APPENDIX A3.2.1-24

SCIENTIFIC REPORT – AMMONIA

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NEW ANALYTICAL METHOD FOR THE QUANTIFICATION OF AMMONIA IN AEROSOL

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SUMMARY

In the context of the improvement and revalidation of the methods used for the PMI58 list, the method related to the analysis of ammonia in aerosol by LC-MS/MS, has been recently reviewed. In addition, it should be also pointed out that the strategy of the Analytical Labs (AL) is to align as much as possible our methodologies to International Standards (ISO, Coresta) (if any). In this context, the Coresta Recommended Method (CRM81) using Ion Chromatography (IC) has been considered as a good opportunity for such alignment. Moreover, this methodology is used by most of the tobacco companies, as well as by commercial laboratories as Labstat.

The improvement of the method using IC leads to a difference on the quantification of ammonia when compared to the current method used in routine. The impact of these changes on the final quantifications for 3R4F and Platform 1 (P1 or THS) aerosol samples using current and new method was evaluated and results are presented in this report.

1 INTRODUCTION

The current method for the determination of ammonia by LC-MS/MS used in the Analytical Labs (AL), provides higher results for ammonia in comparison with competitors and commercial laboratories such as Labstat. It was suspected that higher values of ammonia were obtained due to a potential problem with the derivatization step.

As previously mentioned, most of the tobacco companies follow the Coresta Recommended Method (CRM81) which uses Ion Chromatography (IC) for the determination of ammonia in conventional products. This method was tested within Analytical labs and results obtained where lower than those generated with the current method and fully in line with external laboratories. Consequently, in the context of the

improvement of this method, we also decided to change the technology from High Performance Liquid Chromatography - Tandem Mass Spectrometry (HPLC-MS/MS) to Ion Chromatography (IC) and follow the Coresta Recommended Method (CRM81). However, small modifications were introduced in respect to the CRM81 methodology such as:

- decreasing the calibration range for a more suitable calibration for reduced risk products (RRP) with concentrations distributed evenly within the calibration range;
- modifications introduced on the aerosol collection such as a different trapping solution (0.1N H_2SO_4 instead of 0.02N H_2SO_4) and use of microimpingers with smaller volume of trapping solution instead of large impingers.

A short description of changes in comparison with Coresta, PPQ (PMI Internal Laboratory) and Labstat is presented in Table 1.

Table 1: Summary of Major Changes Applied to New Method Implemented in AL

Parameter	R&D	PPQ	Coresta	Labstat
impinger volume	2x10	2x15	2x15	2x15
Eluent: H_2SO_4 concentration	0.1N	0.1 N	0.02 N	0.1 N
Accumulation for 3R4F	3	5	3	3
Cambridge	44	92	44	44
smoking machine	linear	rotary	linear/rotary	linear
calibration range	0.2-4µg/mL	0.2-8µg/mL	0.2-10µg/mL	info not available
column	CS16, 3 x 250mm	CS16A, 5 x 250mm	CS12A, 4 x 250mm	info not available

2 OBJECTIVE

The purpose of this document is to describe the tests performed on 3R4F and P1 matrices in order to assess the impact on the quantification of ammonia when using the new method by IC. In addition, the impact on product specifications was also assessed.

3 MATERIAL AND METHOD

3.1 Samples

The samples used for this comparative study, sample traceability and related internal project per type of sample are described in Table 2.

Table 2: Test Items Used for the Comparison

Test item	Description	Batch number	RDLIMS project
3R4F	Reference item, conventional cigarette	N/A	RLS-VALID-2015-34
P1	Reduce Risk Product, Platform 1	B-03669	RLS-VALID-2015-34

3.2 Analytical method(s)

The samples were generated on a linear smoking machine (Borgwaldt KC) under HC (Health Canada) smoking regimen.

Samples were analyzed using the current method and the new one. For each type of analytical method, we applied the related aerosol collection and analytical procedure as described in Table 3. Samples were smoked and analyzed at different days according to the availability of instruments and methods.

Table 3: Summary of Instruments Used per Method

Method	Instrument
Current	ID11190
New	ID9306

3.3 Data reprocessing

- Current method by LC-MS/MS analyses were carried out on the HPLC-MS/MS Thermo instrument PMI ID9306 using the Xcalibur software version 2.2 SP1 for data acquisition and the LC Quan software version 2.7.0.20 for data processing.

- New method: analyses were carried out on the Thermo ICS-5000+ instrument PMI ID11190 using Chromeleon Workstation version 7.2.2 for both data acquisition and data processing.

4 RESULTS AND DISCUSSION

4.1 Comparison Current and New Method With External Laboratories

All aerosol samples for 3R4F and P1 were generated on a linear smoking machine as per internal procedures.

Analytical results for ammonia for 3R4F and P1 using current method and compared with external laboratories are summarized in Table 4. Overall results obtained with current method using LC-MS/MS technology are higher than for the rest of the laboratories. It should be highlighted that Coresta, PPQ and Labstat use Ion Chromatography for the determination of ammonia.

Table 4: Ammonia Results When Using Current Method by LC-MS/MS

Compound	3R4F				P1	
	Labstat (ug/item)	**Coresta (ug/item)**	**PPQ* (ug/item)**	**Current* (ug/item)**	**Labstat (ug/item)**	**Current* (ug/item)**
Ammonia	30.3**	30.2 ± 5.3	32.5 ± 5.1	38.8 ± 7.9	12.2 ± 1.3	15.5 ± 0.7

ug = µg

* Data from internal control charts

**stdev not available

Another set of data for 3R4F and P1 was generated on a linear smoking machine but applying the new trapping conditions and analysed using the new method by IC implemented in AL (procedures described in Table 3). Results were compared with existing data from Coresta, PPQ and Labstat and are summarized in Table 5. Results using the new method were fully in line with data from external laboratories and consequently, it was decided at this stage to move forward with the validation of this new method.

Table 5: Ammonia Results When Using New Method by IC

Compound	3R4F				P1	
	Labstat (ug/item)	Coresta (ug/item)	PPQ* (ug/item)	New (ug/item)	Labstat (ug/item)	New (ug/item)
Ammonia	30.3**	30.2 ± 5.3	32.5 ± 5.1	30.7 ± 1.5	12.2 ± 1.3	13.4 ± 0.5

* Data from internal control charts

**stdev not available

4.2 Comparison of Current Versus New Method for 3R4F and P1

Ammonia results obtained with current and new method were compared for 3R4F and P1. Results are summarized in Table 6. Overall the current method provides higher results for the two products with an important impact for 3R4F (22.4%) and lower impact for P1 (12.8%).

Table 6: Comparison Current Versus New on 3R4F and P1 Matrices

Compound	3R4F			P1			
	Current method (ug/item)	New method (ug/item)	Current vs New (%)	Current method (ug/item)	New method (ug/item)	Current vs New (%)	Current method (ug/item)
Ammonia	39.64 ± 2.54	30.76 ± 1.48	22.4	15.40 ± 0.43	13.42 ± 0.54	12.8	7.03 ± 0.38

4.3 Impact on reduction for P1

As shown in Table 6, a notable decrease on ammonia can be noticed when the new method is used. This decrease can be explained by the fact that the new method by IC does not require a derivatization step which was identified as a potential issue with the current method by LC-MS/MS.

In spite of the differences observed between the two methods, the percentage reduction for P1 concentration relative to 3R4F is similar when using the new method as described in Table 7.

Table 7: Ammonia Results – Impact on Reduction to 3R4F

Compound	3R4F (ug/item)		P1 (ug/item)		Reduction to 3R4F	
	Current method (ug/item)	New method (ug/item)	Current method (ug/item)	New method (ug/item)	Current (%)	New (%)
Ammonia	39.64	30.76	15.4	13.42	61.2	56.4

Statistical differences between the two methods for ammonia for 3R4F and P1 are described in chapter 5.

5 STATISTICAL ASSESSMENT

As a consequence to the newly validated method for the quantification of ammonia in aerosol of tobacco product, a descriptive statistical analysis was performed in order to evaluate the quantitative differences between the old and the new method thus the impact on product specifications for carbonyls being used as product performance specifications. Derived for this statistical analysis, new specification's upper limits are proposed for P1.

5.1 Statistical Methods

5.1.1 Statistical Assessment

The statistical analysis presented was performed according to the United States Pharmacopeia (USP) General chapter <1010> Comparison of Analytical Methods.

The 95% confidence interval for the difference between the yields of the methods was calculated and the upper boundary was used to estimate the correction factor to be applied to the specification limits (section 6.1)

The generation of the data for the comparison was not performed on the same smoke collection as it is not the same extraction method, but done using the same product batch.

For 3R4F and P1, 16 replicates were generated with the old method and 16 with the new.

In order to increase the power or the robustness of the conclusions, statistical technics used to assess the precision and accuracy where based in confidence intervals.

5.1.2 Precision of the Methods

The assessment of the new method's precision compared to the reference one was performed by estimating the variance for each method and calculating a two-sided upper confidence interval for the ratio of the variances, where the ratio is defined as the variance of the new method to that of the reference method.

If the two-sided upper confidence limit is less than this upper acceptable limit, then the precision of the new method is considered acceptable in the sense that the use of the new method will not lead to an important loss in precision. If the one-sided upper confidence limit is less than one, then the new method has been shown to have improved precision relative to the current method.

$$\left[\frac{S_2^2}{S_1^2}\frac{1}{F_{(1-\alpha),2,n2-1,n1-1}}, \frac{S_2^2}{S_1^2}\frac{1}{F_{\alpha,2,n2-1,n1-1}}\right]$$

Where S_1^2 is the variance of the old method and S_2^2 is the variance of the new method. F is Fisher's distribution,

n1 is the number of results with old method and n2 the number of results with the new method.

5.1.3 Accuracy

To assess if the difference between the 2 methods is significantly different than 0 (meaning methods with equivalent accuracy) the confidence interval for the difference between the 2 methods was calculated. When the difference is significant this confidence interval defines the range of bias between the 2 methods.

$$Lower\ limit = (M_2 - M_1) - t_{1-\alpha/2,(n_1-1)+(n_2-1)} * \sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

$$Upper\ limit = (M_2 - M_1) + t_{1-\alpha/2,(n_1-1)+(n_2-1)} * \sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}$$

Where n is the number of results, and t is the Student distribution.

5.1.4 New Upper Specification Limit Calculation.

The product performance specifications are only upper limits; no target value is used. Therefore only upper limits correction shall be implemented.

In order to estimate the new upper specification limits the correction factor is applied by using the upper difference at 95% confidence interval so as to capture most of the population of the range of difference between the methods:

New upper limit specification = Old upper limit specification + upper difference at 95%

5.2 Descriptive Statistics for 3R4F

Table 8: Summary of Results

Testing Method	Average	Stdev	RSD	Minimum	Maximum	Difference in average	Ratio of Variances
Current Method	39.64	2.542	6.41%	32.94	43.60	-8.88	0.34
New Method	30.76	1.481	4.81%	28.31	33.30	Different	Different

A significant difference in term of accuracy was found for ammonia. In term of precision the new method was found significantly more precise. The Figure 1 below shows a graphical comparison of the two method for 3R4F.

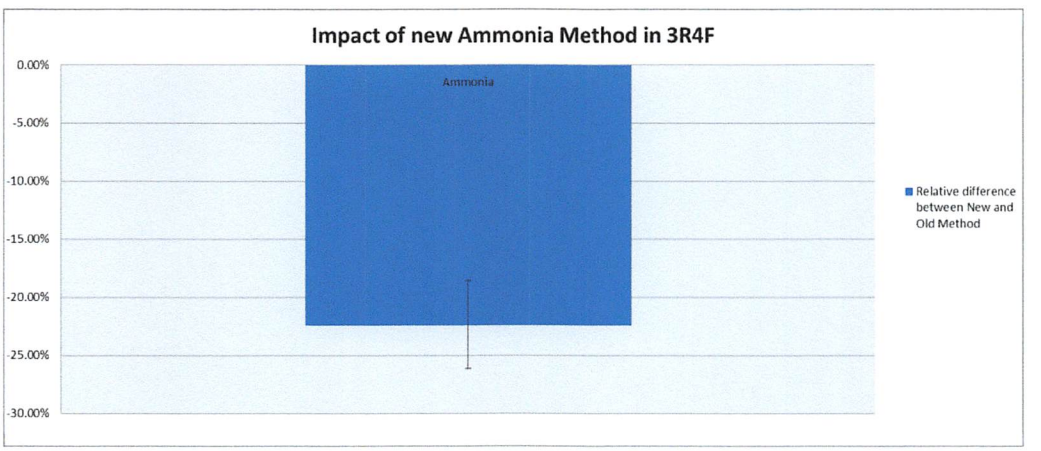


Figure 1: Graphical Comparison of the Two Methods

If error bar crosses the x-axe at 0 this means that there is no different between the two methods.

5.3 Descriptive Statistics for Platform 1

Table 9: Summary of Results

Testing Method	Average	Stdev	RSD	Minimum	Maximum	Difference in average	Ratio of Variances
Current Method	15.40	0.833	5.41%	13.75	16.65	-1.98	0.40
New Method	13.42	0.527	3.93%	12.06	14.08	Different	Not different

A significant difference in term of accuracy was found for ammonia. In term of precision both methods were found equivalent. The Figure 2 below shows a graphical comparison of the tow method for Platform 1.

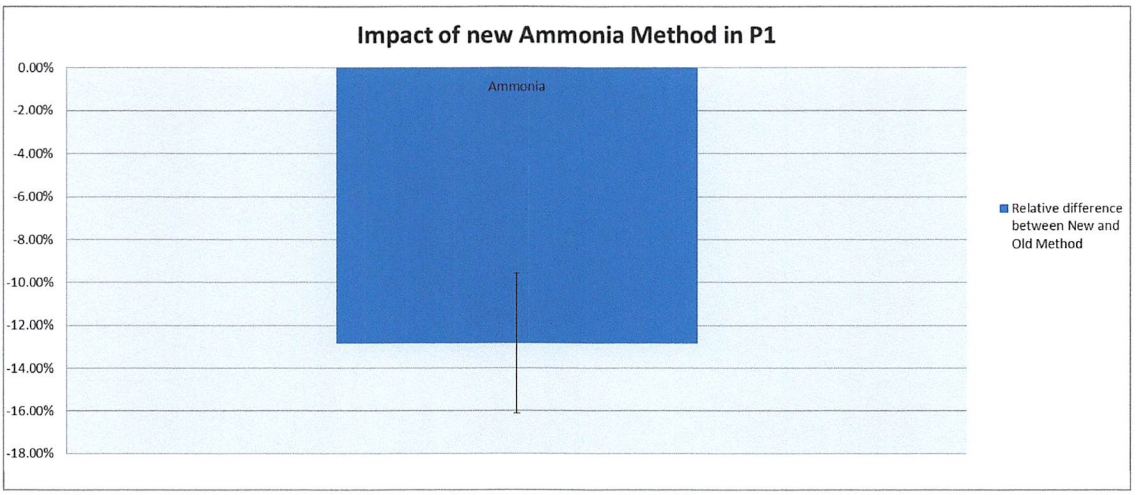


Figure 2: Graphical Comparison of the Two Methods.

If error bar crosses the x-axe at 0 this means that there is no different between the two methods.

6 IMPACT ON SPECIFICATIONS

6.1 New Specifications for Platform 1

Table 10: Statistical Comparison

Lower 95% absolute Diff	Upper 95% absolute Diff	Average absolute diff	Average relative diff	Lower 95% relative Diff	Upper 95% relative Diff
-2.480	**-1.473**	-1.976	-12.83%	-16.10%	-9.56%

Ammonia specification:

Old specification – 1.473 = new specification to be applied for all P1 product specifications.

7 CONCLUSION

The new method by IC has shown to be suitable for the quantification of ammonia in 3R4F and Platform 1 matrices and leads to an improvement on data accuracy and robustness. In addition data are fully in line with external laboratories, including Coresta.

In addition, it was shown that the percentage reduction for Platform 1 to 3R4F is maintained when using the new method.

Recommendation is made to:

- Include this new method in the AL method portfolio and replace the current one.
- Review the performance specifications for P1 based on statistical results.

8 REVISION HISTORY

Version N°	Description of change	Type of change
1.0	Original issue	1
1.0 SV	Submission version (content redefined to include relevant information only, formatting and removal of hyperlinks)	2

(1. Major change/new version; 2. Minor change; 3. Review without change)

9 APPROVALS

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Author	Eva Garcia	Manager Central Analytical Laboratories		14-Jul-2016
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QA Review	Florence Kapp	QA Officer		14 JUL 2016