PHILIP MORRIS INTERNATIONAL

NTDS LC-HR-MS PROGENESIS QI (RDNEU)

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1 Purpose

This document describes an explorative assay for the detection of the most relevant differences between samples by liquid chromatography-high resolution accurate mass-mass spectrometry (LC-HRAM-MS). The assay is based on a comprehensive chemical characterization of complex mixtures with no target compounds defined, and subsequent comparison of the test items.

2 Scope and Applicability

The method provides reproducible and robust measurements for cigarette derived total particulate matter (TPM) or nicotine free dry particulate matter (NFDPM) and cryogenically trapped mainstream aerosol samples (Cold Trap) of both conventional cigarettes (CC) and modified risk tobacco products (MRTP), but can also be applied to other complex mixtures (e.g. body fluids, e-liquids).

The described method may be considered as a generic approach for comparing different kinds of test matrices in an unbiased way.

3 Responsibilities

Task/Activity	Responsible
Sample generation	Lab technician trained at the specific site of sample generation
Sample preparation	Lab technician trained
Instrument handling	Lab technician trained
Data acquisition	Lab technician trained
Data evaluation	Lab technician trained
Data reporting	Lab technician trained and supervisor

Table 1 Tasks and Responsibilities

4 Description of the Method

4.1 Principle

The first step in the method workflow is a comprehensive screening in full scan mode of samples using the high resolution accurate mass-mass spectrometer QExactive™. The samples are analyzed 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. For the subsequent identification of relevant compounds, samples are measured using a data dependent fragmentation method complementary to the full scan analysis. The operation of the high resolution accurate mass-mass spectrometer QExactive™ is described in detail within the instrument Work instruction: *PMI-RRP-WKI-111570*.

The accurate mass measurements allow the determination of elemental composition for precursor ions derived from the full scan analyses, and the elemental composition of the fragments using data dependent fragmentation experiments. 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.

4.2 Sample Requirements and Workload

Task/Activity	Workload
Number of sample per day:	40
Sample preparation:	2 h (for 40 analytical replicates)
Run time 1 injection:	*20* min
Data evaluation/reporting	350 h

Table 2 Sample Requirements and Workload

4.3 Material, Equipment, Chemicals, Standards and References

Detailed descriptions concerning the preparation of solutions and mobile phases are given in ***Section 4.4.12***

4.3.1 Materials

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

Table 3 Materials

4.3.2 Equipment

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	-

Table 4 Equipment

4.3.3 Chemicals

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
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

Table 5 Chemicals

4.3.4 Standards and References

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

Table 6 Standards and References

4.4 Procedure

4.4.1 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 (see ***Section 4.4.4***) 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.

4.4.2 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*.

4.4.3 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 as described in ***Section 4.4.1***. 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 as described in ***Section 4.4.2***.

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

4.4.4 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)

4.4.5 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 IS$_1$* internal standard solution
- Mixing the closed vial using ThermoMixer (5 min, 5 °C, 2000 rpm)

4.4.6 Sample Preparation: Pool Sample

In order to have a single sample representing the entire trapped chemical space, a pool sample is prepared, which comprises equal volumes of the extracts described in ***Section 4.4.4*** and ***Section 4.4.5*** transferred into a fresh pre-cleaned Pyrex® tube and mixed well. An aliquot (200 µL) of this pool sample is transferred into a silanized chromatographic vial and diluted with methanol (700 µL) for RP analysis or acetonitrile (700 µL) for HILIC mode respectively. 100 µL of *WS* IS_1 internal standard solution has to be added. The vial is closed and then mixed for 5 minutes using an Eppendorf ThermoMixer (5 °C; 2000 rpm). Aliquots (1.5 µL) of the diluted extract are injected and analyzed by LC-HRAM-MS in full scan mode and in data-dependent fragmentation mode for compound identification. The pool sample is analyzed 5 times (analytical replicates) in both full scan mode and data-dependent fragmentation mode.

- Mix of equal volumes (200 µL of each sample) in new a fresh pre-cleaned Pyrex® tube
- Vortex for 5 min
- Pipette 200 µL pooled 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)

4.4.7 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* IS_1 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* IS_1 internal standard solution
- Mixing the closed vial using ThermoMixer (5 min, 5 °C, 2000 rpm)

4.4.8 Sample Storage/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

Table 7 Sample Storage and Stability

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

4.4.9 Preparation of Calibration and QC Samples

Non-targeted differential screening (NTDS) assay using LC-HRAM-MS with 3 different ionization modes and 2 different chromatography modes provides a comprehensive chemical picture regarding the composition of samples and information regarding differences in chemical composition between samples. The NTDS assay is an explorative, unbiased approach that is not limited to a fixed selection of compounds and delivers comparative results. Accordingly, there is an opportunity to discover new compounds for every test item, however only qualitative and (semi-) quantitatively estimated information can be generated. No calibration using reference compounds is performed and no QC samples are required.

4.4.10 Instrument Set-up

4.4.10.1 HPLC Reversed Phase Mode (RP) ESI positive and APCI positive

Instructions for preparing the mobile phases can be found in ***Section 4.4.12.1***.

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 8 HPLC RP Gradient for positive ionization modes

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 9 HPLC RP Parameters for positive ionization modes

4.4.10.2 HPLC Reversed Phase Mode (RP) ESI negative

Instructions for preparing the mobile phases can be found in ***Section 4.4.12.1***.

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 10 HPLC RP Gradient for negative ionization modes

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 11 HPLC RP Parameters for negative ionization modes

4.4.10.3 HPLC Hydrophilic Interaction Mode (HILIC)

Instructions for preparing the mobile phases can be found in ***Section 4.4.12.3***.

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 12 HPLC HILIC Gradient

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 13 HPLC HILIC Parameters

4.4.11 Mass Spectrometry Settings

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 14 Detector 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

Table 15 Global Ion Source Settings for RP ESI positive

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

Parameter	Value
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 16 Detector 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

Table 17 Global Ion Source Settings for RP ESI negative

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

Parameter	Value
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 18 Detector 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

Table 19 Global Ion Source Settings for RP APCI positive

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 20 Detector 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

Table 21 Global Ion Source Settings for HILIC ESI positive

4.4.12 Preparation of Chemicals, Solvents and Solutions

All solutions are clearly and permanently labelled according to *PMI-RRP-WKI-111814*, including preparation date, expiration date and initials of the operator. The stock and working solutions are only prepared using measuring flasks of accuracy degree A with quality certificate and volumetric pipettes of accuracy degrees AS with quality certificate, microliter syringes or adjustable electronic pipettes. For all preparations the form *PMI-RRP-FOR-111501* is used. Actual Lot No. of used solvents, chemicals and reference standards are documented in the form. Storage details are recorded using form *PMI-RRP-FOR-111506*.

4.4.12.1 Mobile Phase RP ESI positive and RP APCI positive

All weighing are performed according to *PMI-RRP-WKI-111726*.

Name	Concentration	Approach	Stability/Storage
Mobile phase A NH_4Ac in Water	10mM	770 mg ammonium acetate (MW 77.08) using glass weighing funnel dissolved in 1000 mL water	2 weeks / RT
Mobile phase B NH_4Ac in Methanol	1mM	77 mg ammonium acetate (MW 77.08) using glass weighing funnel dissolved in 1000 mL methanol	6 months / RT

Table 22 Mobile Phases RP ESI positive and RP APCI positive

4.4.12.2 Mobile Phase RP ESI negative

All weighing are performed according to *PMI-RRP-WKI-111726*.

Name	Concentration	Approach	Stability/Storage
Mobile phase A NH_4F in Water	1mM	37 mg ammonium fluoride (MW 37.04) using glass weighing funnel dissolved in 1000 mL water	2 weeks / RT
Mobile phase B Methanol	N/A	1000 mL methanol	6 months / RT

Table 23 Mobile Phases RP ESI negative

4.4.12.3 Mobile Phase HILIC ESI positive

All weighing are performed according to *PMI-RRP-WKI-111726*.

Name	Concentration	Approach	Stability/Storage
Mobile phase A NH_4Ac in Water	10mM	770 mg ammonium acetate (MW 77.08) using glass weighing funnel dissolved in 1000 mL Water	2 weeks / RT
Mobile phase B NH_4Ac in Acetonitrile	10mM	770 mg ammonium acetate (MW 77.08) using glass weighing funnel dissolved in 1000 mL Acetonitrile	6 months / RT

Table 24 Mobile Phases HILIC ESI positive

4.4.12.4 Internal Standard Stock Solutions (*IS_0*)

All weighing are performed according to *PMI-RRP-WKI-111726*.

Name	Target Concentration	Approach	Stability/Storage
Decanoic-d19 acid *IS_0*	1.0 mg/mL	25 mg DA using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Diisobutyl Phthalate-d4 *IS_0*	1.0 mg/mL	25 mg DP using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Ethyl Nicotinate-d4 *IS_0*	1.0 mg/mL	25 mg EN using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Isonicotinamide-2,3,5,6-d4 *IS_0*	1.0 mg/mL	25 mg IN using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
d8-Isophorone *IS_0*	1.0 mg/mL	25 mg IP using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
isoquinoline-d7 *IS_0*	1.0 mg/mL	25 mg IQ using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Methyl Linoleate-d3 *IS_0*	1.0 mg/mL	25 mg ML using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Myosmine-2,4,5,6-d4 *IS_0*	1.0 mg/mL	25 mg MY using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
(±) Nicotine-d7 *IS_0*	1.0 mg/mL	25 mg NI using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Nicotine-1'-Oxide-d3 *IS_0*	1.0 mg/mL	25 mg NO using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C

Name	Target Concentration	Approach	Stability/Storage
α-tocopherol-d6 IS_0	1.0 mg/mL	25 mg TP using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
β-Sitosterol-d7 IS_0	1.0 mg/mL	25 mg SI using glass weighing funnel is weighed into a 25 mL volumetric flask and made up to volume with methanol.	1 year / -20°C

Table 25 Internal Standard Stock Solutions IS_0

Actual amounts of *used* reference compounds used are recorded on form *PMI-RRP-FOR-111501*.

4.4.12.5 Working Solution for Internal Standards (WS $\boldsymbol{IS_1}$)

Name	Target Concentration	Approach	Stability/Storage
Decanoic-d19 acid IS_0	200 µg/mL	1000 µL of the DA IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Diisobutyl Phthalate-d4 IS_0	50 µg/mL	250 µL of the DP IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Ethyl Nicotinate-d4 IS_0	50 µg/mL	250 µL of the EN IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Isonicotinamide-2,3,5,6-d4 IS_0	50 µg/mL	250 µL of the IN IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
d8-Isophorone IS_0	100 µg/mL	500 µL of the IP IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
isoquinoline-d7 IS_0	50 µg/mL	250 µL of the IQ IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Methyl Linoleate-d3 IS_0	50 µg/mL	250 µL of the ML IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Myosmine-2,4,5,6-d4 IS_0	50 µg/mL	250 µL of the MY IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
(±) Nicotine-d7 IS_0	50 µg/mL	250 µL of the NI IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
Nicotine-1'-Oxide-d3 IS_0	50 µg/mL	250 µL of the NO IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C
α-tocopherol-d6 IS_0	50 µg/mL	250 µL of the TP IS_0 stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C

Name	Target Concentration	Approach	Stability/Storage
β-Sitosterol-d7 *IS₀*	50 µg/mL	250 µL of the SI *IS₀* stock solution is pipetted into a 5 mL volumetric flask and made up to volume with methanol.	1 year / -20°C

Table 26 Preparation of Working Solution (WS *IS₁*) of Internal Standards

Actual concentrations *of used* reference compounds are recorded using form *PMI-RRP-FOR-111501*.

4.4.12.6 Equations

$$Concentration\ [mg/ml] = \frac{Compound\ weight\ [mg] * chemical\ purity\ [\%] * isotopic\ purity\ [\%]}{final\ volume\ [ml]\ * 100}$$

$$Concentration\ [mM] = \frac{Compound\ weight\ [mg] * purity\ [\%]}{Molecular\ weight\ [mg/mmol] *\ final\ volume\ [ml]\ * 100}$$

4.4.13 Number of Determinations

- Aerosol replicates/sample replicates (technical replicates): 3
- Blank samples: 1
- Injection replicates (analytical replicates): 5

4.4.14 Daily Verification or According to Use

All steps required prior to the start of an analytical sequence are recorded using form *PMI-RRP-FOR-111502*.

4.4.14.1 System Suitability Test (SST)

Prior to initiating a sample series, the status of the chromatographic system must be assessed via injection of a reference cigarette sample (matrix sample) spiked with internal standards (*WS IS₁*), to ensure suitable sensitivity and chromatographic separation in all 3 ionization modes and the two chromatography modes (3 analytical replicates). Form *PMI-RRP-FOR-111503* must be completed, printed out and signed.

For preparation of system suitability samples and acceptance criteria see ***Section 4.4.14.2*** and ***Section 4.4.14.3***

Method	Compound	RT [min]	Formula	Q-Ion	Quantitation mass [*m/z*]
RP ESI (+)	d8-Isophorone	4.50 – 6.50	$C_9H_6D_8O$	(M+H)	147.16196
RP ESI (-)	Decanoic-d19 acid	6.35 – 8.35	$C_{10}HD_{19}O_2$	(M-H)	190.25831
RP APCI (+)	d8-Isophorone	4.50 – 6.50	$C_9H_6D_8O$	(M+H)	147.16196
HILIC ESI (+)	Myosmine-2,4,5,6-d4	0.70 – 2.70	$C_9H_6D_4N_2$	(M+H)	151.11678

Table 27 Internal Standards per Ionization Mode

In the case of failure of any system suitability parameter, troubleshooting will be initiated (e.g., new analytical column, new pre-column, cleaning of the ion-source, etc.).

4.4.14.2 Preparation of System Suitability Test

An aliquot (200 µL) of the reference matrix extract solution (methanol for RP, acetonitrile for HILIC) is transferred into a silanized autosampler vial and diluted with either methanol (700 µL) for RP SST or acetonitrile (700 µL) for HILIC SST. 100 µL of *WS IS₁* internal standard solution has to be added. The vial is closed and then mixed for 5 minutes using an Eppendorf ThermoMixer (5 °C; 2000 rpm). The prepared SST sample is then analyzed in triplicate for each ionization mode.

- Pipette 200 µL reference matrix 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)

4.4.14.3 Acceptance Criteria for System Suitability Test

	RP ESIpos (+)	RP ESIneg (-)	RP APCIpos (+)	HILIC ESIpos (+)
Internal Standard	d8-Isophorone	Decanoic-d19 acid	d8-Isophorone	Myosmine-2,4,5,6-d4
Quantitation mass [m/z]	147.16196	190.25831	147.16196	151.11678
Typical Mass Accuracy [ppm]	< 2.00	< 2.00	< 2.00	< 2.00
Accepted Mass Accuracy [ppm]	*±2.00*	*±2.00*	*±2.00*	*±2.00*
Typical RT [min]	5.60	7.35	5.60	2.10
Accepted RT [min]	4.50 – 6.50	6.35 – 8.35	4.50 – 6.50	1.10 – 3.10
Typical RT RSD [%]	0.50%	0.50%	0.50%	0.50%
Accepted RT RSD [%]	±15.00%	±15.00%	±15.00%	±15.00%
Typical injected amount [ng]	15	30	15	8
Typical area / injected amount	2’200’000’000	11’000’000’000	6’000’000’000	30’000’000’000
Accepted Area RSD [%]	±15.00%	±15.00%	±15.00%	±15.00%
Typical area / injected ng	140’000’000	370’000’000	457’000’000	3’750’000’000
Accepted area / injected ng	>70’000’000	>180’000’000	>220’000’000	>1’800’000’000

Table 28 Acceptance Criteria for System Suitability Test

Form *PMI-RRP-FOR-111503* must be completed, printed and signed.

4.4.15 Testing Procedure

4.4.15.1 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 in ***Section 4.4.10***. A sequence must be generated as shown in the example in ***Section 4.4.15.2*** and recorded using form *PMI-RRP-FOR-111502*.

4.4.15.2 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.

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

Table 29 Example of Sequence Order

4.4.16 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 (**Section 4.5**)
- Semi-quantification of compounds (**Section 4.5**)
- extraction of obviously different compounds (compounds of interest) (**Section 4.5**)
- sorting of information according to RANK parameters (**Section 4.5**)
- manual verification of results **(Section 4.5)**

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

4.4.16.1 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 (\\rd-share.app.pmi\Studies*Project Number*[1]*\Instrument*[2]*\Primary Raw Data*[3]*\Method*[4]). 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 (e.g. E:\Studies\RLS-ASC-2015-18\LC-HRAM-MS\Primary Raw Data\RP ESI pos).

On the data evaluation workstation, all data evaluations are performed and stored using a separate secondary raw data (processed data) folder (e.g. E:\Studies\RLS-ASC-2015-18\LC-HRAM-MS\Secondary Raw Data\RP ESI pos).

After completion of data evaluation, processed data are transferred to the backed up and secured server environment (\\rd-share.app.pmi\Studies*Project Number*[15]*\Instrument*[16]*\Secondary Raw Data*[17]*\Method*[18])

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

4.4.16.2 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 (e.g. RLS-ASC-2015-18_TankSystemsLifecycle), the method (e.g. RP ESI pos) and the storage location (defined within the specifications of raw data location on data evaluation unit in ***Section 4.4.16.1***, e.g. E:\Studies\RLS-ASC-2015-18\LC-HRAM-MS\Secondary Raw data\RP ESI pos). 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 (*see ***Figure 1***). The list of possible adducts to be used, depending upon the applied method, is presented in ***Table 30***.

[1] The folder has to be created according to the naming convention considering ARMS request ID, PDIMS ID or the LIMS ID (e.g. RLS-ASC-2015-18_TankSystemsLifecycle)

[2] The folder has to be created according to the used instrument (e.g. LC-HRAM-MS)

[3] The folder has to be created

[4] The folder has to be created according to the used method (e.g. RP ESI pos)

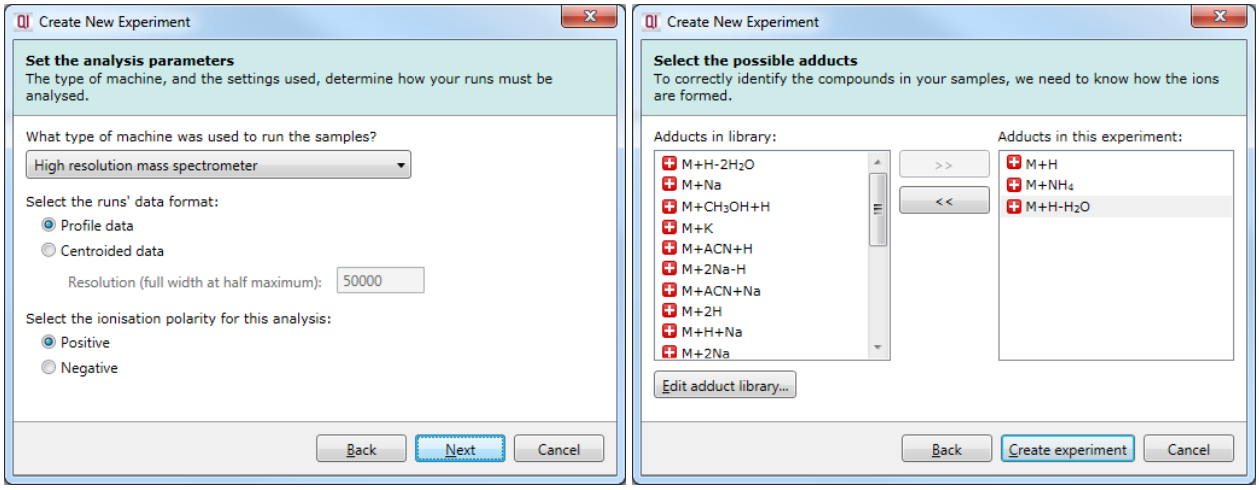


Figure 1 Create new Experiment sections in Progenesis® QI

	RP ESIpos (+)	RP ESIneg (-)	RP APCIpos (+)	HILIC ESIpos (+)
Adducts	M+H M+NH4	M-H M+F-H	M+H M+H-H2O	M+H M+NH4

Table 30 Possible Adducts by Method

Xcalibur™ raw data can be imported directly into Progenesis® QI, the data format should be selected as *Thermo (.raw).* After selection of raw files, data alignment can be started by pressing the *Start alignment process* (**Figure 2**). The data import warning should be ignored. A warning message will appear if any *m/z* values within a scan range are slightly lower than preceding ones. In this case the lower m/z values are skipped, which does not affect the raw data. The selected alignment reference should be the pool sample, which comprises all features from the entire sample set.

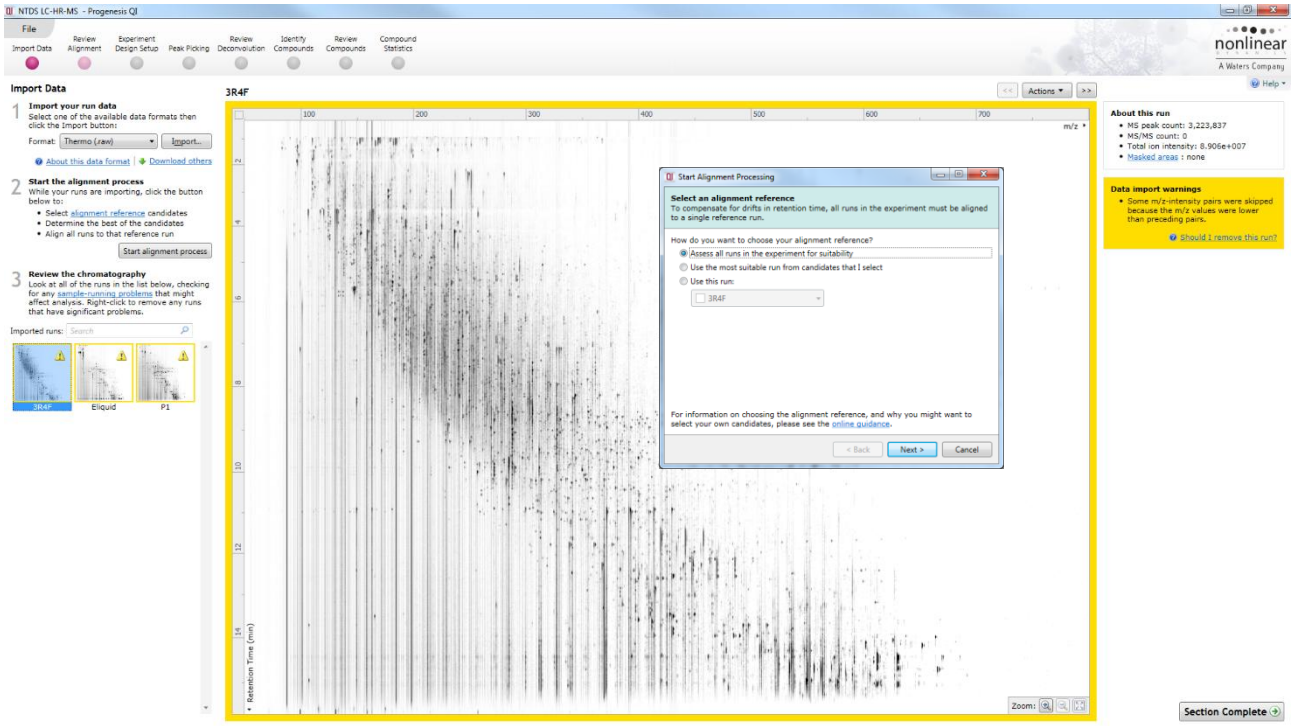


Figure 2 Data Import Window for Progenesis® QI

4.4.16.3 Raw Data Processing by Metabolomics Software Progenesis ® QI

Detailed information about the use of the metabolomics software Progenesis® QI may be found in the software manual.

4.4.16.3.1 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 (**Figure 3**). 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.

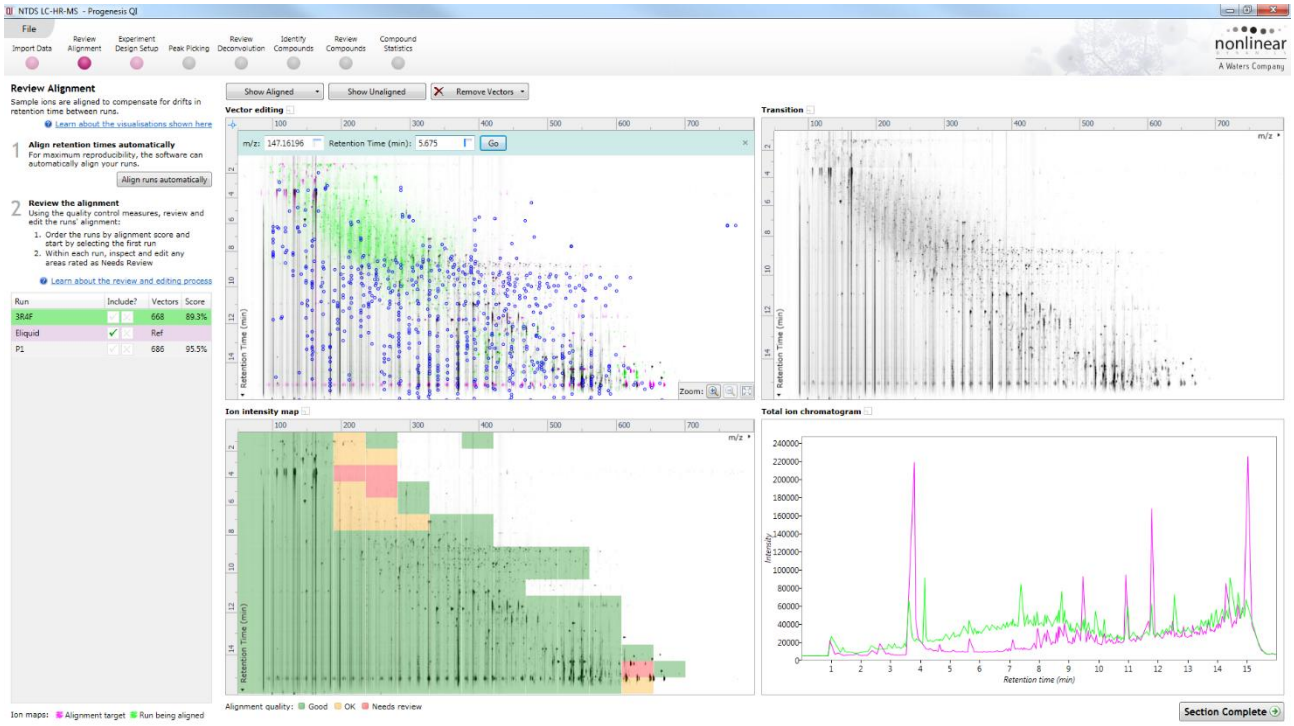


Figure 3 Alignment Window for Progenesis® QI

4.4.16.3.2 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.

4.4.16.3.3 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*.

4.4.16.3.4 Normalization

The *Normalize to set of housekeeping compounds* method is selected for normalization (**Figure 4**). 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.

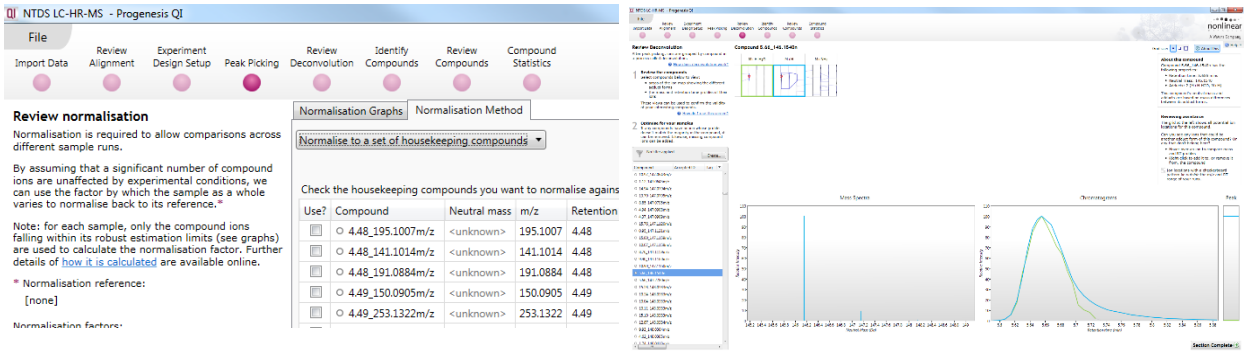
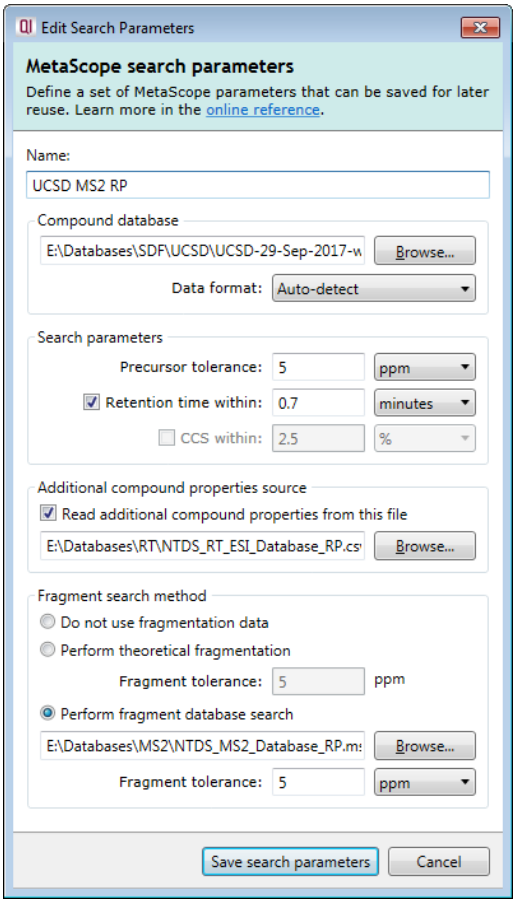


Figure 4 Normalization and Deconvolution Window for Progenesis® QI

4.4.16.3.5 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 *6* steps.

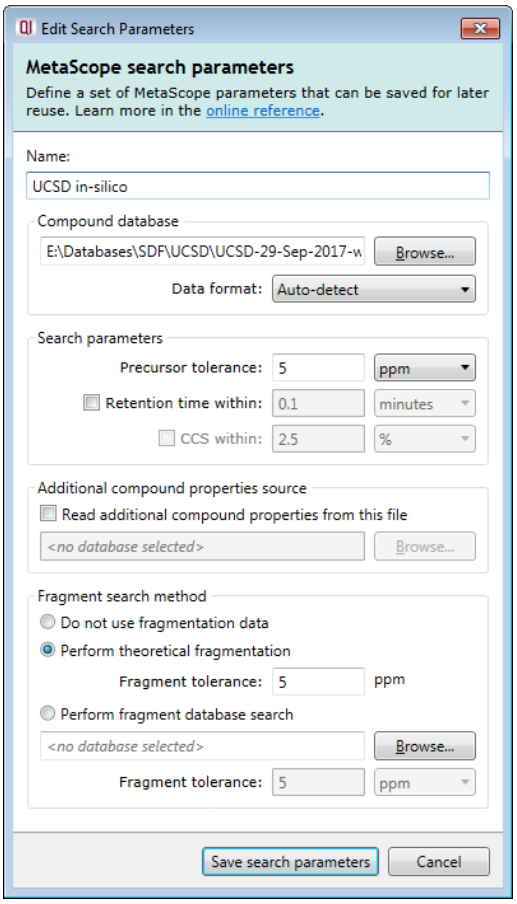
- Compound Identification using UCSD Database Fragmentation (Step I)



The first identification step *(UCSD MS2 RP, UCSD MS2 HILIC)* 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 *0.7* minutes). All acquired MS^2 spectra are compared against in-house MS^2 library (Fragment tolerance: *5* ppm).

Figure 5 UCSD Database Fragmentation Search Parameters for Progenesis® QI

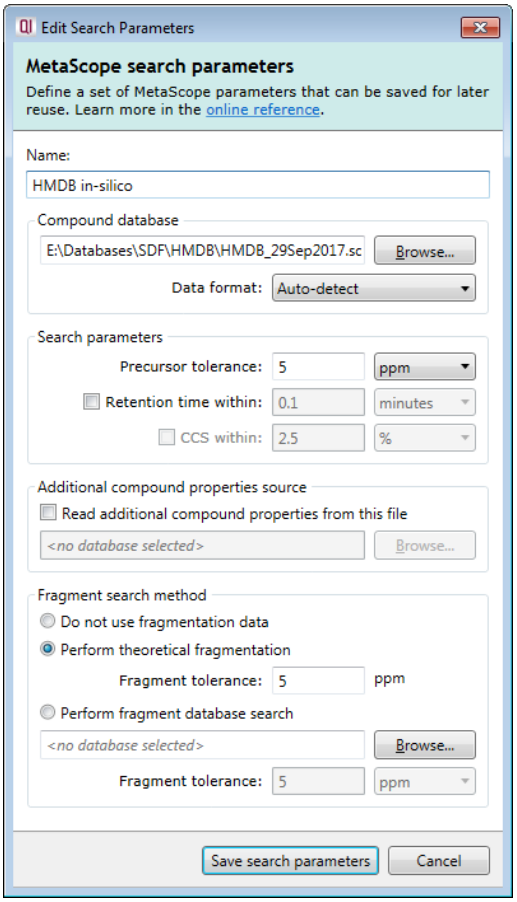
- Compound Identification using UCSD Theoretical Fragmentation (Step II)



The second identification step *(UCSD in-silico)* comprises the theoretical fragmentation of the in-house UCSD database via MetaScope algorithm and MetFrag algorithm *(Precursor tolerance: 5ppm)*. The actual acquired MS^2 spectra are matched against in-silico (theoretical) fragmented spectra of the UCSD candidate compounds (Fragment tolerance: *5* ppm).

Figure 6 UCSD Theoretical Fragmentation Search Parameters for Progenesis® QI

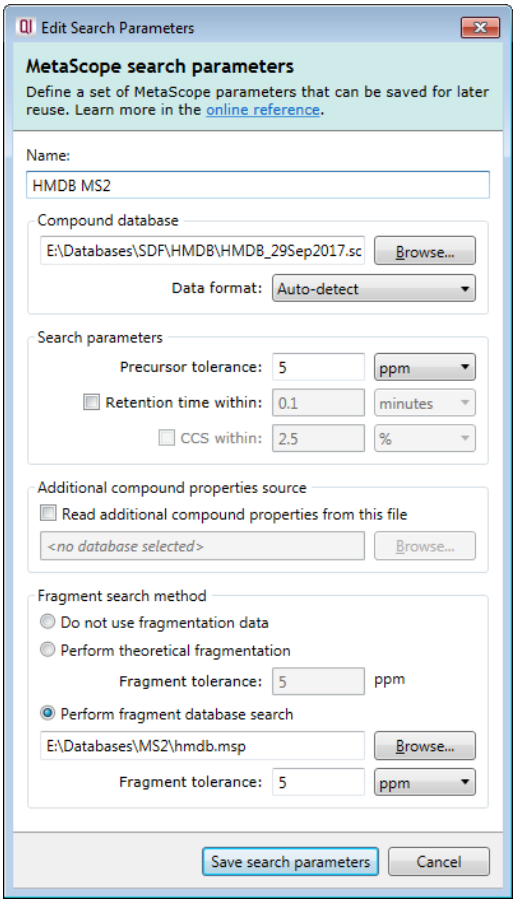
- Compound Identification using HMDB Theoretical Fragmentation (Step III)



The third identification step *(HMDB in-silico)* comprises the theoretical fragmentation of the HMDB database via MetaScope algorithm *(Precursor tolerance: 5ppm)* and MetFrag algorithm. The actual acquired MS^2 spectra are matched against in-silico (theoretical) fragmented spectra of the HMDB candidate compounds (Fragment tolerance: *5* ppm).

Figure 7 HMDB Theoretical Fragmentation Search Parameters for Progenesis® QI

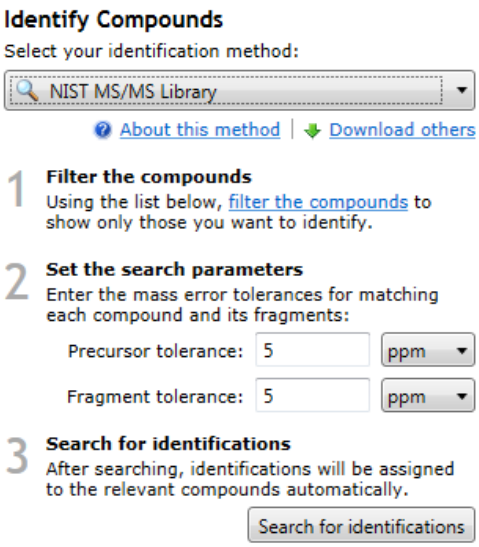
- *Compound Identification using HMDB Database Fragmentation (Step IV)*



The fourth identification step (HMDB MS2) comprises the accurate mass comparison of the acquired data against the MS^2 database of HMDB via MetaScope algorithm (Precursor tolerance: 5ppm). All acquired MS^2 spectra are compared against MS^2 library spectra (Fragment tolerance: 5 ppm).

Figure 8 HMDB Database Fragmentation Search Parameters for Progenesis® QI

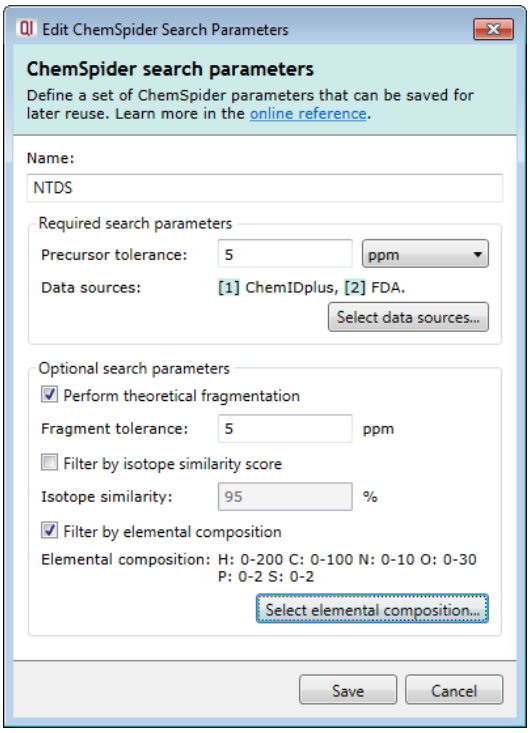
- *Compound Identification using NIST MS/MS Library (Step V)*



The fifth identification step (NIST MS/MS Library) comprises the accurate mass comparison of the acquired data against the MS^2 database of NIST MS/MS (Precursor tolerance: 5ppm). All acquired MS^2 spectra are compared against MS^2 library spectra (Fragment tolerance: 5 ppm).

Figure 9 *NIST MS/MS Library Search Parameters for Progenesis® QI*

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



The sixth identification step *(NTDS)* comprises the theoretical fragmentation of ChemSpider with ChemIDplus, FDA as connected data sources via MetFrag algorithm. The actual acquired MS^2 spectra are matched against in-silico (theoretical) fragmented spectra of the ChemIDplus [16] *and* FDA [17] ~~*and NIST*~~ candidate compounds *(Fragment tolerance: 5 ppm)*. 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.

Figure 10 ChemSpider Theoretical Fragmentation Search Parameters for Progenesis® QI

Database / Data source	Number of Compounds / Data Sets
UCSD	*11,810*
HMDB	*74,435*
ChemIDplus	*207,097*
FDA	*1,524*
NIST *MS/MS*	*13,808*

Table 31 Number of Compounds per Database

4.4.16.3.6 Compound Review

The results retrieved from compound identification (***Section 4.4.16.3.5***) 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.

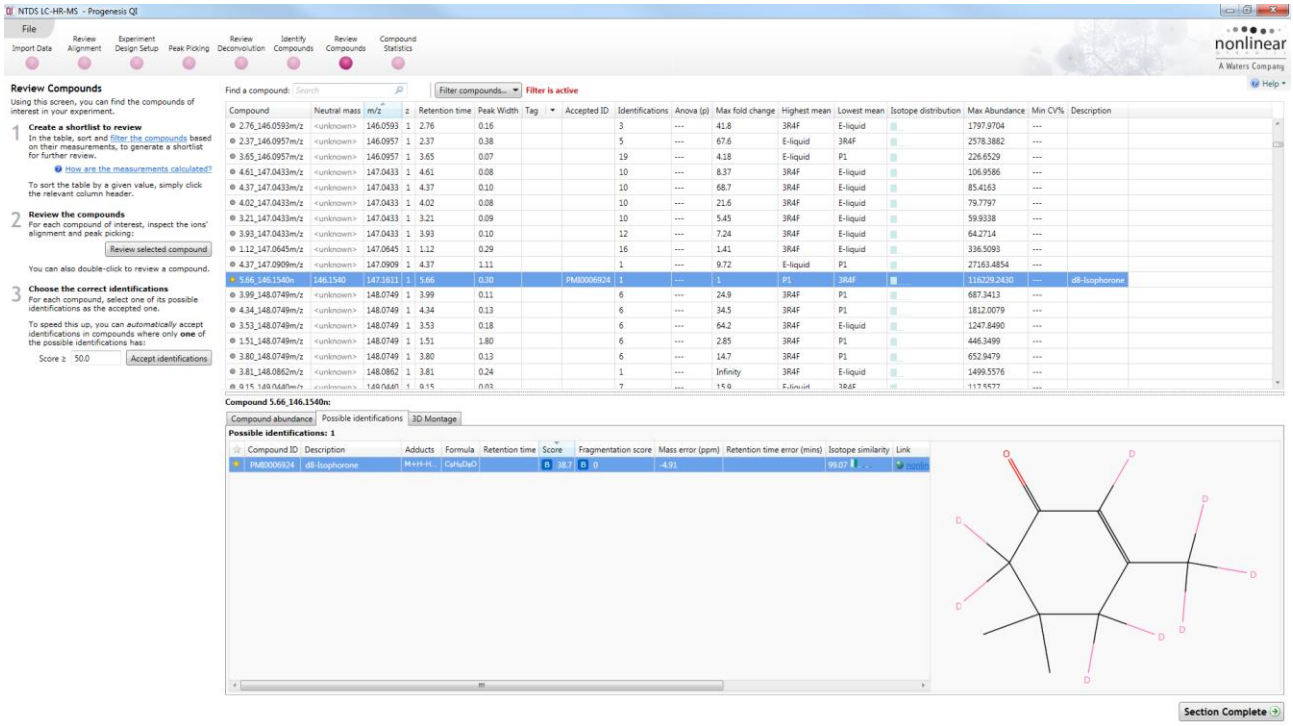


Figure 11 Compound Review in Progenesis® QI

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).

4.5 Calculation and Records

For further calculations the .csv file retrieved from Progenesis® QI is used.

4.5.1 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.

4.5.2 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.

4.5.3 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).

4.5.3.1 Equation for the RANK Parameter

The RANK formula mathematically combines two criteria:

- Abundance of the variable (“Average Concentration” (µg/Item))

- Difference of the variable (“Effect” (%))

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

$$\boldsymbol{Effect} = \frac{\boldsymbol{Ly - Lx}}{\boldsymbol{Ly + Lx}} * \boldsymbol{100}$$

$$\boldsymbol{RANK} = \frac{\boldsymbol{Effect^3}}{\boldsymbol{1000}} \boldsymbol{x\ Average\ Concentration}$$

REMARK:

Lx: measured concentration values for sample x to be compared with sample y

Ly: measured concentration values for sample y to be compared with sample x

The data set is sorted in the order of decreasing RANK-values.

4.5.4 Manual Verification of Results

Verification of the result-table is performed manually with the help of a suitable data browser, e.g., Xcalibur™.

Possible reasons for undesirable Hits in the result table are:

(1) picking noise as a peak

(2) several different adducts of the same molecule

(3) in-source fragmentation products, e.g., thermal degradation in APCI

The first factor can be reduced by adjusting Progenesis® QI peak picking parameters (***Section 4.4.16.3.3***) from default to less sensitive parameters for data analysis. Factor (2) can be optimized by, e.g., reducing contamination of the system by alkali metal cations or avoiding eluents which deliver adducting cations. Factor (3) can be reduced by adjusting source parameters of the mass spectrometer.

4.5.5 Records

Procedure	Acquisition of Results/Data Transfer	Decimal Places
Raw Data	Acquisition of raw data and transfer to network share (primary raw data folder of the project)	all
Raw Data Processing	Processed raw data results are stored in the secondary raw data folder of the project	all
Results	MS Excel calculations and transfer to network share (results folder of the project)	all
Data Transfer	Automatic export of raw data and files to network share	all

Table 32 Records Scheme

Initial raw data are generated on the instrument acquisition computer, which are then transferred to a central data repository (currently HPC data share \\rd-share.app.pmi, within the respective study data folder). Once successful transfer has been confirmed (the number of files and the size is checked) the data on the acquisition computer are deleted. A unique identifier is used for each raw data file respectively. The raw data are then copied to a data evaluation workstation for further data processing (e.g. Progenesis® QI). The processed data are stored in the secondary raw data folder named _processed. Processed data files are transferred back to the HPC data share \\rd-share.app.pmi, respective secondary raw data study folder. After a check for successful file transfer (the number of files and the size is checked) the processed data are deleted from the data evaluation workstation. Thus a single copy of raw data and processed data are ultimately maintained on the HPC data share environment. During the whole study all data transfer steps and the final storage is recorded using form *PMI-RRP-FOR-111511* (see also ***Section 4.4.16.3***)

4.5.6 Documentation

Procedure	Documentation	ID
Preparation of IS stock and working solutions	Balance print-out + description + form	*PMI-RRP-FOR-111501*
Request of Smoke Generation	Form	ARMS ID + *PMI-RRP-FOR-111314*
Generation of smoke related samples	Print-out smoking machine	LIMS ID
Generation of smoke related samples	Smoking protocol	LIMS ID
Sample Preparation	Form	*PMI-RRP-FOR-111504*
Storage of samples and study related materials	Form	*PMI-RRP-FOR-111506*
Instrument Preparation	Form	*PMI-RRP-FOR-111502*
System Suitability Test (SST)	Form	*PMI-RRP-FOR-111503*

Procedure	Documentation	ID
Acquisition methods	Thermo Xcalibur™ report print-out + form	*PMI-RRP-FOR-111502*
Thermo Xcalibur Sequence	Thermo Xcalibur™ sequence report print-out + form	*PMI-RRP-FOR-111502*
Instrument Calibration	Thermo Xcalibur™ calibration report print-out	-
Data Processing	Form	*PMI-RRP-FOR-111505*
Data Transfer and Storage	Form	*PMI-RRP-FOR-111511*
Results	MS Excel print-out	-

Table 33 Documentation

4.6 Testing Scope, Repeatability, Reproducibility

N/A

4.7 Safety

- The usual good laboratory practices are required.
- Work in an exhaust hood and wear safety glasses.
- Use nitrile gloves.
- Store inflammable products away from a heat source or a flame.

Comments:

Take care of the relevant MSDS (material safety data sheets).

4.8 Calibration and Maintenance of Instruments

For most calibration applications, a ProteoMass™ LTQ/FT-Hybrid Cal Mix calibration solution for the respective ionization mode (see ***Section 4.3.3***) is infused directly into the ion source while running the automatic calibration options provided within the data system. Use the syringe pump, located on the top of the Q Exactive™ mass spectrometer, to infuse solution with 5µL/min flow for calibration.

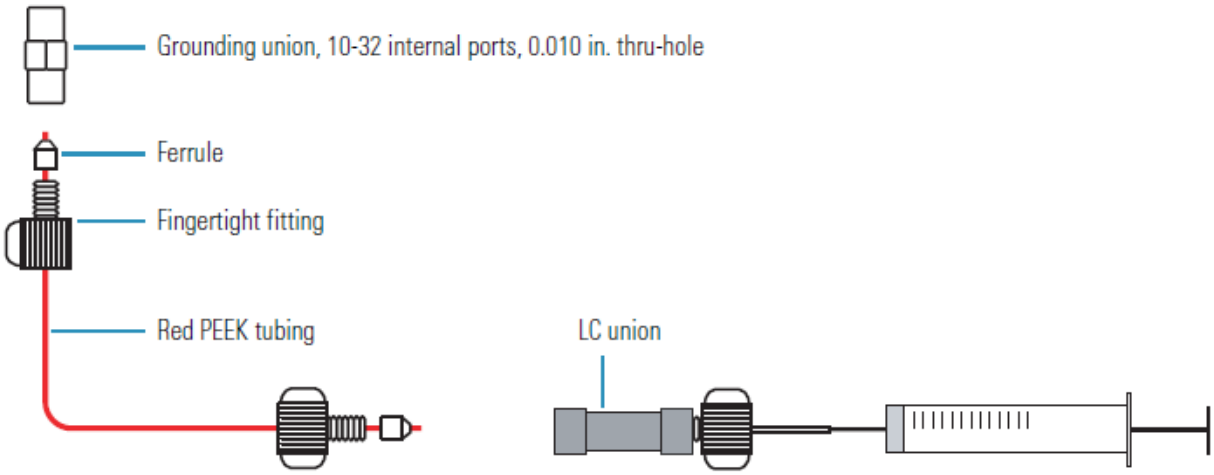


Figure 12 Syringe Setup

For a detailed description use the hardware manual.

4.8.1 Instrument Mass Calibration

To ensure the highest mass accuracy for the LC-HRAM instrument (liquid chromatography – high resolution accurate mass), which is essential for compound identification and possible subsequent structural elucidation, it is mandatory to run a semi-automatic mass calibration prior to initiating a sequence for each respective ionization mode.

For positive ionization mode the ProteoMass LTQ/FT-Hybrid ESI Pos. Mode CalMix solution has to be used. For negative ionization mode the ProteoMass LTQ/FT-Hybrid ESI Neg. Mode CalMix solution has to be used. *In order to achieve better mass accuracy in the lower mass regions 10µL of a 10mg/mL Decanoic-d19 acid has to be spiked to 990µL of the standard ProteoMass LTQ/FT-Hybrid ESI Neg. Mode CalMix solution according to PMI-RRP-WKI-111570.*

The mass calibration has to be performed only for ESI ionization mode.

To perform the mass calibration, the sweep cone at the entrance of the capillary must be removed and a syringe (**Section 4.8**) with the calibration solution mounted to the ESI Source to enable direct infusion. Following ion source parameter are given as an example.

Parameter	Value
Vaporizer Temperature [°C]	40
Sheath Gas Flow Rate	8
Aux Gas Flow Rate	0
Sweep Gas Flow Rate	0
Discharge Current [µA]	3.5

Parameter	Value
Capillary Temp [°C]	280
S-Lens RF Level [%]	55

Table 34 MS Settings for Calibration

Each calibration has to be printed and has to be documented in the raw data.

4.8.2 Cleaning the Ion Sweep Cone, Spray Cone, and Ion Transfer Tube

The Ion Sweep Cone is a metal cone over the API ion transfer tube and acts a physical barrier that protects both the spray cone and the entrance of the ion transfer tube and increases source robustness. The ion transfer tube is a metal, cylindrical tube that assists in desolvating ions produced by the API probe while transferring them into the vacuum system (see ***Figure 11***).

Before removing the parts for cleaning let the system cool down by setting the source parameters to room temperature (see ***Section 4.8.1***, Source Parameter).

Remove the Ion Sweep Cone wearing fresh and clean cotton gloves and place it into a 400 mL beaker. Remove the Ion Transfer Tube using the Ion Transfer Tube removal tool and place in the beaker as well. Fill the beaker with water containing 2% Mucasol and sonicate for 10 min. at 60˚C.

Rinse the parts with deionized water. Repeat the sonication procedure with deionized water for 10 min. at 60˚C.

Rinse the parts with methanol. Repeat the sonication procedure with methanol for 10 min.

Remove the parts from the beaker and dry them with nitrogen prior to the installation to the mass spectrometer.

Clean the Spray Cone with a fresh and clean cotton tip and methanol.

- Setting the source parameters to room temperature for cooling down to room temperature
- Remove Ion Sweep Cone (wearing fresh and clean cotton gloves) and place it into a 400 mL beaker
- Remove the Ion Transfer Tube using the Ion Transfer Tube removal tool and place in the beaker
- Fill the beaker with aqueous 2% Mucasol
- Sonicate for 10 min. at 60˚C
- Rinse the parts with deionized water
- Repeat the sonication procedure with deionized water for 10 min. at 60˚C
- Rinse the parts with methanol
- Repeat the sonication procedure with methanol for 10 min. at 60˚C
- Dry all parts with nitrogen
- Clean the spray cone with methanol using a fresh and clean cotton tip

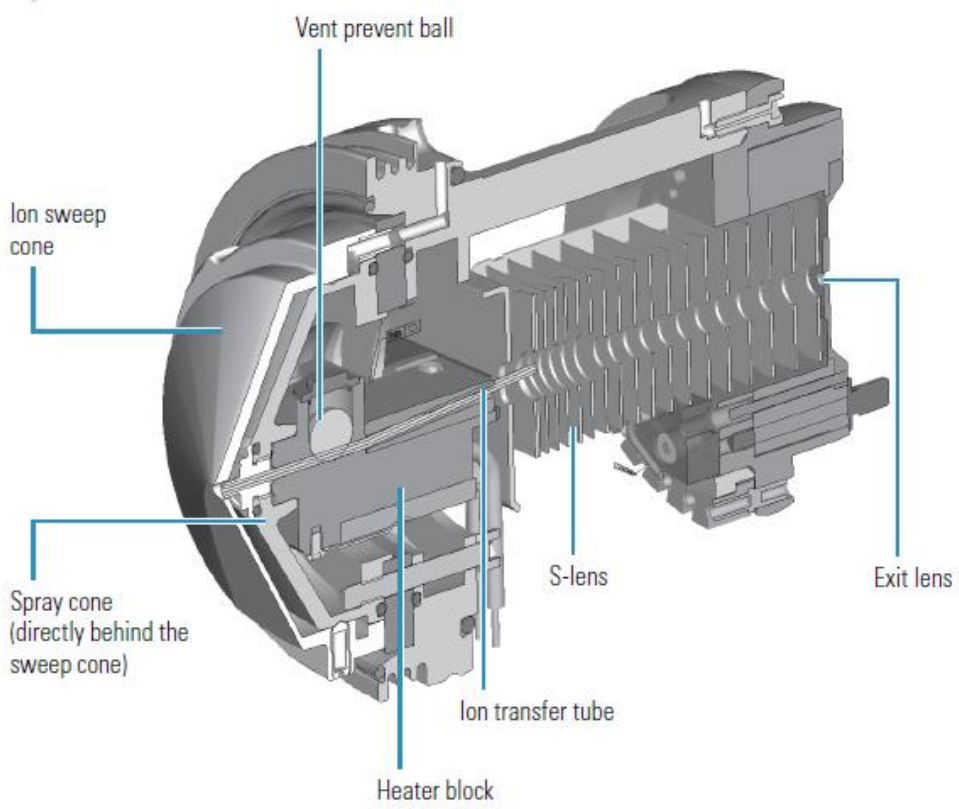


Figure 13 Schematic Overview of Ion Inlet System and First Stage Vacuum Environment

5 Reference Documents

- MetaScope, Algorithm of the Systems Biology Research Group at the University of California, San Diego, USA
- Wolf S., Schmidt S., Müller-Hannemann M., Neumann S.; '*In silico fragmentation for computer assisted identification of metabolite mass spectra*' , 11:48, BMC Bioinformatics 2010
- Wishart DS, Jewison T., Guo AC, Wilson M., Knox C., et al., '*HMDB 3.0 — The Human Metabolome Database in 2013'*. Nucleic Acids Res. 2013. Jan 1;41(D1):D801-7
- ChemIDplus database, U.S. National Library of Medicine
- FDA database, U.S. Food and Drug Administration
- NIST database compiled from NIST/EPA/NIH Mass Spectral Library Data Version: NIST 05, Software Version 2.0d , U.S. National Institute of Standards and Technology

6 Related Documents

- *PMI-RRP-WKI-111570* 'Operation of LC-HRAM-MS Thermo Q Exactive™ Platforms'
- *PMI-RRP-WKI-111801* 'Trappage en phase particulaire pour la détermination des constituants de l'aerosol'
- *PMI-RRP-FOR-111506* 'Storage of samples and study related materials for NTDS LC-HRAM-MS'
- *PMI-RRP-WKI-111626* 'Trappage des composés aromatiques'
- *PMI-RRP-FOR-111314* 'Tracking smoke analysis form'
- *PMI-RRP-FOR-111504* 'Sample preparation for NTDS LC-HRAM-MS'
- *PMI-RRP-FOR-111501* 'Chemicals, solvents, solutions and internal standard amounts used for NTDS LC-HRAM-MS'
- *PMI-RRP-FOR-111502* 'Preparation of QExactive LC-HRAM-MS for NTDS'
- *PMI-RRP-FOR-111503* 'System suitability test LC-HRAM-MS for NTDS'
- *PMI-RRP-FOR-111505* 'Data processing in Progenesis QI for NTDS LC-HRAM-MS'
- *PMI-RRP-FOR-111511* 'Data Transfer and Storage for NTDS LC-HRAM-MS'
- *PMI-RRP-WKI-111726* 'Management of Balances'
- *PMI-RRP-WKI-111784* 'Equipment logbook creation and content'
- *PMI-RRP-WKI-111840* 'Management of calibration and maintenance'
- *PMI-RRP-WKI-111768* 'Management of laboratory fridges and freezers'
- *PMI-RRP-WKI-111774* 'Management of Datalogger'
- *PMI-RRP-WKI-111700* 'Management of pipettes'
- *PMI-RRP-WKI-111814 'Management and labelling of chemicals'*
- Nonlinear Dynamics Progenesis® QI User Guide
- User Manual Progenesis® QI
- Hardware Manual QExactive®
- Hardware Manual HESI Probe Ion Source

7 Revision History

Version No.	Description of change (including reason for change)	Type of change
1.0	Original issue	1
2.0	*Change of gradients for RP ESI pos, RP APCI pos and RP ESI neg in Chapter 4.4.10.1 and 4.4.10.2* *Change of instrument run times for RP ESI pos, RP APCI pos and RP ESI neg in Chapter 4.4.11*	*2*
3.0	*Content added in the new OMSP template* *Modification of EDMS ID of all documents* *Adjusted instrument run time in Chapter 4.2* *Modification of System Suitability Acceptance Criteria (Chapter 4.4.14.3)* *Revision of Compound Identification in Chapter 4.4.16.3.5* *Additional compound for ESI neg mass calibration added (Chapter 4.8.1)*	*2*

(1. Major change/new version; 2. Minor change); at least the last three major versions (i.e. 1.0, 2.0. etc.) are listed in the Revision History.

8 Abbreviations

Abbreviation/Definition	
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

Abbreviation/Definition	
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
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

Abbreviation/Definition	
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
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

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Author Approval	Daniel Arndt	04-Dec-2017 16:22:29 CET	Author Approval
Owner Approval	Mark Bentley	04-Dec-2017 16:14:13 CET	Owner Approval