APPENDIX A3.2.2-11

ANALYSIS OF CARBONYLS IN AEROSOL

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1. ANALYSIS OF CARBONYLS IN AEROSOL

1.1. Applicability

The method described is used to determine acetaldehyde and butyraldehyde in mainstream aerosol from conventional cigarettes and the Tobacco Heating System (THS) under Health Canada Intense (HCI) smoking conditions.

The method also quantitates other carbonyls (formaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, and methyl ethyl ketone [MEK]). Results are expressed as µg/stick for THS and µg/cigarette for 3R4F.

1.2. Analytical Procedure

Aerosol from a smoking machine is collected in 2 micro-impingers, each containing 10 mL of acidified dinitrophenylhydrazine (DNPH) derivatization solution 25 mM in acetonitrile, to which 0.5 mL pyridine is added per impinger. In order to obtain similar concentrations for analysis, 3R4F samples are further diluted with the same solution. Analysis is performed using Ultra-Performance Liquid Chromatography coupled to tandem Mass Spectrometry (MS/MS) with an Electrospray Ionization (ESI) source in negative mode. MS/MS detection, and the equipment and conditions, are described in Sections 1.2.2.1, 1.2.2.2 and 1.2.2.3.

The 3R4F and THS are conditioned at least 48h at target conditions of 22 ± 1 °C and a relative humidity of 60 ± 3%, before being used for aerosol generation.

Preceding each batch, column conditioning and calibration are performed according to the details given in Section 1.2.3. Typically, four sequences of measurement are performed per batch. Each sequence consists of a reference run, followed by a number of sample runs, and is terminated by a standard solution with a medium concentration to validate the results and verify the calibration (requirement: deviation of not more than ± 15% from the theoretical value).

1.2.1. Preparation of Solutions

1.2.1.1. Calibration Solutions

Seven standard solutions containing different concentrations of the target compounds (highest:lowest = 78:1 for acetaldehyde and 30:1 for butyraldehyde) and acetone are prepared by diluting acetaldehyde stock solution, butyraldehyde stock solution and acetone stock solution with 2,4-dinitrophenylhydrazine (DNPH) solution 25 mM containing 5% w/w pyridine. Fresh calibration vials are prepared by diluting 1 mL of each calibration standard with 500 µL internal standard (ISTD) stock solution 2 (Propionaldehyde-D_2 1 µg/mL). The ranges of concentrations cover the ranges relevant for analysis and are provided in

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Table **1**.

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Table 1: Calibration Standards Ranges

Target Compounds	Acetaldehyde	0.256 µg/mL – 20.000 µg/mL
	Butyraldehyde	0.081 µg/mL – 2.398 µg/mL
Internal Standard	Propionaldehyde-D_2	1.0 µg/mL

1.2.1.2. Derivatization Solution

Aerosol is collected in an acidified 25 mM DNPH/acetonitrile derivatization solution and stabilized by addition of 4.76% v/v (5% w/w) pyridine. Replicates with one accumulation each for 3R4F and THS are prepared. DNPH with at least 99% purity from the same lot is used for aerosol collection and preparation of the standard solutions.

1.2.2. Experimental Conditions

1.2.2.1. Equipment

UPLC	A suitable UPLC equipped with a mass spectrometer detector and data acquisition software
Column	Waters Acquity BEH C18, 2.1 x 50 mm x 1.7 µm, or equivalent
Guard-Column	Waters Acquity BEH C18, 2.1 x 5 mm x 1.7 µm, or equivalent

1.2.2.2. UPLC Conditions

Filter	In-Line filter 2 µm, 2.1 mm with guard-filter, Waters Acquity or equivalent
Injection volume	1 µL
Flow rate	500 µL/min
Post-column flow split	1:5
Column oven temperature	40°C
Eluent A1	10 mM ammonium acetate in water/acetonitrile 65/35 v/v
Eluent B1	Acetonitrile

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Flow mode: Gradient

Time	Eluent A	Eluent B
0.0 min	100%	0%
2.0 min	77%	23%
3.7 min	77%	23%
3.8 min	0%	100%
4.8 min	0%	100%
5.0 min	100%	0%

1.2.2.3. Mass Spectrometer Settings

Ionization mode and polarity	ESI negative
Scan type	MRM
Acquisition time	4.5 min
Capillary voltage	2.3 kV
Source offset	40 V
Source temperature	150°C
Desolvation temperature	400°C
Cone gas flow	150 L/h
Desolvation gas flow	800 L/h
Collision gas	Argon, flow 0.17 mL/min
Nebulizer gas pressure	7 bar

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1.2.3. Calibration

Calibration is performed with seven standard solutions described in section 1.2.1.1 and deuterated propionaldehyde (D_2) as internal standard.

Linear (butyraldehyde) and quadratic (acetaldehyde) regressions against concentration are performed, with statistical weights of 1/concentration, according to:

$$\frac{A_a}{A_{iStd}} = p \times C_a + b$$

and

$$\frac{A_a}{A_{iStd}} = q \times C_a^2 + p \times C_a + b$$

where

A_a area of target analyte

A_{iStd} area of internal standard

C_a concentration of target analyte

Calibration is considered valid if a coefficient of determination $r^2 > 0.995$ is obtained and the individual data points do not deviate by more than ± 10% from their theoretical value (± 15% for the lowest concentration).

1.2.4. Example Chromatogram

An example chromatogram is provided in Figure **1**. The chromatogram provided shows the carbonyls which are detected in mainstream aerosol by this procedure.

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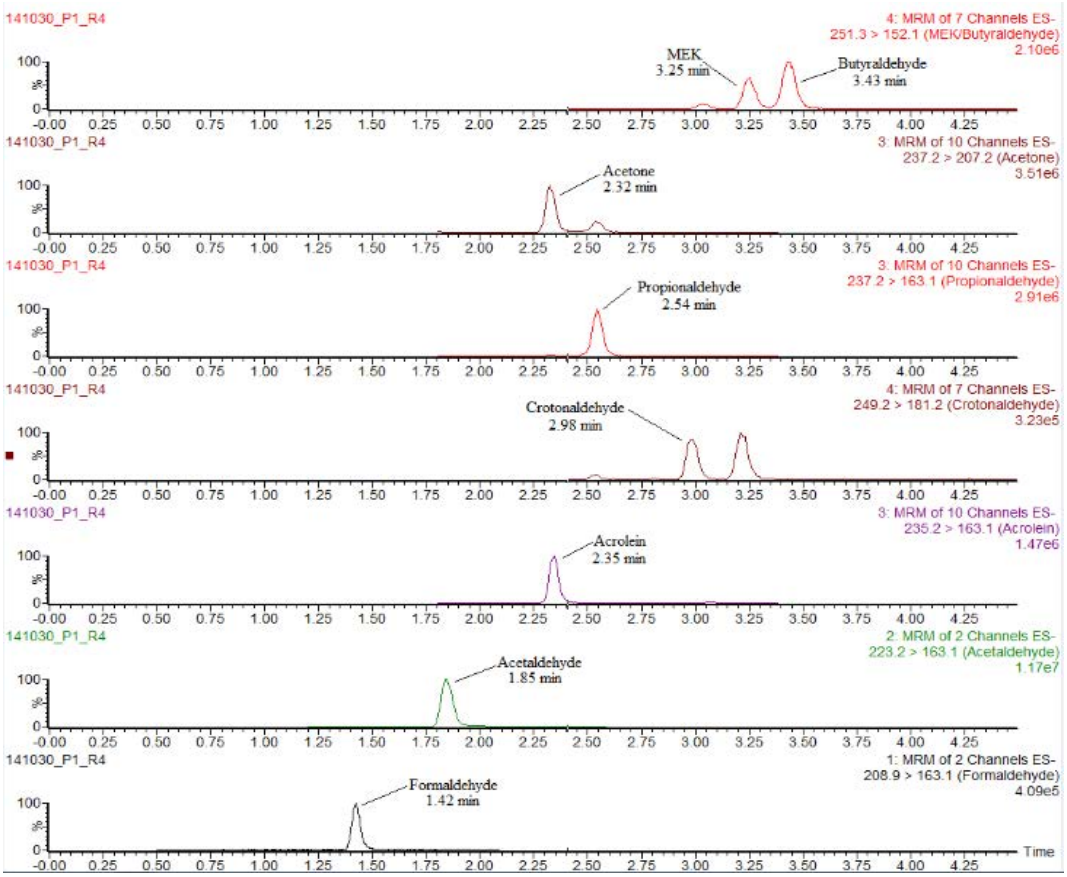


Figure 1: Example Chromatogram Showing the Carbonyls Compounds

1.2.5. Retention Times

Typical retention times for acetaldehyde, butyraldehyde, and internal standard are provided in Table **2**.

Table 2: Typical Retention Times for Target and Internal Standard Compounds

	Name	Retention Time (Minutes)
Target Compounds	Acetaldehyde	1.85
	Butyraldehyde	3.43
Internal standard	Propionaldehyde-D_2	2.53

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1.2.6. System Suitability

A control solution (Medium Standard 4) is used to assess instrument performance evaluation and acetone is used as a representative compound for carbonyls. The acceptance criteria are provided in Table 3.

Table 3: System Suitability Acceptance Criteria

Parameter	Value
Acetone peak area	x > 426202
Acetone peak width	x < 4.367

1.3. Validation

1.3.1. Specificity (Selectivity)

The selectivity of target compounds in the matrices was evaluated by adding at least 3 different amounts of spiking solutions to the matrix and evaluation of the resulting signal. The addition of different amounts of a spiking solution to the matrix resulted in an increase of the peak corresponding to the specific masses of the compounds at corresponding retention times.

The selectivity of target and internal standard compounds in the matrices was evaluated by comparing the response ratio for qualifier to quantifier in matrices with the response ratio obtained in standards. Response ratio for quantifier to qualifier in matrices were within ± 25% of the response ratio for quantifier to qualifier in standards. Matrix samples without internal standard demonstrated lack of interfering peaks in the chromatographic regions of interest for the internal standards.

Selectivity of internal standard compound: matrix and blank samples without internal standard demonstrated lack of interfering peaks (less than 5% compared to matrix and blank samples with internal standard) in the chromatographic regions of interest for the internal standard.

Selectivity of target compounds in blanks: the requirements were that the concentration of target compounds in blank should be lower than 20% compared to the first calibration level for both analytes. The requirements were fulfilled for all matrices.

1.3.2. Linearity

Goodness-of-fit was confirmed by injecting calibration standards covering the target concentration range, as shown in Table **4**. The deviation of the determined concentration from the back calculated concentrations (residuals) for at least five out of seven standard levels were within ± 15% for the lowest calibration level and within ± 10% for the other calibration levels (including the lowest and the highest standards).

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Table 4: Regression Parameters Under Repeatability Conditions (n = 7x5 = 35)

Compound	Range Tested (µg/mL)	Intercept	Slope	q	r	SS
Acetaldehyde	0.26 – 20.00	0.1957	1.6672	-0.0255	0.9981	1.9379
Butyraldehyde	0.08 – 2.40	-0.0098	2.9400	N/A	0.9997	0.1353

q: quadratic coefficient, r: correlation coefficient, SS: residual sum of squares, N/A: not applicable

1.3.2.1. Sample Calibration Plots

Sample calibration plots are provided in Figure **2** and 3.

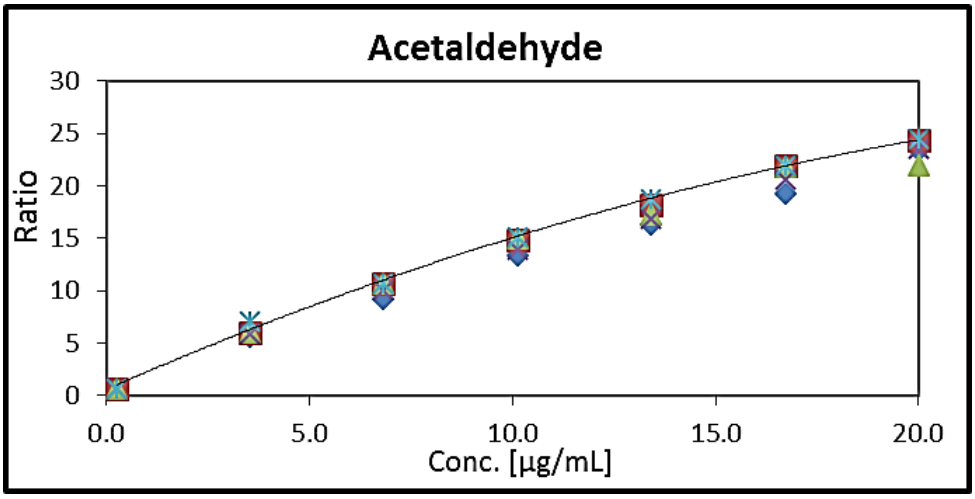


Figure 2: Sample Calibration Plot for Acetaldehyde

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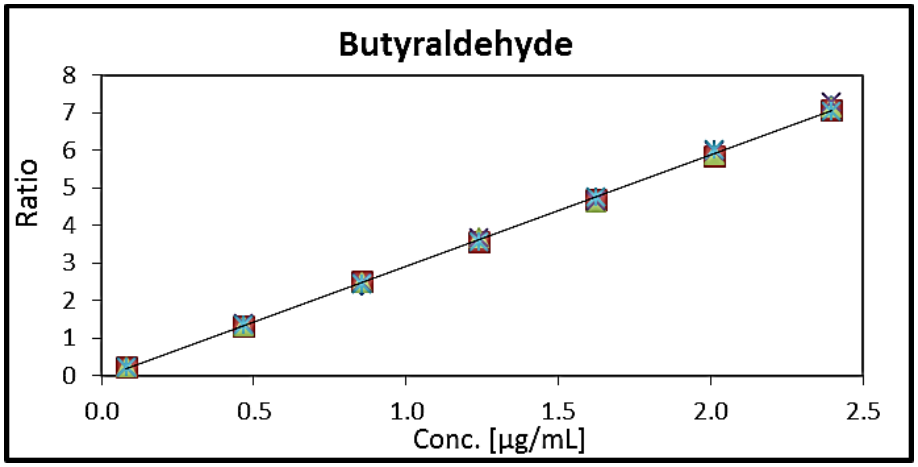


Figure 3: Sample Calibration Plot for Butyraldehyde

1.3.2.2. Working Range

The range is the interval between the upper and lower levels that have been demonstrated to be determined using the accuracy profiles methodology for each matrix studied.

The intersection point of the 95% beta-expectation tolerance intervals with the acceptance intervals of 75-125% corresponds to the smallest quantity measurable with the desired accuracy. The corresponding concentration of the intersection point represents the lower working range limit (LWRL) in the matrix.

The working ranges for the individual compounds are provided in Table **5**.

Table 5: Working Range

Compound	**3R4F (µg/cig)**	**THS (µg/stick)**
Acetaldehyde	446.05 – 3569.71	67.43 – 353.34
Butyraldehyde	15.48 – 150.87	4.94 – 22.86

1.3.3. Accuracy and Precision

1.3.3.1. Repeatability

Within-instrument repeatability is assessed by evaluation of the intra-assay precision of the method under the same operating conditions using a short period of time (same sequence injected on the same day). All coefficients of variation were well within the acceptance range.

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Results for instrumental repeatability limits are given in Table 6.

Table 6: Within-Instrument Repeatability (same day, n=10)

Compound	3R4F (µg/cig)				THS (µg/stick)			
	Mean	**r**	**std**	**CV**	**Mean**	**r**	**std**	**CV**
Acetaldehyde	1899.95	88.946	25.08	1.3%	225.45	6.316	6.68	3.0%
Butyraldehyde	59.75	0.142	1.00	1.7%	16.22	0.008	0.24	1.5%

r = instrumental repeatability, std = standard deviation, CV = coefficient of variation

Table 7 provides the results of repeatability limits in aerosol matrices from the 4 individual smoking replicates collected for matrices over 4 different days with at least 2 different operators obtained in the HCI smoking regime.

Table 7: Repeatability Limits in Aerosol Matrices (Health Canada Intense, n=16)

Compound	3R4F (µg/cig)				THS (µg/stick)			
	Mean	**r**	**SDr**	**CV_r**	**Mean**	**r**	**SDr**	**CV_r**
Acetaldehyde	1778.4	294.5	104.1	5.9%	227.5	38.4	13.6	6.0%
Butyraldehyde	61.9	10.4	3.7	6.0%	16.5	2.6	0.9	5.5%

r = repeatability limit, SDr = standard deviation of repeatability, CVr = coefficient of variation of repeatability

1.3.3.2. Intermediate Precision Limit

The intermediate precision limit was evaluated at nominal content by injecting each smoking replicate for each matrix (one single analytical replicate) once. Health Canada Intense smoking regime is used on different days. Results are given in Table 8. Both the intra-days and inter-days variability are assessed for the calculation of intermediate precision limits.

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Table 8: Intermediate Precision Limit at Nominal Content (Health Canada Intense, n=16)

Compound	3R4F (µg/cig)				THS (µg/stick)			
	Mean	IP	SD_{IP}	CV_{IP}	Mean	IP	SD_{IP}	CV_{IP}
Acetaldehyde	1778.4	294.5	104.1	5.9%	227.5	45.6	16.1	7.1%
Butyraldehyde	61.9	10.4	3.7	6.0%	16.5	2.7	0.9	5.7%

IP = intermediate precision, SD_{IP} = standard deviation of intermediaite precision, CV_{IP} = coefficient of variation of intermediaite precision

1.3.3.3. Reproducibility

No inter-laboratory study was performed for this validation..

1.3.3.4. Combined Uncertainty

The uncertainty of concentration determination of each analyte in the sample was determined. Table **9** reports the minimal and maximal combined uncertainties obtained for 3R4F and THS.

Table 9: Expanded Combined Uncertainty (k=2, 95%)

	3R4F	THS
Acetaldehyde	14 – 24%	12 – 23%
Butyraldehyde	16 – 47%	14 – 46%

1.3.4. Limit of Detection (LOD)/Limit of Quantitation (LOQ)

The LOD and LOQ are both calculated in terms of the standard deviation of the lowest calibration level as determined during validation.

$$LOD = 3 \cdot std_{level1}$$
$$LOQ = 10 \cdot std_{level1}$$

Detailed results are provided in Table 10.

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Table 10: Limits of Detection and Quantitation (Health Canada Intense regime)

Compound	3R4F (µg/cig)			THS (µg/stick)		
	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ
Acetaldehyde	3.44	11.5	4199	0.344	1.15	420
Butyraldehyde	2.89	9.64	504	0.289	0.964	50.4

LLOQ = lower limit of quantitation, ULOQ = upper limit of quantitation (equivalent to STD7)

1.3.5. Robustness

The following parameters evaluated for robustness are available in Table 11.

Table 11: Robustness of Method

Parameter	Recommendation/Comment
Stability of DNPH solution	The DNPH solution is stable 5 days at room temperature and 21 days in the freezer.
Derivatization time	The derivatization time before stabilization with pyridine must not exceed 15 min after aerosol collection. To allow the operators to handle a maximum of replicates at a time in routine conditions, the maximal derivatization time before stabilization with pyridine was not reduced below 15 min.

1.3.6. Stability of Solutions

1.3.6.1. Stability of Sample Extracts

The sample extracts of 3R4F and THS are stable up to 21 days when kept refrigerated to -20°C.

1.3.6.2. Stability of Analytical Stock Solutions

Carbonyls stock solution and acetaldehyde stock solution are stable up to 7 months when kept refrigerated to -20°C.

1.3.6.3. Stability of Internal Standard Stock Solution

The internal standard stock solution 1 is stable 3 weeks when kept refrigerated to -20°C. The internal standard stock solution 2 is stable 3 months when kept refrigerated to -20°C.

1.3.6.4. Stability of Standard Solutions and Samples in Autosampler

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Standard solutions and samples from 3R4F and THS are stable for up to 75h in the autosampler at 15°C in pierced and in unpierced vials.

1.3.6.5. Stability of Calibration Standards

All the calibration standards are stable in the freezer -20°C for 21 days.

1.3.6.6. Stability of Mobile Phase *A*

The mobile phase A is stable when kept at room temperature for 2 months.

1.3.6.7. Stability of Perchloric Acid Solution

The perchloric acid solution is stable for up to 5 months when kept at room temperature.

2. APPROVALS

	Name	Role	Signature	Date
Author (Validation Report)	Audrey Debrick	Supervisor Aerosol Chemistry		22-JUL 2016
Owner	Serge Maeder	Director PMI Testing Laboratories Governance		26.7.2016
QA Review	Florence Kapp	QA Officer		26 JUL 2016

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