LABORATORY INFORMATION BULLETIN

Determination of *N*-nitrosonornicotine (NNN) in Smokeless Tobacco and Tobacco Filler by HPLC-MS/MS[1]

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Abstract

A method for the target compound N-nitrosonornicotine (NNN) was validated. The same extraction process based on CORESTA Method No. 72, Determination of Tobacco-Specific Nitrosamines in Smokeless Tobacco Products by LC-MS/MS, was used with modifications to the calibration ranges and instrumental approach. The validation data were obtained from the replicate measurements of NNN in calibration standards and reference products. The validation results confirm that the modifications to the method were acceptable.

Introduction

CORESTA recommended method No. 72 describes the extraction and analysis of NNN in smokeless tobacco by liquid chromatography-tandem mass spectrometry. Method modifications were validated with updates to the calibration standard preparation and concentrations, in addition to changing the high-performance liquid chromatography (HPLC) method.

The target concentration was established at 1000 ng/g with a quantitative range from 400 to 1600 ng/g based on a 0.25 g sample weight. The ^{13}C isotopically labeled IS concentration was established at 800 ng/g. The standard preparation process was updated by spiking the blank and working standard in separate vials then diluting these mixtures at proportional ratios to create a five point calibration curve. The reversed-phase HPLC procedure was modified with several changes including the use of aqueous ammonium acetate and updating the gradient. The HPLC pump program was adapted for a higher flow rate with an initial hold, increased gradient rate, longer mid-point hold time and equilibration stage, which permitted decreasing the overall run time. The validation was performed using the updated method to determine NNN in CORESTA smokeless reference products and cigarette tobacco filler as received covering five different matrices.

The NNN method described within this LIB provides the NNN quantity on a wet weight basis. In order to determine the NNN levels on a dry weight basis, it will be necessary to determine the water content of the smokeless product being tested using one of the two following International Organization

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[1] This edition revises LIB No. 4620, which issued January 2017. The revision is described on page 2 of this document.

for Standardization (ISO) standards : (1) ISO 6488:2004, Tobacco and tobacco products – Determination of water content – Karl Fischer method and ISO 6488:2004/Cor 1:2008, Technical Corrigendum 1 to ISO 6488:2004 or (2) ISO 16632:2013, Tobacco and tobacco products – Determination of water content – Gas-chromatographic method. The smokeless tobacco products will need to be split in order to determine the NNN measurements on a wet weight basis and measurements of moisture content. The NNN level in the smokeless tobacco product on a dry weight basis should be calculated using the following formula[2]:

$$NNN_{dry} = \frac{NNN_{wet} * 100}{(100 - \%_{water\ content})}$$

Experimental
Reagents

- Ammonium acetate ($NH_4C_2H_3O_2$) (CAS# 631-61-8), HPLC grade
- Water (H_2O) (CAS# 7732-18-5), HPLC grade
- Acetonitrile (C_2H_3N) (CAS# 75-05-8), HPLC grade
- Methanol (CH_4O) (CAS# 67-56-1), HPLC grade
- NNN (N'-Nitrosonornicotine); single element standard, Purity: 95%-100%
- Second source of NNN for use as internal calibration verification (ICV) spiking standard.
- $^{13}C_6$-NNN isotopically labeled internal standard, Purity: 95%-100%

Equipment

- AB Sciex API 6500 Q-Trap® mass spectrometer and Analyst operating software or equivalent
- Shimadzu Nexera UHPLC system with gradient programmable pump, integrated vacuum degasser, autosampler capable of accommodating up to 25 µL volume injection, and heated column compartment or equivalent
- Adjustable Pipetters, ranges: 10-100 µL, 100-1000 µL, 1-10 mL.
- Coffee or spice mills for homogenizing tobacco product.
- Eberbach 6010 2-speed laboratory shaker or equivalent (configured to hold vials in horizontal position).
- Analytical Balance, XP205 Mettler Toledo or equivalent.

Materials

- Amber vials appropriate for holding a tobacco sample submersed in 10 mL of liquid.
- Amber HPLC vials.
- 0.45 µm polytetrafluoroethylene (PTFE) or polyvinyldifluoride (PVDF) membrane syringe filters.
- Waters 2.1 x 50 mm, 2.5 µm Xterra MS C18 HPLC column (P/N: 186000594)
- Tobacco filler from 3R4F cigarettes, University of Kentucky and CORESTA smokeless reference product (CRP) from North Carolina State University: Snus: CRP-1 (filler only), Moist Snuff: CRP-2, Dry Snuff: CRP-3, or Loose Leaf Chewing Tobacco: CRP-4.

Procedures

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[2] The formula in LIB No. 4620 contained a typographical error – some of the terms and variables in the numerator and denominator were inadvertently switched. The formula has been corrected in this edition.

A. Solutions

1. 100 mM ammonium acetate stock solution
    a. Weigh 7.7 ± 0.3 g of ammonium acetate.
    b. Dissolve measured ammonium acetate in 1 L of H_2O in a glass bottle.
    c. Cap the bottle tightly and shake the contents to ensure complete mixing.
    d. Transfer and label as appropriate (concentration, initials, date prepared, and expiration date).

2. HPLC mobile phase A (5 mM Ammonium Acetate in H_2O)
    a. Using graduated cylinders, measure 50 mL of the 100 mM ammonium acetate solution and 950 mL of HPLC grade water, respectively.
    b. Pour into a 1 L glass bottle.
    c. Cap the bottle tightly and shake the contents to ensure complete mixing and label as appropriate.

3. HPLC mobile phase B (5 mM Ammonium Acetate in Acetonitrile (ACN))
    a. Using graduated cylinders, measure 50 mL of the 100 mM ammonium acetate solution and 950 mL of acetonitrile.
    b. Pour into a 1 L glass bottle.
    c. Cap the bottle tightly and shake the contents to ensure complete mixing and label as appropriate.

B. Standards

1. Standards Preparation

a. Isotopically Labeled NNN Internal Standards

1. Isotopically labeled NNN internal standards can be purchased with an appropriate $^{13}C_6$-NNN concentration and purity.

2. Preparation of labeled NNN standard stock (0.2 mg/mL)

    i. Measure 2 ± 0.2 mg of $^{13}C_6$-NNN standard to make 10 ml of standard.

    ii. Transfer $^{13}C_6$-NNN standard to a volumetric flask and fill with methanol to the mark to achieve a target concentration of about 0.2 mg/mL.

    iii. Transfer mixture to amber vial and label as appropriate.

b. Spiking solution: Isotopically Labeled NNN Internal Standards (IS)

1. Preparation of IS spiking solution, 2 μg/mL $^{13}C_6$-NNN

    i. Transfer appropriate amount of isotopically labeled NNN into volumetric flask, dilute with methanol using the following guides for 25 mL; adjust volumes as necessary.

       • mL to transfer = (target concentration * volume) / (purity * source concentration)

        a. To prepare 25 mL of 2 μg/mL, <u>98% purity</u>; transfer 0.255 mL of the <u>0.2 mg/mL</u> standard to 25 mL volumetric flask and dilute with methanol.

           • mL = (2 μg/mL * 25 mL) / (0.98 * 200 μg/mL) = 0.255 mL

    ii. Transfer mixture to separate amber vials for use with appropriate number of samples; approximately 2 mL per 10 samples and label each as appropriate.

c. **Individual NNN Standard Stocks**

1. Purchase or prepare NNN spiking standard.

2. Individual NNN standards may be purchased with an appropriate NNN concentration and purity. If using a higher concentration, dilute appropriately with methanol.

3. Preparation of individual NNN standard stocks (0.2 mg/mL)

   i. Measure 2 ± 0.2 mg of NNN to make 10 mL of stock standard.

   ii. Transfer measured NNN standards to a volumetric flask and fill with methanol to the mark to achieve a target concentration of about 0.2 mg/mL.

   iii. Transfer mixtures into amber vials and label as appropriate.

d. **Spiking Standard Solutions**

1. Preparation of spiking standard solution using stock standard.

   i. Transfer appropriate amount of NNN standard stocks into a volumetric flask to yield 10 μg/mL NNN in methanol using the following guide based on 10 mL.

      • mL to transfer = (target concentration * volume) / (purity * source concentration)

         a. To prepare 10 mL of 10 μg/mL, <u>98% purity</u>; transfer 0.521 mL of the <u>0.2 mg/mL</u> standard to 25 mL volumetric flask and dilute with methanol.

      • mL = (10 μg/mL * 10 mL) / (0.98 * 200 μg/mL) = 0.510 mL

   ii. Transfer to separate amber vials for individual or daily use and label as appropriate.

Calibration Standards

a. Working Standards
   1. Label one amber extraction vial as Blank.
      i. Add 9900 μL of the 100 mM ammonium acetate solution and 100 μL of the internal standard solution. Cap and mix well.

   2. Label one amber extraction vial as 2000 Std.
      i. Add 9850 μL of the 100 mM ammonium acetate solution, 100 μL of the internal standard solution, and 50 μL of the standard spiking solution. Cap and mix well.

   3. To five HPLC vials, add the appropriate volume of the 2000 ng/g standard and blank solutions detailed in the table below, and mix well.

The calibration standards prepared by dilution from a mixture of 2000 ng/g NNN.			
Standard Name	Vol. 2000 ng/g Standard (μL)	Vol. Solvent Blank (μL)	Concentration ng/g
Blank	0	1000	0
400	200	800	400
800	400	600	800
1000	500	500	1000
1200	600	400	1200
1600	800	200	1600

The calibration standards are based on a 0.25 g sample weight.

Sample Preparation

1. Select approximately 0.5 g of smokeless tobacco or cigarette tobacco filler per replicate for analysis. The tobacco filler from smokeless product pouches should be removed, homogenized, and weighed for analysis. TSNAs are light sensitive and samples should be protected from light and stored in the dark. For protection, amber vials are used in sample preparation.

2. Include a QC sample consisting of either a matrix spike and/or a reference tobacco, such as a CRP smokeless tobacco or cigarette tobacco filler, with each batch of samples.

3. Grind each sample for at least 15 seconds, until the sample appears visually uniform.

4. Label the amber sample vials and autosampler vials with identifying numbers and designation for replicates.

5. Weigh approximately 0.25 g of smokeless tobacco or cigarette tobacco filler or adjusted amount into an amber sample preparation vial, record sample weight to nearest 0.01 g using an analytical balance. Weigh a minimum of 0.05 g.

6. Add 100 μL of $^{13}C_6$-NNN isotopically labeled IS solution to each vial.

7. Extract each sample with 10 mL of 100 mM aqueous ammonium acetate on a shaker on high setting for 60 minutes.

8. Filter the extract using a 0.45 μm membrane filter and transfer the filtrate to the appropriately labeled autosampler vial for injection.

Instrument Parameters

HPLC Settings
- HPLC System: Shimadzu UHPLC, or equivalent
- Column: Waters 2.1 x 50 mm, 2.5 μm Xterra MS C18 column P/N: 186000594
- Column Temperature: 60°C
- Flow Rate: 0.5 mL/minute
- Mobile Phase A (See section *A. Solutions* above): 5 mM aqueous ammonium acetate
- Mobile Phase B: 95% acetonitrile with 5% aqueous 5 mM ammonium acetate (See section *A. Solutions* above)
- HPLC Gradient Program:

Time (minutes)	Mobile Phase A (%)	Mobile Phase B (%)
0	95	5
1	95	5
2.4	50	50

4.4	50	50
4.5	95	5
8.5	95	5

Tandem Mass Spectrometer Settings
- Mass Spectrometer: ABI 6500 Q-trap, or equivalent
- Ionization Source: Positive Ion Electrospray
- Ionspray Voltage: 3500 V
- Curtain Gas: 35 psi
- Nebulizer Gas: 45 psi
- Heater Gas: 45 psi
- Source Temperature: 500 ∘C
- Selected Ion Reaction Monitoring (SRM) Settings:

ABI 6500 Q-trap SRM Settings			
Analyte	Precursor/Product Ion pair	Declustering Potential (DP)	Collision Energy (CE)
NNN	178/148	45	15
NNNv	178/120	45	26
$^{13}C_6$-NNN	184/154	45	15
v: Confirmation Ion			

Instrument Setup:
- Create a sequence file with the appropriate naming of project and files. The method sequence should include the bracketing of a Continuing Calibration Verification (CCV) every 10 injections ending with blank and final CCV.
- Select the Standards sample type for five standards from 400 to 1600 ng/g and QC as the sample type for the CCV standard to later assess statistics for these injections.

Data Analysis
  A. Quantitation Method and Parameters
      Note: these instructions were established for ABI Sciex Analyst software, if other instrument used, convey instructions to obtain desired data and achieve quality assurance (QA).

  1. Create quantitation method to calculate NNN concentration with following parameters.
      a. Establish internal standard quantitation method by assigning precursor/product ions.
          i. Integration parameters: Smoothing and/or bunching factors: 3.
          ii. Linear regression between analyte peak area to internal standard ratio and NNN concentration (ng/g).
          iii. 1/X Weighting (X=concentration).
  2. Perform quantitation of each data file.
  3. Review data following system suitability and QA guidance.
  4. Transfer Quantitation Set data by export, print, or other appropriate reporting document.

  B. Calculate sample concentration by correcting for recorded sample weight using the following equation from the calculated concentration from reporting software:

1. Sample Concentration = Calculated Concentration * (0.25/Actual recorded sample weight)

Quality Assurance (QA)
1. Ensure system suitability requirements were met:
    a. Examine the peak shape of the standard, ICV, CCV, and blank injections. Ensure retention time and peak shape are consistent and no interfering peaks are present.
    b. Ensure linear fit of all curves.
        - The correlation coefficient for the NNN calibration curve is at least ≥0.995.
    c. Confirm that CCV and ICV values are within acceptable range of target value.
        - Quantitate the ICV sample. The result is within ±10% of the certified value. If not, verify the standard concentrations and re-prepare solutions if the problem persists
        - System Reproducibility is tested using all CCV areas and the analytical sequence must have at least 5 CCV injections for this calculation. The %RSD must be less than 2%.
        - Quantitate each of the calibration standards as a sample. The results should be within ±10% of expected value.

2. Reference Products or Matrix Spikes
    a. Ensure that recoveries fall within acceptable range of target concentration.

3. If QA requirements are not met, investigate problem and choose appropriate corrective action.

Method Performance Criteria.

The following method performance criteria are considered the minimum needed to consider the procedure acceptable for regulatory purposes.

1. The standard calibration curve must be linear from 400 ng/g to 1600 ng/g. The calibration curve should have at least 5 separate concentration levels with not less than (NLT) 6 independent measurements at each concentration level. The precision (calculated as % CV) at each concentration level is not more than (NMT) 6% and the linear least squares fit of the data should have an r value ≥ 0.995.

2. An external source of accuracy, such as the ICV, must be included in every analytical run and must pass within the certified limits of that product. If certified limits are not available, then the limit will be set at 94%-106% of the expected value.

3. Continuing calibration verifications (CCVs) must be used every 10 or fewer sample injections. In addition, a CCV must be used at the end of each run. A CCV is typically selected to be a calibration standard solution at a concentration level equivalent to 1000 ng/g NNN. The CCV accuracy during the run must be within 94% - 106% of the expected value.

4. Precision of the analytical procedure is assessed using the CCV standard solution. Not less than six (6) replicate standard solutions having NNN concentrations of about 1000 ng/g should be prepared. The standard solutions are measured and % CV calculated. The % CV of NMT 6 is necessary.

5. The accuracy and precision of the method as a whole is measured using the NNN spiked sample solutions. Six multiples of the spiked sample solutions are prepared and measured using the validated analytical procedure with a %CV of NMT 15% (precision) and the percent recovery is NLT 85% (accuracy).

6. The laboratory must establish statistical control charts for matrix spike recoveries using a recognized procedure (e.g., the Shewhart Method). The historical data must support matrix spike recovery control limits of 85% - 115% for all of the data.

7. The limit of quantification (LOQ) is calculated by preparing replicate solutions of NNN standard solutions (NLT 6) having a concentration of 300 ng/g. The replicate solutions are measured and the % CV calculated: % CV of NMT 5% is expected.

Validation Results

The TSNA method was modified and validated for NNN at the target range of 1000 ng/g (based on 0.25 g sample). NNN was determined in replicate measurements of the calibration standards, reference smokeless products, and cigarette filler to validate that the method modifications were acceptable. The validation data for this study were acquired over several days by different analysts using different instruments. The stability of the prepared calibration standards and filtered reference product samples was evaluated by holding the samples at 4°C for 28 days resulting in consistent recovery and precision.

The concentration results were compared to target values to calculate accuracy expressed as the percent recovery (%) and precision in terms of relative standard deviation (%RSD). The system suitability was evaluated using solution based calibration standards at the minimum detection limit (MDL), limit of quantitation (LOQ) and target level expressed as the continuing calibration verification (CCV). The results from the assessment of the NNN levels in the five matrices and different instruments was satisfactory and met the validation criteria to establish the quantitative range from 400 ng/g to 1600 ng/g.

System Suitability

The NNN linearity was evaluated daily by using solution based calibration standards from 400 to 1600 ng/g based on a 0.25 g sample. The calibration curve was determined from the peak area ratio of NNN and IS, weighted by 1/X (X = concentration), and NNN concentration (ng/g). Each day, the linearity was acceptable with linear regression correlation coefficients; $(r^2) \geq 0.995$.

The accuracy and precision results from the calibration standards, met the specified validation and system suitability criteria. The blank calibration standard internal standard peak shape appeared Gaussian and did not contain interfering peaks. The averaged recovery for the CCV (1000 ng/g) and LOQ (400 ng/g) mixtures were 101 to 103% with daily precision values ≤4% RSD (n≥7) and overall precision ≤5% RSD (n≥50). The MDL was determined to be 100 ng/g by using the %RSD and signal-to-noise (S/N) ratio by extrapolation from the calibration curve. The precision was determined to be ≤19% RSD overall (n≥50). The extracted ion chromatograms (EIC) of the 100 ng/g standard presented in Figure 1 was used to illustrate the signal-to-noise (S/N) ratio where the NNN peak in the EIC of the quantification ion S/N exceeded 2000 and qualifier ion S/N exceeded 1000.

Reference Product Analysis

To validate the method modifications, the NNN concentration was determined in the reference products. The sample weights were reduced in the CRP-2 (moist snuff), CRP-3 (dry snuff), CRP-4 (loose leaf chewing tobacco) and 3R4F tobacco filler samples to achieve a target NNN level of approximately 1000 ng/g to evaluate the method accuracy. From multiple sets of replicate measurements, the percent recovery and precision (%RSD) results were determined daily and an overall summary was provided. The averaged NNN recovery from individual reference products ranged from 101 to 109% and the precision was ≤8% RSD (n≥20). Overall, the accuracy was determined to be 105% with precision ≤7% RSD.
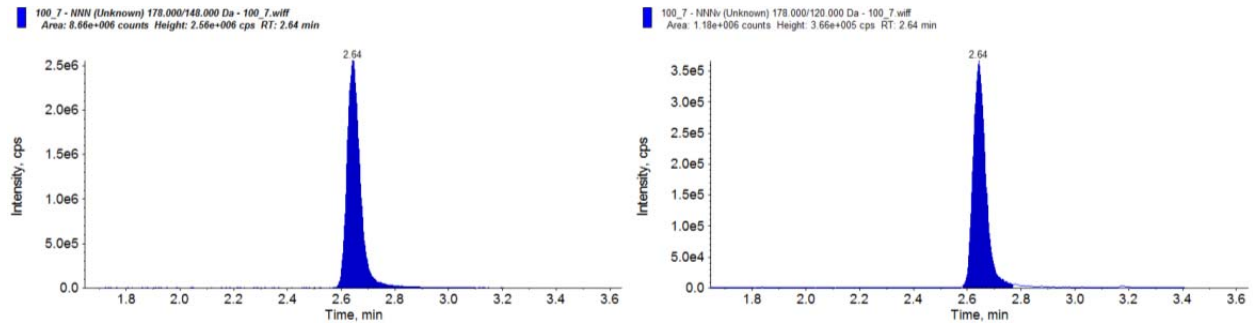
Figure 1. Method detection limit (MDL) for 100 ng/g solution of NNN illustrating extracted ion chromatograms (EIC) of the quantification ion on the left and confirmation ion EIC on the right.

Reference

CORESTA Recommended Method N[o] 72, Determination of Tobacco-Specific Nitrosamines in Smokeless Tobacco Products by LC-MS/MS, July 2013.