Cooperation Centre for Scientific Research Relative to Tobacco

Tobacco and Tobacco Products Analytes Sub-Group

CORESTA Recommended Method No. 86

DETERMINATION OF SELECT CARBONYLS IN TOBACCO AND TOBACCO PRODUCTS BY UHPLC-MS/MS

January 2018

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0. INTRODUCTION

In 2016/2017, the CORESTA Smokeless Tobacco Sub-Group (STS), now named Tobacco and Tobacco Products Analytes Sub-Group (TTPA), conducted a collaborative study for the determination of formaldehyde, acetaldehyde and crotonaldehyde in smokeless tobacco products and cigarette filler. A total of 12 laboratories submitted results. The method specified in this study was shown to be appropriate for the determination of the select carbonyls (aldehydes) in smokeless tobacco products and cigarette fillers and this CORESTA Recommended Method (CRM) was drafted.

1. FIELD OF APPLICATION

This CRM is applicable to the determination of formaldehyde, acetaldehyde and crotonaldehyde in smokeless tobacco products (e.g. moist snuff, snus, chewing tobacco and dry snuff) and cigarette filler.

2. NORMATIVE REFERENCES

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

3. PRINCIPLE

Formaldehyde, acetaldehyde and crotonaldehyde are extracted and derivatized in a two-phase system consisting of aqueous buffer and isohexane using 2,4-Dinitrophenylhydrazine (DNPH) as the derivatizing agent. DNPH both stabilizes the volatile carbonyls and increases the mass of the target analytes to improve detection by mass spectrometry. The extraction of the carbonyls from the tobacco and the subsequent derivatization occurs in the buffer phase and the resulting carbonyl-DNPH derivatives are concentrated in the isohexane phase. Extraction and derivatization is facilitated by mechanical sample rotation for 60 minutes. After derivatization, an aliquot of the isohexane phase is transferred to an autosampler vial for analysis with Ultra High Performance Liquid Chromatography - Tandem Mass Spectrometry (UHPLC-MS/MS). The results are reported in units of micrograms per gram tobacco on a wet–weight basis.

4. APPARATUS

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

4.1. Ultra High Performance Liquid Chromatograph coupled to tandem mass spectrometer (UHPLC-MS/MS) with an electrospray ionization (ESI) source consisting of:

4.1.1. Binary pump

4.1.2. Cooled Autosampler

4.1.3. Column oven

4.1.4. Tandem mass spectrometer. Capable of performing Multiple Reaction Monitoring (MRM)

4.1.5. Data collection system

4.2. UHPLC-column: C18 column, 2,1 mm × 100 mm, 1,7 µm particle size, or equivalent[1]

4.3. In-line filter to analytical column

4.4. Orbital shaker or similar

4.5. Amber volumetric flasks, Class A

4.6. Amber autosampler vials with PTFE lined caps

4.7. Analytical balance with the ability to measure to the nearest 0,1 mg, placed in an enclosure with suitable exhaust

4.8. Mechanical pipettes (adjustable volume): 200 µl and 1000 µl capacity

4.9. Repeater Pipettes: 100 µl and 1000 µl

4.10. Dispensette: 10 ml and 50 ml capacity

4.11. Class A Volumetric flasks; 10, 25, 50, 100 and 2000 ml

4.12. 50 ml glass Erlenmeyer-flasks (E-flasks) with PTFE lined screw caps, or equivalent extraction vessel

4.13. Gas tight glass syringes, 1 ml

4.14. Glass or plastic Pasteur pipettes

4.15. Amber glass storage bottles with PTFE lined screw caps in the range of 10 ml to 125 ml

4.16. pH meter

5. REAGENTS

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

5.1.	Reagent grade water (Deionized)	(resistivity ≥ 18,2 MΩ × cm)
5.2.	Methanol	LC/MS grade
5.3.	2-Propanol	HPLC grade
5.4.	Acetonitrile	LC/MS grade
5.5.	Ammonium acetate	HPLC grade
5.6.	Acetic acid	HPLC grade

[1] Waters Acquity® UPLC BEH C18 column, 2,1 mm x 100 mm, 1,7 µm particle size, Part # 186002352C18 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.

5.7. Ammonium formate HPLC grade

5.8. Formic acid ≥ 98 % purity

5.9. Ammonium hydroxide Puriss. p.a

5.10. Isohexane (contains < 5 % n-Hexane) HPLC grade

5.11. 2,4-Dinitrophenylhydrazine ≥ 98 % purity

5.12. Formaldehyde ≥ 37 wt. % in water solution, stabilized with 10-15 % methanol

5.13. Acetaldehyde ≥ 98 wt. %

5.14. Crotonaldehyde ≥ 98 wt. %

NOTE: Instead of 5.12-5.14, a custom aldehyde standard in water may be purchased (1000 µg/ml Formaldehyde, 1000 µg/ml Acetaldehyde, 100 µg/ml Crotonaldehyde)

5.15. Formaldehyde-d2 approx. 20 wt. % in D_2O ≥ 98 atom % D

5.16. Acetaldehyde-d4 ≥ 98 wt. % ≥ 98 atom % D

5.17. Crotonaldehyde 2,4-Dinitrophenylhydrazone-3,5,6-d3 ≥ 98 atom % D

NOTE: Formaldehyde and Acetaldehyde are carcinogens or are suspected carcinogens. Crotonaldehyde might be lethal when inhaled. Appropriate safety precautions shall be taken when handling these compounds or any solution containing these compounds.

6. PREPARATION OF SOLUTIONS

6.1. DNPH-solution, 5 mg/ml

Add 0,5 g ± 0,02 g of DNPH to a 100-ml volumetric flask. Fill with approximately 50 ml of acetonitrile. Use an orbital shaker set to ~250 rpm to dissolve the DNPH. Fill to volume with acetonitrile and mix well. Transfer to an amber glass bottle.

NOTE: If the background levels of the target analytes are unacceptably high, the DNPH may be purified by recrystallization according to Appendix II. It is not necessary to recrystallize if the DNPH purity is ≥ 98 %.

6.2. Extraction solution, 100 mM ammonium formate, pH 3,0 (± 0,1)

To make a 2-liter solution, weigh and transfer 12,6 g ± 0,05 g of ammonium formate into a 2-liter volumetric flask containing approximately 1 liter of reagent grade water. Use a stir bar to dissolve the solids. Add approximately 0,9 liter of reagent grade water to the bottle and adjust the pH to 3,0 ± 0,1 with formic acid. Adjustment of pH will take approximately 34 ml of formic acid. Fill to volume with reagent grade water and mix well. The solution is stable for up to 2 weeks when stored at ambient temperature.

6.3. Mobile Phase A, 10 mM ammonium acetate buffer, pH 4,7 (± 0,1)

To make a 2-liter solution, weigh and transfer 1,54 ± 0,02 g of ammonium acetate (CH_3COONH_4) into a 2-liter volumetric flask containing approximately 1 liter of reagent grade water. Use a stir bar to dissolve the solids. Add approximately 0,9 liter of reagent grade water to the bottle and adjust the pH to 4,7 ± 0,1 with acetic acid. Adjustment of pH will take approximately 0,82 ml acetic acid. Fill to volume with reagent grade water and mix well. Filter the solution through a 0,45 µm membrane filter. The solution is stable for up to 2 weeks when stored at ambient temperature.

7. STANDARDS

All standards should be prepared in amber, or light protected glassware. Produce a series of enough calibration standards to cover the range of expected results to be found in the test samples, as in the example given below. For each standard, calculate the exact concentration based on actual amount weighed.

7.1. Internal standard solutions

The stock and working internal standard solutions are stable for approximately 4 weeks when stored at 4-6 °C.

7.1.1. Stock Internal Standard Solution Formaldehyde-d2, 10 mg/ml

NOTE: Formaldehyde-d2 solution is approximately 20 wt. %. Remember to compensate concentration according to the Certificate of Analysis when calculating the 10 mg/ml concentration.

Using the appropriate gastight syringe, weigh 1250 mg ± 50 mg of formaldehyde-d2 into a 25-ml volumetric flask containing approximately 10 ml of reagent grade water. Use an analytical balance placed in a fume hood or equipped with local exhaust ventilation to determine the exact mass to 0,0001 g. Dilute to volume with reagent grade water and mix well. Store in a 60-ml amber glass bottle with PTFE lined cap. Storage: Refrigerated.

7.1.2. Stock Internal Standard Solution Acetaldehyde-d4, 10 mg/ml

NOTE: Acetaldehyde-d4 should be refrigerator-cold when weighing it. Otherwise it will boil at room temperature.

Using the appropriate gastight syringe, weigh 250 mg ± 20 mg of acetaldehyde-d4 into a 25-ml volumetric flask containing approximately 10 ml of reagent grade water. Use an analytical balance placed in a fume hood or equipped with local exhaust ventilation to determine the exact mass to 0,0001 g. Dilute to volume with reagent grade water and mix well. Store in a 60-ml amber glass bottle with PTFE lined cap. Storage: Refrigerated.

7.1.3. Stock Internal Standard Solution Crotonaldehyde-DNPH-d3, 0,2 mg/ml

Weigh 10 mg ± 2 mg (determine the exact mass to 0,0001 g) of crotonaldehyde-DNPH-d3 into a 50-ml volumetric flask containing approximately 25 ml of acetonitrile using an analytical balance. Dilute to volume with acetonitrile and mix well. Store in a 60-ml amber bottle with PTFE lined cap. Storage: Refrigerated.

7.1.4. Working Internal Standard Solution (IS): Formaldehyde-d2 500 µg/ml, Acetaldehyde-d4 100 µg/ml, Crotonaldehyd-DNPH-d3 5,6 µg/ml (free aldehyde basis)

Transfer 5 ml formaldehyde-d2 stock internal standard solution, 1 ml acetaldehyde-d4 stock internal standard solution, and 10 ml of crotonaldehyde-DNPH-d3 stock internal standard solution into a 100-ml volumetric flask containing approximately 50 ml of acetonitrile. Dilute to volume with acetonitrile and mix well. Transfer the internal standard solution into a 125-ml amber bottle with PTFE lined cap. Storage: Refrigerated.

7.2. Calibration standard solutions

The stock calibration standard solutions are stable for approximately 2 weeks when stored at 4-6 °C. The working calibration standards are stable for approximately 24 hours at 4-6 °C.

7.2.1. Formaldehyde Stock Solution, 10 mg/ml

NOTE: Formaldehyde solution is approximately 37 wt. %. Remember to compensate concentration according to wt. % in Certificate of Analysis when calculating the 10 mg/ml.

Using the appropriate gastight syringe, weigh 2700 mg ± 50 mg of formaldehyde into a 100-ml volumetric flask containing approximately 50 ml of reagent grade water. Use an analytical balance placed in a fume hood or equipped with local exhaust ventilation to determine the exact mass to 0,0001 g. Dilute to volume with reagent grade water and mix well. Store in a 125-ml amber glass bottle with PTFE lined cap. Storage: Refrigerated.

7.2.2. Acetaldehyde Stock Solution; 10 mg/ml

NOTE: Acetaldehyde should be refrigerator-cold when weighing it. Otherwise it will boil at room temperature.

Using the appropriate gastight syringe, weigh 1000 mg ± 25 mg of acetaldehyde into a 100-ml volumetric flask containing approximately 50 ml of reagent grade water. Use an analytical balance placed in a fume hood or equipped with local exhaust ventilation to determine the exact mass to 0,0001 g. Dilute to volume with reagent grade water and mix well. Store in a 125-ml amber glass bottle with PTFE lined cap. Storage: Refrigerated.

7.2.3. Crotonaldehyde Stock Solution, 1 mg/ml

Using the appropriate gastight syringe, weigh 100 mg ± 5 mg of crotonaldehyde into a 100-ml volumetric flask containing approximately 50 ml of reagent grade water. Use an analytical balance placed in a fume hood or equipped with local exhaust ventilation to determine the exact mass to 0,0001 g. Dilute to volume with reagent grade water and mix well. Store in a 125-ml amber glass bottle with PTFE lined cap. Storage: Refrigerated.

7.2.4. Stock Calibration Standard Solution 1, 1000 µg/ml formaldehyde, 1000 µg/ml acetaldehyde, and 100 µg/ml crotonaldehyde

Using the appropriate mechanical pipette or gastight syringe, weigh 10,0 g ± 0,1 g of each of the stock solutions in 7.2.1-7.2.3, into a 100-ml volumetric flask containing approximately 50 ml of reagent grade water. Record the exact weights. Dilute to volume with reagent grade water and mix well. Store in a 125-ml amber glass bottle with PTFE lined cap.
Storage: Refrigerated.

7.2.5. Stock Calibration Standard Solution 1 from purchased custom Aldehyde solution

Instead of preparing solutions 7.2.1-7.2.4, a custom aldehyde solution, a standard solution containing 1000 µg/ml formaldehyde, 1000 µg/ml acetaldehyde, and 100 µg/ml crotonaldehyde in water may be used. The expiration date and storage conditions are provided by the manufacturer.

7.2.6. Stock Calibration Standard Solution 2

Using the appropriate gastight syringe, weigh 1,00 g ± 0,01 g Stock Calibration Standard Solution 1 (from 7.2.4 or 7.2.5) on an analytical balance using a gastight syringe and record the weight. Then transfer the material into a 10-ml volumetric flask containing ~5 ml of reagent grade water. Dilute to volume with reagent grade water and mix well.. Store in a 10-ml amber bottle with PTFE lined cap. Storage: Refrigerated.

7.2.7. Stock Calibration Standard Solution 3

Transfer 5,0 ml of Stock Calibration Standard Solution 2 into a 25-ml volumetric flask containing ~15 ml of reagent grade water using the appropriate mechanical pipette or gastight syringe. Dilute to volume with reagent grade water and mix well. Store in a 30-ml amber bottle with PTFE lined cap. Storage: Refrigerated.

7.2.8. Stock Calibration Standard Solution 4

Transfer 5,0 ml of Stock Calibration Standard Solution 3 into a 50-ml volumetric flask containing ~25 ml of reagent grade water using the appropriate mechanical pipette or gastight syringe. Dilute to volume with reagent grade water and mix well. Store in a 60-ml amber bottle with PTFE lined cap. Storage: Refrigerated.

7.2.9. Working Calibration Standards

The Working Calibration Standards are prepared at the same time as samples and according to the same procedure as the samples, but without the tobacco. See section 8.3 for a detailed description. Working Calibration Standards 1-8 are prepared using Stock Calibration Standard Solutions 2, 3, and 4 according to Table 1 in combination with 100 µl Working Internal Standard solution per standard. The calibration range for crotonaldehyde begins at calibration level 3 and therefore does not include calibration level 2. Prepared calibration standards should immediately be transferred to amber autosampler vials with PTFE lined caps and placed in the autosampler set to ~5 ºC. Storage: Refrigerated.

Stability studies should be performed by the laboratory to determine the standard's shelf life.

NOTE: The linearity range should be determined for each lab/instrument to fit the instruments capabilities and the range of samples measured in that laboratory.

NOTE: Calibration standards and samples must be prepared and analyzed together using the same procedure described in section 8.3. A batch of standards and samples has been shown to be stable for up to 24 h after preparation, when analyzed together. For this reason, instrumental analysis should be initiated as soon after sample and standard preparation as possible, preferably, within one hour.

Table 1. Concentration and preparation of Carbonyl Calibration Standards

Calibration Level	Stock Calibration Standard Used	Volume Stock Calibration Std (µl)	Form-aldehyde (µg/ml)	Acet-aldehyde (µg/ml)	Croton-aldehyde (µg/ml)
1	NA	0	0	0	0
2	4	50	0,010	0,010	NA
3	4	250	0,050	0,050	0,005
4	4	500	0,100	0,100	0,010
5	3	100	0,200	0,200	0,020
6	3	300	0,600	0,600	0,060
7	3	500	1,000	1,000	0,100
8	2	200	2,000	2,000	0,200

8. PROCEDURES

8.1. Sampling

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

8.2. Sample preparation

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

8.2.1. Smokeless tobacco products in the form of plug, flake, bits, loose-leaf or pellets shall be ground prior to analysis. The sample should be reduced in size to pass through a 4-mm screen. It is important that the size reduction procedure should be performed so it does not generate excessive heat. After grinding the samples should be analyzed directly or within two days if stored in refrigerator. Otherwise the samples need to be stored in freezer.

NOTE: Do not grind samples more than 30 seconds at room temperature. Longer grinding time at room temperature might cause evaporation of carbonyls.

8.2.2. The recommended procedure for portioned products is to analyze unit pouches by cutting the pouch in half and adding the tobacco and pouch material to the extraction vessel.

8.2.3. Smokeless tobacco in a form which would pass through a 4-mm screen does not need to be ground prior to analysis.

NOTE: Insufficient equilibration time for tobacco 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.

8.2.4. The recommended procedure for cigarette fillers is to slit 20 cigarettes, and place the filler in a sealed bottle and mix. Do not include the cigarette paper in the analysis. Samples shall not be ground.

8.2.5. Samples shall be mixed immediately prior to weighing to ensure sample homogeneity.

8.2.6. The test samples shall be stored in an airtight container and protected from light.

8.3. Sample extraction

Samples and Working Calibration Standards are always prepared at the same time.

8.3.1. Using an analytical balance, weigh 1,0 g ± 0,2 g of sample into a tared extraction vessel (e.g. 50 ml E-flask) and record the exact weight to 0,001 g.

8.3.2. Add 40,0 ml of 100 mM ammonium formate (pH 3,0) to the extraction vessels with tobacco using a dispensette.

8.3.3. Add 40,0 ml of 100 mM ammonium formate (pH 3,0) to the extraction vessels for preparation of the Working Calibration Standards (without tobacco) using a dispensette.

8.3.4. Accurately pipette the specified volumes of Stock Calibration Standards given in Table 1 to prepare the Working Calibration Standards.

8.3.5. Add 100 µl of Working Internal Standard Solution using a repeater pipette to all samples and Working Calibration Standards.

8.3.6. Add 1.0 ml of DNPH-solution using a repeater pipette to all samples and Working Calibration Standards.

8.3.7. Add 10 ml of isohexane using a repeater pipette or dispensette to all samples and Working Calibration Standards.

8.3.8. Cap all extraction vessels immediately after addition of isohexane.

8.3.9. Shake the sample(s) for 60 ± 5 minutes at a rate to ensure sufficient mixing.

8.3.10. It is important to start transferring isohexane from the extraction vessel to the autosampler vials within 5 minutes from the completion of shaking and to finish the isohexane transfer within 30 minutes.

Transfer approximately 1,5 ml of the isohexane extracts from each extraction vessel , using a glass or plastic Pasteur pipette (without filtering), to amber autosampler vials. Ensure that no buffer solution or tobacco particles are transferred into the autosampler vials.

8.3.11. The extract is ready for injection into the UHPLC-MS/MS system. The Working Calibration Standards and samples must be prepared and analyzed together using the same procedure. A set of standards and samples is stable for up to 24 hours after preparation when analyzed together. For this reason, instrumental analysis should be initiated as soon after sample and standard preparation as possible, preferably within one hour.

9. DETERMINATION

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

9.1. Suggested UHPLC parameters

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

- Column Temperature: 60,0 °C
- Autosampler Temperature: 4-6 °C
- Injection Volume: 1 µl
- Flow rate: 0,45 ml/min
- Mobile phase A: 10 mM Ammonium acetate, pH 4,7
- Mobile phase B: Acetonitrile

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

Table 2 - UHPLC gradient

Time (min)	Flow (ml/min)	Mobile Phase A (%)	Mobile Phase B (%)	Gradient type
0	0,45	45	55	Initial
0,2	0,45	45	55	Linear
1,0	0,45	0	100	Linear
1,5	0,45	0	100	Linear
1,8	0,45	45	55	Linear
4,1	0,45	45	55	Linear

9.2. MS/MS parameters

The triple quadrupole mass spectrometer shall be operated in negative 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. The dwell times need to have been optimized to achieve accurate quantification, the number of data points across each peak should be approximately 15 to 20. Once optimized, the same UHPLC-MS/MS conditions must be used for the analysis of all standards and samples.

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

9.2.1. 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 product ion, known as the qualifier ion, listed. 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 carbonyls. In order to calculate the ion ratio, the ions used in the calculations must be present and have a signal-to-noise ratio ≥10.

Table 3 - Quantification and Qualification transitions for Carbonyls

Name	Quantification Transition (m/z)	Qualification Transition (m/z)	Internal Standard Reference
Formaldehyde-DNPH	209 > 163	209 > 151	Formaldehyde-d2-DNPH
Formaldehyde-d2-DNPH	211 > 133	n/a	n/a
Acetaldehyde-DNPH	223 > 151	223 > 122	Acetaldehyde-d4-DNPH
Acetaldehyde-d4-DNPH	227 > 151	n/a	n/a
Crotonaldehyde-DNPH	249 > 172	249 > 163	Crotonaldehyde-DNPH-d3
Crotonaldehyde-DNPH-d3	252 > 175	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.

9.3. System Suitability

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

9.4. Calibration

Set the quantitation method to perform an internal standard linear calibration with 1/y weighting. The calibration graph is a response of the area ratio of each analyte to the corresponding internal standard. The linear correlation shall not be forced through the origin. Standards are analyzed at the beginning and the end of the analytical run and the values for each level are averaged to generate the calibration curve.

9.5. Calculations

9.5.1. 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

9.5.2. The concentration of the target analyte in a sample (µg/g) is determined using the 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)

9.6. Quality Control

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

10. SUGGESTED SPECIAL PRECAUTIONS

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.

11. REPEATABILITY AND REPRODUCIBILITY

An international collaborative study involving 12 laboratories that used the specified UHPLC-MS/MS method was conducted by the CORESTA TTPA in 2016/2017[2]. The study included the analysis of seven smokeless tobacco products and two cigarette fillers. Results were analyzed in basic conformance with ISO 5725-2:1994 and ISO/TR 22971:2005. The mean values, %r, and %R for formaldehyde and acetaldehyde are presented in Tables 4 and 5. The value of 'N' is the number of the laboratories used to determine the statistics after the removal of outliers.

[2] CORESTA Tobacco and Tobacco Products Analytes Sub-Group Technical Report – Select Carbonyls in Tobacco and Tobacco Products, 2016/2017 Collaborative Study - January 2018

Table 4 - Results from the 2016/2017[2], Collaborative Studies for Formaldehyde

Sample Type	N	Mean Form-aldehyde (µg/g)	Repeatability		Reproducibility	
			r	%r	R	%R
CRP1 - Swedish style snus pouch	9	1,03	0,151	14,7	0,623	60
CRP2 - American-style loose moist snuff	9	1,18	0,263	22,2	0,675	57
CRP3 - American-style loose dry snuff powder	9	7,77	1,142	14,7	4,737	61
CRP1.1 - Swedish style snus pouch	11	1,34	0,361	27,0	0,890	67
CRP2.1 - American-style loose moist snuff	11	2,43	0,378	15,6	1,440	59
CRP3.1- American-style loose dry snuff powder	11	3,39	0,485	14,3	1,736	51
CRP4.1 – American-style loose-leaf chewing tobacco – long cut format	10	0,36	0,134	37,0	0,272	75
3R4F Cigarette Filler	8	1,34	0,248	18,5	1,171	87
1R6F Ground Cigarette Filler	9	1,26	0,230	18,2	0,962	76

Table 5 - Results from the 2016/2017[2], Collaborative Studies for Acetaldehyde

Sample Type	N	Mean Acet-aldehyde (µg/g)	Repeatability		Reproducibility	
			r	%r	R	%R
CRP1 - Swedish style snus pouch	9	10,11	1,686	16,7	5,438	54
CRP2 - American-style loose moist snuff	9	3,74	0,573	15,3	2,489	67
CRP3 - American-style loose dry snuff powder	9	3,02	0,540	17,9	3,140	104
CRP1.1 - Swedish style snus pouch	11	7,37	1,330	18,1	3,838	52
CRP2.1 - American-style loose moist snuff	11	4,65	0,760	16,3	1,822	39
CRP3.1- American-style loose dry snuff powder	11	6,93	1,014	14,6	3,063	44
CRP4.1 – American-style loose-leaf chewing tobacco – long cut format	11	1,34	0,355	26,6	1,032	77
3R4F Cigarette Filler	9	1,40	0,444	31,7	1,749	125
1R6F Ground Cigarette Filler	10	1,33	0,406	30,5	1,436	108

12. VALIDATION OF CROTONALDEHYDE

In this collaborative study, crotonaldehyde was generally not reported, either because it was not detected or was detected below the limit of quantitation. Consequently, no statistical data analysis could be performed for crotonaldehyde. For this reason, a limited validation of crotonaldehyde involving repeatability and accuracy has been performed by one participating laboratory. The repeatability and accuracy study of crotonaldehyde is described in CORESTA Tobacco and Tobacco Products Analytes Sub-Group Technical Report – "2016/2017 Select Carbonyls in Tobacco and Tobacco Products - Collaborative Study", November 2017.

12.1. Repeatability and Accuracy by fortified matrix spikes

As described in the 2017 Technical Report, an experiment using laboratory fortified matrix spikes was conducted to determine if the analytical method accurately measures the concentration of the analyte in the presence of sample matrix components. The investigated sample types were CRP1, CRP2, CRP3, CRP4 and 3R4F cigarette filler.

The repeatability for crotonaldehyde in the fortified samples was in the range 1 - 10 %RSD, which would correspond to r% = 2,8 - 28. The accuracy for crotonaldehyde in the fortified samples was in the range 84 % - 102 %.

The unfortified analyte levels for crotonaldehyde were below the LOQ for all reference products except CRP3. Therefore, the sample concentration of all unspiked samples, except CRP3, were not quantified. Without having a quantitative level of crotonaldehyde for the unspiked samples, actual recoveries may be more variable than in the spiked samples and potentially overestimated in the spiked samples. The recovery data presented here fell within the range of 80 % - 120 % of the target value for all fortification levels which meets the acceptance criteria. Based on these results, the method is able to quantify crotonaldehyde but with an unknown level of confidence since no statistical data evaluation from the collaborative study data could be performed.

13. REPORT

The test report shall state the yield of carbonyls in micrograms per gram tobacco (wet weight) and the method used shall include all conditions which may affect the results. 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.

APPENDIX I – EXAMPLE CHROMATOGRAMS

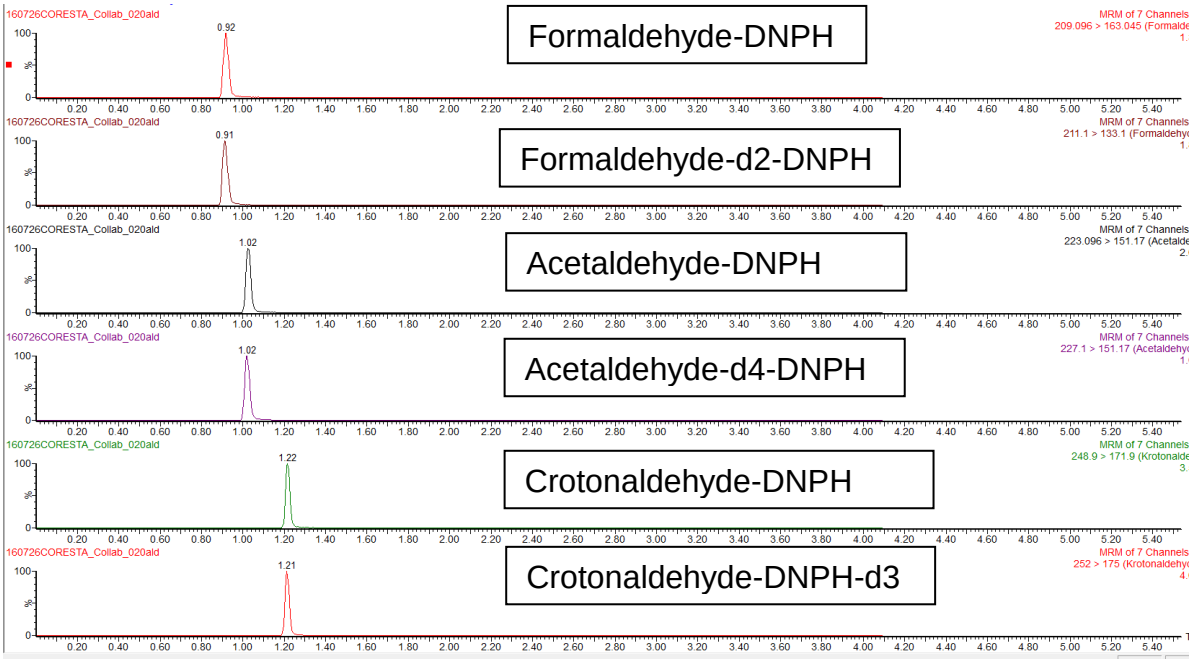

Figure 1 - Example of a MRM-chromatogram for a Formaldehyde, Acetaldehyde and Crotonaldehyde standard (0,1 µg/ml).

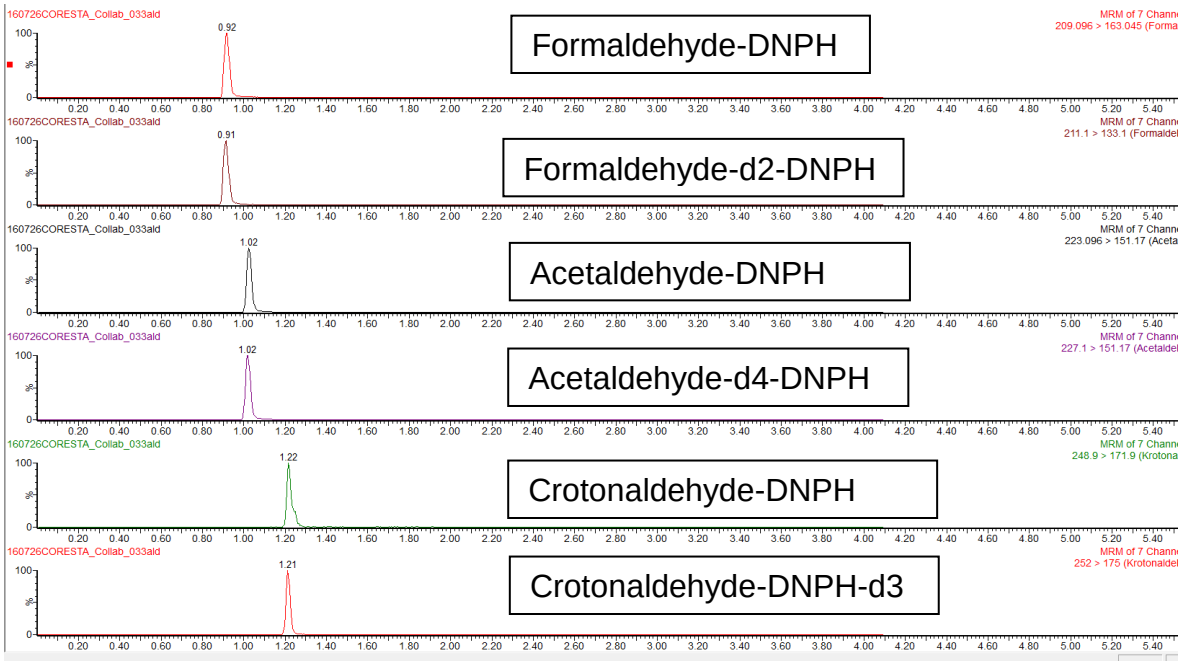

Figure 2 - Example of a MRM-chromatogram for a tobacco product (CRP2).

APPENDIX II – DNPH RECRYSTALLIZATION PROCEDURE

The procedure describes the process for purification 2,4-Dinitrophenylhydrazine (DNPH) by recrystallization.

1. EQUIPMENT REQUIRED

1.1. Hot plate with magnetic stirrer

1.2. Magnetic stir bar

1.3. Analytical balance

1.4. Watch glass

1.5. 0,5-liter beaker

1.6. Thermometer

2. CHEMICALS AND REAGENTS REQUIRED

2.1. Acetonitrile

2.2. 2,4-Dinitrophenylhydrazine (DNPH)

3. PURIFICATION RINSE

3.1. Add approximately 0,5 g of DNPH to a watch glass and allow to dry for approximately 24 hours at ambient conditions.

4. FIRST RECRYSTALLIZATION

4.1. Rinse all glassware to be used with acetonitrile. Work in ventilated hood.

4.2. Weigh approximately 10 g DNPH (~30 % water) into a 0,5-liter beaker containing approximately 150 ml acetonitrile. Add a magnetic stirrer bar.

4.3. Cover beaker with a watch glass and heat on hot plate to a slow boil (~84 ºC) using magnetic stirring for 1 hour.

4.4. After 1 hour, remove the watch glass and continue to boil until approximately 30 ml of liquid is left on top of the crystals.

4.5. Lower heat to between 40 ºC and 60 ºC, and let evaporate until approximately 5 ml of liquid is left.

NOTE: Do not bring the crystals to dryness.

4.6. Let cool.

4.7. Decant the liquid to waste and wash twice with 5 ml acetonitrile.

4.8. Remove about 200 mg of washed crystals for purity testing.

4.9. Let the 200 mg crystals dry protected but with access to air at ambient temperature for 24 hours.

4.10. Recrystallize the remaining crystals a second time following the procedure described in section 5, below.

5. SECOND RECRYSTALLIZATION

5.1. Add another 150 ml acetonitrile to the 0,5-liter beaker containing the washed and recrystallized DNPH (section 4.7).

5.2. Repeat steps 4.3 to 4.9 described in the first recrystallization.

5.3. Let all of the crystals dry on a watch glass at ambient temperature for 24 hours.

6. PURITY TESTING

6.1. The three dried DNPH samples are to be tested; crude DNPH, 1-recrystallization and 2-recrystallizations.

6.2. Weigh 50 mg ± 0,2 mg of DNPH in a 10-ml measuring flask. Fill with approximately 50 ml of acetonitrile. Use an orbital shaker set to ~250 rpm for 1 hour to dissolve the DNPH. Fill to volume with acetonitrile and mix well.

6.3. Prepare DNPH regent blanks (Cal 1) according to method (1 ml DNPH solution, 40 ml Extraction solution, 100 µl ISTD, 10 ml Isohexane) and test on UHPLC-MS.

6.4. Calculate the Purification (%) for 1- and 2-recrystallizations for each of formaldehyde, acetaldehyde , and crotonaldehyde. Use the peak areas in the equation below.

$$\textbf{Purification}\ (\%) = \textbf{100} \times \frac{(\textbf{Crude} - \textbf{Recrystillized})}{\textbf{Crude}}$$