Cooperation Centre for Scientific Research Relative to Tobacco

E-Vapour Sub-Group

CORESTA Recommended Method No. 102

DETERMINATION OF TOBACCO-SPECIFIC NITROSAMINES IN E-LIQUID BY LC-MS/MS

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0. INTRODUCTION

In 2021, the CORESTA E-Vapour (EVAP) Sub-Group conducted a collaborative study for the determination of tobacco-specific nitrosamines (TSNAs) in e-liquids using liquid chromatography with mass spectrometry. The TSNAs examined as part of this study included *N*-nitrosonornicotine (NNN), 4-(*N*-methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB). Nine laboratories participated in the study. The method used for this study was shown to be appropriate for the analysis of TSNAs in e-liquids [1].

1. SCOPE

The purpose of this document is to describe the procedures used for the determination of tobacco-specific nitrosamines (TSNAs) in electronic cigarette liquids (e-liquids). The TSNAs determined with this method are *N*-nitrosonornicotine (NNN), 4-(*N*-methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB). The e-liquids used may be of any flavour, including extracted tobacco-derived flavours.

2. NORMATIVE REFERENCES

2.1 CORESTA Guide N° 18 Technical Guide for Sample Handling and Sample Collection of E-Cigarettes and E-Vapour Generating Products

3. PRINCIPLE

Following the addition of deuterium-labelled internal standards, aqueous buffer solution is added and the sample is shaken. An aliquot is filtered and analysed by Liquid Chromatography – Triple Quadrupole Mass Spectrometry (LC-MS/MS). The results are reported in ng/g of e-liquid.

4. SAFETY

All laboratory personnel performing this method should be familiar with appropriate laboratory practices and health and safety measures. Prior to the handling of any of the chemicals described within this document, personnel should familiarize themselves with the appropriate safety data sheets (SDS). Appropriate personal protective equipment (PPE) should be worn at all times and should include, but is not limited to, laboratory coat, safety glasses/goggles, and gloves.

The TSNAs discussed in this method (NNN, NNK, NAT, and NAB) are carcinogens or suspected carcinogens. As such, appropriate safety measures, including appropriate PPE, must be taken when handling neat materials or any solution which contains the TSNAs analysed in this method.

5. EQUIPMENT AND SUPPLIES

5.1 High performance liquid chromatograph coupled to tandem mass spectrometer (LC-MS/MS) with an electrospray ionization source capable of performing the method described herein.

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

5.3 Analytical balance capable of 0,1 mg accuracy.

5.4 Vortex mixer or similar.

5.5 Class A glassware (volumetric flasks, volumetric pipettes, graduated cylinders).

5.6 Gas-tight syringes.

5.7 Amber vials with PTFE lined caps, 8 mL, or similar.

5.8 Adjustable pipettes.

5.9 Amber autosampler vials with PTFE lined caps.

5.10 0,45 µm Nylon filter or equivalent.

5.11 Disposable syringes.

6. REAGENTS

Unless specified, all reagents should be recognized as analytical grade or better where available. Solvents should be HPLC-grade or better.

Note: ISO 17034 reference solutions may be used in place of neat standard materials.

6.1 *N*-Nitrosonornicotine (NNN, CAS-No: 80508-23-2), w ≥ 98 % (mass fraction).

6.2 *N*-Nitrosoanatabine (NAT, CAS-No: 71267-22-6), w ≥ 98 %.

6.3 *N*-Nitrosoanabasine (NAB, CAS-No: 1133-64-8), w ≥ 98 %.

6.4 4-(*N*-Methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK, CAS-No: 64091-91-4), w ≥ 98 %.

6.5 Deuterated *N*-Nitrosonornicotine-2,4,5,6-d4 (NNN-d4, CAS-No: 66148-19-4), w ≥ 98 %, isotopic purity w ≥ 99 %.

6.6 Deuterated *N*-Nitrosoanatabine-2,4,5,6-d4 (NAT-d4, CAS-No: 1020719-69-0), w ≥ 98 %, isotopic purity w ≥ 99 %.

6.7 Deuterated *N*-Nitrosoanabasine-2,4,5,6-d4 (NAB-d4, CAS-No: 1020719-68-9), w ≥ 98 %, isotopic purity w ≥ 99 %.

6.8 Deuterated 4-(*N*-Methylnitrosoamino)-1-(3-pyridyl)-1-butanone-2,4,5,6-d4, (NNK-d4, CAS-No: 764661-24-7), w ≥ 98 %, isotopic purity w ≥ 99 %.

[1] Waters XBridge BEH C18® is an example of a commercially available column which is suitable for use. Note that this example does not constitute an endorsement of this product, but is merely given as an example. Other columns may be used with this method, provided they have been demonstrated to be fit for analysis and that analytes and internal standards are sufficiently resolved from interferences.

6.9 Ammonium acetate (CAS-No: 631-61-8), w ≥ 98 %

6.10 Acetic acid, (CAS No : 64-19-7), w ≥ 98 %.

6.11 Acetonitrile, (HPLC-grade, CAS No: 75-05-8).

6.12 Methanol, (HPLC-grade, CAS No: 67-56-1).

6.13 Deionized water, ≥ 18,20 MΩ·cm.

7. PREPARATION OF SOLUTIONS

7.1 Extraction Solution (100 mM ammonium acetate in water)

Weigh 15,4 g ± 0,05 g of ammonium acetate and transfer into a 2000 mL volumetric flask. Dilute to mark with deionized water and mix well.

7.2 Mobile Phase A (10 mM ammonium acetate in water)

Weigh 0,77 g ± 0,02 g of ammonium acetate and transfer into a 1000 mL volumetric flask. Dilute to mark with deionized water and mix well.

7.3 Mobile Phase B – 0,1 % v/v acetic acid solution in methanol

Add 1 mL of acetic acid into a 1000 mL volumetric flask containing 500 mL of HPLC-grade methanol. Dilute to mark with methanol and mix well.

7.4 30/70 v/v Acetonitrile/Deionized Water

Combine 150 mL acetonitrile with 350 mL of deionized water in a 500 mL glass screw cap bottle.

Mix well prior to use.

7.5 10/90 v/v Acetonitrile/10 mM ammonium acetate

Combine 100 mL acetonitrile with 900 mL of 10 mM ammonium acetate solution (prepared in section 7.2) in a 1000 mL glass screw cap bottle.

Mix well prior to use.

8. PREPARATION OF STANDARDS

8.1 Preparation of Internal Standard Solutions

8.1.1 Primary Internal Standard Solutions

Weigh approximately 10 mg each of NNN-d4, NAT-d4, NAB-d4, and NNK-d4 into individual 10 mL volumetric flasks.

Fill to volume with acetonitrile and mix well.

The concentration will be approximately 1000 µg/mL.

8.1.2 Combined Secondary Internal Standard Solution

Transfer 2 mL of each primary solution of NNN-d4, NAT-d4, NAB-d4 and NNK-d4 into a 100 mL volumetric flask.

Dilute to volume with acetonitrile and mix well.

The concentration will be approximately 20 µg/mL.

8.1.3 Internal Standard Spiking Solution

Transfer 2 mL of the combined Secondary Internal Standard Solution into a 250 mL volumetric flask.

Dilute to volume with acetonitrile and mix well.

The concentration will be approximately 160 ng/mL.

8.2 Preparation of Calibration Standard Solutions

8.2.1 Primary Stock Solutions

Weigh approximately 10 mg each of NNN, NAT, NAB, and NNK into individual 10 mL volumetric flasks and record the weight to 0,1 mg.

Fill to volume with acetonitrile and mix well.

The concentration should be approximately 1000 µg/mL.

8.2.2 Secondary Stock Solution

Transfer 4 mL of each primary stock solution of NNN, NAT, NNK, and 1 mL of NAB into a 100 mL volumetric flask.

Dilute to volume with acetonitrile and mix well.

The concentration is approximately 40 µg/mL for NNN, NAT, NNK and 10 µg/mL for NAB.

8.2.3 Tertiary Stock Solution

Transfer 2,5 mL of the secondary stock solution into a 250 mL volumetric flask.

Dilute to volume with 30/70 acetonitrile/deionized water and mix well.

The concentration is approximately 400 ng/mL for NNN, NAT, NNK, and 100 ng/mL for N.

8.2.4 Quaternary Stock Solution

Transfer 10 mL of the tertiary stock solution into a 100 mL volumetric flask.

Dilute to volume with 30/70 acetonitrile/deionized water and mix well.

The concentration is approximately 40 ng/mL for NNN, NAT, NNK, and 10 ng/mL for NAB.

8.3 TSNAs Calibration Standards

Prepare seven (7) calibration standards that cover the range of interest. An example of calibration standard preparation can be found in Table 1. The TSNA standards are prepared in separate flasks each containing a small amount of 10/90 acetonitrile/10 mM ammonium acetate solution (prepared in section 7.5) and the Internal Standard Spiking Solution amount listed in Table 1. Transfer the appropriate volume of the quaternary stock solution given in Table 1 and fill to volume with 10/90 acetonitrile/10 mM ammonium acetate solution.

Table 1: Calibration Standard Preparation

Calibration Standard	Flask (mL)	Quaternary Stock Solution (mL) [see 8.2.4]	Internal Standard Spiking Solution (mL) [see 8.1.3]	Calculated NAT, NNK, NNN Conc. (ng/mL)	Calculated NAB Conc. (ng/mL)
1	25	0,03	0,25	0,048	0,012
2	25	0,06	0,25	0,096	0,024
3	10	0,10	0,10	0,400	0,100
4	10	0,20	0,10	0,800	0,200
5	10	0,40	0,10	1,60	0,400
6	10	0,80	0,10	3,20	1,20
7	25	7,50	0,25	12,0	3,00

Note: Stock solutions of the individual TSNAs or internal standards in acetonitrile may be purchased at required levels.

Note: Linearity and range should be determined for every lab/instrument used for the quantitation of samples and should be appropriate for the types of samples to be analysed.

8.4 Storage

All standards and stocks prepared in this procedure are stable for up to six months when stored at or below 5 °C.

9. PROCEDURES

9.1 Samples are to be stored and conditioned according to CORESTA Guide No. 18, *Technical Guide for Sample Handling and Sample Collection of E-Cigarettes and E-Vapour Generating Products*.

9.2 Sample Extraction

9.2.1 Using an analytical balance, accurately transfer 200 µL of sample to a tared extraction vessel and record the weight to the nearest 0,1 mg.

9.2.2 Add 50 µL of the internal standard spiking solution using a calibrated pipette or equivalent.

9.2.3 Add 5 mL of extraction solution to each vessel and cap.

9.2.4 Vortex samples until mixed (at least 5 seconds).

9.2.5 Aliquot samples directly into an amber autosampler vial and cap.

9.2.5.1 If filtration is required, 0,45 µm Nylon syringe filters may be used and samples filtered directly into amber vials.

9.2.6 Sample extract is ready for analysis.

Samples that exceed calibration range for any TSNA compound require either of the following:

9.2.7a Sample preparation may be repeated using a greater volume of extraction solution, provided the appropriate amount of internal standard is used to give a concentration of 1,6 ng/mL.

9.2.7b Extracted samples may be diluted with extraction solution containing internal standard at a concentration of 1,6 ng/mL. Mix samples and proceed with reinjection.

10. DETERMINATION

Set up and operate the LC-MS/MS in accordance with the manufacturer's instructions.

10.1 Suggested Parameters

The following parameters are recommended for the LC system and may be modified to achieve better performance.

- Column temperature: 55,0 °C
- Injection volume: 10 µL
- Flow rate: 0,35 mL/min
- Mobile Phase A: 10 mM Ammonium Acetate in Water
- Mobile Phase B: 0,1% (v/v) acetic acid in methanol

The following HPLC column gradient is suggested, but it may be modified as needed to achieve better performance

Table 2: HPLC Gradient

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)	Curve	Flow Rate (mL/min.)
Initial	97	3	Initial	0,35
1,00	97	3	Linear	0,35
4,00	10	90	Linear	0,35
4,01	1	99	Linear	0,35
5,75	1	99	Linear	0,35
5,90	97	3	Linear	0,35
9,00	97	3	Linear	0,35

10.2 MS/MS Parameters and Transitions

For this method, a triple quadrupole mass spectrometer shall be used and operated in positive electrospray mode using multiple reaction monitoring (MRM). The triple quadrupole should be carefully optimized for each analyte prior to use. Recommended precursor and product ions can be found in Table 3. Cone voltages and collision energies should be optimized on each instrument prior to analysis.

Table 3 – Suggested mass transitions for TSNAs

Analyte	Precursor Ion	Product Ion	Suggested Internal Standard
NAB	192	162	NAB-d4
NAB-d4	196	166	-
NAT	190	160	NAT-d4
NAT-d4	194	164	-
NNK	208	122	NNK-d4
NNK-d4	212	126	-
NNN	178	148	NNN-d4
NNN-d4	182	152	-

10.3 System Suitability

Prior to injection of samples, system performance must be evaluated. This evaluation should include assessing sensitivity, chromatographic performance, and any other criterion to ensure optimal performance of the LC-MS/MS system.

10.4 Calibration Curve

The initial calibration curve must consist of at least five calibration standards for each analyte. The recommended curve type is linear with the origin excluded. A 1/x weighting is used. The calibration curve must have a coefficient of determination (r^2) of 0,995 or greater. For the calibration curve to be considered acceptable, each standard must be within 15 % of their target value.

10.5 Calculations

Data are obtained from the instrument in units of ng/mL. The concentration of each TSNA present in the sample extract is determined by using the internal standard calibration curve. The value is converted to sample concentration as follows:

$$C_{liquid} = \frac{C_{extract} \times V}{M}$$

Where:

C_{liquid} is the concentration of the analyte in the tested e-liquid, reported in units of ng/g.

$C_{extract}$ is the concentration of the analyte, in ng/mL from the linear regression reported by the software in units of ng/mL.

V is the volume of extraction solution in units of mL

M is the weight of e-liquid in units of g.

10.6 Quality Control

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

11. REPEATABILITY AND REPRODUCIBILITY

In 2021, the CORESTA EVAP Sub-Group conducted an international collaborative study involving nine (9) laboratories for the determination of tobacco-specific nitrosamines (TSNAs) in e-liquids following a proposed method. Four different e-liquids were used, each with a different flavour profile and each fortified with a different concentration of the target TSNAs. Also supplied to each participating laboratory were the corresponding non-fortified samples for analysis. Results were reported in ng/g. The majority of the non-fortified results were below the limit of detection (LOD) or limit of quantitation (LOQ) and therefore no repeatability and reproducibility estimates were calculated. Results reported for the fortified e-liquid samples were analysed in basic conformance with ISO 5725-5:1998. Repeatability and reproducibility values are provided in Tables 4 through 7.

Table 4: Repeatability (r) and Reproducibility (R) Limits for NAT (ng/g)

Sample	Nominal Conc. (ng/g)	N° of Labs	Average (ng/g)	Repeatability		Reproducibility	
				r	r %	R	R %
3128701-Unflavoured	3,2	9	3,44	0,49	14,2	1,31	38,1
3128702-Tobacco	12	9	12,06	1,13	9,4	3,8	26,4
3128703-Sweet	80	9	77,94	4,03	5,2	18,77	24,1
3128704-Menthol	48	9	51,19	3,38	6,6	6,70	13,1

Table 5: Repeatability (r) and Reproducibility (R) Limits for NAB (ng/g)

Sample	Nominal Conc. (ng/g)	N° of Labs	Average (ng/g)	Repeatability		Reproducibility	
				r	r %	R	R %
3128701-Unflavoured	0,8	9	0,82	0,14	16,5	0,50	60,4
3128702-Tobacco	3,0	9	2,85	0,48	16,8	1,48	52,0
3128703-Sweet	20	9	19,86	2,28	11,5	7,46	37,6
3128704-Menthol	12	9	12,56	0,72	5,7	3,69	29,3

Table 6: Repeatability (r) and Reproducibility (R) Limits for NNK (ng/g)

Sample	Nominal Conc. (ng/g)	N° of Labs	Average (ng/g)	Repeatability		Reproducibility	
				r	r %	R	R %
3128701-Unflavoured	3,2	9	3,21	0,26	8,2	1,46	45,5
3128702-Tobacco	12	9	11,84	1,26	10,7	2,60	22,0
3128703-Sweet	80	9	80,55	3,61	4,5	15,79	19,6
3128704-Menthol	48	9	49,90	3,33	6,7	9,79	19,6

Table 7: Repeatability (r) and Reproducibility (R) Limits for NNN (ng/g)

Sample	Nominal Conc. (ng/g)	N° of Labs	Average (ng/g)	Repeatability		Reproducibility	
				r	r %	R	R %
3128701-Unflavoured	3,2	9	3,19	0,60	18,9	1,81	56,7
3128702-Tobacco	12	9	11,08	1,11	10,0	2,84	25,7
3128703-Sweet	80	9	78,25	5,22	6,7	18,15	23,2
3128704-Menthol	48	9	47,88	3,81	8,0	11,44	23,9

12. TEST REPORT

The test report shall state the amount of TSNAs in nanograms per gram e-liquid and shall include all conditions not specified in this Recommended Method or regarded as optional that may have influenced the results. The report shall also give all the details necessary for the identification of each sample.

13. REFERENCES

[1] CORESTA E-Vapour Sub-Group Technical Report, 2021 Collaborative Study for the Determination of Tobacco-Specific Nitrosamines in E-liquids, (EVAP-304-1)

[2] CORESTA Recommended Method N° 72 Determination of Tobacco-Specific Nitrosamines in Tobacco and Tobacco Products by LC-MSMS

[3] CORESTA Recommended Method N° 75 Determination of Tobacco-Specific Nitrosamines in Mainstream Smoke by LC-MSMS

APPENDIX A – EXAMPLE CHROMATOGRAMS

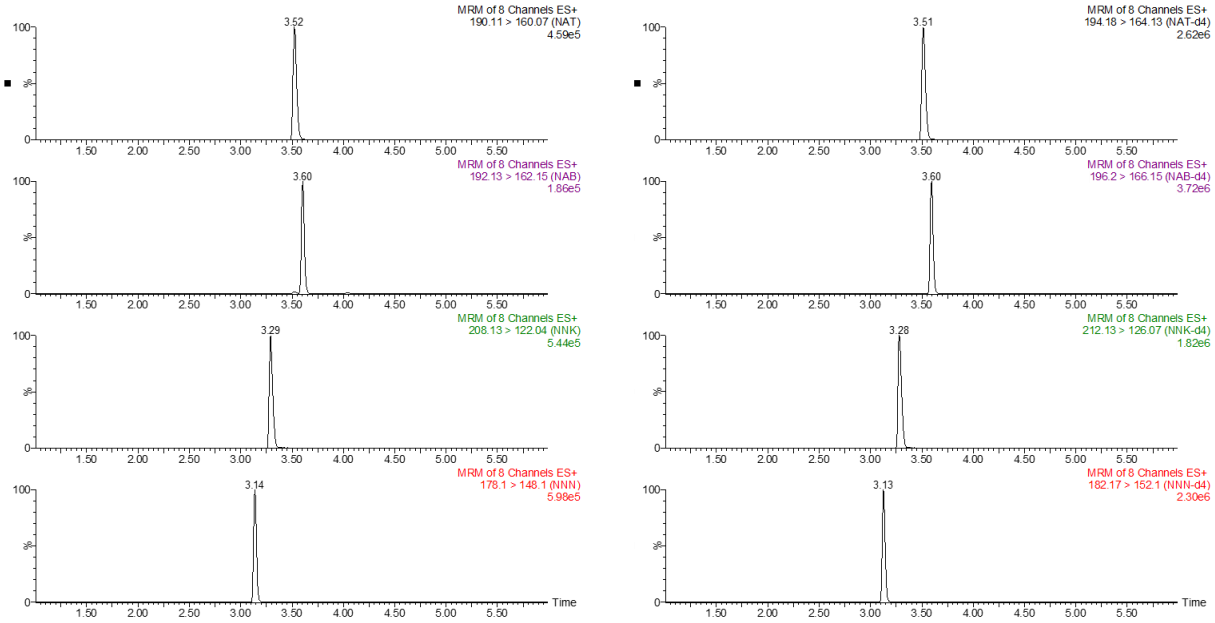

Figure 1 – Example of a MRM-chromatogram for a TSNA standard.

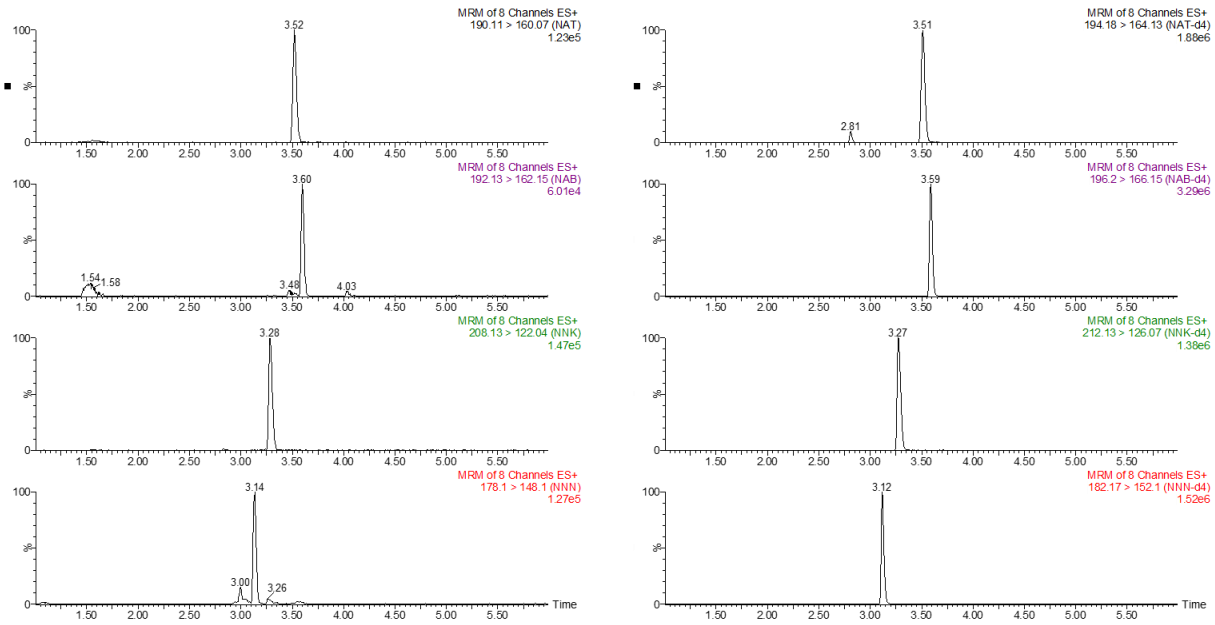

Figure 2 – Example of a MRM-chromatogram for an e-liquid fortified with NAT, NAB, NNK and NNN.