Cooperation Centre for Scientific Research
Relative to Tobacco

Tobacco and Tobacco Products Analytes Sub-Group

CORESTA Recommended Method No. 72

DETERMINATION OF TOBACCO-SPECIFIC NITROSAMINES IN TOBACCO AND TOBACCO PRODUCTS BY LC-MS/MS

July 2017

CORESTA RECOMMENDED METHOD N° 72

Title:

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0. INTRODUCTION

In 2009, the CORESTA Smokeless Tobacco Sub-Group (STS), now named Tobacco and Tobacco Products Analytes Sub-Group (TTPA), conducted a collaborative study to evaluate several different methodologies for the determination of tobacco-specific nitrosamines (TSNAs) in smokeless tobacco products in order to adopt a procedure as the CORESTA Recommended Method (CRM). The study included nine commercial smokeless tobacco products covering 8 different product styles. Nine laboratories generated results using a supplied LC-MS/MS method that proved to be the most consistent and was used as the basis for this CRM.

In 2015, this CRM was updated to include repeatability and reproducibility for the four CORESTA Reference Products manufactured in 2009. In 2017, the TTPA conducted a collaborative study for TSNAs in ground tobacco, cigarette filler, and cigar filler in order to expand the scope of this CRM. This recommended method has been shown to be fit for the analysis of the aforementioned matrices.

1. FIELD OF APPLICATION

This Recommended Method is applicable to the determination of TSNAs in ground tobacco, cigarette filler, cigar filler, and smokeless tobacco products (e.g. moist snuff, snus, chewing tobacco and dry snuff). The TSNAs determined with this method are: *N*-nitrosonornicotine (NNN), 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB).

2. NORMATIVE REFERENCES

2.1. CORESTA Guide N° 11 - Technical Guideline for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products.

3. PRINCIPLE

After addition of deuterium labelled internal standards the sample is extracted into an aqueous buffer and filtered. The filtrate is analyzed by Liquid Chromatography - Triple Quadrupole Mass Spectrometry (LC-MS/MS). The results are reported in units of nanograms per gram tobacco as is, wet weight.

4. APPARATUS

Normal laboratory apparatus and equipment including the following items:

4.1. High performance liquid chromatograph coupled to tandem mass spectrometer (LC-MS/MS) with an electrospray ionization (ESI) source consisting of:

- **4.1.1** Binary pump
- **4.1.2** Autosampler
- **4.1.3** Column oven
- **4.1.4** Tandem mass spectrometer
- **4.1.5** Data collection system

4.2. C18 HPLC Column, 2,5 µm particle size, 2,1 mm × 50 mm, or equivalent[1]

4.3. Analytical balance (0,0001 g resolution).

4.4. Orbital shaker, wrist action shaker, or similar

4.5. Amber autosampler vials and caps

4.6. Disposable syringes, 5 ml

4.7. Syringe filter, 0,45 µm polytetrafluoroethylene (PTFE) or equivalent

Note: Various filter materials were evaluated during the collaborative study and PTFE had the highest recovery from those verified. Other filter materials may also be suitable; however, recovery should be evaluated before routine use.

4.8. Amber volumetric flasks, Class A

4.9. Extraction containers, glass 50 ml - 100 ml, or equivalent

5. REAGENTS

During the analysis, use only reagents of recognized analytical grade. Solvents should be of HPLC-grade or better.

5.1.	Water	(Deionized) (resistivity ≥ 18,2 MΩ)
5.2.	Acetonitrile	(HPLC-grade)
5.3.	Methanol	(HPLC-grade)
5.4.	Ammonium acetate	(≥98 %)
5.5.	Acetic acid	(≥98 %)
5.6.	N-nitrosoanabasine (NAB)	(≥98 %)
5.7.	N-nitrosoanatabine (NAT)	(≥98%)

[1] Waters XTerra MS C18® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of this product. Other column(s) may be suitable for use with this method; however, laboratories must verify that the analytes and internal standards are sufficiently resolved from interferences.

5.8. 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (≥98 %)

5.9. N-nitrosonornicotine (NNN), (≥98 %)

5.10. N-nitrosoanabasine – Deuterated (NAB-d4)
(≥ 98 %, Isotopic purity ≥99 %)

5.11. N-nitrosoanatabine –Deuterated (NAT-d4)
(≥ 98 %, Isotopic purity ≥99 %)

5.12. 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone-Deuterated (NNK-d4)
(≥ 98 %, Isotopic purity ≥99 %)

5.13. N-nitrosonornicotine-Deuterated (NNN-d4)
(≥ 98 %, Isotopic purity ≥99 %)

Note: NNN, NNK, NAT, and NAB are carcinogens or are suspected carcinogens. Appropriate safety precautions shall be taken when handling these compounds or any solution containing these compounds.

6. STANDARDS

All standards should be prepared in amber, or light protected glassware and stored in a freezer. Produce a series of enough calibration standards to cover the range of expected results to be found in the test portions, as in the example given below.

6.1. Internal standard solutions

6.1.1 Stock Solution

In four different 10 ml volumetric flasks, weigh approximately 10,0 mg with the weight recorded to 0,01 mg of NNN-d4, NNK-d4, NAT-d4 and NAB-d4 respectively. Dilute to volume with acetonitrile. The concentration in each solution is approximately 1000 µg/ml.

6.1.2 Mixed Internal Standard Solution

Using a volumetric class A pipette (or calibrated pipette), transfer 4,00 ml of each of the single internal standard stock solutions into a 100 ml volumetric flask and dilute to volume with acetonitrile, mix well. The concentration is approximately 40 µg/ml of NNN-d4, NNK-d4, NAT-d4 and NAB-d4.

6.1.3 Internal Standard Spiking Solution

Using a volumetric class A pipette (or calibrated pipette), transfer 5,00 ml of mixed internal standard solution into a 100 ml volumetric flask and dilute to volume with acetonitrile, mix well. The concentration is approximately 2000 ng/ml of NNN-d4, NNK-d4, NAT-d4 and NAB-d4.

6.2. Calibration standard solutions

6.2.1 Stock Solution

In four different 10 ml volumetric flasks, weigh approximately 10,0 mg with the weight recorded to 0,01 mg of NNN, NNK, NAT and NAB respectively. Dilute to volume with acetonitrile. The concentration in each solution is approximately 1000 µg/ml.

6.2.2 Primary Mixed TSNA Standard Solution

Using a volumetric class A pipette (or calibrated pipette), transfer 4,00 ml of each of the single TSNA stock solutions NNN, NNK, NAT, and 1,00 ml of the TSNA stock solution NAB into a 100 ml volumetric flask and dilute to volume with acetonitrile, mix well. The concentration is approximately 40 µg/ml of NNN, NNK, NAT and 10 µg/ml of NAB.

6.2.3 Secondary Mixed TSNA Standard Solution

Using a volumetric class A pipette (or calibrated pipette), transfer 2,50 ml of the primary mixed TSNA standard solution into a 250 ml volumetric flask and dilute to volume with 30/70 v/v acetonitrile/de-ionized water, mix well. The concentration is approximately 400 ng/ml of NNN, NNK, NAT and 100 ng/ml of NAB.

6.2.4 TSNA Calibration Standards

Prepare 7 working calibration standard solutions that cover the concentration range of interest. An example calibration standard preparation table is provided in Table 1. The TSNA calibration standards are prepared in seven separate 100 ml volumetric flasks, each containing 10 ml of 100 mM ammonium acetate solution. Add 1,00 ml of the Internal Standard Spiking Solution (2000 ng/ml) to each of the seven volumetric flasks using a class A pipette (or a calibrated pipette). Next, the appropriate volume of Intermediate TSNA Standard Solution 2, given in Table 1 is added. Next, add the volume of acetonitrile, given in Table 1 below. Finally each of the seven flasks is diluted to volume with 100 mM ammonium acetate, and mixed well. Calculate the exact concentrations for each calibration standard.

Note: Stock solutions of the individual TSNAs and deuterated internal standards in acetonitrile can be purchased at the required levels.

Note: The linearity range should be determined for each lab/instrument to fit the instruments capabilities and the range of samples usually being measured in that laboratory. Samples can be diluted in extraction solution if necessary.

Table 1 - Concentration and preparation of TSNA Calibration Standards

Cal. Std.	Volume of Mixed TSNA std. Nr. 2 (ml)	Volume of Internal std spiking solution 2000 ng/ml (ml)	Volume acetonitrile (ml)	Conc. NNN (ng/ml)	Conc. NNK (ng/ml)	Conc. NAT (ng/ml)	Conc. NAB (ng/ml)
Cal 1	0,125	1,00	22	0,5	0,5	0,5	0,125
Cal 2	0,250	1,00	22	1,0	1,0	1,0	0,250
Cal 3	0,50	1,00	22	2,0	2,0	2,0	0,50
Cal 4	1,00	1,00	22	4,0	4,0	4,0	1,00
Cal 5	2,00	1,00	22	8,0	8,0	8,0	2,00
Cal 6	5,00	1,00	21	20	20	20	5,00
Cal 7	25,0	1,00	15	100	100	100	25,0

6.2.5 All standard solutions shall be prepared in amber, or light protected glassware and stored at about -20 °C, except the calibration standards shall be stored in a refrigerator. Stability studies should be performed by the laboratory to determine the shelf life of the standards and internal standards.

7. PROCEDURES

7.1. Sampling

Sampling is conducted such that the laboratory test sample is representative of the population to be tested.

7.2. Sample preparation

7.2.1 A homogeneous test portion shall be prepared for each test sample.

7.2.2 Tobacco and tobacco products shall be ground unless the samples are homogeneous and have a particle size <4 mm. It is important that the grinding procedure does not generate excessive heat or cause sample degradation. For further information, see CORESTA Guide no. 11 [4].

7.2.3 Smokeless tobacco products supplied in the form of pouches shall be analyzed together with their pouch (paper) and shall be cut into two halves directly into the extraction flask.

7.2.4 Cigar and cigarette filler will typically need to be ground prior to analysis to ensure test aliquots are removed from a homogenous sample. Testing may also involve the analysis of the entire cigar where the wrapper and filler are ground together.

Note: Insufficient equilibration time for samples removed from the freezer has been identified as a source of variability. Samples removed from the freezer should be placed unopened in the refrigerator for approximately 24 hours to ensure water has sufficient time to fully equilibrate throughout the sample. At the time of analysis, samples should be allowed to equilibrate to room temperature before being opened for weighing.

7.2.6 Samples should be mixed prior to weighing to ensure sample homogeneity.

7.2.7 The test samples shall be stored protected from light.

7.3. Sample extraction

7.3.1 Using an analytical balance, weigh approximately 1,000 g (note the exact weight with 3 decimals) of sample into the extraction vessel.

7.3.2 Add 0,300 ml of the 2000 ng/ml Internal Standard Spiking Solution (using a calibrated pipette (or equivalent).

7.3.3 Add 30 ml of 100 mM ammonium acetate and cap the conical flask.

7.3.4 Shake the sample(s) for 40 min ± 5 min at a rate to ensure sufficient mixing.

7.3.5 Filter each sample using a 25 mm, 0,45 µm PTFE syringe filter directly into amber vials and cap each vial. (Note: the samples can be extracted in a centrifuge tube and be centrifuged after shaking.)

7.3.6 The extract is ready for injection into the LC-MS/MS system.

Note: Sample clean-up using solid phase extraction (SPE) prior to injection into the LC-MS/MS has been shown to reduce contamination of the ion source and reduce the need for routine instrument maintenance. See Appendix II for a suggested sample clean-up procedure using SPE.

Samples that exceed the quantitation range of any TSNA compound require the following:

- **Samples prepared without SPE:** Extracted samples that exceed the calibration range for any TSNA may be diluted with extraction solution (100 mM ammonium acetate solution) containing 20 ng/ml of each internal standard. Mix well then conduct the reanalysis.
- **Samples prepared with SPE:** SPE samples that exceed the calibration range must be prepared again using a reduced sample mass. It is not acceptable to dilute prepared samples. When using a reduced sample mass it is important to ensure the sample is sufficiently homogeneous and if in doubt, the sample should be ground. Alternatively, sample retains of the extracted samples (prior to SPE) may be diluted with extraction solution (100mM ammonium acetate solution) containing 20ng/ml of each internal standard. This diluted extract would then be taken through the SPE procedure detailed in Appendix II prior to analysis.

8. DETERMINATION

Set up and operate the LC-MS/MS system in accordance with the manufacturer's instructions. Equilibrate the system prior to use.

8.1. Suggested HPLC parameters

The following are recommended conditions for the LC system and may be modified to achieve acceptable performance:

- Column Temperature: 60,0 °C
- Injection Volume: 10 µL
- Flow rate: 0,22 ml/min
- Mobile phase A: Water
- Mobile phase B: 0,1 % (v/v) acetic acid in methanol

Depending on the HPLC column that is used, it may be necessary to adjust the HPLC gradient provided in Table 2.

Table 2 - HPLC gradient

Time (min)	Flow (ml/min)	Mob. Ph. A (%)	Mob. Ph. B (%)	Gradient type
0	0,22	100	0	Initial
3,0	0,22	10	90	Linear
4,0	0,22	10	90	Linear
5,0	0,22	0	100	Linear
6,0	0,22	100	0	Linear
10,0	0,22	100	0	Linear

8.2. MS/MS parameters

The triple quadrupole mass spectrometer shall operate in positive electrospray mode using multiple reaction monitoring (MRM). It is necessary that the triple quadrupole mass spectrometer has been carefully optimized for sensitivity of each analyte before analysis can occur. The dwell times need to have been optimized to achieve accurate quantification, the number of data points across each peak should be 15 to 20. Once optimized, the same LC-MS/MS conditions must be used for the analysis of all standards and samples, including the same injection volume.

Note: The parameters need to be optimized for each instrument.

8.2.1 Quantification and qualification transitions

The quantification is done by using MRM-data of the transition of the precursor ion and the product ion recommended in Table 3. For confirmation each analyte has an additional transition ion, known as qualification transition, monitored. Calculate the ion ratios between the quantification ion and the qualifier ion, as percent relative abundances. The overall ion ratio of the quantifier to the qualifier ions is fixed and applied the first time a brand is tested for confirming the presence and purity of the TNSAs. The ions used in the calculations must be present and have a signal-to-noise ratio ≥ 10. The ion ratios must be within ± 20% of the average of the calibration standards in a given sequence.

Table 3 - Quantification and qualification transitions for TSNAs

Name	Quantification Transition (m/z)	Qualification Transition (m/z)	Internal Standard Reference
NNK	208 > 122	208 > 79	NNK-d4
NNK-d4	212 > 126	n/a	n/a
NNN	178 > 148	178 > 105	NNN-d4
NNN-d4	182 > 152	n/a	n/a
NAT	190 > 160	190 > 79	NAT-d4
NAT-d4	194 > 164	n/a	n/a
NAB	192 > 162	192 > 133	NAB-d4
NAB-d4	196 > 166	n/a	n/a

Note: The transitions provided in Table 3 are for guidance purposes only and the actual optimized values may vary from instrument to instrument.

The performance of the system should be sufficient to achieve MRM chromatograms similar to those given in Appendix I.

8.3. System Suitability

The system performance must be evaluated for sensitivity, chromatographic performance, carry over and any other criteria necessary to ensure optimization of the LC-MS/MS system.

8.4. Calibration

Set the quantitation method to perform an internal standard linear calibration with $1/x^2$ weighting. The calibration graph is a response of the area ratio of each analyte to the corresponding internal standard. It is recommended that the linear correlation not be forced through the origin. Inject all calibration standards and then proceed to the samples.

8.5. Calculations

8.5.1 All calibration standards and sample calculations utilize relative response Factors (RRF). The RRF for each injection is calculated using the equation:

$$\mathrm{RRF} = \frac{\mathrm{Area_A}}{\mathrm{Area_{IS}}} \times \mathrm{C_{IS}}$$

Where:

RRF = Relative response factor

$\mathrm{Area_A}$ = Area of the target analyte

$\mathrm{Area_{IS}}$ = Area of the corresponding internal standard

8.5.2 The concentration of the target analyte in a sample (ng/g) is determined using calculated RRF for the sample, the slope and intercept obtained from the corresponding calibration curve, and the following equation:

$$\text{Analyte Concentration} = \frac{\mathrm{RRF} - \mathrm{Int}}{\mathrm{Slope}} \times \frac{\mathrm{Vol\ (ml)}}{\mathrm{Mass\ (g)}}$$

Where:

Int = The y-intercept from the calibration curve

Slope = The slope from the calibration curve

Vol = the final volume of extraction solution (ml)

Mass = the weight of tobacco sample (g)

8.6. Quality Control

Each laboratory should perform quality control procedures per their quality system requirements.

9. SUGGESTED SPECIAL PRECAUTIONS

9.1. Experience has shown that the complex tobacco matrix will lead to contamination of the ion source resulting in poor response and elevated background noise. One way to decrease contamination of the ion source is to use a switch between the column and the ion source to divert the flow prior to the analytes eluting from the column.

10. REPEATABILITY AND REPRODUCIBILITY

An international collaborative study involving 9 laboratories who used the specified LC-MS/MS method was conducted by the CORESTA Smokeless Tobacco Sub-Group (STS) in 2009[2]. This study included nine commercial smokeless tobacco products. Results were analyzed according to ISO 5725-2 (1994). After removal of outlying data, the final repeatability (r) and reproducibility (R) results were calculated. This information is presented in Tables 4-7 for the individual TSNAs. The value of 'N' is the number of the laboratories used to determine the statistics after the removal of outliers.

In 2015, the STS conducted a collaborative study[3] involving 14 laboratories that specified the use of this CRM. This study included the analysis of the four CORESTA Reference Products that were manufactured in 2009. In 2017, the TTPA conducted a collaborative study[4] involving 18 laboratories in order to expand the scope of the recommended method beyond just smokeless tobacco. This study included the analysis of ground tobacco, ground cigarette fillers and ground cigar fillers. Results were analyzed in basic conformance with ISO 5725-2:1994 and ISO/TR 22971:2005. The mean values, %r, and %R are presented in Tables 4-7. The value of 'N' is the number of the laboratories used to determine the statistics after the removal of outliers.

[2] CORESTA Smokeless Tobacco Sub-Group Technical Report - Working Group 2, 2009 Collaborative Study Report, July 2010 (Updated 2016).
[3] CORESTA Smokeless Tobacco Sub-Group Technical Report - CORESTA Reference Products 2015 Analysis, December 2015.
[4] CORESTA Tobacco and Tobacco Products Sub-Group Technical Report – 2017 TSNA, pH, and Moisture (Oven Volatiles) Collaborative Study, July 2017.

Table 4 - Results from the 2009[2], 2015[3], and 2017[4] Collaborative Studies for NNN

Sample Type	N	Mean NNN (ng/g)	Repeatability		Reproducibility	
			r (ng/g)	%r (%)	R (ng/g)	%R (%)
Nasal Snuff[2]	8	1089	69	6,3	372	34,2
Loose Snus[2]	8	276	21	7,6	78	28,3
Chewing Tobacco – Bits[2]	9	555	93	16,8	256	46,1
Chewing Tobacco – Flake[2]	8	480	64	13,3	164	34,2
Pellet[2]	8	213	47	22,1	137	64,3
Chewing Tobacco – Loose Leaf[2]	8	511	52	10,2	109	21,3
Loose Moist Snuff[2]	9	2171	94	4,3	1034	47,6
Loose Moist Snuff[2]	8	2496	175	7,0	730	29,2
Pouched Snus[2]	9	694	108	15,6	259	37,3
CRP1 - Pouched Snus[3]	12	671	72	10,7	102	15,2
CRP2 - Loose Moist Snuff[3]	13	1823	127	7,0	286	15,7
CRP3 - Loose Dry Snuff Powder[3]	13	8249	612	7,4	1247	15,1
CRP4 - Chewing Tobacco, Loose Leaf[3]	11	1948	143	7,3	270	13,9
1R6F Ground Cigarette Filler[4]	18	2294	237	10,3	756	32,9
1R5F Ground Cigarette Filler[4]	17	3221	292	9,1	1141	35,4
RTDAC - Dark Air-Cured Ground Tobacco[4]	17	3947	367	9,3	1717	43,5
RT2 - Flue-Cured Ground Tobacco[4]	16	117	17	14,6	54	45,8
NIST SRM 3222 Cigarette Cut Filler[4]	14	1541	118	7,7	523	33,9
Flavoured Ground Cigar Filler[4]	16	5509	339	6,1	1762	32,0
Dark Air-Cured Ground Cigar (Wrapper and Filler)[4]	15	3534	275	7,8	1359	38,5

Table 5 - Results from 2009[2], 2015[3], and 2017[4] Collaborative Studies for NAT

Sample Type	N	Mean NAT (ng/g)	Repeatability		Reproducibility	
			r (ng/g)	%r (%)	R (ng/g)	%R (%)
Nasal Snuff[2]	9	647	68	10,5	373	57,7
Loose Snus[2]	7	176	12	6,8	122	69,3
Chewing Tobacco – Bits[2]	9	305	75	24,6	311	102,0
Chewing Tobacco – Flake[2]	8	123	19	15,4	91	74,0
Pellet[2]	8	171	45	26,3	142	83,0
Chewing Tobacco – Loose Leaf[2]	7	287	17	5,9	182	63,4
Loose Moist Snuff[2]	9	2091	174	8,3	1290	61,7
Loose Moist Snuff[2]	9	3151	376	11,9	1943	61,7
Pouched Snus[2]	9	529	67	12,7	358	67,7
CRP1 - Pouched Snus[3]	13	516	61	11,8	157	30,4
CRP2 - Loose Moist Snuff[3]	13	1725	101	5,9	405	23,5
CRP3 - Loose Dry Snuff Powder[3]	13	5566	428	7,7	1557	28,0
CRP4 - Chewing Tobacco, Loose Leaf[3]	12	1222	94	7,7	373	30,5
1R6F Ground Cigarette Filler[4]	18	2093	219	10,4	602	28,7
1R5F Ground Cigarette Filler[4]	17	2026	178	8,8	495	24,5
RTDAC - Dark Air-Cured Ground Tobacco[4]	17	4351	416	9,6	1046	24,0
RT2 - Flue-Cured Ground Tobacco[4]	17	198	45	22,9	81	41,2
NIST SRM 3222 Cigarette Cut Filler[4]	14	48	9	19,2	30	62,5
Flavoured Ground Cigar Filler[4]	16	2813	233	8,3	614	21,8
Dark Air-Cured Ground Cigar (Wrapper and Filler)[4]	15	1740	255	14,6	481	27,7

Table 6 - Results from the 2009[2], 2015[3], and 2017[4] Collaborative Studies for NAB

Sample Type	N	Mean NAB (ng/g)	Repeatability		Reproducibility	
			r (ng/g)	%r (%)	R (ng/g)	%R (%)
Nasal Snuff[2]	8	39	6	15,4	13	33,3
Loose Snus[2]	8	15	3	20,0	6	40,0
Chewing Tobacco – Bits[2]	8	16	5	31,3	8	50,0
Chewing Tobacco – Flake[2]	8	48	7	14,6	22	45,8
Pellet[2]	8	19	5	26,3	13	68,4
Chewing Tobacco – Loose Leaf[2]	8	14	2	14,3	6	42,9
Loose Moist Snuff[2]	8	167	15	9,0	68	40,7
Loose Moist Snuff[2]	8	189	15	7,9	100	52,9
Pouched Snus[2]	8	38	5	13,2	15	39,5
CRP1 - Pouched Snus[3]	12	34	7	20,6	12	35,3
CRP2 - Loose Moist Snuff[3]	13	152	14	9,2	31	20,4
CRP3 - Loose Dry Snuff Powder[3]	13	396	43	10,9	105	26,5
CRP4 - Chewing Tobacco, Loose Leaf[3]	12	60	6	10,0	18	30,0
1R6F Ground Cigarette Filler[4]	17	101	16	16,0	39	38,1
1R5F Ground Cigarette Filler[4]	16	111	22	19,8	32	29,2
RTDAC - Dark Air-Cured Ground Tobacco[4]	16	231	26	11,3	45	19,5
RT2 - Flue-Cured Ground Tobacco[4]	16	14	4	26,6	11	78,1
NIST SRM 3222 Cigarette Cut Filler[4]	13	7	4	49,9	6	74,1
Flavoured Ground Cigar Filler[4]	15	188	21	10,9	53	28,0
Dark Air-Cured Ground Cigar (Wrapper and Filler)[4]	14	134	24	17,5	42	31,5

Table 7 - Results from the 2009[2], 2015[3], and 2017[4] Collaborative Studies for NNK

Sample Type	N	Mean NNK (ng/g)	Repeatability		Reproducibility	
			r (ng/g)	%r (%)	R (ng/g)	%R (%)
Nasal Snuff[2]	9	482	46	9,5	131	27,2
Loose Snus[2]	8	133	14	10,5	43	32,3
Chewing Tobacco – Bits[2]	8	78	16	20,5	31	39,7
Chewing Tobacco – Flake[2]	8	152	26	17,1	44	28,9
Pellet[2]	7	246	32	13,0	71	28,9
Chewing Tobacco – Loose Leaf[2]	8	94	14	14,9	25	26,6
Loose Moist Snuff[2]	9	729	56	7,7	505	69,3
Loose Moist Snuff[2]	9	583	75	12,9	372	63,8
Pouched Snus[2]	8	265	14	5,3	73	27,5
CRP1 - Pouched Snus[3]	13	205	25	12,2	48	23,4
CRP2 - Loose Moist Snuff[3]	13	437	33	7,6	82	18,8
CRP3 - Loose Dry Snuff Powder[3]	13	4138	498	12,0	1088	26,3
CRP4 - Chewing Tobacco, Loose Leaf[3]	12	440	61	13,9	109	24,8
1R6F Ground Cigarette Filler[4]	18	675	76	11,2	182	27,0
1R5F Ground Cigarette Filler[4]	17	781	76	9,7	179	22,9
RTDAC - Dark Air-Cured Ground Tobacco[4]	17	1908	360	18,9	624	32,7
RT2 - Flue-Cured Ground Tobacco[4]	17	107	18	17,1	53	49,4
NIST SRM 3222 Cigarette Cut Filler[4]	14	32	10	31,3	21	65,7
Flavoured Ground Cigar Filler[4]	15	1782	150	8,4	328	18,4
Dark Air-Cured Ground Cigar (Wrapper and Filler)[4]	15	901	87	9,7	220	24,4

11. REPORT

The test report shall state the yield of TSNAs in nanograms per gram tobacco (wet weight) and the method used shall include all conditions which may affect the result. The report shall also give all details necessary for the identification of each sample. Moisture content may be determined on separate tobacco aliquots if it is necessary to present the final results on a dry-weight basis. The determination of moisture is detailed in CORESTA Recommended Method N° 76: Determination of Moisture Content (Oven Volatiles) of Tobacco and Tobacco Products.

12. BIBLIOGRAPHY

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APPENDIX I – Example Chromatograms

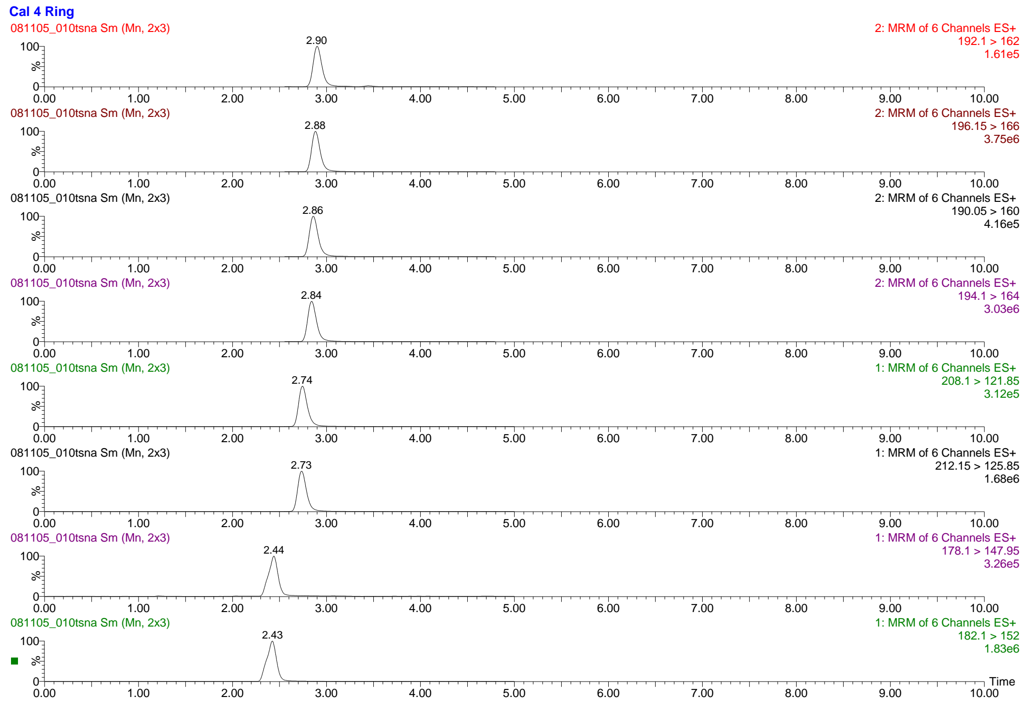

Figure 1 - Example of a MRM-chromatogram for a TSNA standard.

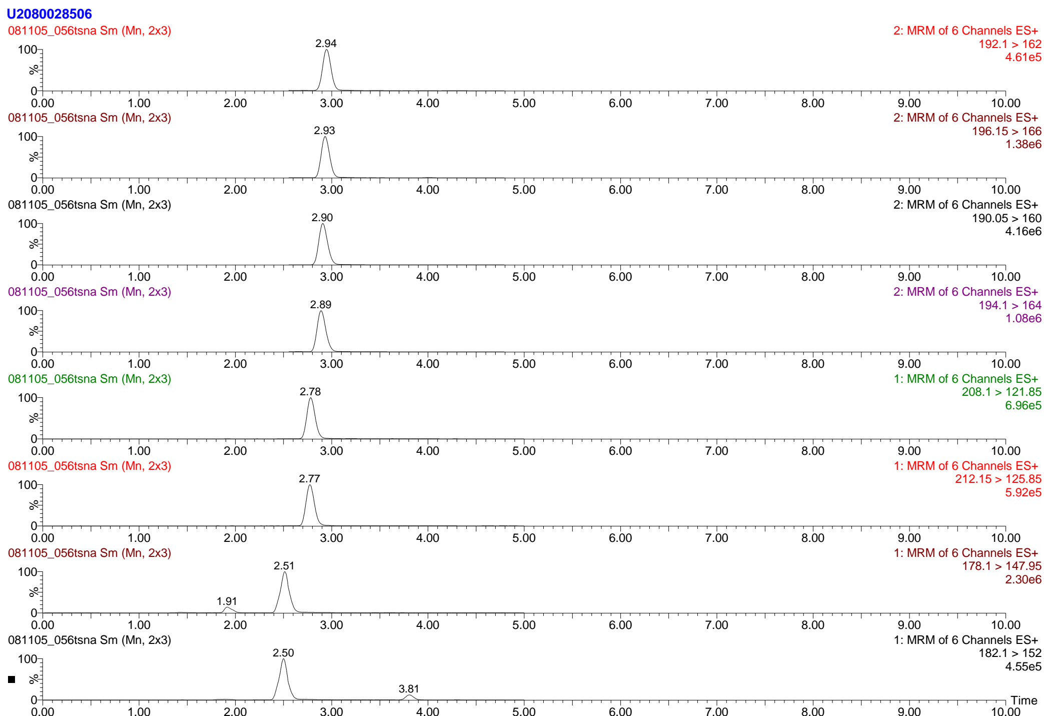

Figure 2 - Example of a MRM-chromatogram for a tobacco product.

APPENDIX II – Sample Clean-Up using Solid Phase Extraction (SPE)

A. Reagents and Supplies

1. De-ionized water, > 18,8 MΩ.
2. Methanol (HPLC grade)
3. Ammonium hydroxide, concentrated (reagent grade or better)
4. Formic Acid (≥98 %)
5. Acetic acid (≥98 %)
6. SPE cartridges, polymer reversed-phase sorbent 3cm3 (60mg), or equivalent[5]

B. Reagent Preparation

1. **Wash No. 1: 95 % Water:5 % Methanol (0,5 % Ammonium Hydroxide (v/v))**
   Combine approximately 100 ml of de-ionized water, 45 ml of methanol and 5 ml of concentrated ammonium hydroxide in a 1 L volumetric flask. Dilute to volume with de-ionized water, and mix solution well.
2. **Wash No. 2: 0,01% Formic Acid in Water (v/v)**
   Add 0.100 ml of formic acid to a 1 L volumetric flask containing approximately 500 ml of de-ionized water. Dilute to volume with de-ionized water and mix well.
3. **70 Methanol: 30 Water (0,1% Acetic Acid (v/v))**:
   Add 300 ml of de-ionized water, 300 ml of methanol, and 1 ml of acetic acid to a 1 L volumetric flask. Dilute to volume with methanol and mix well.

C. SPE Procedure

Perform Sample Extraction as described in section 7.3.1 to 7.3.4

a) Filter approximately 4 ml of sample directly into labelled disposable culture tubes using a 25mm, 0,2 µm PVDF syringe filter.
b) Add 0,250 ml of concentrated ammonium hydroxide to each sample and vortex for 1-5 seconds. The apparent pH of the samples at this point will be approximately 9.0 – 9.5.
c) Precondition the SPE cartridges with ~2,0 ml of methanol. A flow rate of 4-5 drops per second is recommended.
d) Precondition the SPE cartridges with ~2,0 ml of de-ionized water. A flow rate of 4-5 drops per second is recommended.
e) Load 1,5 ml of sample from step C.b on SPE cartridge. A flow rate of 1-2 drops per second is recommended.
f) Wash SPE cartridges with 3,0 ml of Wash No. 1. A flow rate of 4-5 drops per second is recommended.
g) Wash SPE cartridges with 3,0 ml of Wash No. 2. A flow rate of 4-5 drops per second is recommended. This will remove slightly basic and neutral non-target analytes.
h) Allow the SPE cartridges to dry under vacuum (~10-15 psi.) for 3,0 minutes.
i) Elute the analytes from the SPE cartridges using 1,5 ml of 70 Methanol:30 Water (/0,1% Acetic Acid (v/v)). A flow rate of 1-2 drops per second is recommended.
j) Cap and vortex autosampler vials prior to analysis.

Note: For SPE samples that exceed the quantitation range see note for samples prepared with SPE (section 7).

[5] Oasis® HLB is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute endorsement of this product.