APPENDIX A3.2.2-10

ANALYSIS OF PYRIDINE IN AEROSOL

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Contents

APPENDIX A3.2.2-10 ANALYSIS OF PYRIDINE IN AEROSOL 1
1. ANALYSIS OF PYRIDINE IN AEROSOL 4
1.1. Applicability 4
1.2. Analytical Procedure 4
1.2.1. Preparation of Solutions 4
1.2.2. Experimental Conditions 5
1.2.3. Calibration 6
1.2.4. Example Chromatogram 7
1.2.5. Retention Times 8
1.2.6. System Suitability 8
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 11
1.3.5. Limit of Detection (LOD) / Limit of Quantitation (LOQ) 13
1.3.6. Robustness 13
1.3.7. Stability of Solutions 14
2. APPROVALS 15

Tables

Table 1: Calibration Standards Ranges 5
Table 2: Typical Retention Times for Target and Internal Standard Compounds 8
Table 3: System Suitability Acceptance Criteria for Pyridine 9

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Table 4: Regression Parameters Under Repeatability Conditions (n = 6x5 = 30) 10
Table 5: Within-Instrument Repeatability (Same Day) 11
Table 6: Repeatability Limits in Aerosol Matrices (ISO) 12
Table 7: Repeatability Limits in Aerosol Matrices (HCI) 12
Table 8: Intermediate Precision Limit at Nominal Content and Critical Difference (HCI) 12
Table 9: Expanded Combined Uncertainty (k=2, 95%) 13
Table 10: Limits of Detection and Quantitation 13
Table 11: Robustness of method 13

Figures

Figure 1: Example Chromatogram 8
Figure 2: Sample Calibration Plot 11

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1. ANALYSIS OF PYRIDINE IN AEROSOL

1.1. Applicability

This method is used to determine the level of pyridine in aerosol from conventional cigarettes (3R4F) and the Tobacco Heating System (THS) under Health Canada Intense (HCI) and International Organization for Standardization (ISO) smoking conditions. Please note that this method also quantitates styrene, naphthalene, and quinoline. Results are expressed as µg/stick for THS and µg/cigarette for 3R4F.

1.2. Analytical Procedure

Aerosol from a smoking machine is collected using a Cambridge Filter Pad (CFP) and two serially connected impingers, each containing 10 mL of methanol (first impinger at 0°C, second impinger at below -70°C).

The CFP is immersed in the impinger contents and the whole is stirred at 250 rpm for at least 10 minutes. The liquid is then passed through a 0.45 µm disposable filter. GC vials are prepared by pipetting 1 mL of the THS aerosol extract, or standard solution, and 50 µL of internal standard (ISTD) solution. In the case of 3R4F, 920 µL of methanol and 80 µL of aerosol extract with 50 µL of ISTD, which is then analyzed by Gas Chromatography (GC) using Mass Spectrometry (MS) in Selective Ion Monitoring (SMI) mode according to USP <621>. The equipment and conditions are described in Sections 1.2.2.1, 1.2.2.2, 1.2.2.3 and 1.2.2.4.

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 monitor (3R4F) run followed by four samples of the same replicate, and is terminated by a standard solution run with a medium concentration (standard 4) 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

Six standard solutions containing different concentrations of the target compound (highest: lowest = 200:1) and a fixed concentration of standard pyridine-D_5, are prepared in methanol. The ranges of concentrations covers the ranges relevant for analysis and is provided in Table 1.

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Table 1: Calibration Standards Ranges

	Compound	Value (µg/mL)
Target compound Range	Pyridine	0.017– 3.465
Internal standard	Pyridine-D_5	0.381

1.2.1.2. Extraction Solution

The extraction solution is methanol (99.9% pure).

THS: 5 accumulations per replicate, extracted with 20 mL of methanol.

3R4F: 2 accumulations per replicate, extracted with 20 mL of methanol and diluted by a factor of 12.5x.

1.2.2. Experimental Conditions

1.2.2.1. Equipment

Gas chromatograph	Suitable gas chromatograph equipped with a MS detector and data acquisition software
Liner	3.4 mm, Supelco 2877605-U or equivalent
Column	DB-WAXETR, 30m × 0.25 mm × 0.5 µm, or equivalent
Pre-column	2 m of deactivated fused silica T 0.25mm.

1.2.2.2. Instrumental Conditions

Column oven temperature	40°C
Injection temperature	250.0°C
Injection mode	Splitless
Injection volume	1 µL
Flow control mode	Linear velocity
Pressure	90.0 kPa
Total flow	154.0 mL/min
Column flow	1.49mL/min

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Linear velocity	42.7 cm/sec
Purge flow	3.0 mL/min
Split ratio	100.0
Carrier gas saver	Split ratio: 20.0; Time: 5.0 min

Temperature program

Rate (°C/min)	Final Temperature (°C)	Hold Time (min)
N/A	40.0	2.00
15.00	150.0	0.00
30.00	250.0	15.00

Total program time 27.67 min

1.2.2.3. MS Method Parameters

Ionization mode	EI
Ion Source temperature	230°C
Interface temperature	240°C
Solvent cut time	6 min
Micro-scan width	0 atomic mass unit (amu)
Detector gain mode	Relative to the Tuning Result

1.2.2.4. MS-SIM Parameters

Compound	Start Time (min)	End Time (min)	Acq. Mode	Event Time (sec)	Ch 1 m/z	Ch 2 m/z	Ch 3 m/z	Ch 4 m/z
Pyridine	6.75	7.75	SIM	0.20	84	56	79	52
Pyridine-D_5								

1.2.3. Calibration

Calibration is performed with 6 standard solutions containing different concentrations (highest: lowest = 200:1) and a fixed concentration of pyridine-D_5.

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

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

where

A_a integrated area of target analyte

A_{iStd} integrated area of the corresponding ISTD

C_a concentration of the target analyte

b intercept

p slope

Calibration is considered valid if a correlation with $r^2 > 0.995$ is obtained and the deviation of residuals is within ± 25% for the lowest calibration level and within ± 10% for all other calibration levels. Out of 6 standard levels, 5 have to fulfill these criteria, including the lowest and highest standards. If one standard is out of criteria, it must be excluded from calibration curve for calculations.

The calibration curve is continuously validated throughout the analytical sequence by means of injection of control standard.

1.2.4. Example Chromatogram

An example chromatogram is provided in Figure 1.

Note: this method also detects styrene, naphthalene, and quinoline.

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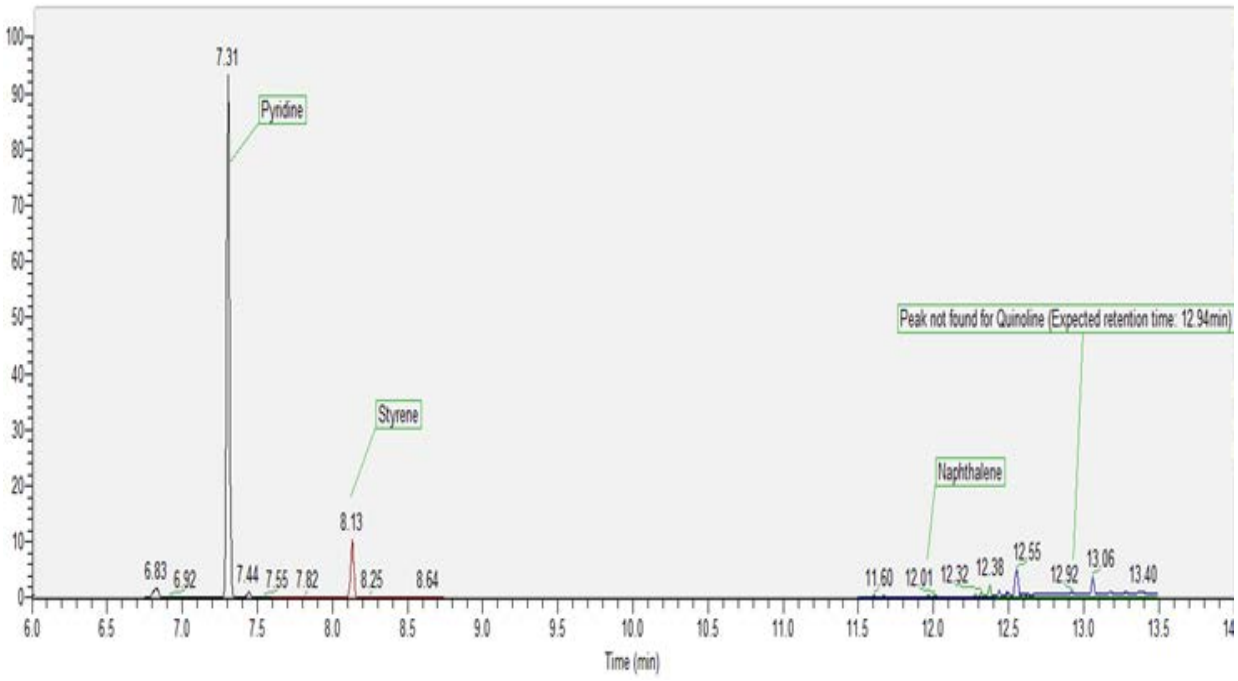


Figure 1: Example Chromatogram

1.2.5. Retention Times

Typical retention times for pyridine and its Internal Standard (ISTD) are provided in Table 2

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

	Compound	**Retention Time (min)**
Target	Pyridine	7.31
Internal standard	Pyridine-D_5	7.34

1.2.6. System Suitability

A control solution (standard 4) is used to assess instrument performance. The following performance indices are considered:

1) Tailing Factor to monitor the injection and the column performance,

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2) Peak Width at 50% height to control the peak shape and monitor the column performance,

3) Signal to noise to monitor instrumental sensitivity.

The acceptance criteria are provided in Table 3.

Table 3: System Suitability Acceptance Criteria for Pyridine

Tailing Factor [NA]	Peak Width [min]	S/N [dB]
$0.95 \leq x \leq 1.16$	$x \leq 0.023$	$x \geq 11498$

S/N = signal to noise ratio

1.3. Validation

Validation of the analytical method is performed for each piece of equipment. PMI equipments ID 6357 and PMI ID 6375 were used to obtain the information provided in this section.

1.3.1. Specificity (Selectivity)

Selectivity of pyridine was demonstrated for aerosol collected under the HCI smoking regimeby adding 4 different amounts of a spiking solution containing a known concentration of pyridine 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 pyridine at the corresponding retention time.

Selectivity of pyridine in the analytical blanks was demonstrated by comparison of resulting signal for injection of solvent with ISTD and solvent without ISTD. Although peak area for pyridine in blank with ISTD cannot be considered neglible when compared with blank without ISTD, this contribution was demonstrate to come from the ISTD solution. As same amount of ISTD solution is added to the calibration standards and samples, the contribution from the ISTD is accounted for on regression's intercept. This correction is demonstrated by quantitation of pyridine in blank with ISTD using the regression. Back-calculated concentration in blank with ISTD is found to be well below STD1 concentration (around 9%) demonstrating selectivity for pyridine.

Selectivity of ISTD was demonstrated in aerosol samples and analytical blanks by comparison of resulting signal for injection of aerosol sample/solvent with and without ISTD.

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The selectivity of the ISTD compound in the matrix was evaluated by dividing the response (peak area) for pyridine in matrices without ISTD with the response (peak area) in matrices with ISTD. Interfering peak at pyridine's retention time in matrix without ISTD was lower than 1.5% of response in matrix with ISTD for both 3R4F and THS samples.

The selectivity of ISTD compound in analytical blank was evaluated by comparing blank with ISTD and blank without ISTD. No significant interference was observed at the retention time of ISTD in blank without ISTD.

1.3.2. Linearity

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

1.3.2.1. Regression Parameters

The linearity of response functions was assessed using the range of concentrations shown in Table 4. The deviation of the determined concentration from the back calculated concentrations (residuals) were within ± 20% for the lowest calibration level and within ± 15% for the other calibration levels.

Table 4: Regression Parameters Under Repeatability Conditions (n = 6x5 = 30)

Range Tested (µg/mL)	**Slope**	**Intercept**	**r**	**SS**
0.017 – 3.465	1.756	0.1830	0.9999	2.05E-02

r = correlation coefficient, SS = residual sum of squares

1.3.2.2. Sample Calibration Plots

A sample calibration plot is provided in Figure 2.

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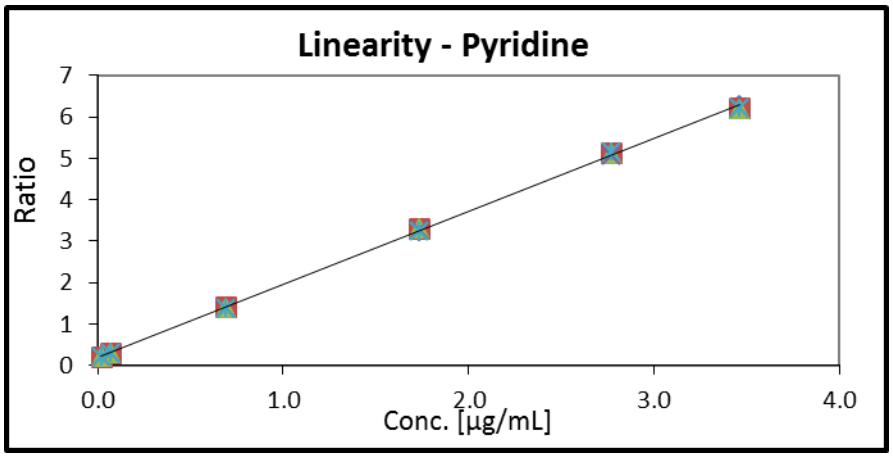


Figure 2: Sample Calibration Plot

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.

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 range for the target compound is 1.12 – 10.89 µg/stick.

1.3.4. Accuracy and Precision

1.3.4.1. 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) on a single instrument. All coefficients of variation were well within the acceptance range. Results for repeatability limits are given in Table 5.

Table 5: Within-Instrument Repeatability (Same Day)

	Mean	**r**	**std**	**CV**
3R4F (µg/cig)	43.267	0.8625	0.3049	0.7%
THS (µg/stick)	10.769	0.1011	0.0357	0.3%

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

The results of repeatability limits in aerosol matrices from the 4 individual smoking replicates collected for matrices over 1 day obtained under the ISO smoking regime are provided in Table 6. Results from the 5 individual aerosol collection replicates collected for matrices over 4 different days obtained under the HC smoking regime are provided in Table 7.

Table 6: Repeatability Limits in Aerosol Matrices (ISO)

	Mean	**r**	**std**	**CV**
3R4F (µg/cig)	7.465	0.837	0.296	4.0%
THS (µg/stick)	6.450	1.158	0.410	6.3%

Table 7: Repeatability Limits in Aerosol Matrices (HCI)

	Mean	**r**	**std**	**CV**
3R4F (µg/cig)	52.09	9.74	3.45	10.2%
THS (µg/stick)	10.798	1.278	0.452	6.6%

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

1.3.4.2. Intermediate Precision

The intermediate precision was evaluated at nominal content by injecting 5 individual replicates for 3R4F and THS matrices under the HCI smoking regime over 4 different days. Results are given in Table 8.

Table 8: Intermediate Precision Limit at Nominal Content and Critical Difference (HCI)

	Mean	**IP**	**std**	**CV**
3R4F (µg/cig)	52.09	15.06	5.32	10.2%
THS (µg/stick)	10.798	2.011	0.711	6.6%

IP = intermediate precision limit, std = standard deviation, CV = coefficient of variation

Reproducibility

No other laboratory is currently using this method.

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1.3.4.3. Expanded 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. The results were obtained for concentration covering the whole calibration curve range for pyridine and are provided in Table 9.

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

Compound	**3R4F (%)**	**THS (%)**
Pyridine	12-26	10-25%

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

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

$$LOD = 3 \times SD_{level\,1}$$

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

The LLOQ found to be lower than the concentration of the lowest calibration level for the target compound. Results are provided in Table 10.

Table 10: Limits of Detection and Quantitation

	LOD	**LLOQ**	**ULOQ**
3R4F (µg/cig)	0.135	0.452	432.57
THS (µg/stick)	0.004	0.01	13.84

ULOQ = upper limit of quantitation (equivalent to STD6)

1.3.6. Robustness

The parameters evaluated for robustness are described in Table 11.

Table 11: Robustness of method

Parameter	**Recommendation/Comment**
Extraction time at 250 rpm for 7 min, 10 min and 15 min for 3R4F	No significant difference was observed for the percentage recoveries (or percentage differences) [limits 80-120%], for the sample extracted at 250 rpm for 7 min or 15 min.

(table continues)

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Parameter	Recommendation/Comment
	The acceptance criteria for the extraction time are fulfilled and samples in routine can be extracted for 7 to 15 minutes.
Standards preparation	No significant difference was observed between the recovery of the theoretical concentrations of STD 2 and STD 5 prepared from the new commercial solution prepared in methanol produced by Restek and actual standard solution, which is ranged between 86.7% and 100%. The linearity criteria were fulfilled from STD1 to STD6. No significant bias was observed for the new preparation of standard with respect to actual standard solution.
Dilution factor for 3R4F	No significant difference was observed between two dilutions of concentration of the compound. The concentration difference for the 3 different days of analysis for the compound ranges between -1.24 to 1.37 % which fulfill the acceptance criteria.

1.3.7. Stability of Solutions

1.3.7.1. Stability of Sample Extracts and Calibration Standards in Unpierced Vials in Autosampler at Room Temperature

Sample extracts and calibration standard solutions are stable up to 144 hours when kept in unpierced vials in autosampler at room temperature.

1.3.7.2. Stability of Standard Mix and ISTD-VOC/ISTD-SVC Solutions in the Freezer at -20 °C

The standard mix and ISTD-VOC/ISTD-SVC solutions are stable up to 18 days when kept in the freezer at -20°C in amber vials or equivalent.

1.3.7.3. Stability of the Filtered Sample in Freezer at -80°C

The filtered sample in freezer is stable up to 17 days when stored at -80°C for 3R4F and THS.

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2. APPROVALS

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