



Title: **Determination of Select Carbonyls in Tobacco by UPLC-MS/MS**

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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 (UPLC-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 autosampler vial for analysis.

B. DEFINITIONS

1. **CCS:** Calibration Check Standard
2. **DNPH:** 2,4-dinitrophenylhydrazine
3. **DNPH RB:** 2,4-dinitrophenylhydrazine 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. RESPONSIBILITIES

1. The designated trained analyst performing the method is responsible to follow all steps of the procedure and to document and report any procedural deviations from the method to laboratory management.
2. Personnel using this procedure are responsible for conducting the analysis in a manner consistent with Altria safety policies.

D. VALIDATION

1. Internal Validations:
 - a. This test Method was validated in October – November 2015 using CRP1, CPR2, CPR3, CRP4, and 3R4F cigarette filler.
 - b. The coefficients of determinations (R^2) for calibration curves were greater than 0.990. The repeatability (within-day) and reproducibility (between-day) of the method ranged from 2 – 20 % RSD for the five reference products, over three different days.

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- c. Samples and working standards are stable for up to 24 hours only when analyzed as a complete preparation set.
- d. The LOQ for formaldehyde, acetaldehyde, and crotonaldehyde are 0.0096 µg/mL (0.096 µg/g), 0.0095 µg/mL (0.095 µg/g), and 0.0058 µg/mL (0.058 µg/g) respectively.

E. EQUIPMENT AND APPARATUS

1. Equipment and Apparatus Required: equivalent materials may be substituted
 - a. UPLC system such as Waters Acquity I-Class. The UPLC should be equipped with a binary solvent delivery system, temperature controlled autosampler, and temperature controlled column compartment. Waters Corp., Milford, MA
 - b. Analytical UPLC Column: Waters Acquity UPLC BEH Shield RP18 column (2.1 x 100 mm, 1.7 µm), Part # 186002854, Waters Corp., Milford, MA
 - c. Guard column: Waters Acquity UPLC BEH C18 VanGuard guard column, (2.1 x 5 mm, 1.7 µm), Part # 186003975, Waters Corp., Milford, MA
 - d. Amber autosampler vials, Fisherbrand, Part # 03-391-6, Fisher Scientific; Atlanta, GA
 - e. Teflon/red rubber-lined 11 mm crimp seal autosampler vial caps, Part # C4011-1AP, Thermo Scientific
 - f. 11 mm electronic crimper, Part # 5190-3188, Agilent Technologies
 - g. Analytical balance with the ability to measure to the nearest 0.1 mg, placed in an enclosure with suitable exhaust
 - h. Dispensette 50-mL capacity, Part # 13-688-224, Fisher Scientific
 - i. Dispensette, 10-mL capacity, Part # 13-688-222, Fisher Scientific
 - j. Sample rotator capable of rotating the samples at 15 rpm ± 2 rpm, custom
 - k. Class A Volumetric flasks, (10, 25, 50, 100, and 5000 mL)
 - l. 60 mL glass bottles with open-top white polypropylene cap containing 0.125 in. thick PTFE lined silicone septum, Part # S136-0060, Thermo Scientific
 - m. Mechanical pipettes and tips in the range of 50-5000 µL
 - n. Repeater Pipette, Part # 4560237, Eppendorf Repeater, Eppendorf
 - o. Combitips (1 mL- 50 mL), Eppendorf
 - p. gas tight glass syringes, 1 mL

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- q. Glass or plastic Pasteur pipettes
- r. Amber glass storage bottles with Teflon-lined screw caps in the range of 10 mL to 125 mL
- s. pH meter, S20 SevenEasy™ pH, Mettler Toledo
- t. pH probe, pH InLab Expert Pro Part # 51343101, Mettler Toledo

2. Instrument Setup

Suggested instrumental parameters for the UPLC and mass spectrometer are listed below. The mass spectrometer settings are suggested starting points and may be modified in order to obtain acceptable performance.

Table 1A: UPLC Parameters

Parameter	Setting
Mobile Phase A	Reagent Water (Milli-Q 18.2 MΩ)
Mobile Phase B	0.1% Acetic Acid in Methanol
Column Temperature	60 °C
Flow Rate	0.3 mL/min
Autosampler Temperature	5 °C
Injection Volume	2 µL
HPLC Run Time	4.5 min

Table 1B: Mobile Phase Gradient

Time (min)	Flow (mL/min)	A (%)	B (%)	Curve
Initial	0.3	50	50	6
0.2	0.3	50	50	6
0.8	0.3	0	100	6
1.4	0.3	0	100	6
1.6	0.3	50	50	6
4.1	0.3	50	50	6

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Table 1C: Sample Manager Settings

Waters Classic UPLC	Waters Acquity UPLC-I Class
Weak wash: 1:1:1:1 water, methanol, acetonitrile, isopropanol and 20 mL ammonium hydroxide Wash Volume 1200µL	Wash Solvent: 1:1:1:1 water, methanol, acetonitrile, isopropanol and 20 mL ammonium hydroxide
Strong wash: 1:1:1:1 water, methanol, acetonitrile, isopropanol and 20 mL formic acid Wash Volume 400µL	Purge Solvent: 10% Acetonitrile Needle Wash: 10% Acetonitrile
Injection loop type: PLNO	Injection loop type: Flow through Needle
	Pre-Injection wash 0 sec
	Post-Injection wash 6 sec.

Table 1D: Sample Manager Settings*

Parameter	Setting
Classic UPLC	Multiplier 650
Acquity UPLC-I Class	Gain 1
Ionization mode:	Electrospray Negative
MS Mode:	MRM
Capillary Voltage:	2.0 kV
Cone Voltage:	30 V
RF Len:	2.5
Source Temperature:	150 °C
Desolvation Temperature:	500 °C
Desolvation Gas:	1000 L/hr
Cone Gas	0 L/hr
LM Resolution 1	10.6
HM Resolution 1	14.9
Ion Energy 1	0.3
LM Resolution 2	12.7
HM Resolution 2	14.9
Ion Energy 2	0.6
Collision Energy	8 V
Source Temperature	150 °C
Extractor	3 V
Entrance	1
Exit	1

*Note: These are suggested settings and may require optimization

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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	209.00	162.96	auto
Formaldehyde-d2-DNPH	30	8	211.10	133.10	auto
Acetaldehyde-DNPH	26	10	223.10	151.06	auto
Acetaldehyde-d4-DNPH	28	12	227.10	151.10	auto
Crotonaldehyde-DNPH	32	14	248.94	172.06	auto
Crotonaldehyde-d3-DNPH	20	12	252.00	175.00	auto

*Note: These are suggested settings and may require optimization

3. Instrument Maintenance

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

F. CHEMICALS AND REAGENTS

- Chemicals Required: equivalent materials may be substituted
 - Methanol, Optima grade, Part # A454-4, Fisher Scientific
 - 2-Propanol, Optima grade, Part # A461-500, Fisher Scientific
 - Acetonitrile, Optima grade, Part # A996-4, Fisher Scientific
 - Acetic Acid, Optima grade, Part # A113-50, Fisher Scientific
 - Ammonium formate, HPLC grade, Part # 401152500, Acros Organics
 - Formic Acid (88%), Laboratory grade, Part # A119P – 500, Fisher Scientific

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- g. Ammonium hydroxide, Optima grade, Part # A-470-250, Fisher Scientific
- h. Type 1 or reagent water, water purification system capable of producing 18.2 MΩ water
- i. Isohexane (contains < 5% n-Hexane), HPLC grade, Catalog #383820025, Acros Organics
- j. Acetaldehyde-d4, 98%+ purity, Part # D-163, CDN Isotopes
- k. Crotonaldehyde 2,4-Dinitrophenylhydrazone-, 5,6-d3, 98% purity, Part # D-7604, CDN Isotopes
- l. Formaldehyde-d2, 98%+ purity, Part # D-5105, CDN Isotopes
- m. 2,4-Dinitrophenylhydrazine ~30% water, Reagent grade, Catalog # D-199303, Sigma-Aldrich. This reagent will need to be purified by recrystallization.
- n. 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. **DNPH-solution, 5 mg/mL**

Add 0.5±0.02g of recrystallized DNPH (see WI 097-6044 for recrystallization procedure) to a 100 mL volumetric flask filled with approximately 50 mL of acetonitrile. Use an orbital shaker set to ~250 rpm for 15 min 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: Analyze a DNPH RB of freshly prepared DNPH solution prior to being used for sample preparation to ensure that background contamination is sufficiently low. The DNPH background levels for formaldehyde and acetaldehyde must be less than 0.005 µg/mL. Crotonaldehyde must have background levels less than 0.0005 µg/mL to be acceptable.

b. **Extraction solution, 100 mM ammonium formate, pH 3.0 (± 0.1)**

To make a 5L solution, weigh and transfer 32g ±0.05g of ammonium formate into a 5 L volumetric flask containing approximately 2 L of reagent grade water. Use a stir bar to dissolve the solids. Add approximately 2L 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 85 mL of formic acid. Fill to volume

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with reagent grade water and mix well. The solution is stable for up to 2 weeks when stored at ambient temperature.

c. Mobile Phase A

Use reagent grade water. The water should be changed every 2 days.

d. Mobile phase B, 0.1% (v/v) acetic acid in methanol

Transfer 1 mL of acetic acid using the appropriate mechanical pipette to a 1 L volumetric flask containing approximately 500 mL of Methanol. Fill to volume with Methanol and mix well. The solution is stable for 1 month 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 of **Stock Calibration Standard Solution 1** on an analytical balance using a tared 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 099-5031 Standard Preparation for Carbonyls in Smokeless Tobacco. Store in a 10 mL amber bottle with PTFE lined cap. The solution is stable for 16 days when stored in the refrigerator (at approximately 4 °C).
- 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 in the refrigerator (at approximately 4 °C).
- 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 in the refrigerator (at approximately 4 °C).
- 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



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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 \pm 0.01g of **Stock Calibration Check Standard Solution 1** on an analytical balance using a tared 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 099-5031 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 in the refrigerator (at approximately 4 °C).
- 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 in the refrigerator (at approximately 4 °C).
- 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 in the refrigerator (at approximately 4 °C) or until the expiration date stated on the purchased material, whichever is first.
- i. **Stock Internal Standard Solution Acetaldehyde-d4, 10 mg/mL**
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 in the refrigerator (at approximately 4 °C) 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 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

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amber bottle with PTFE lined cap. The solution is stable for up to 5 weeks when stored in the refrigerator (at approximately 4 °C) 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, Crotonaldehyde-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 in the refrigerator (at approximately 4 °C).

Working Calibration Standards: The calibration standards are prepared at the same time as the samples and according to the same procedure as the samples excluding the tobacco. See [section H.1](#) 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 5 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 crimp caps and placed in the autosampler set to ~5°C.

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 5. 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

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- I. **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. See [section H.1](#) for a detailed description. Calibration Check Standards are prepared using the Stock Calibration Check Standard Stock 3 in combination with the Working Internal Standard solution according to Table 6. Store in amber bottles at ~ 4°C. 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 6. 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 7.

Table 7 . 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 and 1 µg/mL of acetaldehyde-d4 as the derivatized hydrazone, and 0.056 µg/mL of crotonaldehyde-d3 as the free carbonyl.

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

G. SAMPLE REQUIREMENTS

1. Sample Storage Conditions

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

- a. While waiting to be tested, samples must be stored in the refrigerator (at approximately 4 °C) for short term storage (0-4 weeks). Prior to opening the container, the samples must be allowed to equilibrate to ambient conditions for a minimum of 1hour before sample preparation.
- b. Samples that will not be analyzed within 4 weeks must be stored unopened at approximately -20 °C.
- 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 1hour 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 should 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, and other products with a particle size not exceeding long cut MST should be analyzed without further sample grinding.
- c. Cigarette filler and samples with particle sizes larger than long cut MST should be cryoground with liquid nitrogen using a Retsch Grindomix GM200 at a grinding speed 10 for 1 minute.

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.

H. PROCEDURE

1. Sample Preparation

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Note: Prepare all solutions and equipment in order to be able to run all operations as quickly as possible without time interruptions. Finish operation (h) within 15± 5 minutes after the 60 min shaking in step (g) has been completed. Batch sizes should be adjusted in order to meet time limitations.

- a. Weigh 1.00 ± 0.20 g of sample into a 60 mL glass vial.
- b. Add 40.0 mL of 100 mM ammonium formate (pH 3.0) to the 60 mL glass bottles using a dispensette.
- c. Pipette the calibration standards, see [Table 5](#).
- d. Add 100 µL of Working Internal Standard Solution using a repeater pipette to all samples 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 a rotator set to approximately 15 rpm ±2 rpm.

Important! Continue to step (h) within 15± 5 minutes from the completion of rotation.

- 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 UPLC method
- d. Perform the following operations to prime the instrument:

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- 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 from a single vial of calibration standard level 3
 - 3) Isohexane blank
 - 4) Calibration standards 1 – 8; beginning curve
 - 5) Isohexane blank
 - 6) CCS
 - 7) Set of approximately 10 samples which includes the monitor sample
 - 8) Repeat CCS after each batch of approximately 10 samples and after the last sample batch
 - 9) Isohexane blank
 - 10) 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.

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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 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

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

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

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g. Analyte Amount

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

Where:

Sample Conc = the calculated concentration ($\mu\text{g/mL}$)

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:

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

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 standard level 3. The %RSD of the injections ($n=5$) should be ≤ 10 . These values should be plotted in a control chart so that the laboratory can verify that these are suitable limits. At the time this SOP was written, limited sample data had been generated for the method. To this end, it is recommended that the laboratory determine if other parameters regarding system suitability are required or more appropriate as larger amounts of data are generated. 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) Three calibration points at different concentrations or two points at the same concentration plus a point at another concentration may be removed

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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) Slope and intercept: Since this method is an internal standard method and the UPLC-MS/MS response may drift from day to day, a stable and clean analytical instrument should provide consistent slopes and intercepts without large deviation or offset from day to day.
- 4) % Deviation : For formaldehyde and acetaldehyde the calibration curves should have % deviation values not exceeding $\pm 30\%$ for STD 2 and $\pm 20\%$ for STD 3-8. For crotonaldehyde, since STD 2 is not used, the calibration curves should have % deviation values not exceeding $\pm 30\%$ for STD 3 and $\pm 20\%$ for STD 4-8.
- 5) 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 $\pm 15\%$ of the nominal concentrations for each analyte. If % deviation results for a CCS fall outside of $\pm 15\%$, the samples bracketed by that CCS must be reanalyzed.
- 6) Monitor sample: Results from the appropriate monitor sample (method process control) must be compared to historical control charts for all analytical sample batches. Analytical results for the monitor sample must be plotted on the control charts for each analyte. Samples bracket by a failing CCS should be considered suspect and discarded unless an assignable cause can be identified. Data approval with failing a CCS must be authorized by laboratory management. All samples that are discarded must be prepared and analyzed with new calibration standards and new CCSs.
- 7) 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 prepared again and reanalyzed.
- 8) The LOQ will be 0.01 $\mu\text{g/mL}$ (0.1 $\mu\text{g/g}$) for formaldehyde and acetaldehyde and 0.005 $\mu\text{g/mL}$ (0.05 $\mu\text{g/g}$) for crotonaldehyde.

I. REFERENCES

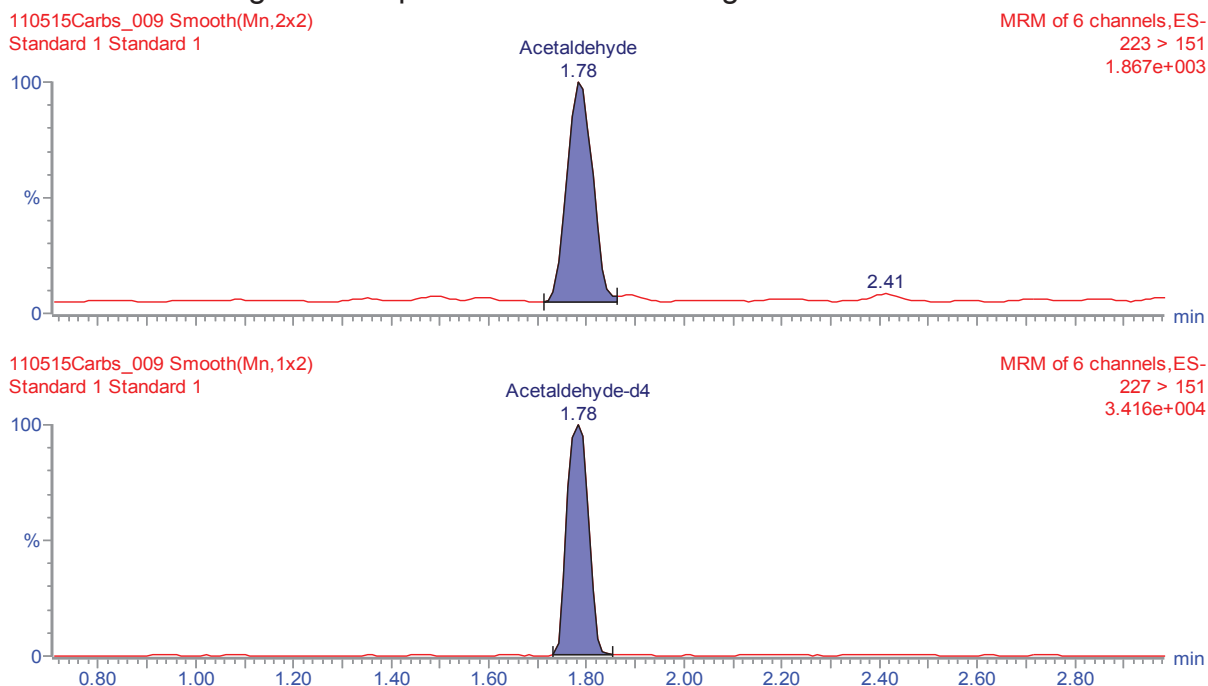
1. Analytical Sciences Test Method Validation Report, "SOP 096-5020 Determination of Select Carbonyls in tobacco by UPLC-MS/MS," January 12, 2016.

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2. Determination of Volatile Aldehydes in Tobacco by HPLC MS/MS. Electronic Laboratory Notebook: Ballentine, Regina-2009-03-30-060
3. Electronic Laboratory Notebook: Ballentine, Regina-2009-03-30-061
4. Form: 099-5031 Calibration Standard Preparation Template
5. WI 097-6044 DNPH Recrystallization
6. WI 097-1007 Regulatory Tobacco Product Sample Storage

J. APPENDICES

Figure 1. Representative Chromatogram of Standard 1



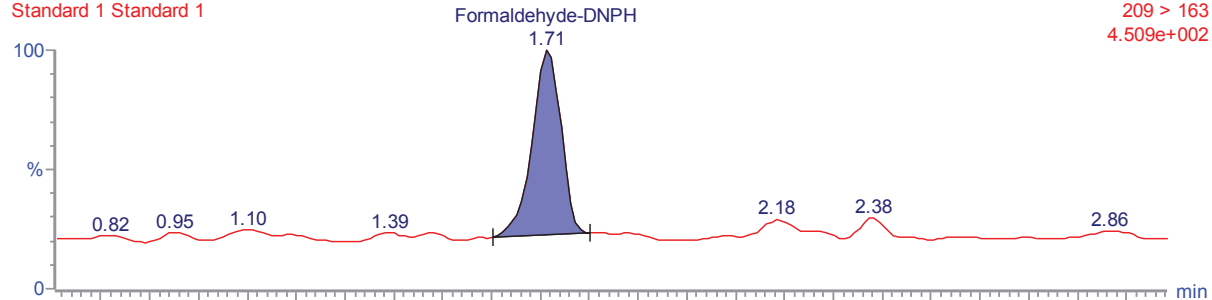
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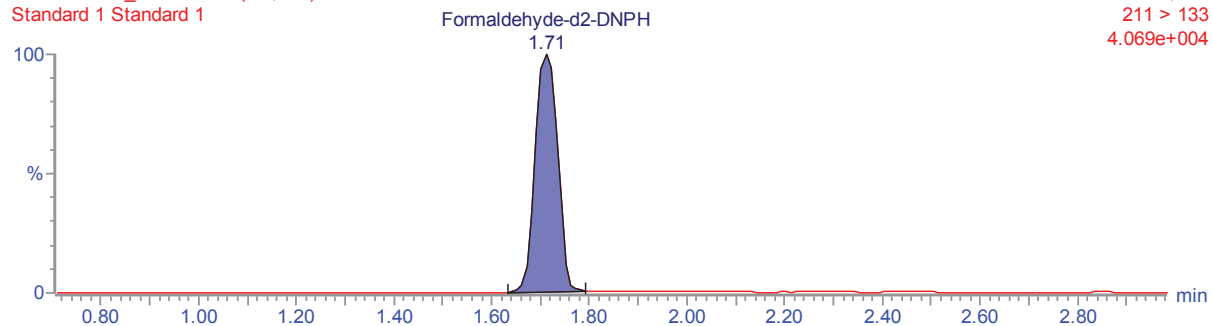
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Standard 1 Standard 1

MRM of 6 channels, ES-
209 > 163
4.509e+002



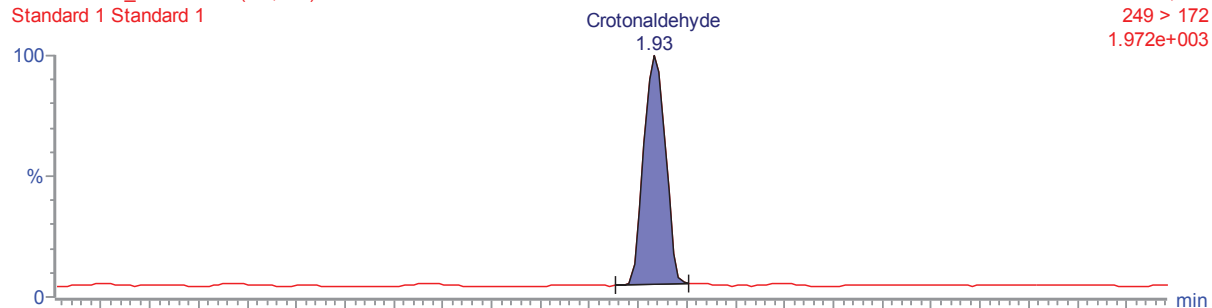
110515Carbs_009 Smooth(Mn,1x2)
Standard 1 Standard 1

MRM of 6 channels, ES-
211 > 133
4.069e+004



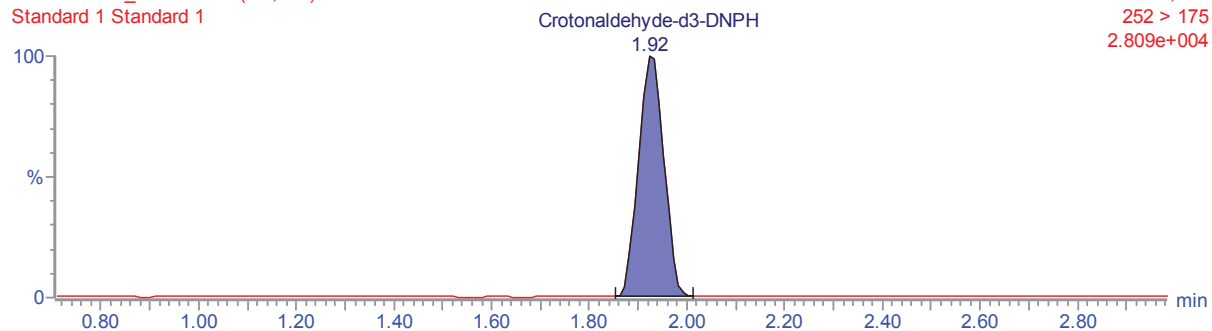
110515Carbs_009 Smooth(Mn,1x2)
Standard 1 Standard 1

MRM of 6 channels, ES-
249 > 172
1.972e+003



110515Carbs_009 Smooth(Mn,2x2)
Standard 1 Standard 1

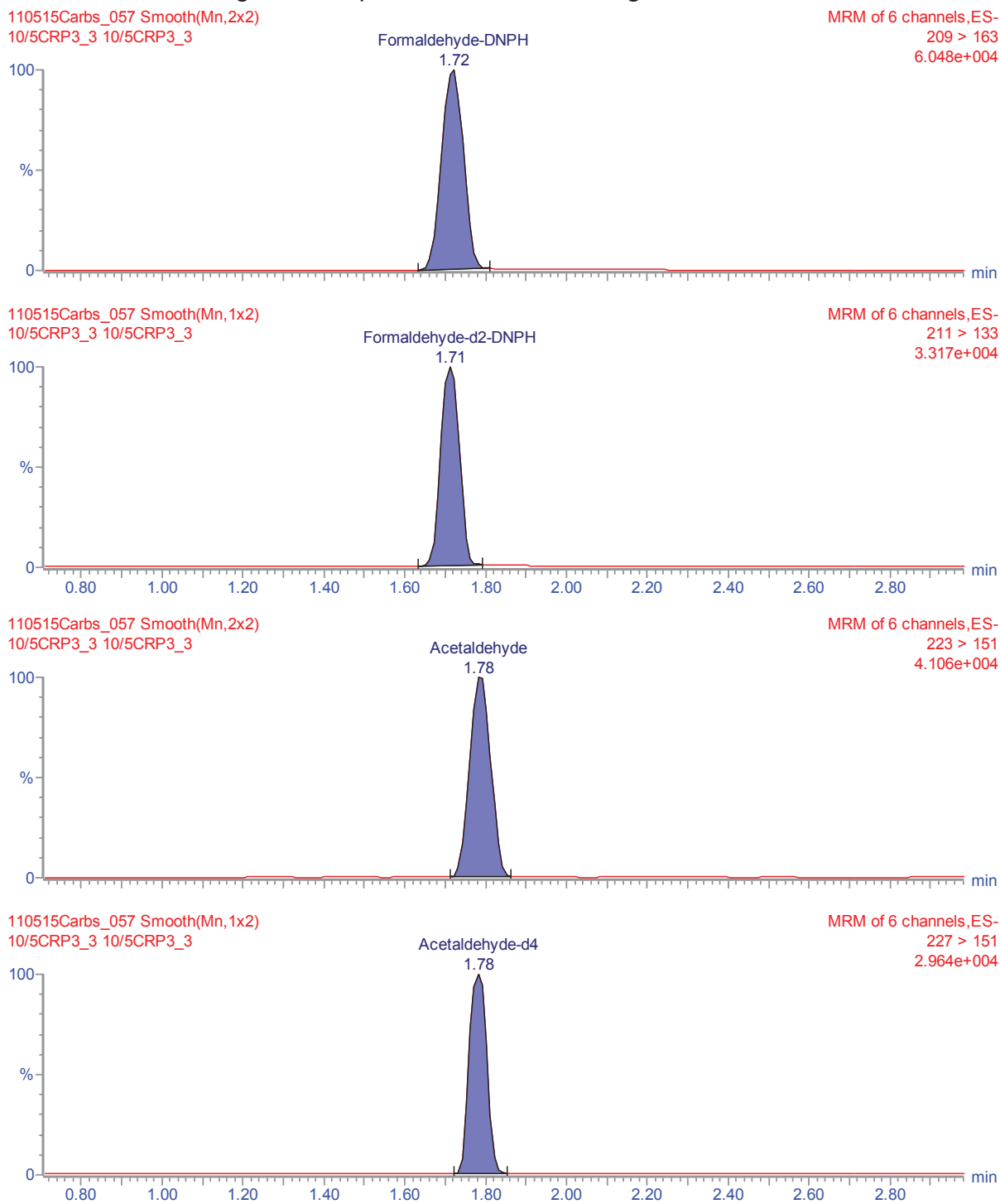
MRM of 6 channels, ES-
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2.809e+004



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Figure 2. Representative Chromatogram of CRP3



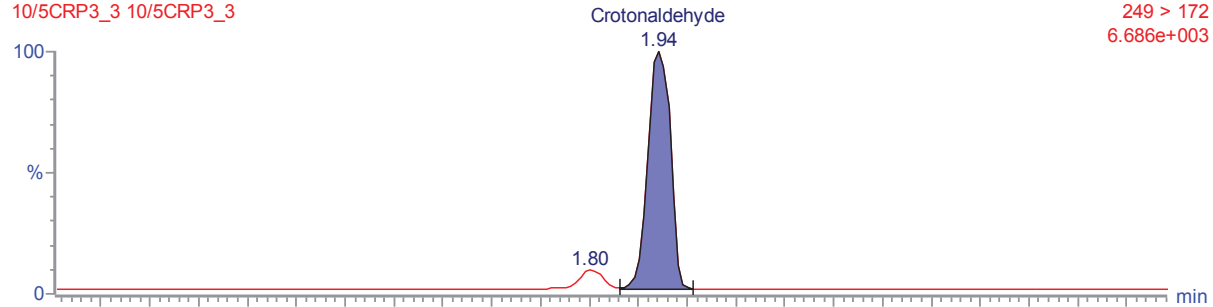
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Title: Determination of Select Carbonyls in Tobacco by UPLC-MS/MS

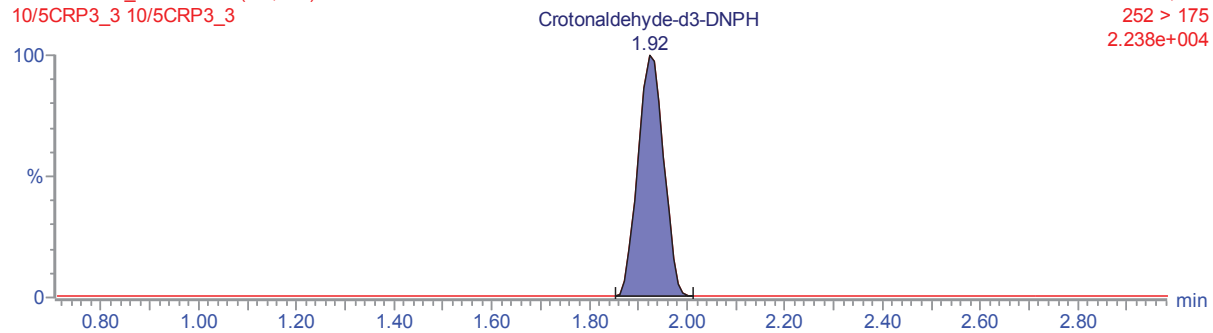
110515Carbs_057 Smooth(Mn,1x2)
10/5CRP3_3 10/5CRP3_3

MRM of 6 channels, ES-
249 > 172
6.686e+003



110515Carbs_057 Smooth(Mn,2x2)
10/5CRP3_3 10/5CRP3_3

MRM of 6 channels, ES-
252 > 175
2.238e+004



****Uncontrolled Copy****