Tobacco and Tobacco Products Analytes Sub-Group

Technical Report

2016/2017 Select Carbonyls in Tobacco and Tobacco Products Collaborative Study

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1. Summary

At the Smokeless Tobacco Sub-Group (STS) meeting (now named Tobacco and Tobacco Products Analytes Sub-Group, TTPA) in Lausanne in April 2016 it was decided that a final collaborative study for the following carbonyls (aldehydes) in tobacco and tobacco products would be initiated: formaldehyde, acetaldehyde and crotonaldehyde. Seven smokeless tobacco products and two cigarette fillers were included in the study. The collaborative study protocol and draft method were distributed to the laboratories in May 2016. Twelve laboratories submitted study results, which were presented at the TTPA-meeting held in Charlottesville in May 2017. This Technical Report includes tabulated data, repeatability (r%) and reproducibility (R%) values as well as an investigation of the stability of carbonyls affected by transportation, grinding, and refrigerated storage after weighing aliquots.

2. Introduction

In 2012, FDA provided an abbreviated list of Harmful and Potentially Harmful Constituents (HPHC) that included three carbonyls (aldehydes) in smokeless tobacco products: formaldehyde, acetaldehyde, and crotonaldehyde. Therefore, the Smokeless Tobacco Sub-group (STS, later renamed TTPA) set an objective to develop a CORESTA Recommended Method (CRM) for these select carbonyls. The development of the CRM for select carbonyls in tobacco and tobacco products originated with an investigational study at the end of 2012. In the investigational study, all participating labs used their own in-house methods, which included several derivatization techniques and instrumental platforms (GC-MS, HPLC-UV, HPLC-MS/MS and UHPLC-MS/MS). The study gave highly variable results with up to 10-fold differences in concentration of the carbonyls in CORESTA Reference Product 2 (CRP2).

As a consequence of the highly variable results, a group of four laboratories formed a method development group to investigate two of the proposed methods which included a GC-MS method using pentafluorobenzylhydroxyl amine (PFBHA) as a derivatizing agent and a UHPLC-MS/MS method utilizing 2,4-dinitrophenyl hydrazine as a derivatizing agent. The two methods gave equivalent results and a Sub-Group decision was taken to move forward with the UHPLC-MS/MS method due to higher throughput. At this stage, there were requests to transform the UHPLC-method into an HPLC-method using the same column and preferably the same mobile-phases as in the TSNA-method (CRM No.72) in order to facilitate the work-flow in the laboratories. Such an HPLC-MS/MS method was developed and evaluated in a collaborative study with seven participating laboratories and the results were presented at the STS-meeting in Hangzhou in April 2015. However, the crotonaldehyde standard curve linearity was not satisfactory and there were also concerns regarding the robustness of the HPLC-method. In addition, the group agreed at the meeting to investigate the stability of carbonyl concentrations to determine if they are affected by transportation, grinding, and refrigerated storage after weighing out aliquots.

The results of the carbonyl stability tests were presented at the STS meeting in Jeju, South Korea in October 2015. At this meeting, the method development group also suggested to switch back to an UHPLC-MS/MS method, which utilizes an internal standard for crotonaldehyde. The new method solved the linearity problems for crotonaldehyde and improved the robustness for all carbonyls. At the following STS meeting in Lausanne, April 2016 it was decided that a final collaborative study would be performed.

2.1 Objective

The objective of this study was to evaluate the method developed within the STS/TTPA for the determination of carbonyls in tobacco products including smokeless tobacco products and cigarette filler. The data were statistically evaluated in basic conformance with the recommendations of ISO 5725-2 to assess within (r) and between laboratory (R) variability. A CRM was to be drafted if the results of the study suggested the method is sufficiently robust for the determination of carbonyls in tobacco products.

The objective of the carbonyl stability investigation was to investigate how the carbonyl concentrations were affected by transportation, grinding, and refrigerated storage after weighing out aliquots and if these parameters contributed to the variability of the method. The carbonyl stability investigation is presented in Appendix C.

3. Organization

3.1 Participants

A list of the 14 participating laboratories is provided in Table 1. One laboratory was excluded from the statistical evaluation because of non-compliance with the protocol. The laboratories are listed in alphabetical order in Table 1. Number codes were assigned to each laboratory and do not correspond to the order shown in the table below.

Table 1: Participating laboratories in the 2016/2017 select carbonyls in tobacco and tobacco products study

2016/2017 Carbonyl Study Participants
Altria Client Services LLC, ACE, United States
Altria Client Services LLC, ELLPSS, United States
Enthalpy Analytical Inc., United States
Essentra Scientific Services, United Kingdom
Eurofins Food & Feed Testing Sweden AB, Sweden
Global Laboratory Services Inc., United States
Imperial Tobacco, Germany
R.J. Reynolds Tobacco Company, United States
Shanghai Tobacco Group Co. Ltd., China
Swedish Match North Europe, Sweden
Swisher International, United States
ZTRI Zhengzhou, China
Swisher International, United States
University of Kentucky, United States

3.2 Protocol

The study protocol is provided in Appendix A and the UHPLC-MS/MS method is provided in Appendix B. Specific details from the protocol are described below:

3.2.1 Sample Shipment

Laboratories were responsible for procuring the seven CORESTA Reference Products (CRP) from North Carolina State University and preground 1R6F filler and 3R4F cigarettes from the University of Kentucky. Laboratories were requested to store the samples at approximately −20 °C upon receipt. Laboratories were requested to conduct the study between June 1 and August 15, 2016, and report data by August 15, 2016. Later the date for data reporting was extended to December 21, 2016. The samples are identified in Table 2.

Table 2: Sample Identification

Samples
2009 CRP1 - Swedish style snus pouch
2009 CRP2 - American-style loose moist snuff
2009 CRP3 - American-style loose dry snuff powder
2016 CRP1.1 - Swedish style snus pouch
2016 CRP2.1 - American-style loose moist snuff
2016 CRP3.1 - American-style loose dry snuff powder
2016 CRP4.1 - American-style loose-leaf chewing tobacco – long cut format
1R6F ground filler-Lot RT1
3R4F cigarettes

3.2.2 Within Laboratory Sample Preparation

The laboratories were directed to remove samples from the −20 °C freezer and place the unopened samples in a refrigerator for 24 to 48 hours to ensure water was fully equilibrated. Samples could then be removed from the refrigerator for 1 to 2 hours prior to analysis and allowed to equilibrate to room temperature before opening. Special handling requirements are described below:

CRP1 and CRP1.1: Unit pouches of portioned smokeless tobacco products were to be analyzed. The pouches were cut in half and the tobacco and pouch were added directly to the digestion vessel. The three (3) aliquots were taken from the same can.

All other CRPs and 1R6F: The tobacco in the can or bottle was mixed with a spatula before weighing out the aliquots. The three (3) aliquots were taken from the same can.

3R4F: The filler from 20 cigarettes was removed from the cigarette paper and filter and placed in a bottle and mixed. The three (3) aliquots were taken from the same bottle. Samples were not ground.

3.2.3 Sample Analysis and Data Reporting

The participating laboratories were instructed to conduct triplicate analyses (individual tobacco weighing) for the carbonyls using the method given in Appendix B.

Participating laboratories were requested to document any deviations from the protocol and the method and submit the deviations with their results. As stated in the protocol, data submitted with significant deviations from the method would be excluded from the study. Deviations reported by the laboratories are identified below. Lab 12 was excluded from the study because of the reported deviations. The other laboratory deviations were minor, so no other data sets were excluded from the study for protocol deviations.

- Lab 2: Minor deviations. The laboratory did not recrystallize the DNPH and a chromatographic flow rate of 0,15 ml/min was used instead of the specified flow rate of 0,45 ml/min.
- Lab 4: Minor deviation. The laboratory did not use an in-line column filter, instead the lab used a guard column Waters Acquity UPLC BEH C18 VanGuard column, (2,1 mm × 100 mm X 1,7 µm), part #186003975.
- Lab 7: Minor deviations. The laboratory did not recrystallize the DNPH and used C13-marked internal standard instead of deuterated.
- Lab 8: Minor deviation. The laboratory did not recrystallize the DNPH.
- Lab 9: Minor deviation. The laboratory did not recrystallize the DNPH.
- Lab 10: Minor deviations. The laboratory did not recrystallize the DNPH and did not use a tandem mass spectrometer. Instead the laboratory used a Q-Exactive, which is a hybrid Quadrupole-Orbitrap. Samples CRP1, CRP2 and CRP3 had been stored too long in room temperature. For these reasons, the results for CRP1, CRP2 and CRP3 were excluded from the statistical evaluation.
- Lab 11: Minor deviation. The laboratory did not recrystallize the DNPH.
- Lab 12: Major deviation. The laboratory used pre-derivatized formaldehyde and acetaldehyde internal standards instead of the specified free carbonyl forms. No results were included in the statistical evaluation.

All test results were to be reported on an *as-is* basis without correction for moisture content. The results were not to be rounded and ideally reported to at least one more digit than typically required. The study results and the comments were to be sent by e-mail to the study coordinators.

4. Data – Raw

The full data set for the study is provided in Appendix D, except for Lab 12 data which were excluded from the study. The results are presented on an as-is basis, without correction for moisture. Each analysis includes three replicates. Not all laboratories provided data for all samples. Data sets were removed from the repeatability (r) and reproducibility (R) (r & R) portion of the study if the data were identified as outlying data. Those data are included in Appendix D, but were eliminated prior to the r & R analysis. Raw data plots that include all replicates, without removal of outliers, are given in Appendix E.

5. Data – Statistical Analysis

The statistical analysis was conducted in basic conformance with ISO 5725-2:1994 and ISO/TR 22971:2005. A summary of the results from outlier detection and the calculated results for repeatability (r) and reproducibility (R) are given below in sections 5.1 and 5.2, respectively. Raw data plots that include all replicates, without removal of outliers, are shown in Appendix E. Crotonaldehyde was generally not reported, either because it was not detected or was detected below the limit of quantitation. For that reason, the statistical analysis only includes acetaldehyde and formaldehyde.

5.1 Exclusion of Outliers

Procedures outlined in ISO 5725-2:1994 and ISO/TR 22971:2005 were generally used for the exclusion of outliers. An adaptation of Levene's Test was used for eliminating laboratories with overly large repeatability standard deviations and Grubbs' Test was used to eliminate laboratories with outlying mean values.

ISO 5725(2) also recommends the use of Mandel's h and k plots. Mandel's h statistic is the same as the statistic used in Grubbs' Test. Similarly, Mandel's k statistic, associated with within lab standard deviation, is statistically equivalent to the c-value calculated in Cochran's Test ($k = \sqrt{n_{labs}c}$). However, the critical values associated with Mandel's h and k statistics do not make allowance for multiple testing and can therefore, give a false impression of statistical significance. Thus, Mandel's h and k statistics do not add fundamentally new information and may lead to incorrect conclusions. For those reasons, we do not include Mandel's h and k plots.

The intent of ISO 5725-2:1994 is to eliminate outliers that exceed a 1 % critical value. This was accomplished by an adaptation of Levene's Test. Levene's Test is preferable to Cochran's Test, which is recommended in ISO 5725-2:1994, because of Cochran's Test's extreme sensitivity to deviations from normality. Levene's Test is mentioned in ISO/TR 22971:2005 as an alternative to Cochran's Test. However, Levene's Test does not directly apply without adaptation. For more details, see the footnote below.[1]

Grubbs' Test and an adaptation of Levene's Test were applied at the standard nominal 1 % significance level to determine outliers and the results are shown in Table 3.

Table 3: Outliers

Product	Analyte	Levene's Outlier Lab	Grubbs' Outlier Lab
CRP 1	Formaldehyde	2[a]	–
CRP 2.1	Acetaldehyde	4[b]	–
1R6F	Formaldehyde	4	–
3R4F	Formaldehyde	3	–
CRP 4.1	Formaldehyde	–	5

The (**–**) symbol indicates there was not an outlier of the indicated type.
a. Rep 2 was dropped as a single-point outlier for both formaldehyde and acetaldehyde.
b. Rep 3 was dropped as a single-point outlier for acetaldehyde.

[1] Levene's Test is commonly used to determine if each of several subpopulations have the same variance. Since it was designed to test for overall differences, not to determine if the largest variance is significantly greater than the others, some adaptation is necessary to use the approach to eliminate laboratories whose within lab variation is too large. Levene's Test was adapted to this purpose by Morton, who presented the approach utilized in this report at the 2014 CORESTA Congress (Quebec, Canada, presentation ST28, October 14, 2014). Specifically, the approach taken here is a two-step process with a lab being eliminated as an outlier if both steps are statistically significant. First, Levene's Test was run at a nominal α-level of 0,02. Second a comparison of the largest variance to the remaining variances is carried out at a one-sided nominal level of α=0,01/number of labs. Dividing by the number of labs is to account for multiple testing, since it is not known a priori which lab will have the largest variance. Simulation studies were carried out by Morton and presented at the 2014 CORESTA Congress and these results demonstrated that this process has an overall α-level near 0,01 and is robust to deviations from normality.

5.2 Calculation of Repeatability (r) and Reproducibility (R)

After removal of outlying data based on numerical data consistency methods (Grubbs' Test and Levene's Test), the final repeatability and reproducibility (r & R) results were calculated. The r & R results are shown in Table 4. The r & R results reflect both laboratory variability and product consistency. As stated above, crotonaldehyde was generally not reported, either because it was not detected or was detected below the limit of quantitation. For that reason, the r & R results are not presented for crotonaldehyde.

Table 4: Repeatability (r) and Reproducibility (R) Limits

Analyte	Product	N° of Labs*	Mean	Repeatability		Reproducibility	
				r	% r	R	% R
Formaldehyde	CRP1	9	1,03	0,151	14,7	0,623	60
Formaldehyde	CRP2	9	1,18	0,263	22,2	0,675	57
Formaldehyde	CRP3	9	7,77	1,142	14,7	4,737	61
Formaldehyde	CRP1.1	11	1,34	0,361	27,0	0,890	67
Formaldehyde	CRP2.1	11	2,43	0,378	15,6	1,440	59
Formaldehyde	CRP3.1	11	3,39	0,485	14,3	1,736	51
Formaldehyde	CRP4.1	10	0,36	0,134	37,0	0,272	75
Formaldehyde	3R4F filler	8	1,34	0,248	18,5	1,171	87
Formaldehyde	1R6F ground filler	9	1,26	0,230	18,2	0,962	76
Acetaldehyde	CRP1	9	10,11	1,686	16,7	5,438	54
Acetaldehyde	CRP2	9	3,74	0,573	15,3	2,489	67
Acetaldehyde	CRP3	9	3,02	0,540	17,9	3,140	104
Acetaldehyde	CRP1.1	11	7,37	1,330	18,1	3,838	52
Acetaldehyde	CRP2.1	11	4,65	0,760	16,3	1,822	39
Acetaldehyde	CRP3.1	11	6,93	1,014	14,6	3,063	44
Acetaldehyde	CRP4.1	11	1,34	0,355	26,6	1,032	77
Acetaldehyde	3R4F filler	9	1,40	0,444	31,7	1,749	125
Acetaldehyde	1R6F ground filler	10	1,33	0,406	30,5	1,436	108

*This is the number of laboratory data sets reported as values and after removal of outliers.

6. Validation of Crotonaldehyde

As seen in Appendix D, crotonaldehyde was generally not reported, because it was detected near or below the quantitation limit. Consequently, no statistical data analysis could by performed for crotonaldehyde. Several other options were considered in order to generate crotonaldehyde data for a statistical analysis, but none of the following were believed to offer value: First, no other tobacco samples have been found that contain measurable levels of crotonaldehyde (above 0,05 µg/g). Secondly, the group considered fortifying or spiking tobacco with crotonaldehyde for distribution to the participating members for analysis, but crotonaldehyde would not be stable in the spiked tobacco. Instead of validation with data from a collaborative study, a limited validation of crotonaldehyde involving repeatability and accuracy was performed by one laboratory.

6.1 Crotonaldehyde Repeatability and Accuracy by Fortified Matrix Spikes

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 method specified in this study was followed except that the know quantities of analyte were added during the extraction process. Specifically, this experiment was conducted by adding the tobacco, extraction solution, and fortification solution to the extraction vessel. Next the internal standard, DNPH, and isohexane were added and the samples were rotated for the specified time.

Each sample was fortified at three levels and each fortification level was prepared in triplicate. The average value (n=3) of analyte determined in the unfortified matrix was used for the unspiked sample concentration in the equation below. The percent recovery for the individual replicates as well as the mean % recovery, standard deviation, and % relative standard deviation (%RSD) for each level, and each analyte are listed in Appendix F.

$$\text{Accuracy (\%)} = 100\text{x}\ \frac{(\text{Spiked Sample Conc} - \text{Unspiked Sample Conc})}{\text{Theoretical conc}}$$

Spiked Sample Conc = concentration determined experimentally for the fortified sample
Unspiked Sample Conc = concentration determined experimentally for the unfortified sample
Theoretical Conc = the theoretical concentration of the spike in the resulting sample

The relative standard deviation 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 set to zero. Without having a quantitative level of crotonaldehyde for the unspiked samples, recoveries may be more variable and potentially overestimated. 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.

7. Data Interpretations

Generally speaking, the variability of this analytical method for the quantification of formaldehyde and acetaldehyde is relatively large. Nonetheless the method specified in the enclosed protocol represents a very large improvement over the years of development and variability has been reduced to the lowest possible level for a consensus standardized method. For these reasons, this method is believed to be fit for its intended purposes. Due to the fact that the levels of crotonaldehyde were at or below the limit of quantitation, a statistical analysis could not be performed on this analyte. Instead internal validation data of repeatability and accuracy has been performed. Repeatability results of the crotonaldehyde-spiked samples were in line with the repeatability of formaldehyde and acetaldehyde and the accuracy results were within the accepted range of 80 % - 120 %. Based on these results, the method is able to quantify crotonaldehyde, but with an unknown level of confidence.

8. Recommendations

The consensus of the STS/TTPA is to recommend the analytical procedure used in this collaborative study as a CORESTA Recommended Method.

9. Appendices

Appendix A: Study Protocol

Appendix B: Method

Appendix C: Carbonyl Stability Affected by Transportation, Knife-grinding and Storage in Refrigerator after Weighing

Appendix D: Full Data Set

Appendix E: Raw Data Plots

Appendix F: Repeatability and Accuracy of Crotonaldehyde Using Laboratory Fortified Matrix Spikes

APPENDIX A: Study Protocol

CORESTA SMOKELESS TOBACCO SUB-GROUP

Project Title: 2016 Carbonyl Study

Type of Document: Draft Protocol

Revision Date: May 24, 2016

Study Coordinator: David Ericsson

1. Introduction

In 2013, the CORESTA Smokeless Tobacco Subgroup (STS) proposed the development of a CORESTA Recommended Method (CRM) for the determination of formaldehyde, acetaldehyde, and crotonaldehyde (carbonyls) in tobacco products. The STS has been developing a method supplied by a participating member since that time.

2. Objective

The Objective of this study is to evaluate the carbonyl method developed within the STS for the determination of carbonyls in tobacco products including smokeless tobacco products and cigarette filler. The data will be statistically evaluated in basic conformance with the recommendations of ISO 5725 to assess within (r) and between laboratory (R) variability. A CRM will be drafted if the results of the study suggest the method is sufficiently robust for the determination of carbonyls in tobacco products.

3. Time schedule and Data Reporting

Laboratories are urged to order the 2016 CRPs immediately to ensure there are minimum delays due to importing the samples though customs.

Date	Activity
Immediately	Laboratories order reference products
June 1 – Aug. 15	Laboratories conduct the study
August 15	Laboratories submit results by this date
October 9	Discuss results at 2016 fall STS meeting

Note: Although each participant should read the applicable methods to determine what supplies are needed in order to participate in the study, the following supplies may need to be ordered. Use of other suppliers will not disqualify a laboratory from the study.

1. **Standards:**
   - Mixed formaldehyde, acetaldehyde, and crotonaldehyde standard: Accustandard, Catalog # S-27484-R1
2. **Internal Standards:**
   - Formaldehyde-d2: 98 %+ purity, CDN Isotopes, Part # D-5105
   - Acetaldehyde-d4: 98 %+ purity, CDN Isotopes, Part # D-163
   - Crotonaldehyde-d3-DNPH: CDN Isotopes, Part # D-7604
3. **Reagents:**
   - Isohexane: (contains < 5 % n-Hexane), HPLC grade, CAS # 73513-42-5 or 107-83-5 (Catalog #383820025, Acros Organics or Product code 11488343, Fisher Chemical)
   - 2,4-Dinitrophenylhydrazine: ~30 % water, Reagent grade, Catalog # D-199303, Sigma-Aldrich. This reagent will need to be purified by recrystallization.

4. **UHPLC Column:**

- Analytical UHPLC Column: Waters Acquity UPLC BEH C18 column, 2.1mm x 100mm, 1.7µm particle size, Part # 186002352, Waters
- In-line filter to analytical column: Acquity UPLC. In-Line Filter, Part # 205000343, Waters

4. Participating Laboratories:

Following receipt of this protocol, the participating laboratories will notify the Study Coordinators of their intent to participate. Please include your complete company name and location.

5. Samples

The samples listed in the table below will be analyzed. Samples should be ordered from the University of Kentucky and North Carolina State University:

- Coresta Reference Products (CRPs) - North Carolina State University
- 1R6F preground cigarette filler and 3R4F cigarettes - University of Kentucky

Participants may use an internal supply of these products assuming the samples have been stored unopened and under suitable conditions. It is critical that the CRPs have been stored at the recommended temperature of −20 °C, or they should not be used.

Table 1: Samples

Product Type	Quantity to Request
2009 CRP1 - Swedish style snus pouch	5 cans
2009 CRP2 - American-style loose moist snuff	5 cans
2009 CRP3 - American-style loose dry snuff powder	5 cans
2016 CRP1.1 - Swedish style snus pouch	5 cans
2016 CRP2.1 - American-style loose moist snuff	5 cans
2016 CRP3.1 - American-style loose dry snuff powder	5 cans
2016 CRP4.1 - American-style loose-leaf chewing tobacco – long cut format	5 cans
1R6F filler - participants will order pre-ground cigarette filler	5 bottle
3R4F cigarettes - participants will remove the filler from the cigarettes- Do not grind!	5 carton

6. Analysis

6.1 Analytes: formaldehyde, acetaldehyde, and crotonaldehyde will be determined in each sample.

6.2 Method: Participating laboratories must use the supplied method "Determination of Select Carbonyls in Tobacco by UHPLC-MS/MS" for the determination of the analytes. Please keep in mind that data generated from methods other than

specified in this protocol do not support the study objectives and cannot be included.

6.3 Replicates and Sample Handling: Conduct three (3) independent replicate analyses for each sample. The replicates should be determined from independent tobacco extractions and conducted in the same run.

6.4 Sample equilibration: The CRPs should be stored in the freezer.

- **Step 1:** Samples shall be removed from the freezer and placed in the refrigerator 24 to 48 hours before analysis.
- **Step 2:** Samples shall be removed from the refrigerator 1 to 2 hours prior to analysis and allowed to equilibrate to room temperature before opening.

An insufficient equilibration time has been identified as a source of variability.

6.5 Sample preparation:

CRP1 and CRP1.1: Unit pouches of portioned smokeless tobacco products shall be analyzed. Determine how many pouches need to be extracted to come closest to the target sample weight of 1g. Cut the pouch(es) in half and add both the tobacco and pouch to the extraction vessel for preparation. The three (3) aliquots should be from the same can.
All others CRPs and 1R6F: Mix the tobacco in the can or bottle with a spatula before weighing out the aliquots. The three (3) aliquots should be from the same can.
3R4F: Remove the filler from 20 cigarettes and place the filler in a sealed bottle and mix. All three (3) aliquots should be analyzed by removing aliquots directly from the bottle. Samples shall not be ground.

6.6 CRP 1, CRP 2 and CRP3 target values

As a guide for method performance control, Table 2 presents acceptable concentration ranges for CRP1-3 for the three carbonyls.

	Formaldehyde (as-is µg/g)	Acetaldehyde (as-is µg/g)	Crotonaldehyde (as-is µg/g)
CRP1	0.7-1.3	6.0-12.0	< 0.05
CRP2	1.0-1.6	2.0-4.5	< 0.05
CRP3	7.0-11.0	1.5-4.0	0.05-0.15

6.7 Data Reporting: The analytes shall be reported in units of µg/g, on an as-is basis, in the attached data reporting spreadsheet. Data shall be reported to two decimal places. The data reporting spreadsheet should not be modified. The method, data reporting sheet and standard preparation for carbonyls spreadsheet are provided below. The standard preparation sheet is used to calculate the precise standard concentrations of the working calibration standards.

Report data in the provided data reporting sheet to [redacted] ([redacted]) and [redacted] ([redacted]).

1. Presentation of the Results

The final output will be a presentation for discussion at the 2016 fall STS meeting.

APPENDIX B: Method

Draft Method with the CORESTA Smokeless Tobacco Sub-Group

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A. SCOPE

1. This method is applicable to the quantitation of formaldehyde, acetaldehyde and crotonaldehyde in tobacco and smokeless tobacco products by ultra-high performance liquid chromatography with triple quadrupole mass spectrometry detection (UHPLC-MS/MS).

2. Formaldehyde, acetaldehyde and crotonaldehyde are derivatized with 2,4-dinitrophenylhydrazine (DNPH) in order to improve sample stability and method sensitivity. The extraction and derivatization are done in a single step, using a two-phase system consisting of aqueous buffer and isohexane. The extraction of the aldehydes from the tobacco and the derivatization with DNPH occurs in the buffer phase and the resulting aldehyde-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.

B. DEFINITIONS

1. **CCS:** Calibration Check Standard
2. **DNPH:** 2,4-dinitrophenylhydrazine
3. **DNPH RB:** 2,4-dinitrophenylhydrazine reagent blank. **Cal 1 is a reagent blank.**
4. **LOQ:** Limit of Quantitation
5. **RB:** Reagent blank
6. **%Deviation:** Percent Deviation from Theoretical. %Deviation is calculated to show the degree of deviation of individual concentration points to the established calibration equation

C. VALIDATION

To be determined.

D. EQUIPMENT AND APPARATUS

1. Equipment and Apparatus Required

   a. UHPLC system such as Waters Acquity I-Class. The UHPLC should be equipped with a binary solvent delivery system, temperature controlled autosampler, and temperature controlled column compartment.
   b. Detector: Triple quadrupole mass spectrometer capable of performing Multiple Reaction Monitoring (MRM)
   c. Analytical UHPLC Column: Waters Acquity UPLC BEH C18 column, 2.1mm x 100mm, 1.7µm particle size, Part # 186002352, Waters Corp., Milford, MA
   d. In-line filter to analytical column: Acquity UPLC Col. In-Line Filter, Part # 205000343, Waters Corp., Milford, MA
   e. Amber autosampler vials with PTFE lined caps
   f. Knife-grinder, Retsch Grindomix GM200, Retsch
   g. Analytical balance with the ability to measure to the nearest 0.1 mg, placed in an enclosure with suitable exhaust
   h. Mechanical pipettes (adjustable volume): 200-µL and 1000-µL capacity
   i. Repeater Pipettes: 100-µL and 1000-µL
   j. Dispensette: 10-mL and 50-mL capacity
   k. Orbital shaker
   l. Class A Volumetric flasks; 10, 25, 50,100 and 2000 mL

m. 50 mL glass Erlenmeyer-flasks (E-flasks). Preferably with PTFE lined screw caps. Otherwise glass stoppers.
n. Gas tight glass syringes, 1 mL
o. Glass or plastic Pasteur pipettes
p. Amber glass storage bottles with PTFE lined screw caps in the range of 10 mL to 125 mL
q. pH meter

2. Instrument Setup

Suggested instrumental parameters for the UHPLC and mass spectrometer are listed below. The mass spectrometer settings are suggested starting points (originating from Waters TQ-S) and may be modified in order to obtain acceptable performance.

Table 1A: UHPLC Parameters

Parameter	**Setting**
Mobile Phase A	10 mM Ammonium acetate, pH 4.7
Mobile Phase B	Acetonitrile
Column Temperature	60 ºC
Flow Rate	0.45 mL/min
Autosampler Temperature	5 ºC
Injection Volume	1 µL
UHPLC Run Time	5 min per sample

Table 1B: Mobile Phase Gradient

Time (min)	**Flow (mL/min)**	**A (%)**	**B (%)**	**Curve**
Initial	0.45	45	55	linear
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

Table 1C: Sample Manager Settings

UHPLC with Loop injection	**UHPLC with Flow through needle**
Weak wash: 250ml water, 250 ml methanol, 250 ml acetonitrile, 250 ml isopropanol, 20 ml ammonium hydroxide Wash Volume 1200µL	**Wash Solvent**: 250ml water, 250 ml methanol, 250 ml acetonitrile, 250 ml isopropanol, 20 ml ammonium hydroxide
Strong wash: 250ml water, 250 ml methanol, 250 ml acetonitrile, 250 ml isopropanol, 20 mL formic acid. Wash Volume 400µL	**Purge Solvent**: 10 % Acetonitrile Needle Wash: 10 % Acetonitrile
Injection type: Partial Loop No Overfill (PLNO)	**Injection type**: Flow through Needle
	Pre-Injection wash 0 sec
	Post-Injection wash 6 sec.

Table 1D: Mass Spectrometer Settings*

Parameter	Setting
Ionization mode:	Electrospray Negative
MS Mode:	MRM
Capillary Voltage:	2.0 kV
Cone Voltage:	30 V
Source Temperature:	150ºC
Desolvation Temperature:	600ºC
Desolvation Gas:	800 L/hr
Cone Gas	150 L/hr

*Note: These are suggested settings on a Waters Xevo Triple Quadrupole mass spectrometer and may require optimization

Table 1E: Multiple Reaction Monitoring (MRM) Parameters*

Analyte	Cone Voltage (V)	Collision Energy (V)	Precursor Ion (m/z)	Product Ions (m/z)	Dwell Time (msec)
Formaldehyde-DNPH	24	6	209.10	163.10	auto
Formaldehyde-d2-DNPH	30	8	211.10	133.10	auto
Acetaldehyde-DNPH	26	10	223.10	151.10	auto
Acetaldehyde-d4-DNPH	28	12	227.10	151.10	auto
Crotonaldehyde-DNPH	32	14	249.00	172.10	auto
Crotonaldehyde-d3-DNPH	20	12	252.00	175.00	auto

*Note: These are suggested settings on a Waters Xevo Triple Quadrupole mass spectrometer and may require optimization.

3. Instrument Maintenance

   a. An in-line column filter is required to extend the life of the analytical column. The in-line column filter should be replaced when analytical performance (peak shape, resolution, increased pressure) has degraded. If changing the in-line column filter does not resolve chromatographic issues, it may be necessary to replace the analytical column.

   b. The ion source (sample cone and cone gas nozzle assembly) should be cleaned when the instrument precision for standard 2 for formaldehyde and acetaldehyde and standard 3 for crotonaldehyde does not pass suitability criteria or as part of routine maintenance. Consult the instrument manual for the manufacturer's recommended instructions on removing and cleaning the ion source. A general procedure consists of first sonicating in 50/50 MeOH/water containing 10 % formic acid for approximately 15 minutes. Next, sonicate for 5 minutes in reagent grade water to remove formic acid residues. Finally, sonicate the parts in methanol for approximately 15 minutes. Dry the parts by blowing with a stream of clean, dry nitrogen before reinstalling on the source. Consult the manufacturer's manual for instructions on installing the parts on the instrument.

E. CHEMICALS AND REAGENTS

1. Chemicals Required: equivalent materials may be substituted
   a. Methanol, LC/MS grade
   b. 2-Propanol, HPLC grade
   c. Acetonitrile, LC/MS grade
   d. Ammonium acetate, HPLC grade
   e. Acetic Acid, HPLC grade
   f. Ammonium formate, HPLC grade
   g. Formic Acid (88 %), Laboratory grade
   h. Ammonium hydroxide, Optima grade
   i. Reagent water: HPLC grade or water purification system capable of producing 18.2 MΩ water
   j. Isohexane (contains < 5 % n-Hexane), HPLC grade, CAS # 73513-42-5 or 107-83-5 (Catalog #383820025, Acros Organics or Product code 11488343, Fisher Chemical)
   k. Acetaldehyde-d4,98 %+ purity, Part # D-163, CDN Isotopes
   l. Crotonaldehyde 2,4-Dinitrophenylhydrazone-,5,6-d3, 98 % purity, Part # D-7604, CDN Isotopes
   m. Formaldehyde-d2, 98 %+ purity, Part # D-5105,CDN Isotopes
   n. 2,4-Dinitrophenylhydrazine ~30 % water, Reagent grade, Catalog # D-199303, Sigma-Aldrich. This reagent will need to be purified by recrystallization.
   o. Custom Aldehyde Standard in water,1000 µg/mL Formaldehyde, 1000 µg/mL Acetaldehyde, 100 µg/mL Crotonaldehyde, Catalog # S-27484-R1 (order two different lots for the standards and CCS), AccuStandard

2. Reagent Preparation

   Depending on the requirements of the particular analysis, different quantities than those suggested below, may be prepared by applying the appropriate scaling ratio to the procedure.

   a. **Recrystallization of DNPH**

      See Appendices for DNPH recrystallization procedure.

   b. **DNPH-solution, 5 mg/mL**

   c. Add 0.5±0.02g of dry recrystallized 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. The solution is stable for 36 days when stored at room temperature and protected from light.

   Note**:** Remember to analyze DNPH RB of freshly prepared DNPH solutions from crude DNPH, one recrystallization and two recrystallizations in order to estimate the Purification % of each recrystallization step (See Appendice).

   d. **Extraction solution, 100 mM ammonium formate, pH 3.0 (± 0.1)** To make a 2 L solution, weigh and transfer 12.6g ±0.05g of ammonium formate into a 2 L volumetric flask containing approximately 1L of reagent grade water. Use a stir bar to dissolve the solids. Add approximately 0.9 L 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.

e. **Mobile Phase A, 10 mM ammonium acetate buffer, pH 4.7 (± 0.1)**
To make a 2 L solution, weigh and transfer 1.54±0.02g of ammonium acetate (CH_3COONH_4) into a 2 L volumetric flask containing approximately 1L of reagent grade water. Use a stir bar to dissolve the solids. Add approximately 0.9 L 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.

3. Standard Preparation

a. **Stock Calibration Standard Solution 1:** Stocks are purchased solutions of 1000 µg/mL formaldehyde, 1000 µg/mL acetaldehyde, and 100 µg/mL crotonaldehyde in water. The expiration date and storage conditions are provided by the manufacturer.

b. **Stock Calibration Standard Solution 2:**
Using the appropriate gastight syringe, weigh 1.00 g ±0.01g **Stock Calibration Standard Solution 1** on an analytical balance using a tarred 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. Calculate the exact concentrations based on actual amount weighed. Store in a 10 mL amber bottle with PTFE lined cap. The solution is stable for 16 days when stored at ~5 ºC.

c. **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. The solution is stable for 16 days when stored at stored at ~5 ºC.

d. **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. The solution is stable for 16 days when stored at stored at ~5 ºC.

e. **Stock Calibration Check Standard Solution 1:** Stocks are purchased solutions of 1000 µg/mL Formaldehyde, 1000 µg/mL Acetaldehyde, and 100 µg/mL Crotonaldehyde in water. Separate lots of stock solutions must be used for calibration standards and check standards. The expiration date and storage conditions are provided by the manufacturer.

f. **Stock Calibration Check Standard Solution 2:**
Using the appropriate gastight syringe, weigh 1.00 g ±0.01g **Stock Calibration Check Standard Solution 1** on an analytical balance using a tarred 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. Calculate exact concentrations based on actual amount weighed using the template Standard Preparation for Carbonyls in Smokeless Tobacco. Store in a 10 mL amber bottle with a PTFE lined cap. The solution is stable for 16 days when stored at stored at ~5 ºC.

g. **Stock Calibration Check Standard Solution 3:** Transfer 5.0 mL of **Stock Calibration Standard Solution 2** 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. The solution is stable for 16 days when stored at stored at ~5 ºC.

h. **Stock Internal Standard Solution Formaldehyde-d2, 10 mg/mL**
Using the appropriate gastight syringe, weigh approximately 1000 mg of formaldehyde-d2 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. Dilute to volume with reagent grade water and mix well. Store in a 125 mL amber glass bottle with PTFE lined cap. The solution is stable for up to 5 weeks when stored at ~5 ºC or until the expiration date stated on the purchased material, whichever is first.

i. **Stock Internal Standard Solution Acetaldehyde-d4, 10 mg/mL**

Note: **The Acetaldehyde-d4** should be refrigerator-cold when weighing it. Otherwise it will boil at room temperature! Using the appropriate gastight syringe, weigh approximately 1000 mg of acetaldehyde-d4 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. Dilute to volume with reagent grade water and mix well. Store in a 125 mL amber glass bottle with PTFE lined cap. The solution is stable for up to 5 weeks when stored at ~5 ºC or until the expiration date stated on the purchased material, whichever is first.

j. **Stock Internal Standard Solution Crotonaldehyde-d3-DNPH, 0.2 mg/mL**
Weigh approximately 10 mg of crotonaldehyde-d3-DNPH 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. The solution is stable for up to 5 weeks when stored at ~5 ºC or until the expiration date stated on the purchased material, whichever is first.

k. **Working Internal Standard Solution (IS): Formaldehyde-d2 500 µg/mL, Acetaldehyde-d4 100 µg/mL, Crotonaldehyd-d3-DNPH 5.6 µg/mL (free aldehyde basis)**
Transfer 5mL Formaldehyde-d2 stock internal standard solution, 1mL Acetaldehyde-d4 stock internal standard solution, and 10 mL of crotonaldehyde-d3-DNPH 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. The solution is stable for up to 5 weeks when stored at ~5 ºC.

l. **Working Calibration Standards**
The calibration standards are prepared at the same time as samples and according to the same procedure as the samples excluding the tobacco. See section G. for a detailed description. Working Calibration Standards 1-8 are prepared using Stock Calibration Standard Solution 2, 3, and 4 in combination with the Working Internal Standard solution according to Table 2 below. The working 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.

Note: Calibration standards, the CCS, and samples must be prepared and analyzed together using the same procedure. A batch of standards and samples is stable for up to 24h 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 2. Working Calibration Standard Preparation

Calibration Level	Stock Calibration Standard Used	Volume Stock Calibration Std (µL)	Volume Working Internal Std (µL)
1	NA	0	100
2	4	50	100
3	4	250	100
4	4	500	100
5	3	100	100
6	3	300	100
7	3	500	100
8	2	200	100

m. **Calibration Check Standards (CCS)**: The calibration check standards are prepared at the same time as samples and according to the same procedure as the samples excluding the tobacco. Calibration Check Standards are prepared using the Stock Calibration Check Standard Stock 3 in combination with the Working Internal Standard solution according to Table 3. Store in amber bottles at ~ 5ºC. As stated above with the calibration standards, calibration check standards are stable for up to 24 hours when analyzed with the same batch of standard and samples that were prepared with the CCS.

Table 3. Calibration Check Standard Preparation

Calibration Level	Stock Calibration Check Standard Used	Volume Stock Calibration Std (µL)	Volume Working Internal (µL)
CCS	3	500	100

The target concentrations of the calibration standards and CCS are shown in Table 4.

Table 4. Target Concentrations of Working Calibration and Check Calibration Standards

Calibration Level	Formaldehyde (µg/mL)	Acetaldehyde (µg/mL)	Crotonaldehyde (µg/mL)
Cal 1	0	0	0
Cal 2	0.010	0.010	NA
Cal 3	0.050	0.050	0.005
Cal 4	0.100	0.100	0.010
Cal 5	0.200	0.200	0.020
Cal 6	0.600	0.600	0.060
Cal 7	1.000	1.000	0.100
Cal 8	2.000	2.000	0.200
CCS	0.500	0.500	0.050

NA: the first calibration level for crotonaldehyde is Cal 3.

Each calibration standard solution contains 5 µg/mL of formaldehyde-d2, 1 µg/mL of acetaldehyde-d4 and 0.056 µg/mL of crotonaldehyde-d3 as the free carbonyl.

Note: Due to background contamination of the analytes of interest in DNPH, there will be measurable peaks for the analytes in Calibration level 1.

F. SAMPLE REQUIREMENTS

1. Sample Storage Conditions

   Note**:** Smokeless tobacco samples should be stored unopened in the freezer (at approximately -20 ºC) for long term storage.

   a. While waiting to be tested, samples must be stored in the refrigerator (at approximately 4 ºC) for short term storage (1-3 days). Prior to opening the container, the samples must be allowed to equilibrate to ambient conditions for a minimum of 1 hour before sample preparation.
   b. Samples that will not be analyzed within 3 days must be stored unopened at approximately -20 ºC.
   c. Samples removed from the freezer must be stored unopened in the refrigerator for a minimum of 24 hours. Prior to opening the container, the samples must be allowed to equilibrate to ambient conditions for a minimum of 1 hour before sample preparation.
   d. Proper planning must be done to avoid multiple freeze thaw cycles.

2. Sample Handling

   a. Unit pouches of portioned smokeless tobacco products shall be analyzed. Determine how many pouches need to be extracted to come closest to the target sample weight of 1g. Cut the pouch(es) in half and add both the tobacco and pouch to the extraction vessel for preparation.
   b. Moist smokeless tobacco (MST), dry snuff, cigarette filler and other products with a particle size not exceeding long cut MST should be analyzed without further sample grinding.

c. Samples with particle sizes larger than long cut MST should be ground frozen, when taken directly from the freezer (-20ºC), using a Retsch Grindomix GM200 at a grinding speed 10 for 15 seconds. Use a PTFE-top to push the tobacco close to the cutting knives.
d. Cigarette fillers do not need to be ground. Just remove the paper around the tobacco.

Note: Aldehydes will vaporize if samples are at room temperature or if the grinding time is longer than 30 seconds.

Note**:** Samples that need to be ground should be ground and prepared the same day. Samples may be stored in a tightly sealed container between grinding and preparation.

G. PROCEDURE

1. Sample Preparation

Note**:** Prepare all solutions and equipment in order to be able to run all operations as quickly as possible without time interruptions. Begin step (h) within **5 minutes** from the completion of rotation in step (g). Finish step (h) within **30 minutes** from the completion of rotation in step (g)

a. Weigh 1.00 ± 0.20 g of sample into a 50 mL Erlenmeyer flask.
b. Add 40.0 mL of 100 mM ammonium formate (pH 3.0) to the 50 mL E-flasks using a dispensette. Please note! Also add 40.0 mL to E-flasks for calibration standards and CCS (without tobacco).
c. Pipette the calibration standards, see Table 2.
d. Add 100 µL of Working Internal Standard Solution using a repeater pipette to all samples, CCS and calibration standard solutions.
e. Add 1.0 mL of DNPH-solution using a repeater pipette to all samples and calibration standard solutions.
f. Add 10 mL of isohexane using a repeater pipette or dispensette to all samples and calibration standard solutions.
g. Rotate the samples for 60 min using an Orbital shaker set to approximately 130 rpm.

*Important! Begin step (h) within **5 minutes** from the completion of rotation in step (g). Finish step (h) within **30 minutes** from the completion of rotation in step (g).*

h. After the isohexane and aqueous layers have separated and the tobacco has settled, transfer approximately 1.5 mL of the isohexane extracts, 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.
i. The Calibration standards, the CCS, and samples must be prepared and analyzed together using the same procedure. A set of standards and samples is stable for up to 24h 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.

2. Calibration

a. New calibration standards, the CCS, and a new calibration curve must be generated with each sample batch.
b. The calibration curve is composed of the average values for each level generated from an opening and closing curve.
c. Load the UHPLC method.

d. Perform the following operations to prime the instrument:
    1) Prime mobile phase solvents A and B
    2) Prime the sample syringe
    3) Prime the wash needle
e. Allow the column to equilibrate to 60 °C and the instrument to stabilize for approximately 30 minutes before initiating the calibration.
f. Inject a standard or sample to equilibrate the system before running the sequence.
g. Inject the system suitability standards
h. Create the sequence table. An example of a typical run sequence is as follows:
    1) Isohexane blank
    2) 5 injections of a 0.01 µg/mL standard for formaldehyde and acetaldehyde
    3) 5 injections of a 0.005 µg/mL standard for crotonaldehyde
    4) Isohexane blank
    5) Calibration standards 1 – 8; beginning curve
    6) Isohexane blank
    7) CCS
    8) Set of approximately 10 samples
    9) Repeat CCS after each batch of approximately 10 samples and after the last sample batch
    10) Isohexane blank
    11) Calibration standards 1 – 8; ending curve
i. Linear regression ($y = mx + b$) is used for calibration. Using peak area, set the quantitation method to perform a linear calibration with the origin excluded and a weighting factor of 1/x. 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. Formaldehyde-d2 is used as the internal standard for formaldehyde, acetaldehyde-d4 is used as the internal standard for acetaldehyde, and crotonaldehyde-DNPH-d3 is used as the internal standard for crotonaldehyde. Quantify the resulting data against the calibration curves using the instrument data acquisition software. Ensure that all reports are appropriately labeled to provide traceability to the sample and calibration curves. The resulting analyte concentrations will be calculated in units of µg/mL.

3. Calculations and Reporting

a. Before analyzing samples, enter the sample weights and dilutions into the appropriate fields of the acquisition software.
b. Quantify the resulting data against the average of the beginning and ending calibration curves using the data acquisition software and generate Sample Summary and Compound Summary reports for the analysis batch. The resulting Carbonyl concentrations will be the concentration on an ‘as- is’ tobacco weight basis (µg/g).
c. The individual carbonyl concentrations in each sample must be inspected to determine if the calibration range for any analytes has been exceeded. If the individual concentration of any carbonyl analyte has been exceeded, then that sample needs to be re-weighed, using less tobacco, and taken through the sample preparation procedure. The preparation is then reanalyzed with a new batch of calibration standards.
d. The person performing the analysis is responsible for determining if the run meets all of the acceptance criteria stated in the Quality Control and Acceptance Criteria section.

e. All calibration and sample analysis calculations utilize Relative Response Factors (RRF). The RRF is calculated using the equation:

$$RRF = \frac{Area_a}{Area_{IS}} \times Conc_{IS}$$

Where:
$Area_a$ = integrated area of the target analyte.
$Area_{IS}$ = integrated area of the corresponding internal standard
$Conc_{IS}$ = concentration of the corresponding internal standard in the standard

f. Sample Concentration

$$\text{Sample Conc } (\mu g/mL) = \frac{RRF - \text{y-intercept}}{\text{slope}}$$

Where:
Sample Conc = the calculated concentration (µg/mL)
y-intercept = the y-intercept from the average of the beginning and ending calibration curves
Slope (m) = the slope from average of the beginning and ending calibration curves
RRF = the relative response factor

g. Analyte Amount

$$\text{Analyte Amount } (\mu g/g) = \text{Sample Conc} \times \frac{Vol}{W}$$

Where:
Sample Conc = the calculated concentration (µg/mL)
Vol = the final volume of isohexane extraction solution in mL (10 mL)
W = the weight of the tobacco sample

h. Percent deviation from theoretical is calculated to show the degree of deviation of individual concentration points from the established calibration equation:

$$\%\text{ Deviation from theoretical} = \frac{RC\text{-}NC}{NC} \times 100$$

Where:
RC = the concentration calculated from the calibration curve
NC = the nominal or theoretical concentration

4. Quality Control and Acceptance Criteria

a. Chromatogram Evaluation: The standard and sample chromatograms should be reviewed to verify typical peak shape, proper assignment and integration.

b. System Suitability Test: Five injections from a single vial of a standard with concentration 0.01 µg/mL for formaldehyde and acetaldehyde as well as five injections of a standard with concentration 0.005 µg/mL for crotonaldehyde. The %RSD of the injections (n=5) should be ≤ 20 %. Deviations from these values would require an investigation. The system will be checked against this specification before the analysis of unknowns to ensure that the system performance meets the intended purpose.

c. Calibration: A calibration curve is considered valid if the following conditions are met:

1) Coefficient of determination (R^2): The average of the beginning and ending calibration curves for each analyte should have R^2 values of 0.990 or higher. All curves should be visually inspected for linearity. Recalibrate if R^2 falls below 0.990.
2) Up to three calibration points, two points at the same concentration plus a point at another concentration, may be removed before approving the standard curve according to the criteria above. If the calibration curve does not meet the requirements, new calibration standards and samples should be prepared and analyzed.
3) % Deviation: For formaldehyde and acetaldehyde the calibration curves should have % deviation values not exceeding ±30 % for STD 2 and ± 20 % for STD 3-8. For crotonaldehyde, since STD 2 is not used, the calibration curves should have % deviation values not exceeding ±30 % for STD 3 and ± 20 % for STD 4-8.
4) Calibration Check Standard (CCS): The validity of the calibration curves must be checked routinely during an analysis batch by injecting the calibration check standard. All samples must be bracketed by passing CCSs. Analyze the CCSs as samples. The % deviation for the CCS should be within ±15 % of the nominal concentrations for each analyte. If % deviation results for a CCS fall outside of ±15 %, the samples bracketed by that CCS must be reanalyzed.
5) In the event that any of the quality control measures outlined in this section pass acceptance criteria for some but not all of the analytes in this method, data for analytes with acceptable quality controls can be accepted, but analytes which fail quality control measures must be reprepared and analyzed.
6) The LOQ was determined to be 0.01 µg/mL (0.1 µg/g) for formaldehyde and acetaldehyde and 0.005 µg/mL (0.05µg/g) for crotonaldehyde.

H. APPENDICE- DNPH recrystallization procedure

The purpose of the procedure is to describe the process for 2, 4 –Dinitrophenyl hydrazine (DNPH) purification by recrystallization in acetonitrile.

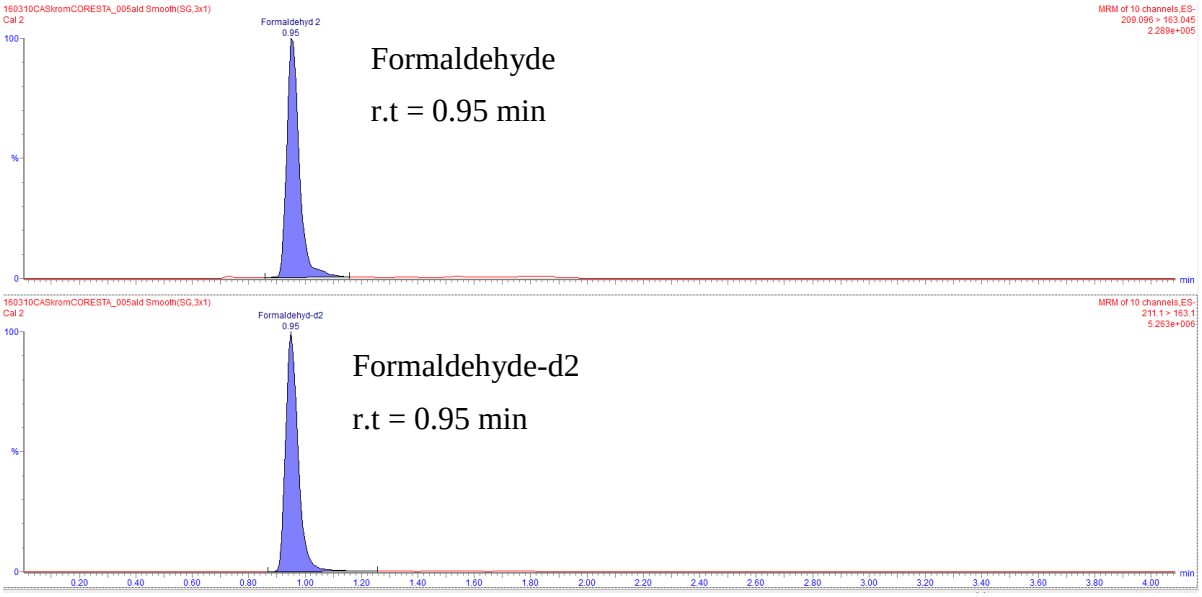
5. Equipment Required
    a. Hot Plate with a Magnetic Stirrer.
    b. Magnetic stir bar
    c. Analytical Balance
    d. Watch Glass
    e. 0.5-L beaker
    f. Thermometer
6. Chemicals and Reagents Required
    a. Acetonitrile
    b. 2,4-DINITROPHENYLHYDRAZINE (DNPH).
7. Purification Reference
    a. Let about 0.5 g of crude DNPH dry at ambient temperature on a watch glass for 24 hours.
8. First Recrystallization
    a. Rinse all glassware to be used with acetonitrile. Work in ventilated hood.
    b. Weigh approximately 10 grams DNPH (~30 % water) into 150 mL acetonitrile in a 0.5 L beaker. Add a magnetic stirrer bar.
    c. Cover beaker with a watch glass and heat on hot plate to a slow boil (~84 ºC) using magnetic stirring for 1 hour.
    d. After 1 hour, let boil with watch glass off until approximately 30 mL of liquid is left on top of the crystals.
    e. Lower heat to between 40 ºC and 60 ºC, and let evaporate until approximately 5 mL of liquid is left.
    f. Let cool
    g. Decant the liquid to waste and wash twice with 5 mL acetonitrile
    h. Remove about 200 mg of washed crystals for purity testing.
    i. Let the 200 mg crystals dry at ambient temperature for 24 hours.
    j. The rest of the crystals are recrystallized a second round.
9. Second Recrystallization
    a. Add another 150 mL acetonitrile to the 0.5 L beaker.
    b. Repeat steps 4c. to 4g. described in First recrystallization.
    c. Let all of the crystals dry on a watch glass at ambient temperature for 24 hours.
10. Testing of purity
    a. The three dried samples of DNPH from 3-5 are to be tested; crude DNPH, 1-recrystallization and 2-recrystallizations.
    b. Weigh 50 ± 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.
    c. Prepare DNPH regent blanks (Cal 1) according to method (1 mL DNPH solution, 40 mL Extraction solution, 100 uL ISTD, 10 mL Isohexane) and test on UHPLC-MS.

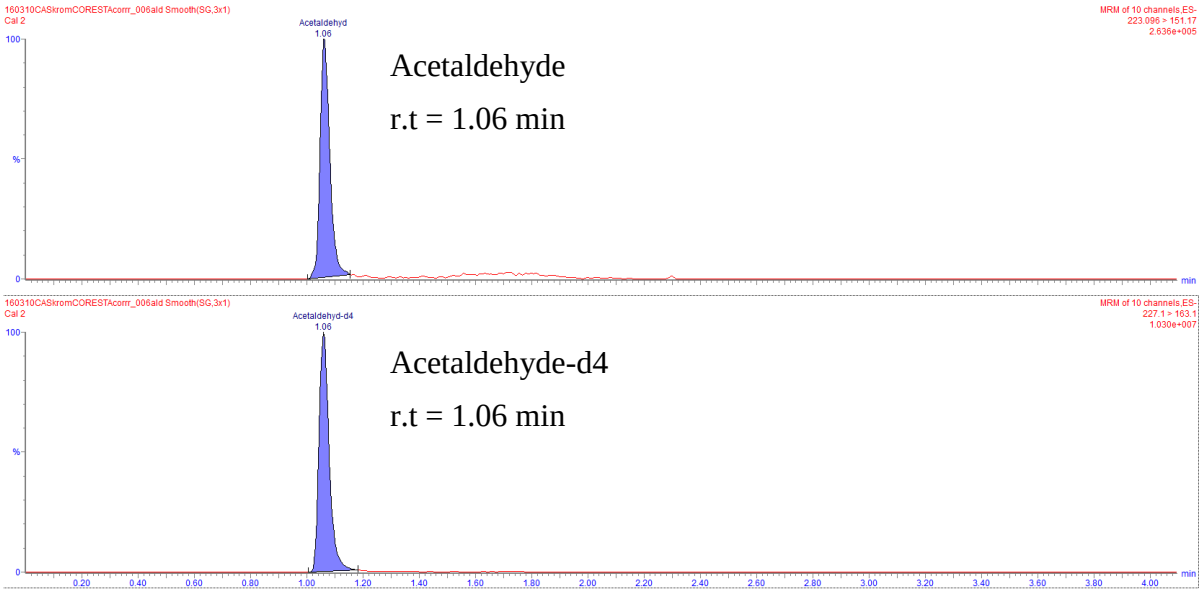
d. Calculate the Purification (%) for 1- and 2-recrystallizations for each of form-, acet- and crotonaldehyde. Use the peak areas in the equation below.

$$\text{Purification (\%)} = 100 \text{ x } \frac{(\text{Crude } - \text{Recrystillized})}{\text{Crude}}$$

I. APPENDICE- Representative Chromatograms

Figure 1. Representative Chromatogram of the lowest calibration level (Cal 2)





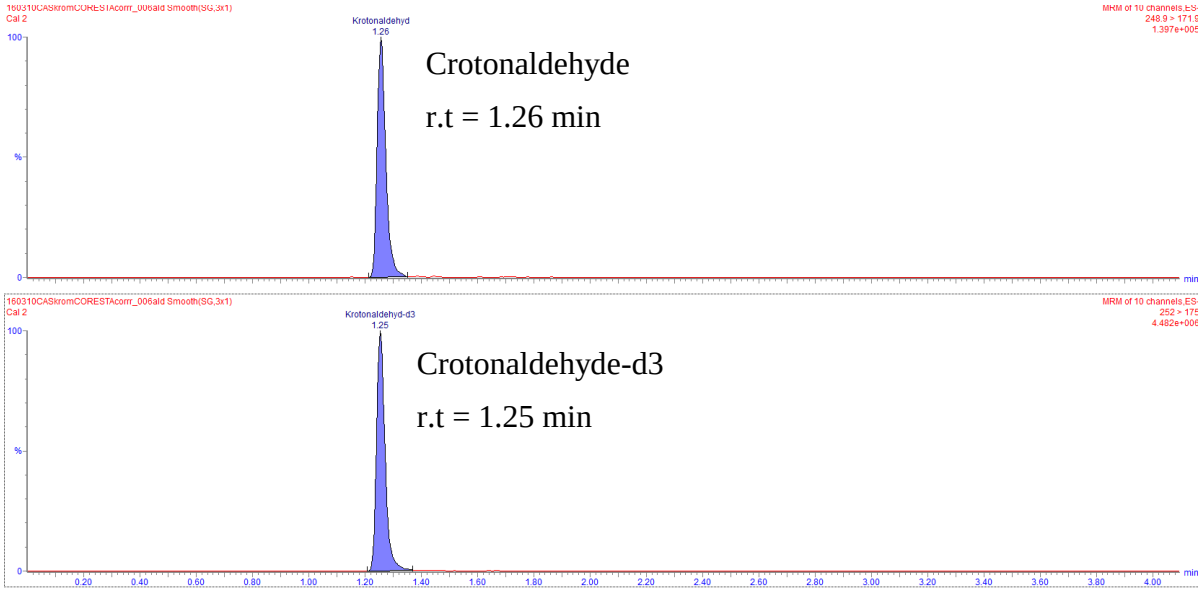
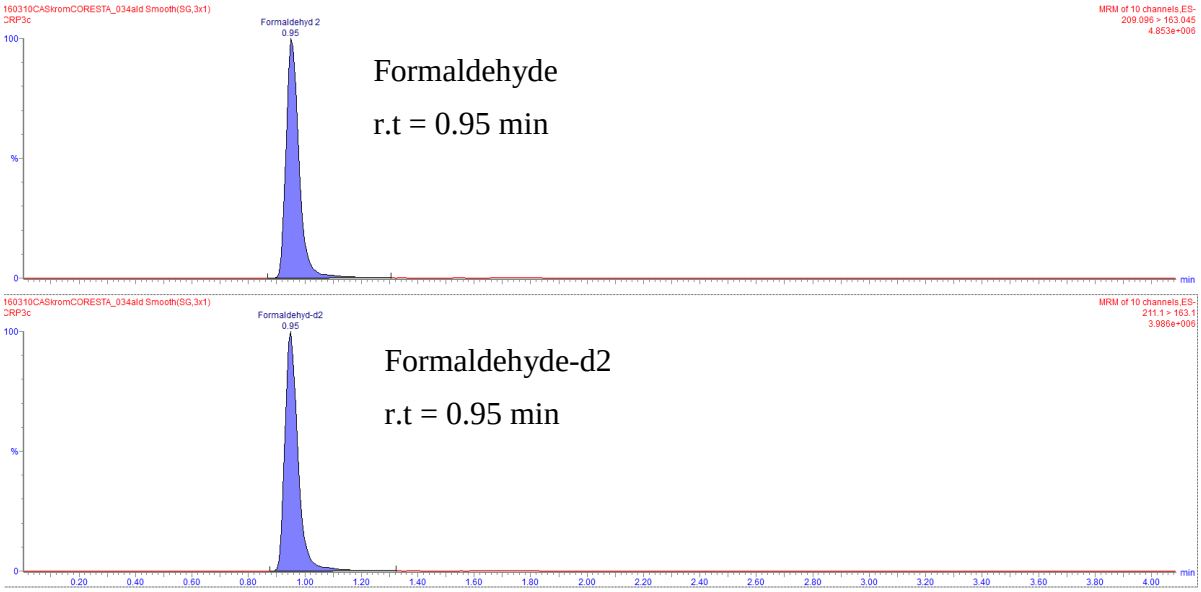
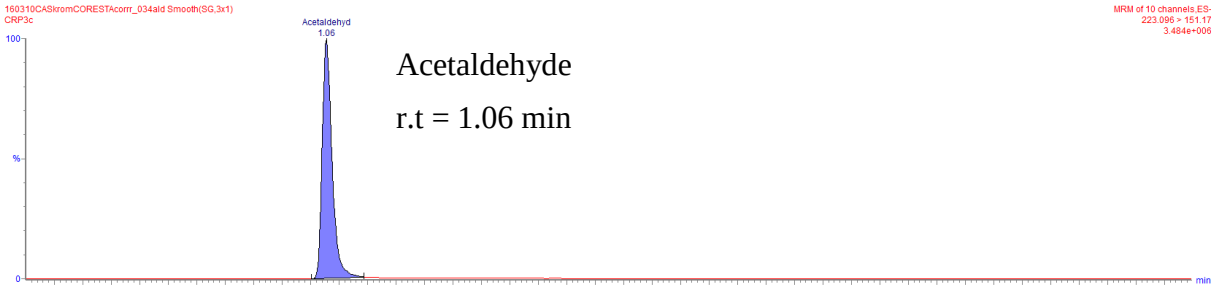


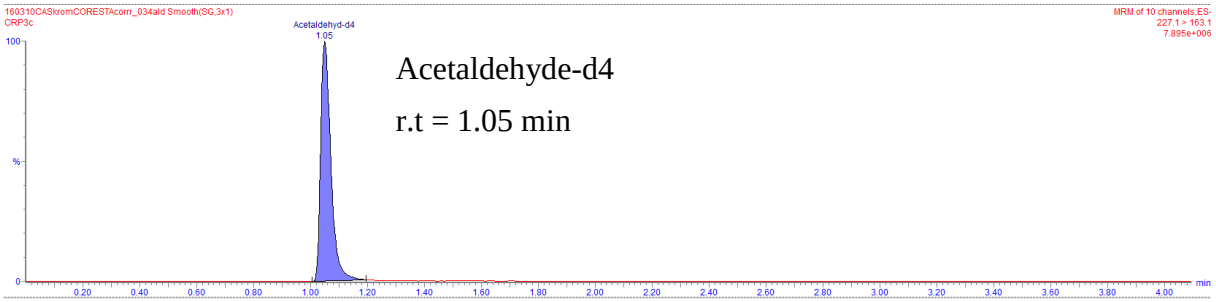
Figure 2. Representative Chromatogram of CRP3





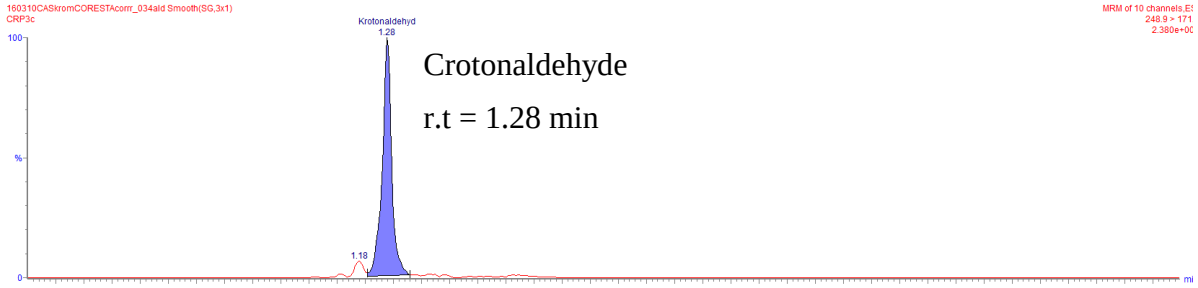
Acetaldehyde

r.t = 1.06 min



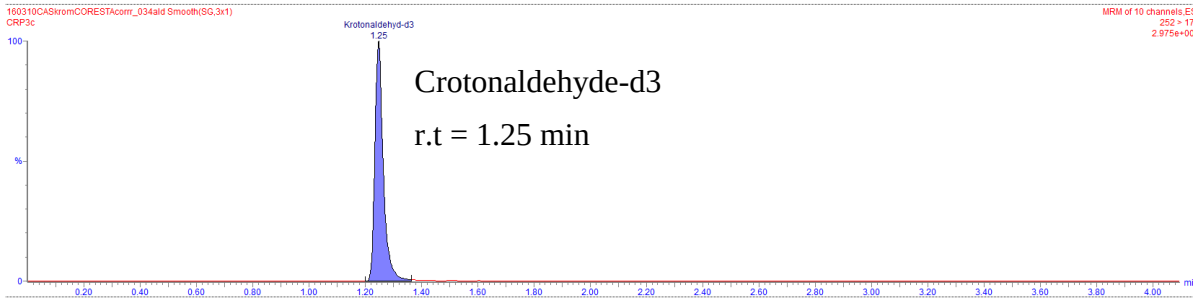
Acetaldehyde-d4

r.t = 1.05 min



Crotonaldehyde

r.t = 1.28 min



Crotonaldehyde-d3

r.t = 1.25 min

APPENDIX C: Carbonyl Stability Affected by Transportation, Knife-grinding and Storage in Refrigerator after Weighing

Three limited robustness experiments were carried out in order to investigate the carbonyl stability affected by transportation, knife-grinding of CRP4 and 3R4F cigarette filler and the effect of storage in refrigerator after weighing of aliquots for analysis.

C.1 Carbonyl stability during transportation from Sweden to the United States and back

Purpose: To investigate the stability of carbonyl concentrations in tobacco and tobacco products during air transportation (no temperature control).

Tobacco products: CRP1, CRP2, CRP3, CRP4, 3R4F cigarette filler and ground raw tobacco.

Experiment: Three packages of each of the six tobacco products were sent by air from Sweden to the United States and back again. The total travel time was about one week with uncontrolled storage temperature. Three packages of each of the six tobacco products were stored in -20 ºC and used as control samples.

Analysis: All transported samples and control samples were analyzed on the same day with one replicate per package. All CRP4 and 3R4F samples were ground before analysis.

Comment: The CRP1-4 and 3R4F samples were previously received from the distributors in the United States prior to initiating the experiment.

Results: This was a limited robustness test from which no certain conclusion could be drawn. The test suggests that the concentration of acetaldehyde was unaffected by uncontrolled temperatures during one week of transportation, see **Figure 2**. Regarding formaldehyde, CRP3 seems to be much lower after transportation but the other samples did not change significantly in concentration, see **Figure 1**.

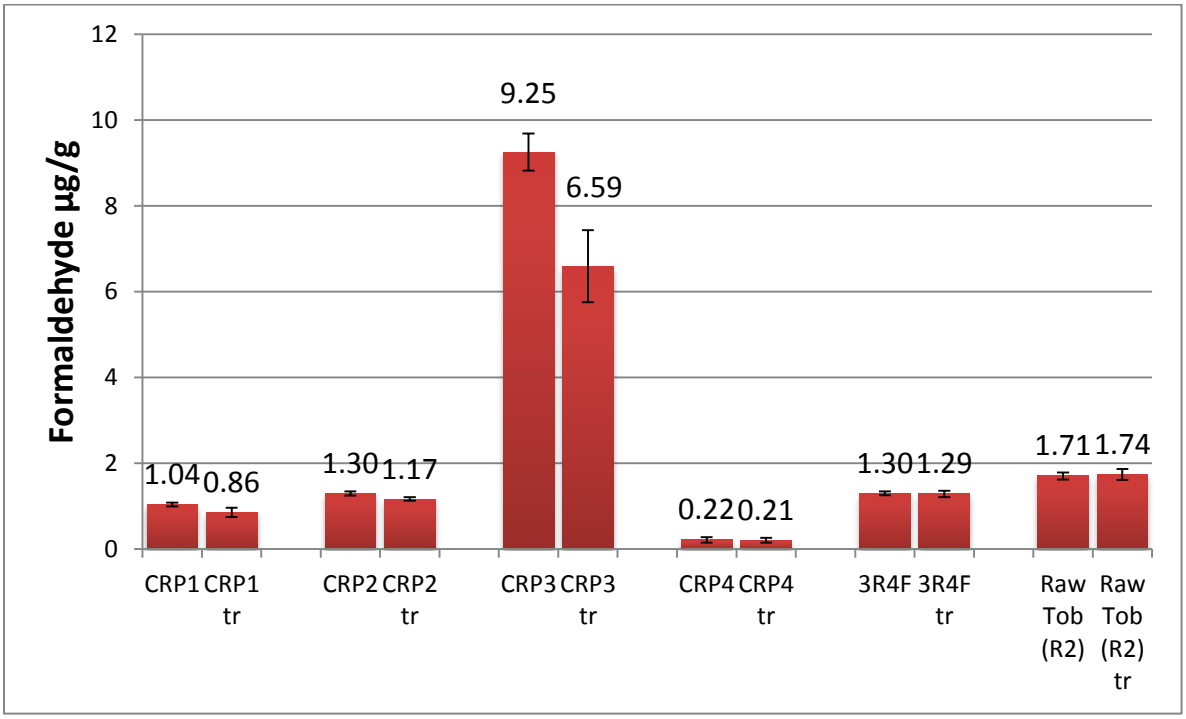


Figure 1. Effect of transportation Sweden-USA-Sweden on Formaldehyde concentrations. (tr = transported)

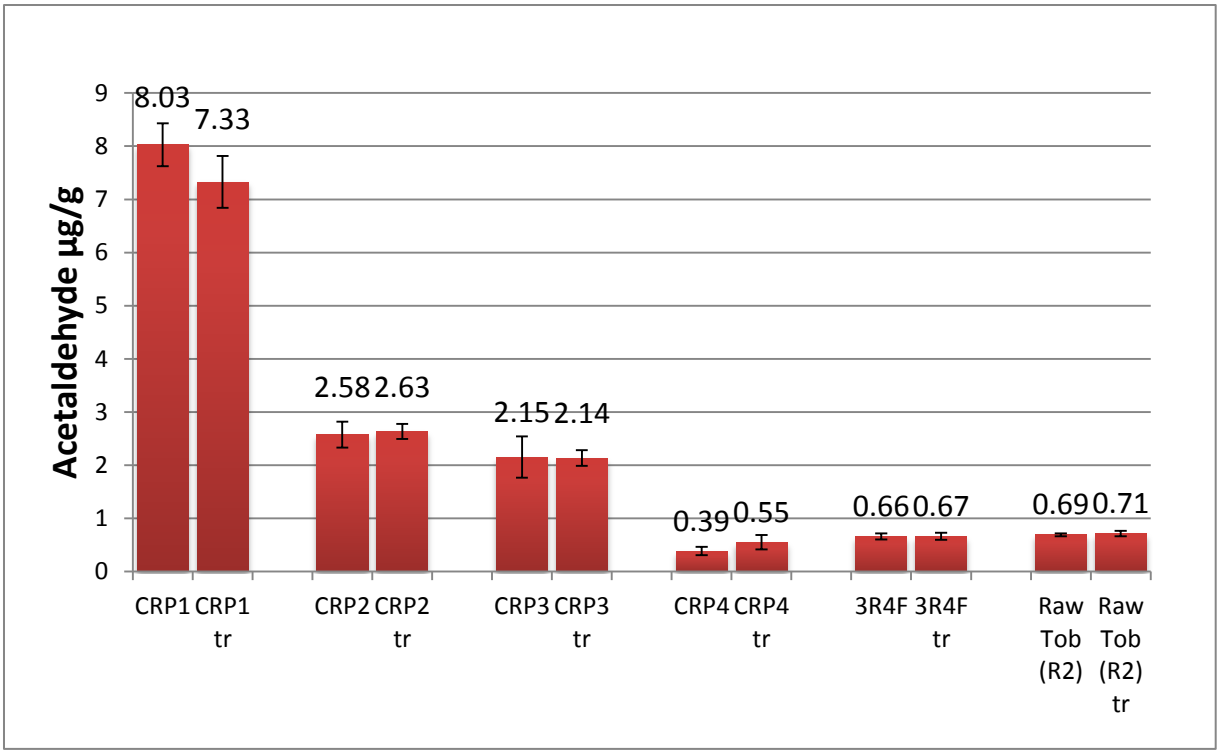


Figure 2. Effect of transportation Sweden-USA-Sweden on Acetaldehyde concentrations. (tr = transported)

C.2 Stability during knife-grinding

Purpose: To investigate the effect of different grinding times (knife-grinder) on carbonyl concentrations in tobacco products.

Tobacco products: Chewing tobacco CRP4 and cigarette filler 3R4F.

Knife-grinder: Retsch GM 300

Experiment: Triplicate samples at each grinding time of both tobacco products. The samples were stored in -20 ºC until immediately before grinding

Grinding times: 0 sec - no grinding, 5 sec, 15 sec, 30 sec, 60 sec, 240 sec

Results: This was a limited robustness test from which no certain conclusion could be drawn. The general trend with increased grinding time seems to be carbonyl-evaporation except for formaldehyde in CRP4, see **Figure 3**. The effect of knife-grinding on the RSD% of analyte concentration with different grinding times is shown in **Figure 4**. It is hard to see any general trends regarding the effect on RSD% with increasing grinding times.

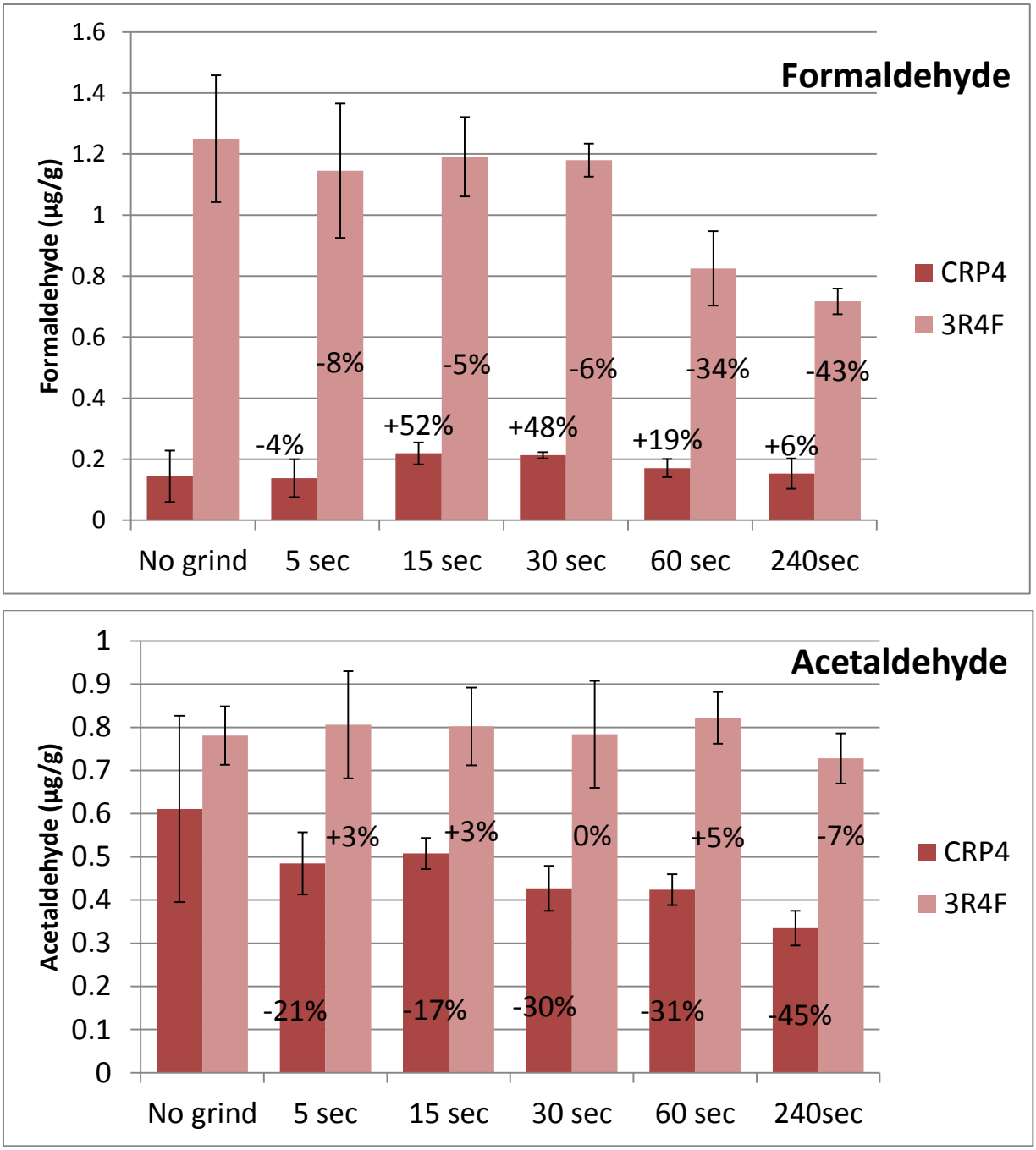


Figure 3. Effect of knife-grinding on the formaldehyde (top) and acetaldehyde (bottom) concentrations when grinding CRP4 and cigarette filler 3R4F for 0-240 seconds. The %-number illustrates % increase or decrease after x sec of grinding compared to No grinding (reference).

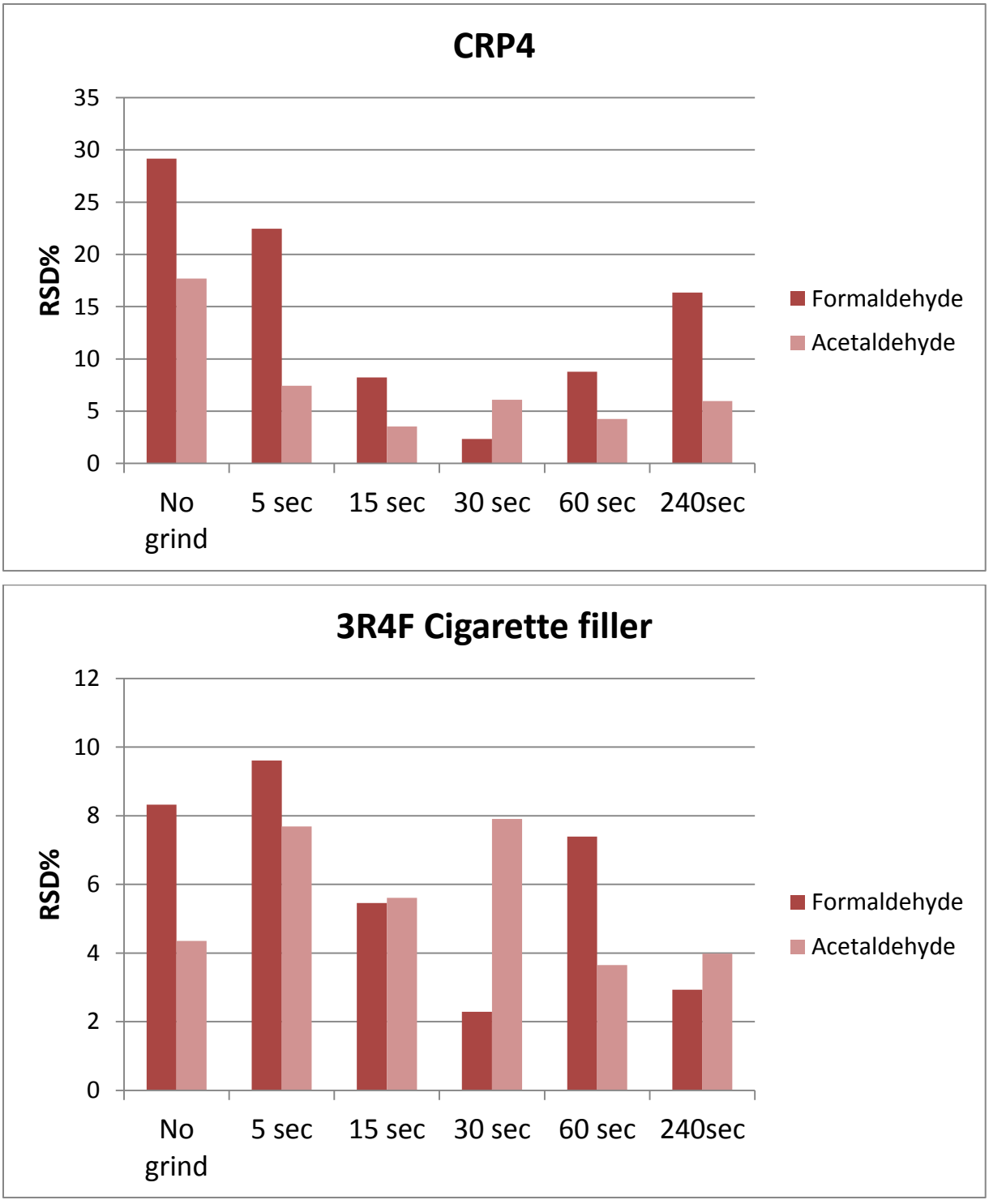


Figure 4. Effect of knife-grinding on the RSD% of analyte concentration with different grinding times. CRP4 (top) and 3R4F (bottom).

C.3 Stability- storage in sealed flask in refrigerator after weighing

Purpose: To investigate the stability of carbonyl concentrations after weighing out tobacco and tobacco products into E-flasks (sealed with stopper after weighing) and stored for up to two days in refrigerator.

Tobacco products: CRP1, CRP2, CRP3, CRP4, 3R4F cigarette filler and ground raw tobacco.

Experiment: Triplicate analysis from same package for each storage time and sample. The

Storage time after weighing: 0 days (direct analysis), 1 day, 2 days and 3 days.

Analysis: The samples were weighed out at different days and then analyzed at the same day.

Results: This was a limited robustness test from which no certain conclusion could be drawn. The results suggest that there are no changes in formaldehyde or acetaldehyde concentrations for any samples when they were stored up to two days in sealed E-flasks in refrigerator. On the third day, the concentrations might decrease.

APPENDIX D: Full Data Set

Full Data Set (results are presented on an as-is basis)

		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
1	1R6F ground filler	1,1500	1,0100	<0,05
1	1R6F ground filler	1,1200	0,9900	<0,05
1	1R6F ground filler	1,1400	0,9400	<0,05
2	1R6F ground filler	1,0533	1,6872	<0,05
2	1R6F ground filler	1,1288	2,3685	<0,05
2	1R6F ground filler	1,0547	1,9727	<0,05
3	1R6F ground filler	1,0627	1,0916	<0,05
3	1R6F ground filler	0,9982	1,0644	<0,05
3	1R6F ground filler	1,2306	1,1013	<0,05
4	1R6F ground filler	0,9150	1,6610	<0,05
4	1R6F ground filler	1,4340	1,5560	0,12
4	1R6F ground filler	1,0860	1,6830	0,07
5	1R6F ground filler	1,9989	1,2404	<LOQ
5	1R6F ground filler	1,9871	1,2351	<LOQ
5	1R6F ground filler	2,0076	1,2105	<LOQ
6	1R6F ground filler	1,3865	1,7971	<0,05
6	1R6F ground filler	1,4413	1,9824	<0,05
6	1R6F ground filler	1,3636	1,8309	<0,05
7	1R6F ground filler	0,8296	1,8226	<0,05
7	1R6F ground filler	0,7115	1,9153	<0,05
7	1R6F ground filler	0,9970	1,8790	<0,05
8	1R6F ground filler	1,6401	0,6480	ND
8	1R6F ground filler	1,3776	0,4395	ND
8	1R6F ground filler	1,5152	0,3352	ND
9	1R6F ground filler	1,0200	1,1700	0,14
9	1R6F ground filler	1,0400	1,1500	0,15
9	1R6F ground filler	1,1500	1,4200	0,21
10	1R6F ground filler	1,2470	0,8830	ND
10	1R6F ground filler	1,2430	1,0960	ND
10	1R6F ground filler	1,2400	0,7740	ND
11	1R6F ground filler	–	–	–
11	1R6F ground filler	–	–	–
11	1R6F ground filler	–	–	–
1	3R4F filler	1,4000	1,0200	<0,05

		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
1	3R4F filler	1,4000	0,9900	<0,05
1	3R4F filler	1,3600	0,9400	<0,05
2	3R4F filler	0,9918	1,7274	<0,05
2	3R4F filler	0,9715	1,9726	<0,05
2	3R4F filler	0,9908	1,9339	<0,05
3	3R4F filler	0,6174	1,1208	<0,05
3	3R4F filler	1,1326	1,0838	<0,05
3	3R4F filler	0,8117	1,3560	<0,05
4	3R4F filler	0,9580	1,0180	<0,05
4	3R4F filler	0,9180	1,0360	<0,05
4	3R4F filler	0,9600	1,1170	<0,05
5	3R4F filler	2,0632	1,5025	<LOQ
5	3R4F filler	2,0424	1,4175	<LOQ
5	3R4F filler	1,9915	1,4865	<LOQ
6	3R4F filler	1,4974	1,9398	<0,05
6	3R4F filler	1,2190	1,9995	<0,05
6	3R4F filler	1,6456	1,9845	<0,05
7	3R4F filler	0,8970	2,7715	<0,05
7	3R4F filler	1,0668	2,3862	<0,05
7	3R4F filler	0,8984	2,3498	<0,05
8	3R4F filler	1,1576	0,5700	ND
8	3R4F filler	1,1159	0,6222	ND
8	3R4F filler	1,1881	0,5151	ND
9	3R4F filler	–	–	–
9	3R4F filler	–	–	–
9	3R4F filler	–	–	–
10	3R4F filler	1,7680	0,7990	ND
10	3R4F filler	1,8200	1,3740	ND
10	3R4F filler	1,8650	0,7430	ND
11	3R4F filler	–	–	–
11	3R4F filler	–	–	–
11	3R4F filler	–	–	–
1	CRP1	1,1900	11,3000	<0,05
1	CRP1	1,1200	11,2000	<0,05
1	CRP1	1,1600	11,0000	<0,05
2	CRP1	1,0398	10,6952	<0,05

		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
2	CRP1	0,6158	5,9567	<0,05
2	CRP1	1,0850	11,2483	<0,05
3	CRP1	0,9746	9,1394	<0,05
3	CRP1	1,0731	10,0605	<0,05
3	CRP1	0,9804	8,7132	<0,05
4	CRP1	0,8650	8,2280	<0,05
4	CRP1	0,8400	8,0070	<0,05
4	CRP1	0,6990	8,6190	<0,05
5	CRP1	1,3209	9,4333	<LOQ
5	CRP1	1,3818	9,0285	<LOQ
5	CRP1	1,4014	8,8296	<LOQ
6	CRP1	1,3109	13,6837	<0,05
6	CRP1	1,2737	13,6185	<0,05
6	CRP1	1,3365	14,6556	<0,05
7	CRP1	0,7389	9,1404	<0,05
7	CRP1	0,7114	8,2512	<0,05
7	CRP1	0,8174	8,0971	<0,05
8	CRP1	0,8286	8,5308	<LOQ
8	CRP1	0,8622	7,4564	ND
8	CRP1	0,9651	10,0019	ND
9	CRP1	0,8900	10,9900	0,21
9	CRP1	0,9500	10,8900	0,21
9	CRP1	0,9500	11,2300	0,22
10	CRP1	–	–	–
10	CRP1	–	–	–
10	CRP1	–	–	–
11	CRP1	–	–	–
11	CRP1	–	–	–
11	CRP1	–	–	–
1	CRP1.1	1,7200	8,2100	<0,05
1	CRP1.1	1,2900	7,0300	<0,05
1	CRP1.1	1,4900	7,7500	<0,05
2	CRP1.1	1,0369	7,1072	<0,05
2	CRP1.1	1,1492	7,2714	<0,05
2	CRP1.1	1,3484	7,0135	<0,05
3	CRP1.1	1,4522	6,2845	<0,05

		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
3	CRP1.1	1,7023	7,1887	<0,05
3	CRP1.1	1,6683	6,6709	<0,05
4	CRP1.1	1,1290	5,1640	<0,05
4	CRP1.1	0,8940	5,3010	<0,05
4	CRP1.1	1,0610	5,0120	<0,05
5	CRP1.1	1,4027	6,6663	<LOQ
5	CRP1.1	1,6569	7,2404	<LOQ
5	CRP1.1	1,6776	7,2343	<LOQ
6	CRP1.1	1,6963	10,2616	<0,05
6	CRP1.1	2,0270	10,2269	<0,05
6	CRP1.1	1,7392	10,0255	<0,05
7	CRP1.1	1,1961	6,7750	<0,05
7	CRP1.1	1,2568	6,9725	<0,05
7	CRP1.1	1,2077	5,8058	<0,05
8	CRP1.1	1,0365	6,4171	<LOQ
8	CRP1.1	1,2243	7,5042	ND
8	CRP1.1	1,0006	5,5425	ND
9	CRP1.1	1,0400	7,4500	0,19
9	CRP1.1	1,0000	7,3100	0,17
9	CRP1.1	1,0700	7,4100	0,19
10	CRP1.1	1,0720	8,6420	ND
10	CRP1.1	0,9740	7,9080	ND
10	CRP1.1	0,9300	7,5470	ND
11	CRP1.1	1,6220	8,7630	0,02
11	CRP1.1	1,6840	9,0510	0,02
11	CRP1.1	1,6580	8,4570	0,02
1	CRP2	1,5000	4,1800	<0,05
1	CRP2	1,4400	3,9100	<0,05
1	CRP2	1,3800	3,9200	<0,05
2	CRP2	1,2151	3,2436	<0,05
2	CRP2	1,1083	3,7235	<0,05
2	CRP2	1,0588	3,8172	<0,05
3	CRP2	0,9959	3,8367	<0,05
3	CRP2	0,9259	4,0162	<0,05
3	CRP2	1,0165	3,8074	<0,05
4	CRP2	0,8990	4,6720	<0,05

		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
4	CRP2	0,8310	4,2410	<0,05
4	CRP2	0,9190	3,9000	<0,05
5	CRP2	1,6929	2,1574	<LOQ
5	CRP2	1,6102	2,1508	<LOQ
5	CRP2	1,5984	2,3373	<LOQ
6	CRP2	1,2325	4,2948	<0,05
6	CRP2	1,1569	4,3313	<0,05
6	CRP2	1,2790	4,1706	<0,05
7	CRP2	0,9894	3,2092	<0,05
7	CRP2	1,0883	3,1010	<0,05
7	CRP2	1,2845	2,9601	<0,05
8	CRP2	0,9982	3,0313	ND
8	CRP2	0,9861	3,3221	0,05
8	CRP2	1,3121	2,9157	ND
9	CRP2	1,1400	5,1000	0,19
9	CRP2	1,1100	5,3700	0,21
9	CRP2	1,2000	5,2600	0,18
10	CRP2	–	–	–
10	CRP2	–	–	–
10	CRP2	–	–	–
11	CRP2	–	–	–
11	CRP2	–	–	–
11	CRP2	–	–	–
1	CRP2.1	2,5900	4,8200	<0,05
1	CRP2.1	2,5900	5,1300	<0,05
1	CRP2.1	2,5400	4,5400	<0,05
2	CRP2.1	2,0402	4,4072	<0,05
2	CRP2.1	2,0672	4,3381	<0,05
2	CRP2.1	2,0885	4,3947	<0,05
3	CRP2.1	2,2133	4,0328	<0,05
3	CRP2.1	2,3676	4,3151	<0,05
3	CRP2.1	2,3534	7,5776	<0,05
4	CRP2.1	1,9720	4,2660	<0,05
4	CRP2.1	1,8710	4,0400	<0,05
4	CRP2.1	1,8780	4,3190	<0,05
5	CRP2.1	2,1200	3,7760	<LOQ

		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
5	CRP2.1	2,0678	3,8587	<LOQ
5	CRP2.1	2,6015	4,5006	<LOQ
6	CRP2.1	2,7808	5,7726	<0,05
6	CRP2.1	2,7187	5,7132	<0,05
6	CRP2.1	2,7493	5,6143	<0,05
7	CRP2.1	2,1603	4,7382	<0,05
7	CRP2.1	2,1503	4,6895	<0,05
7	CRP2.1	2,3852	4,8953	<0,05
8	CRP2.1	1,8064	3,2413	ND
8	CRP2.1	2,1132	4,2481	<LOQ
8	CRP2.1	1,7246	3,6881	ND
9	CRP2.1	2,4100	4,9600	0,22
9	CRP2.1	2,6000	5,0700	0,21
9	CRP2.1	2,4500	4,9300	0,2
10	CRP2.1	2,5840	5,2390	ND
10	CRP2.1	2,5180	4,9370	ND
10	CRP2.1	2,5970	4,5400	ND
11	CRP2.1	3,5320	5,4630	0,021
11	CRP2.1	3,8740	5,1810	0,022
11	CRP2.1	3,6650	5,7610	0,021
1	CRP3	10,4000	2,6800	0,061
1	CRP3	10,5000	2,6300	0,063
1	CRP3	10,6000	2,5400	0,064
2	CRP3	9,6079	3,7717	<0,05
2	CRP3	9,0207	3,7571	<0,05
2	CRP3	8,9279	3,4775	<0,05
3	CRP3	6,9222	2,3911	<0,05
3	CRP3	6,8692	2,3731	<0,05
3	CRP3	6,9699	2,4075	<0,05
4	CRP3	6,8520	2,4740	<0,05
4	CRP3	7,4850	2,7280	<0,05
4	CRP3	7,2330	2,5980	<0,05
5	CRP3	8,4513	2,6991	0,11
5	CRP3	7,4037	2,0506	0,09
5	CRP3	7,7937	2,2262	0,09
6	CRP3	8,5810	2,7337	<0,05

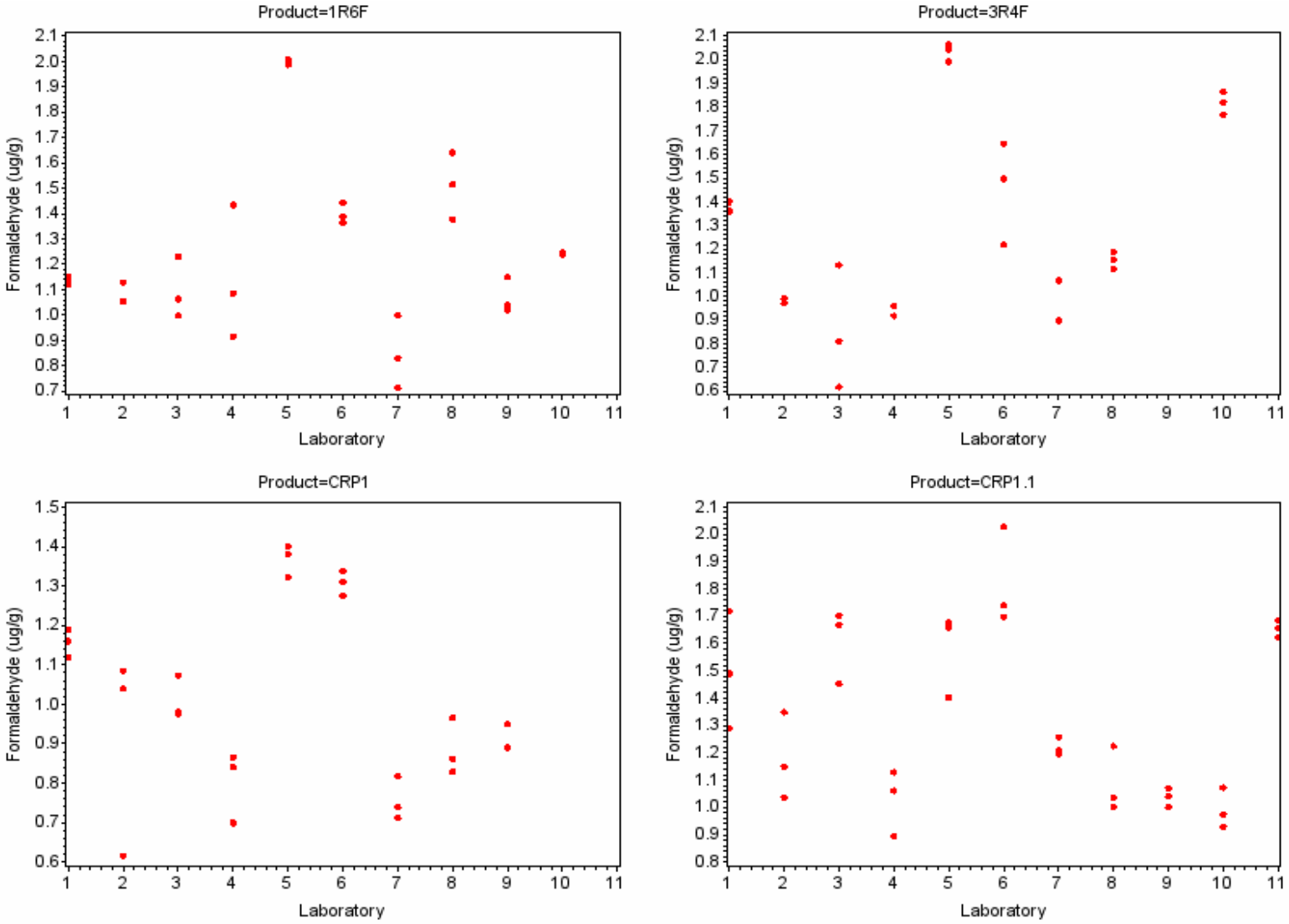
		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
6	CRP3	8,7669	2,3712	<0,05
6	CRP3	7,3312	2,1780	<0,05
7	CRP3	9,0146	3,6166	<0,05
7	CRP3	8,2709	3,6422	<0,05
7	CRP3	9,0264	3,8900	<0,05
8	CRP3	6,7996	1,7166	ND
8	CRP3	6,0620	2,1776	ND
8	CRP3	6,1433	1,8264	ND
9	CRP3	5,0000	5,3800	0,22
9	CRP3	4,8600	5,5100	0,24
9	CRP3	4,8500	5,6100	0,21
10	CRP3	–	–	–
10	CRP3	–	–	–
10	CRP3	–	–	–
11	CRP3	–	–	–
11	CRP3	–	–	–
11	CRP3	–	–	–
1	CRP3.1	3,6300	7,4700	<0,05
1	CRP3.1	3,6400	7,4800	<0,05
1	CRP3.1	3,6300	7,3300	<0,05
2	CRP3.1	3,6987	8,6334	<0,05
2	CRP3.1	3,6584	8,7981	<0,05
2	CRP3.1	3,8212	8,5363	<0,05
3	CRP3.1	3,0788	6,0185	<0,05
3	CRP3.1	2,8821	5,1931	<0,05
3	CRP3.1	3,2760	5,3961	<0,05
4	CRP3.1	2,7070	5,9780	<0,05
4	CRP3.1	2,8250	5,7510	<0,05
4	CRP3.1	2,7700	5,4770	<0,05
5	CRP3.1	4,0805	6,9302	<LOQ
5	CRP3.1	4,0780	6,8547	<LOQ
5	CRP3.1	4,1717	6,9145	<LOQ
6	CRP3.1	2,7504	5,9684	<0,05
6	CRP3.1	3,3512	6,9220	<0,05
6	CRP3.1	2,7383	5,9060	<0,05
7	CRP3.1	3,5000	7,1809	<0,05

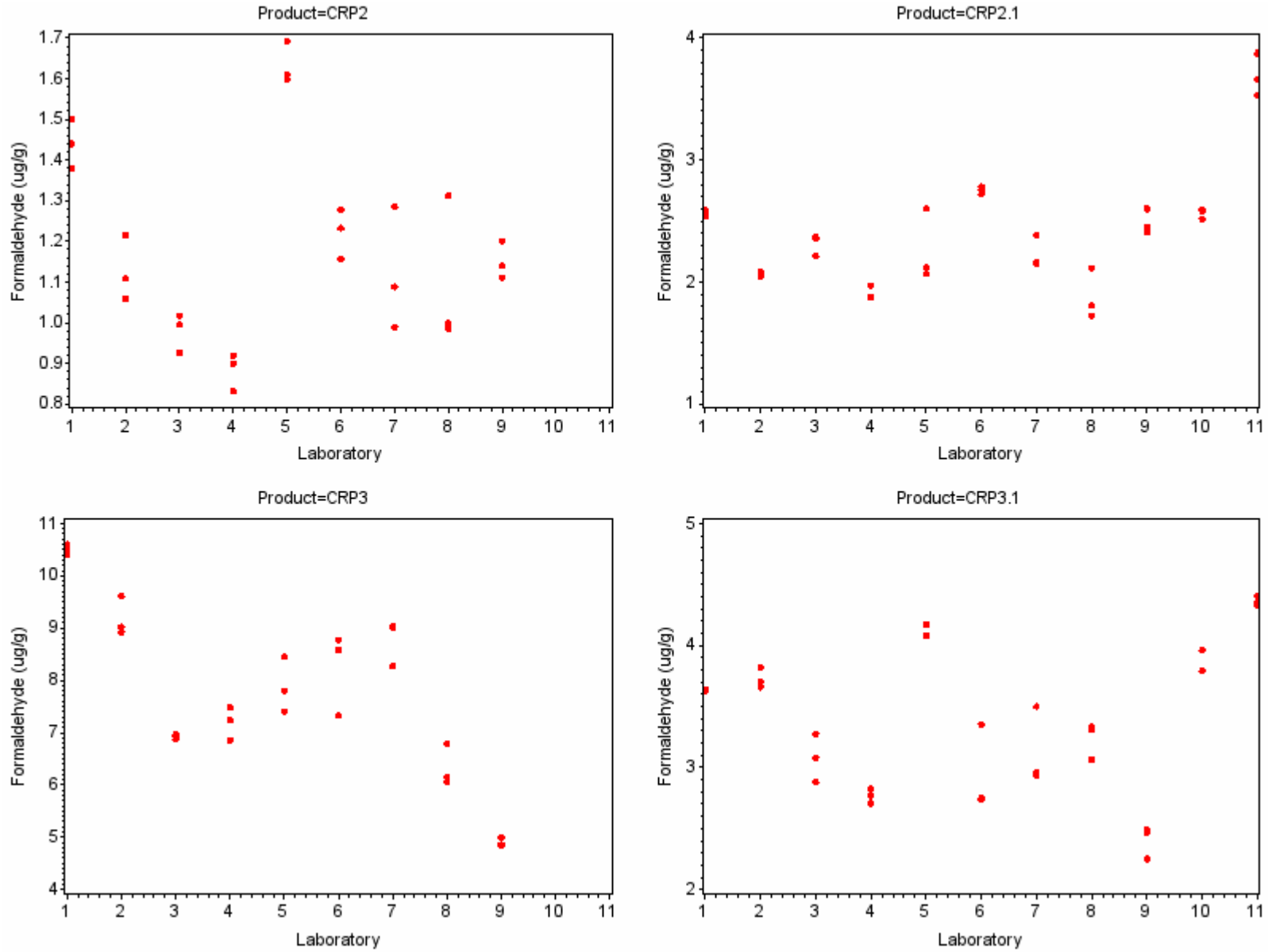
		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
7	CRP3.1	2,9574	7,4041	<0,05
7	CRP3.1	2,9312	7,4438	<0,05
8	CRP3.1	3,3360	7,4202	<LOQ
8	CRP3.1	3,3076	5,8371	<LOQ
8	CRP3.1	3,0648	6,2330	ND
9	CRP3.1	2,4700	6,8600	0,14
9	CRP3.1	2,4900	6,7800	0,14
9	CRP3.1	2,2500	6,1900	0,17
10	CRP3.1	3,9630	6,5880	ND
10	CRP3.1	3,9610	6,5190	ND
10	CRP3.1	3,7890	6,4460	ND
11	CRP3.1	4,3520	8,7790	0,019
11	CRP3.1	4,4050	8,8650	0,02
11	CRP3.1	4,3240	8,6970	0,02
1	CRP4.1	0,4700	1,0800	<0,05
1	CRP4.1	0,5200	1,0800	<0,05
1	CRP4.1	0,5200	1,0200	<0,05
2	CRP4.1	0,2970	2,2921	<0,05
2	CRP4.1	0,2901	2,0634	<0,05
2	CRP4.1	0,3630	2,2874	<0,05
3	CRP4.1	0,4286	0,9080	<0,05
3	CRP4.1	0,3135	0,8610	<0,05
3	CRP4.1	0,4542	0,9628	<0,05
4	CRP4.1	0,2330	1,1540	<0,05
4	CRP4.1	0,2950	1,1480	<0,05
4	CRP4.1	0,2850	1,0100	<0,05
5	CRP4.1	1,4670	1,3444	<LOQ
5	CRP4.1	1,3002	1,2976	<LOQ
5	CRP4.1	1,2911	1,3710	<LOQ
6	CRP4.1	0,4210	1,2747	<0,05
6	CRP4.1	0,4729	1,3283	<0,05
6	CRP4.1	0,3880	1,2770	<0,05
7	CRP4.1	0,3336	1,5141	<0,05
7	CRP4.1	0,2852	1,6259	<0,05
7	CRP4.1	0,2169	1,5300	<0,05
8	CRP4.1	0,2104	0,9513	ND

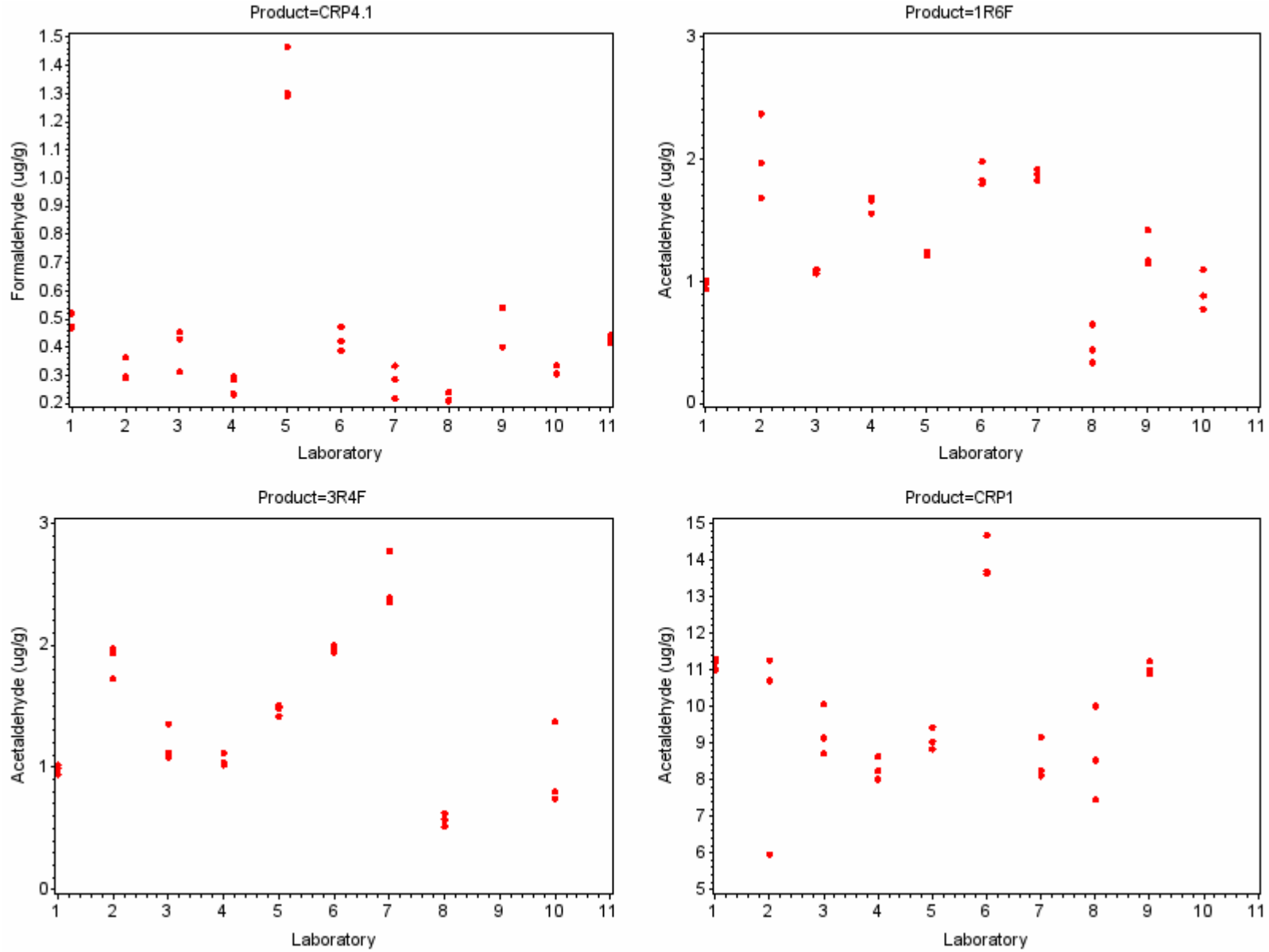
		Formaldehyde	Acetaldehyde	Crotonaldehyde
Lab Code	**Product**	**µg/g**	**µg/g**	**µg/g**
8	CRP4.1	<LOQ	1,3461	ND
8	CRP4.1	0,2403	1,2812	ND
9	CRP4.1	0,4000	1,4000	0,18
9	CRP4.1	0,4000	1,4200	0,18
9	CRP4.1	0,5400	1,6500	0,22
10	CRP4.1	0,3050	1,2550	ND
10	CRP4.1	0,3360	1,7740	ND
10	CRP4.1	0,3080	1,3280	ND
11	CRP4.1	0,4310	1,0860	0,006
11	CRP4.1	0,4410	1,1050	0,006
11	CRP4.1	0,4160	1,0800	0,006

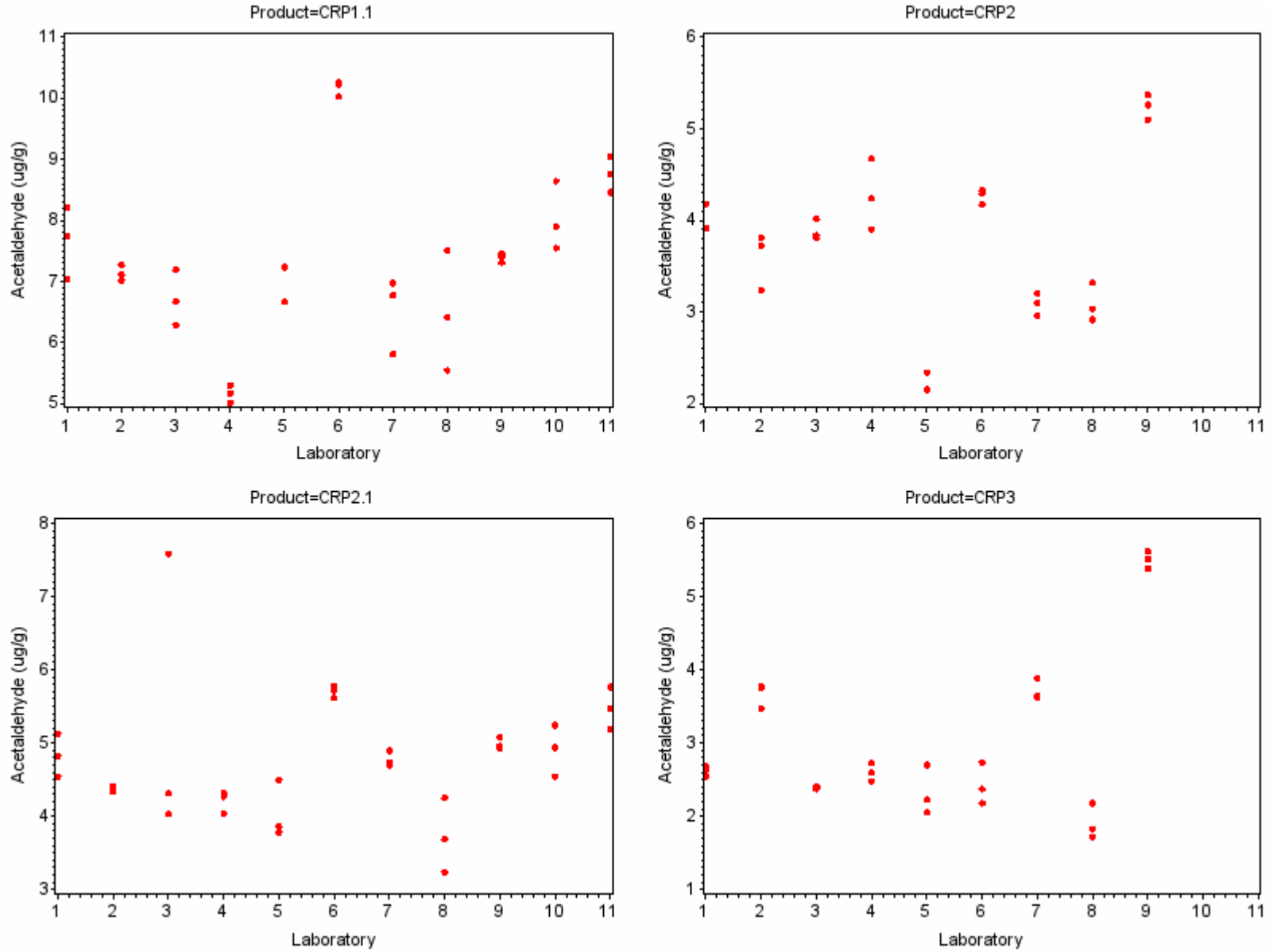
The (–) symbol indicates the laboratory did not submit a value for that sample analysis.
<LOQ and <x.xx indicates there was a detectable peak but it was below the method Limit of Quantitation (LOQ).
ND indicates there was no detectable peak (Not Detected).

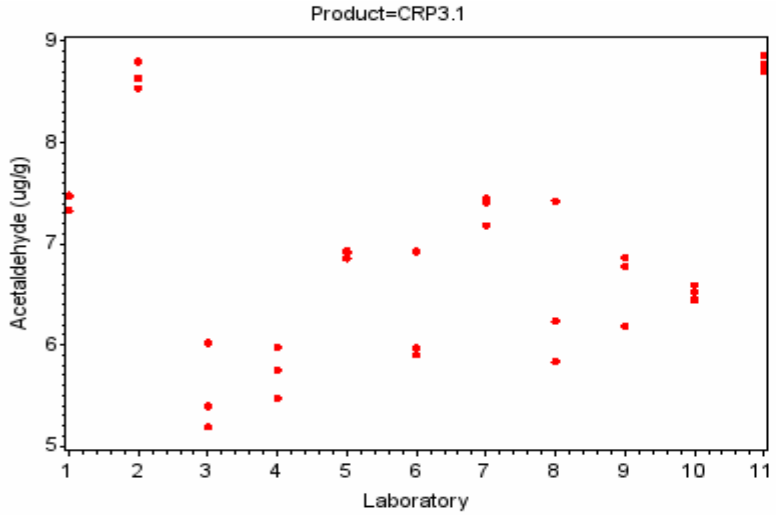
APPENDIX E: Raw Data Plots

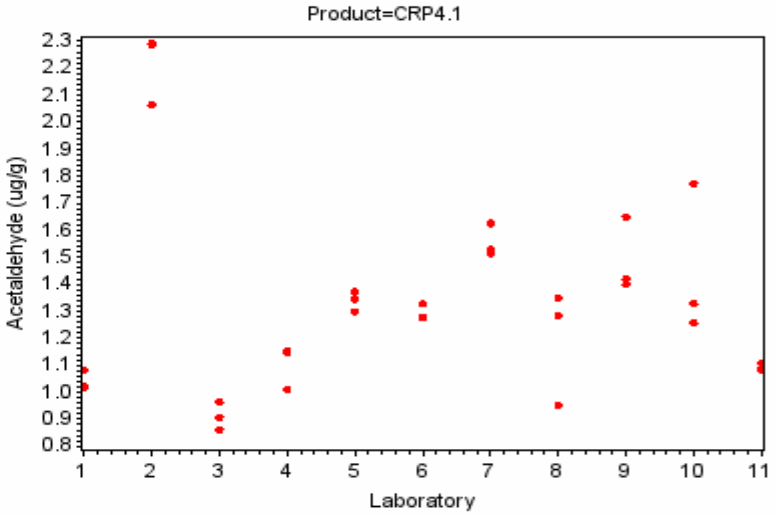



Product=CRP2
Formaldehyde (ug/g)
Laboratory
Product=CRP2.1
Formaldehyde (ug/g)
Laboratory
Product=CRP3
Formaldehyde (ug/g)
Laboratory
Product=CRP3.1
Formaldehyde (ug/g)
Laboratory


Product=CRP4.1
Formaldehyde (ug/g)
Laboratory
Product=1R6F
Acetaldehyde (ug/g)
Laboratory
Product=3R4F
Acetaldehyde (ug/g)
Laboratory
Product=CRP1
Acetaldehyde (ug/g)
Laboratory


Product=CRP1.1
Acetaldehyde (ug/g)
Laboratory
Product=CRP2
Acetaldehyde (ug/g)
Laboratory
Product=CRP2.1
Acetaldehyde (ug/g)
Laboratory
Product=CRP3
Acetaldehyde (ug/g)
Laboratory


Product=CRP3.1
Acetaldehyde (ug/g)
Laboratory


Product=CRP4.1
Acetaldehyde (ug/g)
Laboratory

APPENDIX F: Repeatability and Accuracy of Crotonaldehyde Using Laboratory Fortified Matrix Spikes

Crotonaldehyde	**CRP1**						
Fortification Amount (µg/g)		0,3		0,5		1,0	
Replicate	Unfortified (µg/g)	Low (µg/g)	Low %Rec	Med (µg/g)	Med %Rec	High (µg/g)	High %Rec
1	<LOQ	0,28	93	0,49	98	0,99	99
2	<LOQ	0,27	90	0,48	96	0,97	97
3	<LOQ	0,28	93	0,44	88	0,93	93
Average	NA	0,28	92	0,47	94	11,05	96
Standard Deviation	NA	0,01	0,02	0,03	0,05	0,36	0,03
%RSD	NA	4,3	2,1	4,3	5,6	3,2	3,2

Crotonaldehyde	**CRP2**						
Fortification Amount (µg/g)		0,3		0,5		1,0	
Replicate	Unfortified (µg/g)	Low (µg/g)	Low %Rec	Med (µg/g)	Med %Rec	High (µg/g)	High %Rec
1	<LOQ	0,26	87	0,51	102	0,92	92
2	<LOQ	0,30	100	0,50	100	0,98	98
3	<LOQ	0,30	100	0,50	100	0,96	96
Average	NA	0,29	96	0,50	101	0,95	95
Standard Deviation	NA	0,02	0,08	0,01	0,01	0,03	0,03
%RSD	NA	8,1	8,1	1,1	1,1	3,2	3,2

Crotonaldehyde	**CRP3**						
Fortification Amount (µg/g)		0,30		0,50		1,0	
Replicate	Unfortified (µg/g)	Low (µg/g)	Low %Rec	Med (µg/g)	Med %Rec	High (µg/g)	High %Rec
1	0,11	0,39	86	0,64	101	0,97	84
2	0,14	0,42	96	0,62	97	0,93	80
3	0,14	0,34	70	0,59	92	1,01	88
Average	0,13	0,38	84	0,61	97	0,97	84
Standard Deviation	0,02	0,04	0,13	0,02	0,05	0,04	0,04
%RSD	16	10	15	4	5	4	5

Crotonaldehyde	**CRP4**						
Fortification Amount (µg/g)		0,3		0,5		1	
Replicate	Unfortified (µg/g)	Low (µg/g)	Low %Rec	Med (µg/g)	Med %Rec	High (µg/g)	High %Rec
1	<LOQ	0,32	107	0,52	104	0,93	93
2	<LOQ	0,29	97	0,46	92	0,91	91
3	<LOQ	0,31	103	0,49	98	1,04	104
Average	NA	0,31	102	0,49	98	0,96	96
Standard Deviation	NA	0,02	0,05	0,03	0,06	0,07	0,07
%RSD	NA	5,0	5,0	6,1	6,1	7,3	7,3

Crotonaldehyde	**3R4F filler**						
Fortification Amount (µg/g)		0,3		0,5		1	
Replicate	Unfortified (µg/g)	Low (µg/g)	Low %Rec	Med (µg/g)	Med %Rec	High (µg/g)	High %Rec
1	<LOQ	0,258	86	0,450	90	0,901	90
2	<LOQ	0,243	81	0,479	96	0,840	84
3	<LOQ	0,272	91	0,487	97	0,880	88
Average	NA	0,26	86	0,472	94	0,87	87
Standard Deviation	NA	0,01	0,05	0,02	0,04	0,03	0,03
%RSD	NA	5,6	5,6	4,1	4,1	3,5	3,5