APPENDIX A3.2.2-9

ANALYSIS OF ACETAMIDE AND ACRYLAMIDE IN AEROSOL

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1. ANALYSIS OF ACETAMIDE AND ACRYLAMIDE IN AEROSOL

1.1. Applicability

This method is used to quantitate acetamide and acrylamide compounds in mainstream aerosol from conventional cigarettes (3R4F) and the reduced risk product (THS) under Health Canada Intense (HCI) and International Organization for Standardization (ISO) conditions.

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 a Cambridge 44 mm diameter glass fiber filter pad (Cambridge Filter Pad, CFP) and extracted with a 2-butanone solution containing internal standard compounds. The extract is then filtered with a PTFE filter into 20 mL vials and analyzed according to USP <621> Gas Chromatography (GC) using MS-EI detection; 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 the analysis of each batch, calibration is 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 ± 10% from the theoretical value).

1.2.1. Preparation of Solutions

1.2.1.1. Calibration Solutions

Eight standard solutions containing different concentrations of each target compound (highest:lowest = 67:1) and fixed concentrations of internal standards (related deuterated compounds, see Table 1) are prepared in 2-butanone. The ranges of concentrations cover the ranges relevant for analysis and are provided in Table 1.

Table 1: Calibration Standards Ranges

Target Compound	Range (µg/mL)	Internal Standard	Value (µg/mL)
Acetamide	0.105 – 7.000	Acetamide-D_3	0.800
Acrylamide	0.075 – 5.000	Acrylamide-D_3	0.800

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1.2.1.2. Extraction Solution

The extraction solution is 2-butanone containing the internal standards.

THS: five accumulations on one pad for HCI and ISO, extracted with 10 mL

3R4F: two accumulations on one pad for HCI and five accumulations on one pad for ISO, extracted with 10 mL

1.2.2. Chromatographic Conditions

1.2.2.1. Equipment

Gas chromatograph	Suitable gas chromatograph with a Mass Spectrometer Detector and data acquisition software
Capillary column	DBWAXETR, 30 m x 0.25 mm x 0.25 µm, or equivalent

1.2.2.2. GC Conditions

Injection temperature	230°C
Injection volume	0.5 µL
Injection mode	Pulsed splitless
Carrier gas	Helium
Purge flow to split vent	50mL/min for 0.5 min
Flow control mode	Constant flow
Temperature program	65°C, hold 0.5 min Heating, 20°C/min, from 65°C to 120°C Heating, 10°C/min, from 120°C to 200°C Heating, 50°C/min, from 200°C to 260°C
Column flow	1.1 mL/min
Post run	10 min at 2.0 mL/min and 260°C

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1.2.2.3. Mass Spectrometer Settings

Ionization mode	EI		
MS Quad temperature	150°C		
Ion source temperature	230°C		
Transfer line temperature	250°C		
Solvent delay	7.0 min		
EM setting	Delta EMV		
Relative voltage	0.000V		
Acquisition type	Selective ion monitoring (SIM)		
Mass parameters	Acetamide	Quantifier	m/z = 59
		Qualifier	m/z = 43
	Acrylamide	Quantifier	m/z = 71
		Qualifier	m/z = 55

1.2.3. Calibration

Calibration is performed with eight standard solutions containing different concentrations of each compound (highest:lowest = 67:1) and fixed concentrations of internal standards (Table 1). Acetamide-D_3 is used as the internal standard for the quantification of acetamide and acrylamide-D_3 for the quantification of acrylamide.

A linear regression against concentration is performed for each compound, with statistical weights of 1/concentration, according to:

$$\frac{A_a}{A_{ISTD}} = b + pC_a$$

where

A_a integrated area of the target analyte

A_{ISTD} integrated area of corresponding internal standard

C_a concentration of the 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 from the calibration line by more than ± 15% for the lowest calibration level and ± 10% for all other calibration levels.

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1.2.4. Example Chromatogram

An example chromatogram is provided in Figure 1.

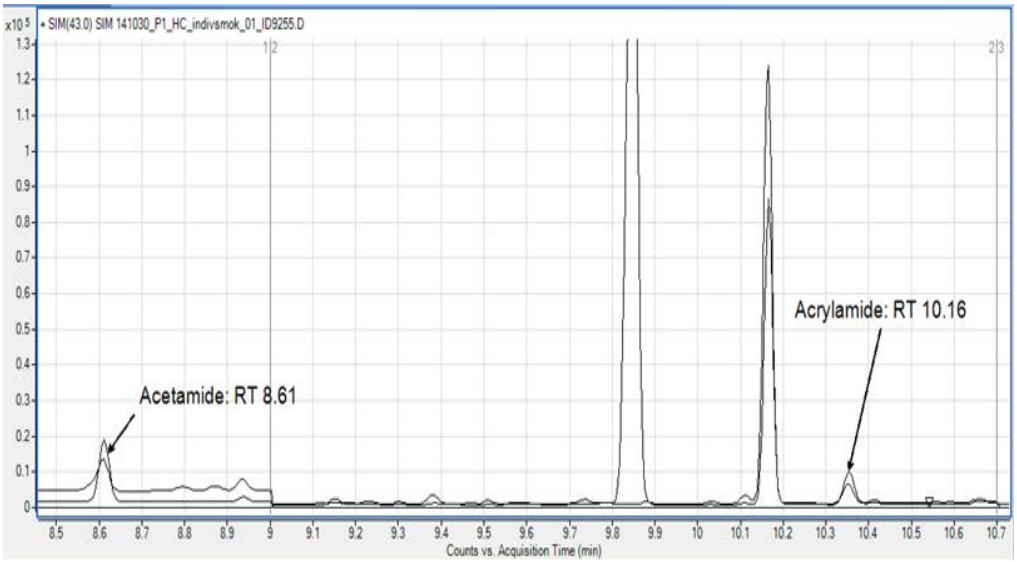


Figure 1: Example Chromatogram

1.2.5. Retention Times

Typical retention times for acetamide, acrylamide, and the internal standards are provided in Table 2.

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

Target Compound		**Internal Standard**	
Acetamide	8.43	Acetamide-D_3	8.41
Acrylamide	10.17	Acrylamide-D_3	10. 17

1.2.6. System Suitability

A medium standard (STD 5) is used to assess the symmetry (tailing factor), full width at half maximum (FWHM) and height of the peak for acrylamide. System suitability criteria are specific and based on the method validation performed using the equipment. Example of acceptance criteria are provided in Table 3.

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Table 3: System Suitability Acceptance Criteria (Example for Equipment ID 10809)

Criteria	Value
Symmetry	$0.793 \leq x \leq 1.305$
FWHM	$x \leq 0.0028$
Peak height	$x \geq 8007$

FWHM = full width at half maximum

1.3. Validation

Validation of the analytical method is performed for each equipment used. PMI equipment ID 9255 and ID 10809 was used to obtain the information provided in this section.

1.3.1. Specificity (Selectivity)

The selectivity of target compounds in the matrices (aerosol collected under HCI and ISO smoking regimes) was evaluated by adding at least 3 different amounts of a spiking solution to the matrix. 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 the internal standard compounds was evaluated. Matrix samples without internal standards demonstrated lack of interfering peaks in the chromatographic regions of interest for the internal standards.

The selectivity of the target compounds and internal standard compounds in blanks was evaluated. The concentrations in blanks (measurement solutions without internal standards) were lower than 20% compared to the first calibration level for all analytes and internal standards. The concentration of analytes in the blank measurement solutions with internal standards was lower than 20% compared to the first calibration level (≤ Lower Limit of Quantitation, LLOQ).

1.3.2. Linearity

The linearity of the response function was demonstrated for the range of concentrations planned to be used in routine under repeatability conditions and during accuracy profile studies under intermediate precision conditions.

The linearity of the response function was assessed using the range of concentrations shown in Table 4. The deviation of the determined concentration from the back calculated concentrations (residuals) for at least 4 out of 6 standard levels are within ± 15% for the lowest

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calibration level and within ± 10% for the other calibration levels (including LLOQ and highest standard). No obvious curvature was observed in the residuals plots.

Table 4: Regression Parameters

Compound	Range Tested (µg/mL)	Slope	Intercept	r	SS
Acetamide	0.105 – 7.024	1.203	0.014	1.000	0.009
Acrylamide	0.074 – 4.903	1.324	0.006	1.000	0.007

r = correlation coefficient, SS = residual sum of squares

1.3.2.1. Calibration Curve Parameters Under Repeatability Conditions

Calibration curve parameters under repeatability conditions using the selected regression model (linear regression with 1/concentration weighing) were evaluated using two instruments. The deviation of the lowest determined concentration from the back calculated concentrations met the same criteria as above. The average slope, intercept, correlation coefficient (r) and residual sum of squares (SS) for each compound and instrument are provided in Table 4.

Sample calibration plots are provided in Figures 2 and 3.

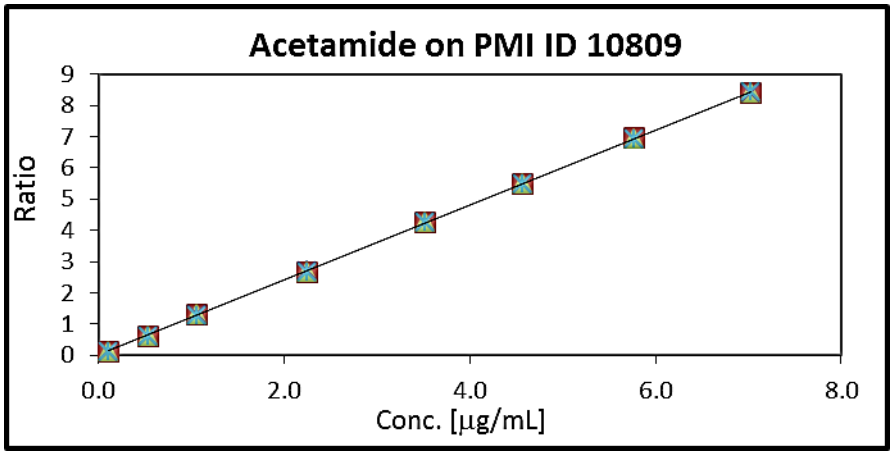


Figure 2: Sample Calibration Plot for Acetamide

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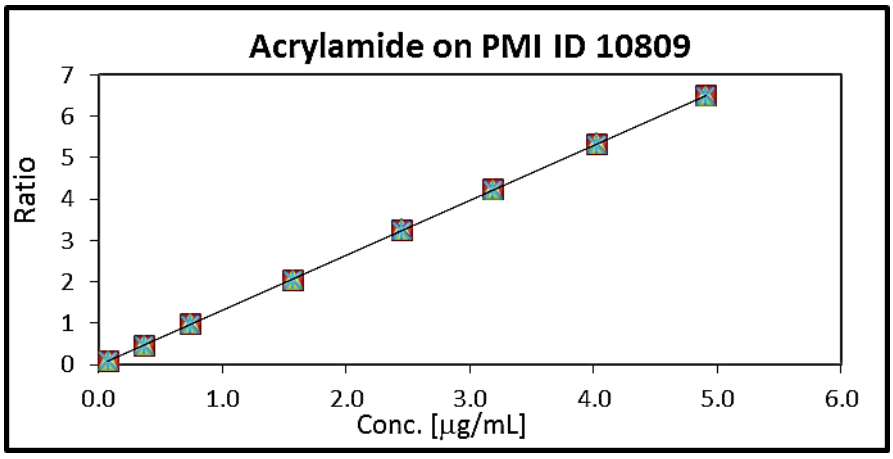


Figure 3: Sample Calibration Plot for Acrylamide

1.3.3. Range

Aerosol collection was performed under Health Canada Intense (HCI) conditions on two days. Samples were homogenized, spiked and diluted, and analyzed by two operators on two different (but functionally equivalent) instruments. The homogenized extracts at different levels of concentration were analyzed on four different series for the evaluation of working range. The method is also valid at concentrations which are typical for ISO conditions.

The working 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 75% – 125% acceptance intervals 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 Ranges

Compound	3R4F/HCI (µg/cig)	3R4F/ISO (µg/cig)	THS (µg/stick)
Acetamide	7.02 – 33.4	2.81 – 13.4	0.442 – 12.0
Acrylamide	2.41 – 18.7	0.97 – 7.47	0.475 – 7.70

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1.3.4. Accuracy and Precision

1.3.4.1. Instrument Repeatability

The instrumental repeatability, determined for 10 injections of the same homogenized aerosol extract of 3R4F generated under HCI smoking regime on two instruments on the same day, is provided in Table 6. As acetamide and acrylamide concentrations in THS matrix are roughly equivalent to concentration in 3R4F aerosol in µg/mL (around 1 ppm for acrylamide, 2-3 ppm for acetamide), the injection repeatability was only verified for 3R4F.

Table 6: Instrumental Repeatability (µg/cig)

Compound	**Mean**	**r**	**std**	**CV**
Acetamide	14.71	0.26	0.09	0.63%
Acrylamide	4.98	0.34	0.12	2.41%

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

1.3.4.2. Repeatability Limit and Intermediate Precision at Nominal Content

The repeatability and intermediate precision limits for the whole process were evaluated at nominal content by injecting once each smoking replicate for each matrix on two instruments, collected under HCI and ISO smoking regimes on four different days, and varying the smoking machine, operators and standard solutions preparation. Only intra-day variability was considered in the calculation for repeatability limit, and intra-day and inter-day variabilities for the calculation of intermediate precision limit.

Table 7: Repeatability Limits at Nominal Content (n=16)

Compound		**3R4F (µg/cig)**				**THS (µg/stick)**			
		Mean	**r**	**SDr**	**CVr**	**Mean**	**r**	**SDr**	**CVr**
HCI	Acetamide	13.7	3.15	1.12	8.13%	4.12	0.504	0.178	4.33%
	Acrylamide	4.55	0.961	0.340	7.46%	2.10	0.314	0.111	5.29%
ISO	Acetamide	3.28	0.530	0.187	5.72%	1.22	0.273	0.096	7.92%
	Acrylamide	1.43	0.225	0.079	5.57%	0.562	0.1552	0.0549	9.76%

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

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Table 8: Intermediate Precision Limits at Nominal Content (n=16)

Compound		3R4F (µg/cig)				THS (µg/stick)			
		Mean	**IP**	**SD_{IP}**	**CV_{IP}**	**Mean**	**IP**	**SD_{IP}**	**CV_{IP}**
HCI	Acetamide	13.7	3.95	1.40	10.2%	4.12	0.504	0.178	4.33%
	Acrylamide	4.55	1.168	0.413	9.07%	2.10	0.314	0.111	5.29%
ISO	Acetamide	3.28	0.702	0.248	7.57%	1.22	0.328	0.116	9.54%
	Acrylamide	1.43	0.225	0.079	5.57%	0.562	0.1620	0.0573	10.19%

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

1.3.4.2.1. Repeatability and Intermediate Precision Limits at Different Concentrations

Coefficients of variation have been determined for repeatability and intermediate precision of the analytical method (excluding the aerosol collection and product variabilities; covered by 1.3.4.2) over a wide range of concentrations for both matrices.

Coefficients of variation for repeatability of the analytical method were found to be very low for 3R4F at all concentrations tested. For THS, coefficients of variation for repeatability were generally higher, especially for the lower levels. Overall, all coefficients of variation for repeatability were found to be lower than 7% for all tested levels in both matrices. Maximum coefficient of variation for repeatability was below 3% for 3R4F and below 7% for THS.

Coefficients of variation for intermediate precision of the analytical method were found between 1.6% and 3.7% for 3R4F. For THS and linked to the much lower levels of concentration found in this type of matrix,coefficient of variation for intermediate precision values were found higher than those obtained for 3R4F, ranging from 3.3% to 9.9% for THS.

1.3.4.3. Reproducibility

No inter-laboratory study was performed for this validation.

1.3.4.4. Combined Uncertainty

The uncertainties (either calculated or estimated) of each identified source of uncertainty in the analytical process are associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to a measurand. A measurand is defined as a particular quantity subject to measurement analyte in matrix in this case. Table **9** reports the 95% uncertainties in 3R4F and THS.

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

	3R4F	THS
Acetamide	22.6%	15.2%
Acrylamide	20.1%	18.9%

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

The LOD and lower limit of quantitation (LLOQ) were both calculated in terms of the standard deviation of the lowest calibration level as determined during validation.

$$LOD = 3 \times std_{level1}$$

$$LLOQ = 10 \times std_{level1}$$

Both were found to be lower than the concentration of the lowest calibration level for all compounds. Detailed results are provided in Table **10**.

Table 10: Limits of Detection and Quantitation

		3R4F (µg/cig)			THS (µg/stick)		
		LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ
HCI	Acetamide	0.083	0.275	35.8	0.033	0.110	14.32
	Acrylamide	0.091	0.302	26.55	0.036	0.121	10.62
ISO	Acetamide	0.033	0.110	14.32	0.033	0.110	14.32
	Acrylamide	0.036	0.121	10.62	0.036	0.121	10.62

ULOQ = upper limit of quantitation (highest calibration level)

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1.3.6. Robustness

The parameters found for robustness are described in Table 11.

Table 11: Robustness of Method

Parameter	**Recommendation/Comment**
Extraction time at 250 rpm for 20 min, 30 min (target) and 40 min	An extraction time of 30±10 minutes is recommended.
Column lot number on both instruments	No clear limitation on number of injections was found, only the resolution between acrylamide m/z specific peak and the impurity eluting just before gives an indication of aging of the column.
Liner with or without glass wool	No significant difference was found in percentage recoveries for samples injected with a liner with and without glass wool and results are observed in-between 80-120%. It is recommended to use the liner with glass wool to avoid GC system clogging.
Volume of extraction solution (10 mL, 20 mL)	No statistically significant difference was observed in concentration of acetamide and acrylamide; therefore, 10 mL extraction volume is sufficient for extraction of analyte.

1.3.7. Stability of Solutions

1.3.7.1. Stability of Individually Prepared Internal Standard (ISTD) Compounds in Stock and Extraction Solutions

The stability of individually prepared ISTD and extraction solutions in 2-butanone was evaluated. The individual ISTD stock solutions are stable up to 129 days and the extraction solution are stable up to 78 days when kept at -20°C.

1.3.7.2. Stability of Samples and Standards in the Auto-Sampler at Room Temperature

Samples and standards vials are stable for up to 79 hours (h) in unpierced GC vials placed in the auto-sampler at room temperature. Samples and standard 5 (used as control) are stable for up to 71 h in pierced GC vials. Vials for calibration standards must be re-prepared in case of re-injection.

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1.3.7.3. Stability of Primary and Secondary Mix Solutions

The primary and secondary mix solutions (acrylamide and acetamide in extraction solution with internal standards) are stable for 129 days when stored at -20°C.

1.3.7.4. Stability of Working Standards

The calibration standards were evaluated by comparison of the calculated concentration at each testing point with the theoretical concentration. The calibration standard solutions are stable for 78 days when stored at -20°C.

1.3.7.5. Stability of Aerosol Sample Extracts at -20°C

The aerosol extracts (3R4F and THS) are stable for 35 days at -20°C.

1.3.7.6. Stability of Target Compounds in Cambridge filter pad (CFP) After Aerosol Collection

Acetamide and acrylamide compounds are stable in the CFP in capped amber vials at -20°C for 3R4F and THS for up to 5 days.

2. APPROVALS

	Name	Role	Signature	Date
SME	Audrey Debrick	Supervisor Aerosol Chemistry		22 JUL 2016
Owner	Serge Maeder	Director PMI Testing Laboratories Governance		26.7.1.2016
QA Review	Florence Kapp	QA Officer		26JUL2016

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