P1 AEROSOL CHARACTERIZATION - GCXGC-TOF-MS

STUDY PLAN / EXPERIMENTAL PLAN & REPORT

PROGRAM NAME	ANALYTICAL CAPABILITY
PROJECT NAME	BRIDGING METHODOLOGY
WORK PACKAGE NAME	P1 AEROSOL CHARACTERIZATION
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1 Abstract

This document describes the planning and deliverables for the characterization of the most abundant P1 aerosol constituents using non-targeted screening (NTS) methodologies by 2-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-TOF-MS). The primary objectives are to determine the major constituents present in P1 NFDPM, in order to provide supporting information for PMI's position regarding the absence of "Tar" in P1 and to deliver product developers with a list of the top 500 most abundant compounds in the aerosol from the THS 2.2 Dorado II Ron as a benchmark for PMI's heat-not-burn portfolio

2 Introduction

The objective of this study is to identify and semi-quantify heat-not-burn aerosol constituents from the THS 2.2 Dorado II Regular (THS regular) using all available non-targeted methodologies for the analytical platform GCxGC-TOF-MS.

A set of 3 non-targeted methods using GCxGC-TOF-MS with a focus on volatile, non-polar and polar compounds are currently being developed and validated. These complementary methods, designed to cover the broadest possible range of chemical classes present in conventional and RRP aerosols using 2-dimensional gas chromatography, will be applied. The methods will be used in parallel with their development, to characterize the most abundant constituents in the aerosol of the THS 2.2 Dorado II Regular heat-not-burn product generated using the Health Canada Intense (HC) smoking regime. The methods will not have been formally validated at the point of analysis, however, a work instruction is available for each approach in advance.

Identified compounds will be classified in accordance with structure, origin and their potential role within the aerosol.

3 Samples definition

Aerosol will be generated using the THS 2.2 tobacco heating system comprising a commercially produced tobacco heating device (THD 2.4; PDIMS Device Batch B23172; Device Version DV.000174(7)) and the regular version of the THS HeatStick as described in **Table 1**. Mainstream aerosol will be generated and trapped in accordance with [1] PMI_RD_WKI_000530[1] and [2] PMI_RD_WKI_000518[2].

[1] PMI_RD_WKI_000530 'Trappage en phase particulaire pour la détermination des constituants de l'aérosol'

[2] PMI_RD_WKI_000518 'Trappage des volatiles et semi-volatiles'

Table 1 Test Items

Short Name	Description	Product Code	Consumable Batch Number	Manufacturing Date	Batch Size (sticks)
THSR	THS 2.2 Dorado II Ron	ME000004.02	B-25906 / 41-2382704	14.01.2016	(b) (4)
3R4F	Reference	K1908	N/A	N/A	N/A

The commercialized THS 2.2 (THSR) test articles were produced at Intertaba SpA, Via Fratelli Rosselli 4, 40069 Zola Predosa, Italy. The samples will be stored in the climatic chambers in packs. The packs are polypropylene wrapped Mini princess packs consisting of two collations, each collation containing 10 heat sticks.

The Reference Cigarette 3R4F was purchased from the University of Kentucky, Kentucky Tobacco Research and Development Center (for specifications see [3] (http://www2.ca.uky.edu/refcig/3R4F20Preliminary20Analysis.pdf)).

Mass-produced test items and cigarettes are generally homogeneous. However, most constituents involved in the manufacture are derived from natural products, and therefore result in a final product which is intrinsically variable.

Table 2 Experimental Groups of Study Samples

Description	Smoking Regimen	Type	Short Name
THS 2.2 regular	HC	test	THSR
Blank_THS	HC	reference	BLP1
3R4F	HC	reference	3R4F
Blank_3R4F	HC	reference	BLCC

The aerosol will be trapped and treated in accordance with the requirements for each of the analytical methods.

4 Research Questions

- What is the identity and the quantity (semi-quantitatively determined) of the most abundant compounds in the THS 2.2 Dorado II Regular amenable to 2-dimensional gas chromatography coupled to time-of-flight mass-spectrometry (GCxGC-TOF-MS)?
- What is the relative distribution of compounds between the particulate phase (nicotine free dry particulate matter, NFDPM) and gas vapor phase (GVP)?

5 Methods

5.1 Design

The latest version of THS 2.2 Dorado II Regular (THSR) together with the device THD 2.4 will be used to generate aerosols for the chemical characterization of the most abundant compounds present in NFDPM and GVP. The reference cigarette 3R4F will be used to qualify the system and to serve as a comparator for the most abundant chemical constituents found in the aerosol of the P1. Blank aerosol samples for P1 as well as for 3R4F will be used to exclude any potential impact of smoke machines, trapping approaches or analytical methods on the results.

A set of 3 non-targeted methods using GCxGC-TOF-MS with a focus on volatile, non-polar and polar compounds will be used to analyze the samples using a non-targeted screening approach (NTS).

5.2 Procedure

5.3 Analytical Procedures

5.3.1 Test Item Conditioning

All test articles will be stored in a cooling chamber at 4 ± 3 °C with uncontrolled humidity. Prior to aerosol generation the test articles THSR, THSM and 3R4F will be conditioned according to [4] ISO 3402[3] and [5] PMI_RD_WKI_000489[4] for a minimum of 48h and a maximum of 10 days at 22 ± 1 °C and 60 ± 3% relative humidity (RH). The conditioning will be performed in open packages for all test items.

5.3.2 Aerosol Generation

Mainstream aerosol from THS 2.2 regular and 3R4F will be generated under HC smoking conditions (**Table 3**) and trapped in accordance with [1] PMI_RD_WKI_000530[5] and [2] PMI_RD_WKI_000518[6]. For the HC smoking protocol (Health Canada, T-115, 1999) [6], the 3R4F cigarettes will be 100% vent-blocked by taping in accordance with [7] PMI_RD_WKI_000399[7]. THSR items will not be taped due to absence of ventilation holes in the filter region. The room conditions for aerosol generation will be 22 ± 2 °C and 60 ± 5% RH.

Table 3 - Smoking Regime

Short Name	Puff Volume [mL]	Duration [s]	Puff interval [s]	Frequency [min^{-1}]	Puff Count [n]
THSR	55	2	30	2	12
BLP1	55	2	30	2	12
3R4F	55	2	30	2	10 *
BLCC	55	2	30	2	12

* smoked to a fixed butt length of 35mm (normally achieved in approximately 10 puffs)

The aerosol generation and sample analyses will be conducted by Product Testing labs. Blank samples are generated using the same aerosol collection configuration without test items. The number of replicates

[3] ISO 3402 'Tobacco and tobacco products -- Atmosphere for conditioning and testing 4th edition'

[4] PMI_RD_WKI_000489 'Preparation of items'

[5] PMI_RD_WKI_000530 'Trappage en phase particulaire pour la détermination des constituants de l'aérosol'.

[6] PMI_RD_WKI_000518 'Trappage des volatiles et semi-volatiles'.

[7] PMI_RD_WKI_000399 'Blocage de la ventilation du papier de bout des cigarettes'

and accumulations required per replicate are presented in Table 4. The required trapping configurations are summarized in Table 5.

Table 4 - Experimental Details for the Preparation of Aerosol Samples

Short Name	Methods	Items per Replicate [accumulations]	Replicates (n)
THSR	Nonpolar and Polar	5	3
	Volatile	5	4
BLP1	Nonpolar and Polar	-	3
	Volatile	-	4
3R4F	Nonpolar and Polar	3	3
	Volatile	3	4
BLCC	Nonpolar and Polar	-	3
	Volatile	-	4

Table 5 - Trapping Setup for NFDPM and GVP Samples

	GCxGC-TOF-MS nonpolar + polar	GCxGC-TOF-MS volatile
For trapping of NFDPM samples:		
GF-Filter	44mm	44mm
For trapping of gas vapor phase (GVP) samples, consecutive:		
No. Impinger traps	2	2
Impinger solvent	2x10ml dichloromethane / acetone (80:20 v/v) - containing internal standard and retention index marker compounds	2x10ml N,N-Dimethylformamide - containing internal standard and retention index marker compounds
Impinger Temperature	dry ice / isopropanol (approx.-80°C)	dry ice / isopropanol (approx.-60°C)

5.4 Analytical Methods

The NTS assay using GCxGC-TOF-MS will be performed to evaluate the most abundant compounds in the NFDPM and GVP aerosol fraction of the THS 2.2 Dorado II Regular.

The NTS assay using GCxGC-TOF-MS consists of 3 analytical methods focused on a) nonpolar, b) polar and c) highly volatile compounds. The methods will be conducted according to the following WKIs, which contain all necessary information related to required instrumentation and materials:

- PMI_RD_WKI_001229 NTDS GC×GC-TOFMS Nonpolar
- PMI_RD_WKI_001353 NTDS GC×GC-TOFMS Volatile
- PMI_RD_WKI_001354 NTDS GC×GC-TOFMS Polar

The non-targeted screening methodology represents a subset of the non-targeted differential comparison, described in the WKIs. The comparative evaluation is not in scope for this study.

5.4.1 GCxGC-TOF-MS nonpolar/polar

Aerosol Trapping

Whole aerosol is collected on a glass fiber filter pad with two micro-impingers connected in series. Each micro-impinger is filled with 10mL dichloromethane/acetone (80/20, v/v) containing internal standard (IS) and retention index (RI) marker compounds. The micro-impingers are cooled using a dry ice/isopropanol mixture.

Analysis of NFDPM fraction

GCxGC-TOF-MS nonpolar

NFDPM collected on the glass fiber filter pad is extracted with 10mL of a solution of dichloromethane/acetone (80/20, v/v) containing IS and RI marker compounds. Water is added to the extracts in equal volume amounts, then the sample is shaken and centrifuged. The dichloromethane layer is separated, dried with sodium sulfate, and analyzed by GCxGC-TOF-MS in full scan mode.

GCxGC-TOF-MS polar

The remaining water layer from the nonpolar sample preparation is used for the analysis of the polar compounds. IS and RI marker compounds are added to the water layer and analyzed by GCxGC-TOF-MS in full scan mode.

Analysis of GVP fraction

GCxGC-TOF-MS nonpolar

For the analysis of the GVP fraction, the content of the two micro-impingers are combined. Water is added to the extracts in equal volume amounts, then the sample is shaken and centrifuged. The dichloromethane layer is separated, dried with sodium sulfate, and analyzed by GCxGC-TOF-MS in full scan mode.

GCxGC-TOF-MS polar

The remaining water layer from the nonpolar sample preparation is used for the analysis of the polar compounds. IS and RI marker compounds are added to the water layer and analyzed by GCxGC-TOF-MS in full scan mode.

5.4.2 GCxGC-TOF-MS volatile

Aerosol Trapping for GCxGC-TOF-MS volatile

Whole aerosol is collected using a glass fiber filter pad with two micro-impingers connected in series directly after. Each micro-impinger is filled with 10mL N,N-dimethylformamide (DMF) containing internal standard (IS) and retention index (RI) marker compounds. The micro-impingers are cooled using a dry ice/isopropanol mixture adjusted to a temperature of approx. -50 to -60°C.

Analysis of NFDPM fraction

NFDPM collected on the glass fiber filter pad is extracted with 10mL DMF containing internal standard (IS) and retention index (RI) marker compounds and analyzed by GCxGC-TOF-MS in full scan mode.

Analysis of GVP fraction

For the analysis of the GVP fraction, the content of the two micro-impingers are combined and analyzed by GCxGC-TOF-MS in full scan mode.

5.5 Data Processing

Data acquisition (GCxGC-TOFMS) is followed by advanced raw data processing using ChromaTOF software for automatic peak finding, spectral deconvolution and peak alignment, resulting in an aligned peak table. Data processing is divided into multiple steps, (1) ChromaTOF processing, (2) CASI Pre-processor, (3) CASI and (4) CASI Post-processor (see Figure 1). The software tools CASI Pre-/Post-Processor and CASI automate a sequence of important data evaluation steps, e.g. batch processing, data alignment, compound identification and semi-quantification.

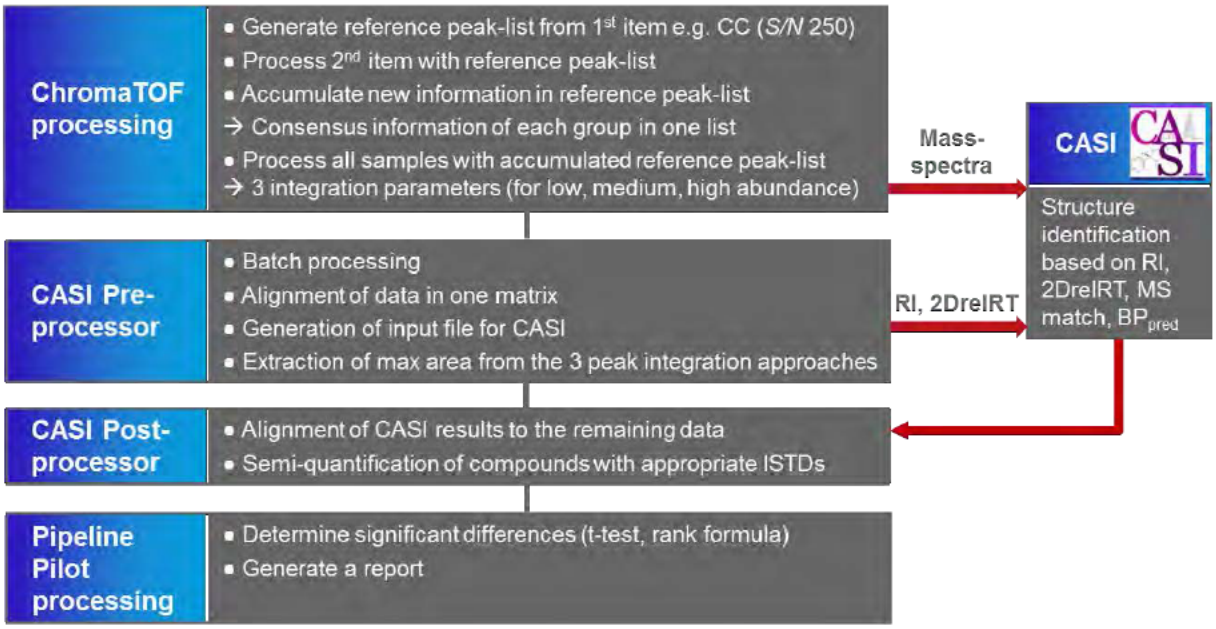

Figure 1. Overview of the data processing steps

5.6 Data Evaluation

Data Fusion and Sorting for Abundance

A meta-result list will be prepared separately for NFDPM and for GVP analysis, either using the software tools Excel or Pipeline Pilot using the fused dataset of the 3 available methods (nonpolar, polar and volatile) and sorted for abundance.

Cleaning for Systemic Compounds (Blank)

The meta-result list will be cleaned for compounds present in blank samples with a concentration more than 50% of the aerosol result.

Retrieving additional Structural Information

The cleaned meta-result list will be complemented by PhysChem properties and other relevant information on a proposed chemical structure that is available in UCSD (Unique Compound and Spectral Database) with the help of chemoinformatic tools.

Meta-Analysis: Abundance Profile of P1

A cumulative plot is prepared from the cleaned meta-result list addressing cumulative mass versus compound number from the abundance ranked list. An example for the analysis of the abundance profile for P1 using the nonpolar method (COMPARATIVE STUDY: NON-TARGETED DIFFERENTIAL SCREENING OF YVERDON, 3R4F AND ZUERICH (ALL HC) BY GCXGC-TOF) is given in **Figure 2**.

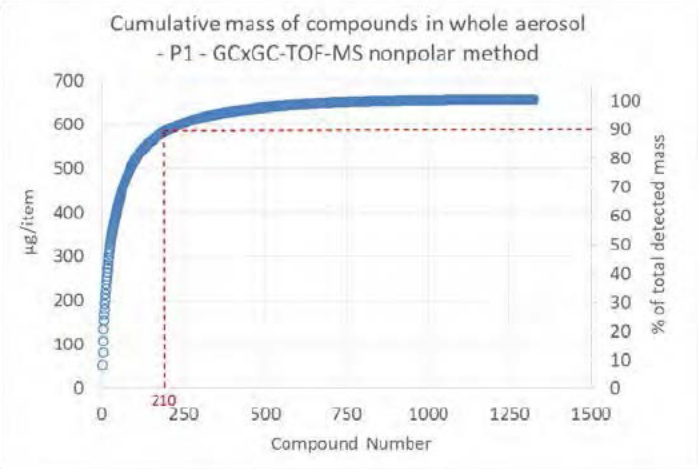

Figure 2. Accumulated mass of compounds detected in P1 whole aerosol – nonpolar method (Nicotine, Water, Propyleneglycol (PG) and Glycerin not included)

In addition a plot of measured concentration of compounds detected will be prepared and the number of compounds above a threshold of 100ng/item determined, an example is given in **Figure 3**.

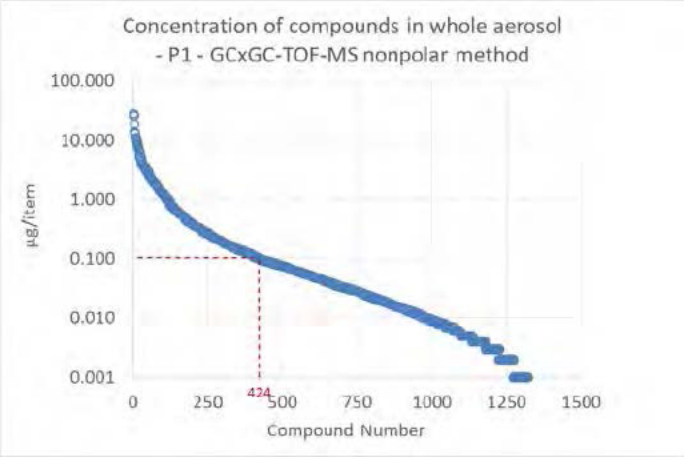

Figure 3. Measured concentration of compounds detected in P1 whole aerosol – nonpolar method (Nicotine, Water, Propyleneglycole (PG) and Glycerin not included)

Defining Compound Scope

The results of the meta-analysis will be considered for defining the number of compounds to be identified using reference standards.

Current planning:

- Confirmed identification using reference standards (if practicable) for the top 500 constituents of the fused dataset of LC-HRAM-MS, GC-HR-TOF-MS and GCxGC-TOF-MS platforms, semi-quantified to be above 100 ng/stick
- Tentative identification for constituents semi-quantified to be above 10 ng/stick (per method)

Alternative planning:

- Confirmed identification using reference standards (if practicable) for all constituents semi-quantified to be above 100 ng/stick (per method)
- Tentative identification for constituents semi-quantified to be above 10 ng/stick (per method)

5.7 Structural Identification

For each compound detected in the P1, a structural proposal is generated by using a platform developed by PMI, called Computer Assisted Structure Identification (CASI), which is dedicated to identify chemical structures using Mass Spectrometry (MS) data in combination with 2-dimensional chromatographic data. Structural proposals finally will be confirmed by measuring reference standards of the proposed structure in pure solution as well as added to the aerosol matrix in relevant concentration.

Principle description of CASI

CASI automatically searches mass spectral libraries for matches using a NIST MS Search algorithm, which proposes structural candidates for experimental spectra from two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC-TOF-MS) measurements, each with an associated match factor. Next, quantitative structure-property relationship (QSPR) models implemented in CASI predict three specific parameters to enhance the confidence for correct compound identification, which were Kovats Index (KI) for the first dimension (1D) separation, relative retention time for the second dimension separation (2DrelRT) and boiling point (BP). In order to reduce the impact of chromatographic variability on the second dimension retention time, a concept based upon hypothetical reference points from linear regressions of a deuterated n-alkanes reference system was introduced, providing a more stable relative retention time measurement. Predicted values for KI and 2DrelRT were calculated and matched with experimentally derived values. Boiling points derived from 1D separations are matched with predicted boiling points, calculated from the chemical structures of the candidates. As a last step, CASI combines the NIST MS Search match factors (NIST MF) with up to three predicted parameter matches from the QSPR models to generate a combined CASI Score representing the measure of confidence for the identification.

Threshold values are applied to the CASI Scores assigned to proposed structures, which improves the accuracy for the classification of true/false positives and true/false negatives. Results for the identification of compounds have been validated.

For providing confirmed identifications reference standards (as possible) of the structural proposals for the compounds in scope will be purchased and tentative identifications will be confirmed. The status of the provided structural identifications is given in **Table 7**.

Table 1 – Classification of Identification Status

CASI Score	Confidence Level of CASI Proposal	Confirmation by Reference Standard	Identification Status
≥795	High	Yes	**Identified**
		No (standard not available)	**Tentative – high confidence**
		Negative	**Not Identified**
700≤X<795	Medium	Yes	**Identified**
		No (standard not available)	**Tentative – medium confidence**
		Negative	**Not Identified**
<700	Low	Yes, if proposal meaningful (expert decision)	**Identified**
		No (standard not available)	**Not Identified**
		Negative	**Not Identified**

5.8 Statistical Analysis

Mean and standard deviation values only will be calculated.

6 Administrative Aspects

6.1 Experiment Timelines

No.	Tasks / **Deliverables**	Description	Responsible	Delivery date
1.	**Work package in place**	**Work package description P1 aerosol characterization – GCxGC-TOF approved**	**A. Knorr**	**Q2, 2015**
2.	**Study Plan in place**	**Study Plan P1 aerosol characterization – GCxGC-TOF approved**	**A. Knorr**	**Q1, 2016**
3.	Start Date Experimental	Start aerosol sample generation	A. Knorr	Q1, 2016 (Mar)
4.	End Date Experimental	Sample generation, preparation, acquisition and data processing finished	A. Knorr	Q2, 2016 (Mar)
5.	Data Evaluation finished	Data evaluation finished (generation of data table)	A. Knorr	Q3, 2016 (Jul)
6.	**Data Summary I (Generic Identification Volatiles)**	**Data summary for volatile compounds**	**A. Knorr**	**Q3, 2016 (Aug)**
7.	**Data Summary II (Generic Identification Nonpolar)**	**Data summary for nonpolar compounds**	**A. Knorr**	**Q3, 2016 (Sep)**
8.	**Data Summary III (Generic Identification Polar)**	**Data summary for polar compounds**	**A. Knorr**	**Q3, 2016 (Oct)**
9.	Confirmed Identification finished	Reference standards measured and proposed structures (generic identification) confirmed	A. Knorr	Q4, 2016 (Dec)
10.	**Data Summary Final (Confirmed Identifications all Methods)**	**Final data summary for volatile, nonpolar and polar compounds**	**A. Knorr**	**Q4, 2016 (Dec)**

6.2 Roles and Responsibilities

The internal key contributors are as follows:

Name	Function in the project
Mark Bentley	Summary WP Owner/Manager
Arno Knorr	WP Manager – P1 Aerosol Characterization - GCxGC-TOF-MS
Martin Almstetter	Scientist - SME, WP Manager – NTDS - GCxGC-TOF-MS
Daniel Arndt	WP Manager – LC-HRAM-MS
Philippe Guy	WP Manager – GC-HR-QTOF-MS
Pavel Pospisil	Manager Computational Chemistry – Data Management
Quentin Dutertre	Scientist – SME NTDS - GCxGC-TOF-MS
Elyette Martin	Scientist Computational Chemistry
Antonio Castellon	Scientist Computational Chemistry

7 References

[1] Computer-Assisted Structure Identification (CASI)—An Automated Platform for High-Throughput Identification of Small Molecules by Two-Dimensional Gas Chromatography Coupled to Mass Spectrometry, Arno Knorr, Aurelien Monge, Markus Stueber, André Stratmann, Daniel Arndt, Elyette Martin, and Pavel Pospisil, Anal. Chem., 2013, 85 (23), pp 11216–11224

[2] University of Kentucky (www.2r4f.com/3r4f.pdf)

[3] THS 2.2 COMPARABILITY STUDY REPORT DORADO II RON - batch release attributes and sensorial evaluation.docx, (b) (4)

[4] Health Canada, T-115, 1999

[5] NTDS_GCxGC-TOF-YVDreg-ZRHreg-3R4F_HC_07Feb2014.pptx, (b) (4)

8 Related Documents

- PMI_RD_WKI_000489 Reception and preparation of test and reference item
- PMI_RD_WKI_000380 Préparation des moniteurs pour le fumage (R2540.M050)
- PMI_RD_WKI_000399 Blocage de la ventilation du papier de bout des cigarettes (R2540.M037)

- PMI_RD_WKI_000529 Guide d'utilisation de la machine à fumer linéaire à 20 canaux KC
- PMI_RD_WKI_000530 Trappage en phase particulaire pour la détermination des constituants de l'aérosol
- PMI_RD_WKI_000518 Trappage des volatiles et semi-volatiles
- PMI_RD_WKI_001229 NTDS GC×GC-TOFMS Nonpolar
- PMI_RD_WKI_001353 NTDS GC×GC-TOFMS Volatile
- PMI_RD_WKI_001354 NTDS GC×GC-TOFMS Polar
- CASI 2 - User Guide, (b) (4)
- CASI 2.1 Pre&Post-Processors User Guide, (b) (4)
- WPD_P1 Characterization_GCxGC-TOF.doc, (b) (4)
- WPD_P1 Characterization_LC-HRAM-MS.docx, (b) (4)
-

9 Abbreviations

Abbreviation/Term	Explanation
CASI	Computer-Assisted Structure Identification
EDMS	Electronic Document Management System
GCxGC-TOF-MS	2-Dimensional Gas Chromatography coupled to time-of-flight Mass Spectrometry
GC-HR-TOF-MS	Gas Chromatography coupled to High Resolution time-of-flight Mass Spectrometry
GVP	Gas Vapor Phase
HC	Health Canada
ISO	International Standard Organization
IS	Internal standard
LC-HRAM-MS	Liquid Chromatography High Resolution Accurate Mass Spectrometry
LIMS	Laboratory Information Management System
NFDPM	Nicotine free dry particulate matter

Abbreviation/Term	Explanation
NTDS	Non-targeted differential screening
NTS	Non-targeted screening
P1	Platform 1
PT	Product Testing
RI	Retention Index
RRP	Reduced Risk Products
SME	Subject Matter Expert
THD	Tobacco Heating Device
THS	Tobacco Heating System
UCSD	Unique Compound Spectra Database
WKI	Work Instruction

For complete definition, refer to PMI OPS Glossary and PMI RD Glossary.

Place abbreviations in alphabetical order and remove non-relevant abbreviations.

10 Review and Approval

This document has been approved by:

Name	Function	Date / Signature
Mark Bentley Manager Complex Matrix Analysis	Summary Work Package Owner/Manager	P.P. 24. Mar. 2016
Arno Knorr Supervisor Xenobiotic Metabolism	Associated WP Owner	22. Mar. 2016
Martin Almstetter Scientist Xenobiotic Metabolism	SME	22. Mar. 2016
Deborah Forte Supervisor Test Item Management	SME	22 Mar 2016

Electronic signatures, please refers to EDMS

11 Quality Assurance Documentation

This study will not be conducted in accordance with Good Laboratory Practice (GLP).