

CORESTA RECOMMENDED METHOD N° 63

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

(June 2005)

0. INTRODUCTION

Between 1999 and 2005, a Task Force composed of CORESTA members has 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 is at present, the most widely used in laboratories analysing nitrosamines and only in this 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 uses a liquid chromatography alumina column. This procedure is operated successfully by several laboratories, however some others have reported losses of TSNAs during the clean-up. These difficulties are 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, a second clean-up procedure using a liquid chromatography combined silica-alumina column was included in the proposed method. Laboratories implementing the method may use either clean up procedure but should test and validate thoroughly the chosen method. The comparison studies performed by the Task Force have 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, 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 3308:2000 smoking parameters, but is technically compatible with other smoking regimes.

2. REFERENCES

ISO 3308:2000

Routine analytical smoking machine – Definition and standard conditions

ISO 4387:2000

Cigarettes – Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine

ISO 3402:1999

Tobacco and tobacco products – Atmosphere for conditioning and testing

CORESTA Recommended Method N°21 (August 1991)

Atmosphere for conditioning and testing tobacco and tobacco products.

CORESTA Recommended Method N°22 (August 1991)

Routine analytical cigarette-smoking machine specifications, definitions and standard conditions.

CORESTA Recommended method N°23 (August 1991)

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.

3. PRINCIPLE

Sampling of the test cigarettes. Conditioning of the test cigarettes. Smoking of the test cigarettes according to the smoking procedure specified in ISO 4387:2000. After addition of an internal standard, extraction of the total particulate matter collected on the glass-fibre filter pad with dichloromethane. Clean-up of the extraction solution 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 a 8% methanol in dichloromethane solution. Concentration of the extract. Analytical determination of NNN, NAB, NAT and NNK by Gas Chromatography with a Thermal Energy Analyser detector (GC-TEA).

4. APPARATUS

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

- 4.1. Routine analytical cigarette-smoking machine
Complying with the requirements of ISO 3308:2000 and equipped for smoking according to ISO 4387:2000.
- 4.2. Gas chromatograph – Thermal Energy Analyser
Equipped with its computerised control and data acquisition and processing system. The gas chromatograph must 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 or equivalent) allowing concentration of sample extract solutions without losses 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

All reagents shall be of analytical grade quality.

- 5.1. Dichloromethane
- 5.2. Acetone
- 5.3. Methanol
- 5.4. N-Nitrosornicotine (NNN)
- 5.5. N-Nitrosoanabasine (NAB)
- 5.6. N-Nitrosoanatabine (NAT)
- 5.7. 4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)
- 5.8. N-Nitrosopentyl-(3-picolyl)-amine (NNPA)
- 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) (clean-up procedure n°2)

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

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

6. 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.

7. SAMPLE PREPARATION

7.1. Sampling

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

Note: ISO 8243 provides comprehensive sampling procedures for cigarettes. However such procedures may be unpractical in the case of the application of this method and simpler sampling plans may be sufficient.

7.2. Smoking

Cigarettes are smoked according to ISO 4387:2000. 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 shall 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.

7.3. Filter pads extraction

- 7.3.1.** 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.
- 7.3.2.** Transfer the filter pad into an Erlenmeyer flask (300 ml if 10 cigarettes are smoked, 500 ml if 20 cigarettes are smoked).
- 7.3.3.** If 10 cigarettes have been smoked (7.2), add 400 μ l 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 μ l of the working NNPA solution (6.1.3) with a suitable syringe and add 200 ml of dichloromethane (7.2).
- 7.3.4.** 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.
- 7.3.5.** 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.
- 7.3.6.** Take an aliquot of the obtained solution and concentrate it to approximately 5 ml using a rotary evaporator or equivalent equipment (4.4). The volume of this aliquot shall not exceed 150 ml, which is convenient for this procedure.

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

- 7.4.1.** 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 dessicator 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.
- 7.4.2.** Clean-up procedure
 - 7.4.2.1.** Place a piece of cotton at the bottom of the liquid chromatographic column.
 - 7.4.2.2.** In a beaker, add 50 ml of dichloromethane and 10 g (\pm 0,2 g) of basic alumina (7.4.1) in order to obtain an alumina slurry.
 - 7.4.2.3.** 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.
 - 7.4.2.4.** Add the extract sample obtained in 7.3.6 on the top of the alumina with a pipette taking care not to disturb the alumina packing.
 - 7.4.2.5.** 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.

7.4.2.6. 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)

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

7.5.1. 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.

7.5.2. Add the extract sample obtained in 7.3.6 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.

7.5.3. 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 (7.4.2.5 and 7.5.2), through an analysis of these solutions by GC-TEA. Percentage recovery of each of the TSNAs may be 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.

7.6. Final sample preparation

7.6.1. Using a concentrating system (4.6), concentrate the TSNAs elution solution (7.4.2.6 for procedure n°1 and 7.5.3 for procedure n°2) to a volume below 2 ml.

7.6.2. 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.

8. DETERMINATION

8.1. 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 shall be adapted in order to obtain a correct resolution of the NNN, NAB, NAT, NNK, and NNPA peaks. A typical chromatogram is given in Annex 1.

8.2. Calibration

Inject successively each working standard solution (6.2.3) in the GC/TEA system. Record the area of each of the TSNA and the NNPA peaks. A calibration curve for each of the TSNA 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 (6.2.3) 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.

8.3. Determination of the TSNA

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.

8.4. Calculation

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

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

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 (7.3.5)
- V_c is the volume of the aliquot of the extraction solution used during the clean-up (7.3.6).

9. REPEATABILITY AND REPRODUCIBILITY

A major international study involving 9 laboratories and 7 cigarette samples including the 2R4F (a reference cigarette 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.

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:

	NNN (ng/cigarette)		
Cigarette sample	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

	NAB (ng/cigarette)		
Cigarette sample	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

	NAT (ng/cigarette)		
Cigarette sample	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

	NNK (ng/cigarette)		
Cigarette sample	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.

10. REPORT

The test report shall indicate the method used and the results obtained. It shall also mention any operating conditions not specified in this recommended method, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details required for complete identification of the sample. Where appropriate, record the information in 10.1 to 10.4.

10.1. Characteristic data about the cigarette

Cigarette identification. In the case of a commercial cigarette this may include:

- Name of manufacturer, country of manufacture;
- Product name;
- Packet number (of that product sampled that day);
- Marks on any tax stamp;
- Printed mainstream smoke yields (if any);
- Length of cigarette;
- Length of filter;
- Length of overwrap.
- Diameter

10.2. Sampling

- Type of sampling procedure.
- Number of cigarettes in laboratory sample.
- Date and location of purchase.
- Place of purchase or sampling;
- Kind of sampling point;
- Sampling point (e.g. address of retail outlet or machine number);

10.3. Description of Test

- Date of test.
- Type of smoking machine used.
- Type of smoke trap used.
- Number of cigarettes smoked into each smoke trap.
- Butt length.
- Room temperature (°C) during smoking operation.
- Relative humidity (%) during smoking operation.

- Atmospheric pressure (kPa) during smoking operation.

10.4. Test Results

The expression of the laboratory data depends on the purpose for which the data are required, and the level of laboratory precision. Confidence limits shall be calculated and expressed on the basis of the laboratory data before any rounding has taken place.

- Amount of NNN, NAB, NAT and NNK in the mainstream smoke of cigarette in ng/cig to the nearest 0.1 ng

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

