



Title: **Determination of TSNA in Tobacco and Tobacco Products by LC-MS/MS**

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Status (Initial/Revision/ Retired)	Document Revision Number	Issue/Revision Date	Revision Identification	Revision Author
Initial Release	1	02/21/2014	Format changed from PPI to SOP.	M. Noe, D. Self
Revision	2	03/01/2017	The document was rewritten with the following updates: 1. Update Instrument settings for optimized performance 2. Add NAB-d4 as internal standard for NAB determination 3. Expand method scope to include cigar filler 4. Update sample preparations for tobacco leaf samples and cigarette filler 5. Add portion sample preparations	Cathy Jin
Revision	3	05/17/2017	Sample requirements and handling were updated to provide clarity for pouched product sample preparation.	Tammy Blake

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**A. SCOPE**

1. This method is used to quantitatively determine the concentrations of four tobacco specific n-nitrosamines (TSNAs) in tobacco and tobacco products using liquid chromatography with tandem mass spectrometry (LC/MS/MS). The TSNAs determined with this method are:

N-nitrosonornicotine	NNN	CAS # 16543-5-8
4-(N-Methyl-N-Nitrosamino)-1-(3-Pyridyl)-1-Butanone	NNK	CAS # 64091-91-4
N-nitrosoanatabine	NAT	CAS # 71267-22-6
4-nitrosoanabasine	NAB	CAS # 1133-64-8

The TSNAs are extracted with an aqueous buffer and analyzed by LC/MS/MS. Results are reported in units of nanograms per gram (ng/g) on a dry tobacco weight or “as-is” bases.

2. This method is applicable to the analysis of ground tobacco, cigarette filler, cigar filler, pipe tobacco, and smokeless tobacco (dry snuff, MST, (b) (4), (b) (4) sample).
3. This method applies to sample concentrations of NNN, NNK, and NAT in an approximate range of 160 ng/g to 12,000 ng/g and NAB concentrations in an approximate range of 40 ng/g to 3,000 ng/g.

**B. DEFINITIONS**

1. Calibration Check Standard (CCS) – A standard solution that is prepared independently of the calibration solutions from a different source/lot of material.
2. Internal Quality Control Samples (IQCS): A process control sample, such as ground 3R4F filler, that is prepared with each batch of samples in order to monitor the performance of the method overtime.
3. Reagent blank with internal standard (RBIS): The RBIS is extraction solution with internal standard that is prepared following the same procedures as is used for samples.

**C. RESPONSIBILITIES**

1. The designated trained analyst performing this method is responsible for following all the procedures, including data review and approval, and to document and report any procedural deviations to lab management.
2. Personnel using this test method are responsible for conducting the analysis in a manner consistent with the safety policies of ALCS.

**D. VALIDATION**

1. The June 6, 2002 validation report for Tobacco Filler using 1R4F and low TSNA filler determined that the repeatability ranged from 2.56 to 10.3 %CV and the reproducibility ranged from 2.44 to 14.8 % CV<sup>2</sup>.

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2. The March 20, 2007 validation report for Bright tobacco, Burley tobacco, 2R4F filler, Dry snuff, Wet snuff and Reconstituted Tobacco determined that the repeatability and reproducibility were less than 5.5% CV<sup>3</sup>.
3. The December 20, 2010 validation report for (b) (4) and (b) (4) determined that the repeatability and reproducibility were 2% and 4% CV respectively<sup>5</sup>.
4. The November 2016 validation report for cigar filler determined that repeatability ranged from 0.5%-4% and intermediate precision ranged from 1%-4%<sup>6</sup>.

**E. EQUIPMENT AND APPARATUS**

1. Required Equipment and Apparatus: equivalent items may be substituted. Critical substitutions must have management approval.
  - a. LC/MS/MS-Liquid Chromatographic system with quadrupole mass spectrometer with electrospray ionization (ESI) source: such as Waters UPLC with a chilled autosampler, binary pump, and column oven; Waters triple quadrupole mass spectrometer with ESI source, MassLynx control and quantitative analysis software. Waters Corporation; Milford, MA
  - b. Nitrogen, high purity (99.99% or better)
  - c. Argon, high purity (99.99% or better)
  - d. HPLC analytical column: Waters XBridge BEH C18, 2.1 mm x 50 mm, 2.5 µm particle size. Part # 186003085, Waters Corporation
  - e. HPLC guard column: Waters XBridge BEH C18 guard cartridge, 2.1 mm x 10 mm, 3.5 µm particle size. Part # 186003059, Waters Corporation or equivalent.
  - f. Brinkman Dispensette, 50 mL capacity, Cat. # 13-688-224, Fisher Scientific
  - g. Gilson 215 SPE Liquid Handler with software version 735 or higher. Gilson Corporation
  - h. Rotary extractor capable of holding test tube racks used to extract samples by repeated inversion of the sample tubes. Sample rotator capable of 15 revolutions per minute (rpm)
  - i. Thermo Scientific HyperSep Retain-PEP 60mg/3mL SPE column, product # 60107-203
  - j. 60 mL amber vials with Teflon lined caps, e.g. Part # S146-0040, I Chem Brand Products
  - k. Eppendorf repeater (to deliver 10-1000 µL volume), Cat. #21-380-9, Fisher Scientific

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- l. Eppendorf pipette tips, Repeater plus series, e.g. Cat. #21-381-329, Fisher Scientific
  - m. Eppendorf pipette, 100-1000 µL. e.g. Cat. # 05-402-50, Fisher Scientific
  - n. 5ml disposable syringe with luer-lock tip, e.g. Cat. #14-829-4F, Fisher Scientific
  - o. Polyvinylidene difluoride (PVDF) syringe filters, 25 mm diameter, 0.2 µm pore size, e.g. Cat. #6872-2502, Whatman/Fisher Scientific
  - p. Disposable Culture Tubes, Borosilicate Glass, 16x100mm, Cat.#14-961-29, Fisher Scientific
  - q. Amber autosampler vials with PTFE lined caps, e.g. Cat.#03-391-6 for vials, Cat# C4011-1AP for caps, Fisher Scientific
  - r. Vortex mixer, e.g. Cat.# 02215365, Fisher Scientific
  - s. Autosampler vial inserts, Glass, e.g., Cat.# 03-375-3B, Fisher Scientific
2. Instrument Setup
- Typical mass spectrometer settings are listed below. Minor modifications may be made in order to improve analyte sensitivity. Changes shall be discussed with laboratory management and documented.
- a. Liquid Chromatograph
    - 1) HPLC parameters

Liquid Chromatograph Parameters	
Mobile Phase A	Reagent Water (Milli-Q 18.2 M)
Mobile Phase B	0.1% Acetic Acid in Methanol (MeOH)
Column Temperature	60°C
Flow Rate	0.300 mL/min
Autosampler	10°C
Injection Volume	2 µL
HPLC Run Time	8 min



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2) Mobile phase gradient

Time (min)	Mobile Phase A (Water) (%)	Mobile Phase B (0.1% Acetic acid in MeOH) (%)	Flow Rate (mL/min)
0.00	98	2	0.300
0.10	98	2	0.300
3.50	45	55	0.300
4.00	0	100	0.300
5.55	0	100	0.300
6.00	98	2	0.300
8.00	98	2	0.300

b. Mass Spectrometer

1) Typical mass spectrometer parameters are listed below.

Electrospray and Source Parameters	
Source Temperature	150 °C
Desolvation Temperature	500 °C
Desolvation Gas (Nitrogen)	1000 L/Hour
Collision Gas (Argon)	regulator set to 7±2 psi
Cone Gas (Nitrogen) flow	0
Capillary	0.5 kV
Cone	19V

- Place the mass spectrometer in "Operate" mode and allow the instrument to warm up with HPLC mobile phase flowing at 0.300 mL/min for at least 30 minutes before instrument tuning or analysis.
- The TSNA analytes are detected using Multiple Reaction Monitoring (MRM) mode. The Acquisition Span is set at 0; Inter-Channel Delay and Inter-Scan Delay are set to "Automatic". The MRM acquisition channels are as follows and may be changed to optimize instrument performance:

Analyte	Cone Voltage (V)	Collision Energy (EV)	Parent Ion (m/z)	Daughter Ion (m/z)	Dwell Time
NNK	24	10	208.0	122.0	AutoDwell
NNK-d4	24	10	212.0	126.0	AutoDwell
NNN	24	10	178.0	148.0	AutoDwell
NNN-d4	24	10	182.0	152.0	AutoDwell
NAT	22	10	190.0	160.0	AutoDwell
NAT-d4	24	8	194.0	164.0	AutoDwell
NAB	24	10	192.0	162.0	AutoDwell
NAB-d4	26	10	196.0	166.0	AutoDwell

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- 4) The mass spectrometer settings, such as capillary voltage, cone voltage, collision energy, parent ion and daughter ions are to be optimized using "IntelliStart- Sample Tune and Develop Method" option in the software or using manual tune to optimize instrument performance. The optimization procedures are:
  - a) Set the HPLC to deliver 50% Mobile Phase A and 50% Mobile Phase B at 0.300 mL/min.
  - b) Set the column temperature at 60°C and equilibrate the HPLC for at least 30 min.
  - c) Fill the 250 µL syringe with a TSNA standard solution CAL6 or a higher concentration standard or internal standard.
  - d) Start the syringe pump at 20 µL/mL.
  - e) Set flow path as "combined" and sample flow rate at 20 µL/mL in the "Fluidics" section.
- 5) The Method Events option of MS method is used to direct the column flow to waste except for the time that the analytes of interest elute from the column from 1.5 min to 3.0 min

**3. Instrument Maintenance****a. HPLC maintenance**

- 1) Prior to turning on the HPLC, ensure that there are sufficient volumes of mobile phases A and B and both mobile phases are within their expiration dates. Purge the LC pumps to remove any air in the solvent lines.
- 2) The HPLC system pressure should be less than 3500 PSI at the initial mobile phase conditions (98% mobile phase A and 2% mobile phase B, flow rate 0.300mL/min, and the column pre-warmed to 60°C). If the column pressure exceeds 3500 PSI, replace the guard and analytical columns and re-equilibrate the HPLC system for at least 30minutes.
- 3) The frits are used to protect the precision pumping system and analytical column from small particulate matter contaminating the solvents (mobile phases). Replace solvent reservoir bottle frits as needed.

**b. Mass Spectrometer maintenance**

- 1) The ion source (sample cone and cone gas nozzle assembly) shall be cleaned as needed. Consult the manufacturer's manual for instructions on removing and installing instrument parts and proper care/cleaning.



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- 2) Sample cone cleaning procedures:
  - a) Sonicate with 50% formic acid in Milli-Q water for 20 minutes.
  - b) Sonicate the parts in Milli-Q water for 10 minutes.
  - c) Sonicate the parts in methanol for 10 minutes.
  - d) Dry the parts with dry nitrogen before reinstalling on the ion source.  
Alternatively, the parts may be air dried.
- 3) After PM, mass spectrometer will be re-tuned. Calibration standards and IQCS monitors will be injected to check acceptance with the new instrument parameter.

## **F. CHEMICALS AND REAGENTS**

### **1. Chemicals Required**

**Note:** The grade or purities for the reagents specified below are minimum requirements. Higher purity reagents may be substituted. The suppliers listed below are recommendations. Equivalent alternative suppliers may be used with approval of laboratory management.

- a. Methanol - Optima grade. Cat.# A454-4, Fisher Scientific
- b. Acetonitrile, Optima grade. Cat.# A996-4, Fisher Scientific
- c. Acetic Acid, glacial. HPLC grade, Cat.# A35-500, Fisher Scientific
- d. Formic Acid (88%), Certified ACS Plus, Cat.# A118P-500, Fisher Scientific
- e. Ammonium Acetate, HPLC Grade, Cat.# A639-500, Fisher Scientific
- f. Ammonium hydroxide, Certified ACS Plus, Cat. # A6695-500, Fisher Scientific, or equivalent.
- g. Reagent Water: HPLC grade water or a water purification system with 18.2 MΩ product
- h. ISO Guide 34 Certified stock solution of N-Nitrosonornicotine (NNN), Purity 98%, 1000µg/mL in acetonitrile, Cat.# LC-FSPA-103-30, Spex Certiprep, Metuchen, NJ
- i. ISO Guide 34 Certified stock solution of 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyridyl)-1-Butanone (NNK), Purity 98%, 1000µg/mL in acetonitrile, Cat.# LC-FSPA-102-30, Spex Certiprep
- j. ISO Guide 34 Certified stock solution of (R,S) N-Nitrosoanatabine (NAT), Purity 98%, 1000µg/mL in acetonitrile, Cat.# LC-FSPA-105-30, Spex Certiprep
- k. ISO Guide 34 Certified stock solution of N-Nitrosoanabasine (NAB), Purity 98%, 1000µg/mL in acetonitrile, Cat.# LC-FSPA-104-10, Spex Certiprep



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- l. ISO Guide 34 Certified stock solutions of NNN, NNK, NAT, and NAB from Cerilliant Corporations (811 Paloma Drive, Suite A, Round Rock, TX 78665) standards used for calibration check standards

Standards	Description	Cat.#
NNN	1mg/mL in methanol	N-075
NNK	1mg/mL in methanol	N-076
NAT	1mg/mL in acetonitrile	N-078
NAB	1mg/mL in methanol	N-077

- m. N-Nitrosornicotine-2,4,5,6-d<sub>4</sub> (NNN-d<sub>4</sub>), Chemical purity 98%, Isotopic purity 99%. 0.1mg/mL in methanol, Cat.# KIT0765, Toronto Research Chemicals, North York, ON, Canada
- n. 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyridyl-d<sub>4</sub>)-1-Butanone (NNK-d<sub>4</sub>), Chemical purity 98%, Isotopic purity 99%, 0.1mg/mL in methanol, Cat.# KIT0770, Toronto Research Chemicals
- o. (R,S)-N-Nitrosoanatabine-2,4,5,6-d<sub>4</sub> (NAT-d<sub>4</sub>), Chemical purity 98%, Isotopic purity 99%, 0.1mg/mL in methanol, Cat.# KIT0780, Toronto Research Chemicals
- p. (R,S)-N-Nitroso Anabasine-d<sub>4</sub> (NAB-d<sub>4</sub>), Chemical purity 98%, Isotopic purity 99%, 0.1mg/mL in methanol, Cat.# KIT0775, Toronto Research Chemicals.

2. Reagent Preparation

The amount of solution prepared may be adjusted by appropriately scaling the reagents specified below. All solutions should be prepared using appropriately sized volumetric glassware (Class A pipettes and Class A volumetric flasks) or calibrated mechanical pipettes. Record all preparations in the Reagent Preparation logbook.

- a. **Extraction Solution:** 100 mM Ammonium Acetate: Using an analytical balance, weigh 15.40 (±0.05g) grams of ammonium acetate to the nearest 0.01 grams. Transfer the ammonium acetate into a 2000-mL volumetric flask containing ~1500 mL of water. Dilute to volume with water and mix well. Store the solution in a closed glass bottle at room temperature. The solution expires two months after the preparation date.
- b. **Mobile Phase A:** Reagent water (Milli-Q 18.2 M). The bottle should be rinsed with Milli-Q water and refilled at least weekly.
- c. **Mobile Phase B:** 0.1% acetic acid in methanol (v/v). Using a mechanical or volumetric pipette, transfer 1.00 mL of glacial acetic acid into a 1000-mL volumetric flask containing ~ 500 mL of methanol, mix well. Dilute to volume with methanol and mix well. Store in a closed glass amber bottle



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at room temperature. The solution expires one month after the preparation date.

- d. **30:70 acetonitrile:Milli-Q water:** This solution is used as the diluent for the intermediate standard solution. To a 500 mL beaker, add 350 mL Milli-Q water and 150 mL acetonitrile using an appropriately sized graduated cylinder and mix well. This solution is used as the diluent for Intermediate Standard Solutions 2 and Intermediate Check Standard Solution 2. Store the solution in a glass bottle at room temperature. Solution expires one month after preparation date.

e. **SPE Solutions**

- 1) **Wash1 (95:4.5:0.5 Milli-Q water: methanol:ammonium hydroxide):**  
To a 1 L volumetric flask containing approximately 100 mL Milli-Q water, add 45 mL methanol and 5 mL ammonium hydroxide using graduated cylinders. Bring the solution to volume with Milli-Q water and mix well. Store in a closed glass bottle at room temperature when not in use. The solution expires one month after preparation date.
- 2) **Wash2 (0.01% Formic Acid in Milli-Q water, v/v):** To a 1 L volumetric flask containing approximately 500 mL Milli-Q water, add 0.100 mL of formic acid. Bring to volume with Milli-Q water and mix well. Store in a closed glass bottle at room temperature when not in use. Solution expires one month after preparation date.
- 3) **Eluent Solution (70:30 methanol: 0.1% Acetic Acid in Milli-Q water, v/v):** To a 1 L volumetric flask containing approximately 300 mL Milli-Q water and 1.000 mL of glacial acetic acid. Bring to volume with methanol and mix well. Store in a closed glass bottle at room temperature when not in use. Solution expires one month after preparation date.

3. Standard Preparation

**Note:** See TSNA Standards Solutions Preparation worksheet. The amounts of solutions may be adjusted as needed. All standard preparations should be carried out by fully trained proficient personnel with minimum personal protective equipment such as safety glasses, lab coat, and nitrile gloves.

a. Internal Standard Solution

- 1) **TSNA Internal Standard Stock Solutions: NNN-d4, NNK-d4, NAT-d4, and NAB-d4** stock solutions are purchased from Toronto Research Chemicals (TRC) at concentrations of about 0.1mg/mL. Store at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ . The Certificate of Analysis (CoA) for each target compound specifies what the exact concentration and expiration/retest date. TSNA Internal Standard stock solutions expire on the CoA expiration/retest date.



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- 2) **Intermediate Internal Standard Stock Solution (IS#1)/spiking solution (~2000ng/mL):** In an amber 100-mL volumetric flask containing ~50 mL of acetonitrile, add 2.0 mL of the NNN-d4, NNK-d4, NAT-d4, and NAB-d4 Internal Standard Stock Solutions. Dilute to volume with acetonitrile and mix well. Store in an amber bottle at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ . The IS#1 solution expires on the CoA expiration/retest date.

**Note:**

1. The lab may re-test the internal standard purchased stocks to qualify use after the CoA expiration dates. The IS#1 expiration dates may be extended 2 years after the original CoA expiration/retest dates if the peak area ratios of analytes/internal standards are within 15% of those of the fresh prepared calibration standards.
2. The same batch of internal standard spiking solution must be used for preparing working calibration standard, calibration check standards and samples.

b. TSNA Standard Solutions

- 1) **TSNA Stock Solutions:** NNN, NNK, NAT, and NAB stock solutions are purchased from Spex CertiPrep at certified concentrations of about 1000 $\mu\text{g/mL}$ . The Certificate of Analysis (CoA) for each target compound specifies what the exact concentration and expiration/retest date. Store at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ . All purchased stock solutions expire on the CoA expiration/retest date.
- 2) **Intermediate TSNA Standard Solution 1(Interm#1):** In an amber 100 mL volumetric flask containing ~50 mL of acetonitrile, add 4.0 mL each of the purchased NNN, NNK, NAT Stock Standard Solutions and 1.0 mL of the purchased NAB Stock Standard Solution. Dilute to volume with acetonitrile and mix well. Store in an amber bottle at  $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ . The Interm#1 solution expires on the CoA expiration/retest date.
- 3) **Intermediate Standard Solution 2 (Interm#2):** In an amber 200 mL volumetric flask containing ~50 mL of 30:70 acetonitrile/water, add 2.0 mL of Interm#1. Dilute to volume with 30:70 acetonitrile/water and mix well. Store in an amber bottle at  $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$ . The Interm#2 expires on the CoA expiration/retest date.
- 4) **Calibration Standards:** The TSNA calibration standards are prepared in 100 mL volumetric flasks containing 10 mL of 100 mM ammonium acetate solution. Add 1.0 mL of IS#1. Add appropriate volumes of Interm#2 and acetonitrile as shown in the following table. Dilute to the volume with water and mix well. Store the standards in amber bottles at  $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$ . The calibration standards expire on the CoA expiration/retest date. See [table](#) below:

**Altria**

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	Vol. of 100mM Ammonium Acetate (mL)	Vol. of IS #1 (mL)	Vol. of Acetonitrile (mL)	Vol. of Interm. #2 (mL)	NNN (ng/mL)	NNK (ng/mL)	NAT (ng/mL)	NAB (ng/mL)
Cal1	10.0	1.00	10.0	1.00	4.00	4.00	4.00	1.00
Cal2	10.0	1.00	10.0	2.00	8.00	8.00	8.00	2.00
Cal3	10.0	1.00	9.00	5.00	20.0	20.0	20.0	5.00
Cal4	10.0	1.00	7.00	10.0	40.0	40.0	40.0	10.0
Cal5	10.0	1.00	3.00	25.0	100	100	100	25.0
Cal6	10.0	1.00	1.00	75.0	300	300	300	75.0

**c. Calibration Check Standards (CCS):**

1) **Check Standard Stock Solution:** NNN, NNK, NAT and NAB stock solutions (about 1000µg/mL) are purchased from Cerilliant (Stock Solutions purchased from Spex CertiPrep may also be used). Store at -20°C ± 5°C. All check standard stock solutions expire on the CoA expiration/retest date.

2) **Intermediate TSNA Check Standard Solution 1(CS#1):** In an amber 100 mL volumetric flask containing ~50 mL of acetonitrile, add 4.0 mL each of the NNN, NNK, NAT Stock Standard Solutions and 1.0 mL of the NAB Stock Standard Solution. Dilute to volume with acetonitrile and mix well. Store in an amber bottle at -20°C ± 5°C. The CS#1 solution expires on the CoA expiration/retest date.

**Note:** The lab may re-test the purchased stocks after the CoA expiration dates. The CS#1 expiration dates may be extended 2 years after the original CoA expiration/retest dates if the results meet the CCS acceptance criteria (The %RCR for both CCS1 and CCS2 should be within ±15% of the nominal concentration for each analyte).

3) **Intermediate Check Standard Solution 2 (CS#2):** In an amber 100 mL volumetric flask containing ~50 mL of 30:70 acetonitrile/water v/v, add 1.0 mL of CS#1. Dilute to volume with 30:70 acetonitrile/water and mix well. Store in an amber bottle at 4°C ± 3°C. The CS#2 solution expires on CoA expiration/retest date.

**Note:** The lab may re-test the purchased stocks after the CoA expiration dates. The CS#2 expiration dates may be extended 2 years after the original CoA expiration/retest dates if the results meet the CCS acceptance criteria (The %RCR for both CCS1 and CCS2 should be within ±15% of the nominal concentration for each analyte).

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- 4) **Calibration Check Standards:** Two CCS of different concentrations are to be prepared. The CCS are prepared in 100 mL volumetric flasks containing 10 mL of 100 mM ammonium acetate solution and 1 mL of IS#1. Add the appropriate volumes of CS#2 and acetonitrile as shown in the following table. Dilute with water and mix well. The internal standard concentrations in each CCS is approximately 20 ng/mL for NAT-d4, NNN-d4, NNK-d4, and NAB-d4. Store the standards in amber bottles at 4°C ± 3°C. The calibration check standards expire on CoA expiration/retest date.

**Note:** The lab may re-test the purchased stocks after the CoA expiration dates. The calibration check standards expiration dates may be extended 2 years after the original CoA expiration/retest dates if the results meet the CCS acceptance criteria (The %RCR for both CCS1 and CCS2 should be within ±15% of the nominal concentration for each analyte). See table below:

	Vol. of 100mM Ammonium Acetate (mL)	Vol. of IS#1 (mL)	Vol. of Acetonitrile (mL)	Vol. of CS#2 (mL)	NNN (ng/mL)	NNK (ng/mL)	NAT (ng/mL)	NAB (ng/mL)
CCS	10.0	1.00	10.0	2.00	8.00	8.00	8.00	2.00
CCS	10.0	1.00	3.00	25.0	100	100	100	25.0

**G. SAMPLE REQUIREMENTS**

1. The target tobacco weight for a replicate analysis of loose/ground tobacco is 0.75 g ± 0.05 g. Samples that require grinding and/or removal of filler from cigarettes or cigars should be prepared according to the following instructions. A minimum of 15 g of ground composite should be prepared.
  - a. Tobacco shall be ground at ambient temperature as described in WI 097-1108<sup>10</sup> "Sample Preparation".
  - b. Cigarette filler: cigarette filler shall be ground at ambient temperature as described in WI 097-1108 "Sample Preparation".
  - c. Cigar filler: cigar filler shall be freeze ground as described in WI 097-1108 "Sample Preparation".
  - d. Pipe Tobacco: pipe tobacco shall be freeze ground as described in WI 097-1108 "Sample Preparation".
  - e. Portioned smokeless tobacco products (pouches)
    - 1) Routine sample requests and Stability Testing: Manually open each pouch using scissors, a knife, or tearing by hand. Use nitrile gloves when handling pouches. Transfer the tobacco material into a sample bottle to

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make a composite sample and discard the pouch material. Bottle a minimum of 15 grams; the composite tobacco sample may be used for other tests in addition to TSNA, e.g. OV and/or pH. Mix the composite sample by shaking or stirring before removing sample aliquots.

Note: The composite sample amount will vary depending on the customer test request and the number of replicates per test. Sample amounts may be communicated on the Sample Preparation Sheet.

- 2) FDA Analysis: unit pouches shall be analyzed and include both the paper and tobacco. Cut the pouches in half and add the tobacco and paper directly into the extraction vessel.
- f. Monitor sample (IQCS): A monitor sample such as ground 3R4F filler is prepared as a method control with each batch of samples in order to monitor the performance of the method overtime.
- g. (b) (4) the tobacco shall be removed from the (b) (4) and ground as described in WI 097-1108.

**Note:** Consult laboratory management for instructions on preparing samples not described above.

## **H. PROCEDURE**

1. Sample Handling
  - a. Loose Tobacco: Weigh 0.75 g  $\pm$  0.05 g into a tared 60 mL amber vial and record the exact weight to the nearest 0.0001g.
  - b. Portion product (pouches, and novel portions):
    - 1) Routine sample requests and Stability Testing: Follow the Loose Tobacco sample handling procedure above using the composite tobacco sample.
    - 2) FDA Analysis: Unit portions are analyzed. The target weight range for portioned products is between 0.65 to 1.2 grams. Preweigh a portion to determine the number of portions required to reach the target sample weight for each replicate. Cut the portion(s) in half and add the tobacco and pouch paper directly to the extraction vessel. Record the exact weight to the nearest 0.0001g.
2. Sample extraction **without SPE**: Used for all matrices **except** smokeless tobacco<sup>6,9</sup>
  - a. Prepare a reagent blank with internal standard (RBIS). The RBIS is prepared following the same procedures as the samples except that tobacco is not added to the extraction vial.
  - b. Prepare 3 IQCS sample for each batch of samples, following the same procedure as the samples.



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- c. Add 300 µL of the 2000 ng/mL Internal Standard Spiking solution (IS#1) to each sample vial using a Repeater.
- d. Add 30.0 mL of 100 mM ammonium acetate Extraction Solution to each vial using the verified dispenser.
- e. Extract the samples on a rotary extractor set to 15 RPM for at least 30 minutes. Proceed to the next step on the same day.
- f. Filter approximately 1 mL of sample directly into an amber autosampler vial using a 25mm, 0.2 µm PVDF syringe filter and a 5 mL disposable syringe and then cap the vials.
- g. The prepared samples should be stored 4°C ± 3°C if not analyzed the same day. These prepared samples are stable for 5 days.
- h. The remaining sample extracts may be stored in the 60mL amber extraction vials at 4°C ± 3°C until the analytical results have been approved and released. The stored extracts can be used for subsequent analysis up to 5 days after extraction.
- i. For samples which exceed the quantitation range for any target TSNA compound, the filtered sample in the autosampler vial or stored extract solution containing tobacco may be filtered and then diluted with a diluent made from Extraction Solution containing 20ng/ml of each internal standards (e.g. add 0.1mL spiking solution into 10mL extraction solution). Reanalyze the diluted extract.

**Note:** The concentration of NNN, NNK, NAT are typically much greater than NAB. Therefore, a low level analyte may be diluted below the LOQ when performing a dilution for a high level analyte. If this occurs, the undiluted result for the low level analyte must be reported.

3. Sample extraction **with SPE**: Used for smokeless tobacco:
- a. Prepare a reagent blank with internal standard (RBIS). The RBIS is prepared following the same procedures as the samples except that tobacco is not added to the extraction vial.
  - b. Prepare 3 IQCS sample following the same procedure as the samples.
  - c. Add 300 µL of the 2000 ng/mL Internal Standard Spiking solution (IS#1) to each sample vial containing samples using a Repeater.
  - d. Add 30.0 mL of 100 mM ammonium acetate Extraction Solution to each vial using the verified dispenser.
  - e. Extract the samples on a rotary extractor set to 15 RPM for at least 30 minutes. Proceed to the next step on the same day.
  - f. Filter approximately 4 mL of sample directly into labeled disposable culture tubes using a 25mm, 0.2 µm PVDF syringe filter and a 5 mL disposable



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syringe.

- g. Add 0.250 mL of concentrated ammonium hydroxide to each sample using an Eppendorf Repeater and vortex for 3-5 seconds.
- h. Proceed to SPE procedure following WI 097-1519<sup>11</sup>. Alternatively, the samples may be manually prepared following the procedure described in Appendix 1.
- i. Cap and vortex the amber autosampler vials prior to analysis. The prepared samples should be stored  $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$  if not analyzed the same day. These prepared samples are stable for 5 days.
- j. The remaining sample extracts may be stored in the 60mL amber extraction vials at  $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$  until the analytical results have been approved and released. The stored extracts can be used for subsequent analysis up to 5 days after extraction.
- k. For samples which exceed the quantitation range for any target TSNA compound, the extract solution containing tobacco may be diluted with a diluent made from Extraction Solution containing 20ng/mL of each internal standards (e.g. add 0.1mL spiking solution into 10mL extraction solution), and then repeat steps f-i before analysis. For example, filter 4 mL of extract from step J (extract containing tobacco), transfer 2 mL of the filtered extract, dilute with calculated amount of diluent (e.g. 2 mL for 2:1 dilution), mix well, and then repeat steps f-i before analysis.

**Note:** The concentration of NNN, NNK, and NAT are typically much greater than the concentration of NAB. Therefore, a low level analyte may be diluted below the LOQ when performing a dilution for a high level analyte. If this occurs, the undiluted result for the low level analyte must be reported.

#### 4. Calibration

- a. System suitability must be evaluated as described in the Quality Control and Acceptance Criteria section.
- b. A new calibration curve must be acquired daily before performing a sample analysis. In order to conserve the working standards, low volume autosampler vial insert (fill at least half full) may be used.
- c. Generation of Calibration Curves:
  - 1) When analyzing the working standards, the individual analyte concentrations are to be entered into appropriate fields in the sample list (MassLynx Batch page). "Standard" should be selected for the sample type of each calibration standard.
  - 2) Set up the quantitation software to perform internal standard calibration based on peak areas. Enter names, appropriate retention times, and

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internal standard references as shown in the example table below.

Quantitation Parameter Settings

Name	Retention Time (min)	Quantitation Trace	Internal Standard Reference
NNK	~ 2.46	208.0 > 122.0	NNK-d4
NNN	~ 2.01	178.0 > 148.0	NNN-d4
NAT	~ 2.60	190.0 > 160.0	NAT-d4
NAB	~ 2.68	192.0 > 162.0	NAB-d4
NNK-d4	~ 2.45	212.0 > 126.0	N/A
NNN-d4	~ 1.99	182.0 > 152.0	N/A
NAT-d4	~ 2.57	194.0 > 164.0	N/A
NAB-d4	~2.66	196.0 > 166.0	N/A

- 3) Set the quantitation method to perform a linear calibration with the origin excluded and a weighting factor of  $1/x^2$ .
- 4) Set the smoothing integration parameter to perform Mean Average Smoothing with "Smoothing Iterations" of 2 and "Smoothing Width" of 2.
- 5) Use MassLynx to integrate the standard chromatograms and generate the corresponding calibration curves for each compound. During internal standard calibration, MassLynx uses relative response factors (RRF) for the y-axis of the calibration curve. Consult the Calculations section of the SOP for an explanation of the RRF. The calibration curve is based on least squares regression with  $1/x^2$  weighting, for the data plotted using the target analyte concentration as the x-variable and the RRF as the y-variable.

## 5. Analysis

There is not a specific requirement for the frequency CCSs are incorporated into a run sequence; however, samples must be bracketed by a Low and High CCS that pass the acceptance criteria (see Quality Control section). A typical sample run order is as following:

- a. Wash or test injection
- b. Reagent Blank (100 mM ammonium acetate/Extraction Solution)
- c. Wash or Test Injection (acetonitrile blank)
- d. RBIS (The Reagent Blank with Internal Standard)
- e. System Suitability sample (calibration standard 1)
- f. TSNA calibration standards (Cal 1 through Cal 6)
- g. RBIS

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- h. CCS Low
  - i. CCS High
  - j. IQCS-1
  - k. Approximately half the samples in the batch if there are more than 20 samples in the batch)
  - l. CCS Low
  - m. CCS High
  - n. IQCS-2
  - o. Second half of the samples in the batch
  - p. IQCS-3
  - q. CCS Low
  - r. CCS High
6. Calculations and Reporting
- a. Percent relative concentration residual (%RCR) is calculated to show the degree of deviation of individual concentration points from the established calibration equation. It is calculated using the following equation:

$$\%RCR = \frac{RC-NC}{NC} \times 100\%$$

Where: RC=The concentration calculated from the calibration curve  
NC=The nominal or theoretical concentration

- b. All calibration and sample analysis calculations utilize Relative Response Factors (RRF). The RRF is calculated using the equation:

$$RRF = \frac{Area_a}{Area_{IS}} \times Conc_{IS}$$

Where: Area<sub>a</sub> = area of the target analyte peak  
Area<sub>IS</sub> = area of the corresponding internal standard peak  
Conc<sub>IS</sub> = concentration of the corresponding internal standard

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- c. The concentration of the target analytes in tobacco sample (ng/mL), is determined using the calculated RRF for the sample, the slope and intercept obtained from the appropriate calibration curve, and the equation:

$$\text{Conc} = \frac{\text{RRF-Int}}{\text{slope}}$$

Where: Conc = calculated concentration (ng/mL)

Int = y-intercept from the calibration curve

Slope = the slope from calibration curve

- d. The concentration of analyte is converted to a gram of sample basis using the following equation:

$$\text{Amount (ng/g)} = \text{Conc (ng/mL)} \times \frac{\text{Vol}}{\text{Wt}}$$

Where: Amount (ng/g) = The amount of analyte in one gram of sample

Conc (ng/mL) = The sample concentration obtained from the instrument

Vol = volume of the extraction solution (30mL) in mL

Wt = sample mass weight in g (e.g. 0.75 g)

- e. An analyte response that falls below the lowest calibration standard will be reported as less than the limit of quantitation (<LOQ). Data will be reported as not detected (N.D.) if an analyte response is not detected.

## 7. Quality Control and Acceptance Criteria

- a. System Suitability Test: System suitability should be evaluated prior to calibration using the criteria below:
- 1) Sensitivity: Suitable instrument sensitivity is determined using calibration standard 1. The signal to ratio for all analytes in calibration standard 1 should exceed 10:1.
  - 2) Peak shape and width: Using the chromatogram for calibration standard 1, examine the peak shape for unacceptable chromatography (e.g. poor symmetry); Select "display mass 182 > 152" for NNN-d4 under the "Chromatogram" dropdown (MassLynx Batch page), measure the peak width at 10% peak height. The peak width must be less than 0.22 minutes for NNN-d4. Record the data in the system suitability logbook. If any abnormalities exist, investigate the cause and correct before proceeding to the analytical sample run.

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- b. Chromatogram Evaluation: Evaluation of every standard and sample chromatogram after analysis is required to assure proper peak assignments and integration. Integration must be checked and corrected to reflect accurate peak baselines. Chromatograms of calibration standard 1 and samples are included in [Appendix 2](#).
- c. Calibration: A calibration curve is considered valid if the following conditions are met:
  - 1) Coefficient of determination ( $R^2$ ): Calibration curves for each analyte should have  $R^2$  values of 0.990 or higher. Recalibrate if  $R^2$  falls below 0.990.
  - 2) %RCR: All calibration curves should have %RCR values not exceeding  $\pm 20\%$  for CAL 1 and  $\pm 15\%$  for CAL 2-6.
  - 3) The peak area ratios of the calibration standards, prepared from new stock or fresh dilution from existing stock, should be verified against the peak area ratios of the current calibration standards. The peak area ratios should agree within  $\pm 15\%$ .
  - 4) Calibration curves may be truncated by removing either the low or high standard as long as the samples remain bracketed by the calibration curve. The calibration level must be removed for all analytes. Other calibration levels may be replaced by either reinjection of the same standard or repreparing and reinjecting the new standard. Consult laboratory management for guidance.
- d. Calibration Check Standard (CCS): The validity of the calibration curves must be checked after calibration and during an analysis batch by injecting CCS Low and CCS High. All samples must be bracketed by passing CCSs. The %RCR for both CCSs should be within  $\pm 15\%$  of the nominal concentration for each analyte. The CCS results that do not pass the acceptance criteria suggest that the calibration curves are no longer valid. The samples analyzed after the last passing CCS must be reanalyzed with a new calibration unless there is an assignable cause.
- e. IQCS sample: the results for the IQCS samples are used to monitor the performance. These results must be tracked and compared to a control chart. If the TSNA values of the control samples are not within the control limits, data from the given run batch should be considered suspect and must be investigated. If an assignable cause cannot be identified the entire run shall be discarded and re-prepared.
- f. RBIS: the RBIS is used to check for contamination and any carryover originating from sample preparation or the analytical procedure. If the calculated concentrations of TSNA in the RBIS exceeds half of the

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concentration of standard 1, appropriate corrective measures should be conducted. The RB should also be evaluated for analyte peaks.

- g. In the event that any of the quality control measures outlined in this section pass acceptance criteria for some but not all of the analytes in this method, data for analytes with acceptable quality controls can be accepted, but analytes which fail quality control measures must be re-analyzed before reporting. Consult laboratory management for assistance.

## **I. REFERENCES**

1. Analytical Method SOP 095-5519 "Determination of Tobacco Specific N-Nitrosamines in Tobacco", Effective February 21, 2014
2. Validation report "Validation of Determination of Tobacco Specific N-Nitrosamines by LC/MS/MS in Tobacco Filler," June 6, 2002.
3. Validation report "LC/MS/MS Analysis of Tobacco Specific Nitrosamines in Tobacco," March 30, 2007.
4. Internal Memo: Use of New Reference Materials for "Determination of Tobacco Specific N-Nitrosamines in Tobacco", Sept 24, 2010
5. Validation report "Validation of Determination of Tobacco Specific N-Nitrosamines by LC/MS/MS in (b) (4) December 20, 2010
6. Supplemental validation report "Determination of Tobacco Specific N-Nitrosamines in Tobacco and Tobacco Products\_Cigar", November 22, 2016
7. LPSS Analytical Sciences Communication form (TSNA) for SOP-095-5519 modification, February 25, 2015
8. Analytical Sciences Communication form "FDA for Portion Samples", January 4, 2016
9. Method improvement report "Determination of Tobacco Specific N-Nitrosamines in Tobacco", November 2, 2016
10. WI 097-1108, Sample Preparation
11. WI 097-1519, Gilson Operation for TSNA SPE Clean-up
12. Waters MassLynx Software manuals

## **J. FORMS**

1. TSNA in Tobacco and Tobacco Products System Suitability
2. TSNA Standard Solutions Preparation Worksheet
3. TSNA Reagent Prep templates
4. TSNA Standards Validation template
5. TSNA spiking solution validation template

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**K. APPENDICES**

1. Manual SPE sample preparation procedures
  - a. Precondition the SPE cartridges with ~2.0 mL of Milli-Q water at a flow rate of 4-5 drops per seconds and discard to waste.
  - b. Load 2.0 mL of sample from step H.3.f. and let the waste drain at 1-2 drops per second.
  - c. Wash the SPE cartridge with 3.0 mL of Wash1 at a flow rate of 4-5 drops per seconds and discard to waste
  - d. Wash the SPE cartridge with 3.0 mL of Wash2 using a flow rate of 4-5 drops per seconds.
  - e. Dry the SPE cartridge under vacuum at ~10-15 psi for approximately 3 minutes.
  - f. Elute the analytes from the SPE cartridges using 1.0 mL of Eluent Solution using a flow rate of 1-2 drops per seconds.





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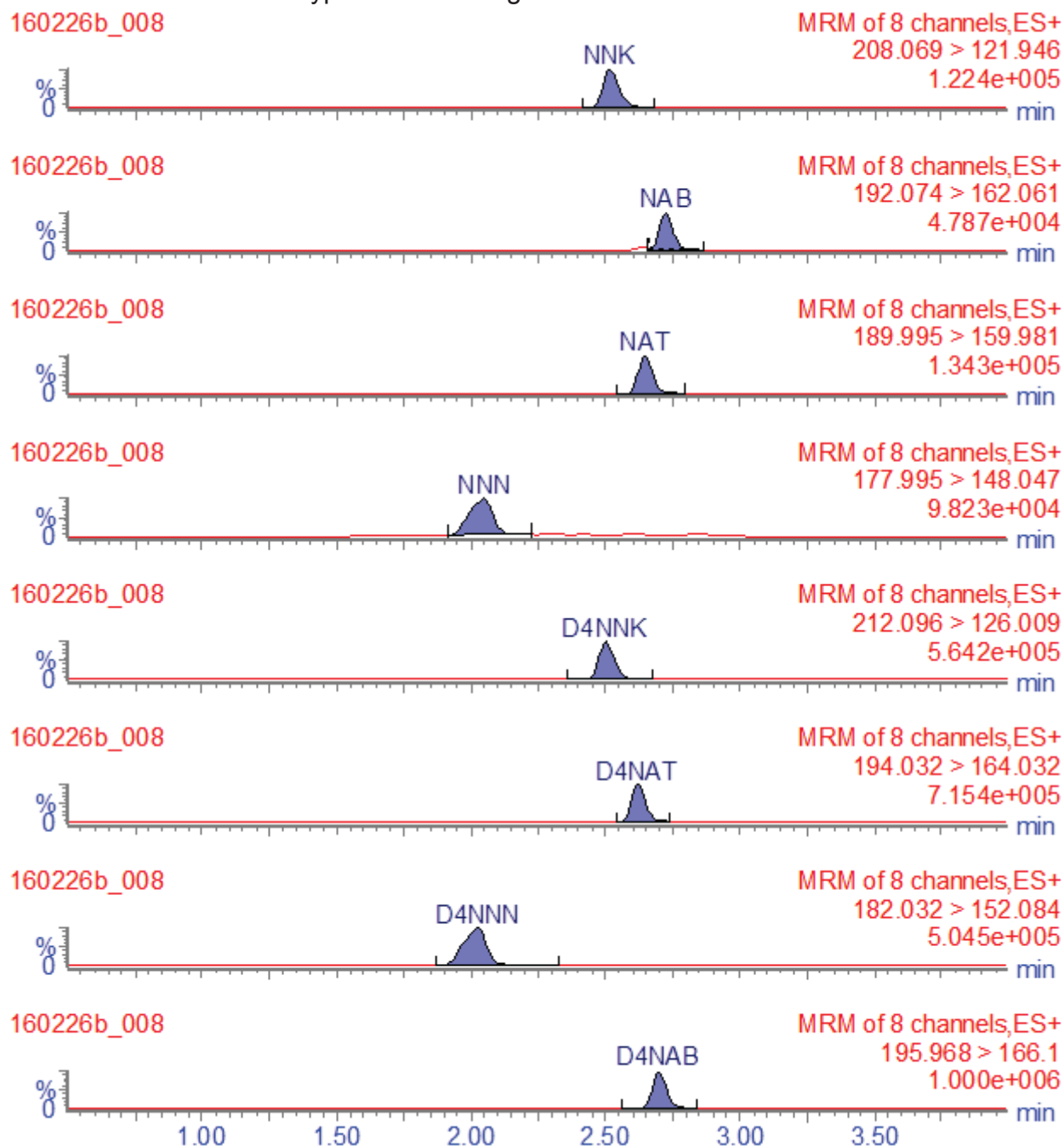
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2 Example Chromatography

2a Typical Chromatogram of Calibration Standard1



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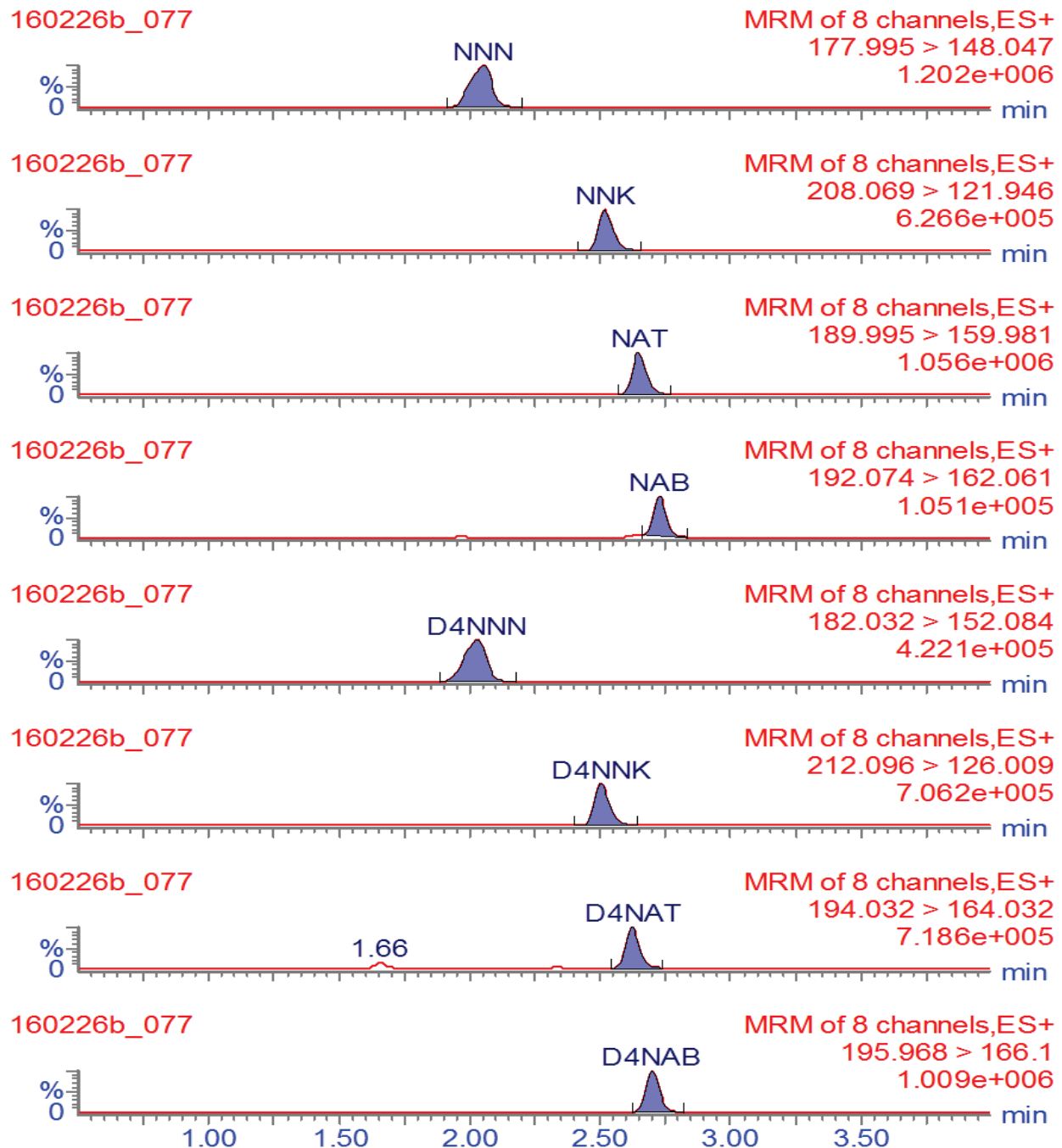
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2b. Typical Chromatogram in [REDACTED] Cigar Filler Sample



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