



**Cooperation Centre for Scientific Research
Relative to Tobacco**

Smoke Analytes Sub-Group

**CORESTA Recommended Method
No. 63**

**DETERMINATION OF TOBACCO
SPECIFIC NITROSAMINES IN
CIGARETTE MAINSTREAM SMOKE –
GC-TEA METHOD**

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0. INTRODUCTION

Between 1999 and 2005, a Task Force composed of CORESTA members studied the existing methodologies for the determination of the Tobacco Specific Nitrosamines (TSNAs) in the mainstream smoke of cigarettes. Several methods have been proposed for this determination, which are mainly based on two types of analytical methodologies: GC-TEA (Gas Chromatography with a Thermal Energy Analyser as detector) and LC/MS/MS.

The Task Force decided in the first instance to develop a method using GC-TEA, because this methodology was the most widely used in laboratories analysing nitrosamines and only in that case was it possible to obtain the collaboration of a sufficient number of experienced laboratories to develop a Recommended Method. In the course of the development of the method, it was recognised that a clean-up step should be used after extraction of the smoke condensate from the glass fibre filter. Normally the TEA detector provides a specific detection of Nitrosamines and clean-up is theoretically not necessary. However, several laboratories reported a rapid deterioration of the chromatographic performance when injecting total smoke extracts into the system and the Task Force decided to include a clean-up procedure to avoid this problem. The Task Force performed several studies in order to develop and specify this procedure, investigating two different methods. The first one used a liquid chromatography alumina column. This procedure was operated successfully by several laboratories, however some others reported losses of TSNAs during the clean-up. These difficulties were certainly linked with the quality and preparation of the alumina packing. However, despite several investigations, the Task Force was not able to specify with sufficient precision the preparation mode of the alumina column for obtaining correct results in every instance. Thereafter, an alternative clean-up procedure using a liquid chromatography combined silica-alumina column was included in the proposed method. The comparison studies performed by the Task Force demonstrated that laboratories performing correctly one of these methods obtain equivalent results with laboratories performing correctly the other one.

This recommended method, produced through collaborative experiments involving many laboratories in many countries, using cigarettes across Virginia and US blend styles of low, medium, and high ‘tar’ yields provides an optimised procedure for the determination of TSNAs in cigarette mainstream smoke, and constitutes the accepted reference procedure by the Task Force. The repeatability and reproducibility of this method have been assessed according to ISO recommendations and are included.

1. FIELD OF APPLICATION

This recommended method is applicable to the determination of four Tobacco Specific Nitrosamines (TSNAs) in the total particulate matter of cigarette mainstream smoke. The determined TSNAs are: N-Nitrosornicotine (NNN), N-Nitrosoanabasine (NAB), N-Nitrosoanatabine (NAT) and 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK).

The described method is specified using ISO 3308 smoking parameters, but is technically compatible with other smoking regimes.

2. NORMATIVE REFERENCES

- 2.1 ISO 3308 Routine analytical smoking machine – Definition and standard conditions
- 2.2 ISO 4387 Cigarettes – Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine
- 2.3 ISO 3402 Tobacco and tobacco products – Atmosphere for conditioning and testing
- 2.4 CORESTA Recommended Method N°21 *Atmosphere for conditioning and testing tobacco and tobacco products.*
- 2.5 CORESTA Recommended Method N°22 *Routine analytical cigarette-smoking machine specifications, definitions and standard conditions.*
- 2.6 CORESTA Recommended Method N°23 – *Determination of total and nicotine-free dry particulate matter using routine analytical cigarette-smoking machine – Determination of total particulate matter and preparation for water and nicotine measurements.*
- 2.7 *Determination of Tobacco Specific Nitrosamines in Cigarette Mainstream Smoke: The CORESTA 2011 Collaborative Study.* Intorp, M; Purkis, S; Wagstaff, W., Beitr. zur Tabac, Vol. 25, No. 4, December 2012.

3. METHOD SUMMARY

Conditioned cigarettes are smoked using standard procedures. Mainstream smoke is trapped on a glass fiber filter pad. After addition of an internal standard, the filter pad is extracted with dichloromethane. Sample clean-up of the extraction solution is accomplished with one of the following methods: a) Elution of the extract through an alumina column, followed by the elution of the TSNAs with an acetone/dichloromethane (50:50 v/v) mixture; b) Elution of the extract through a combined silica-alumina column, followed by the elution of the TSNAs with an 8 % methanol in dichloromethane solution. The extract is concentrated followed, by quantitative analysis using gas chromatography with a thermal energy analyser for detection (GC-TEA).

4. APPARATUS AND EQUIPMENT

Normal laboratory apparatus and equipment and in particular the following items:

4.1 Routine analytical cigarette-smoking machine

Complying with the requirements of ISO 3308 and equipped for smoking according to ISO 4387.

4.2 Gas chromatograph – Thermal Energy Analyser

Equipped with its computerised control, data acquisition and processing system. The gas chromatograph is to be configured to perform splitless injections on a capillary column. It is recommended to equip the gas chromatograph with an autosampler for sample injection.

4.3 Column

Fused silica capillary column with a (50 %) methyl/(50 %) phenyl polysiloxane stationary phase. A 30 m, 0,53 mm internal diameter column with a 1 µm film thickness is suitable for this analysis.

4.4 Rotary evaporator or equivalent equipment

4.5 Liquid chromatographic column (e.g.: 300 mm, 22 mm OD, 15 mm ID)

4.6 Concentrating system (TurboVap^[1] or equivalent) allowing concentration of sample extract solutions without loss of TSNAs.

4.7 Gas tight syringes

4.8 General laboratory equipment

For the preparation of samples, standards and reagents: all glassware shall be cleaned before use to avoid any contamination.

5. REAGENTS AND SUPPLIES

All reagents shall be of analytical grade quality.

5.1 Dichloromethane, CAS # 75-09-2

5.2 Acetone, CAS # 67-64-1

5.3 Methanol, CAS # 67-56-1

5.4 N-Nitrosornicotine (NNN), CAS # 80508-23-2

5.5 N-Nitrosoanabasine (NAB), CAS # 37620-20-5

5.6 N-Nitrosoanatabine (NAT), CAS # 887407-16-1

5.7 4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), CAS # 64091-91-4

5.8 N-Nitrosopentyl-(3-picolyl)-amine (NNPA), CAS # 124521-15-9

5.9 Basic alumina (Activity Super I) (clean-up procedure n°1)

5.10 Alumina (Activity II-III) (clean-up procedure n°2)

5.11 Silica (100-120 active 60 Å) (clean-up procedure n°2)

5.12 Anhydrous sodium sulphate (clean-up procedure n°2)

Note: The use of this method involves hazardous materials and potentially hazardous operations and equipment. It is the responsibility of the user to establish appropriate safety and health practices and to determine the applicability of regulatory limitations prior to use.

^[1] This information is provided as an example for the convenience of the users of this CORESTA Recommended Method and does not constitute endorsement of this product.

6. PREPARATION OF STANDARDS

6.1 Internal standard solutions

6.1.1 Primary NNPA solution

Dissolve approximately 40 mg NNPA, weighed exactly to 0,01 mg, in 10 mL of dichloromethane. The NNPA concentration in this solution is approximately 4000 µg/mL.

6.1.2 Secondary NNPA solution

Dilute 5 mL of the primary NNPA solution to 200 mL with dichloromethane. The NNPA concentration in this solution is approximately 100 µg/mL.

6.1.3 Working NNPA solution

Dilute 50 mL of the secondary NNPA solution to 500 mL with dichloromethane. The NNPA concentration in this solution is approximately 10 µg/mL.

6.2 Calibration standard solutions

6.2.1 Primary single TSNA solutions

In four different volumetric flasks, dissolve approximately 10 mg of NNN, NAB, NAT and NNK respectively, weighed exactly to 0,01 mg, in 10 mL of dichloromethane. The TSNA concentration in each solution is approximately 1000 µg/mL

6.2.2 Mixed TSNA stock solution

Using a volumetric pipette, transfer 1 mL of each of the primary single TSNA solutions into a 100 mL volumetric flask and complete to the mark with dichloromethane. This solution has a concentration of approximately 10 µg/mL of NNN, NAB, NAT and NNK.

6.2.3 Working standard solutions

Prepare 6 working standard solutions that cover the concentration range of interest. For example, transfer 2 mL of the secondary NNPA solution (6.1.2) and 0,5, 1, 3, 5, 10 and 20 mL of the mixed TSNA solution (6.2.2) into 100 mL volumetric flasks and complete to the mark with dichloromethane. These solutions have a concentration of approximately 2 µg/mL of NNPA and concentrations from 50 to 2000 ng/mL of NNN, NAB, NAT and NNK.

6.2.4 Storage

The above standard solutions are stable for up to six months if stored at around –20 °C.

Table 1. Preparation of Working Calibration Standards (Example)

Calibration Standards	Volume of Mixed TSNA Stock (mL)	Volume of Secondary NNPA solution (mL)	Final Conc. of TSNAs (ng/mL)	Final Conc. of NNPA (µg/mL)
1	0,5	2,0	50,0	2,0
2	1,0	2,0	100,0	2,0
3	3,0	2,0	300,0	2,0
4	5,0	2,0	500,0	2,0
5	10,0	2,0	1000,0	2,0
6	20,0	2,0	2000,0	2,0

7. SAMPLE PROCEDURE

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

Note: ISO 8243 provides comprehensive sampling procedures for cigarettes. These procedures are optional depending on study objectives.

8. SAMPLE GENERATION – SMOKING OF CIGARETTES

Cigarettes are smoked according to ISO 4387. Typically 10 cigarettes should be smoked onto a 44 mm glass fibre filter pad, and 20 cigarettes onto a 92 mm glass fibre filter pad (however, smoking 10 cigarettes onto a 92 mm glass fibre filter pad is acceptable). Glass fibre filter pads of 44 mm diameter are capable of retaining up to 150 mg of total particulate matter (TPM) and pads of 92 mm diameter up to 600 mg. If this mass is exceeded, the number of cigarettes is to be reduced. For low tar products, a greater number of cigarettes may be smoked to achieve a nominal TPM of 10 mg for a 44 mm pad and 20 mg for a 92 mm pad.

9. SAMPLE ANALYSIS

9.1 Filter pad extraction

- 9.1.1 Remove the filter pad from its holder, fold the filter two times (with the condensate inside) and wipe the inside of the holder with the pad.
- 9.1.2 Transfer the filter pad into an Erlenmeyer flask (300 mL if 10 cigarettes are smoked, 500 mL if 20 cigarettes are smoked).
- 9.1.3 If 10 cigarettes have been smoked, add 400 µl of the working NNPA solution (6.1.3) with a suitable syringe and add 100 mL of dichloromethane. If 20 cigarettes have been smoked, add 800 µl of the working NNPA solution with a suitable syringe and add 200 mL of dichloromethane.
- 9.1.4 Shake the flask on a suitable shaker for 30 minutes and filter the solution through a glass suction filter or using paper filtration into a 500 mL round-bottomed flask.
- 9.1.5 Wash the Erlenmeyer flask three times with approximately 15 mL of dichloromethane. Add the washing solutions to the filter extract. Complete to a volume which is 150 mL if 10 cigarettes have been smoked and 300 mL if 20 cigarettes have been smoked.
- 9.1.6 Take an aliquot, not to exceed 150 mL, of the solution and concentrate it to approximately 5 mL using an evaporator or equivalent equipment.

9.2 Sample clean-up: procedure n°1 (alumina)

9.2.1. Preparation of the basic alumina: Introduce 500 g of basic alumina in an Erlenmeyer flask. Place the flask in an oven and dry the alumina at 110 °C for more than 16 hours. Place the alumina in a desiccator and let it cool down to room temperature. Add 20 mL of distilled water and shake for 30 minutes. The prepared alumina has a moisture content of approximately 4 % and a grade II activity.

9.2.2 Clean-up procedure

- 9.2.2.1 Place a piece of cotton at the bottom of the liquid chromatographic column.
- 9.2.2.2 In a beaker, add 50 mL of dichloromethane and 10 g (\pm 0,2 g) of basic alumina (9.2.1) in order to obtain an alumina slurry.
- 9.2.2.3 Introduce the alumina slurry into the column, using a vibrator to eliminate any possible air pockets. Let the liquid phase pass through the column until it reaches the top of the alumina packing. Close the column with the stopcock. Discard the liquid phase to waste.
- 9.2.2.4 Add the extracted sample obtained in 9.1.6 on the top of the alumina with a pipette taking care not to disturb the alumina packing.
- 9.2.2.5 Let the liquid phase pass through the column and close the stopcock when it reaches the top of the alumina packing. Add 30 mL of dichloromethane and let the liquid phase pass through the column and close the stopcock when it reaches the top of the alumina packing. Discard liquid phases to waste.
- 9.2.2.6 Place a clean 300 mL Erlenmeyer flask beneath the column. Elute the TSNAs with 100 mL of a solution of acetone – dichloromethane (50 : 50 v/v)

9.3 Sample clean-up: procedure n°2 (alumina - silica)

- 9.3.1 In the liquid chromatographic column, place approximately 2 g of anhydrous sodium sulphate, 15 g of alumina, 15 g of silica and 2 g of anhydrous sodium sulphate, in that order ensuring each material forms a uniform layer.
- 9.3.2 Add the extracted sample obtained in 9.1.6 on the top of the packing with a pipette and allow all dichloromethane to soak into the column packing. Add 100 mL of dichloromethane and let the liquid phase pass through the column. Collect the eluent and discard to waste.
- 9.3.3 Place a clean 300 mL Erlenmeyer flask beneath the column. Elute the TSNAs with 160 mL of a solution of 8 % methanol in dichloromethane.

Note: The selection of one of the above clean-up procedures should be made through a complete method checking and validation procedure. In particular, it is important to verify the absence of any of the target TSNAs and of the internal standard in the washing solutions (9.2.2.5 and 9.3.2), through an analysis of these solutions by GC-TEA. Percentage recovery of each of the TSNAs are determined by analysing properly spiked solutions. Finally, the quality of the chromatographic separation of the peaks of interest may be another criterion for selecting a particular method.

9.4 Final sample preparation

- 9.4.1 Using a concentrating system (4.6), concentrate the TSNAs elution solution (9.2.2.6 for procedure n°1 and 9.3.3 for procedure n°2) to a volume below 2 mL.
- 9.4.2 Complete with dichloromethane to obtain a final volume of 2 mL. Transfer to autosample vial for analysis.

Note: In case of products with low TSNAs content, a final volume of 1 mL may be required.

9.5 Determination

9.6 GC/TEA operating conditions

Set up and operate the GC/TEA system in accordance with the manufacturer's instructions.

The following conditions are suitable for this analysis:

Injector temperature:	230 °C
Mode:	constant flow, 5 psi at 150 °C
Injection:	2 µl splitless (purge time: 1 min)
Column temperature:	150 °C for 2 min 3 °C/min to 230 °C 20 °C/min to 250 °C hold at 250 °C for 3 min
TEA interface temperature:	240 °C
TEA pyrolyser temperature:	500 °C

These chromatographic conditions are to be adapted in order to obtain a correct resolution of the NNN, NAB, NAT, NNK, and NNPA peaks. A typical chromatogram is given in Appendix 1.

9.7 Calculations

9.7.1 Calibration

Inject successively each working standard solution (6.2.3) in the GC/TEA system. Record the area of each of the TSNAs and the NNPA peaks. A calibration curve for each of the TSNAs is generated by calculating a linear equation regression of the area ratios of TSNA to NNPA peaks in function of the TSNA concentrations. The intercept of these regression lines should be close to zero.

Inject one mid-range working standard solution after every 10 sample analyses. If any of the measured concentrations for this solution are different by more than 15 % from the nominal calibration values then repeat the calibration procedure.

9.7.2 Determination of the TSNAs

Inject the sample, calculate the area ratio of TSNA to NNPA for each TSNA and obtain the concentration of each TSNA in the solution by comparing this ratio with the corresponding calibration line.

9.7.3 Sample Quantification

The amount of each TSNA per cigarette is calculated as follows:

Equation 1:

$$M = \frac{C * V * V_e}{n * V_c}$$

Where:

- M is the mass of TSNA in cigarette smoke expressed in ng/cig
- C is the concentration of TSNA in the sample solution expressed in ng/mL.
- V is the final volume of the sample solution expressed in mL (V = 2mL)
- n is the number of cigarettes smoked
- V_e is the volume of the extraction solution (9.1.5)
- V_c is the volume of the aliquot of the extraction solution used during the clean-up (9.1.6)

10. REPEATABILITY AND REPRODUCIBILITY

A major international study involving nine laboratories and seven cigarette samples including the 2R4F (a reference cigarette previously available from the University of Kentucky) and covering a wide range of blends and construction, was conducted in 2005 and the following values for repeatability (r) and reproducibility (R) were obtained for this method under the ISO smoking regime.

The difference between two single results found on matched cigarette samples by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability value (r) on average not more than once in 20 cases in the normal and correct operation of this method.

Single results on matched cigarette samples reported by two laboratories will differ by more than the reproducibility (R) on average not more than once in 20 cases in the normal and correct operation of the method.

Data analysis for the 7 cigarette samples gave the estimates as summarised in the following tables:

Table 2. ISO Smoking Regime - NNN (ng/cigarette)

Cigarette sample	NNN (ng/cigarette)		
	Mean	r	R
2R4F	146,01	10,75	32,26
A	179,55	28,90	44,63
B	274,21	53,96	89,54
C	42,15	10,19	22,37
D	10,62	2,88	5,43
E	26,38	7,00	14,95
F	84,59	11,76	45,98

Table 3. ISO Smoking Regime - NAB (ng/cigarette)

Cigarette sample	NAB (ng/cigarette)		
	Mean	r	R
2R4F	16,60	5,15	10,70
A	20,39	4,93	11,84
B	27,30	6,41	12,43
C	6,96	2,32	5,21
D	2,23	1,43	3,28
E	4,36	2,18	3,44
F	12,41	3,67	6,22

Table 4. ISO Smoking Regime - NAT (ng/cigarette)

Cigarette sample	NAT (ng/cigarette)		
	Mean	r	R
2R4F	143,38	20,38	63,62
A	173,22	27,10	84,59
B	133,60	21,95	75,46
C	49,83	13,86	34,86
D	9,44	3,00	4,90
E	26,93	6,69	14,34
F	86,74	17,95	44,63

Table 5. ISO Smoking Regime - NNK (ng/cigarette)

Cigarette sample	NNK (ng/cigarette)		
	Mean	r	R
2R4F	141,39	15,68	43,88
A	87,73	13,30	52,72
B	201,60	37,04	146,24
C	35,95	8,37	23,41
D	5,38	1,82	5,85
E	23,40	6,97	12,40
F	51,68	8,48	22,90

Note: Several laboratories reported results below their quantification or detection limits in the following cases: NAB (samples C, D, and E) and NNK (sample D). From the valid data points, 5,3 % were found to be outliers after performing the statistical procedure for outliers' detection according to ISO 5725 recommendations. As a consequence, most of the above r & R values were calculated taking into account the results of 8 laboratories. This is generally considered as the minimum sufficient number for a validation study. In the four cases stated above, the r & R values were obtained taking into account the results of a lower number of laboratories because the mean values were close to the limit of quantification of the method as reported by participating laboratories.

11. REPORT

The test report shall state the analyte yields rounded to the nearest 0,1 ng/cig and shall include all conditions which may affect the results. The report shall also give all details necessary for the identification of each sample. Additional specific information depends on the purpose for which the data are required.

Appendix 1: Example of a chromatogram of a cigarette smoke extract

