APPENDIX A3.2.2-7

ANALYSIS OF GLYCERIN AND MENTHOL IN AEROSOL

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

Contents

APPENDIX A3.2.2-7 1
1. ANALYSIS OF GLYCERIN AND MENTHOL IN AEROSOL 4
1.1. Applicability 4
1.2. Analytical Procedure 4
1.2.1. Non *in-situ* extraction 4
1.2.2. *In-situ* extraction 4
1.2.3. Preparation of Solutions 5
1.2.4. Experimental Conditions 6
1.2.5. Calibration 6
1.2.6. Example Chromatogram 8
1.2.7. Retention Times 8
1.2.8. System Suitability 9
1.3. Validation 9
1.3.1. Specificity (Selectivity) 9
1.3.2. Linearity 10
1.3.3. Range 11
1.3.4. Accuracy and Precision 13
1.3.5. Limit of Detection (LOD) / Limit of Quantitation (LOQ) 16
1.3.6. Robustness 16
1.3.7. Stability of Solutions 17
2. APPROVALS 17

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

Tables

Table 1: Calibration Standards Ranges 5
Table 2: Typical Retention Times for Target and ISTD Compounds (Minutes) 8
Table 3: System Suitability Acceptance Criteria 9
Table 4: Regression Parameters (PMI ID 6398 – reference instrument) 10
Table 5: Working Range 13
Table 6: Instrumental Repeatability 13
Table 7: Repeatability Limits in Aerosol Matrices 14
Table 8: Intermediate Precision 15
Table 9: Combined Uncertainty (Standard Deviation) 15
Table 10: Limits of Detection and Quantitation (HCI and ISO Regimes) 16
Table 11: Robustness of Method 16

Figures

Figure 1: Example Chromatogram of Glycerin and Menthol 8
Figure 2: Sample Calibration Plot for Glycerin 11
Figure 3: Sample Calibration Plot for Menthol 11
Figure 4: Example of an Accuracy Profile 12

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

1. ANALYSIS OF GLYCERIN AND MENTHOL IN AEROSOL

1.1. Applicability

This method is used to determine glycerin and menthol 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/cig for 3R4F and mg/stick for THS.

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) and for menthol in THS. 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.

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.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

The samples are then analyzed by Gas Chromatography (GC) with a Flame Ionization Detector (FID) GC-FID according to USP <621>.

For the glycerin analysis, 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.

For the menthol analysis, THS are conditioned in closed 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 and 1.2.4.2 respectively.

Preceding each batch, calibration is performed according to the details given in Section 1.2.3. Typically, 4 sequences of measurement are performed per batch. Each sequence consists of suitability test (SSTs), reference runs (glycerin and menthol), monitor runs followed by a number of sample runs and is terminated by a standard (STD) 3 run to validate the results and verify the calibration (requirement: deviation of not more than ± 10% on two of the three controls from the theoretical value for glycerin and menthol).

1.2.3. Preparation of Solutions

1.2.3.1. Calibration Solutions

Five standard (STD) solutions containing different concentrations of each target compound (highest:lowest = 20:1) and fixed concentrations of ISTD *n*-heptadecane are prepared. The ranges of concentrations cover the ranges relevant for analysis and are provided in Table **1**.

Table 1: Calibration Standards Ranges

Compound		**Internal Standard**	
Glycerin	0.0000 mg/mL – 1.250 mg/mL	*n*-heptadecane	0.3
Menthol	0.0202 mg/mL – 0.5000 mg/mL		

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.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

1.2.4. Experimental Conditions

1.2.4.1. Equipment

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

1.2.4.2. Chromatographic Conditions

Column	Restek Capillary column RTx-35, 30 m x 0.25 mm, ID x 1 μm film thickness
Carrier gas	Helium
Carrier gas	1.2 mL/min
Flow mode	Ratio
H_2 flow (FID)	40 mL/min
Air flow (FID)	350 mL/min
Split ratio	10
Oven temperature program	110°C, 1 min 10°C/min → 150°C, 2 min 30°C/min → 230°C, 3 min
Run time	12.67 min
Injection temperature	230°C
Detector temperature	300°C
Injection volume	1 μL

1.2.5. Calibration

Calibration is performed with five STD solutions for glycerin and menthol. Each standard contains different concentrations of the compound (highest:lowest = 20:1) and ISTDs of 2.0 mg/mL for ethanol and 0.3 mg/mL for *n*-heptadecane.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

A ratio of each compound (*n*-heptadecane for glycerin and menthol) is determined according to:

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

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

The concentration of menthol in the extract in (mg/mL) is calculated using the equation y = ax+b using a linear regression without a weighting factor.

Note: The use of a weighting factor is conditioned to the instrument used.

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:

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

Where,

V Extraction volume

n Number of sticks or THS per replicate

D Dilution factor

Glycerin calibration is considered valid if a coefficient of determination $r^2 > 0.995$.

Menthol calibration is considered valid if a coefficient of determination $r^2 > 0.995$.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

1.2.6. Example Chromatogram

An example chromatogram is provided in Figure 1.

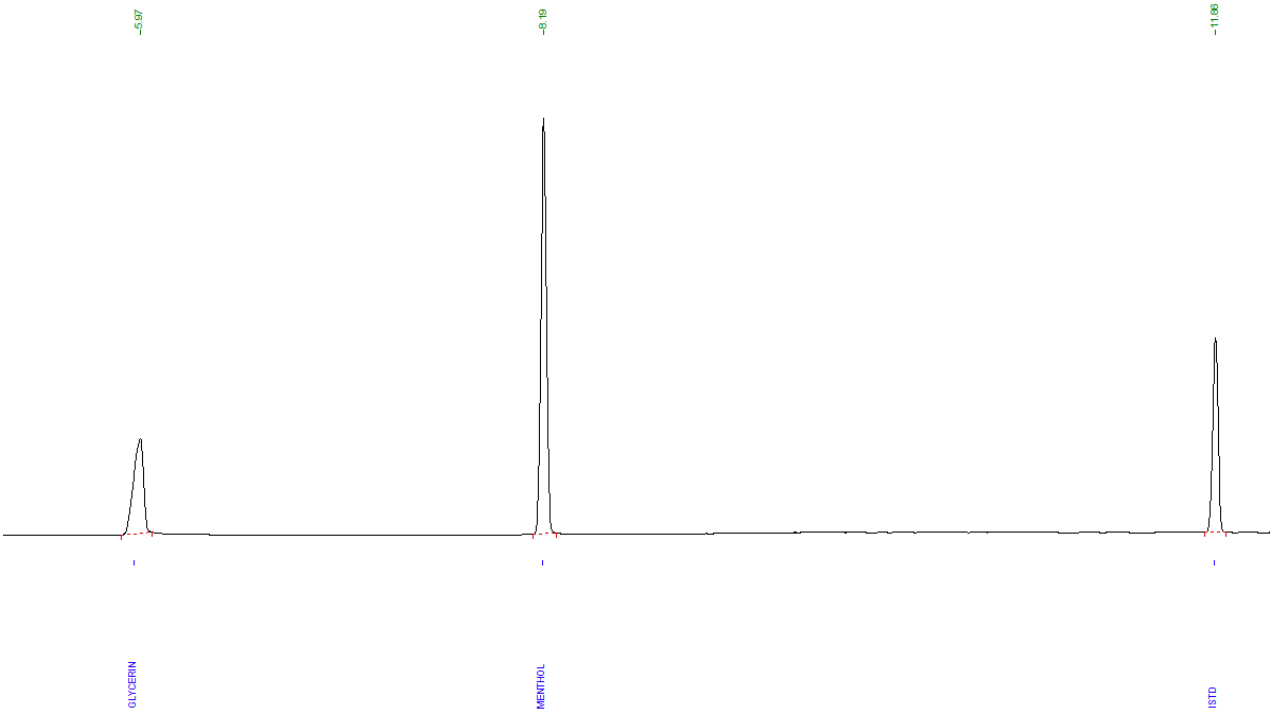


Figure 1: Example Chromatogram of Glycerin and Menthol

1.2.7. Retention Times

Typical retention times for glycerin, menthol and the ISTD are provided in Table 2.

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

Target		**Internal Standard**	
Glycerin	5.95	*n*-heptadecane	11.69
Menthol	8.14		

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

1.2.8. System Suitability

Control solution STD 3 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 6398.

Table 3: System Suitability Acceptance Criteria

Compound	Glycerin	Menthol
Peak Width	< 4.50	< 3.65
Tailing factor	< 1.14	< 1.05
Peak Area	> 7621	> 27161

1.3. Validation

Validation of the analytical method is performed for each piece of equipment. PMI equipment ID 6398 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.

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 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 are 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 was set at 20% and the lower values are considered selective.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

1.3.2. Linearity

The response function was demonstrated using calculated residuals for glycerin and menthol for a range of concentrations with a linear regression model.

1.3.2.1. Regression Parameters

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

The linearity of response functions was assessed on multiple instruments using the range of concentrations shown in Table 4. The relative residuals requirement was ≤ 20% for the lowest calibration level and 10% for all other calibration level and $r^2 > 0.995$.

Table 4: Regression Parameters (PMI ID 6398 – reference instrument)

Compound	Range tested	Slope	Intercept	r^2
Glycerin	0.05 mg/mL – 1.00 mg/mL	0.9366	-0.0094	0.9989
Menthol	0.02 mg/mL – 0.80 mg/mL	2.7836	-0.0049	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) were evaluated. The relative residuals requirement was ≤ 20% for the lowest calibration level and 10% for all other calibration level of the nominal concentrations and $r^2 > 0.995$. The average slope, intercept, and coefficient of determination (r^2) for each compound are provided in Table 4.

1.3.2.2. Sample Calibration Plots

Sample calibration plots are provided in Figures 2 and 3.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

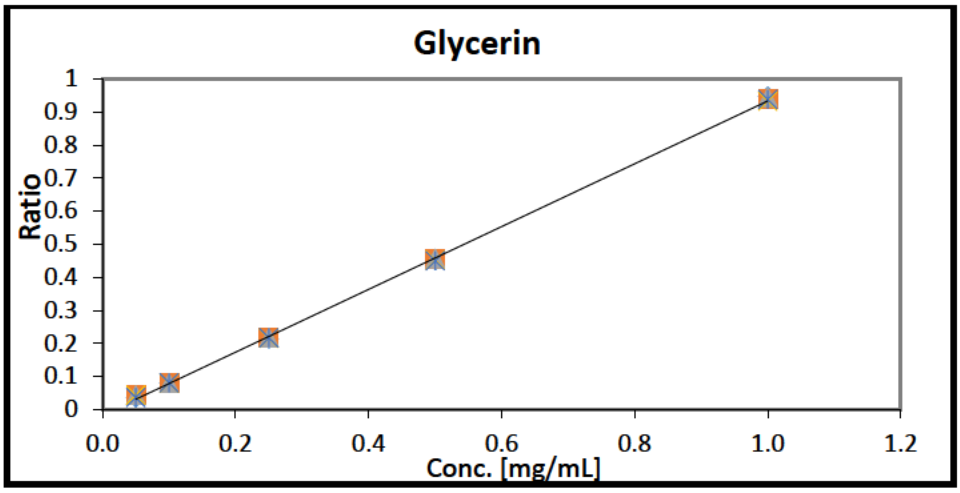


Figure 2: Sample Calibration Plot for Glycerin

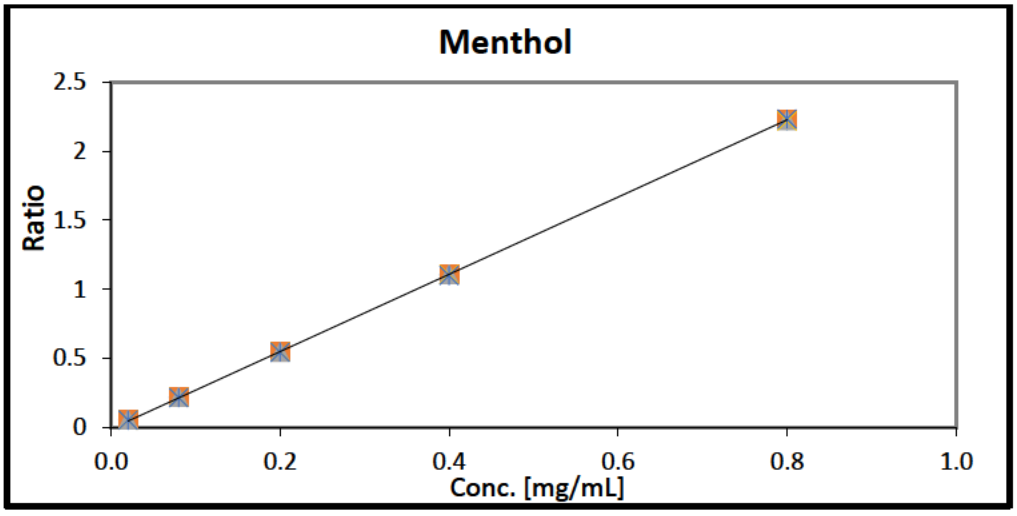


Figure 3: Sample Calibration Plot for Menthol

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 4 for an example).

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

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.

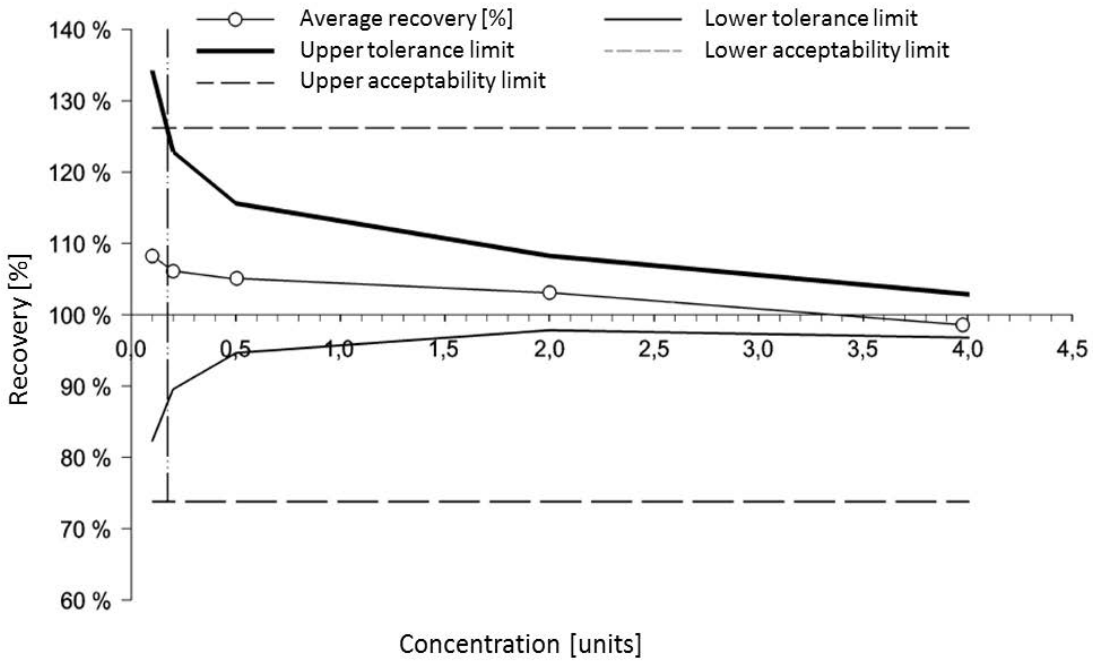


Figure 4: Example of an Accuracy Profile

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

Table 5: Working Range

Compound	Working Range 3R4F (mg/cig)		Working Range THS (mg/stick)	
	HCI	ISO	HC	ISO
Glycerin	0.50 – 8.65	0.55 – 1.15	2.65 – 14.40	0.48 – 2.57
Menthol	1.17 – 5.00	0.30 – 0.66	0.67 – 11.20	0.30 – 2.40

1.3.4. Accuracy and Precision

1.3.4.1. Instrument Repeatability

Instrumental repeatability was determined by analyzing 10 injections of the same vial of the same treated solution for glycerin and menthol for each matrix. Very low coefficients of variation were found for glycerin and menthol in all the matrices. The acceptance criteria for glycerin and menthol were fulfilled All coefficients of variation were well within the acceptance range. Results for repeatability limits are given in Table **6**.

Table 6: Instrumental Repeatability

Compound	3R4F (mg/cig)				THS (mg/stick)			
	Mean	r	std	CV_i [%]	Mean	r	std	CV_i [%]
Glycerin	0.82	0.0000	0.0010	0.49	1.76	0.0001	0.0029	0.66
Menthol	NA	NA	NA	NA	1.71	0.0000	0.0020	0.47

r = repetability, std = standard deviation, CV = 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. All coefficients of variation for the repeatability were lower than 5.3% for menthol and slightly higher than 5.3% for glycerin for

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

most analytes for the complete procedure, therefore the acceptance criterion were increased from 5.3% to 10% to be able to quantify glycerin in 3R4F and in THS.

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 [%]**
Glycerin (HCI)	2.38	0.358	0.127	5.32	4.08	1.076	0.381	9.33
Menthol (HCI)	NA	NA	NA	NA	2.91	0.297	0.105	3.60
Glycerin (ISO)	0.80	0.083	0.029	3.70	1.78	0.287	0.102	5.71
Menthol (ISO)	NA	NA	NA	NA	1.69	0.251	0.089	5.24

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

1.3.4.2. Intermediate Precision

The intermediate precision limit 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 were smoking day, the operator and calibration. Intermediate precision limit was evaluated by injecting each aerosol collection replicate for each matrix generated under the HCI and ISO smoking regimes. Regardless of analytie or matrix, coefficients of variation are lower than the acceptance limit of 15%. Results are given in Table 8.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

Table 8: Intermediate Precision

Compound	3R4F (mg/cig)					THS (mg/stick)				
	Mean	**IP**	**Std_{IP}**	**CV_{IP}**	**CD95 %**	**Mean**	**IP**	**Std_{IP}**	**CV_{IP}**	**CD95 %**
Glycerin (HCI)	2.38	0.373	0.132	5.54	0.208	4.08	1.076	0.381	9.33	0.538
Menthol (HCI)	NA	NA	NA	NA	NA	2.92	0.297	0.105	3.60	0.148
Glycerin (ISO)	0.80	0.148	0.052	6.55	0.129	1.78	0.304	0.107	6.04	0.174
Menthol (ISO)	NA	NA	NA	NA	1.69	1.69	0.347	0.123	7.25	0.271

IP = intermediate precision, std_{IP} = standard deviation, CV_{IP} = coefficient of variation, CD95% = 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 concentrations in 3R4F and THS are provided in Table 9.

Table 9: Combined Uncertainty (Standard Deviation)

	3R4F	**THS**
Glycerin	8% - 18%	8% - 18%
Menthol	6% to 13%	6% - 13%

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

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).

$$LOD = LLOQ / 3.3$$

$$LLOQ = 10 \times SD_{level\,1}$$

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)

		3R4F (mg/cig)			**THS (mg/stick)**		
		LOD	**LLOQ**	**ULOQ**	**LOD**	**LLOQ**	**ULOQ**
HCI	Glycerin	0.044	0.144	10	0.073	0.240	33.3
	Menthol	0.019	0.063	8	0.032	0.105	5.3
ISO	Glycerin	0.017	0.058	4	0.017	0.058	20
	Menthol	0.008	0.025	3.2	0.008	0.025	3.2

ULOQ = upper limit of quantitation (highest calibration level)

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 for the instrument (Increase the split flow of 50%	Regardless of the instrument and matrix, values obtained for glycerine and menthol by increasing split flow were similar compared to the values obtained with initial conditions. Differences arweree lower than 3% and therefore within the acceptance limit of 20%. The acceptance criteria for the modified split flow for robustness were fulfilled.

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.

1.3.7. Stability of Solutions

1.3.7.1. Homogenized Aerosol Solution at Ambient Temperature

The homogenized aerosol solutions are stable in Filtrona tube up to 32 days at ambient temperature.

1.3.7.2. Stability of Glycerin and Menthol STD Solutions in the Refrigerator

The glycerin and menthol STD solutions are stable up to 6 months in the refrigerator.

2. APPROVALS

	Name	Role	Signature	Date
Author	Michel Yerly	Team Leader Aerosol Formers & Special Analyses		22 JUL 2016
Owner	Serge Maeder	Director PMI Testing Laboratories Governance		26.07.2016
QA Review	Shelley Moores	Manager QA Assessment & Quality Systems	PP Florence Kapp	26 JUL 2016

Confidentiality Statement

Confidentiality Statement: Data and information contained in this document are considered to constitute trade secrets and confidential commercial information, and the legal protections provided to such trade secrets and confidential information are hereby claimed under the applicable provisions of United States law. No part of this document may be publicly disclosed without the written consent of Philip Morris International.