



PHILIP MORRIS
INTERNATIONAL

RRP/ Testing Laboratories

Page 1 of 36

Doc. Type: Work Instruction

Status: Production

Doc. ID: PMI-RRP-WKI-111620

Version N°: 4.0.0

Effective date:
28-Feb-2018

NTDS GC×GC-TOFMS Nonpolar (RDNEU)



PHILIP MORRIS
INTERNATIONAL

NTDS GC×GC-TOFMS NonPOLAR (RDNEU)

CONFIDENTIALITY NOTICE

This document is strictly confidential. Information contained in this document may not be reproduced or disclosed to any person without the written permission of Philip Morris International.



 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 2 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

Table of contents

1 Purpose	3
2 Scope and Applicability.....	3
3 Responsibilities.....	4
4 Description of the Method.....	4
4.1 Principle	4
4.2 Sample Requirements and Workload.....	4
4.3 Material, Equipment, Chemicals, Standards and References.....	5
4.3.1 Equipment	5
4.3.2 Chemicals/Reagents	6
4.3.3 Internal Standard Compounds	6
4.3.4 Retention Index Marker Compounds	7
4.4 Procedure	7
4.4.1 Sample Collection	7
4.4.2 Sample Preparation.....	8
4.4.3 Setting-up of Instruments	9
4.4.4 Preparation of Solutions and Media	11
4.4.5 Number of Determinations	13
4.4.6 Daily Verification or According to Use	13
4.4.7 Testing Procedure	13
4.5 Calculation and Records	26
4.5.1 Calculation of the Second Dimension Relative Retention Time.....	26
4.5.2 Semi-quantification of Compounds	27
4.5.3 Extraction of Significant Differences	28
4.5.4 Ranking of Detected Compounds	28
4.5.5 Flexible Filtering	29
4.5.6 Results Recording/Data Transfer/Documentation.....	29
4.6 Testing Scope, Repeatability, Reproducibility	30
4.6.1 Testing Scope.....	31
4.6.2 Repeatability, Reproducibility	31
4.6.3 Acceptability Limits	31
4.7 Safety.....	31
4.8 Calibration and Maintenance of Instruments.....	31
5 Reference Documents	32
6 Related Documents	33
7 Records	34
8 Revision History	35
9 Abbreviations	36

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 3 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

1 Purpose

This method describes an assay for the detection of significant differences between two samples using comprehensive two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer (GC×GC-TOFMS). The assay is based on a comprehensive chemical characterization of complex mixtures with no predefined target compounds.

To maximize the coverage of the chemical space in terms of polarity and volatility, the non-targeted differential screening (NTDS) GC×GC-TOFMS assay consists of three analytical methods, for nonpolar, polar and volatile compounds, respectively. The analytical methods compare two test items by comprehensive chemical screening and subsequent data evaluation to get summary tables with ranked chemical differences. This work instruction describes the method for nonpolar compounds, which is referred to as “Nonpolar method”.

The high resolution power using comprehensive 2-dimensional gas chromatography, combined with spectral deconvolution, results in high quality electron ionization (EI) mass spectra, improving the search against commercial mass-spectral libraries.

Data acquisition is followed by advanced raw data processing using the ChromaTOF software for automatic peak finding, spectral deconvolution and peak alignment, resulting in an aligned peak table. 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, semi-quantification, comparison and ranking.


Ranking of the compounds is done by applying a student's t-test to filter compounds with a significant difference followed by a ranking procedure that was developed empirically. The ranking procedure considers the relative differences in abundance of each compound as well as the absolute abundance. In addition flexible filtering (e.g. fold change, concentration cut-off) can be applied.

NTDS using GC×GC-TOFMS represents a key methodology to not only comprehensively characterize the chemical composition of aerosols derived from different test items, but also to determine significant differences of these complex matrices. Upon comparison of different reduced risk product (RRP) platforms, the detection of novel compounds or elevated levels of specific constituents provides an important basis for subsequent toxicological assessment.

2 Scope and Applicability

This document applies to the analytical laboratories of the Complex Matrix Analysis team, Testing Labs & Analytical Research (TL&AR), at PMI, RRP in Neuchâtel.

The methods were developed for smoke/aerosol-related samples (e.g. TPM, whole smoke) from conventional cigarettes and prototypes (e.g. RRP). Nevertheless, the described method can be taken as a universal approach for comparing samples of different matrices in an unbiased way.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 4 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

3 Responsibilities

Task/Activity	Responsible
Sample collection and sample preparation	Lab technician or equivalent trained
Setting up the instrument and verification of the quality control criteria according to <i>PMI-RRP-WKI-111668</i> - Operation of a LECO Pegasus 4D GC×GC-TOFMS system (<i>RDNEU</i>)	Lab technician or equivalent trained
Reporting of any procedural deviation to study director	Lab technician or equivalent trained
Selection of the detector voltage	Scientist or equivalent trained
Perform analysis	Lab technician or equivalent trained
Data processing and data export using the LECO ChromaTOF software	Lab technician or equivalent trained
Data evaluation using CASI Pre-processor, CASI and CASI Post-Processor	Lab technician or equivalent trained
Verification and finalization of the dataset	Scientist or equivalent trained
Preparation of a report	Scientist or equivalent trained
Release of results	Study director

Table 1. Tasks and responsible persons for the complete workflow of NTDS GC×GC-TOFMS Nonpolar.


4 Description of the Method

4.1 Principle

This method describes an assay for the detection of the most significant differences between two samples using comprehensive two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer. The assay is based on a comprehensive chemical characterization of complex mixtures with no predefined target compounds.

4.2 Sample Requirements and Workload

An example for the workload is shown in **Table 2**. The time is calculated for one full-time equivalent (FTE) and under the assumption that two test items are compared using three replicates per test item.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 5 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

Task	Man-hours (1 FTE)
Sample collection	
ARMS request	2
Preparation of solvent and working solutions	16
Setting up the instrument	8
Analytical quality assurance	
Sensitivity test	6
System suitability test	16
Sample preparation	8
Data processing and evaluation	
Raw data processing	32
CASI Pre-Processor, CASI, CASI-Post-Processor	24
Reporting	48
Archiving of study related data	16
TOTAL	176

Table 2. Workload in man-hours, one FTE, comparison of two test items.

4.3 Material, Equipment, Chemicals, Standards and References

4.3.1 Equipment

GC×GC-TOFMS system 1, PMI ID 7764:


Instrument	Instrument ID	PMI ID
Autosampler	Agilent 7683 Series	3484
Injector	Agilent 7683B Series	11650
Gas chromatograph	Agilent 7890A	7765
Mass spectrometer	LECO Pegasus 4D SN 3390	7766
Dewar	Cryotherm Apollo 350	6478

Table 3. GC×GC-TOFMS system 1.

GC×GC-TOFMS system 2, PMI ID 10606:

Instrument	Instrument-ID	PMI ID
Autosampler	Agilent 7683 Series	11651
Injector	Agilent 7683B Series	0896
Gas chromatograph	Agilent 6890N	2938
Mass spectrometer	LECO Pegasus 4D SN 3284	3103
Dewar	Cryotherm Apollo 200	7771

Table 4. GC×GC-TOFMS system 2.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 6 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

GC×GC-TOFMS system 3, PMI ID 6472:

Instrument	Instrument-ID	PMI ID
Autosampler	Agilent 7683 Series	3494
Injector	Agilent 7683B Series	12457
Gas chromatograph	Agilent 6890N	6474
Mass spectrometer	LECO Pegasus 4D SN 3242	6473
Dewar	Cryotherm Apollo 350	9878

Table 5. GC×GC-TOFMS system 3.

Additional instruments:

Instrument	Instrument-ID (or equivalent)	PMI ID
Analytical balance	Mettler Toledo XP205 Delta Range	3489
Centrifuge	Beckman Coulter Avanti J-E	2132

Table 6. Additional instrumentation.

4.3.2 Chemicals/Reagents


Name	Specification (equivalent or higher)	Supplier (or equivalent)	Product No.
Acetone	Chromasolv plus, p.a. ≥ 99.9%	Sigma-Aldrich	650501
Dichloromethane	ACS reagent, puriss. p.a. ≥ 99.9%	Sigma-Aldrich	32222
Hexachlorobenzene	Pestanal, analytical standard	Fluka	45522
Sodium sulfate	ACS reagent, p.a. ≥ 99.0%	Sigma-Aldrich	238597
2,2,4-Trimethylpentane	Chromasolv plus, p.a. ≥ 99.5%	Sigma-Aldrich	650439
Water	Chromasolv	Fluka	39253

Table 7. List of chemicals/solvents used for the Nonpolar method.

4.3.3 Internal Standard Compounds

Name	Specification (equivalent or higher)	Supplier (or equivalent)	Product No.
4-Aminobiphenyl-d9	≥ 98.0% purity/atom-%d	CDN	D-2638
Benz(a)pyrene-d12	≥ 97.0% purity/atom-%d	CIL	DLM-258
Decanoic acid-d19	≥ 98.0% purity/atom-%d	CDN	D-1616
Isophorone-d8	≥ 98.0% purity/atom-%d	CDN	D-2304
Isoquinoline-d7	≥ 97.0% purity/atom-%d	CDN	D-904
Naphthalene-d8	≥ 98.0% purity/atom-%d	Sigma-Aldrich	176044
Phenol-d6	≥ 98.0% purity/atom-%d	CDN	D-29
Styrene-d8	≥ 98.0% purity/atom-%d	Sigma-Aldrich	338222

Table 8. List of deuterium labeled internal standard (ISTD) compounds used for the Nonpolar method.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 7 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.3.4 Retention Index Marker Compounds

Name	Specification (equivalent or higher)	Supplier (or equivalent)	Product No.
n-Decane-d22	≥ 97.0% purity/atom-%d	CIL	DLM-133
n-Eicosane-d42	≥ 97.0% purity/atom-%d	CIL	DLM-2208
n-Triacontane-d62	≥ 97.0% purity/atom-%d	CIL	DLM-2210

Table 9. List of retention index marker (RIM) compounds used for the Nonpolar method.

4.4 Procedure

No calibration for quantification is used in this assay. The assay uses a semi-quantification procedure described in **Section 4.5.2**.

4.4.1 Sample Collection

4.4.1.1 Generation of Smoke/aerosol-related Samples

Cigarette smoke/aerosol related samples are generated according to *PMI-RRP-WKI-111729* and *PMI-RRP-WKI-111801*. All the necessary parameters for smoke/aerosol collection are described in the respective ARMS request.


Whole smoke is collected on a Cambridge filter pad with two micro-impingers connected in series. The extraction solution is DCM/acetone (80/20, v/v) containing internal standards and retention index markers (ISTDs/RIMs_{nonpolar}). The preparation of stock solutions, working solution (ISTD_{work}) and extraction solution (ISTD_{extract}) is described and has to be documented in *PMI-RRP-FOR-111487* - Chemicals, solvents, solutions and internal standard amount used for NTDS GC×GC-TOF (*RDNEU*). Stock solutions, ISTD_{work} and ISTD_{extract} have to be freshly prepared.

After generation of the smoke/aerosol samples the filter pad is kept in a Pyrex tube. The micro-impingers are sealed and kept in dry ice/isopropanol.

The samples are analyzed as soon as possible after sample generation. In case samples need to be stored storage of the filters/impinger contents/crude extracts or e-liquids/e-liquid extracts has to be documented in *PMI-RRP-FOR-111488* - Storage of samples and study related materials for NTDS (*RDNEU*).

4.4.1.2 Generation of Other Samples

The described method can be used for comparing samples with different kinds of matrices. The samples will be analyzed as soon as possible after sample generation.


 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 8 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.4.2 Sample Preparation

A step-by-step workflow for sample preparation is described and has to be documented in **PMI-RRP-FOR-111489** - Sample preparation NTDS GC×GC-TOF nonpolar (**RDNEU**). The workflow is briefly described here point by point:

- the content of the micro-impingers (twice 10mL DCM/acetone (80/20, v/v)) containing a set of internal standard and retention index marker compounds is added to the Pyrex tube containing the Cambridge filter pad
- the Pyrex tube is shaken by hand until the filter is starting to break
- the extract is centrifuged with 1000 rpm (approximately 233 ×g) for 10 minutes
- a 10 mL aliquot is transferred to a fresh Pyrex tube and 10 mL of water are added to the aliquot
- the sample is vortexed for 20 seconds and centrifuged with 1000 rpm (approximately 233 ×g) for 10 minutes
- while the organic phase (lower) is transferred by means of a pasteur glass pipette into a fresh amber glass vial, the aqueous phase is kept for the Polar method
- in order to dry the organic phase extract sodium sulfate is added in approx. 100 mg portions until the added salt does not agglomerate anymore
- the sample is vortexed for 10 seconds and stored on the bench top for a minimum of 5 min
- an aliquot of the dried extract is transferred into an autosampler vial and analyzed by GC×GC-TOFMS in full scan mode
- pool sample(s) is/are created from equal volumes of aerosol/smoke replicates to represent the chemical space of all sample groups

The aqueous phase is stored until analysis in a freezer at approx. -20°C. Storage of the aqueous phase, remaining extracts plus filter and processed extracts has to be documented in **PMI-RRP-FOR-111488** - Storage of samples and study related materials for NTDS (**RDNEU**). Remaining extracts plus filter are kept until the study is closed.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 9 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.4.3 Setting-up of Instruments

Instrument	Parameter	Settings
Injector	injector	cool-on-column, track-oven mode
	injection	on-column
	injection volume	0.1 µL
Gas chromatograph	carrier gas	helium
	flow	1.0 mL/min (constant flow)
	column 1 (1 st dimension)	30 m DB-5ms, 0.25 mm ID, 0.25 µm d _f
	column 2 (2 nd dimension)	2.2 m DB-17ht, 0.10 mm ID, 0.10 µm d _f
	primary oven temperature program	rate (°C/min) target temp. (°C) duration (min)
		initial 30.0 2.0
		5.0 325.0 15.0
	secondary oven temperature program	rate (°C/min) target temp. (°C) duration (min)
		Initial 35.0 2.0
		5.2 340.0 14.5
Transfer line	temperature	280 °C
Modulator	modulator	enabled
	modulator temperature program	rate (°C/min) target temp. (°C) duration (min)
		Initial 50.0 3.0
		5.0 340.0 15.0
	2-dimension separation time	6 s
	hot pulse time	1.00 s
	cool time between stages	2.00 s
Mass spectrometer	acquisition delay	440 s
	mass range	35-700 u
	data acquisition rate	200 spectra/s
	detector voltage	1450 – 2000 V
	electron energy	-70 V
	temperature ion source	230 °C


Table 10. Setup of the instrument for the Nonpolar method.

Before the main sequence is started, the sensitivity and the chromatographic resolution of the system is tested. In the case of failure of at least one system suitability parameter, a troubleshooting will be initiated (e.g., new analytical column, increase of multiplier voltage, etc.).

Prior to every analysis the instrument has to be checked and the changes documented in **PMI-RRP-FOR-111496** - Preparation of LECO PEGASUS 4D SYSTEM for NTDS GC×GC-TOFMS (**RDNEU**).

4.4.3.1 Sensitivity Test

The operating procedure of the LECO Pegasus 4D GC×GC-TOFMS system is given in **PMI-RRP-WKI-111668** - Operation of a LECO Pegasus 4D GC×GC-TOFMS system (**RDNEU**). The preparation of a hexachlorobenzene (HCB) stock solution/sensitivity test solution and the correct procedure to conduct a sensitivity test is described in the WKI and has to be documented in **PMI-RRP-FOR-111492** - Sensitivity test and system suitability test NTDS GC×GC-TOF nonpolar (**RDNEU**). The final detector voltage chosen for the

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 10 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GCxGC-TOFMS Nonpolar (RDNEU)			

subsequent analysis has to be indicated in the form *PMI-RRP-FOR-111492* and a LECO ChromaTOF report (template name “Detector Sensitivity”) has to be generated, saved electronically in the study folder and printed, signed and stored in the study binder.

Sensitivity testing of the system is done by measuring the sensitivity test solution prior to the analytical series. A sequence with an increasing multiplier voltage in steps of 50-V-increments is prepared. The maximum detector voltage is given in **Section 4.4.3**. The analysis will be performed in 1-dimensional mode. The main criterion for the sensitivity is the **quant signal to noise ratio (quant S/N)** of the m/z 284 ion of HCB. Additionally the correct name has to be assigned (Benzene, hexachloro-) when matching the EI spectrum to the HCB library. In general an increase of the detector voltage leads to an improvement in S/N, yet at some point the improvements are less pronounced as the noise increases along with the signal. Typically the acceptance criterion is a **quant S/N ≥ 30**, however the final detector voltage has to be selected by the scientist. The decision has to be commented in the form *PMI-RRP-FOR-111492*.

4.4.3.2 System Suitability Test

The chromatographic system is tested by injection of aliquots of the extraction solution (ISTD_extract). The samples are called SST (system suitability test) and are distributed equally across the analytical series (SST_1, SST_2, ...). The concentration is the same as in the measured samples. The sample preparation procedures are described in *PMI-RRP-FOR-111487* - Chemicals, solvents, solutions and internal standard amount used for NTDS GCxGC-TOF (*RDNEU*) and briefly in **Section 4.4.4**.


Test parameters are:

- **retention time** of selected compounds to test the flow and temperature programs of the analytical system
→ stability of the retention times is monitored
- **peak shape** to test the inertness of the analytical system, especially of the analytical columns
→ tailing factor is monitored

Compound	1st Dimension RT (s)		2nd Dimension RT (s)		Tailing Factor 2nd Dimension
Phenol-d6	1058	±70 s	3.270	±0.4 s	<5
Isophorone-d8	1346	±80 s	3.170	±0.5 s	<5
Naphthalene-d8	1478	±80 s	3.480	±0.5 s	<5
Decanoic acid-d19	1736	±90 s	2.670	±0.6 s	<5

Table 11. Acceptance criteria for the system suitability test using the Nonpolar method.

ChromaTOF processing and data evaluation of the system suitability test is described in *PMI-RRP-FOR-111492* - Sensitivity test and system suitability test NTDS GCxGC-TOF nonpolar (*RDNEU*). A LECO ChromaTOF (template name “System Suitability Test”) report has to be generated for each SST sample, saved electronically in the study folder and printed, signed and stored in the study binder. The results of the SST have to be documented in *PMI-RRP-FOR-111492*, saved electronically in the study folder and printed and stored in the study binder.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 11 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GCxGC-TOFMS Nonpolar (RDNEU)			

4.4.4 Preparation of Solutions and Media

Preparation of stock solutions, working and extraction solution is described and has to be documented in *PMI-RRP-FOR-111487* - Chemicals, solvents, solutions and internal standard amount used for NTDS GCxGC-TOF (RDNEU). All solutions are labeled with solution name, concentration, type of solvent, preparation date, storage condition and the initials of the person who prepared the solution (in accordance with *PMI-RRP-WKI-111814*). The solutions are stored at -10 to -80 °C. The stock solutions are prepared by adding the required volume of solvent using volumetric pipettes of accuracy degree AS with quality certificate to the weighed standard compound. Working and extraction solutions are only prepared by using measuring flasks of accuracy degree A with quality certificate and volumetric pipettes of accuracy degree AS with quality certificate or piston pipettes according to work instruction *PMI-RRP-WKI-111700* or microliter syringes, respectively. For weighing an analytical balance with an accuracy of at least 0.01 mg has to be used according to *PMI-RRP-WKI-111726*. Exact weights have to be printed and printouts added to the form *PMI-RRP-FOR-111487*.

Calculation of concentrations:

In case of weighing solid compounds:

$$\text{Concentration (mg/mL)} = \frac{\text{Compound weight (mg)} \cdot (\text{purity atom-\%d} \geq \% / 100) \cdot (\text{purity content} \geq \% / 100)}{\text{Total volume (mL)}}$$

In case of adding liquid compounds:

$$\text{Concentration (mg/mL)} = \frac{\text{Compound volume (mL)} \cdot \text{density (g/mL)} \cdot (\text{purity atom-\%d} \geq \% / 100) \cdot (\text{purity content} \geq \% / 100)}{\text{Total volume (mL)}}$$

4.4.4.1 Solvents


Name	Storage/Stability
DCM/acetone (4:1) v/v	1 year at ambient temperature

Table 12. Preparation of the solvent.

4.4.4.2 Stock Solutions of Internal Standards and Retention Index Markers

The appropriate amounts and volumes that need to be weighed and pipetted according to work instructions *PMI-RRP-WKI-111726* and *PMI-RRP-WKI-111700*, respectively, for the ISTD and RIM stock solutions are described in *PMI-RRP-FOR-111487* - Chemicals, solvents, solutions and internal standard amount used for NTDS GCxGC-TOF (RDNEU).

Concentrations depend on the exact weight and the purity (content and atom-%d) of the used compounds. The exact values are documented in *PMI-RRP-FOR-111487*.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 12 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

Solution Name	Weight (mg)	Volume (µL)	Density (g/mL)	Volume (mL)	Solvent
4-Aminobiphenyl-d9	10 ±3	- ^a	- ^a	10	DCM
Benz(a)pyrene-d12	20 ±3	- ^a	- ^a	10	DCM
Decanoic-d19 acid	10 ±3	- ^a	- ^a	10	DCM
Isophorone-d8	- ^a	12.5	0.920	10	DCM
Isoquinoline-d7	12.5 ±3	- ^a	- ^a	10	DCM
Naphthalene-d8	10 ±3	- ^a	- ^a	10	DCM
Phenol-d6	10 ±3	- ^a	- ^a	10	DCM
Styrene-d8	- ^a	12.5	0.979	10	DCM
n-Decane-d22	- ^a	12.5	0.845	10	DCM
n-Eicosane-d42	10 ±3	- ^a	- ^a	10	DCM
n-Triacontane-d62	10 ±3	- ^a	- ^a	10	DCM

Table 13. Target weights and volumes for the ISTD and RIM stock solutions.

4.4.4.3 Working and Extraction Solution of ISTDs and RIMs


The ISTD and RIM working solution (ISTD_work) is prepared in a 50 mL measuring flask which will be filled up to a final volume with dichloromethane.

Concentrations depend on the exact weight and the purity of the used compounds. The exact values are documented in *PMI-RRP-FOR-111487*.

Used Stock Solution	Volume Stock Solution (µL)	Target Concentration (µg/mL)
4-Aminobiphenyl-d9	2000	40
Benz(a)pyrene-d12	100	4
Decanoic-d19 acid	10000	200
Isophorone-d8	2000	46
Isoquinoline-d7	2000	50
Naphthalene-d8	1000	20
Phenol-d6	5000	100
Styrene-d8	2000	49
n-Decane-d22	5000	100
n-Eicosane-d42	5000	100
n-Triacontane-d62	5000	100

Table 14. Target volumes for the working solution of ISTDs and RIMs.

The extraction solution (ISTD_extract) is prepared by diluting the working solution (250µL ISTD_work filled up to 10mL with DCM/acetone (4:1) v/v). Calculation of concentrations is given in *PMI-RRP-FOR-111487*.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 13 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.4.5 Number of Determinations

Each sample will be generated minimum in triplicate and analyzed. Blanks, SST samples, pool sample(s) and one technical replicate of each matrix will be evenly distributed across the sequence. The term technical replicate describes the process of injecting the same sample extract multiple times across a sequence to assess the instrumental variability.

4.4.6 Daily Verification or According to Use

Prior to each study the LECO Pegasus 4D system is verified according to *PMI-RRP-FOR-111496* - Preparation of LECO PEGASUS 4D SYSTEM for NTDS GC×GC-TOFMS (*RDNEU*) and tested in terms of sensitivity and system suitability according to *PMI-RRP-FOR-111492* - Sensitivity test and system suitability test NTDS GC×GC-TOF nonpolar (*RDNEU*).

4.4.7 Testing Procedure

This method describes an assay for detection of significant differences between two samples by GC×GC-TOFMS. The composition of different complex mixtures, like cigarette smoke or RRP aerosol, is compared in a hypothesis-free unbiased way (non-targeted). Compounds found to be different between samples are ranked according to relevance considering the relative difference in abundance of each compound as well as the absolute abundance. Focus of the approach is the comprehensive chemical characterization of a complex mixture using three GC×GC-TOFMS methods, which are allocated to different polarities and volatilities of the constituents. The methods are not intended to assess absolute quantitative amounts of the detected compounds, the concept is rather based on a semi-quantitative assessment.

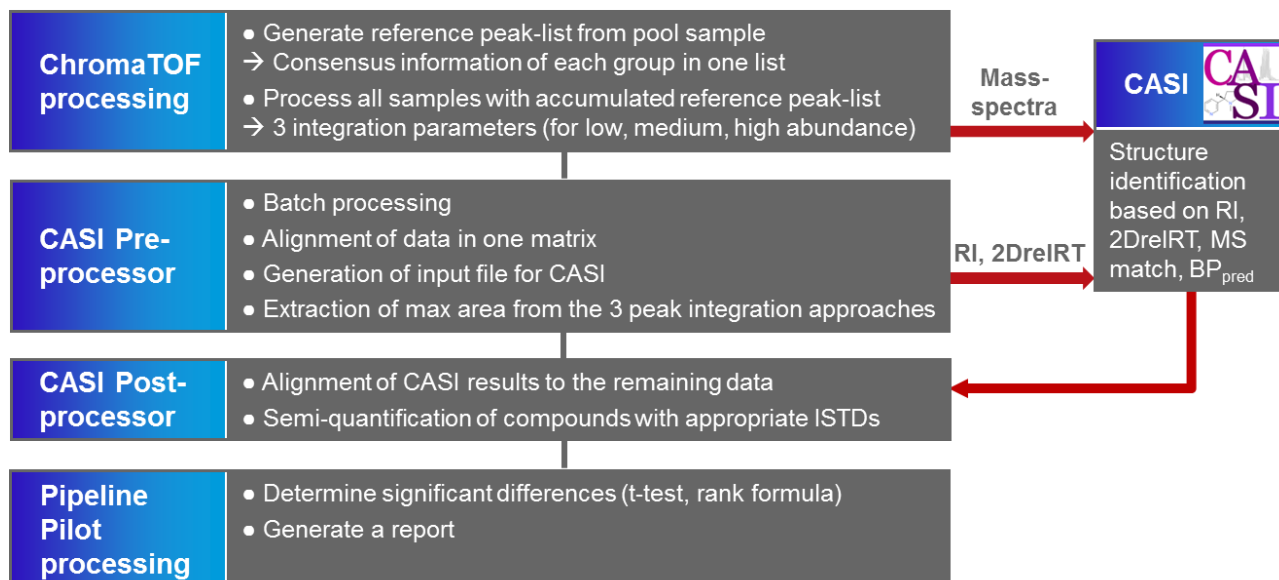



Figure 1. Overview of the data processing steps.

Data processing is divided into multiple steps, (1) ChromaTOF processing, (2) CASI Pre-processor, (3) CASI, (4) CASI Post-processor, and (5) Pipeline Pilot processing (see **Figure 1**).

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 14 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

First the raw data has to be processed by the LECO ChromaTOF software. After assembling all the relevant information each sample is processed with three different parameter settings to optimally assess the peak area of minor, medium and major peaks. Files are exported in .csv format.

The data is further processed using CASI Pre-processor, which processes the batches and aligns the data in one matrix. The maximum area of each peak is determined and an input file for CASI is created. The CASI input file contains the mean of retention indices (RI) and 2nd dimension relative retention times (2DrelRT) for all compounds. Together with the casi_input file the associated EI mass spectral library (converted with lib2nist) is submitted to CASI.

The CASI platform increases the accuracy for analytical identification of compound structures and accelerates and standardizes the identification process. It assures reproducibility and enables scientists to have higher confidence in the correct assignment of mass spectra to the right compounds. CASI automatically identifies, on-the-fly and with highest confidence, possible relevant structures from mass spectra associated with chromatographic values, including models for retention index, 2-dimensional relative retention time and boiling point.

CASI Post-Processor combines the high confidence identifications of CASI to the existing data matrix. Subsequently, semi-quantification is performed according to predefined rules.

In the final step, a Pipeline Pilot script is used to determine the significant differences and to transform the data to a suitable reporting format.

Alternatively, a manual Microsoft Excel process can be used instead of CASI Pre- and Post-processor. This procedure was employed before the automated CASI Pre- and Post-processor procedure, but became obsolete due the enormous time-consumption and the error-proneness. However it will be briefly described in **Section 4.4.7.2.4**.

4.4.7.1 Data Processing using the LECO ChromaTOF Software


A step-by-step description for the ChromaTOF processing is given in *PMI-RRP-FOR-111490* - Data processing in ChromaTOF NTDS GC×GC-TOF nonpolar (*RDNEU*). All the steps have to be documented. In the following an example for two items (e.g. 3R4F versus P1) is shown.

4.4.7.1.1 Generation of the Reference Peak Matrix from the Pool Sample

- **Processing of the pool sample**
 - computing of the baseline
 - finding peaks above the baseline
 - identifying peaks by library search (select only "ISTDs_RIMs" library)
 - integration of the peaks (area, height)

<input checked="" type="checkbox"/>	Baseline - computes baseline
<input checked="" type="checkbox"/>	Peak Find - finds peaks above the baseline
<input checked="" type="checkbox"/>	Library Search - identifies all peaks found
<input checked="" type="checkbox"/>	Calculate Area / Height - computes the area and height of peaks without a calibration

Figure 2. Processing steps that need to be activated in the Data Processing Method (DPM).

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 15 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

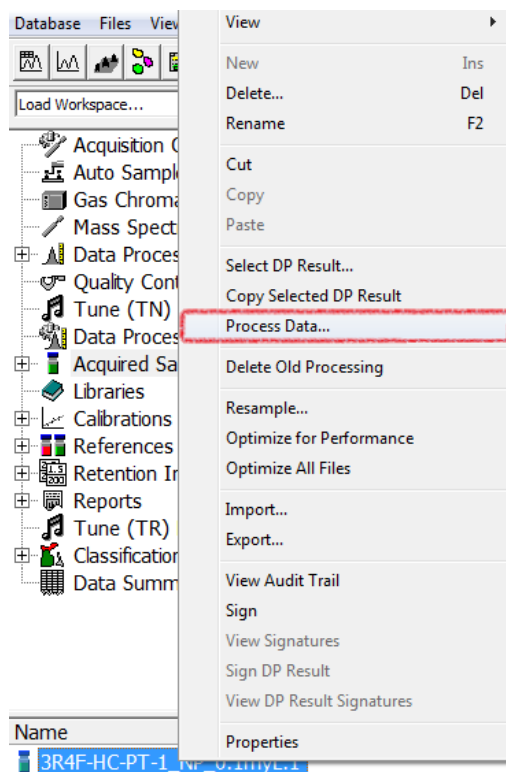



Figure 3. Processing of the pool sample.

- **Repeated processing of the pool sample with classification and RI method**
 - computing of the baseline
 - finding peaks above the baseline
 - identifying peaks by library search (select only “ISTDs_RIMs” library)
 - integration of the peaks (area, height)
 - calculation of retention index
 - classification (exclusion of, e.g. bleed, high abundant compounds triacetine, nicotine, tailing of high abundant fatty acids)

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 16 of 36
	Doc. Type: Work Instruction		Status: Production
	Doc. ID: PMI-RRP-WKI-111620		Version N°: 4.0.0 Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

☒ Baseline - computes baseline
☒ Peak Find - finds peaks above the baseline
☒ Library Search - identifies all peaks found
☒ Calculate Area / Height - computes the area and height of peaks without a calibration
☒ Retention Index Method

☒ Filter peaks by classification

CLASSIFICATION

Add...

Remove

Select the retention index method to convert retention time to retention index:

RIM

Select ...

☒ Check Retention Index Analytes

Criteria:

☒ Maximum allowed retention index variation.

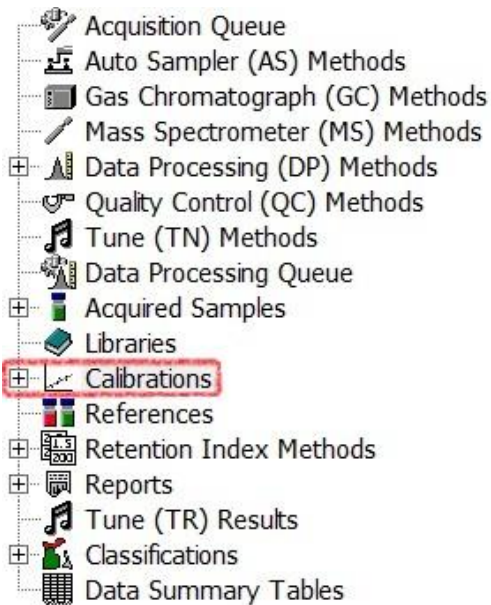
20

Options:

☒ Update the retention times of retention index analytes.

Figure 4. Processing steps that need to be activated in the DPM of the repeated processing.

- **Evaluate the data and create a new calibration of the pool sample**
 - evaluate correct finding of ISTD and RI compounds
 - sort according to Quant S/N, delete peaks with S/N <50
 - flag false/noise peaks
 - import processed and flagged data into new calibration
 - select quantitation parameters in the calibration



Name
 Nonpolar_Pool sample-S/N 250

Figure 5. Creating a “New” calibration under tab “Calibrations”.

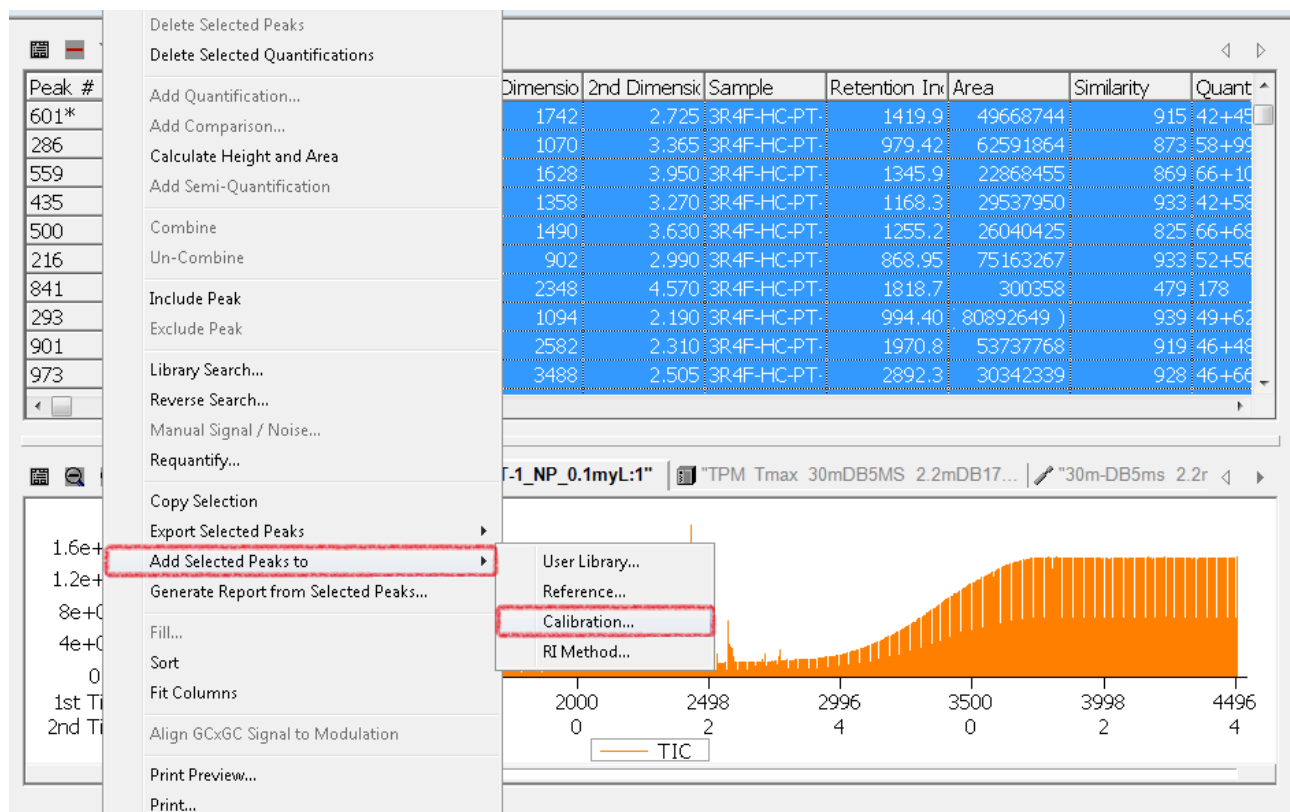



Figure 6. Select the processed pool sample, select all peaks and add selected peaks to calibration.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 18 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018

NTDS GC×GC-TOFMS Nonpolar (RDNEU)

Acquisition Queue

Auto Sampler (AS) Methods

Gas Chromatograph (GC) Methods

Mass Spectrometer (MS) Methods

Data Processing (DP) Methods

Quality Control (QC) Methods

Tune (TN) Methods

Data Processing Queue

Acquired Samples

Libraries

Calibrations

References

Retention Index Methods

Reports

Tune (TR) Results

Classifications

Data Summary Tables

Name

Nonpolar_Pool sample-S/N 250

Nonpolar_Pool sample-S/N...

Analyte	1st Dimension R.I.	Deviation	R.I.	Deviation	S/N Threshold	Force Origin	Min Valid Concentration	Max Valid Concentration
1	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
2	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
3	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
4	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
5	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
6	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
7	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
8	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
9	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
10	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
11	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
12	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
13	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
14	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
15	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
16	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
17	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
18	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		
19	12.000	0.30000	10.000	Force Origin	0.0000	1000000000		

Figure 7. Selection of the quantitation parameters. Values have to be filled down.

4.4.7.1.2 Comparison of the Whole Sample Set against the Reference Peak List and Export Data

- **Prepare a report-file folder structure for the export of the .csv files**

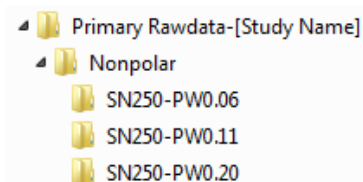



Figure 8. Folder structure for the export of the .csv files.

- **Prepare three quantitation methods with alternating peak widths in the 2nd dimension**
 - computing of the baseline
 - finding peaks above the baseline
 - disable library search
 - calculation of retention index
 - apply calibration
 - enable export of the peak information in ASCII CSV format

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 19 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

Select the task or tasks you wish to perform from the list below.


- ☒ Baseline - computes baseline
- ☒ Peak Find - finds peaks above the baseline
- ☐ Library Search - identifies all peaks found
- ☐ Calculate Area / Height - computes the area and height of peaks without a calibration
- ☒ Retention Index Method
- ☐ Classifications
- ☒ Apply Calibration(s) - computes the absolute concentration of peaks based upon a calibration
- ☐ Apply Reference(s) - computes the relative concentration of peaks with respect to a reference
- ☐ Semi Quantification - computes concentration based on another analytes calibration curve
- ☐ Tune Check
- ☐ Tailing Factor Check - checks to see if the analytes have an acceptable peak shape
- ☐ Calibration Check
- ☐ Blank Check - checks to make sure none of the analytes exceed their blank concentration
- ☐ Report - prints selected reports for each sample
- ☒ Export peak information in ASCII CSV format
- ☐ Export data in Andi MS format (.cdf)
- ☐ Export data file

Add the calibrations to use for quantification to the list below:

Nonpolar_Pool sample-S/N 250	Add...
	Remove
	Promote
	Demote

Figure 9. Processing steps that need to be activated in the DPM of the final three methods.

- method 1: peak width 0.06 sec
- method 2: peak width 0.11 sec
- method 3: peak width 0.20 sec
- keep remaining parameters consistent

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 20 of 36
	Doc. Type: Work Instruction		Status: Production
	Doc. ID: PMI-RRP-WKI-111620		Version N°: 4.0.0 Effective date: 28-Feb-2018
NTDS GCxGC-TOFMS Nonpolar (RDNEU)			

Enter baseline tracking info below:

#	Start	End	Mode
1*	Start of Run	End of Run	Default

Add
Remove

Enter the baseline offset below (0.5-3.0):

0.8

Examples:
 0.5 Through the middle of the noise
 1.0 Just above the noise

Enter the number of data points that should be averaged for smoothing below:

Auto

☒ GCxGC Parameters

1st Dimension

Enter the expected peak width in seconds below.

☐ Peak widths broaden throughout the chromatographic run

Peak Width	Retention Time	Peak Width values should be the expected number of slices multiplied by the modulation period. Typically, 3 to 6 slices per analyte are expected. Example: 6 slices x 4 sec. modulation period = Peak Width of 24
12		

2nd Dimension

Match Required to combine: 850

☒ Override the allowed second dimension R.T shift for combine

Early 0.150

Late 0.050

Enter the expected peak width in seconds below: (as measured from baseline to baseline)

☐ Peak widths broaden throughout the chromatographic run

Peak Width	Retention Time	For broadening, two peak widths may be specified at two different retention times. All peak widths will be extrapolated from these two points.
0.06		

Subpeak Settings

Minimum S/N: 5

Integration Approach:
☒ Traditional
☐ Adaptive


☒ Filter peaks by classification

CLASSIFICATION

Add...

Figure 10. Alternate peak width in 2D (green square); keep remaining parameters constant (red squares).

- choose header "@SampleName[]"
- export all peaks , except "Contaminants / Unknowns"
- ensure export of peak information **in the right order**

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 21 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

Header: (Leave Blank if no header information is desired)

Functions...

@SampleName()

Field Separator

☒ Comma

☐ Tab

Filter

☒ Calculate area percentage from filtered peaks only

☒ Quantifieds

☒ Analytes ☒ Surrogates ☒ Internal Standards

☒ Match

☒ Out of Tolerance

☐ Contaminants / Unknowns

☒ Not Found

Sort by:

Quantification

☒ Ascending

☐ Descending

Then by:

☒ Ascending

☐ Descending

Then by:

☒ Ascending

☐ Descending

Information not exported

Actual Tailing Factor

Analyte Id

Analyte Range

Analyte Type

Apexing Masses

Area %

BaselineModified

Calculated Ion Ratio 1

Calculated Ion Ratio 2

Calculated Ion Ratio 3

Calibration

Classifications

Comment

Conc. Concern

Conc. Conv. Units

Conc. Units

Concerns

Contributor

Conv. Conc.

Add >>

<< Remove

Exported Information

Quantification

Retention Index

Area

1st Dimension Time (s)

2nd Dimension Time (s)

Concentration

R.T. (s)

Expected Analyte R.T. (s)

Similarity

Quant S/N

Type

CAS

standard

Formula

Exact Mass

UniqueMass

Name

Quant Masses


Full Width at Half Height

Promote

Demote

Figure 11. Critical parameters that have to be inserted, deactivated or given in the right order.

- **Process all samples using the final quantitation methods 1, 2 and 3**

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 22 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.4.7.1.3 Transfer of the Final Calibration into a Library

- Create a new user library in ChromaTOF and name it according to the study
- Select all entries of the final ChromaTOF calibration and add them to the newly created user library
- Ensure to deactivate the box “Enter additional user information for each spectrum”

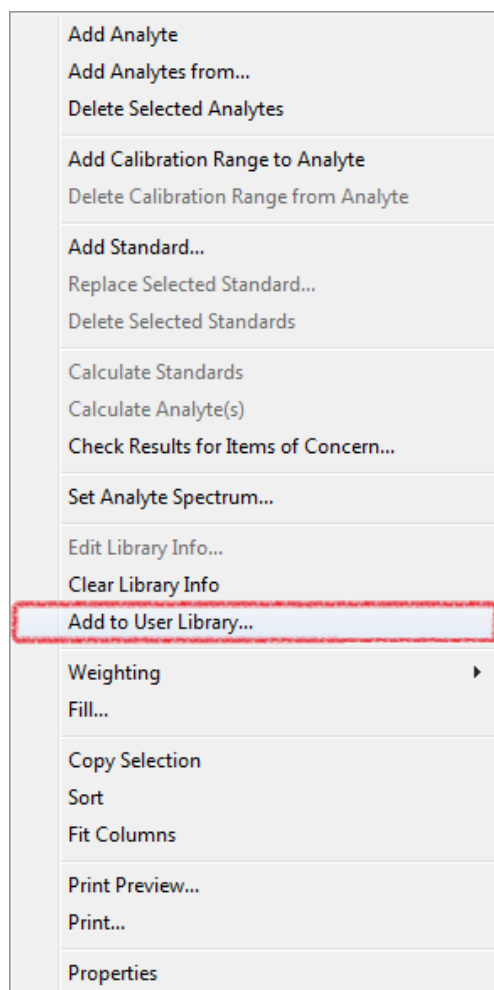



Figure 12. Adding information from a calibration to a user library.

4.4.7.2 Submitting Metadata to CASI Pre-processor, CASI and CASI Post-processor

A step-by-step description is presented in *PMI-RRP-FOR-111491* - Submitting metadata for CASI pre-processor, CASI, CASI post-processor (*RDNEU*). All the steps have to be documented.

Two files are needed as input in order to run through all the CASI processes. Both files have to be saved in the study folder "...\\Primary Raw data-[Study name]\\Nonpolar\\".

The file "Concentration_ISTDs.txt" is generated from the respective nonpolar sheet in *PMI-RRP-FOR-111487* - Chemicals, solvents, solutions and internal standard amount used for NTDS GC×GC-TOF (*RDNEU*). The file has to be saved with the exact name "Concentration_ISTDs.txt". The group names (column → test item) in "Concentration_ISTDs.txt" have to be in accordance with the groups defined in the exported file names.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 23 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

The JCAMP file “Library.HPJ” is generated by converting the library, which was created in **Section 4.4.7.1.3**, with lib2nist converter. Here, no naming convention is necessary.

4.4.7.2.1 CASI Pre-processor

CASI Pre-processor performs a fully automated batch processing, which includes replacement of saturated signals by the approximate value, alignment of the data, calculation of RI and 2DreIRT means, and determination of the maximum peak area for each signal within the three quantitation methods. In case the max area of an ISTD is not between 50 and 200 % of the mean of max area values across all samples the value is replaced by the mean of the max area.

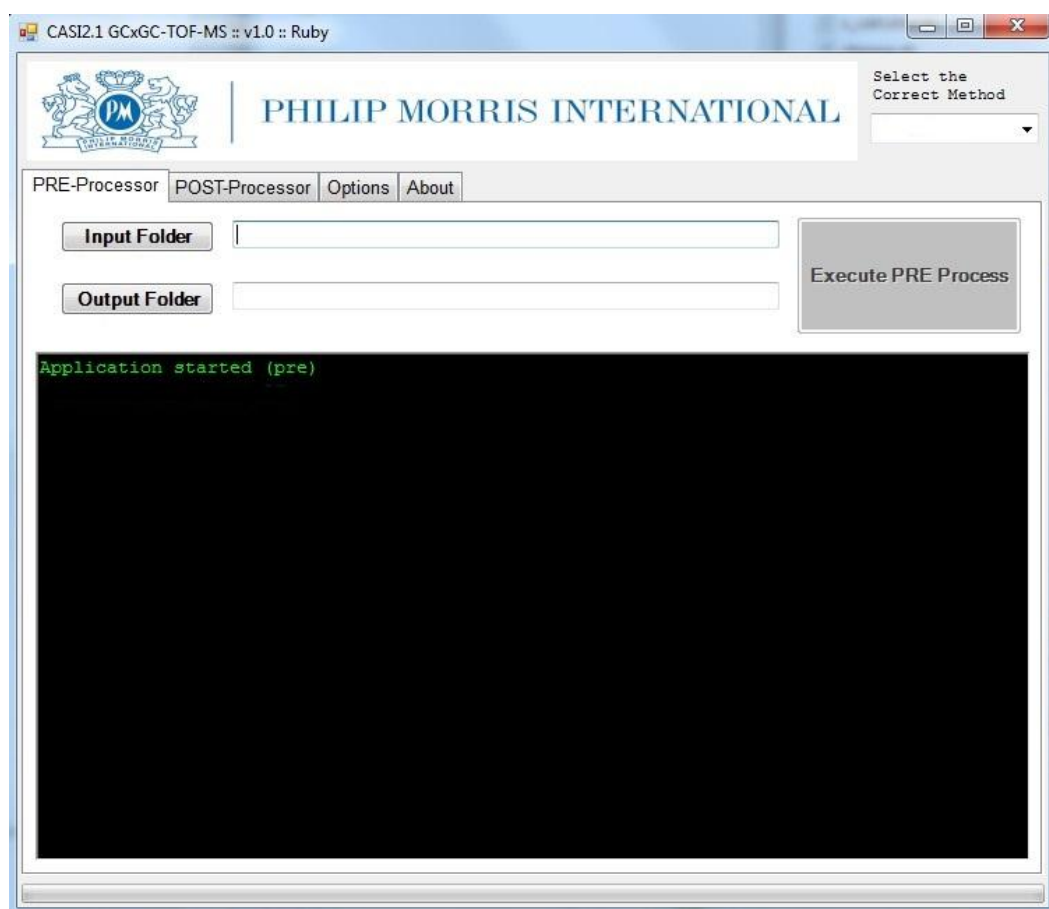



Figure 13. User interface of CASI Pre- and Post-processor.

CASI Pre-processor outputs in total four files, two warning files and two files that are needed for further processes.

The file “warning.txt” lists all signals, which (A) were saturated and therefore replaced or (B) could not be found in respective samples.

The file “warning-area-dcompounds.txt” lists the max areas of the ISTDs that were out of the defined range and thus replaced (original max area values are shown).

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 24 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

The file “input-postprocess.txt” contains the accumulated information and is required as an input file for CASI Post-processor.

The file “casi_input.txt” comprises average RI and 2DrelRT values, which are needed for CASI.

4.4.7.2.2 CASI

Computer Assisted Structure Identification is a powerful platform that enhances the accuracy of compound structure identification and accelerates and standardizes the identification process. CASI's automatic identification process operates on-the-fly and facilitates a higher confidence in the correct assignment of mass spectra to the right compounds as relevant structures are associated with chromatographic values, including models for retention index, 2-dimensional relative retention time and boiling point.

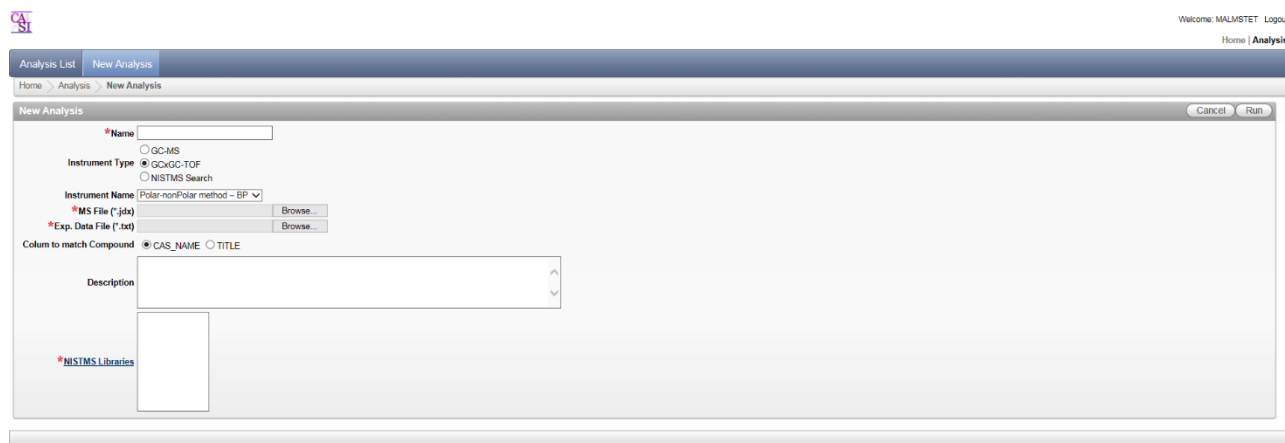


Figure 14. User interface of CASI.


A user manual for CASI is available in EDMS ([PMI-RRP-WKI-111624](#) - User guide for CASI ([RDNEU](#))).

CASI requires two files, the “Library.HPJ”, which was converted from the original library with lib2nist converter, and the “casi_input.txt” generated by the CASI Pre-processor.

When the CASI process is finished the “casi_report_ntds.txt” is exported. The exact name is kept. The file is further required to run CASI Post-processor.

4.4.7.2.3 CASI Post-processor

CASI Post-processor aligns the data comprised in “casi_report_ntds.txt” to the data of the file “input-postprocess.txt”. Semi-quantification is performed according to specific rules, which are defined in **Section 4.5.2**. The output file of CASI Post-processor is the file “concentration.txt”, which contains the final processed data.


 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 25 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.4.7.2.4 Manual Excel Processing

Before the CASI processors were developed the metadata was processed manually in a predefined MS Excel workflow. The individual steps are briefly described point by point in the following:

- open .csv file and delete saturation comments in field "area"
- sort table: field "standard" and then "quantification" ascending
- sort standards for "standard" descending
- verify ISTD and RT-INDEX alignment => correction if necessary
- rename .csv data into [SNx]-[Pwy]-[sample name]-[repetition number]-[method], e.g. SN250-PW0.11-3R4F_HC1-NP.xls
- repeat first 5 steps for all .csv files
- generate EXCEL reference calculation file
- import compound name, area data and information on sample, measurement-no., S/N and PW into EXCEL
- verify correct data-table alignment (conditional format, compare names)
- correction of misalignments in data
- get maximum area of values from the different integration parameters
- prepare NIST format library from final calibration file, prepare chromatography file for CASI, including calculation of 2DrelRT, data have to be averaged
- align CASI results with chromatography data
- perform analysis on elemental composition
- copy worksheet and transform formulas to values
- check ISTD areas according to criteria:
 - area must be within 50% and 200% of mean area
 - non-accepted areas are replaced by mean area of valid dataset
- semiquantify all samples according to rules described in **Section 4.5.2**
- copy worksheet e.g. 3 times (depending on the number of comparisons) and transform formulas to values
- rename as table P1reg vs P2reg / P1reg vs 3R4F / P2reg vs 3R4F
- perform t-test on the data sets
- sort tables for p-values, separate data with p-values >0.05 as "not significantly different"
- calculate EFFECT, INDEX and RANK
- sort for RANK, split tables in positive and negative list, sort for absolute values within categories
- verify results (manually)

The time-consuming manual processing procedure has been replaced and each step verified by CASI Pre- and Post-processor.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 26 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.4.7.3 Pipeline Pilot Processing

The Pipeline Pilot script “NTDS_Comparison and Report” is specifically dedicated to perform t-tests and ranking on the dataset. In addition the script transforms all of the information into a suitable reporting format. Therefore the “concentration.txt” has to be uploaded to the webport, the study name/comparisons entered and the process executed. The output file is a final report file in Excel format.

The user guide “User Guide Pipeline Pilot Web Port Protocols”, Elyette Martin, version 1.0 describes all the available Pipeline Pilot webport protocols (available on DISCO).

4.5 Calculation and Records

4.5.1 Calculation of the Second Dimension Relative Retention Time

For the calculation of the second dimension relative retention time an experimental model was developed. The 2DrelRT is derived from second dimension peaks in relation to hypothetical reference points based upon linear regressions of deuterated n-alkanes (**Figure 15**). The n-alkanes are used to generate a hypothetical second dimension retention time reference system, compensating for systematic shifts (such as different column length or gas flow) but not for any shifts related to analyte-stationary phase interaction, as these shifts are dependent upon individual compound properties.

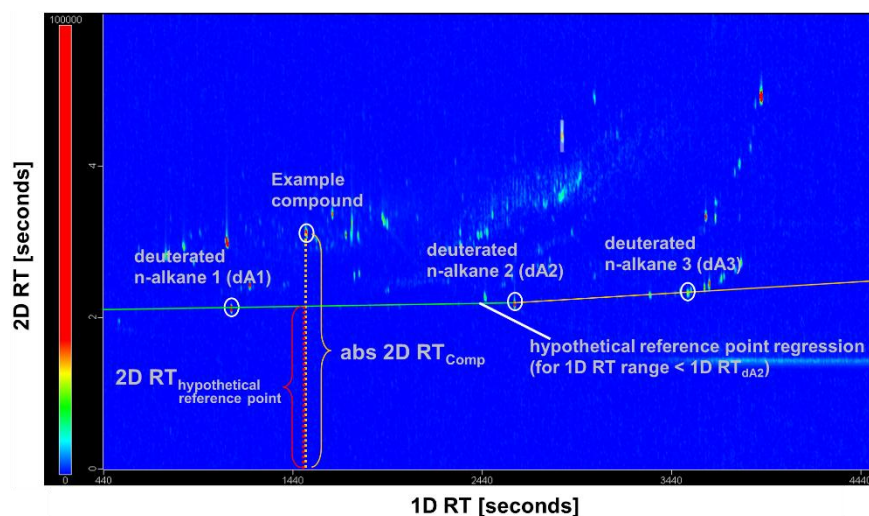



Figure 15. Principals of the second dimension relative retention time generation. Hypothetical reference points (full line) are derived from the linear regression of the experimentally measured retention times of deuterated n-alkanes in the two dimensional separation space.

The second dimension relative retention time of a compound is calculated as follows:

$$2DrelRT = \frac{abs_2DRT}{2DRT_{hrf}} \quad (1)$$

where abs_2DRT is the measured second dimension retention time of the compound and 2DRT_{hrf} is the second dimension retention time of the hypothetical reference point.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 27 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

For a given compound that elutes between deuterated n-alkane standard compound 1 and compound 2, the $2DRT_{hrf}$ is calculated using the linear equation $y=ax+b$:

$$2DRT_{hrf} = \frac{2DRT_{dA2} - 2DRT_{dA1}}{1DRT_{dA2} - 1DRT_{dA1}} \times 1DRT + \left(2DRT_{dA1} - \frac{2DRT_{dA2} - 2DRT_{dA1}}{1DRT_{dA2} - 1DRT_{dA1}} \times 1DRT_{dA1} \right) \quad (2)$$

where $a = \frac{2DRT_{dA2} - 2DRT_{dA1}}{1DRT_{dA2} - 1DRT_{dA1}}$ is describing the slope and b is calculated by substituting x using the known values for $dA1$.

The equation resolved using the known values for $dA1$ for $b=y_{dA1}-ax$ leads to

$$b = 2DRT_{dA1} - \frac{2DRT_{dA2} - 2DRT_{dA1}}{1DRT_{dA2} - 1DRT_{dA1}} \times 1DRT_{dA1}$$


where $dA1$ and $dA2$ are deuterated n-alkane 1 and 2, respectively, and $1DRT$ and $2DRT$ are the first and second dimension retention time of the respective molecules.

4.5.2 Semi-quantification of Compounds

The calculation of peak areas (integration) for a high number of diverse compounds is a critical step due to the different chromatographic behavior of individual compounds. In order to enhance the quality of the integration process, the samples are processed three times using different peak integration parameters as described in **Section 4.4.7.1.2**. Then, the maximum value of the integration results for each component will be used for further calculation.

Compound	Case of Known Compounds	Case of Unknown Compounds
Naphthalene-d8	internal standard for hydrocarbons	-
Isophorone-d8	internal standard for carbonyls	internal standard for compounds with 2nd dimension relative retention time ≤ 1.5 (in general corresponds to nonpolar to medium polar unknowns)
Phenol-d6	internal standard for phenolic compounds	internal standard for compounds with 2nd dimension relative retention time > 1.5 and ≤ 1.8 (in general corresponds to medium polar aromatic unknowns)
Isoquinoline-d7	internal standard for N-containing compounds	internal standard for compounds with 2nd dimension retention time > 1.8 (in general corresponds to basic unknowns)
Decanoic acid-d19	internal standard for acids	-

Table 15. Assignment of compound classes to specific ISTDs.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 28 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

For semiquantification, each compound will be referred to one of the internal standards. Every internal standard is allocated to a certain compound class. If a compound cannot be classified by a corresponding internal standard or is unknown a secondary classification according to 2DrelRT applies. A detailed description of the rules is given in **Error! Reference source not found..**

4.5.3 Extraction of Significant Differences

The extraction of significantly different compounds between the different test items is done by applying a two-tailed two-sample t-test with unequal variances (heteroscedastic) on the data set (2 groups, 3--5 replicates). The results provide the probability (p) of significant differences between two samples and/or test items. Comparisons with $p \leq 0.05$ will be considered to be significantly different. On the contrary, compounds with $p > 0.05$ will be excluded from further calculations/processing.

TTEST(Dataset Lx, Dataset Ly, tails, type)

Lx: measured values of test item 1 to be compared with Ly

Ly: measured values of test item 2 to be compared with Lx

tails = 2; two-tailed distribution

type = 3; heteroscedastic

4.5.4 Ranking of Detected Compounds

The sorting of significantly different compounds by their relevance is done by applying an empirically developed ("RANK") formula on the t-test filtered data set.

This "RANK" formula mathematically combines two criteria:


- difference of the variable ("Effect" (%))
- abundance of the variable ("Average Concentration" (e.g., µg/cig. or µg/article, or µg/mg TPM)).

$$\text{RANK} = \frac{\text{Effect}^3}{1000} \times \text{Average Concentration}$$

$$\text{Effect} = \frac{(\text{Ly}-\text{Lx})}{(\text{Ly}+\text{Lx})} \times 100$$

$$\text{Average Concentration} = \frac{\text{Lx}+\text{Ly}}{2}$$

The data set is divided into positive ($\text{Lx} > \text{Ly}$) and negative ($\text{Lx} < \text{Ly}$) rank values and sorted by increasing absolute rank values for the positive as well as the negative effect. A lower rank value shows more significant differences than a higher rank value.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 29 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

4.5.5 Flexible Filtering

The final processed data can be filtered according to e.g. fold change or a concentration cut-off can be applied depending on the request or individual requirement.

4.5.6 Results Recording/Data Transfer/Documentation

A detailed description for data management is given in **PMI-RRP-FOR-111519** - NTDS GC×GC-TOF-Data Management (**RDNEU**). The form summarizes all the administrative data, certificates, paper-rawdata, method specific forms and sample naming information that are needed for the study, and the GC×GC-TOFMS rawdata, processed data, CASI Processor data and result tables that are generated during the study with the respective location of storage. To conclude the study all steps have to be documented in **PMI-RRP-FOR-111519**, saved electronically in the study folder and printed and stored in the study binder.

Initial raw data are generated on the instrument acquisition computer, which are then copied including all study relevant instrument methods using the ChromaTOF inbuilt archiving functionality to a central data repository (currently HPC data share \\rd-hpc-samba.app.pmi). Autosampler sequences are copied to the same repository.

The initial raw data and all related data are restored on a local data evaluation computer using the ChromaTOF inbuilt restore from archive function. The data are deleted from the acquisition computer when the ChromaTOF data processing is finished successfully, provided that no file was corrupted during transfer.

Intermediate results (.csv files) of the data processing are stored in the primary raw data folder of the study directory in the \\rd-hpc-samba.app.pmi data share. Processed data files including all study relevant files are backed up to the same data repository using the ChromaTOF inbuilt archiving function.


After successful data processing using CASI Pre-processor, CASI and CASI Post-processor and subsequent confirmation of the results the processed data are deleted from the data evaluation workstation. Ultimately the entire study including a complete set of raw data and processed data must be maintained on the Long Term Repository (LTR, currently PMRD LabData_NeuchatelData \\cifs.arch10.store.pmi) environment.

Study related documents	Type
Certificates of ISTDs, syringe and analytical columns	Original/printout
Smoke/aerosol generation request and protocol	Printout/signed printout

Table 16. Study related documents.

Study relevant raw data and instrument related data for backup	File-type
Instrument raw data of samples, SSTs and sensitivity test	ChromaTOF database
Acquisition methods: GC and MS methods	ChromaTOF database
Autosampler methods	ChromaTOF database

Table 17. Study relevant raw data and instrument related data for backup.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 30 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GCxGC-TOFMS Nonpolar (RDNEU)			

Study relevant processed data and intermediate results for backup	File-type
Processed raw data of samples, system suitability tests and sensitivity test	ChromaTOF database
Data Processing methods: Data Processing methods, calibrations, retention index methods, classifications	ChromaTOF database
Results sensitivity test + report	Textfile (.pdf)
Results system suitability tests + report	Textfile (.csv and .pdf), Excel-form
Results sample data processing	Textfiles (.csv)
CASI Pre-processor, CASI, and CASI Post-processor input/output files	Textfiles (.txt)
Converted library	JCAMP (.HPJ)
Further Data Evaluation and Reporting files	Excel-files

Table 18. Study relevant raw data and instrument related data for backup.


Study relevant forms for backup	File-type
<i>PMI-RRP-FOR-111487</i> - Chemicals, solvents, solutions and internal standard amount used for NTDS GCxGC-TOF (RDNEU)	Excel-file, signed printout
<i>PMI-RRP-FOR-111488</i> - Storage of samples and study related materials for NTDS (RDNEU)	Excel-file, signed printout
<i>PMI-RRP-FOR-111489</i> - Sample preparation NTDS GCxGC-TOF nonpolar (RDNEU)	Excel-file, signed printout
<i>PMI-RRP-FOR-111490</i> - Data processing in ChromaTOF NTDS GCxGC-TOF nonpolar (RDNEU)	Excel-file, signed printout
<i>PMI-RRP-FOR-111491</i> - Submitting metadata for CASI pre-processor, CASI, CASI post-processor (RDNEU)	Excel-file, signed printout
<i>PMI-RRP-FOR-111492</i> - Sensitivity test and system suitability test NTDS GCxGC-TOF nonpolar (RDNEU)	Excel-file, signed printout
<i>PMI-RRP-FOR-111496</i> - Preparation of LECO PEGASUS 4D SYSTEM for NTDS GCxGC-TOFMS (RDNEU)	Excel-file, signed printout
<i>PMI-RRP-FOR-111519</i> - NTDS GCxGC-TOF-Data Management (RDNEU)	Excel-file, signed printout

Table 19. Study relevant forms for backup.

4.6 Testing Scope, Repeatability, Reproducibility

Type	Name	Title	Author	Version
Assay Lifecycle Management Process Validation Report	rep_AEC_033	Validation Report (Fast Validation) Non-Targeted Differential Screening (NTDS) Assay Using Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GCxGC-TOF) September 2008 (available on DISCO)	Arno Wittig	1.0

Table 20. Documentation.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 31 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GCxGC-TOFMS Nonpolar (RDNEU)			

4.6.1 Testing Scope

NTDS GCxGC-TOFMS Nonpolar describes an assay for the detection of the most significant differences between two complex samples. The assay is non-targeted, i.e. it is based on a comprehensive chemical characterization of complex mixtures with no predefined target compounds. Depending on the matrix the testing scope can vary and thus cannot be defined specifically.

4.6.2 Repeatability, Reproducibility

Each sample will be generated minimum in triplicate and analyzed. Blanks, SST samples, pool sample(s) and one technical replicate of each matrix will be evenly distributed across the sequence. The repeatability of the SST and technical replicate samples are assessed by means of the area values of the ISTDs.

4.6.3 Acceptability Limits

The acceptability limits of the method are described in **Section 4.4.3.1** (sensitivity test) and **4.4.3.2** (system suitability test).

In case the criteria of the sensitivity test are not met, the multiplier voltage will be increased in steps of 50-V-increments until the acceptance criteria are fulfilled (further described in **Section 4.4.3.1**). If the detector voltage exceeds 1900 V a replacement has to be considered.

If the acceptance criteria of the system suitability test are not met, the deviation needs to be commented in **PMI-RRP-FOR-111492** - Sensitivity test and system suitability test NTDS GCxGC-TOF nonpolar (**RDNEU**). In case of multiple deviations the samples following the respective SST sample in the sequence are rendered invalid.


4.7 Safety

- The usual good practices of a laboratory are required, information is available in the "Laboratory EHS Handbook"
- Be aware of the risk assessment of the labs
- Work in an exhaust hood and wear safety glasses
- Take care of the relevant MSDS (material safety data sheets, storage location: T0.182 - 186)
- Store the inflammable products away from a heat source or a flame

4.8 Calibration and Maintenance of Instruments

Name	Title
PMI-RRP-WKI-111668	Operation of a LECO Pegasus 4D GCxGC-TOFMS system (RDNEU)

Table 21. Documentation for calibration and maintenance of the instruments.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 32 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

5 Reference Documents

Knorr, A., Monge, A., Stueber, M., Stratmann, A., Arndt, D., Martin, E., Pospisil, P., Computer-assisted structure identification (CASI)--an automated platform for high-throughput identification of small molecules by two-dimensional gas chromatography coupled to mass spectrometry, *Analytical Chemistry*, 85(23) (2013) 11216-24


Shellie, R. A., Welthagen, W., Spranger, J., Ristow, M., Zrostlikova, J., Fiehn, O., Zimmermann, R., Statistical methods for comparing comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry results: Metabolomic analysis of mouse tissue extracts, *Journal of Chromatography A*, 1086 (2005) 83-90

Zhu, S., Lu. X., Dong, L., Xing, J., Su, X., Kong, H., Xu, G., Wu, C., Quantitative determination of compounds in tobacco essential oils by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry, *Journal of Chromatography A*, 1086 (2005) 107-114

International Organization for Standardization: International Standard ISO 3308, Routine analytical cigarette-smoking machine - Definitions and standard conditions, 4th ed., 2000

International Organization for Standardization: International Standard ISO 3402, Tobacco and tobacco products – Atmosphere for conditioning and testing, 4th ed., 1999


University of Kentucky, Kentucky Tobacco Research and Development Center: The reference cigarette, Lexington: The University of Kentucky Printing Services, 2003

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 33 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

6 Related Documents

Type	Name	Title
SOP	<i>PMI-RRP-SOP-111561</i>	Change control for CASI (<i>RDNEU</i>)
SOP	<i>PMI-RRP-SOP-111562</i>	Change control for CASI pre&post-processors (<i>RDNEU</i>)
SOP	<i>PMI-RRP-SOP-111686</i>	Perform Analysis (<i>RDNEU</i>)
User Guide	User Guide Pipeline Pilot.docx	User Guide Pipeline Pilot webport protocols, Elyette Martin, version 1.0 (available on DISCO)
Validation Report	rep_AEC_033	Validation Report (Fast Validation) Non-Targeted Differential Screening (NTDS) Assay Using Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GC×GC-TOF) September 2008, Arno Wittig, version 1.0 (available on DISCO)
WKI	<i>PMI-RRP-WKI-111700</i>	Management of pipettes (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111768</i>	Management of laboratory fridges and freezers (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111726</i>	Management of Balances (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111784</i>	Equipment Logbook creation and content (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111729</i>	Trappage des volatiles et semi-volatiles (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111801</i>	Trappage en phase particulaire pour la détermination des constituants de l'aérosol (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111814</i>	Management and labeling of chemicals (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111668</i>	Operation of a LECO Pegasus 4D GC×GC-TOFMS system (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111624</i>	User guide for CASI (<i>RDNEU</i>)
WKI	<i>PMI-RRP-WKI-111625</i>	User guide for CASI Pre&post-processors (<i>RDNEU</i>)


Table 22. Related documents.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 34 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GCxGC-TOFMS Nonpolar (RDNEU)			

7 Records

Type	Name	Title
FOR	<i>PMI-RRP-FOR-111487</i>	Chemicals, solvents, solutions and internal standard amount used for NTDS GCxGC-TOF (<i>RDNEU</i>)
FOR	<i>PMI-RRP-FOR-111488</i>	Storage of samples and study related materials for NTDS (<i>RDNEU</i>)
FOR	<i>PMI-RRP-FOR-111489</i>	Sample preparation NTDS GCxGC-TOF nonpolar (<i>RDNEU</i>)
FOR	<i>PMI-RRP-FOR-111490</i>	Data processing in ChromaTOF NTDS GCxGC-TOF nonpolar (<i>RDNEU</i>)
FOR	<i>PMI-RRP-FOR-111491</i>	Submitting metadata for CASI pre-processor, CASI, CASI post-processor (<i>RDNEU</i>)
FOR	<i>PMI-RRP-FOR-111492</i>	Sensitivity test and system suitability test NTDS GCxGC-TOF nonpolar (<i>RDNEU</i>)
FOR	<i>PMI-RRP-FOR-111496</i>	Preparation of LECO PEGASUS 4D SYSTEM for NTDS GCxGC-TOFMS (<i>RDNEU</i>)
FOR	<i>PMI-RRP-FOR-111519</i>	NTDS GCxGC-TOF-Data Management (<i>RDNEU</i>)


Table 23. Records.

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 35 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

8 Revision History

Version No.	Description of change (including reason for change)	Type of change
1.0	New version	Original issue
2.0	Addition of another instrument, minor changes in the process (improvement of the overall performance)	2
3.0	Improvements of the process by implementing the use of a pool sample, changes in data storage location (LTR)	2
<i>4.0.0</i>	<i>Content transferred in the new OMSP template. Change document names into document OMSP IDs</i>	<i>2</i>

(1. Major change/new version; 2. Minor change)

 PHILIP MORRIS INTERNATIONAL	RRP/ Testing Laboratories		Page 36 of 36
	Doc. Type: Work Instruction	Status: Production	
	Doc. ID: PMI-RRP-WKI-111620	Version N°: 4.0.0	Effective date: 28-Feb-2018
NTDS GC×GC-TOFMS Nonpolar (RDNEU)			

9 Abbreviations

Abbreviation/Definition	
2DreIRT	2 nd dimension relative retention time
ARMS	advanced request management system
CASI	computer-assisted structure identification
DCM	dichloromethane
DISCO	document improvement system customer oriented
DPM	data processing method
EDMS	electronic document management system
EI	electron ionization
FTE	full-time equivalent
GC	gas chromatography, gas chromatograph
GC×GC	comprehensive two-dimensional gas chromatography
HPC	high performance computer
ISTD	internal standard
JCAMP	joint committee on atomic and molecular physical data
LTR	long term repository
MS	Microsoft, mass spectrometer, mass spectrometry
NTDS	non-targeted differential screening
PQ	performance qualification
RIM	retention index marker
RRP	reduced risk product
S/N	signal-to-noise ratio
SOP	standard operating procedure
SST	system suitability test
TOFMS	time-of-flight mass spectrometer
TPM	total particulate matter
WKI	work instruction

Document Name: NTDS GC×GC-TOFMS Nonpolar (RDNEU)

Document Info : Document Number #:PMI-RRP-WKI-111620 Version #:4.0.0 Effective Date: 28-Feb-2018

Signature Trail :

Stage	Signed By	Signed On	Signed
QA Signature	Cecile Panighini	14-Feb-2018 13:57:30 CET	QA Approval
Author Approval	Martin Almstetter	08-Feb-2018 13:33:23 CET	Author Approval
Owner Approval	Arno Knorr	08-Feb-2018 12:16:10 CET	Owner Approval