APPENDIX A3.2.2-8

ANALYSIS OF PHENOL IN AEROSOL

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1. ANALYSIS OF PHENOL IN AEROSOL

1.1. Applicability

This method is used to determine phenol in aerosol from conventional cigarettes (3R4F) and the Tobacco Heating System (THS). This method additionally quantitates the remaining six phenolic compounds in mainstream aerosol. 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 filter pad (CFP) and analytes are extracted with a 2-butanone solution containing an internal standard compound. The extract is derivatized with N,O-Bis(trimethylsilyl)-trifluoracetamide (BSTFA) and analyzed according to USP <621>, Gas Chromatography, using MS-EI detection and the equipment and conditions 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, calibration is performed according to the details given in section 1.2.3 Typically, four sequences of measurements are performed per batch. Each sequence consists of a reference run, followed by a number of samples, 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 the target compound (highest:lowest = 250:1) and a fixed concentration of the internal standard (related deuterated compound) are prepared in 2-butanone. The range of concentrations covers the range relevant for analysis and is provided in Table 1.

Table 1: Calibration Standard Ranges

	Compound	**Value (µg/mL)**
Target Compound Range	Phenol	0.019 – 4.800
Internal Standard	Phenol-D_6	0.800

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1.2.1.2. Extraction Solution

The extraction solution is 2-butanone, containing the internal standard.

THS: five accumulations on one pad, extracted with 10 mL

3R4F: two accumulations on one pad for HCI and five accumulation on one pad for ISO, extracted with 10 mL and diluted by a factor of 4

1.2.2. Experimental Conditions

1.2.2.1. Equipment

Gas Chromatograph	A suitable GC equipped with a MS-EI detector and data acquisition software
Capillary column	DB-624, 30 m x 0.32 mm x 1.8 µm, or equivalent

1.2.2.2. GC Conditions

Injection temperature	225°C
Injection volume	1 µL
Carrier gas	Helium, splitless injection at 59 kPa
Flow control mode	Constant pressure
Pressure	59 kPa
Temperature program	50°C, hold 2 min Heating to 150°C, 30°C/min Heating to 200°C, 4°C/min
Column flow	2.8 mL/min
Post run	5 min at 240°C and 150 kPa

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1.2.2.3. Mass Spectrometer Settings

Ionization Mode	EI
MS Quad temperature	150ºC
Ion source temperature	230ºC
Transfer line temperature	225ºC
Solvent delay	6.5 min
EM setting	Delta EMV
Relative voltage	0.000V
Acquisition type	SIM

1.2.3. Calibration

Calibration is performed with eight standard solutions containing different concentrations of phenol (highest:lowest = 250:1) and fixed concentrations of internal standard, Phenol-D_6 (see Table 1). A linear regression against concentration is performed, with statistical weights of 1/concentration, according to:

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

where

A_a area of target analyte

A_{ISTD} area of corresponding internal standard

C_a concentration of target analyte

b intercept

p slope

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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 phenolic compounds which are detected in mainstream aerosol by this procedure.

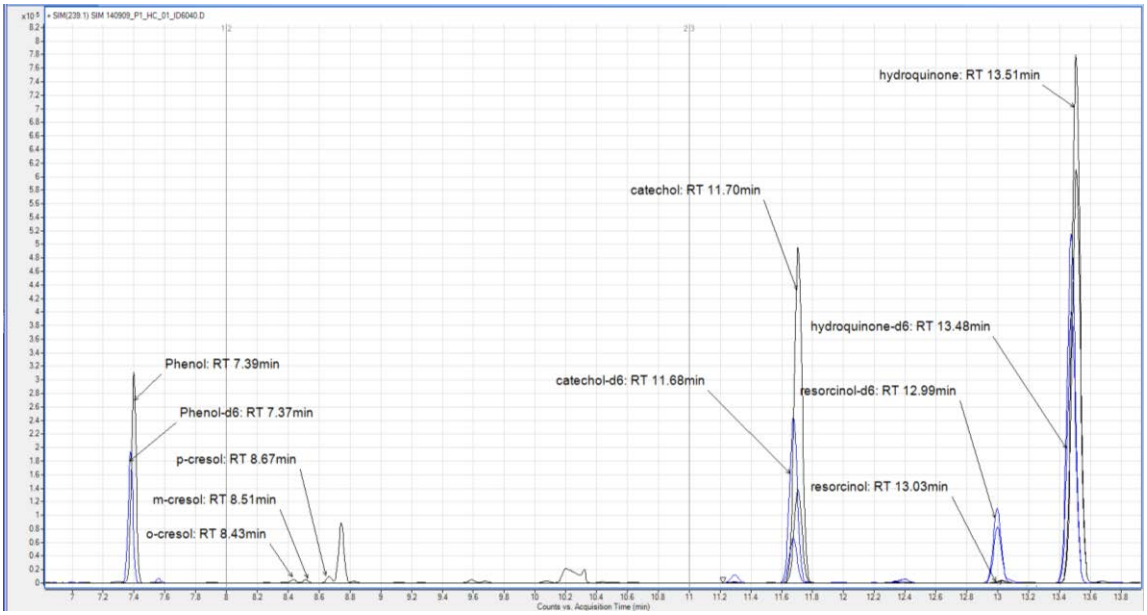


Figure 1: Example Chromatogram Showing the Phenolic Compounds and Their Respective Reference Standards

1.2.5. Retention Times

Typical retention times for phenol and internal standard are provided in Table **2**.

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

	Compound	**Retention Time**
Target	Phenol	7.39
Internal standard	Phenol-D_6	7.37

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1.2.6. System Suitability

A medium standard (STD6) is used to assess the symmetry (tailing factor), full width at half maximum (FWHM) and height of the peak for phenol-D_6 (internal standard) and resorcinol. System suitability criteria are specific and based on the method validation performed using the equipment. Example of acceptance criteria for equipment ID 10809 are provided in Table 3.

Table 3: System Suitability Acceptance Criteria (Agilent PMI ID No. 10809)

Parameter	**Resorcinol**	**Phenol-D_6**
Symmetry	$0.817 \leq x \leq 1.184$	$0.760 \leq x \leq 1.243$
FWHM	$x \leq 0.0602$	$x \leq 0.0333$
Peak Height	$x \geq 3140$	$x \geq 73055$

1.3. Validation

Validation of the analytical method is performed for each equipment used. PMI equipment ID 6040 and ID 10809 was used to obtain the information provided in this section.

1.3.1. Specificity (Selectivity)

Selectivity was demonstrated with analytical blanks and aerosol collected under Health Canada Intense and International Organization for Standardization (ISO) smoking regimes with two different amounts of a spiking solution and evaluation of the resulting signal.

In addition, the response ratio for qualifier to quantifier ions in matrices was compared to standards. 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 the corresponding retention times.

The selectivity of the internal standard compound was also evaluated. Matrix samples without internal standard demonstrated lack of interfering peaks in the chromatographic region of interest for the internal standard. The concentration in double blanks (measurement solution without internal standard) were lower than 20% compared to the first calibration level for analyte and internal standard. The concentration in the blank measurement solution with internal standard was lower than 20% compared to the first calibration level (≤ Lower Limit of Quantitation, LLOQ).

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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 for an extended range used 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 between the determined concentrations and the calculated concentrations (residuals) was within ± 20% for the lowest calibration level and within ± 15% for all the other calibration levels. Out of six standards levels, four had to fulfil these criteria, including the LLOQ and the highest standard. No obvious curvature was observed on the residuals plots when applying a linear regression model with 1/concentration weighing.

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. The average slope, intercept, and correlation coefficient obtained for 5 calibration curves with 8 standard levels each are provided in Table 4.

Table 4: Regression Parameters Under Repeatability Conditions (n = 8x5 = 40)

Range Tested (µg/mL)	Slope	Intercept	r	SS
0.010 – 6.242	1.300	0.027	1	7.62E-03

r = correlation coefficient, SS = residual sum of squares

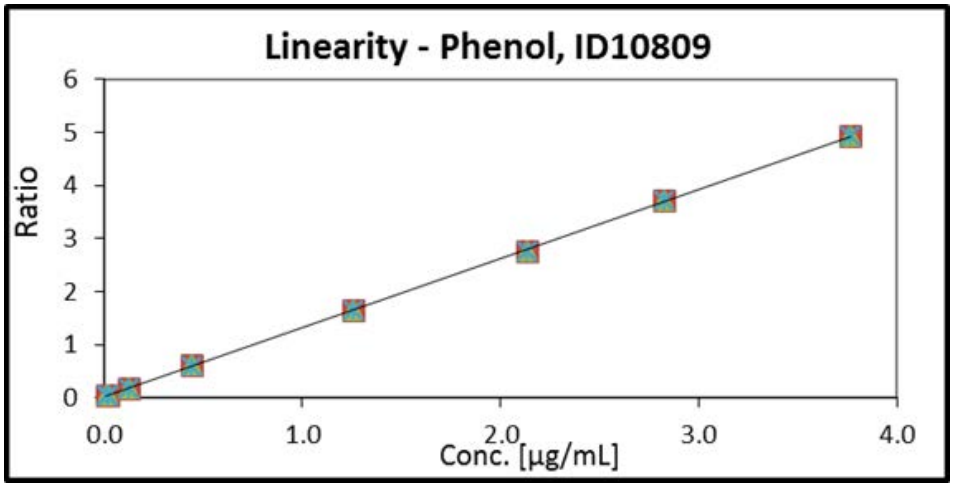


Figure 2: Sample Calibration Plot

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1.3.2.2. Calibration Curve Parameters Variability Under Intermediate Precision Conditions

The variability of the calibration curve parameters under intermediate precision conditions was evaluated. The calibration curves were obtained varying 1) preparation of the stock solutions date, 2) preparation of secondary mix and working standards date, and 3) analyst. The goal of this evaluation is to provide the laboratory with information regarding the variability of the calibration curve under intermediate conditions. In addition, these data allow verification of the linearity of the response covering the extended range of concentrations used during assessment of accuracy. Results are provided in Table 5. Five calibration curves were injected on different days, each including 9 standard levels The same data were used to determine LOD and LLOQ/ULOQ (section 1.3.5).

Table 5: Regression Parameters Under Intermediate Precision Conditions (n = 9x5 = 45)

Range Tested (µg/mL)	**Slope**	**Intercept**	**r**
0.010 – 6.242	1.189 – 1.389	0.030 – 0.143	1

r = correlation coefficient

1.3.3. Range

Aerosol collection was performed under Health Canada Intense (HCI) conditions on three 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 six 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 (Figure 3).

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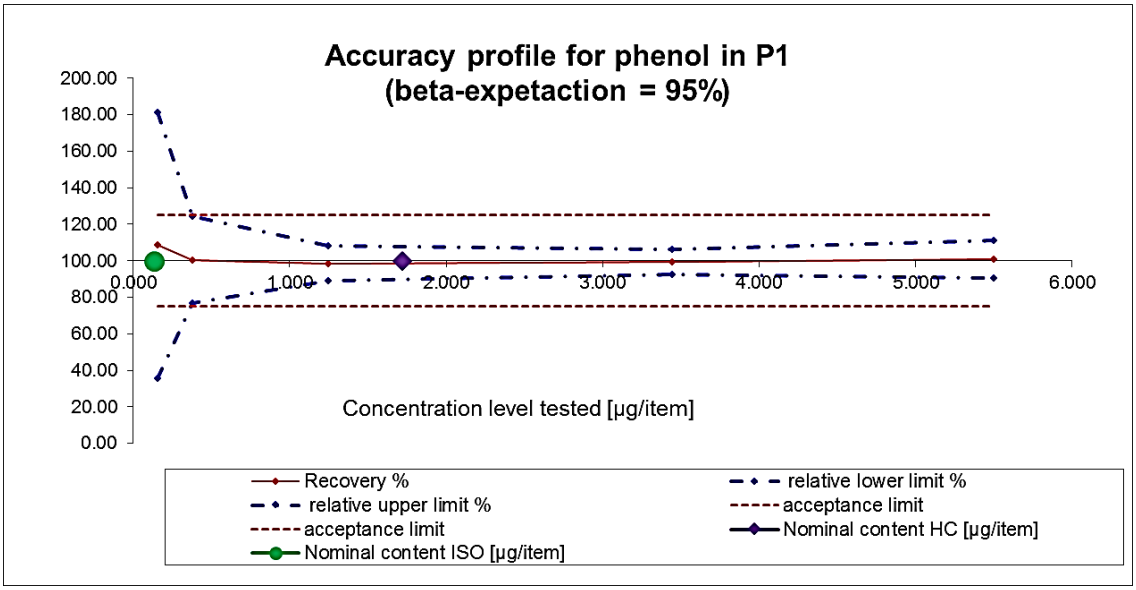


Figure 3: Determination of Working Range

The working ranges are provided in Table 6.

Table 6: Working Ranges for THS and 3R4F

Product	Working Range
THS (µg/stick)	0.371 – 5.50
3R4F HCI (µg/cig)	2.82 – 37.01
3R4F ISO (µg/cig)	1.13 – 14.80

1.3.4. Accuracy and Precision

1.3.4.1. Instrumental Repeatability

Within-instrument repeatability was 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.

Results for instrumental repeatability limits obtained from ten injections of the same aerosol extract are given in Table 7.

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Table 7: Within-Instrument Repeatability (Same Day, n=10)

Phenol	**Mean**	**SD**	**CV**	**r**
3R4F (µg/cig)	15.87	0.017	0.11%	0.0482
THS (µg/stick)	1.67	0.001	0.06%	0.0029

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

1.3.4.2. Repeatability and Intermediate Precision Limits

The repeatability and intermediate precision limits for the whole process were evaluated at nominal content by injecting once each aerosol collection 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.

Repeatability and intermediate precision limits for the whole process are provided in Table 8.

Table 8: Precision Limits, n = 28 for HCI, n= 16 for ISO

Phenol		**Nom.**	**r**	**SD_r**	**CV_r**	**IP**	**SD_{IP}**	**CV_{IP}**
HCI	3R4F (µg/cig)	14.00	3.086	1.091	7.79%	3.344	1.182	8.44%
	THS (µg/stick)	1.72	0.570	0.202	11.73%	0.902	0.319	18.56%
ISO	3R4F (µg/cig)	7.73	1.027	0.363	4.70%	1.559	0.551	7.13%
	THS (µg/stick)	0.136	0.0607	0.0215	15.79%	0.0756	0.0267	19.67%

Nom. = nominal content, r = repeatability limit, SDr = standard deviation of repeatability, CVr = coefficient of variation of repeatability, IP = intermediate precision, SD_{IP} = standard deviation of the intermediate precision, CV_{IP} = coefficient of variation for the intermediate precision

1.3.4.2.1. Intermediate Precision Limit at Different Concentrations

Coefficients of Variation were 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.

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All phenol coefficients of variation of repeatability of the analytical method were lower than 1.5% for 3R4F and THS.

Phenol coefficients of variation for intermediate precision of the analytical method were found to be between 3.3% and 6.5% for 3R4F, and between 2.5 and 8.7% for THS, with the exception of the lowest level of concentration tested.

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 were combined through addition of variances to one single global uncertainty (Table 9).

The results were obtained for two standard solutions which contain the relevant range for that compound.

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

Product	Expanded Combined Uncertainty
3R4F	8% – 9%
THS	8% – 9%

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

The limit of detection and lower limit of quantitation (LLOQ) are both calculated in terms of the standard deviation of the lowest calibration level as determined during validation,

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

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

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Table 10: Limits of Detection and Quantitation

		LOD	LLOQ	ULOQ
3R4F (µg/cig)	Health Canada Intense	0.079	0.263	124.84
	ISO	0.032	0.105	49.93
THS (µg/stick)	Health Canada Intense / ISO	0.008	0.026	12.48

ULOQ = upper limit of quantitation (highest calibration level)

1.3.6. Robustness

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

Table 11: Robustness of Method

Parameter	**Recommendation/Comment**
Derivatization for 30 minutes at ambient temperature, 50ºC and 80ºC	Derivatization does not require elevated temperature.
Column lots	No clear limitation on the number of injections was found.
Different types of BSTFA (different purities, with and without TMCS)	No significant differences are observed if phenolic compounds are derivatized using a solution that does not contain TMCS.
Volume of standard/sample and derivatization solution	Equivalent results were obtained for all volumes tested.
Volume of extraction solution (10 mL, 20 mL)	No statistically significant difference was observed.
Trapping efficiency	Trapping efficiency > 90% was observed.
Injection Volume (0.6, 1 (target), and 2 µL)	Not critical
GC Oven Temperature (45, 50 (target), and 55ºC)	Not critical
Injection Temperature (220, 225 (target), 230ºC)	Not critical

table continues

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Parameter	Recommendation/Comment
Column Pressure (55, 59 (target), 63 kPa)	Not critical

1.3.7. Stability

1.3.7.1. Stability of Individually Prepared Solutions

The stability of individually prepared solutions containing phenolic compounds in 2-butanone was evaluated. The individually prepared stock solutions for the target phenolic compounds are stable up to 119 days when kept at -20ºC in amber headspace vials.

1.3.7.2. Individually Prepared Internal Standard Stock Solutions

The individually prepared internal standard stock solutions are stable up to 119 days when kept at -20ºC in amber headspace vials.

1.3.7.3. Secondary Mix Solution

The stability of phenolic compounds in secondary mix solution was evaluated. The compounds are stable for a longer period if stored in individually prepared solutions. The secondary mix solution storage period is 10 days at -20ºC.

1.3.7.4. Extraction Solution

The extraction solution is stable for at least 2 months in amber bottles at -20ºC; however, it is recommended that when the solution is removed from the freezer, a homogenized aliquot corresponding to the volume of solution needed for preparation that day is immediately transferred to another amber bottle with cap and that the bottle containing the originally prepared solution is kept in the freezer at -20ºC to avoid repeated and unnecessary freeze/thaw cycles. The same extraction solution must be used for standard preparation and sample extraction. Any remaining volume from the aliquot taken each day must be rejected.

1.3.7.5. Phenolic Compounds Derivatized in GC Vial

The maximum length of the sequence for quantitation of samples with calibration at T0 is up to 72 hours if GC vials are kept in an autosampler at room temperature.

It is not recommended to reinject the same GC vials when the preparation of 100 µL of samples/standard and 60 µL of derivatization solution is used. A new set of vials must be prepared if a retest of the batch is deemed necessary.

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1.3.7.6. Target Compounds on CFP after Aerosol Collection

Phenolic compounds are stable in the CFP without extraction in capped amber vials at -20°C for up to 5 days.

1.3.7.7. Aerosol Extracts

The matrix extracts are stable up to 18 days at -20°C in headspace amber vials.

2. APPROVALS

	Name	Role	Signature	Date
Author (Validation)	Daniela Correia	Scientist	DanielaCorreia	22-July-2016
Owner	Serge Maeder	Director PMI Testing Laboratories Governance	S. M. L	26.JUL.2016
QA Review	Shelley Moores	Manager QA Assessment & Quality Systems	S. Moores	27-JUL-2016

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