APPENDIX A3.2.2-6

ANALYSIS OF WATER, NICOTINE, AND TRIACETIN IN AEROSOL

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1. ANALYSIS OF WATER, NICOTINE, AND TRIACETIN IN AEROSOL

1.1. Applicability

The method described is used to determine water, nicotine, and triacetin in mainstream aerosol from conventional cigarettes (3R4F) and the Tobacco Heating System (THS) under Health Canada Intense (HCI) and International Organization for Standardization (ISO) smoking conditions.

Results are expressed as mg/stick for THS and mg/cig for 3R4F.

1.2. Analytical Procedure

The aerosol samples are generated on a linear smoking machine (Borgwaldt KC) under ISO and HCI smoking regimens and collected on a Cambridge filter pad (CFP).

1.2.1. Non *in-situ* extraction

The standard extraction (non *in-situ*) is mainly performed for conventional cigarettes (CCs). In extraction, a Cambridge Filter Pad (CFP) is placed in 20 mL Filtrona tube directly after smoking by opening the plastic filter holder. The appropriate volume of extraction solution containing internal standards (ISTDs) is added directly into Filtrona tubes containing the CFP and these tubes are sealed immediately with a septum and an aluminum ring. Filtrona tubes are stirred at 200 rpm for at least 60 minutes. Dilution of each sample is performed in a new Filtrona tube and both tubes are sealed immediately with a septum and an aluminum ring.

The corresponding blank samples for water content analysis are prepared in the same manner except for the dilution step (blanks are never diluted).

1.2.2. *In-situ* extraction

The *in-situ* extraction procedure is performed for THS. Directly after smoking, the metallic filter holder containing the glass fiber CFP is extracted. The metallic filter holder containing the glass fiber CFP is placed on the extractor and an empty syringe is connected on the top of the metallic filter holder. A syringe containing 10 mL of extraction solution containing ISTDs is connected to the bottom of the metallic filter holder. Afterwards the extractor is started and applies a constant back and forth flow with several cycles on extraction solution through the metallic filter holder for about 30 minutes.

The extracted aerosol replicates are collected in a Filtrona tube and sealed. Dilution of each sample is performed in a new Filtrona tube and both tubes are sealed immediately with a septum and an aluminum ring.

The corresponding blank samples for water content analysis are prepared in the same manner except the dilution step (blanks are never diluted).

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The samples are then analyzed by Gas Chromatography (GC) with a Flame Ionization Detector (FID) GC-FID for nicotine and triacetin and a Thermal Conductivity Detector (TCD) GC-TCD for water according to USP <621>.

CCs and THS are conditioned in opened pack for at least 48 hours at target conditions of 22 ± 1°C and relative humidity of 60 ± 3% before used for aerosol generation. The equipment and conditions are described in Sections 1.2.4.1, 1.2.4.2 and 1.2.4.3 respectively.

Preceding each batch, calibration is performed according to the details given in section 1.2.3.1. Typically, 5 sequences of measurement are performed per batch. Each sequence consists of suitability tests (SSTs), reference runs (water, nicotine and triacetin), blank runs (*in-situ* and non in-*situ*), monitor runs, sample runs and is terminated by a Standard (STD) 4 run to validate the results (see Section 1.2.8) and verify the calibration (requirement: deviation of not more than ± 5% on one of the three controls from the theoretical value for water and ± 3% on one of the three controls from the theoretical value for nicotine and triacetin).

1.2.3. Preparation of Solutions

1.2.3.1. Calibration Solutions

Six standard (STD) solutions (for water) and six standard (STD) solutions (for nicotine and triacetin) containing different concentrations of each target compound (highest:lowest = 25:1) and concentrations of ISTDs and n-heptadecane are prepared. The ranges of concentrations cover the ranges relevant for analysis and are provided in Table 1.

Table 1: Calibration Standard Ranges (mg/mL)

Compound		**Internal standard**	
Water	0.0000 – 2.500	Ethanol	2.0
Nicotine	0.0000 – 1.250	*n*-heptadecane	0.3
Triacetin	0.0202 – 0.5000		

1.2.3.2. Extraction Solution

The extraction solution is a solution containing approximately 0.3 mg/mL of *n*-heptadecane and approximately 2 mg/mL of ethanol in isopropanol.

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1.2.4. Experimental Conditions

1.2.4.1. Equipment

Gas chromatograph	A suitable gas chromatograph equipped with a flame ionization detector and a thermal conductivity detector and data acquisition software

1.2.4.2. Chromatographic Conditions for Determination of Nicotine and Triacetin

Column	Capillary column DB WAXETER, 15 mm × 0.32 mm, ID × 1.0 µm film thickness
Carrier gas	Helium
Carrier gas flow rate	1.8 mL/min
Flow mode	Gradient
H_2 flow (FID)	40 to 80 mL/min
Air flow (FID)	250 to 350 mL/min
Make-up gas (N_2)	10 mL/min
Split flow	36 mL/min
Split ratio	20
Oven temperature program	150°C, 5.50 min 40°C/min → 240°C, 1.6 min
Run time	9.35 min
Injection temperature	250°C
Detector temperature	250°C
Injection volume	1 µL

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1.2.4.3. Chromatographic Conditions for Determination of Water

Column	Packed column HaySep Q 80/100 mesh, 8 feet external diameter of 1/8" and internal diameter of 2.1 mm
Carrier gas	Helium
Carrier gas flow	45 mL/min
Reference gas flow (Helium)	40 mL/min
Air flow (make-up)	10 mL/min
Oven temperature program	150°C, 5.50 min 40°C/min → 240°C, 1.6 min
Run time	9.35 min
Injection temperature	250°C
Detector temperature	250°C
Injection volume	1 µL

1.2.5. Calibration

Calibration is performed with six STD solutions for water, nicotine and triacetin. Each standard contains different concentrations of the compounds (highest: lowest = 25:1) and concentration of ISTDs of 2.0 mg/mL for ethanol and 0.3 mg/mL for *n*-heptadecane.

A ratio of each compound (*n*-heptadecane for nicotine and triacetin; ethanol for water) is determined according to:

$$\text{Ratio area} = \frac{\text{Compounds area}}{\text{Internal Standard area}}$$

The concentration of water in the extract in (mg/mL) is calculated using the equation y = ax+b using a linear regression with a weighting factor 1/x.

STD 1 must be excluded for the water content analysis.

Also the concentration of nicotine and triacetin in the extract (mg/mL) is calculated using the equation y = ax+b using a linear regression without a weighting factor. STD 1 must be excluded for the triacetin content analysis.

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where

a Slope

b Intercept

y Ratio

x Concentration of the compound in the working solution in mg/mL

$$\text{Concentration of the compound } \left({}^{\text{mg}}/_{\text{mL}}\right) = \frac{\text{Compound} - \text{intercept}}{\text{Slope}}$$

The concentration of compound per cigarette or THS is calculated by using the following equation:

$$\text{Concentration of the compound } \left({}^{\text{mg}}/_{\text{stick}}\right) = \left(\text{Concentration of the compound } \left({}^{\text{mg}}/_{\text{mL}}\right) \times \frac{\text{V}}{\text{n}} \times \text{D}\right.$$

Where,

V Extraction volume

n Number of sticks or THS per replicate

D Dilution factor

Calibration is considered valid if the coefficient of determination $r^2 > 0.999$ is obtained for all three compounds. and the relative residuals should be ≤ 5% of the nominal concentration.

1.2.6. Example Chromatogram

An example chromatogram of nicotine and triacetin is provided in Figure 1. An example chromatogram for water is provided in Figure 2.

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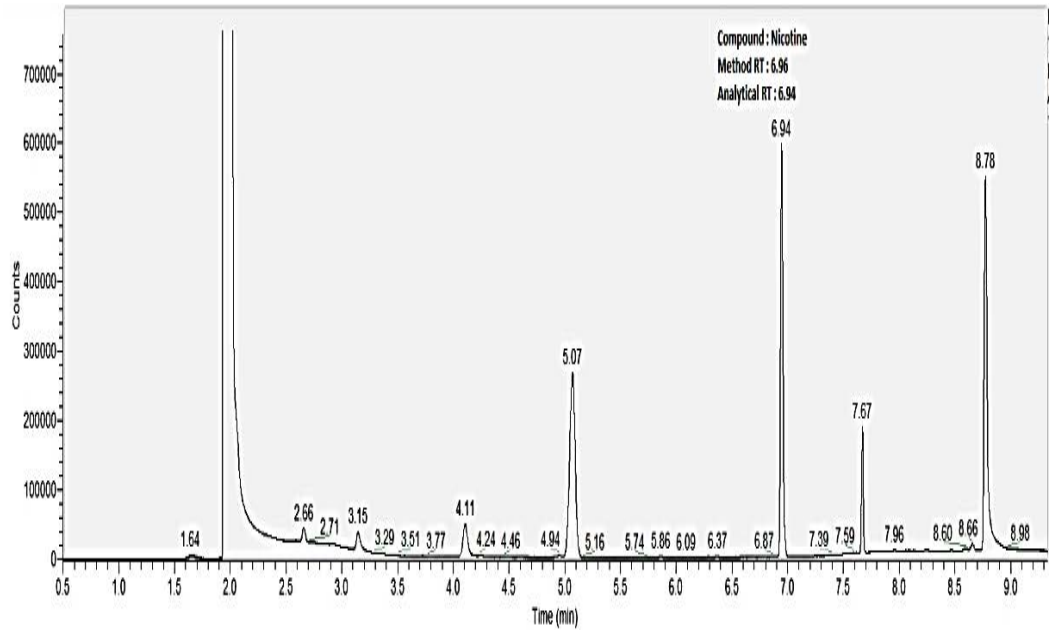


Figure 1: Example Chromatogram of Nicotine and Triacetin

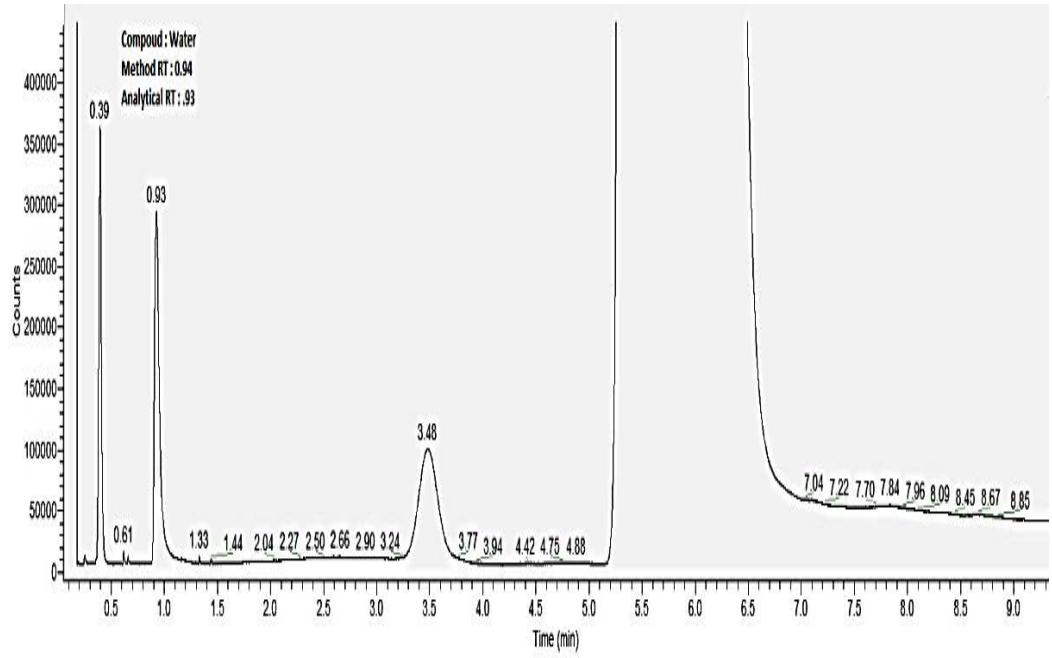


Figure 2: Example Chromatogram for Water

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1.2.7. Retention Times

Typical retention times for water, nicotine, triacetin and the ISTDs are provided in Table 2.

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

Target		**Internal Standards**	
Water	0.94	Ethanol	2.96
Nicotine	6.96	*n*-heptadecane	4.88
Triacetin	7.67		

1.2.8. System Suitability

Control solution STD 4 is used to assess instrument performance. System suitability criteria are specific and based on the method validation performed using the equipment. Example acceptance criteria are provided in Table 3 for PMI equipment ID 10818.

Table 3: System Suitability Acceptance Criteria

Compound	**Water**	**Nicotine**	**Triacetin**
Peak Area [0.1*µV*sec]	Min: 440581	Min: 605852	Min: 64866
Peak Width	Max: 0.28	Max: 0.08	Max: 0.07

1.3. Validation

Validation of the analytical method is performed for each piece of equipment. PMI equipment ID 10818 was used to obtain the information provided in this section.

1.3.1. Specificity (Selectivity)

Selectivity was demonstrated for aerosol collected under the HCI and ISO smoking regime and analytical blank by adding three different amounts of a spiking solution and evaluation of the resulting signal on two instruments.

The selectivity of ISTD compounds was calculated by dividing the response of the pure 2-isopropanol and matrices without ISTD with the response of extraction solution and matrix with ISTD. The addition of different amounts of aerosol collection homogenate tested at

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different levels of concentration resulted in an increase of the peak area response at specific retention time for the target compound. The area response of ISTD compounds calculated by dividing pure 2-isopropanol and matrices without ISTD with the response of extraction solution and matrix with ISTD was lower than 5% and lower values were considered selective.

The selectivity of analytes in aerosol matrices was evaluated by spiking at the lowest, middle and highest calibration levels. The selectivity of analytes was calculated by dividing the response of the extraction solution with the response of matrices. The acceptance threshold for the analytes in the solvent compared to the lowest calibration level is set at 0.2 and the lower values are considered selective. Nicotine and triacetin were found specific in the extraction solution (solvent with ISTD) which is lower than the acceptance threshold 20%.

The selectivity for water was found higher than 20% and did not meet the initial acceptance criteria. To manage a well-known contamination of water in the extraction solution, the same extraction solution was used for the preparation of the calibration solutions as well as for the samples. The contamination of water was around 3 times lower than the lowest calibration level which is considered acceptable for the selectivity of water in the extraction solution. The addition of different amounts of standard solutions to the matrices resulted in an increase of the peak of the corresponding compound. The extraction solution with menthol, propylene glycol and glycerin was injected to demonstrate the separation with the ISTD (*n*-heptadecane) as they are the main sources of contamination coming from THS.

1.3.2. Linearity

The response function was demonstrated using calculated residuals for water, nicotine and triacetin for a range of concentrations with linear regression model.

1.3.2.1. Regression Parameters

The linearity of the response function was demonstrated for the range of concentrations by injecting five calibration standards covering the target concentration range.

The linearity of response functions was assessed using the range of concentrations shown in Table 4. The relative residuals were required to be ≤ 5% of the nominal concentrations and $r^2 > 0.999$.

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Table 4: Regression Parameters (PMI ID 10818)

Compound	Range tested	Slope	Intercept	r^2
Water calibration 1	0.00 mg/mL – 2.50 mg/mL	0.481	0.111	0.9997
Water calibration 2		0.478	0.123	0.9995
Water calibration 3		0.525	0.101	0.9997
Water calibration 4		0.504	0.109	0.9998
Water calibration 5		0.504	0.128	0.9995
Compound	**Range tested**	**Slope**	**Intercept**	**r^2**
Nicotine calibration 1	0.00 mg/mL – 1.25 mg/mL	3.01	-0.0213	0.9999
Nicotine calibration 2		3.03	-0.0242	0.9998
Nicotine calibration 3		3.02	-0.0194	0.9999
Nicotine calibration 4		3.02	-0.0211	0.9999
Nicotine calibration 5		3.02	-0.0212	0.9999
Compound	**Range tested**	**Slope**	**Intercept**	**r^2**
Triacetin calibration 1	0.00 mg/mL – 0.50 mg/mL	1.22	-0.00187	0.9999
Triacetin calibration 2		1.21	-0.00163	1.0000
Triacetin calibration 3		1.22	-0.00272	0.9999
Triacetin calibration 4		1.22	-0.00262	1.0000
Triacetin calibration 5		1.22	-0.00113	1.0000

r^2 = coefficient of determination

1.3.2.1.1. Calibration Curve Parameters Under Repeatability Conditions

Calibration curve parameters under repeatability conditions using the selected regression model (linear regression with 1/x) are evaluated. The relative residuals must be ≤ 5% of the nominal concentrations and $r^2 > 0.999$. The average slope, intercept, and coefficient of determination (r^2) for each compound are provided in Table 4.

1.3.2.1.2. Sample Calibration Plots

Sample calibration plots are provided in Figures 3 to 5.

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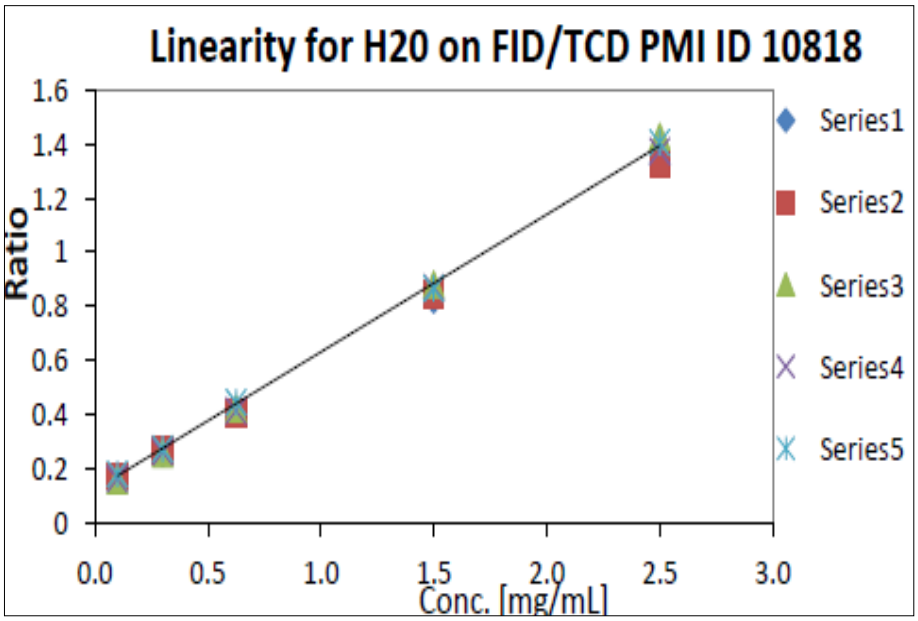


Figure 3: Sample Calibration Plot for Water

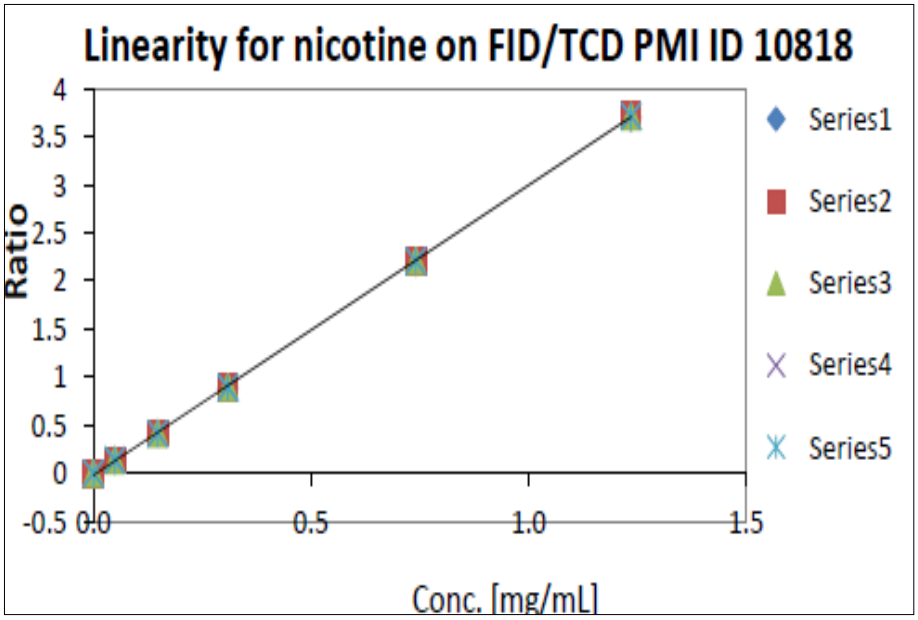


Figure 4: Sample Calibration Plot for Nicotine

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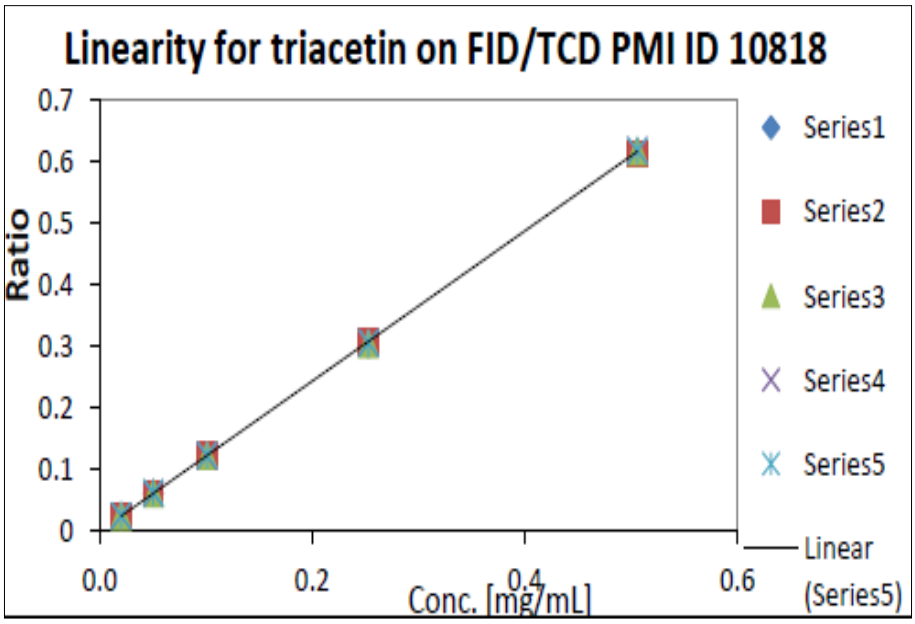


Figure 5: Sample Calibration Plot for Triacetin

1.3.3. Range

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 (see Figure 6 for an example).

The intersection point of the beta-expectation tolerance intervals with the 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.

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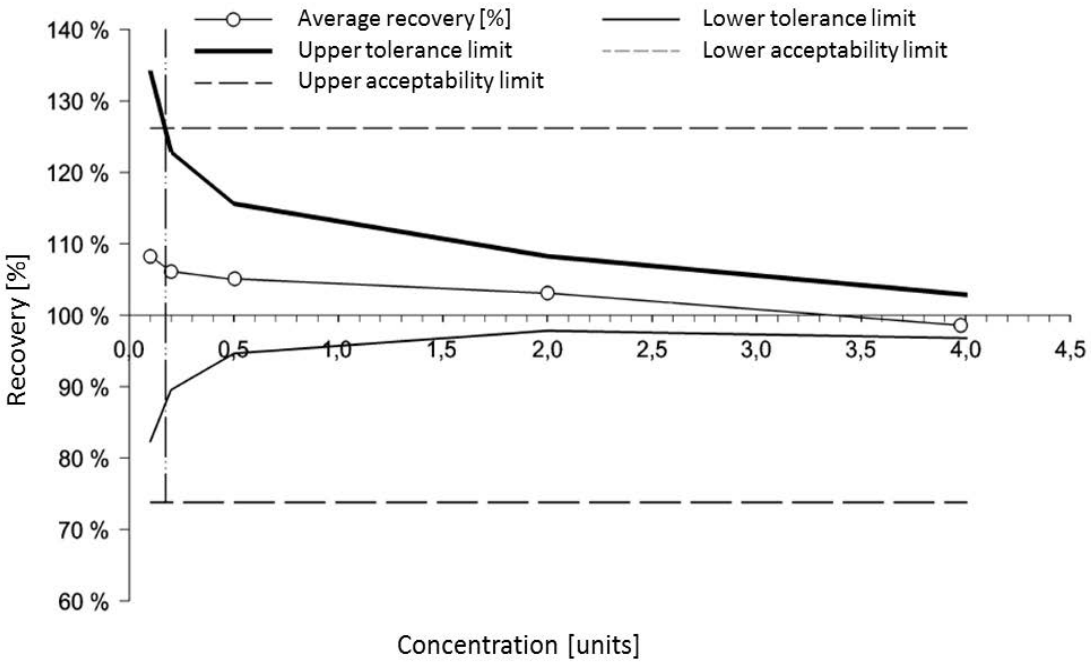


Figure 6: Example of an Accuracy Profile

Table 5: Working Range

Compound	Working Range 3R4F (mg/cig)		Working Range THS (mg/stick)	
	HCI	ISO	HC	ISO
Water	10.6 – 19.3	2.81 – 4.63	36.0 – 66.5	23.4 – 43.0
Nicotine	0.557 – 9.42	0.398 – 3.69	0.382 – 3.99	0.135 – 1.98
Triacetin	0.286 – 4.13	0.123 – 1.45	0.123 – 1.49	0.0725 – 0.892

1.3.4. Accuracy and Precision

1.3.4.1. Instrument Repeatability

Instrument repeatability was determined by analyzing 10 injections of the same vial of the same treated solution for nicotine and triacetin for each matrix. To maintain the contamination of water as low as possible, 10 different vials containing the same treated solution was used for determination of the instrumental repeatability of water. Very low coefficients of variation

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were found for nicotine and triacetin in all the matrices. The acceptance criteria for nicotine and triacetin are fulfilled. All coefficients of variation were well within the acceptance range. The variability of water is matrix dependent: ~5% for 3R4F and ~7% for THS. This led to conclusion that recommended acceptance criteria with a coefficient of variation of 5.3% was too restrictive for water analysis and it was therefore increased to 10% for water. Results for repeatability limits are provided in Table 6.

Table 6: Instrumental Repeatability

3R4F	**Mean [mg/cig]**	**r [mg/cig]**	**std_r [mg/mL]**	**CV_i [%]**
Water	15.1	0.133	0.0686	4.55
Nicotine	2.16	3.36 E-05	0.00109	0.51
Triacetin	1.06	3.47 E-05	0.00111	1.05
THS	**Mean [mg/stick]**	**r [mg/ stick]**	**std_r [mg/mL]**	**CV_i [%]**
Water	38.1	0.757	0.0896	7.83
Nicotine	1.55	1.07 E-04	0.00336	0.72
Triacetin	0.485	1.19 E-05	0.00112	0.77

r = repetability, std_r = standard deviation, CV_r = coefficient of variation

Table 7 provides the results of repeatability limits in aerosol matrices obtained under the HCI and ISO smoking regimes. The repeatability measures the variability observed within a laboratory, over a short time, using a single operator and stick of equipment. The repeatability was assessed under ISO and HCI smoking regimes; 4 individual smoking replicates are performed for all the 3 matrices over 4 different days with different operators for aerosol collection and 2 operators for analytical method. The blank values of water of CFP are consistently subtracted from sample values by performing an outlier test. The acceptance criteria previously set appeared to be constraining for more complex natural products including different analytical procedures which might impact variability of repeatability. Also, the quantification of water is normally subject to environmental contamination because of all the different manipulation that is necessary prior to the analysis. All coefficients of variation for the repeatability were lower than 5% for water, nicotine and triacetin higher than 5% for most analytes for the complete procedure, therefore the acceptance criterion were increased from 5.3% to 10% to be able to quantify water, nicotine and triacetin in 3R4F and in THS.

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Table 7: Repeatability Limits in Aerosol Matrices

Compound		3R4F (mg/cig)				THS (mg/stick)			
		Mean	**r**	**std_r**	**CV_r [%]**	**Mean**	**r**	**std_r**	**CV_r [%]**
HCI	Water	15.1	3.18	1.13	7.43	44.4	3.29	1.16	2.62
	Nicotine	2.06	0.186	0.0658	3.19	1.51	0.180	0.0637	4.22
	Triacetin	1.11	0.27	0.0956	8.65	0.491	0.0993	0.0351	7.15
ISO	Water	0.886	0.218	0.0770	8.70	24.1	5.05	1.78	7.41
	Nicotine	0.801	0.0834	0.0295	3.68	0.546	0.142	0.0503	9.22
	Triacetin	0.244	0.0383	0.0135	5.56	0.281	0.0514	0.0182	6.48

r = repeatability, std_r = standard deviation, CV_r = coefficient of variation

1.3.4.2. Intermediate Precision

The intermediate precision relates to the variation observed when one or more factors such as time, equipment, calibration (between groups of measurements) or operator are varied within laboratory. The varying parameters used were smoking day, the operator and the calibration. Intermediate precision limit was evaluated by injecting each aerosol collection replicate for each matrix generated under the HCI and ISO smoking regimes. As mentioned in repeatability, the mentioned criteria was too restraining for complex natural products. Numerous coefficient of variation were found to be higher than recommended limit. Regardless of compound, instrument and smoking regimen, 16 coefficients of variations were lower and 20 higher than defined limits of ± 8%. For this reason the acceptance criteria was increased from 8 to 15% to quantify water, nicotine and triacetin in 3R4F and THS under HC and ISO smoking regimes. Results are given in Table 8.

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Table 8: Intermediate Precision Limit

Compound		Mean	IP	std_{IP}	CV_{IP}	CD 95%
3R4F (mg/cig)						
HCI	Water	15.1	3.18	1.13	7.43	1.59
	Nicotine	2.06	0.191	0.0675	3.28	0.102
	Triacetin	1.11	0.288	0.102	9.22	0.168
ISO	Water	0.886	0.507	0.179	20.25	0.471
	Nicotine	0.801	0.0950	0.0336	4.19	0.0617
	Triacetin	0.244	0.0429	0.0252	6.23	0.0272
THS (mg/stick)						
HCI	Water	44.4	4.51	1.59	3.59	3.50
	Nicotine	1.51	0.294	0.104	6.89	0.249
	Triacetin	0.491	0.115	0.0406	8.27	0.0762
ISO	Water	24.1	5.05	1.78	7.41	2.52
	Nicotine	0.546	0.142	0.503	9.22	0.0712
	Triacetin	0.281	0.0691	0.244	8.70	0.0528

IP = intermediate precision, std_{IP} = standard deviation, CV_{IP} = coefficient of variation, CD 95% = critical difference 95%

1.3.4.3. Reproducibility

No other laboratory is currently using this method.

1.3.4.4. Estimation of Combined Uncertainty

The uncertainty of concentration of each analyte in the sample was determined. The minimal and maximal combined uncertaintconcentrations in 3R4F and THS are reported in Table 9.

Table 9: Combined Uncertainty (Standard Deviation)

	3R4F	THS
Water	4% - 31%	5% - 31%
Nicotine	1% - 26%	1% - 26%
Triacetin	1% - 10%	1% - 10%

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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 (5 injections over different days by using same calibration standard preparations analyzed on 5 different days).

$$LOD = LLOQ / 3.3$$

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

Level 1 is the lowest calibration level.

The LLOQ was 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 (HCI and ISO Regimes)

Compound	3R4F					
	HCI			ISO		
	LOD	LLOQ	ULOQ	LOD	LLOQ	ULOQ
	(mg/cig)	(mg/cig)	(mg/cig)	(mg/cig)	(mg/cig)	(mg/cig)
Water	0.16	0.527	25	0.064	0.211	10
Nicotine	0.04	0.13	12.35	0.016	0.052	4.94
Triacetin	0.041	0.134	5.06	0.016	0.054	2.024
Compound	**THS**					
	HCI			**ISO**		
	LOD	**LLOQ**	**ULOQ**	**LOD**	**LLOQ**	**ULOQ**
	(mg/stick)	(mg/stick)	(mg/stick)	(mg/stick)	(mg/stick)	(mg/stick)
Water	0.053	0.176	8.333	0.032	0.105	5.000
Nicotine	0.013	0.043	4.117	0.008	0.026	2.470
Triacetin	0.014	0.045	1.687	0.008	0.027	1.012

ULOQ = upper limit of quantitation (highest calibration level)

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1.3.6. Robustness

The robustness of the method was evaluated as described in Table 11.

Table 11: Robustness of Method

Parameter	Recommendation/Comment
Modification of split flow (Increase in split flow for nicotine and triacetin from 36 mL/min to 54 mL/min)	Regardless of the matrix, values obtained for nicotine and triacetin by increasing split flow were similar compared to the values obtained with initial conditions. Differences were lower than 2% and therefore within the acceptance limit of 90-108%. The acceptance criteria for the modified split flow (36 mL/min to 54 mL/min) for robustness were fulfilled.
Modification of injection volume for water (Increase in injection volume from 1 µL to 2 µL)	Regardless of the matrix, values obtained for water by increasing injection volume were similar compared to the values obtained with initial conditions. Differences were lower than 4% and therefore within the acceptance limit of 90-108%. The acceptance criteria for the modified injection volume (1 µL to 2 µL) for robustness were fulfilled.

1.3.7. Stability of Solutions

1.3.7.1. Stability of Homogenized Aerosol Solution at Ambient Temperature

The homogenized aerosol solutions are stable in Filtrona tube up to 9 days at ambient temperature.

1.3.7.2. Stability of Nicotine and Triacetin STD Solutions in the Refrigerator

The nicotine and triacetin STD solutions are stable up to 6 months in the refrigerator.

1.3.7.3. Stability of Water STD Solutions at Ambient Temperature

The water STD solutions are stable up to 69 days at ambient temperature.

The water stock solution is stable up to 6 months at ambient temperature.

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2. APPROVALS

	Name	Role	Signature	Date
Author	Michel Yerly	Team Leader Aerosol Formers & Special Analyses		22 JUL 2016
Author (validation report)	Iris Hofer	Scientist		25-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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