# METHOD SUMMARIES

# NONTARGETED DIFFERENTIAL SCREENING (NTDS) USING GCXGC-TOFMS AND LC-HRAM-MS

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|---|---|
| Summary of Documents | PMI_RD_WKI_001229 'NTDS GC×GC-TOFMS Nonpolar'<br>PMI_RD_WKI_001353 'NTDS GC×GC-TOFMS Volatile'<br>PMI_RD_WKI_001354 'NTDS GC×GC-TOFMS Polar'<br>PMI_RD_WKI_001225 'NTDS LC-HR-MS PROGENESIS QI' |
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## 3 INTRODUCTION

The methods describe a set of assays 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) and liquid chromatography with high resolution accurate mass spectrometry (LC-HRAM-MS). The assays are 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. For LC-HRAM-MS a set of 4 methods have been established using reversed phase chromatography (RP) in positive and negative electrospray ionization (ESI(+/-)) and positive atmospheric pressure chemical ionization (APCI(+)) modes in addition to hydrophilic interaction chromatography (HILIC) positive electrospray ionization (ESI(+)) mode to cover a wide range of substances with different ionization and chromatographic properties.

The analytical methods compare two test items by comprehensive chemical screening and subsequent data evaluation to get summary tables with ranked chemical differences.

For GCxGC-TOFMS, 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.

For LC-HRAM-MS, the accurate mass measurements allow the determination of elemental composition for precursor ions derived from the full scan analyses. For the subsequent identification of relevant compounds, samples are measured using a data dependent fragmentation method complementary to the full scan analysis. Combining these information results in a high certainty for the proposed elemental composition of a compound and additionally identified structural features. Data acquisition is followed by advanced data processing using a data mining software that enables peak alignment, peak detection, experimental design setup, data set filtering, noise reduction, deconvolution, normalization to an internal standards and identification of compounds, creating an aligned peak table. The determination of chemical differences comprises raw data acquisition, semi-quantification based on peak area ratios, extraction of significantly different compounds and finally sorting compounds by relevance according to RANK parameters using Nonlinear Dynamics Progenesis® QI and MS Excel.

# 4 NONTARGETED DIFFERENTIAL SCREENING (NTDS) USING GCXGC-TOFMS

## 4.1 Equipment, Chemicals, Standards and References

### 4.1.1 Equipment

**GC×GC-TOFMS system 1, PMI ID 7764:**

**Table 1. GC×GC-TOFMS system 1.**

| 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 |

**GC×GC-TOFMS system 2, PMI ID 10606:**

**Table 2. GC×GC-TOFMS system 2.**

| 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 |

**GC×GC-TOFMS system 3, PMI ID 6472:**

**Table 3. GC×GC-TOFMS system 3.**

| 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 |

**Additional instruments:**

**Table 4. Additional instrumentation.**

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

### 4.1.2 Chemicals/Reagents

**Table 5. List of chemicals/solvents used for the Nonpolar method.**

| 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 6. List of chemicals/solvents used for the Polar method.**

| Name | Specification (equivalent or higher) | Supplier (or equivalent) | Product No. |
|---|---|---|---|
| Acetone | Chromasolv plus, p.a. ≥ 99.9% | Sigma-Aldrich | 650501 |
| Hexachlorobenzene | Pestanal, analytical standard | Fluka | 45522 |
| 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 Volatile method.**

| Name | Specification (equivalent or higher) | Supplier (or equivalent) | Product No. |
|---|---|---|---|
| N,N-Dimethylformamide | puriss. p.a. ≥ 99.8% | Sigma-Aldrich | 33120 |
| Hexafluorobenzene | 99% | Sigma-Aldrich | H8706 |

### 4.1.3 Internal Standard and Retention Index Marker Compounds

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

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

| 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 10. List of deuterium labeled internal standard (ISTD) compounds used for the Polar method.**

| Name | Specification (equivalent or higher) | Supplier (or equivalent) | Product No. |
|---|---|---|---|
| Furfural-d4 | ≥ 98.0% purity/atom-%d | CDN | D-2115 |
| 4-Hydroxy-4-methyl-2-pentanone-d12 | ≥ 98.0% purity/atom-%d | CDN | D-6323 |
| 5-Hydroxy-2-methyl-d3-pyridine-3,4,6-d3 | ≥ 98.0% purity/atom-%d | CDN | D-7566 |
| N-Methylnicotinamide-2,4,5,6-d4 | ≥ 99.0% purity/atom-%d | CDN | D-7642 |
| 2-Methylbutyric-d9 acid | ≥ 98.0% purity/atom-%d | CDN | D-5267 |
| 2-Methyl-2,4-pentane-d12-diol | ≥ 98.0% purity/atom-%d | CDN | D-5825 |
| Pentanenitrile-d9 | ≥ 98.0% purity/atom-%d | CDN | D-5649 |
| Phenol-d6 | ≥ 98.0% purity/atom-%d | CDN | D-29 |
| N-iso-Propyl-d7-acrylamide | ≥ 99.0% purity/atom-%d | CDN | D-6567 |
| Pyridine-d5 | ≥ 99.0% purity/atom-%d | CDN | D-85 |

**Table 11. List of retention index marker (RIM) compounds used for the Polar method.**

| Name | Specification (equivalent or higher) | Supplier (or equivalent) | Product No. |
|---|---|---|---|
| Methyl hexanoate | ≥ 99.0% purity/atom-%d | Sigma-Aldrich | 21599 |
| Methyl decanoate-d19 | ≥ 98.0% purity/atom-%d | CDN | D-5847 |
| Methyl tetradecanoate-d27 | ≥ 98.0% purity/atom-%d | CDN | D-5854 |
| Methyl hexadecanoate-d31 | ≥ 98.0% purity/atom-%d | CDN | D-1360 |
| Methyl arachidate | ≥ 99.0% purity/atom-%d | Sigma-Aldrich | 10941 |

**Table 12. List of deuterium labeled internal standard (ISTD) compounds used for the Volatile method.**

| Name | Specification (equivalent or higher) | Supplier (or equivalent) | Product No. |
|---|---|---|---|
| Acetone-d6 | ≥ 99.5% purity/atom-%d | Sigma-Aldrich | 151793 |
| Benzene-d6 | ≥ 99.5% purity/atom-%d | Sigma-Aldrich | 175870 |
| 2-Butanone-d5 | ≥ 98.0% purity/atom-%d | CDN Isotopes | D-293 |
| Butyraldehyde-d8 | ≥ 98.0% purity/atom-%d | Sigma-Aldrich | 755532 |
| Cyclohexene-d10 | ≥ 98.0% purity/atom-%d | CDN Isotopes | D-173 |
| Cyclopentane-d10 | ≥ 99.0% purity/atom-%d | CDN Isotopes | D-236 |
| 1,2-Dichloroethane-d4 | ≥ 99.0% purity/atom-%d | Sigma-Aldrich | 396540 |
| Dimethyl sulfide-d6 | ≥ 99.0% purity/atom-%d | Sigma-Aldrich | 416452 |
| Ethyl acetate-d8 | ≥ 99.0% purity/atom-%d | Sigma-Aldrich | 522899 |
| Furan-d4 | ≥ 98.0% purity/atom-%d | Sigma-Aldrich | 338753 |
| Methacrylonitrile-d5 | ≥ 97.0% purity/atom-%d | Sigma-Aldrich | 693480 |
| 3-Methylhexane-d16 | ≥ 98.0% purity/atom-%d | Sigma-Aldrich | 491470 |
| Propylene oxide-d6 | ≥ 98.0% purity/atom-%d | CDN Isotopes | D-1915 |
| Tetrahydrofuran-d8 | ≥ 99.0% purity/atom-%d | Sigma-Aldrich | 184314 |

**Table 13. List of retention index marker (RIM) compounds used for the Volatile method.**

| Name | Specification (equivalent or higher) | Supplier (or equivalent) | Product No. |
|---|---|---|---|
| n-Pentane-d12 | ≥ 98.0% purity/atom-%d | CDN Isotopes | D-28 |
| n-Hexane-d14 | ≥ 99.0% purity/atom-%d | Sigma-Aldrich | 303003 |
| n-Heptane-d16 | ≥ 99.0% purity/atom-%d | CDN Isotopes | D-1142 |

## 4.2 Procedure NTDS GCXGC-TOFMS Nonpolar

### 4.2.1 Generation of Smoke/aerosol-related Samples

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 GCxGC-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)*.

### 4.2.2 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 GCxGC-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.

### 4.2.3 Instrument Set-up and Mass Spectrometry Setting

**Table 14. Setup of the instrument for the Nonpolar method.**

| 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 (1st dimension) | 30 m DB-5ms, 0.25 mm ID, 0.25 µm $d_f$ |
| | column 2 (2nd 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)<br>initial 30.0 2.0<br>5.0 325.0 15.0 |
| | secondary oven temperature program | rate (°C/min) target temp. (°C) duration (min)<br>Initial 35.0 2.0<br>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)<br>Initial 50.0 3.0<br>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 |

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 GCxGC-TOFMS *(RDNEU)*.

#### 4.2.4 Additional Information

For details on Sensitivity Test, System Suitability Test, Preparation of Solutions and Media, Solvents, Stock Solutions of Internal Standards and Retention Index Markers, Working and Extraction Solution of ISTDs and RIMs, Number of Determinations and other information please refer to the corresponding work instruction.

### 4.3 Procedure NTDS GCXGC-TOFMS Polar

#### 4.3.1 Generation of Smoke/aerosol-related Samples

Whole smoke is collected on a Cambridge filter pad with two micro-impingers connected in series. The extraction solution is dichloromethane (DCM)/acetone (80/20, v/v) containing internal standards and retention index markers (ISTDs/RIMs$_{nonpolar}$). After extraction a liquid/liquid extraction with water is performed. A working solution (ISTD_work) of acetone containing internal standards and retention index markers (ISTDs/RIMs$_{polar}$) is added to the aqueous phase. The preparation of stock solutions and working solution is described in *PMI-RRP-FOR-111487* - Chemicals, solvents, solutions and internal standard amount used for NTDS GCxGC-TOF *(RDNEU)* and has to be documented in this form according to *PMI-RRP-WKI-113456*. Stock solutions and ISTD_work 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)*.

### 4.3.2 Sample Preparation

A step-by-step workflow for sample preparation is described and has to be documented in *PMI-RRP-FOR-111513* - Sample preparation for NTDS GCxGC-TOFMS Polar *(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 for the Nonpolar method, the aqueous phase is kept for the Polar method
- 500 µL of ISTD_work are added to the aqueous phase
- the sample is vortexed for 10 seconds
- an aliquot 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

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.

### 4.3.3 Instrument Set-up and Mass Spectrometry Setting

**Table 15. Setup of the instrument for the Polar method.**

| 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) |
| | pre-column | 2 m SLB-IL60, 0.25 mm ID, 0.20 µm $d_f$ |
| | column 1 (1st dimension) | 30 m DB-FFAP, 0.25 mm ID, 0.25 µm $d_f$ |
| | column 2 (2nd dimension) | 1.9 m VF-624ms, 0.15 mm ID, 0.84 µm $d_f$ |
| | primary oven temperature program | rate (°C/min) target temp. (°C) duration (min)<br>initial 35.0 2.0<br>5.0 250.0 23.0 |
| | secondary oven temperature program | rate (°C/min) target temp. (°C) duration (min)<br>Initial 55.0 2.0<br>4.6 285.0 16.0 |
| Transfer line | temperature | 280 °C |
| Modulator | modulator | enabled |
| | modulator temperature program | rate (°C/min) target temp. (°C) duration (min)<br>Initial 65.0 2.0<br>5.0 300.0 19.0 |
| | 2-dimension separation time | 6 s |
| | hot pulse time | 1.00 s |
| | cool time between stages | 2.00 s |
| Mass spectrometer | acquisition delay | 450 s |
| | mass range | 29-700 u |
| | data acquisition rate | 200 spectra/s |
| | detector voltage | 1450 – 2000 V |
| | electron energy | -70 V |
| | temperature ion source | 230 °C |

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 GCxGC-TOFMS *(RDNEU)*.

#### 4.3.4 Additional Information

For details on Sensitivity Test, System Suitability Test, Preparation of Solutions and Media, Solvents, Stock Solutions of Internal Standards and Retention Index Markers, Working and Extraction Solution of ISTDs and RIMs, Number of Determinations and other information please refer to the corresponding work instruction.

### 4.4 Procedure NTDS GCXGC-TOFMS Volatile

#### 4.4.1 Generation of Smoke/aerosol-related Samples

Whole smoke is collected in two micro-impingers connected in series. The extraction solution is N,N-Dimethylformamide (N,N-DMF) containing internal standards and retention index markers ($ISTDs/RIMs_{volatile}$). 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 GCxGC-TOF *(RDNEU)*. Stock solutions, ISTD_work and ISTD_extract have to be freshly prepared.

After generation of the smoke/aerosol samples 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 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)*.

#### 4.4.2 Sample Preparation

A step-by-step workflow for sample preparation is described and has to be documented in *PMI-RRP-FOR-111493* - Sample preparation NTDS GCxGC-TOF volatile *(RDNEU)*. The workflow is briefly described here point by point:

- the content of the micro-impingers (twice 10mL N,N-DMF) containing a set of internal standard and retention index marker compounds is added to a Pyrex tube
- an aliquot 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

Storage of the remaining extracts has to be documented in *PMI-RRP-FOR-111488* - Storage of samples and study related materials for NTDS *(RDNEU)*. Remaining extracts are kept until the study is closed.

### 4.4.3 Instrument Set-up and Mass Spectrometry Setting

**Table 16. Setup of the instrument for the Volatile method.**

| 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 | 2.2 ml/min (68min), 1.2 ml/min (12min) (ramped flow) |
| | column 1 (1st dimension) | 30 m DB-624UI, 0.25 mm ID, 1.4 µm $d_f$ |
| | column 2 (2nd dimension) | 2.4 m DB-FFAP, 0.10 mm ID, 0.1 µm $d_f$ |
| | primary oven temperature program | rate (°C/min) / target temp. (°C) / duration (min)<br>initial / -20.0 / 1.0<br>5.0 / -5.0 / 0.0<br>1.0 / 50.0 / 0.0<br>5.0 / 95.0 / 0.0<br>45.0 / 230.0 / 9.0 |
| | secondary oven temperature program | rate (°C/min) / target temp. (°C) / duration (min)<br>initial / 0.0 / 4.0<br>1.0 / 55.0 / 0.0<br>5.0 / 100.0 / 0.0<br>45.0 / 235.0 / 9.0 |
| Transfer line | temperature | 250 °C |
| Modulator | modulator | enabled |
| | modulator temperature offset to secondary oven | 15 °C |
| | 2-dimension separation time | 6 s |
| | hot pulse time | 1.00 s |
| | cool time between stages | 2.00 s |
| Mass spectrometer | acquisition delay | 0 s |
| | mass range | 29-500 u |
| | data acquisition rate | 200 spectra/s |
| | detector voltage | 1450 – 2000 V |
| | electron energy | -70 V |
| | temperature ion source | 230 °C |

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 GCxGC-TOFMS *(RDNEU)*.

#### 4.4.4 Additional Information

For details on Sensitivity Test, System Suitability Test, Preparation of Solutions and Media, Solvents, Stock Solutions of Internal Standards and Retention Index Markers, Working and Extraction Solution of ISTDs and RIMs, Number of Determinations and other information please refer to the corresponding work instruction.

### 4.5 Data Processing

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.

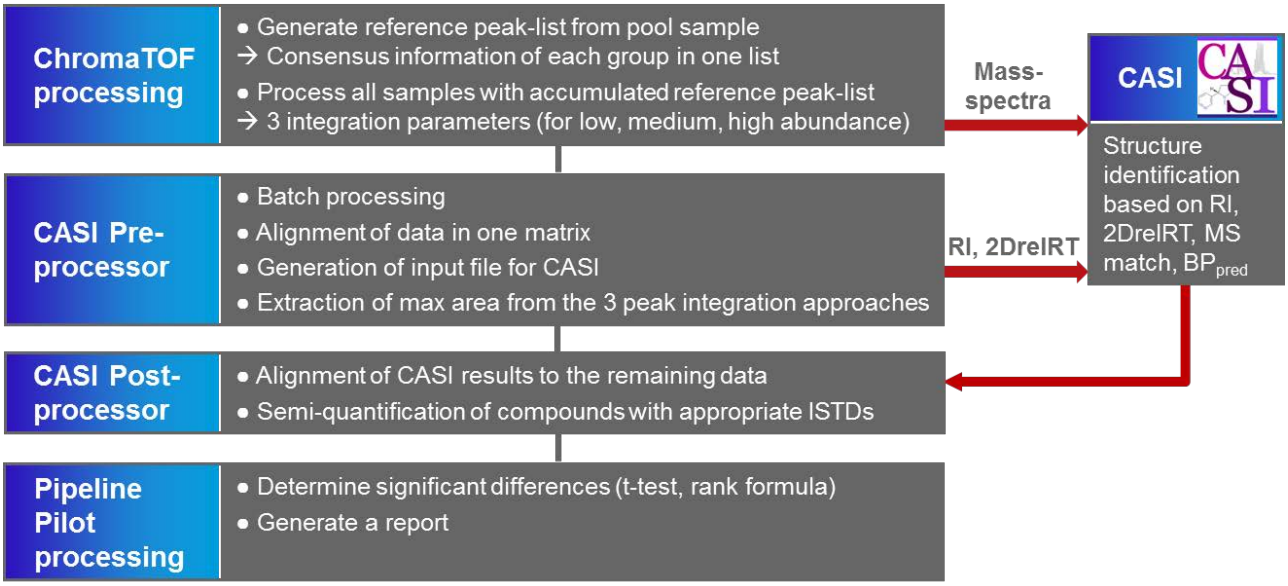


**Figure 1. Overview of the data processing steps NTDS GCXGC-TOFMS.**

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).

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 2$^{nd}$ 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.

#### 4.5.1 Data Processing using the LECO ChromaTOF Software

##### 4.5.1.1 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)
- **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)

- **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

#### 4.5.1.2 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**
- **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

Nonpolar:

- method 1: peak width 0.06 sec
- method 2: peak width 0.11 sec
- method 3: peak width 0.20 sec

Polar:

- method 1: peak width 0.07 sec
- method 2: peak width 0.12 sec
- method 3: peak width 0.23 sec

Volatile:

- method 1: peak width 0.08 sec
- method 2: peak width 0.14 sec

- method 3: peak width 0.28 sec
- keep remaining parameters consistent
- choose header "@SampleName[]"
- export all peaks , except "Contaminants / Unknowns"
- ensure export of peak information **in the right order**

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

#### 4.5.1.3 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"

### 4.5.2 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 GCxGC-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.

The JCAMP file "Library.HPJ" is generated by converting the library with lib2nist converter. Here, no naming convention is necessary.

#### 4.5.2.1 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 2DrelRT 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.

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). 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.

#### 4.5.2.2 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. 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.

#### 4.5.2.3 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. The output file of CASI Post-processor is the file "concentration.txt", which contains the final processed data.

### 4.5.3 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", version 1.0 describes all the available Pipeline Pilot webport protocols (available on DISCO).

### 4.5.4 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. Then, the maximum value of the integration results for each component will be used for further calculation. 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.

**Table 17. Assignment of compound classes to specific ISTDs for the nonpolar method.**

| 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 18. Assignment of compound classes to specific ISTDs for the polar method.**

| Compound | Case of Known Compounds | Case of Unknown Compounds |
|---|---|---|
| Furfural-d4 | internal standard for<br>• compounds with at least one ring (without nitrogen) and one carbonyl function<br>• (thio)ethers (without N-containing rings) | internal standard for compounds with 2DRT > 2.5 and ≤ 4.25 sec |
| 4-Hydroxy-4-methyl-2-pentanone-d12 | internal standard for carbonyls (without a ring) | - |
| 5-Hydroxy-2-methyl-d3-pyridine-3,4,6-d3 | internal standard for compounds with at least one N-containing ring and one oxygen-containing functional group (except amide) | - |
| N-Methylnicotinamide-2,4,5,6-d4 | internal standard for compounds with at least one ring and one amide function | - |
| 2-Methylbutyric-d9 acid | internal standard for acids, thioacids (without N-containing rings) | - |
| 2-Methyl-2,4-pentane-d12-diol | internal standard for alcohols (without a ring) | - |
| Pentanenitrile-d9 | internal standard for nitriles | internal standard for compounds with 2DRT > 4.25 sec |
| Phenol-d6 | internal standard for compounds with at least one ring and one alcohol (without N-containing rings) | internal standard for compounds with 2DRT ≤ 2.5 sec |
| N-iso-Propyl-d7-acrylamide | internal standard for compounds without a ring and at least one amide function | - |
| Pyridine-d5 | internal standard for N-containing rings without oxygen-containing functional groups | - |

**Table 19. Assignment of compound classes to specific ISTDs for the volatile method.**

| Compound | Case of Known Compounds | Case of Unknown Compounds |
|---|---|---|
| Acetone-d6 | internal standard for ketones with RI ≤ 590 | - |
| Benzene-d6 | internal standard for aromatic hydrocarbons | - |
| 2-Butanone-d5 | internal standard for ketones with RI > 590 | - |
| Butyraldehyde-d8 | internal standard for aldehydes | - |
| Cyclohexene-d10 | internal standard for unsaturated cyclic hydrocarbons | internal standard for compounds with 2DrelRT ≤ 1.173 (in general corresponds to nonpolar unknowns) |
| Cyclopentane-d10 | internal standard for saturated cyclic hydrocarbons | - |
| 1,2-Dichloroethane-d4 | internal standard for Cl-containing compounds | - |
| Dimethyl sulfide-d6 | internal standard for S-containing compounds | - |
| Ethyl acetate-d8 | internal standard for esters | internal standard for compounds with 2DrelRT > 1.173 and ≤ 1.788 (in general corresponds to nonpolar to medium polar unknowns) |
| Furan-d4 | internal standard for compounds with at least one ether function and at least one double or aromatic bond | - |
| Methacrylonitrile-d5 | internal standard for N-containing compounds | internal standard for compounds with 2DrelRT > 1.788 (in general corresponds to medium polar to medium polar aromatic or basic unknowns) |
| 3-Methylhexane-d16 | internal standard for branched hydrocarbons (saturated and unsaturated) | - |
| Propylene oxide-d6 | internal standard for ethers (cyclic and non-cyclic), no double or aromatic bond, RI ≤ 600 | - |
| Tetrahydrofuran-d8 | internal standard for ethers (cyclic and non-cyclic), no double or aromatic bond, RI > 600 | - |
| n-Hexane-d14 | retention-index marker 2, internal standard for linear hydrocarbons (saturated and unsaturated) | retention-index marker 2 |

### 4.5.5 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

### 4.5.6 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)).

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

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

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

The data set is divided into positive (Lx > Ly) and negative (Lx < 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.

### 4.6 Additional Information

For details on Calculation of the Second Dimension Relative Retention Time and other information please refer to the corresponding work instruction.

## 5 NONTARGETED DIFFERENTIAL SCREENING (NTDS) USING LC-HRAM-MS

### 5.1 Material, Equipment, Chemicals, Standards and References

5.1.1 Materials

**Table 20. Materials for NTDS LC-HRAM-MS**

| Identity | Specification | Supplier (or equivalent) | Product No. (or equivalent) |
|---|---|---|---|
| Guard Cartridge Holder RP | Thermo UHPLC Guard Cartridge Holder, 10 mm, 2.1 mm ID | Thermo Scientific | 852-00 |
| Guard Cartridge RP | Thermo UHPLC Filter Cartridge, 0.2 µm, 2.1 mm ID | Thermo Scientific | 22180 |
| Column RP | Hypersil GOLD™ (150 × 2.1 mm, 1.9 µm) | Thermo Scientific | 25002-152130 |
| Defender Guard HILIC | Thermo Defender Guard HILIC 10 mm, 2.6µm | Thermo Scientific | 17526-012105 |
| Column HILIC | Accucore HILIC™ (150 x 2.1 mm, 2.6µm) | Thermo Scientific | 17526-152130 |
| Weighing Funnel | Glass Weighing Funnel 65 mm | Fisher Scientific | 11910787 |
| Silanized vials | Silanized 2 mL Amber ID 9 mm X100 | Fisher Scientific | 15388066 |
| Pyrex® Tube | Pyrex® glass culture tube 18 mm x 100 mm | Sigma Aldrich | Z653616 |
| Glass fiber frit | Glass fiber frit 15 mm | Sigma Aldrich | 21537-U |

### 5.1.2 Equipment

**Table 21. Equipment for NTDS LC-HRAM-MS**

| Instrument | Instrument - ID | WKI | Instrument Logbook-ID |
|---|---|---|---|
| Mass spectrometer QExactive™ with UHPLC | QExactive™ LC-HRAM-MS System | *PMI-RRP-WKI-111570* | PMI011636 |
| Mass spectrometer QExactive™ with UHPLC | QExactive™ LC-HRAM-MS System | *PMI-RRP-WKI-111570* | PMI003642 |
| Mass spectrometer QExactive™ Plus with UHPLC | QExactive™ Plus LC-HRAM-MS System | *PMI-RRP-WKI-111570* | PMI009323 |
| Analytical Balance | Mettler Toledo XP205 | *PMI-RRP-WKI-111726* | PMI003489 |
| Centrifuge | Beckman Coulter Allegra XR-22 | N/A | PMI000980 |
| Thermo Mixer | Eppendorf ThermoMixer C | N/A | - |

### 5.1.3 Chemicals

**Table 22. Chemicals for NTDS LC-HRAM-MS**

| Name | Specification/Purity | Supplier (or equivalent) | Product No. (or equivalent) |
|---|---|---|---|
| Ammonium acetate | Eluent additive for LC-MS ≥99.0% | Fluka | 73594 |
| Ammonium fluoride | Eluent additive for LC-MS ≥98.0% | Fluka | 52481 |
| Acetonitrile | LC-MS CHROMASOLV® | Sigma-Aldrich | 34967 |
| Methanol | LC-MS CHROMASOLV® | Sigma-Aldrich | 34966 |

(table continues)

| Name | Specification/Purity | Supplier (or equivalent) | Product No. (or equivalent) |
|---|---|---|---|
| Water | LC-MS CHROMASOLV® | Sigma-Aldrich | 39253 |
| ProteoMass™ LTQ/FT-Hybrid ESI Pos. Mode Cal Mix | N/A | Supelco | MSCAL5 |
| ProteoMass™ LTQ/FT-Hybrid ESI Neg. Mode Cal Mix | N/A | Supelco | MSCAL6 |
| Mucasol | N/A | Fisher Scientific | 10729301 |

5.1.4 Standards and References

**Table 23. Standards and References NTDS LC-HRAM-MS**

| Name | Abbreviation | Specification/Purity | Supplier (or equivalent) | Product No. (or equivalent) |
|---|---|---|---|---|
| Decanoic-d19 acid | DA | ≥98.0 atom%-d | CDN Isotopes | D-1616 |
| Diisobutyl Phthalate-d4 | DP | ≥98.0 atom%-d | TRC Canada | D455212 |
| Ethyl Nicotinate-d4 | EN | ≥98.0 atom%-d | TRC Canada | E925128 |
| Isonicotinamide-2,3,5,6-d4 | IN | ≥98.0 atom%-d | CDN Isotopes | D-6004 |
| d8-Isophorone | IP | ≥98.0 atom%-d | CDN Isotopes | D-2304 |
| isoquinoline-d7 | IQ | ≥98.0 atom%-d | TRC Canada | D-904 |
| Methyl Linoleate-d3 | ML | ≥98.0 atom%-d | TRC Canada | M265192 |
| Myosmine-2,4,5,6-d4 | MY | ≥98.0 atom%-d | TRC Canada | M835010 |
| (±) Nicotine-d7 | NI | ≥98.0 atom%-d | CDN Isotopes | D-6500 |
| Nicotine-1'-Oxide-d3 | NO | ≥98.0 atom%-d | TLC Pharmachem | N-0641 |
| α-tocopherol-d6 | TP | ≥95.0 atom%-d | TRC Canada | T526127 |
| β-Sitosterol-d7 | SI | ≥98.0 atom%-d | TRC Canada | S497052 |

## 5.2 Procedure

### 5.2.1 Sample Collection/Generation: TPM/NFDPM

The crude condensate (TPM/NFDPM amount: approximately 100 to 150 mg) is collected on a Cambridge filter (GF) according to a procedure described in PMI-RRP-WKI-111801. After sample generation the GF is stored in a cleaned (3 times methanol rinsed and dried) Pyrex® tube. Prior to sample preparation the sample is stored at -20 ± 5 °C. Storage details are recorded using form *PMI-RRP-FOR-111506*.

All aerosol samples are generated in triplicate (3 x RP, 3 x HILIC) unless otherwise specified in the study plan.

### 5.2.2 Sample Collection/Generation: Cryogenically Trapped Mainstream Aerosol (Cold Trap)

Mainstream aerosol is trapped using cryogenic trapping (Cold Trap) at -200 °C according to *PMI-RRP-WKI-111626* using inverse mode. Each replicate consists of the accumulated trapped whole aerosol from 2 sticks/cigarettes, which will be subsequently extracted with 2 times 5 mL methanol (for RP chromatography). When P4 aerosol is collected the cold trap is extracted with a single addition of 5 mL extraction solvent. For HILIC chromatography mode, the trapped whole aerosol is extracted with 2 times 5 mL acetonitrile. The extract solution will be provided to the aerosol generation lab on the day of aerosol generation. The two 5 mL extraction volumes, per replicate, may be combined in a single pre-rinsed glass vessel.

All aerosol samples are generated in triplicate (3 x RP, 3 x HILIC).

In addition to the ARMS request, a detailed aerosol generation description must be recorded using form *PMI-RRP-FOR-111314*.

### 5.2.3 Sample Collection/Generation: Blank Samples

In order to exclude background impurities which might be assimilated during sample generation, blank samples must be generated for each type of sample generation. For TPM/NFDPM samples, blanks are generated prior to sample generation by using the actual aerosol collection setup intended for TPM/NFDPM generation (incl. GF) but drawing air instead of using a stick/cigarette. The blank GF is handled like an aerosol sample. For cold trap samples, blanks are generated prior to sample generation by using the actual aerosol collection setup intended for cryogenic trapping but drawing air instead of using a stick/cigarette. The cold trap blanks are handled in the same way an aerosol sample.

All blank samples are generated in single replicates (1 x RP, 1 x HILIC).

### 5.2.4 Sample Preparation: TPM/NFDPM, Collected on Glass Fiber Filter Pad (GF)

TPM/NFDPM, collected on a filter pad, is extracted with 10 mL of extraction solvent (methanol for RP, acetonitrile for HILIC). The extraction solvent is added to the Pyrex® tube containing the GF and the filter is extracted by thoroughly shaking the Pyrex® tube (disintegrating the GF), vortexing for 5 min and finally centrifuging (4500 g, 5 min, 10 °C). The extract is then filtered using a glass fiber frit to avoid any transfer of glass fiber particles. An aliquot (200 µL) of the TPM/NFDPM extract is transferred into a silanized chromatographic vial and diluted with methanol (700 µL) for RP analysis or acetonitrile (700 µL) for HILIC analysis. 100 µL of *WS $IS_1$* internal standard solution has to be added. The vial is sealed and then mixed using an Eppendorf ThermoMixer (5 min, 5 °C, 2000 rpm). An aliquot (1.5 µL) of the diluted extract is injected and analyzed by LC-HRAM-MS in full scan mode and in data-dependent fragmentation mode for compound identification. Each diluted extract will be analyzed 5 times (analytical replicates) in both full scan mode and data-dependent fragmentation mode. All steps for sample preparation are recorded using *form PMI-RRP-FOR-111504*. Storage details are recorded using form *PMI-RRP-FOR-111506*.

- TPM/NFDPM collected on pad is extracted with 10 mL extraction solvent (methanol for RP, acetonitrile for HILIC)
- Thoroughly shaking (disintegrating the GF)
- Vortex for 5 min
- Centrifugation (4500 g, 5 min, 10 °C)
- Transfer of extract in new vial using glass fiber frit
- Pipette 200 µL extract in silanized HPLC vial
- Dilute with 700 µL methanol for RP analysis or 700 µL acetonitrile for HILIC analysis
- Add 100 µL *WS $IS_1$* internal standard solution
- Mixing the closed vial using ThermoMixer (5 min, 5 °C, 2000 rpm)

### 5.2.5 Sample Preparation: Cryogenically Trapped Mainstream Aerosol (Cold Trap)

An aliquot (200 µL) of the combined (2 x 5 mL) cold trap extract will be transferred into a silanized chromatographic vial and diluted with methanol (700 µL) for RP analysis or acetonitrile (700 µL) for HILIC analysis. 100 µL of *WS $IS_1$* internal standard solution has to be added. After vial closure the sample are mixed for 5 minutes using an Eppendorf ThermoMixer (5 °C; 2000 rpm). An aliquot (1.5 µL) of the diluted extract will be injected and analyzed by LC-HRAM-MS in full scan mode and in data-dependent fragmentation mode for compound identification. Each diluted extract will be analyzed 5 times (analytical replicates) in both full scan mode and data-dependent fragmentation mode.

- Pipette 200 µL extract in silanized HPLC vial
- Dilute with 700 µL methanol for RP analysis or 700 µL acetonitrile for HILIC analysis
- Add 100 µL WS IS1 internal standard solution
- Mixing the closed vial using ThermoMixer (5 min, 5 °C, 2000 rpm)

### 5.2.6 Sample Preparation: Blank Sample

An aliquot (200 µL) of the blank is diluted with methanol (700 µL) for RP analysis or acetonitrile (700µL) for HILIC analysis in a silanized autosampler vial. 100 µL of *WS IS1* internal standard solution has to be added. The closed vial is mixed for 5 minutes at 5 °C using an Eppendorf ThermoMixer set at 2000 rpm.

- Pipette 200 µL blank sample in silanized HPLC vial
- Dilute with 700 µL methanol for RP analysis or 700 µL acetonitrile for HILIC analysis
- Add 100 µL WS IS1 internal standard solution

Mixing the closed vial using ThermoMixer (5 min, 5 °C, 2000 rpm)

### 5.2.7 Sample Storage/Stability

**Table 24. Sample Storage and Stability**

| Identity | Storage | Stability | Comments |
|---|---|---|---|
| Methanol-TPM-Extract | -20°C | 1 month | In a 3 times pre rinsed vial |
| Methanol-TPM-Extract | 5°C | 1 week | In cooled autosampler tray |
| Methanol-Cold Trap-Extract | -20°C | 1 month | In a 3 times pre rinsed vial |
| Methanol-Cold Trap-Extract | 5°C | 1 week | In cooled autosampler tray |
| Acetonitrile-TPM-Extract | -20°C | 1 month | In a 3 times pre rinsed vial |
| Acetonitrile -TPM-Extract | 5°C | 1 week | In cooled autosampler tray |
| Acetonitrile -Cold Trap-Extract | -20°C | 1 month | In a 3 times pre rinsed vial |
| Acetonitrile -Cold Trap-Extract | 5°C | 1 week | In cooled autosampler tray |

Storage details are recorded using form *PMI-RRP-FOR-111506*.

### 5.2.8 Instrument Set-up and Mass Spectrometry Setting

#### 5.2.8.1 HPLC Reversed Phase Mode (RP) ESI positive

**Table 25 . HPLC RP Gradient for ESI positive mode**

| Time [min] | Mobile phase A [%] 10mM $NH_4AC$ in water | Mobile phase B [%] 1mM $NH_4AC$ in methanol |
|---|---|---|
| 0 | 85 | 15 |
| 7.00 | 10 | 90 |
| 12.80 | 0 | 100 |
| 18.00 | 0 | 100 |
| 18.10 | 85 | 15 |
| 20.00 | 85 | 15 |

**Table 26. HPLC RP Parameters for ESI positive mode**

| Parameter | Value |
|---|---|
| Precolumn | Thermo UHPLC Filter Cartridge, 0.2 µm, 2.1 mm ID |
| Column | Hypersil GOLD™ (150 × 2.1 mm, 1.9 µm) |
| Mobile phase A | 10mM $NH_4AC$ in water |
| Mobile phase B | 1mM $NH_4AC$ in methanol |
| Flow [µL/min] | 400 |
| Column compartment temperature [°C] | 50 |
| Injection Volume [µL] | 1.5 |
| Autosampler Temperature [°C] | 5 |

**Table 27. Detector Settings for RP ESI positive**

| Parameter | Value |
|---|---|
| **General Parameter** | |
| MS Run Time [min] | 20.00 |
| Detector/Analyzer | HRAM Orbitrap |
| In-source CID [eV] | Off (0.0 eV) |
| Default Charge State | 1 |
| Mode/Type | Full Scan MS / dd-$MS^2$ (TopN) |
| Polarity | positive |
| **Full MS** | |
| Microscans | 1 |
| Resolution | 70000 |
| AGC Target | 3e6 |
| Maximum IT [ms] | 100 |
| Scan Range [Da] | 80 – 800 |
| Data Type | Profile |
| **dd-MS2 (TopN)** | |
| Microscans | 1 |
| Resolution | 17500 |
| AGC Target | 1e5 |
| Maximum IT [ms] | 150 |
| Loop Count | 3 |
| TopN | 3 |
| Isolation Window [m/z] | 4 |
| Scan Range [Da] | 80 - 800 |
| Stepped NCE [eV] | 25, 50, 75 |
| **dd Settings** | |
| Underfill Ratio [%] | 1.00 |
| Intensity Threshold | 6.7e3 |
| Apex Trigger | Off |
| Dynamic Exclusion [s] | 10 |

**Table 28. Global Ion Source Settings for RP ESI positive**

| Parameter | Value |
|---|---|
| Vaporizer Heater Temperature [°C] | 350 |
| Sheath Gas Flow Rate [arb.] | 60 |
| Aux Gas Flow Rate [arb.] | 20 |
| Sweep Gas Flow Rate [arb.] | 0 |
| Spray Voltage [kV] | 3.00 |
| Capillary Temp [°C] | 380 |
| S-Lens RF Level [%] | 55 |

5.2.8.2 HPLC Reversed Phase Mode (RP) APCI positive

**Table 29. HPLC RP Gradient for APCI positive mode**

| Time [min] | Mobile phase A [%]<br>10mM $NH_4AC$ in water | Mobile phase B [%]<br>1mM $NH_4AC$ in methanol |
|---|---|---|
| 0 | 85 | 15 |
| 7.00 | 10 | 90 |
| 12.80 | 0 | 100 |
| 18.00 | 0 | 100 |
| 18.10 | 85 | 15 |
| 20.00 | 85 | 15 |

**Table 30. HPLC RP Parameters for APCI positive mode**

| Parameter | Value |
|---|---|
| Precolumn | Thermo UHPLC Filter Cartridge, 0.2 µm, 2.1 mm ID |
| Column | Hypersil GOLD™ (150 × 2.1 mm, 1.9 µm) |
| Mobile phase A | 10mM $NH_4AC$ in water |
| Mobile phase B | 1mM $NH_4AC$ in methanol |
| Flow [µL/min] | 400 |
| Column compartment temperature [°C] | 50 |
| Injection Volume [µL] | 1.5 |
| Autosampler Temperature [°C] | 5 |

**Table 31. Detector Settings for RP APCI positive**

| Parameter | Value |
|---|---|
| **General Parameter** | |
| MS Run Time [min] | 20.00 |
| Detector/Analyzer | HRAM Orbitrap |
| In-source CID [eV] | Off (0.0 eV) |
| Default Charge State | 1 |
| Mode/Type | Full Scan MS / dd-$MS^2$ (TopN) |
| Polarity | positive |
| **Full MS** | |
| Microscans | 1 |
| Resolution | 70000 |
| AGC Target | 3e6 |
| Maximum IT [ms] | 100 |
| Scan Range [Da] | 80 – 800 |
| Data Type | Profile |
| **dd-MS2 (TopN)** | |
| Microscans | 1 |
| Resolution | 17500 |
| AGC Target | 1e5 |
| Maximum IT [ms] | 150 |
| Loop Count | 3 |
| TopN | 3 |
| Isolation Window [m/z] | 4 |
| Scan Range [Da] | 80 - 800 |
| Stepped NCE [eV] | 25, 50, 75 |
| **dd Settings** | |
| Underfill Ratio [%] | 1.00 |
| Intensity Threshold | 6.7e3 |
| Apex Trigger | Off |
| Dynamic Exclusion [s] | 10 |

**Table 32. Global Ion Source Settings for RP APCI positive**

| Parameter | Value |
|---|---|
| Vaporizer Temperature [°C] | 450 |
| Sheath Gas Flow Rate | 50 |
| Aux Gas Flow Rate | 5 |
| Sweep Gas Flow Rate | 0 |
| Discharge Current [µA] | 5.0 |
| Capillary Temp [°C] | 380 |
| S-Lens RF Level [%] | 55 |

5.2.8.3 HPLC Reversed Phase Mode (RP) ESI negative

**Table 33. HPLC RP Gradient for ESI negative mode**

| Time [min] | Mobile phase A [%] 1mM $NH_4F$ in water | Mobile phase B [%] methanol |
|---|---|---|
| 0 | 85 | 15 |
| 7.00 | 10 | 90 |
| 12.80 | 0 | 100 |
| 18.00 | 0 | 100 |
| 18.10 | 85 | 15 |
| 20.00 | 85 | 15 |

**Table 34. HPLC RP Parameters for ESI negative mode**

| Parameter | Value |
|---|---|
| Precolumn | Thermo UHPLC Filter Cartridge, 0.2 µm, 2.1 mm ID |
| Column | Hypersil GOLD™ (150 × 2.1 mm, 1.9 µm) |
| Mobile phase A | 1mM $NH_4F$ in water |
| Mobile phase B | methanol |
| Flow [µL/min] | 400 |
| Column compartment temperature [°C] | 50 |
| Injection Volume [µL] | 1.5 |
| Autosampler Temperature [°C] | 5 |

**Table 35. Detector Settings for RP ESI negative**

| Parameter | Value |
|---|---|
| **General Parameter** | |
| MS Run Time [min] | 20.00 |
| Detector/Analyzer | HRAM Orbitrap |
| In-source CID [eV] | Off (0.0 eV) |
| Default Charge State | 1 |
| Mode/Type | Full Scan MS / dd-$MS^2$ (TopN) |
| Polarity | negative |
| **Full MS** | |
| Microscans | 1 |
| Resolution | 70000 |
| AGC Target | 3e6 |
| Maximum IT [ms] | 100 |
| Scan Range [Da] | 80 – 800 |
| Data Type | Profile |
| **dd-MS2 (TopN)** | |
| Microscans | 1 |
| Resolution | 17500 |
| AGC Target | 1e5 |
| Maximum IT [ms] | 150 |
| Loop Count | 3 |
| TopN | 3 |
| Isolation Window [m/z] | 4 |
| Scan Range [Da] | 80 - 800 |
| Stepped NCE [eV] | 25, 50, 75 |
| **dd Settings** | |
| Underfill Ratio [%] | 1.00 |
| Intensity Threshold | 6.7e3 |
| Apex Trigger | Off |
| Dynamic Exclusion [s] | 10 |

**Table 36. Global Ion Source Settings for RP ESI negative**

| Parameter | Value |
|---|---|
| Vaporizer Heater Temperature [°C] | 350 |
| Sheath Gas Flow Rate [arb.] | 60 |
| Aux Gas Flow Rate [arb.] | 20 |
| Sweep Gas Flow Rate [arb.] | 0 |
| Spray Voltage [kV] | 3.00 |
| Capillary Temp [°C] | 380 |
| S-Lens RF Level [%] | 55 |

5.2.8.4 HPLC Hydrophilic Interaction Mode (HILIC)

**Table 37. HPLC HILIC Gradient**

| Time [min] | Mobile phase A [%] 10mM $NH_4AC$ in water | Mobile phase B [%] 10mM $NH_4AC$ in acetonitrile |
|---|---|---|
| 0 | 2 | 98 |
| 7.00 | 25 | 75 |
| 8.00 | 2 | 98 |
| 15.00 | 2 | 98 |

**Table 38. HPLC HILIC Parameters**

| Parameter | Value |
|---|---|
| Precolumn | Thermo Defender Guard HILIC 10 mm, 2.6µm |
| Column | Accucore HILIC™ (150 x 2.1 mm, 2.6µm) |
| Mobile phase A | 10mM $NH_4AC$ in water |
| Mobile phase B | 10mM $NH_4AC$ in acetonitrile |
| Flow [µL/min] | 500 |
| Column compartment temperature [°C] | 50 |
| Injection Volume [µL] | 1.5 |
| Autosampler Temperature [°C] | 5 |

**Table 39. Detector Settings for HILIC ESI positive**

| Parameter | Value |
|---|---|
| **General Parameter** | |
| MS Run Time [min] | 15.00 |
| Detector/Analyzer | HRAM Orbitrap |
| In-source CID [eV] | Off (0.0 eV) |
| Default Charge State | 1 |
| Mode/Type | Full Scan MS / dd-$MS^2$ (TopN) |
| Polarity | positive |
| **Full MS** | |
| Microscans | 1 |
| Resolution | 70000 |
| AGC Target | 3e6 |
| Maximum IT [ms] | 100 |
| Scan Range [Da] | 80 – 800 |
| Data Type | Profile |
| **dd-MS2 (TopN)** | |
| Microscans | 1 |
| Resolution | 17500 |
| AGC Target | 1e5 |
| Maximum IT [ms] | 150 |
| Loop Count | 3 |
| TopN | 3 |
| Isolation Window [m/z] | 4 |
| Scan Range [Da] | 80 - 800 |
| Stepped NCE [eV] | 25, 50, 75 |
| **dd Settings** | |
| Underfill Ratio [%] | 1.00 |
| Intensity Threshold | 6.7e3 |
| Apex Trigger | Off |
| Dynamic Exclusion [s] | 10 |

**Table 40. Global Ion Source Settings for HILIC ESI positive**

| Parameter | Value |
|---|---|
| Vaporizer Heater Temperature [°C] | 350 |
| Sheath Gas Flow Rate [arb.] | 60 |
| Aux Gas Flow Rate [arb.] | 20 |
| Sweep Gas Flow Rate [arb.] | 0 |
| Spray Voltage [kV] | 3.00 |
| Capillary Temp [°C] | 380 |
| S-Lens RF Level [%] | 55 |

#### 5.2.9 Additional Information

For details on Preparation of Chemicals, Solvents and Solutions, Internal Standard Stock Solutions (IS0), Working Solution for Internal Standards (WS IS1), System Suitability Test (SST) and other information please refer to the corresponding work instruction.

### 5.3 Procedure NTDS LC-HRAM-MS

#### 5.3.1 Sample Analysis

Processed and prepared samples are injected in 5 replicates via autosampler. Data acquisition is performed by means of Xcalibur™ software and the MS acquisition parameters listed. A sequence must be generated as shown in the following example and recorded using form *PMI-RRP-FOR-111502*.

#### 5.3.2 Sequence Generation

At the beginning of a sequence 3 dummy samples should be injected in order to have an appropriate conditioning of the ion source. Following the dummy samples, the SST samples should be injected. The sequence generated on the data acquisition computer using Xcalibur™ software must be printed and verified by signature of an independent analyst in terms of naming convention and sample order. The naming convention for samples should include either the ARMS request ID, PDIMS ID or the LIMS ID of the respective sample plus the replicate ID (e.g. P1_S2014121207-02_1, P1_S2014121207-02_2, P1_S2014121207-02_3, etc.) The following sequence should be used as an example.

**Table 41. Example of Sequence Order NTDS LC-HRAM-MS**

| Sample Number | Sample Name |
|---|---|
| 1 | Dummy 1 |
| 2 | Dummy 2 |
| 3 | Dummy 3 |
| 4 | Solvent Blank 1 |
| 5 | SST 1 |
| 6 | SST 2 |
| 7 | SST 3 |
| 8 | Blank 1 |
| 9 | Test Item 1 |
| 10 | Reference Item 1 |
| 11 | Solvent Blank 2 |
| 12 | Blank 2 |
| 13 | Test Item 2 |
| 14 | Reference Item 2 |
| 15 | Solvent Blank 3 |
| 16 | Blank 3 |
| 17 | Test Item 3 |
| 18 | Reference Item3 |
| 19 | Solvent Blank 4 |
| 20 | Blank 4 |
| 21 | Test Item 4 |
| 22 | Reference Item 4 |
| 23 | Solvent Blank 5 |
| 24 | Blank 5 |
| 25 | Test Item 5 |
| 26 | Reference Item 5 |

### 5.3.3 Data Evaluation

The data evaluation process consists of several steps:

- Data Transfer and Storage
- Data import into metabolomics data mining software Nonlinear Dynamics Progenesis® QI

- Alignment
- Experimental design setup (defining one or more groups for aligned runs)
- Peak picking
- Normalization using internal standards
- Deconvolution
- Compound identification (accurate mass and adduct search against database)
- Compound review (managing compound identities and exploring identities and expression between conditions)
- Processing of aligned and normalized (csv-)dataset with EXCEL
- Semi-quantification of compounds
- extraction of obviously different compounds (compounds of interest)
- sorting of information according to RANK parameters
- manual verification of results

The required steps for data evaluation are recorded using form *PMI-RRP-FOR-111505*.

#### 5.3.3.1 Data Transfer and Storage

The acquired data (sequence file, raw data and acquisition method) on the respective data acquisition workstation are transferred to the backed up and secured server environment. Once the raw data are successfully copied and the transfer verified by checking the original amount of files and size vs. the actual copied amount of files and size, the raw data on the data acquisition unit are deleted. On the data evaluation workstation the same folder structure is created and the raw data are copied to the local hard disk drive.

On the data evaluation workstation, all data evaluations are performed and stored using a separate secondary raw data (processed data) folder.

After completion of data evaluation, processed data are transferred to the backed up and secured server environment.

All data transfer steps are recorded using form *PMI-RRP-FOR-111511*.

#### 5.3.3.2 Data Import (Progenesis® QI)

Prior to importing raw data, a new project is created in Progenesis® QI by selecting *file/new Create New Experiment*. A new Experiment/Project name is then created, defined by the naming convention considering ARMS request ID, PDIMS ID or the LIMS ID, the method and the storage location. Next, the type of machine (high resolution mass spectrometer), the data format (profile data) and the respective ionization mode (positive or negative) are selected.

Any possible adducts are selected in the *Create New Experiment* window and confirmed by clicking *Create experiment*.

For a more detailed description refer to corresponding work instruction.

#### 5.3.3.3 Raw Data Processing by Metabolomics Software Progenesis ® QI

##### *5.3.3.3.1 Alignment*

The alignment is performed separately for each ionization mode data set. In order to increase the alignment accuracy, an alignment reference vector (i.e. internal standard) is selected by clicking *Vector editing*. The m/z value and retention time of the respective internal standard per ionization mode is selected. By selecting *Align runs automatically* the alignment procedure starts. After successful alignment the section is complete. By pressing *Section Complete* the experimental design setup is initiated.

##### *5.3.3.3.2 Experimental Design Setup*

The acquired / processed raw data must be divided into sample groups (e.g. P1, 3R4F, Pool Samples, Blank). The raw data are grouped according to sample origin. By pressing *Section Complete* the peak picking procedure is initiated.

##### *5.3.3.3.3 Peak Picking*

Default values are used for all peak picking parameters. By pressing *Start peak picking* the automatic peak picking starts. The raw files are analyzed and the peaks are picked for all samples in parallel. If the software picks a peak in one sample the software is looking for the same peak in all other samples. Normalization is initiated by clicking *Review normalization.*

##### *5.3.3.3.4 Normalization*

The *Normalize to set of housekeeping compounds* method is selected for normalization. The table can be sorted according to the m/z values and the internal standard (m/z and retention time) should be selected according to the respective ionization mode. This step is a prerequisite for further semi-quantification.

##### *5.3.3.3.5 Compound Identification*

Compounds are identified based on the accurate mass, isotopic similarity and detected adducts, retention time compared to reference database, $MS^2$ fragments compared to theoretical fragmentation and $MS^2$ compared to reference database. The compound identification comprises the following *4* steps.

- Compound Identification using UCSD Database Fragmentation (Step I)

The first identification step comprises the accurate mass comparison of the acquired data against the in-house UCSD database via MetaScope algorithm (Precursor tolerance: 5ppm). In addition the compound retention times are compared with the in-house retention time database (Retention within 0.5 minutes). All acquired MS2 spectra are compared against in-house MS2 library (Fragment tolerance: 10 ppm).

- Compound Identification using UCSD Theoretical Fragmentation (Step II)

The second identification step comprises the theoretical fragmentation of the in-house UCSD database via MetaScope algorithm and MetFrag algorithm. The actual acquired MS2 spectra are matched against in-silico (theoretical) fragmented spectra of the UCSD candidate compounds (Fragment tolerance: 10 ppm).

- Compound Identification using HMDB Theoretical Fragmentation (Step III)

The third identification step comprises the theoretical fragmentation of the HMDB database via MetaScope algorithm and MetFrag algorithm. The actual acquired $MS^2$ spectra are matched against in-silico (theoretical) fragmented spectra of the HMDB candidate compounds (Fragment tolerance: *10* ppm).

- Compound Identification using ChemSpider Theoretical Fragmentation (*Step IV*)

The sixth identification step comprises the theoretical fragmentation of ChemSpider with ChemIDplus, FDA and NIST as connected data sources via MetFrag algorithm. The actual acquired $MS^2$ spectra are matched against in-silico (theoretical) fragmented spectra of the ChemIDplus, FDA [17] *and NIST* candidate compounds. The elemental composition comprises H: 0-200, C: 0-100, N: 0-10, O: 0-30, P: 0-2 and S: 0-2 is considered as filter.

#### *5.3.3.3.6 Compound Review*

The results retrieved from compound identification must be reviewed. Each line is checked regarding compound abundance, detected adducts, fragmentation score, retention time score, isotope similarity, mass error and overall score. The proposed identification is a useful guide. However, if definitive compound confirmation is required it has to be performed using reference standards matched with fragmentation and retention time.

After completion of compound review all measurements incl. e.g. compound, m/z, retention times, and normalized abundance are exported as a .csv file using *File/Export compound measurements*. The file storage location has to be specified (secondary raw data folder within a project folder).

### 5.3.4 Semi-quantification of Compounds

Although normalization of the peak abundances against an internal standard is performed within Progenesis® QI, semi-quantification of compounds is calculated using EXCEL. Semi-quantification is performed on the basis of peak area ratios between the analytes and an appropriate internal standard (of known concentration), which is chosen depending upon the ionization mode used.

The results are semi-quantitative, since no calibration is performed. The ability for compounds to ionize varies strongly in APCI positive since it is a soft ionization technique, and thus values derived should only be used as a rough estimation of abundance.

If quantitative data are requested by the customer, the respective analytes will be calibrated using a reference substance.

### 5.3.5 Extraction of Obviously Different Compounds (Compounds of Interest)

The processing of aligned and normalized (csv-)data sets for extraction of obviously different compounds (compounds of interest) and filtering of information according to relevance-criteria is performed using EXCEL.

The extraction of obviously different compounds (variables) is carried out by applying a t-test to the data set (2 groups, 5 replicates = 10 observations/variable).

**TTEST(data set Lx, data set Ly, tails, type)**

tails = 2; two-tailed distribution

type = 3; heteroscedastic

Results that yield p values > 0.05 are not considered statistically different and are therefore excluded from further analysis.

### 5.3.6 Sorting of Information According to RANK Parameter

To consider the relevance of each compound, the compounds are ranked according to the relative difference in abundance and the semi-quantitatively estimated absolute abundance using MS Excel.

The sorting of obviously different compounds (variables) by their relevance is done by applying an empirically developed formula (RANK) on the t-test filtered data sets. The relevance of a constituent considers the relative difference of the abundance of the compound as well as the semi-quantitatively estimated absolute abundance (i.e., the greater the difference and absolute abundance, the greater the relevance). For more details on the RANK equation, refer to section NTDS GCXGC-TOFMS.

## ABBREVIATIONS

| Abbreviation/Definition (GCXGC-TOFMS) | |
|---|---|
| 2DrelRT | 2nd 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 |

| Abbreviation/Definition (LC-HRAM-MS) | |
|---|---|
| APCI | Atmospheric Pressure Chemical Ionization |
| API | Atmospheric Pressure Ionization |
| ARMS | Advanced Request Management System |
| Cal Mix | Calibration Solution |
| CC | Conventional cigarette |
| CDOCS | Controlled Electronic Document Management System |
| CID | Collision Induced Dissociation |
| DA | Decanoic-d19 acid |
| Da | Dalton (atomic mass unit) |
| DP | Diisobutyl Phthalate-d4 |
| EN | Ethyl Nicotinate-d4 |
| EPA | U.S. Environmental Protection Agency |
| ESI | Electrospray Ionization |
| FDA | U.S. Food and Drug Administration |
| FOR | Form |
| FWHH | Full Width at Half-Height |
| HILIC | Hydrophilic Interaction Chromatography |
| HPLC | High-Performance Liquid Chromatography |
| HMDB | Human Metabolome Database |
| HRAM | High Resolution Accurate Mass |
| ID | Identification Code |
| IP | d8-Isophorone |
| IN | Isonicotinamide-2,3,5,6-d4 |

(table continues)

| Abbreviation/Definition (LC-HRAM-MS) | |
|---|---|
| IS | Internal standard |
| IQ | isoquinoline-d7 |
| LC | Liquid Chromatography |
| LC-HRAM-MS | Liquid Chromatography coupled to High Resolution Mass Spectrometry |
| LIMS | Laboratory Information Management System |
| MeOH | Methanol |
| ML | Methyl Linoleate-d3 |
| MRTP | Modified Risk Tobacco Product |
| MS | Mass Spectrometry |
| $MS^2$ | First Order Fragmentation |
| MW | Molecular Weight |
| MY | Myosmine-2,4,5,6-d4 |
| m/z | Mass-to-Charge Ratio |
| N/A | Not Avaialble |
| NEG. | Negative |
| NI | (±) Nicotine-d7 |
| NIH | U.S. National Institute of Health |
| NIST | National Institute of Standards and Technology (USA) |
| NO | Nicotine-1'-Oxide-d3 |
| PDIMS | Product Development Information Management System |
| PH | Peak height |
| PH% | Peak height % |
| ppm | Parts per Million (here: mass error) |
| POS. | Positive |

(table continues)

| Abbreviation/Definition (LC-HRAM-MS) | |
|---|---|
| RP | Reversed Phase Chromatography |
| RRP | Reduced Risk Product |
| RT | Retention Time |
| SI | β-Sitosterol-d7 |
| SOP | Standard Operating Procedure |
| SST | System Suitability Test |
| S/N | Signal-to-Noise |
| TP | α-tocopherol-d6 |
| tR | Retention Time |
| TPM | Total Particulate Matter |
| UCSD | Unique Compounds & Spectra Database (PMI) |
| WKI | Work Instruction |
| WS | Working Solution |