Sample Compartment Temp.	NA		
Analytical Run Time	7 min		
Post Run Time	0 min		
Injection Vol.	3 µL		
Data Capture Time:	1.0-7.0 min		

MS Acquire Time	**7 min**
Desolvation Temperature	**~300 °C**
Source Temperature	**~120 °C**
Desolvation Gas (Nitrogen)	**~280 L/Hr**
Nebulizer Gas (Nitrogen)	**open**
Collision Gas (Argon)	**~2.5 mtorr**

Analyte Detection Parameters

Analyte	Parent Ion	Cone Voltage	Collision Energy	Quantitation Mass Range (m/z)	Dwell Time (msec)
NNN	**178.2**	**35 V**	**10.0 V**	**147.7 – 148.3**	**100**
d-NNN	**182.2**	**35 V**	**10.0 V**	**151.7 – 152.3**	**100**
NNK	**208.2**	**35 V**	**10.0 V**	**121.7 – 122.3**	**100**
d-NNK	**212.2**	**35 V**	**10.0 V**	**125.7 – 126.3**	**100**
NAT	**190.1**	**35 V**	**10.0 V**	**159.7 – 160.3**	**100**
NAB	**192.2**	**35 V**	**10.0 V**	**161.7 – 162.3**	**100**

Table IX.2
Mobile Phase Gradient

Time (min)	0.1% AA in H_2O	0.1% AA in MeOH
0	98	2
4.00	50	50
5.00	20	80
6.00	98	2
7.00	98	2

Figure IX.1
Calibration Blank

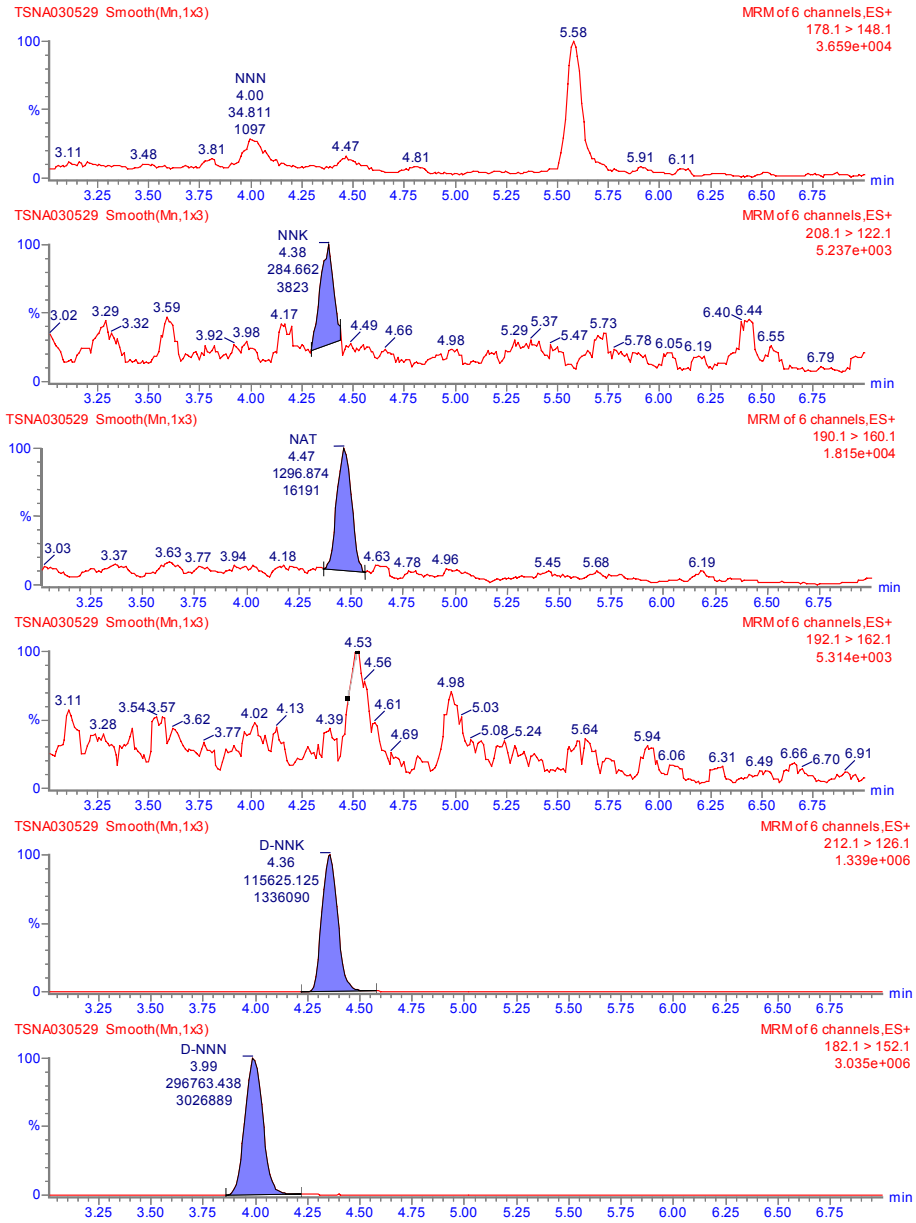


Figure IX.2
High Standard

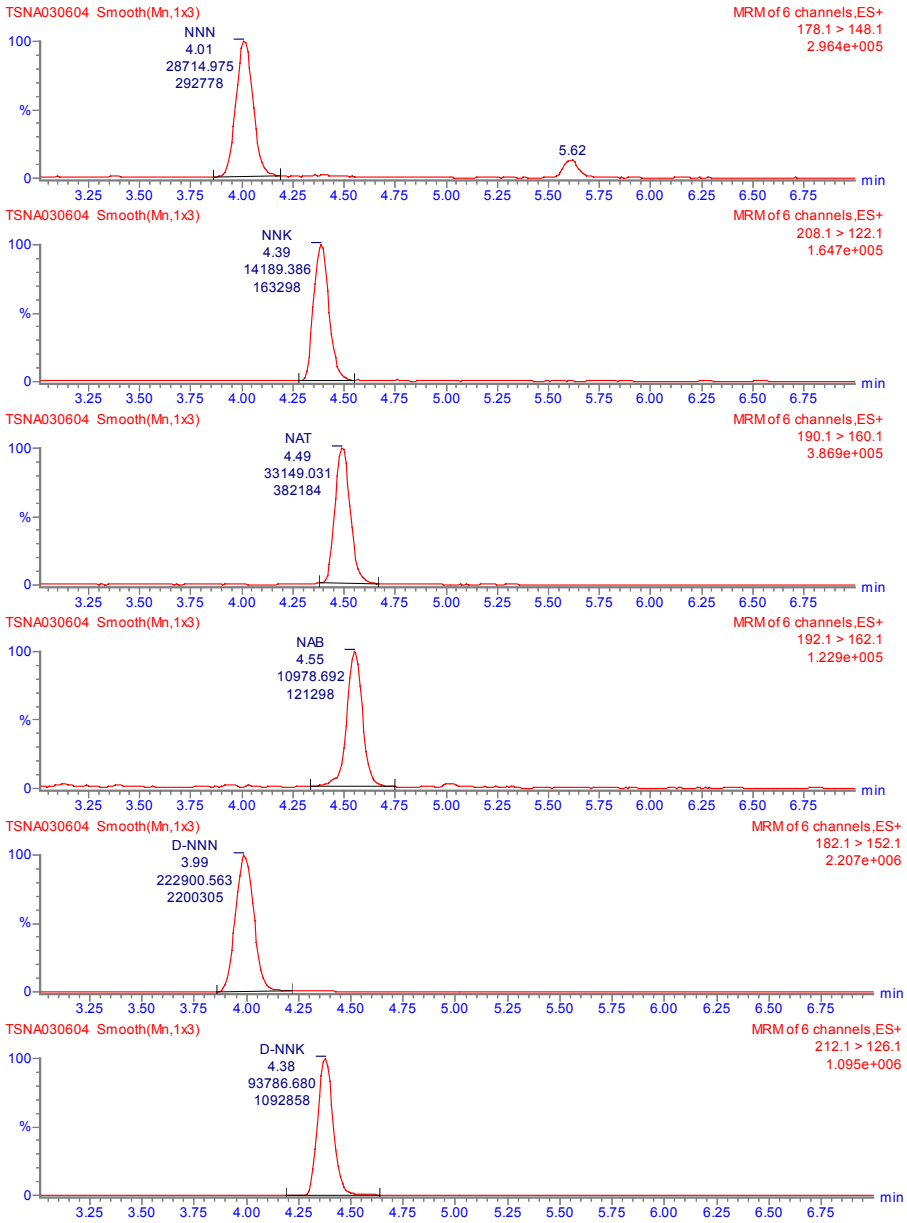
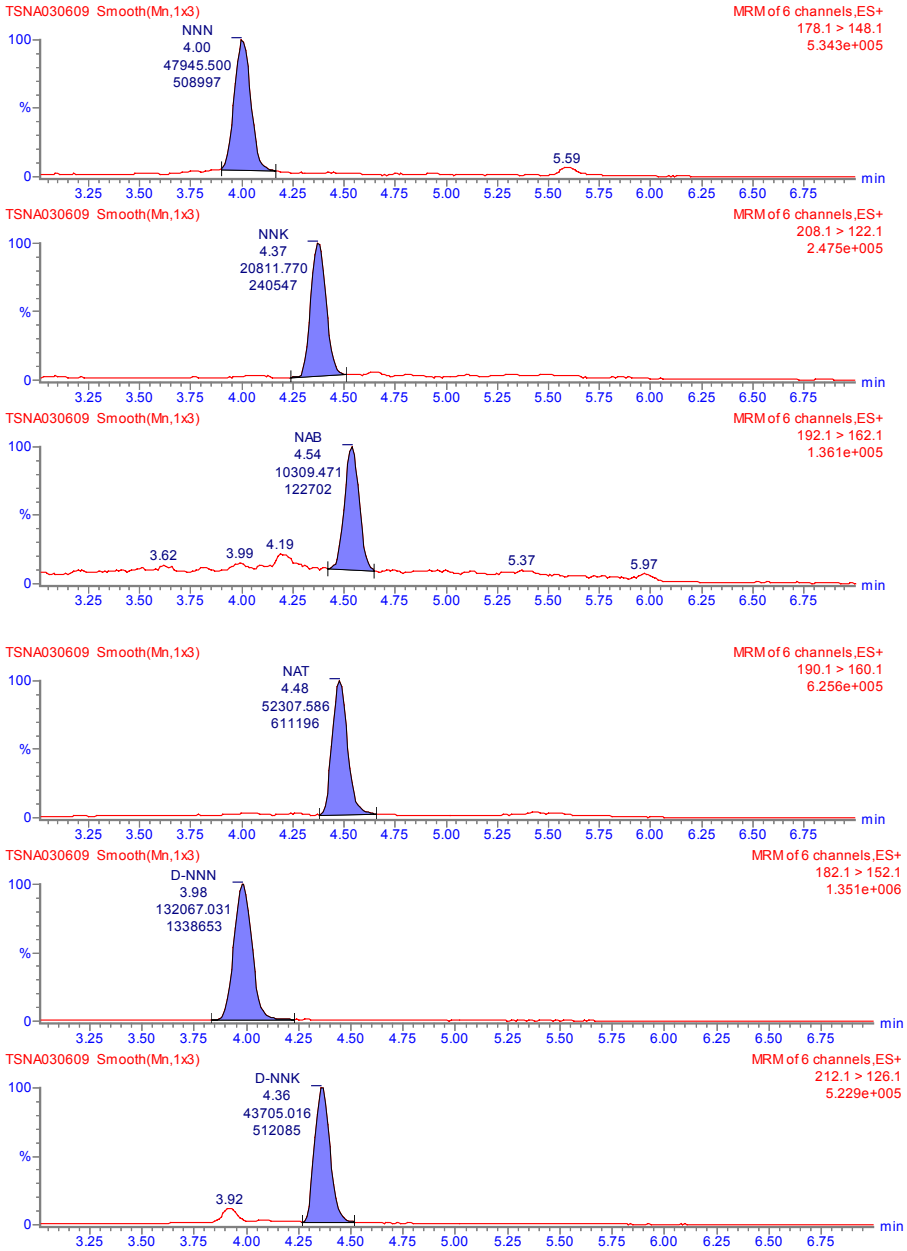


Figure IX.3
1R4F – Mainstream Smoke Sample



VIII. DATA PROCESSING AND EXAMPLE CALCULATIONS

This section describes the method used for determining the amount of TSNAs on a per cigarette basis.

A. Instrument Calibration Calculations

The analysis is carried out using the calibration capabilities of the Micromass MassLynx software and assumes the operator is already familiar with the procedure for setting up a calibration on the software.

B. Processing of Raw Data from the MS/MS

The analyte concentration is determined by the internal calibration method using the regression equation derived from the calibration curve. Calculation of the analyte is obtained by using the calculation capabilities of the Micromass MassLynx software and assumes the operator is already familiar with the procedure for setting up the Micromass MassLynx software**.**

Example Calculation of a mainstream 1R4F sample:

Example calculation:
Mainstream Cigarette Sample Concentration

5 cigarettes were smoked and the pad was extracted in 20mL of buffer. The concentration of NNN was determined to be 4.61 ng/mL.

NNN concentration: (ng/cigt.) = (4.61ng/mL*20mL)/ 5 cigt. = 18.44 ng/cigt.

C. Data Acceptance

In the event of poor chromatography (e.g., bad peak shape, no peaks, bad baseline, etc.), the lead chemist will be contacted. If the poor chromatography can be attributed to a single event, such as a bad injection, the standard or sample exhibiting the bad chromatography will be disregarded from any calculations, and a complete explanation will be included with the data.

D. Calibration and Quality Control Standard Acceptance Criteria

Each calibration curve must have a coefficient of determination (r^2) of 0.995 or better. Calibrations that do not meet these requirements should be brought to the attention of the lead chemist immediately. All quality control standards must be within ±15% RE of their calculated values. All quality control standards that do not meet these requirements should be brought to the attention of the lead chemist immediately. If a quality control standard is not ± 15% RE of their calculated values the quality control standard may be replaced at the operators discretion with a different vial of the same standard and re-analyzed. If the new quality control standard is within ±15% RE of its calculated value then sample analysis may continue. If the new quality control standard is not ± 15% RE of the calculated value then the sample analysis must stop, the problem corrected and the instrument recalibrated. Any samples that are bracketed with a failed quality control standard must be re-analyzed.

Appendix A

A. Preparation of Analytical Solutions

Mobile phase and extraction solutions expire 3 months after the preparation date and are stored at ambient room temperature.

Prepare all solutions in Class A, “to contain” glassware. All pipetting will be performed with Class A, “to deliver” pipettes unless otherwise stated. Transfer all solutions to sealed glass bottles for storage.

0.1% Acetic Acid in Methanol (v/v), (0.1% AA in MeOH)

Using a 2.0-mL volumetric pipette, pipette 2 mL of glacial acetic acid into a 2000-mL volumetric flask containing ~1400 mL of MeOH. Dilute to volume with MeOH and mix the solution well.

0.1% Acetic Acid in Water (v/v), (0.1% AA in Water)

Using a 2.0-mL volumetric pipette, pipette 2 mL of glacial acetic acid into a 2000-mL volumetric flask containing ~1400 mL of Water. Dilute to volume with Water and mix the solution well.

100 mM Ammonium Acetate Acid in Water (v/v) (Extraction Solution)
Using an analytical balance weight 15.4 gm of ammonium acetate acid. Transfer the ammonium acetate into a 2000-mL volumetric flask containing ~1400 mL of water (H_2O). Dilute to volume with water and mix the solution well

APPENDIX B

A. Preparation of Analytical Standards

Prepare all solutions in Class A, "to contain" glassware. All pipetting will be performed with Class A, "to deliver" pipettes unless otherwise stated. Transfer all solutions to sealed glass bottles for storage.

TSNAs Stock Solution

Weigh to the nearest 0.0001g approximately 100 mg of each TSNA in separate 100-mL volumetric flask and make up to the volume with Acetonitrile. This solution is stable for 2 years at -5°C.

TSNAs concentration = 1.0mg/mL

TSNAs Intermediate Solution 1

In the same 100-mL volumetric flask, add the following amounts of standard solutions as indicated in the table below. Dilute to volume with 100% ACN.

Standard	Starting Conc. (mg/mL)	Vol of Stock added (mL)	Final Conc. (µg/mL)
NNN	~1.0	4 .0	~40
NNK	~1.0	4.0	~40
NAT	~1.0	4.0	~40
NAB	~1.0	1.0	~10

TSNAs Intermediate Solution 2

In a 100-mL volumetric flask, add 1.0 mL of TSNA Intermediate solution 1. Dilute to volume with 30% ACN / 70% water.

Analyte	Starting Conc (μg/mL)	Final Conc. (ng/mL)
NNN	~40	~400
NNK	~40	~400
NAT	~40	~400
NAB	~10	~100

Working Standards

To make standard S1 add the amounts listed below to a 250 mL volumetric flask that contains 25 mL of 100mM Ammonia Acetate and 2.5 mL of the internal standard spiking solution. Add the required amount of ACN and then dilute to volume with water. To make the standards S2-S6 add the amounts listed below to a 100 mL volumetric flask that contains 10 mL of 100mM Ammonia Acetate and 1 mL of the internal standard spiking solution. Add the required amount of ACN and then dilute to volume with water. Store the standards at ~5°C.

Standard	Vol of Intermediate Stock 2 (mL)	Vol of ACN (mL)	Nominal Conc. NNN (ng/mL)	Nominal Conc. NNK (ng/mL)	Nominal Conc. NAT (ng/mL)	Nominal Conc. NAB (ng/mL)
S1	0.5	25.0	~0.8	~0.8	~0.8	~0.2
S2	1.0	10.0	~4.0	~4.0	~4.0	~1.0
S3	2.0	10.0	~8.0	~8.0	~8.0	~2.0
S4	5.0	8.0	~20	~20	~20	~5.0
S5	10	7.0	~40	~40	~40	~10
S6	25	2.5	~100	~100	~100	~25
S7	0.5	10.0	~200	~200	~200	~50

NOTE: THESE SOLUTIONS ARE STABLE FOR 2 MONTHS AT ~5°C

B. Preparation of Deuterated Internal Standards

TSNAs Deuterated Internal Standard Stock

Weigh to the nearest 0.0001g approximately 100mg of d-NNN and d-NNK in separate 100mL volumetric flask and make up to the volume with acetonitrile. Store the standards at -5°C in an amber glass bottle. This solution is stable for 2 years at -5°C.

Internal Standard TSNAs concentration = 1.0mg/mL

TSNAs Deuterated Internal Spiking Solution

In the same 100-mL volumetric flask, add the following amounts of standard solutions as indicated in the table below. Dilute all standards with 100% ACN. Store the standards at -5°C in an amber glass bottle. This solution is stable for six months at -5°C.

Standard	Starting Conc. (mg/mL)	Vol of Stock added (mL)	Final Conc. (ng/mL)
d-NNN	~1.0	0.5	5000
d-NNK	~1.0	0.5	5000

C. Preparation of Quality Control Stock Solutions

TSNAs QC Stock Solution

Weigh to the nearest 0.0001g approximately 100mg of each TSNAs in a 100mL volumetric flask and make up to the volume with ACN. This solution is stable for 2 years at -5°C.

TSNAs concentration = 1.0mg/mL

TSNAs QC Intermediate Solution 1

In the same 100-mL volumetric flasks, add the following amounts of standard solutions as indicated in the table below. Dilute all standards with 100% ACN.

Standard	Starting Conc. (mg/mL)	Vol of Stock added (mL)	Final Conc. (µg/mL)
NNN	~1.0	4 .0	~40
NNK	~1.0	4.0	~40
NAT	~1.0	4.0	~40
NAB	~1.0	1.0	~10

TSNAs QC Intermediate Solution 2

In a 100-mL volumetric flask, add 1.0 mL of TSNA Intermediate solution 1. Dilute to volume with 30% ACN / 70% water.

Analyte	Starting Conc (µg/mL)	Final Conc. (ng/mL)
NNN	~40	~400
NNK	~40	~400
NAT	~40	~400
NAB	~10	~100

QC Working Standards

To make the standards add the amounts listed below to a 100 mL volumetric flask that contains 10 mL of 100mM Ammonia Acetate and 1 mL of the internal standard spiking solution. Add the required amount of ACN and then make up to volume in 100 % water. Store the standards at ~5°C.

Standard	Vol of QC Intermediate Stock 2 (mL)	Vol of ACN (mL)	Nominal Conc. NNN (ng/mL)	Nominal Conc. NNK (ng/mL)	Nominal Conc. NAT (ng/mL)	Nominal Conc. NAB (ng/mL)
QC Low	5.0	8.0	~20	~20	~20	~5.0
QC High	10	7.0	~40	~40	~40	~10

NOTE: THESE SOLUTIONS ARE STABLE FOR 2 MONTH AT ~5°C

APPENDIX C

A. Mainstream Sample Collection and Workup Procedure