

MS – Multiplatform Metabolomics Workshop

CEMBIO, Spring 2018

Dr Danuta Dudzik

2. Setting up a Metabolomics experiments

2.3. Quality in Metabolomics

2.3.1 Quality Control and Quality Assurance Procedure in Metabolomics

2.3.2. Data processing

Quality Control and Quality Assurance Procedures in Metabolomics

Metabolomics

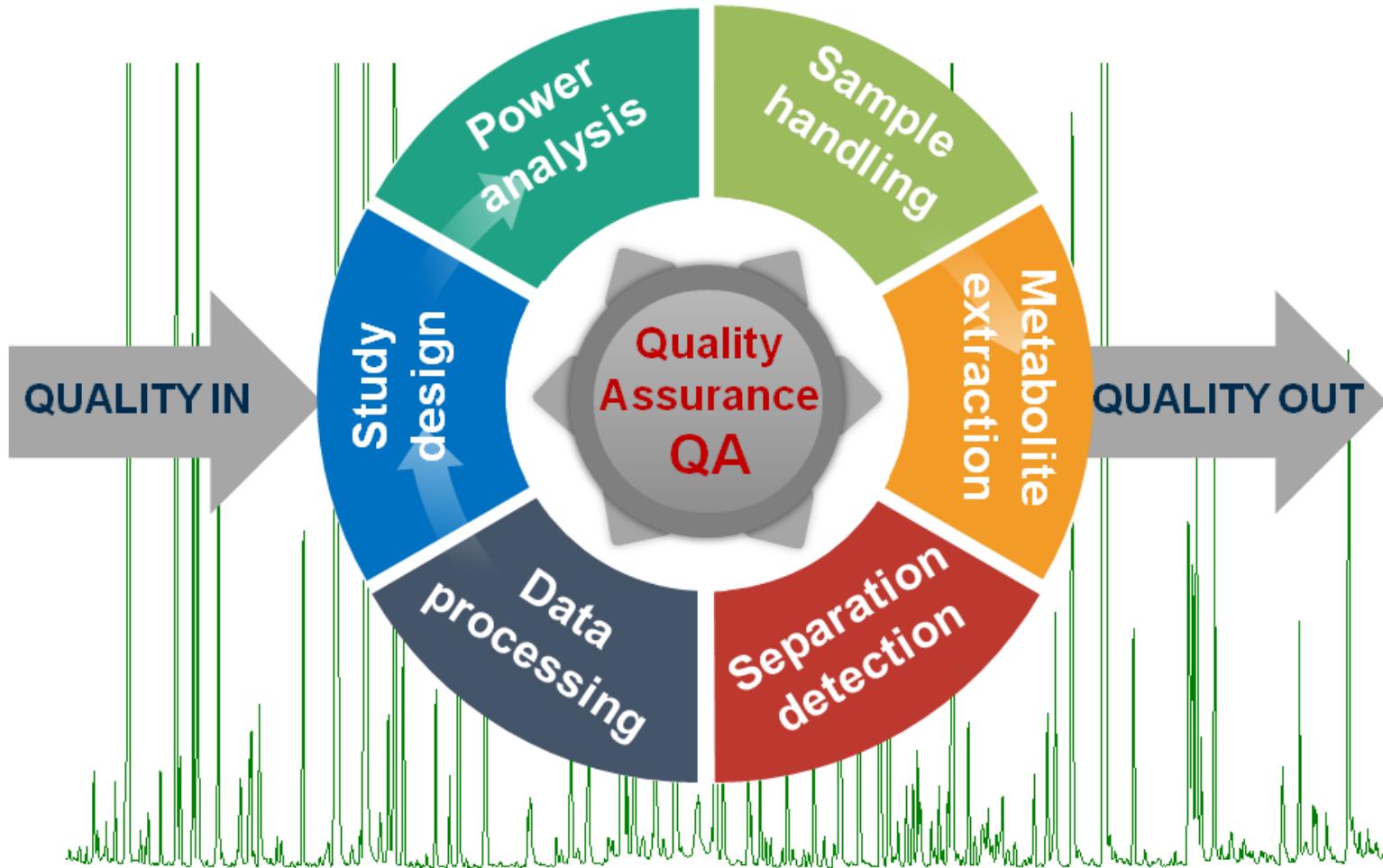


Quality control in metabolomics



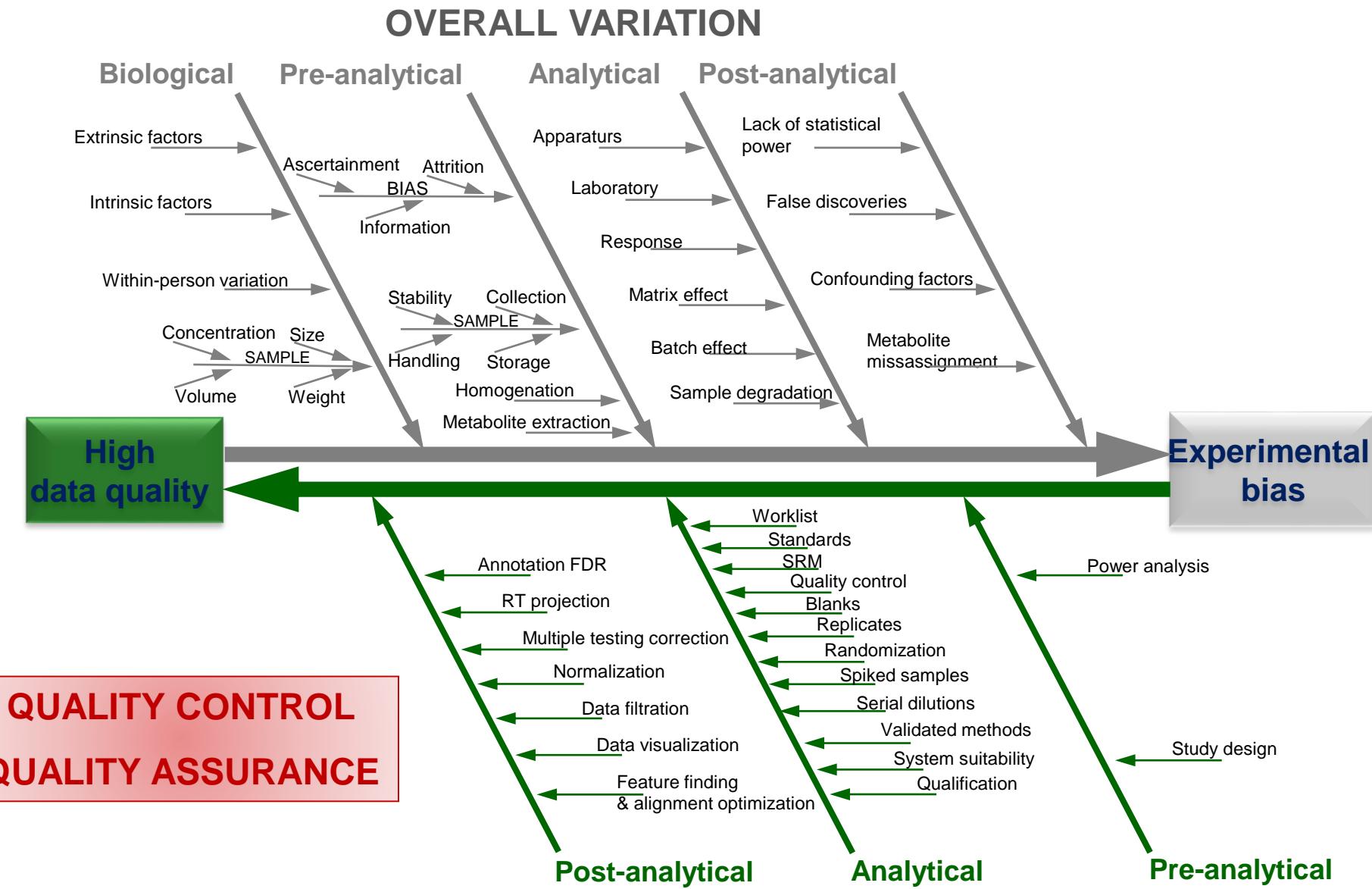
Recommendations and guidelines needed

Metabolomics workflow

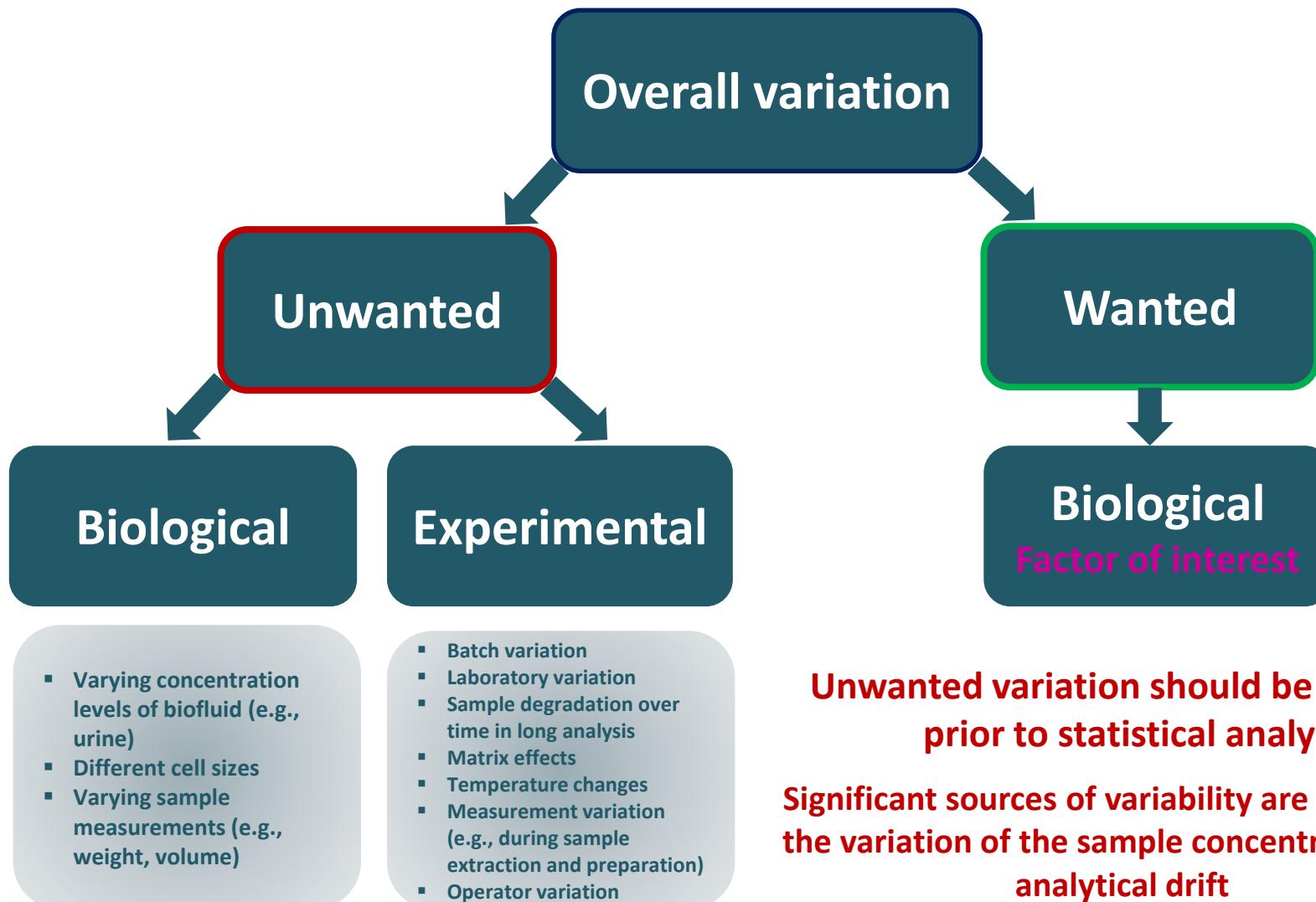


Dudzik D, Barbas-Bernardos C, García A, Barbas C. *Quality assurance procedures for mass spectrometry untargeted metabolomics. A review.* J Pharm Biomed Anal. 2018 Jan 5;147:149-173.

Sources of variation



The identification and reduction of the main sources of unwanted variation of metabolomics experiment is essential to ensure high data quality.



Unwanted variation should be removed prior to statistical analysis

Significant sources of variability are related to the variation of the sample concentration and analytical drift

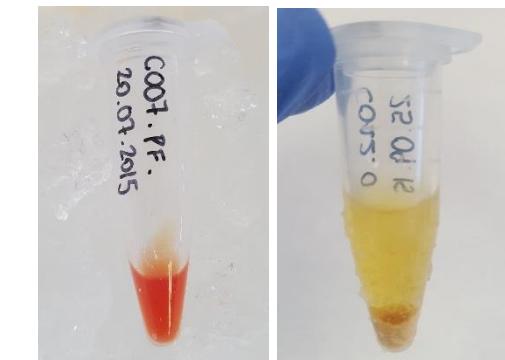
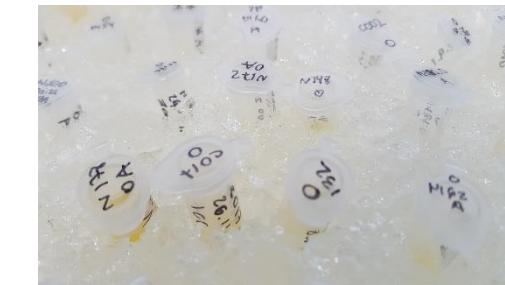
DATA QUALITY ASSESSMENT → NORMALIZATION

Pre-analytical

QA procedure	Controlled factor	General considerations*
Study design	Sample handling Sample collection Sample pre-processing Unwanted biological and analytical variation Sample stability Sample integrity	<ul style="list-style-type: none"> It is essential to develop validated SOPs to ensure that samples across the study are handled and pre-processed in consistent manner. Prospective vs. retrospective study. Population homogeneity: (i) intrinsic factors: (e.g., age, gender, genotype, health status, body composition, diurnal cycle, circadian rhythm resting metabolic rate, tissue turnover); (ii) extrinsic factors: diet, drugs, stress, physical activity, microbiota, smoking, alcohol intake, environmental factors, etc.); (iii) within-person variation; (iv) varying level of biofluids concentrations, cell size, sample measurements (e.g., volume, weight). Mendelian randomization – determination of causal relationship between exposure and outcome of the interest Provide representative samples and appropriate control group. Avoid poor quality samples e.g., hemolytic samples. Collect homogenous samples (especially for tissue samples). Consider addition of additives (e.g., anticoagulants) or preservatives (e.g., sodium azide). Stop metabolic turnover by inhibition of enzymatic activity (metabolism quenching) – LN2 or ice cold organic solvent. Process samples under a controlled temperature (4°C). Minimize time for sample processing. Prepare sample sub-aliquots. Consider type of storage tubes. Label samples in a clear way. Storage condition (recommended temperature -80°C, before and after extraction; be aware of light exposure and oxidation). Avoid freeze-thaw cycles.

Samples

- Samples: Plasma and Urine
- Plasma - Hemolysis or neonatal jaundice
- Urine - vary in concentrations



plasma

urine

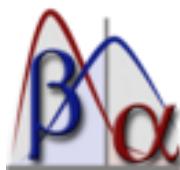
Plasma Sample	excluded CEMS	excluded GCMS	excluded LCMS	Observations
NUT065				
NUT068				
NUT069				
NUT077				
NUT081				
NUT083				
NUT085				
NUT102				
NUT103				
NUT106				low sample volume
NUT117				
NUT118				
NUT120				
NUT121				
NUT124				
NUT122				low sample volume
NUT125				
NUT127				
NUT131				
NUT134				
NUT135				
NUT136				
NUT137				low sample volume
NUT138				low sample volume
NUT143				
NUT144				
NUT146				low sample volume
NUT147				
NUT149				low sample volume
NUT150				
NUT151				
NUT154				
NUT160				sample analysed as plasma
NUT161				
NUT162				low sample volume
NUT165				low sample volume
NUT167				
NUT169				
NUT170				
NUT173				
NUT175				
NUT179				
NUT192				low sample volume
NUT193				
NUT184				
NUT190				
NUT185				
NUT186				
NUT187				
NUT188				
NUT191				
NUT197				
NUT204				low sample volume
NUT206				
NUT201				
NUT202				
C004				
C005				
C007				low sample volume
C008				low sample volume
C010				
C011				low sample volume
C013				low sample volume
C014				
C015				
C017				low sample volume
n analysed	52	66	66	

Colored on red – hemolytic samples?

Urine Sample	excluded CEMS	excluded GCMS	excluded LCMS	Observations
NUT080				
NUT081				
NUT085				
NUT086				
NUT091				
NUT100				
NUT103				
NUT105				
NUT117				
NUT118				
NUT120				
NUT121				
NUT124				
NUT125				
NUT127				
NUT128				
NUT131				
NUT132				
NUT133				
NUT134				
NUT135				
NUT136				
NUT137				
NUT138				
NUT141				
NUT142				
NUT143				
NUT144				
NUT146				
NUT147				
NUT148				
NUT149				
NUT150				
NUT151				
NUT152				
NUT154				
NUT155				
NUT157				low sample volume
NUT160				PLASMA not URINE
NUT161				
NUT162				
NUT163				
NUT166				
NUT167				
NUT168				
NUT170				
NUT172				
NUT173				
NUT174				
NUT176				
NUT177				
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NUT183				
NUT184				
NUT185				
NUT186				
NUT187				
NUT188				
NUT189				
NUT190				
NUT192				
NUT193				
NUT197				
NUT204				low sample volume
NUT201				
NUT202				
C002				
C004				
C005				
C008				
C012				
C014				
C015				
C016				
C017				
n analysed	76	78	78	

Pre-analytical

QA procedure	Controlled factor	General considerations*
Power analysis	Sample size Statistical power	<ul style="list-style-type: none">• Minimum required sample size should be predetermined.• The more inherent variation is controlled, the fewer sample is required to the study.• Power of the statistical test increases with increasing sample size. Large sample size has a greater ability than a small sample to detect an important effect if it exists.• The lower variability of the observations the higher statistical power detected.• The standard deviation for power calculation could be estimated from a pilot study or, if not available, from other published studies on the same subject.• Adjust the sample size by recruiting more patients into the study at the outset (drop-out rate).



G*Power is a free, open source program for power analysis and sample size calculations.

Analytical

QA procedure	Controlled factor	General considerations*
Qualification	System performance	<ul style="list-style-type: none">• Design Q – setting the functional and performance specifications; user requirement specifications, operational specifications, vendor qualification.• Installation Q – performing and documenting installation; check as purchased; check proper installations of hardware and software.• Operational Q – testing the equipment to ensure specifications; performance testing.• Performance Q – testing the system performance according to the specifications and selected application; periodic calibration; preventive system maintenance• <u>Analytical instruments qualifications:</u><ul style="list-style-type: none">➤ Level of qualification depends of the type of instrument and on application.➤ Group A - standard equipment with no measurement capability e.g., vortex mixers and centrifuges.➤ Group B - standard equipment and instruments providing measure values, e.g., balance, pH meters, oven, pipettes. This group also includes equipment controlling physical parameters, such temperature, pressure or flow.➤ Group C - instruments and computerized analytical systems, e.g., HPLC systems and mass spectrometers.

Analytical

QA procedure	Controlled factor	General considerations*
System suitability	System performance Results quality	<ul style="list-style-type: none">Verify that the system will perform in accordance with specified criterial for the chosen analytical procedure.Should be performed at the beginning of each analytical batch including tunning and mass calibration, together with chromatographic column and MS source cleaning. In case of GC-MS injector liner, septum or syringe cleaning or if necessary column cutting should be applied.In case of CE-MS system, that refers to the capillary cutting and ion source cleaning.
Analytical method	Linearity Selectivity Sensitivity Limit of detection Limit of quantification Precision	<ul style="list-style-type: none">Linear response between signal and concentration within the broadest possible range of compound classes.Prove the resolution with isobaric compounds.Calculation of the LOQ (accurately and precisely) when possible, if not, calculation of the LOD.Several measurements of the standards with a concentration in the calibration curve (inter and intraday precision).Introduce intentionally variations to check if the method remains unaffected.
Serial dilutions Spiked samples	Proportionality Matrix effect Selectivity	<ul style="list-style-type: none">For each analyte of interest, use a dilution factor to generate the calibration curve.Spike diluted QC's to check ion suppression, matrix effect and selectivity loss.

Analytical

QA procedure	Controlled factor	General considerations*
Randomization	Unwanted variation Unwanted correlations	<ul style="list-style-type: none"> Level of sample collection – in order to obtain representative and homogenous samples; include participants enrollment, experimental group assignment. Level of sample preparation and analysis - to ensure that the analysis of the resulting data are not biased by sample preparation or analysis order. Constrained randomization – dependent or matched samples. Applied to ensure that the bias related to instrumental drift will not be confounded with the biological variation of interest.

Randomization	
Samples	Rand
1C	0.84
2C	0.38
3C	0.76
4C	0.90
5C	0.61
6C	0.88
7C	0.12
8C	0.47
9C	0.56
10C	0.86
1D	0.36
2D	0.28
3D	0.32
4D	0.04
5D	0.45
6D	0.92
7D	0.71
8D	0.03
9D	0.89
10D	0.54

Homogenization	
Samples	Rand
8D	0.24
1C	0.02
4C	0.02
4D	0.12
10C	0.41
7C	0.69
9C	0.08
10D	0.44
8C	0.67
9D	0.87
1D	0.00
5C	0.89
6D	0.82
6C	0.43
7D	0.97
5D	0.83
2C	0.43
2D	0.82
3C	0.16
3D	0.70

Extraction	
Samples	Rand
3C	0.30
10C	0.86
6D	0.04
7C	0.27
5D	0.69
4D	0.06
4C	0.94
6C	0.28
1C	0.49
7D	0.31
2C	0.64
8D	0.73
3D	0.71
9C	0.11
10D	1.00
9D	0.44
8C	0.47
2D	0.79
5C	0.41
1D	0.68

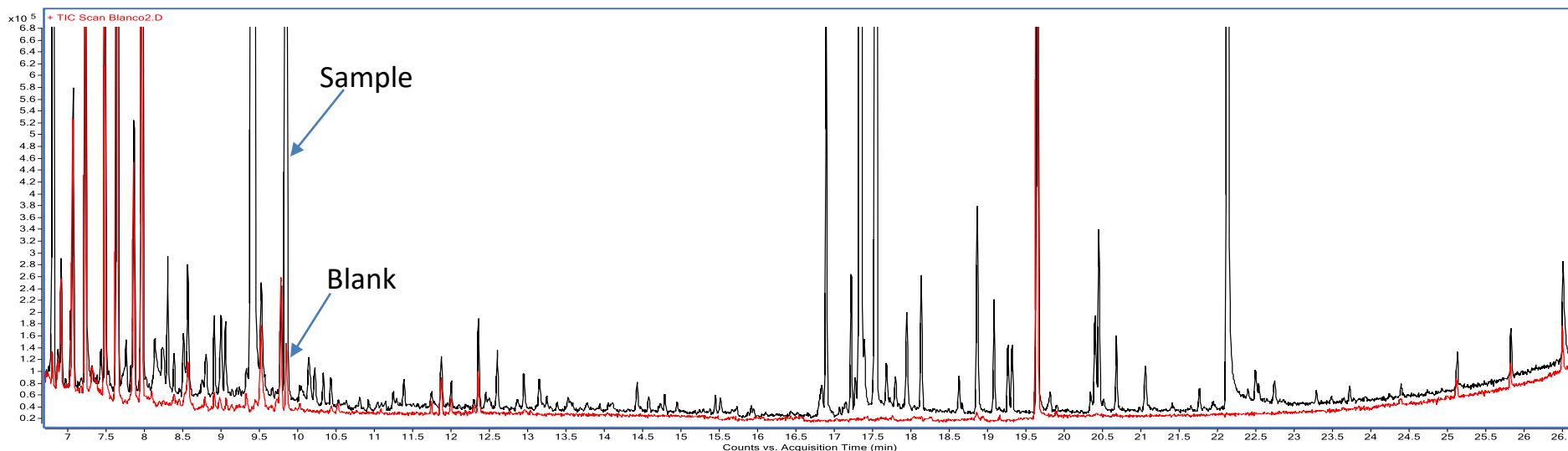
Analysis	
Samples	Rand
10C	0.75
3D	0.33
2D	0.95
4C	0.00
1D	0.66
5D	0.51
1C	0.42
3C	0.36
10D	0.87
7C	0.38
7D	0.53
5C	0.49
9C	0.65
4D	0.79
8C	0.13
2C	0.89
8D	0.29
9D	0.83
6C	0.91
6D	0.53

Analytical

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<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th>No</th> <th>Sample</th> <th>Position</th> <th>Method</th> <th>File</th> </tr> </thead> <tbody> <tr><td>1</td><td>Mix19</td><td>30</td><td>Resp_17s_25MBar_30KV_30min.m</td><td>mix19_2-r002.d</td></tr> <tr><td>2</td><td>Mix19</td><td>30</td><td>Resp_17s_25MBar_30KV_30min.m</td><td>mix19_2-r003.d</td></tr> <tr><td>3</td><td>Mix19</td><td>30</td><td>Resp_17s_25MBar_30KV_30min.m</td><td>mix19_2-r004.d</td></tr> <tr><td>4</td><td>Blank_1</td><td>5</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>Blank_1.d</td></tr> <tr><td>5</td><td>QCeq</td><td>7</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>QCeq_1-r001.d</td></tr> <tr><td>6</td><td>QCeq</td><td>7</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>QCeq_1-r002.d</td></tr> <tr><td>7</td><td>QCeq</td><td>7</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>QCeq_1-r003.d</td></tr> 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<tr><td>17</td><td>13_1</td><td>13</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>13_1.d</td></tr> <tr><td>18</td><td>13_2</td><td>13</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>13_2.d</td></tr> <tr><td>19</td><td>13_3</td><td>13</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>13_3.d</td></tr> <tr><td>20</td><td>1_1</td><td>14</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>1_1.d</td></tr> <tr><td>21</td><td>1_2</td><td>14</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>1_2.d</td></tr> <tr><td>22</td><td>1_3</td><td>14</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>1_3.d</td></tr> <tr><td>23</td><td>QC3</td><td>6</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>QC3.d</td></tr> <tr><td>24</td><td>16_1</td><td>15</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>16_1.d</td></tr> <tr><td>25</td><td>16_2</td><td>15</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>16_2.d</td></tr> <tr><td>26</td><td>16_3</td><td>15</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>16_3.d</td></tr> <tr><td>27</td><td>12_1</td><td>16</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>12_1.d</td></tr> <tr><td>28</td><td>12_2</td><td>16</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>12_2.d</td></tr> <tr><td>29</td><td>12_3</td><td>16</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>12_3.d</td></tr> <tr><td>30</td><td>QC4</td><td>6</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>QC4.d</td></tr> <tr><td>31</td><td>15_1</td><td>17</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>15_1.d</td></tr> <tr><td>32</td><td>15_2</td><td>17</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>15_2.d</td></tr> <tr><td>33</td><td>15_3</td><td>17</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>15_3.d</td></tr> <tr><td>34</td><td>2_1</td><td>18</td><td>Resp_50s_25MBar_30KV_125V_30min.m</td><td>2_1.d</td></tr> 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Analytical

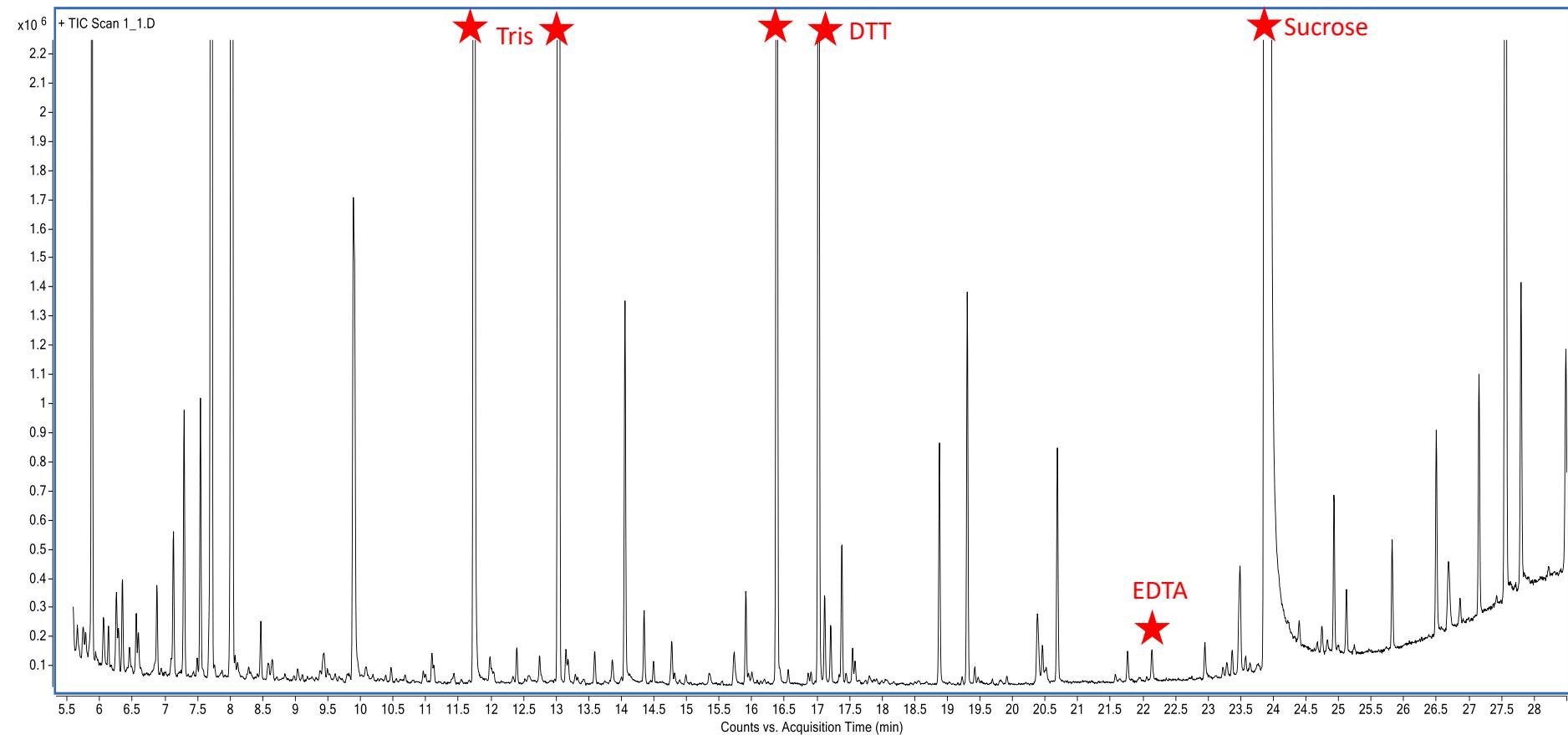
QA procedure	Controlled factor	General considerations*
Blank samples	Signal-to-noise ratio Contaminations Interferences Artifact feature Carry-over Matrix-effect	<ul style="list-style-type: none"> Matrices that have no measurable amount of the analyte of interest. Collection blank - sample that has been collected from the same source as the experimental sample. Extraction blank - prepared following exactly the same procedure as experimental samples. Mobile phase blank - consist only the mobile phase. Cleaning blank - sample of strong solvent e.g., isopropanol introduced in order to clean the system. Can be helpful to identify the artifacts. Consider features with the main values in blank higher than 10% as non-relevant. Essential for any QA procedure.



Analytical

Contaminations

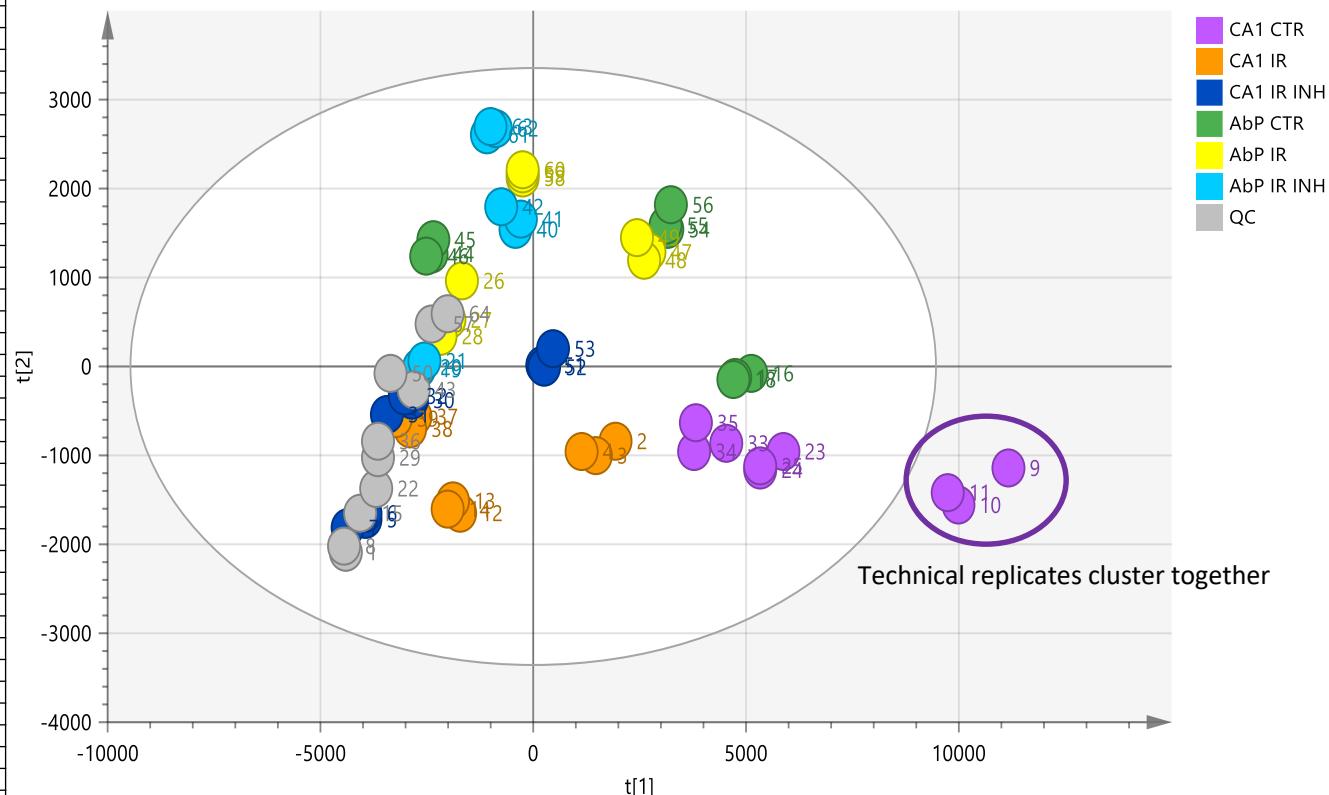
Hippocampi were homogenized in ice cold isotonic **buffer** (15 mM **Tris/HCl** (tromethamine), pH 7.5, 0.25 M **sucrose**, 1 mM **MgCl₂**, 1 mM **EGTA**, 2 mM **EDTA**, 1 mM **PMSF** (phenylmethylsulfonyl fluoride) and 1 mM **DTT** (Dithiothreitol)), prior to centrifugation (1000g, 10 min, 4°C). The supernatant was centrifuged at 11,000g for 20 min at 4 °C to yield a particulate fraction enriched with mitochondria (P2), as well as a soluble fraction (S2). The pure mitochondrial pellet was obtained after centrifugation of P2 (100,000g, 30 min, 4 °C) with 12% **Ficoll**.



Analytical

QA procedure	Controlled factor	General considerations*
Replicates	Unwanted variation	<ul style="list-style-type: none"> Biological: for quantities that vary due to external factors (e.g., blood glucose level which vary with food intake). It may be necessary to take several measurements over a period of time to get an accurate result. Analytical (technical): for quantities that are difficult to measure accurately. Replicate sample – to acquire data regarding sample to sample processing consistency.

No	Sample	Position	Method	File
1	Mix19	30	Resp_17s_25MBar_30KV_30min.m	mix19_2-r002.d
2	Mix19	30	Resp_17s_25MBar_30KV_30min.m	mix19_2-r003.d
3	Mix19	30	Resp_17s_25MBar_30KV_30min.m	mix19_2-r004.d
4	Blank_1	5	Resp_50s_25MBar_30KV_125V_30min.m	Blank_1.d
5	QCeq	7	Resp_50s_25MBar_30KV_125V_30min.m	QCeq_1-r001.d
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9	QC1	6	Resp_50s_25MBar_30KV_125V_30min.m	QC1.d
10	5_1	11	Resp_50s_25MBar_30KV_125V_30min.m	5_1.d
11	5_2	11	Resp_50s_25MBar_30KV_125V_30min.m	5_2.d
12	5_3	11	Resp_50s_25MBar_30KV_125V_30min.m	5_3.d
13	7_1	12	Resp_50s_25MBar_30KV_125V_30min.m	7_1.d
14	7_2	12	Resp_50s_25MBar_30KV_125V_30min.m	7_2.d
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17	13_1	13	Resp_50s_25MBar_30KV_125V_30min.m	13_1.d
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19	13_3	13	Resp_50s_25MBar_30KV_125V_30min.m	13_3.d
20	1_1	14	Resp_50s_25MBar_30KV_125V_30min.m	1_1.d
21	1_2	14	Resp_50s_25MBar_30KV_125V_30min.m	1_2.d
22	1_3	14	Resp_50s_25MBar_30KV_125V_30min.m	1_3.d
23	QC3	6	Resp_50s_25MBar_30KV_125V_30min.m	QC3.d
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26	16_3	15	Resp_50s_25MBar_30KV_125V_30min.m	16_3.d
27	12_1	16	Resp_50s_25MBar_30KV_125V_30min.m	12_1.d
28	12_2	16	Resp_50s_25MBar_30KV_125V_30min.m	12_2.d
29	12_3	16	Resp_50s_25MBar_30KV_125V_30min.m	12_3.d
30	QC4	6	Resp_50s_25MBar_30KV_125V_30min.m	QC4.d
31	15_1	17	Resp_50s_25MBar_30KV_125V_30min.m	15_1.d
32	15_2	17	Resp_50s_25MBar_30KV_125V_30min.m	15_2.d
33	15_3	17	Resp_50s_25MBar_30KV_125V_30min.m	15_3.d
34	2_1	18	Resp_50s_25MBar_30KV_125V_30min.m	2_1.d
35	2_2	18	Resp_50s_25MBar_30KV_125V_30min.m	2_2.d
36	2_3	18	Resp_50s_25MBar_30KV_125V_30min.m	2_3.d
37	QC5	6	Resp_50s_25MBar_30KV_125V_30min.m	QC5.d



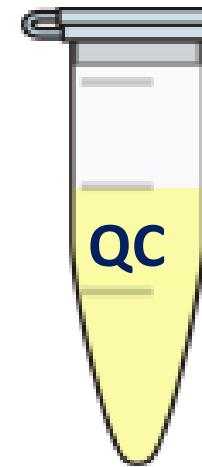
Analytical

QA procedure	Controlled factor	General considerations*
Quality Control	Efficiency of the sample preparation and metabolite extraction System stability and system performance Reliability of the system Reproducibility Variability Data quality Method development Data normalization	<ul style="list-style-type: none">• QC samples should be representative of the qualitative and quantitative composition of the study samples.• Different type of QC sample exist (pooled, extraction, surrogate, commercially available), that should be considered before the study.• QC allows to correct signal drift within batches and between analytical batches.• Should be used for %RSD calculation for both retention time (RSD<1% as general rule) and metabolic features (LC/MS and CE/MS<20%; GC/MS<30%) that allow for estimation of the overall precision.• The results obtained from the QC samples should be monitored by plotting them on a control chart, check for systematic (trend, shift) and random error.• QC gives the highest level of QA.

Quality Control Samples

single sample or a set of samples representative of the qualitative and quantitative composition of the samples under investigation

- QC samples should be **as similar as possible in matrix and metabolite composition** to the real samples.



TYPE of QC samples



raw sample

- pool of all experimental samples (higher dilution of the metabolites of interest)
- pool of each experimental group (avoid metabolite dilution)
- measure of **sample treatment**, data acquisition and data pre-processing



- pool of already prepared samples
- measure of data acquisition and data pre-processing

extracted sample



surrogate

- pool of available sample (other than experimental or samples purchased commercially)
- **risk of loss of very specific markers**
- measure of **sample treatment**, data acquisition and data pre-processing

The use of QC samples

monitor and control

- system stability and reproducibility
- deviations in sample preparation

equilibrium

- to equilibrate the system to achieve fully reproducible conditions

signal correction

- to correct small levels of drift in the measured signal over analysis (within batches) and between analytical batches

batch integration

- integration of different analytical batches – important for large scale studies
- requires the use of the same QC sample across all batches

Quality Assurance

- quantitative calculation of measurement precision of replicate injections of QC
- PCA modelling for quick visualisation and % detection rate and relative standard deviation (RSD)

Data quality assessment

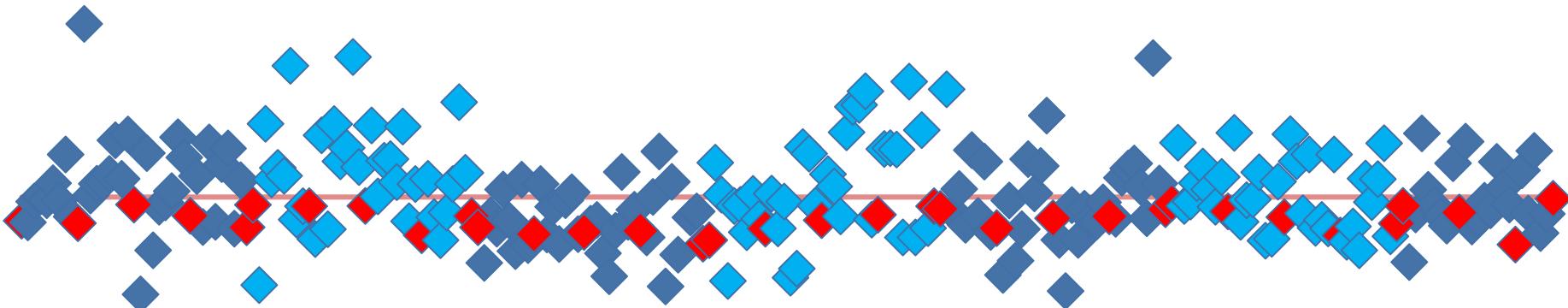
monitor and control

- system stability and reproducibility
- deviations in sample preparation

signal correction

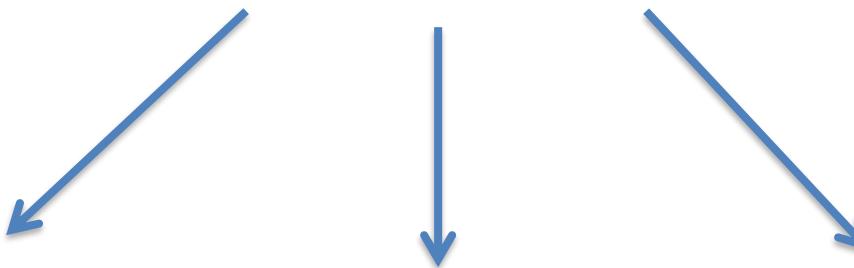
- to correct small levels of drift in the measured signal over analysis (within batches) and between analytical batches

QC samples are theoretically identical and therefore
the signal should be (relatively) constant over time and not show any instrumental drift over time.



Data quality assessment

QC samples inspection



Raw data TIC inspection

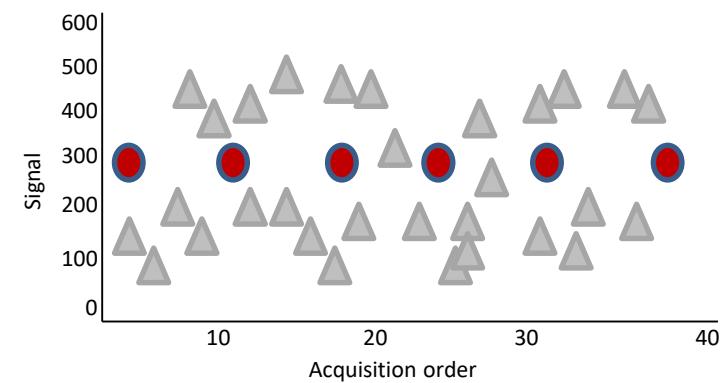
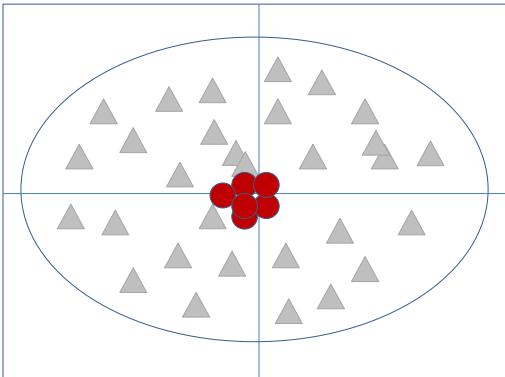
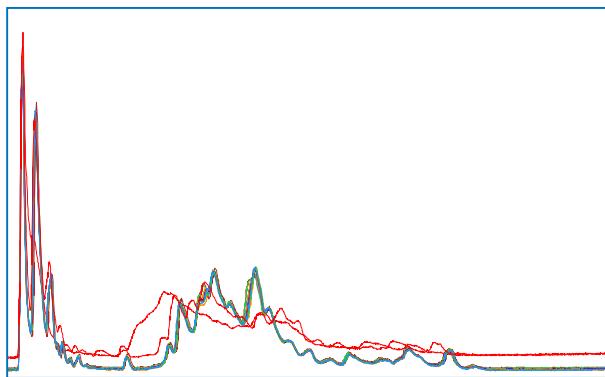
- what is the QCs performance

MVA – PCA projection

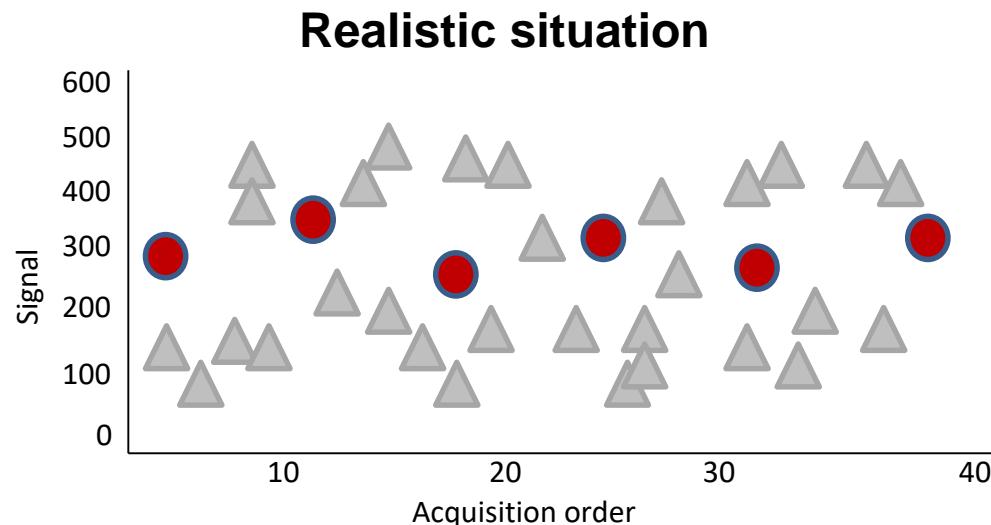
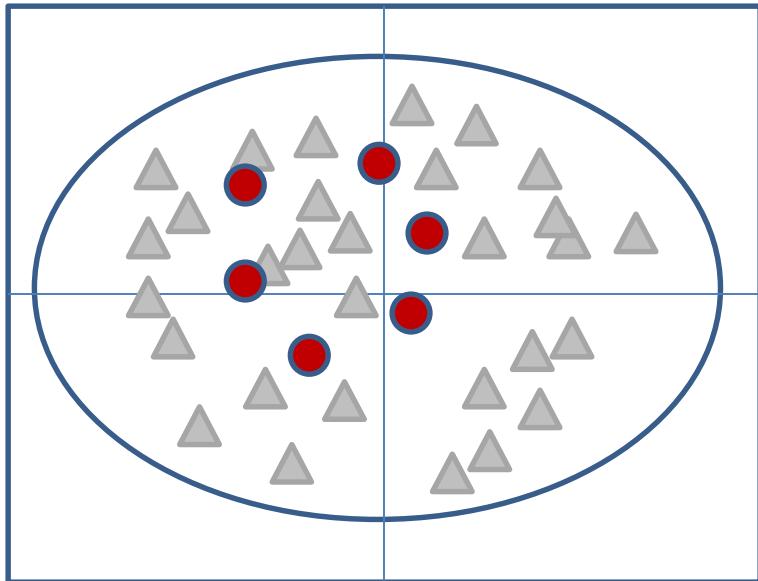
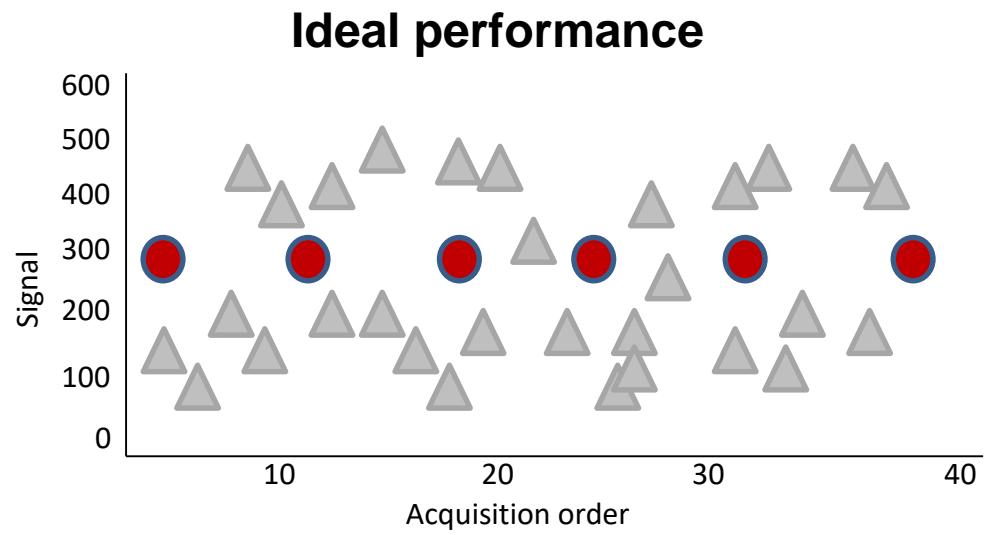
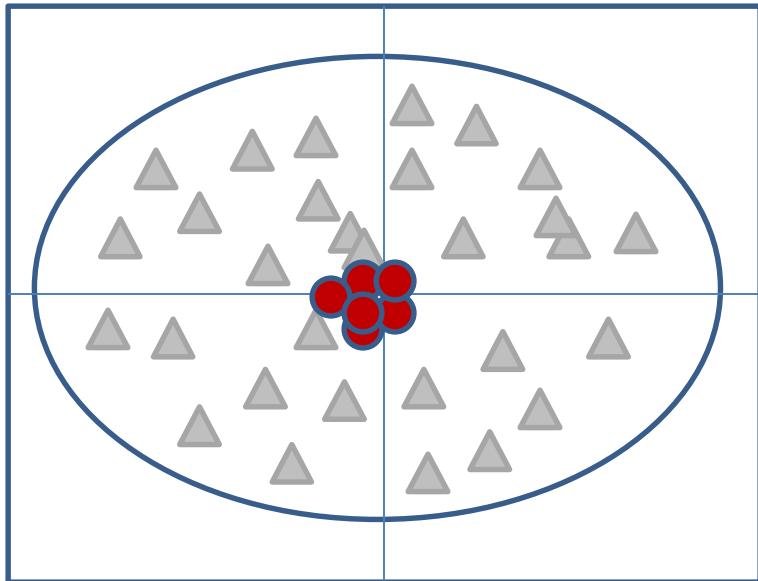
-how/where the QCs cluster

Scatter plot

-how the QCs arrange
-what is the samples distribution across the different STD regions

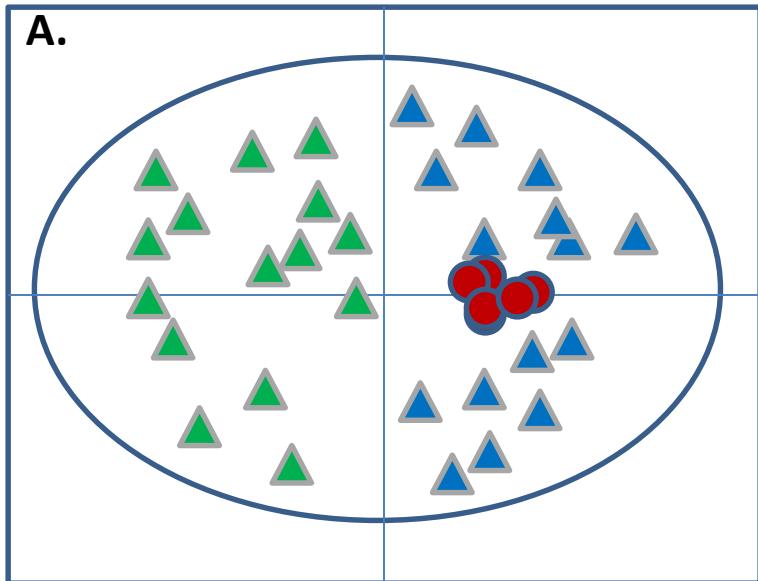


Data quality assessment

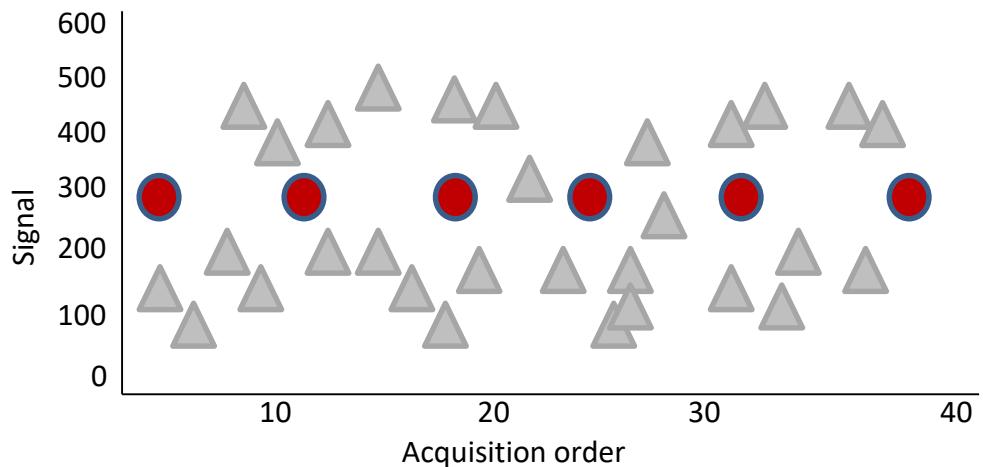


Data quality assessment

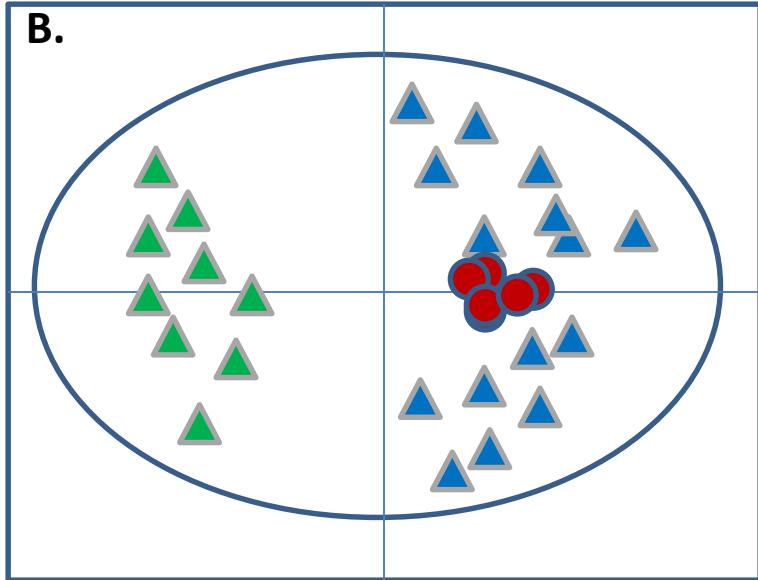
A.



Ideal performance



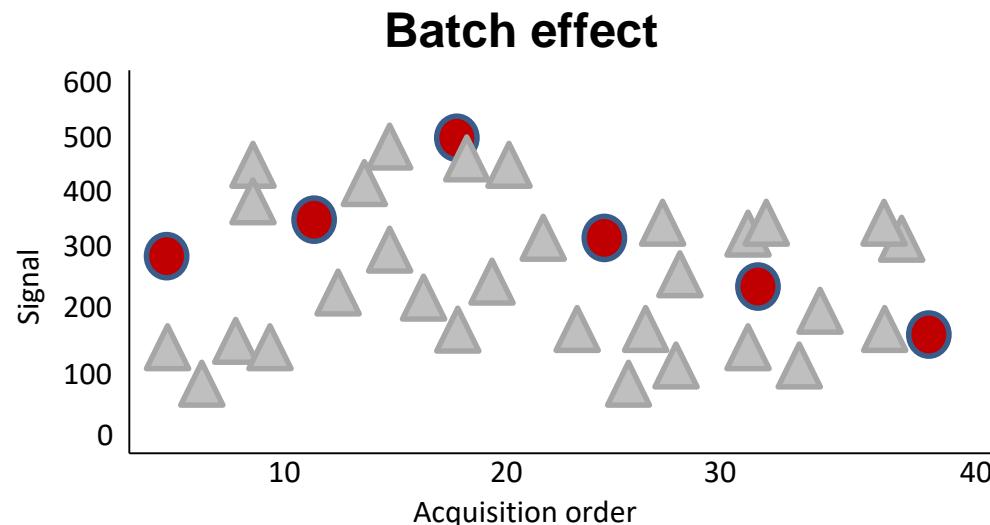
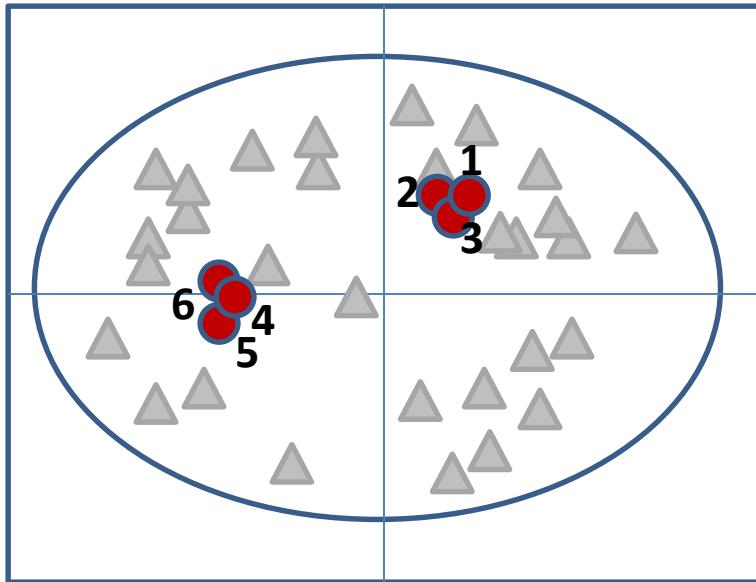
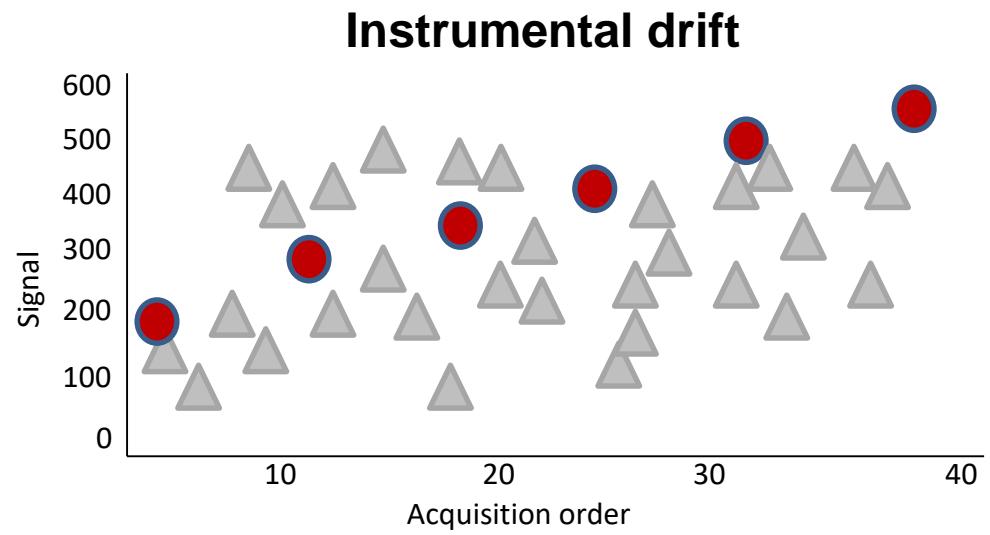
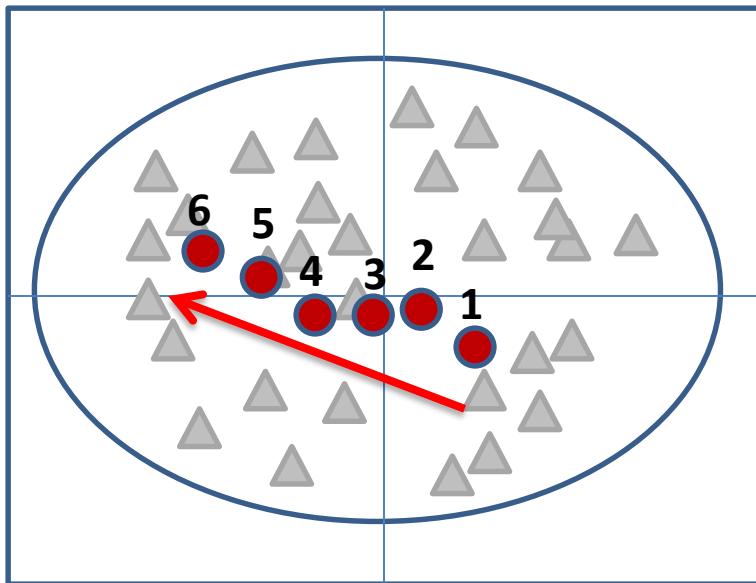
B.



QC centered

- A. QC cluster moved due to disproportion in the number of the samples.
- B. QC cluster moved due to the detected metabolites.

Data quality assessment



Batch effect

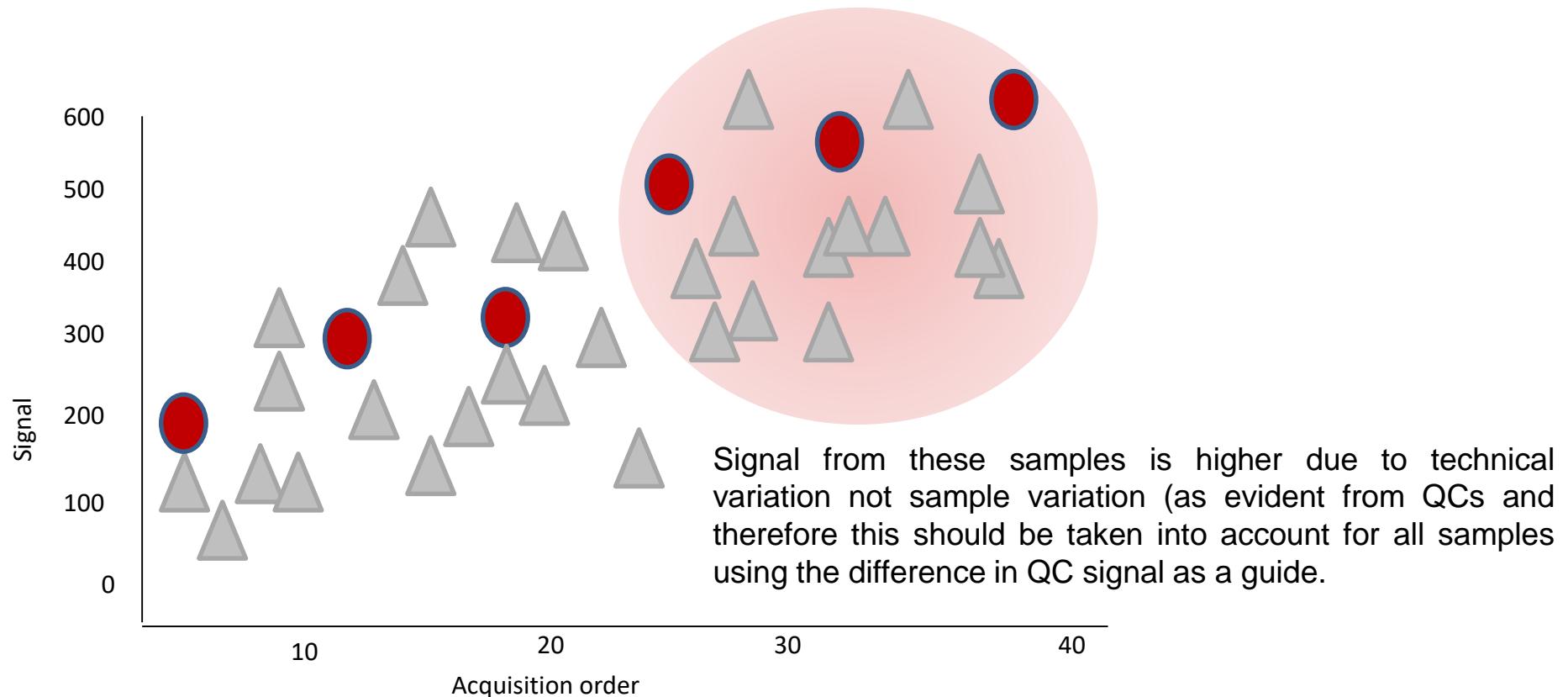
Experimental variation

- The first few injections of sample give unrepresentative results due to small changes in chromatographic RT and/or signal intensity.
- After 5-10 injections of the matrix, RT stabilize as the column becomes “conditioned” and the system then shows little variability through the remainder of the run.
- The source of the mass spectrometer can become contaminated, leading to gradual changes in instrument sensitivity over time.
- Batch effects may occur if a subset of experiments was run on different days, technicians, labs, etc.
- Downstream analysis should consider if differences seen are of biological significance or simply due to two different experiments.

Batch effect

batch integration

- integration of different analytical batches – important for large scale studies
- requires the use of the same QC sample across all batches



Several algorithms are available to correct intra- and inter- batch variation

Analytical

QA procedure	Controlled factor	General considerations*
Standards	Unwanted variation	<ul style="list-style-type: none">Known metabolites added to each biological sample before extraction.IS cannot remove unwanted biological variationVariation captured by IS depends on its chemical properties and could include other source of variation e.g., arising from chromatographical system or ion suppression.

Post-analytical

QA procedure	Controlled factor	General considerations*
Data Visualization	Data variability Signal changes Systematic or random error Unwanted correlations Potential outliers	<ul style="list-style-type: none">Provide a quick graphical summary of data.Help to understand variation, evaluate analytical performance, trends, shift or outlying observations.Raw chromatogram (EIC, BPC, ECC), pressure curve or current flow.Control chart (Shewhart control chart) for understanding process variability. Allow to overview of the analytical precision based on plotting the sum of metabolic feature intensities for every experimental and QC sample against run order.PCA – unsupervised multivariate method that allow for evaluating signal drift, sensitivity loss, variation in QC samples. The score plot can be used to identify the differences or similarities among the samples and identification of the outliers. The loadings plot can be used to identify the signals responsible for the grouping or separation among the samples.

Quality Assurance

Post-analytical

- quantitative calculation of measurement precision of replicate injections of QC
- PCA modelling for quick visualisation and % detection rate and relative standard deviation (RSD)

Data quality assessment

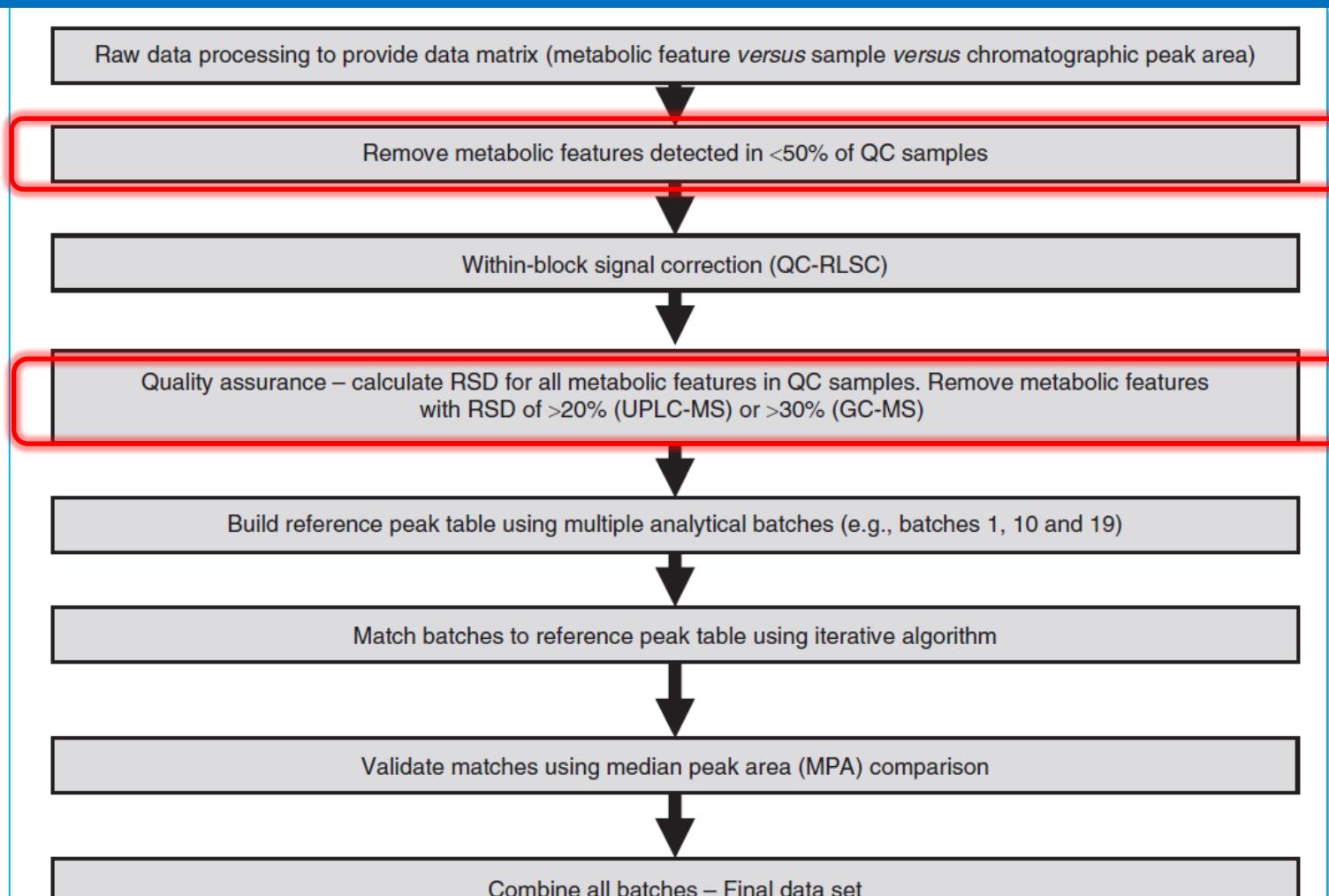


Figure 3 | The data preprocessing workflow for UPLC-MS data. The workflow incorporates QC samples for quality assurance, QC-RLSC and block integration.

Data quality assessment

Exploratory Analysis – Raw data

- 1. Overview of the overall quality of the analytical run.**
 - Inspect TIC (RT precision, peak shape, height).
 - Inspect pressure stability.
- 2. Set the optimum parameters in the data extraction software**
- 2. Evaluate QC samples.**
 - Inspect TIC for all QC (conditioning and analytical) to ensure that the column conditioning has been successful and the system was stable over the duration of the run.
- 3. Perform PCA and plot all samples and QC.**
 - Check for run order effect.
 - Consider excluding samples before and after QC outliers.

Data quality assessment

Review re-processed data matrix

1. Check for features reported with the same mass and proximal RT (indication of failed peak alignment).
2. Overview of the analytical precision based on the QC sample analysis.
 - Sum features intensities for every sample and QC and plot according to run order.
 - Check for signal drift, sensitivity loss, variation in QC
 - Overviewed potential outliers.
 - Estimate the precision of intensity for every feature in the QC by assessing SD and RSD.

Post-analytical

- **Ion:** Atomic, molecular, or radical species with a non-zero net electric charge.
- **Adduct ion.** Ion formed by the interaction of a precursor ion with one or more atoms or molecules to form an ion containing all the constituent atoms of the precursor ion as well as the additional atoms from the associated atoms or molecules. For example, a Na^+ adduct of a molecule (M) that is represented as $[\text{M} + \text{Na}]^+$.
- **Peak:** Localized region of relatively intense detector response in a mass spectrum when ions of a specified m/z are detected. If resolving power is insufficient two or more components of similar m/z may contribute to one unresolved mass peak. Although mass peaks are often associated with particular ions, the terms peak and ion should not be used interchangeably.
- **Base peak (BP):** Peak in a mass spectrum that has the greatest intensity.
- **Metabolic feature.** The combination of the exact m/z and the corresponding RT.
- **m/z (mass-to-charge ratio):** Represents a relationship between the mass of a given ion and the number of elementary charges that it carries.
- **Accurate mass:** Experimentally determined mass of an ion of known charge. Accurate mass and exact mass are not synonymous. Accurate mass refers to a measured mass, and exact mass refers to a calculated mass.
- **Mass chromatogram:** Graphical representation of mass spectrometry (MS) data, where x-axis represents retention time (RT) and y-axis represents signal intensity.

Post-analytical

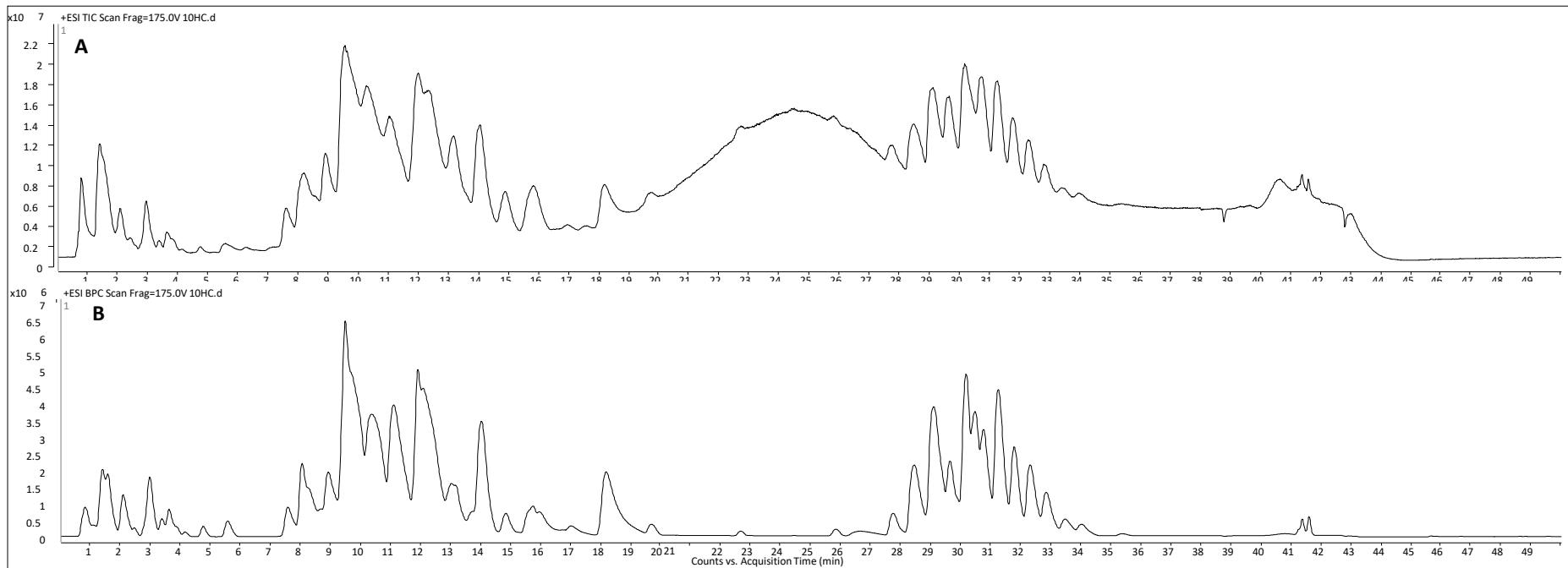


Figure. Representative chromatograms of the **A.** Total ion Chromatogram and **B.** Base Peak Chromatogram

- **Total Ion Chromatogram (Total Ion Current) (TIC):** The TIC shows the sum of the signals from all ions detected (summed intensity of all ions) in any given spectrum, plotted versus time.
- **Base Peak Chromatogram (BPC):** All ions from the base peak (the most intense mass peak in the mass spectrum) are summed and plotted as a function of time. So it is a plot of the intensity of the largest peak in a mass spectrometer scan versus time.

Post-analytical

- **Extracted Ion Chromatogram (EIC).** One or more m/z values representing one or more analytes of interest are recovered ('extracted') from the entire data set for a chromatographic run. Chromatogram created by plotting the intensity of the signal observed at a chosen m/z value or set of values in a series of mass spectra recorded as a function of retention time.
 - **Mass spectrum:** An intensity vs. m/z (mass-to-charge ratio) plot representing a chemical analysis. The mass spectrum of a sample is a pattern representing the distribution of ions by m/z in a sample.
 - **Background mass spectrum:** Mass spectrum observed when no analyte is introduced into the mass spectrometer.
- Centroid acquisition:** Procedure of recording mass spectra in which an automated computer-based system detects peaks, calculates the centroid based on the average m/z value weighted by the intensity, and assigns m/z values based on a calibration file. Only the centroid m/z value and the peak magnitude are stored.
- **Profile mode:** Method for acquiring a mass spectrum where each peak is displayed as a curve, with the data points defining the curve corresponding to the signal intensities at each particular m/z value.

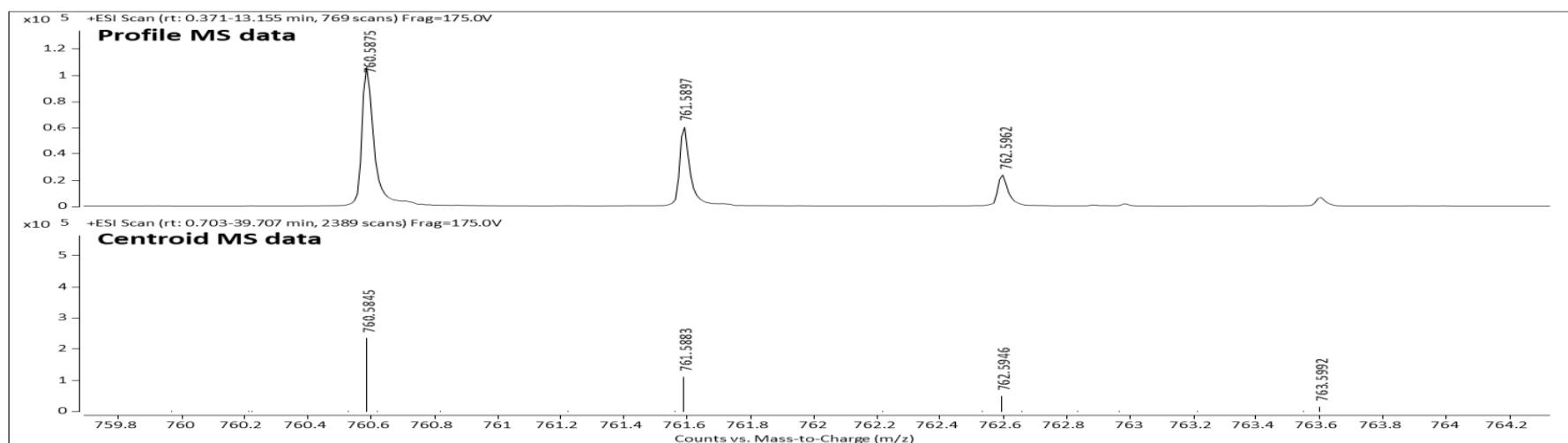
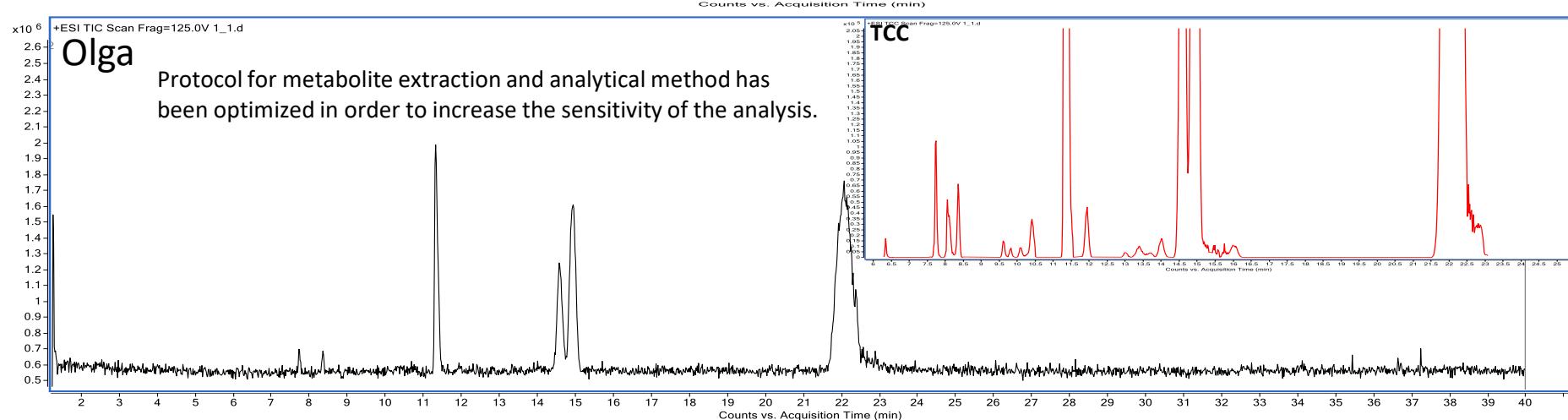
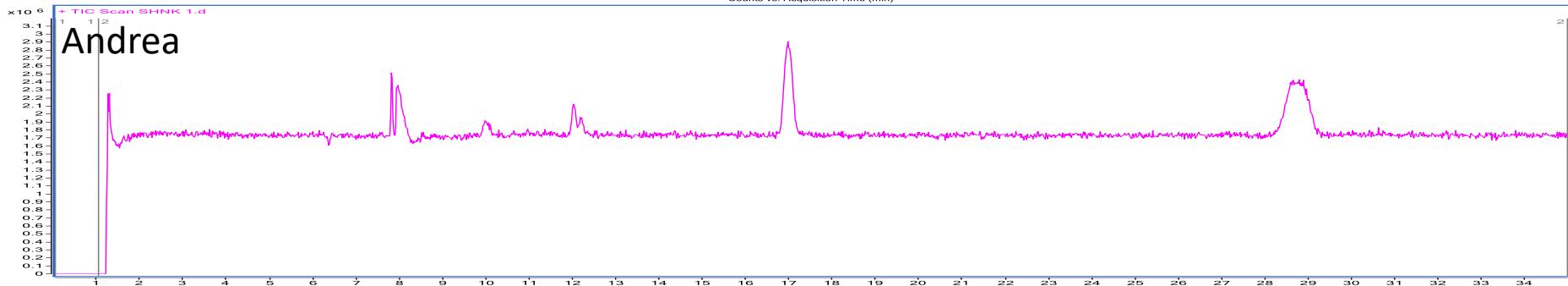
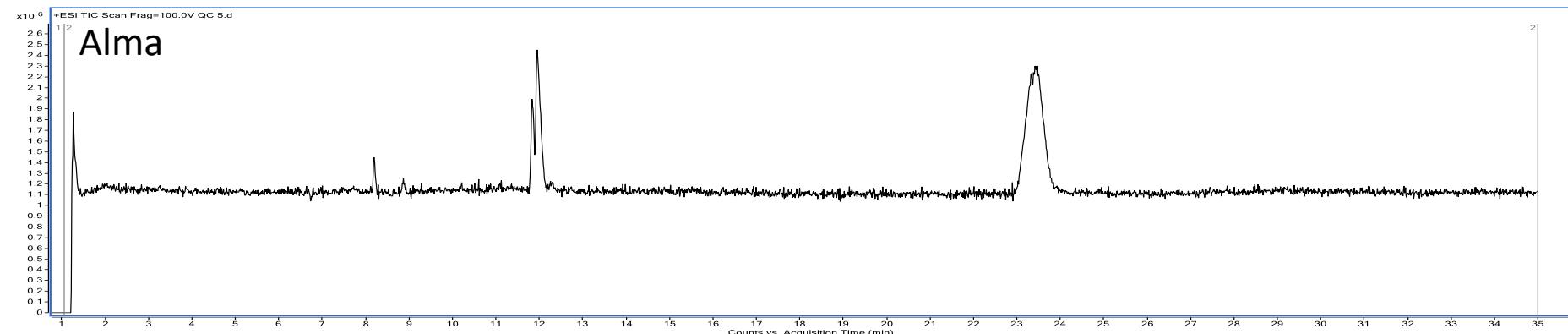
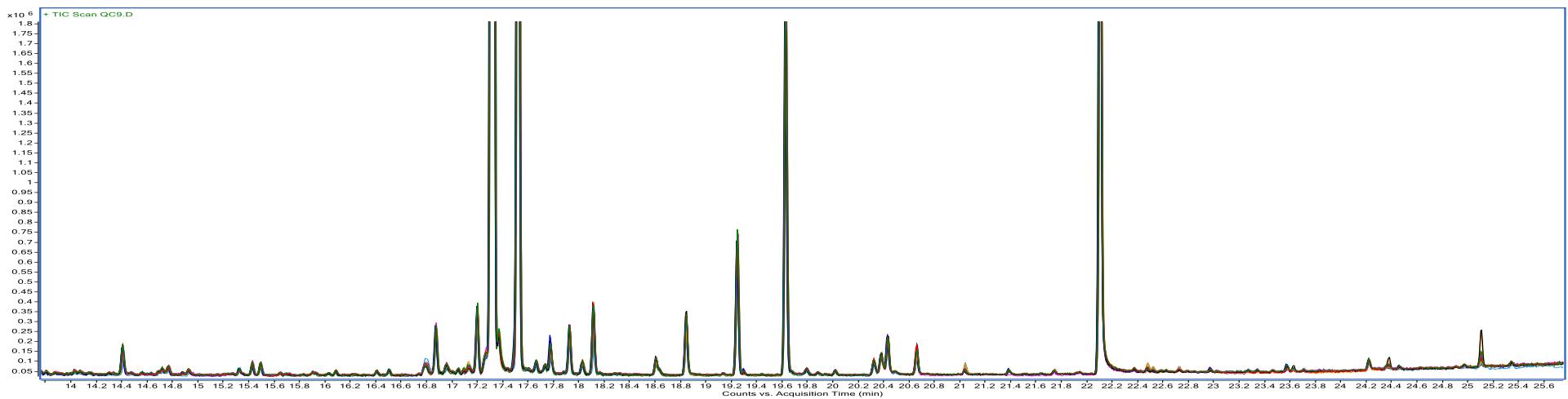
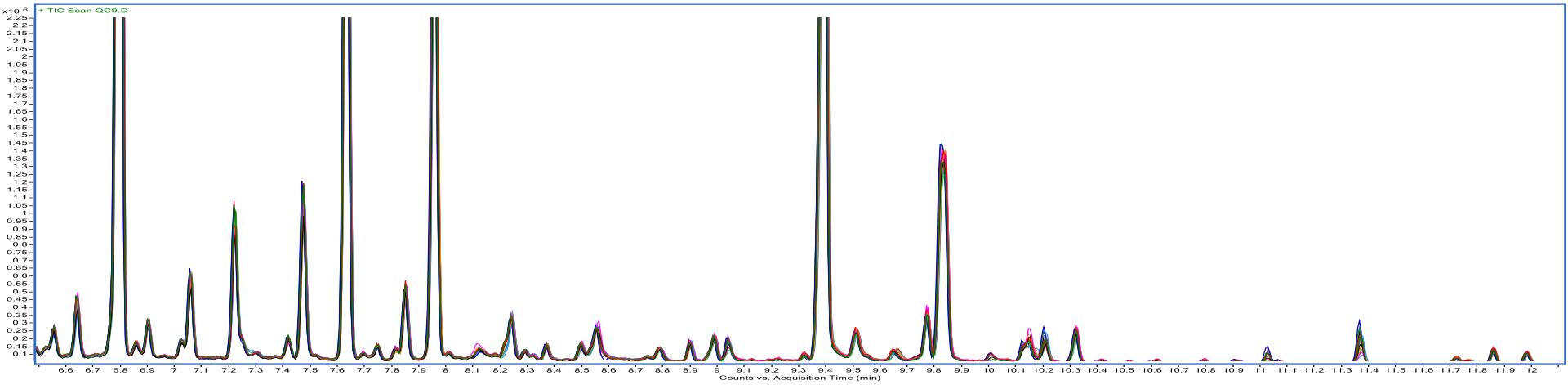
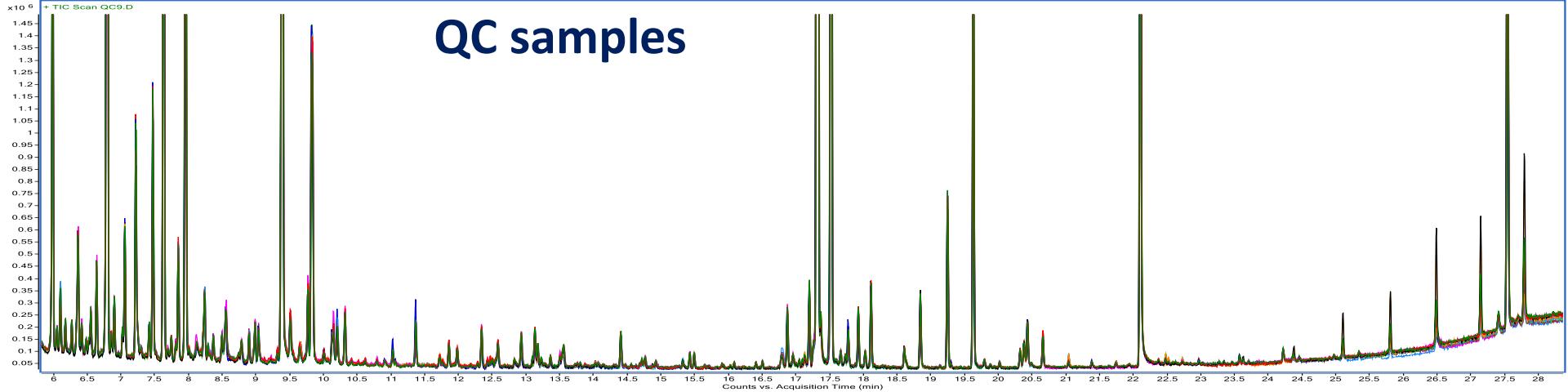


Figure. Representative mass spectra recorded in the profile and centroid acquisition mode.

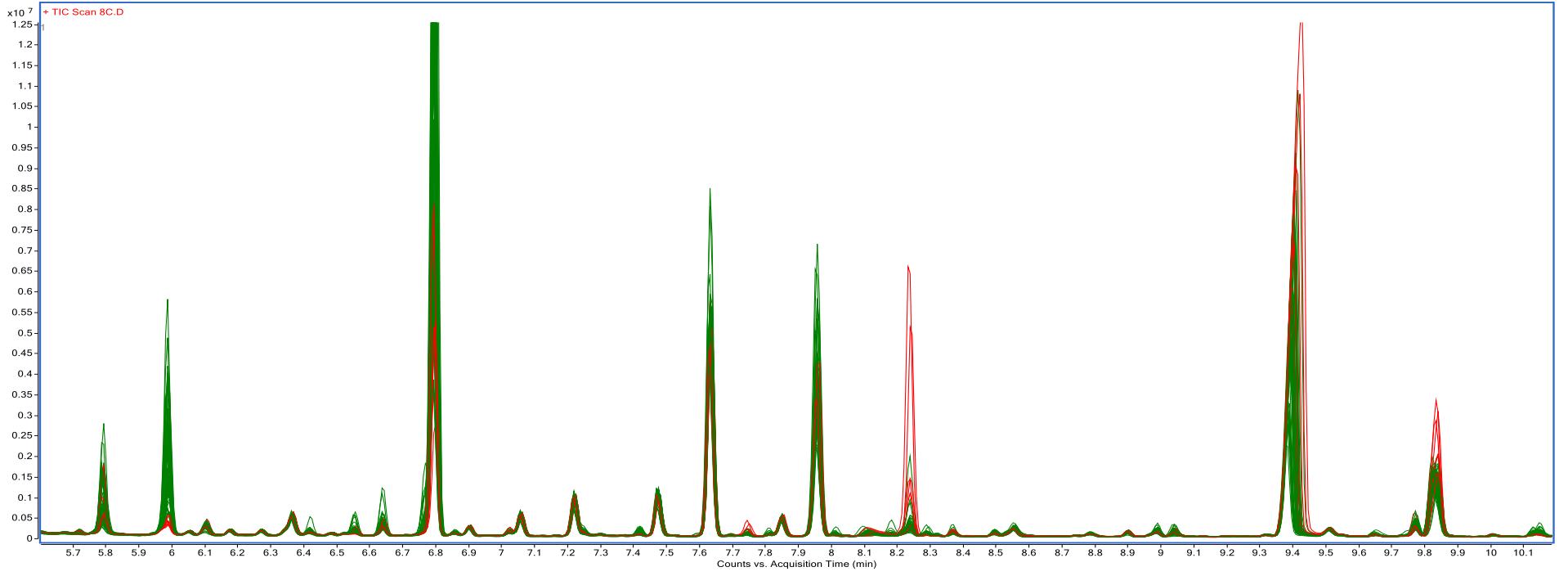
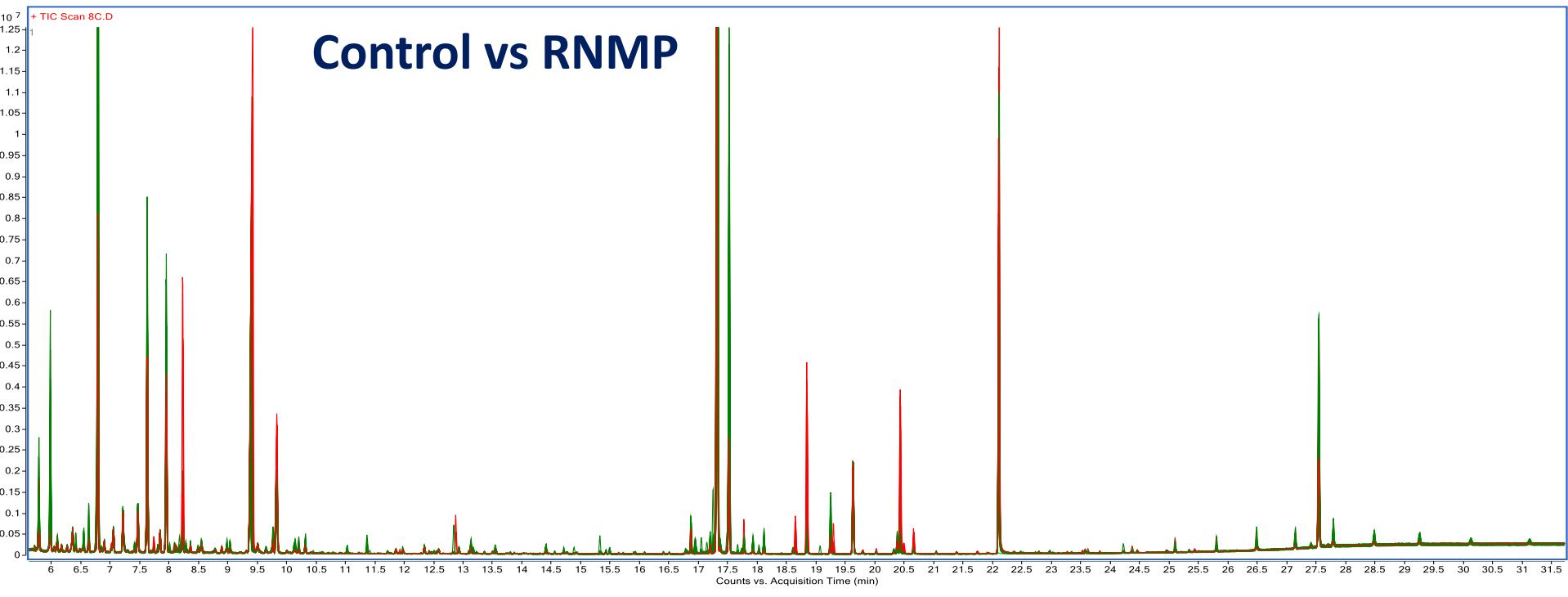
Post-analytical



QC samples

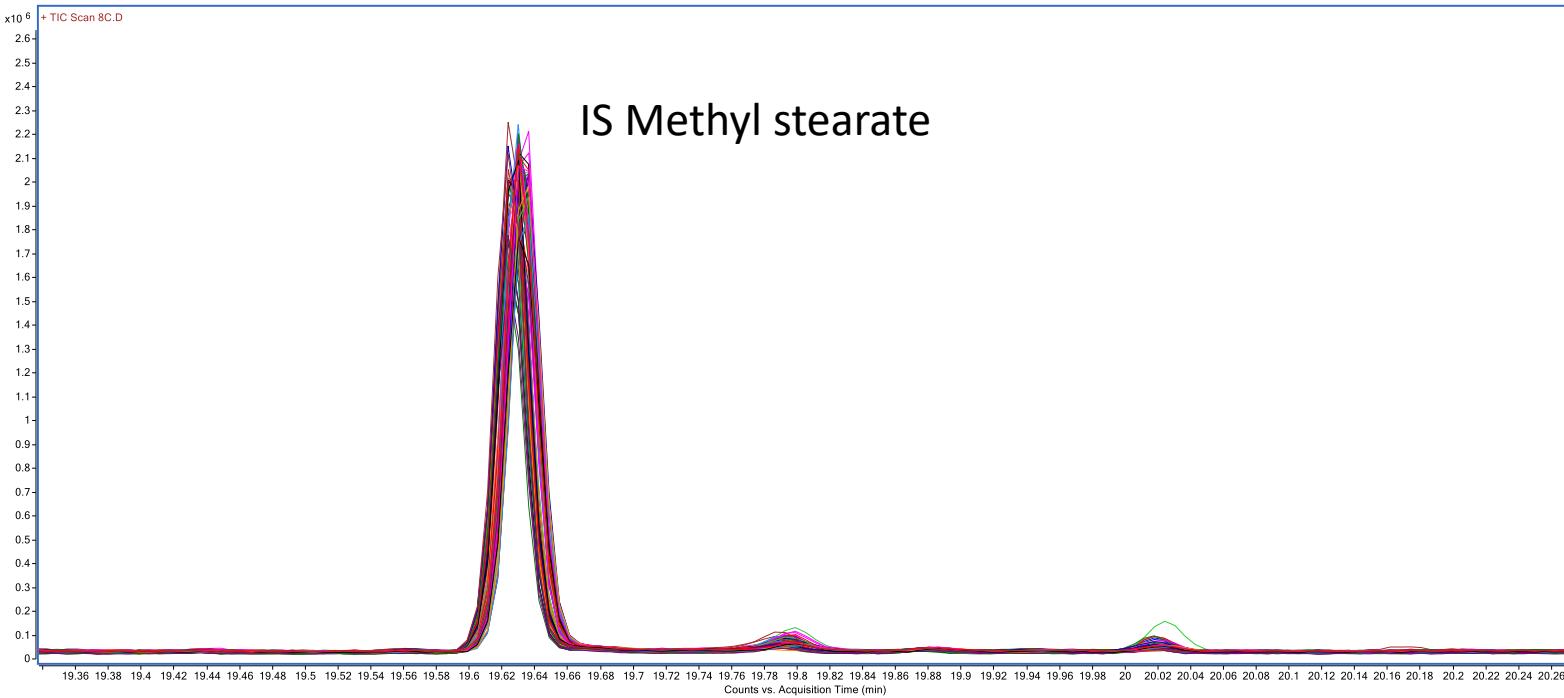
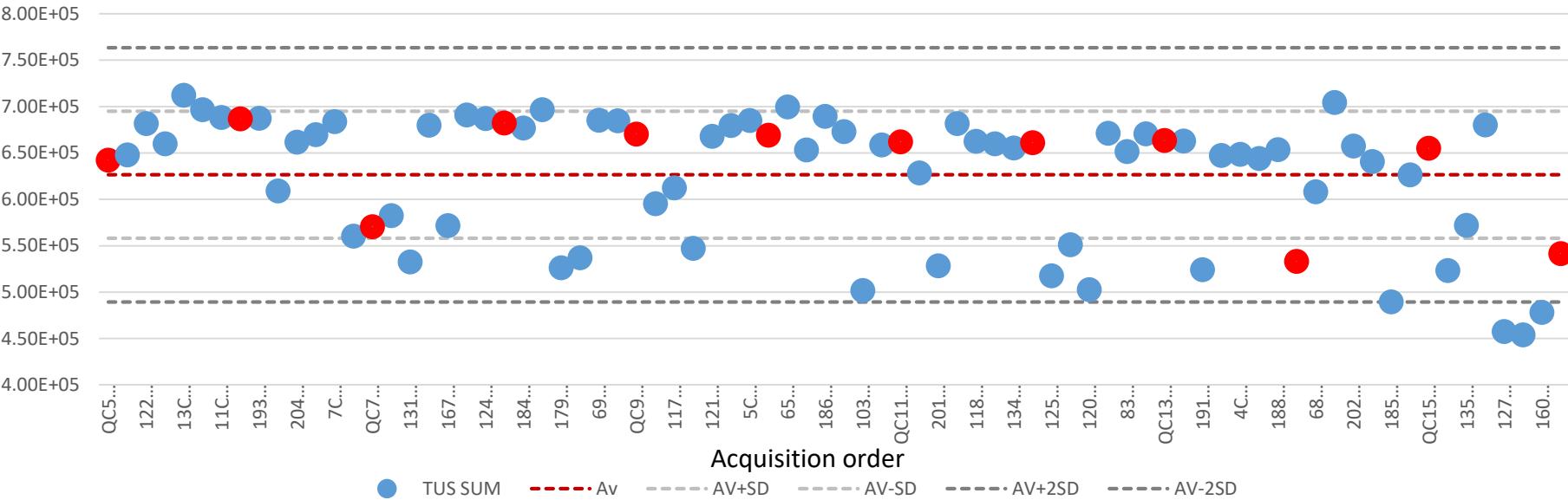


Control vs RNMP

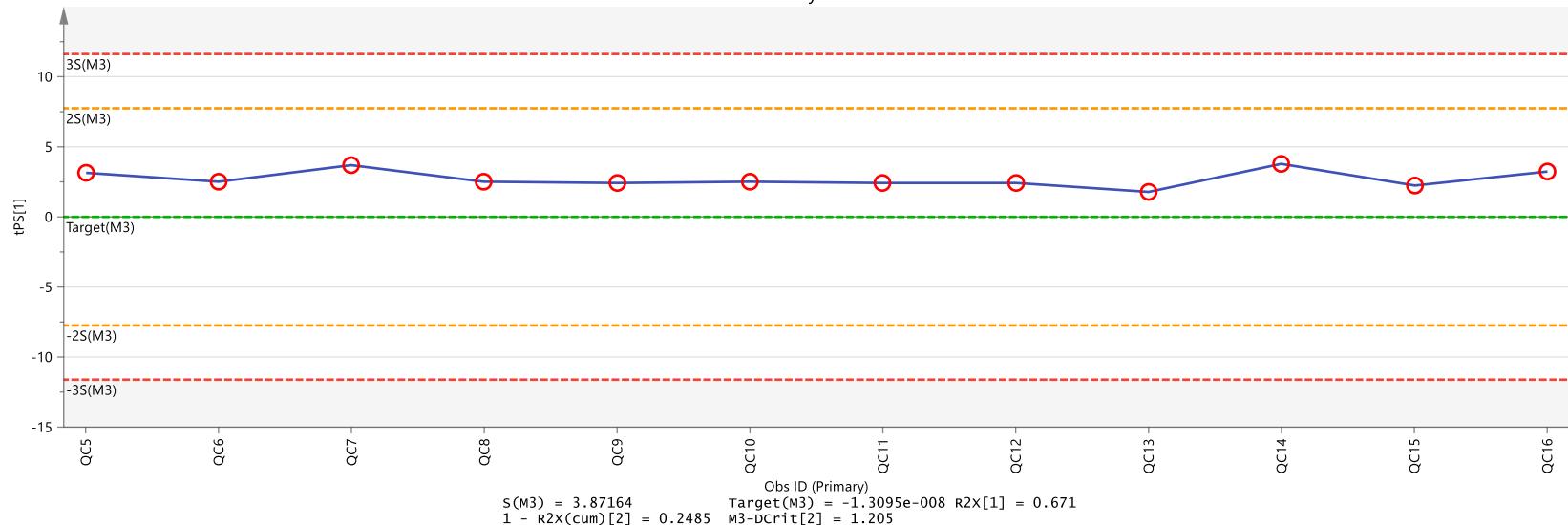
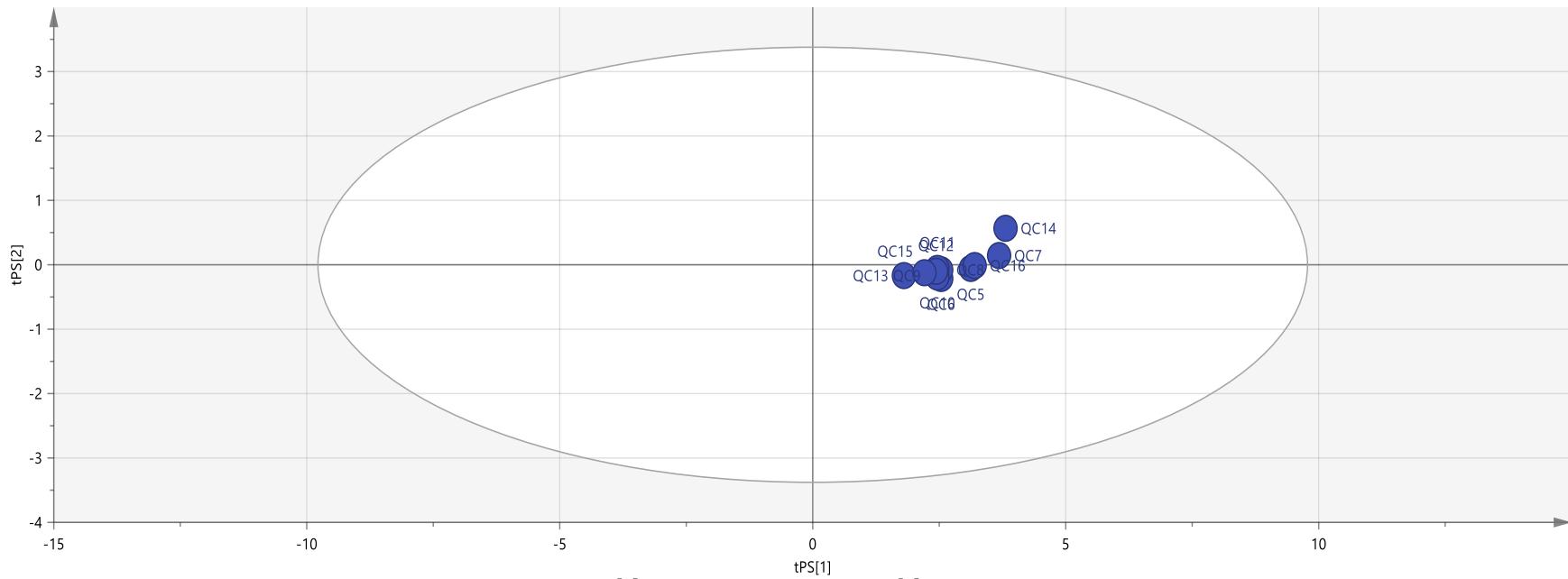


Signal for IS

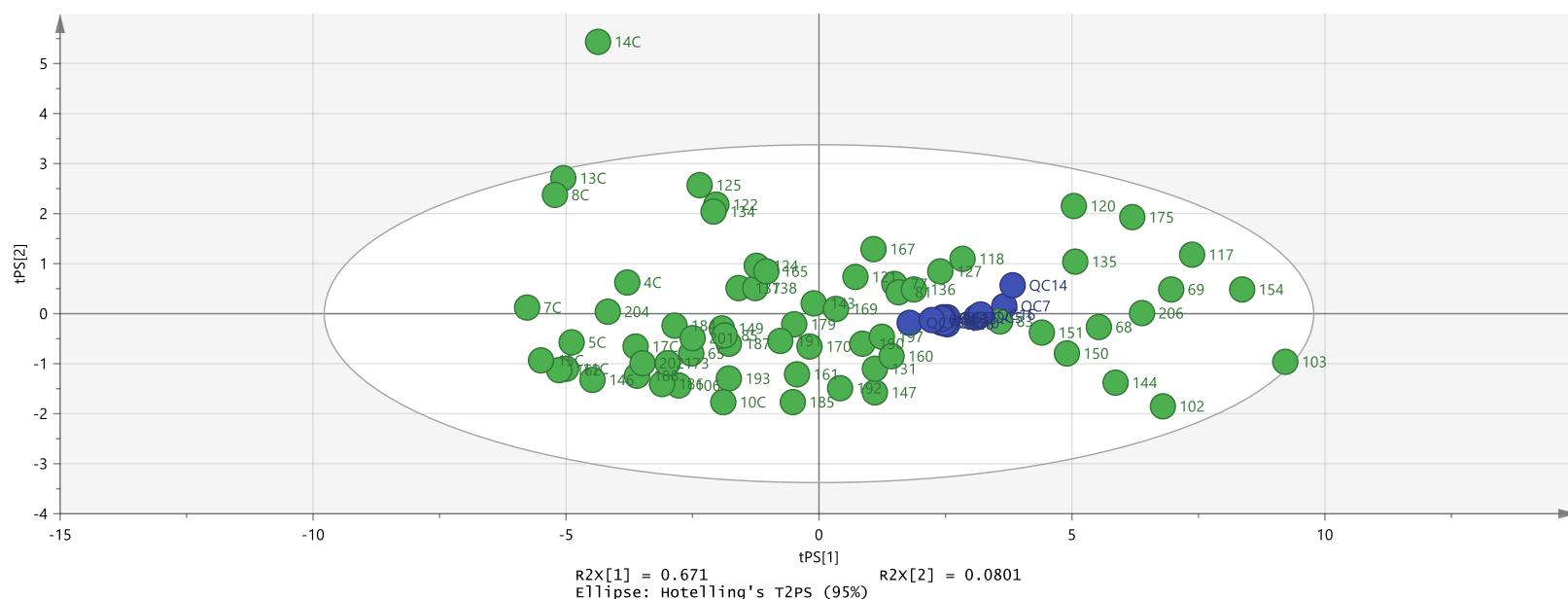
Experimental samples QC samples



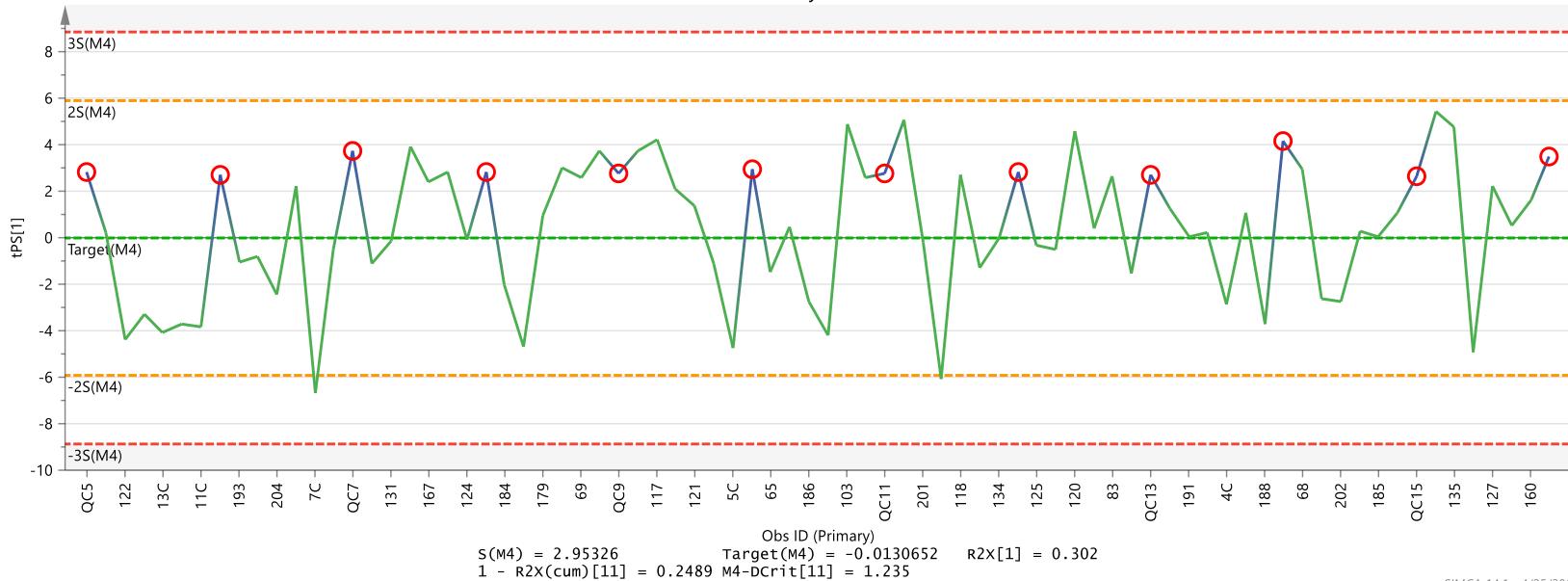
IS norm data_1.M3 (PCA-X), Par, PS-Transpose PS
 Observations are colored if they are available in WS or PS

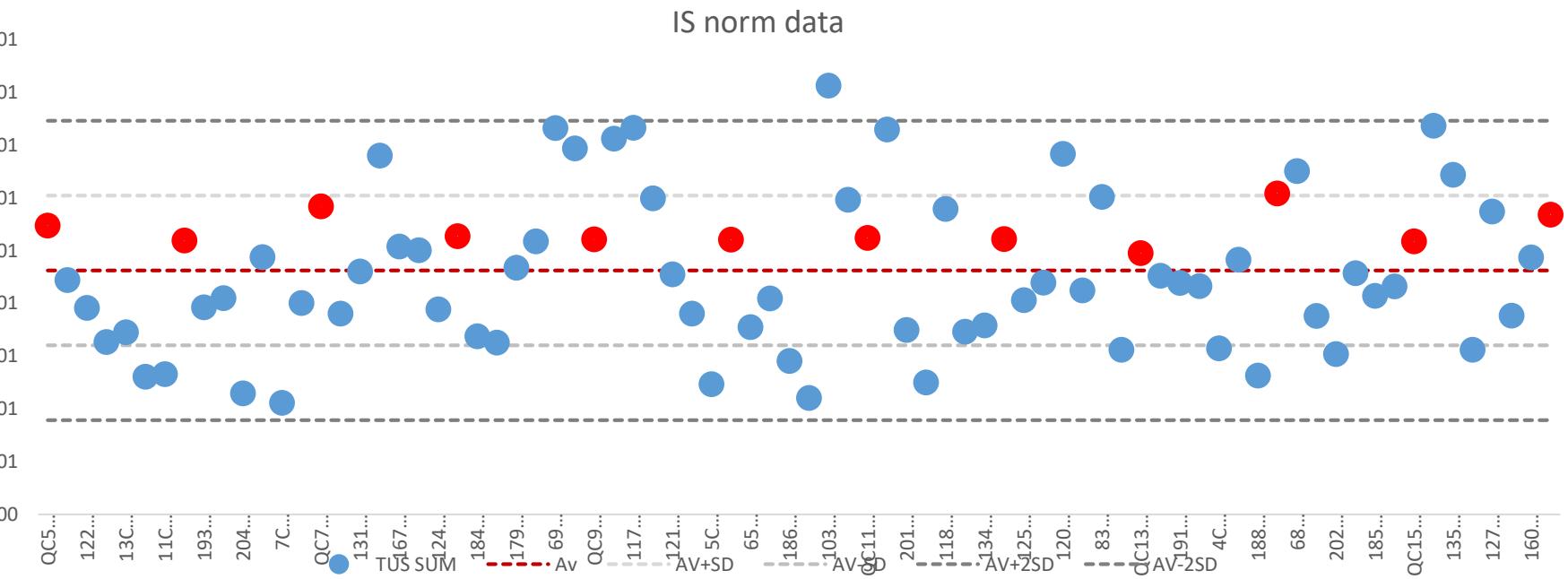
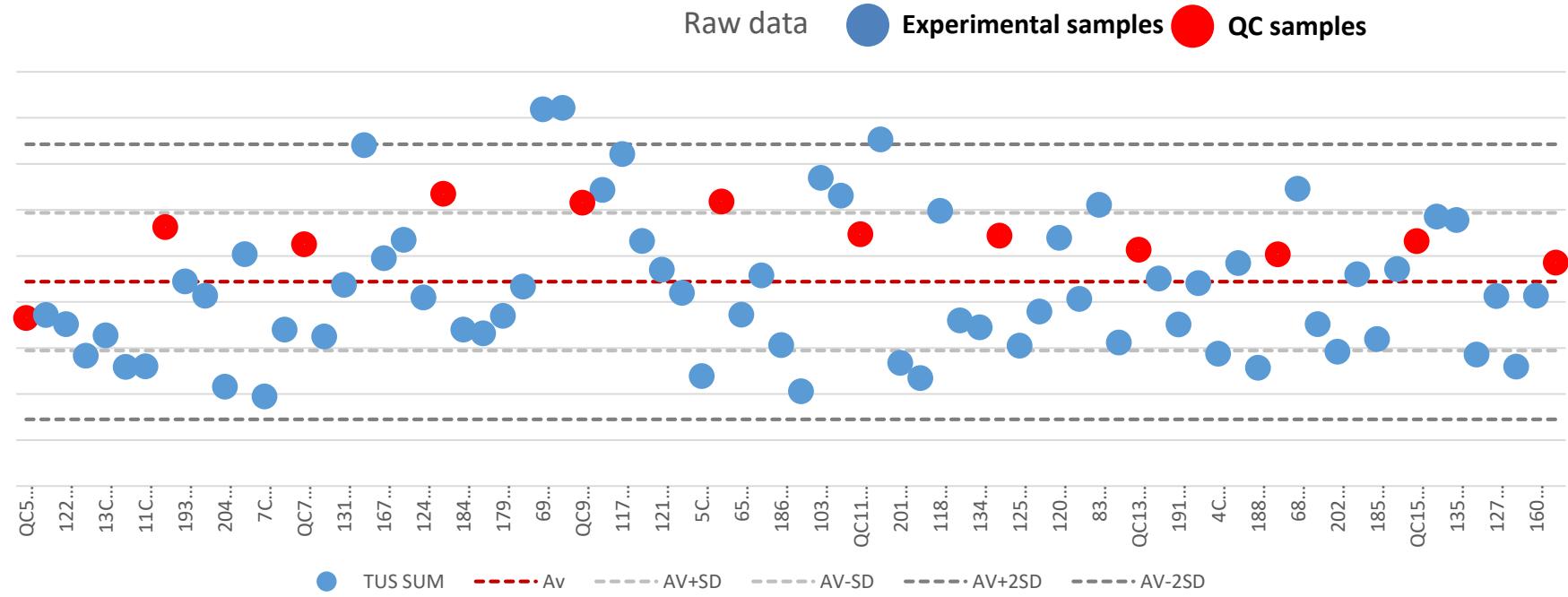


IS norm data_1.M3 (PCA-X), PS-Transpose PS
Observations are colored if they are available in WS or PS



IS norm data_1.M4 (PCA-X), Log Par, PS-Transpose PS
Shewhart (Sample size 1) : tPS[Comp. 1]
Observations are colored if they are available in WS or PS

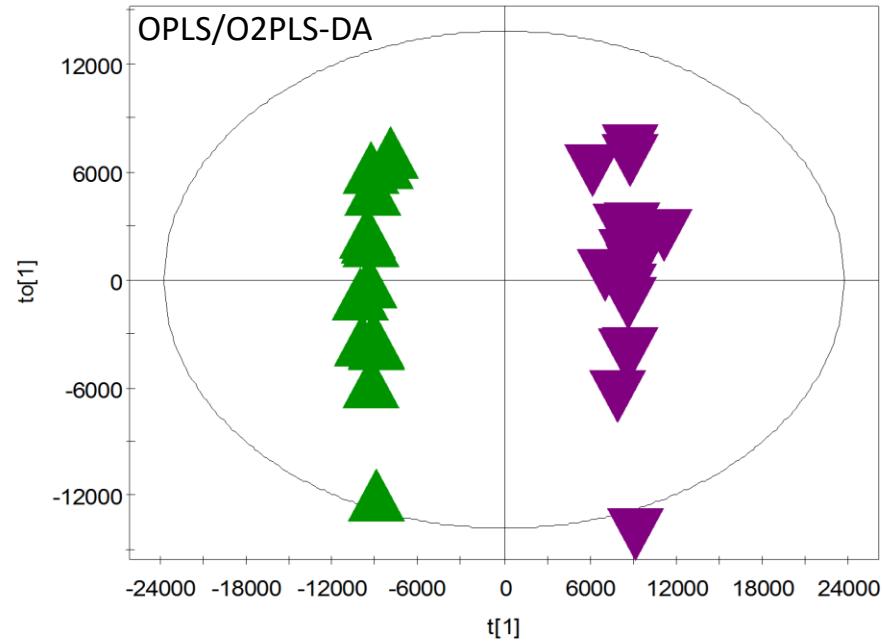
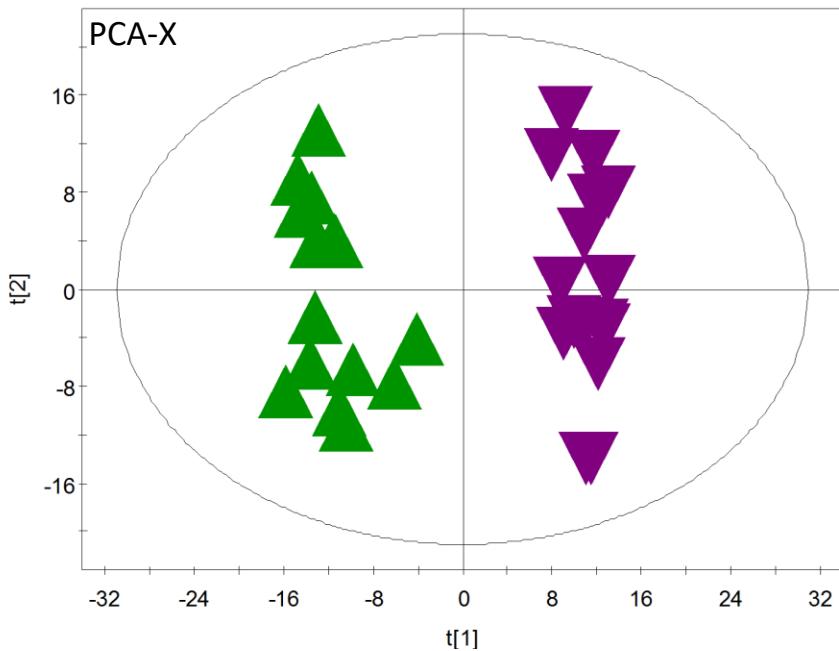




Post-analytical

Study: LC/MS based GDM metabolomics fingerprinting

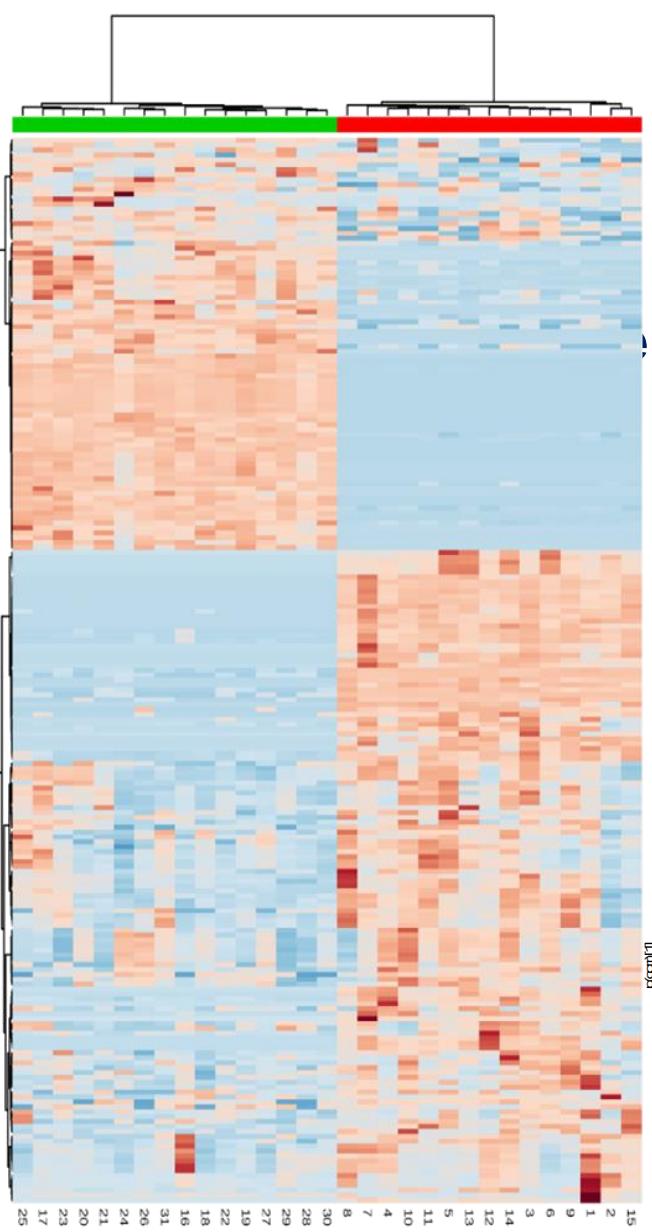
Samples: Control n=15 GDM n=16
Material: Plasma
Platform: LC-QTOF-MS



Comp No.	M4.R2X(cum)	M4.Q2(cum)
Comp[1]	0.522908	0.485716

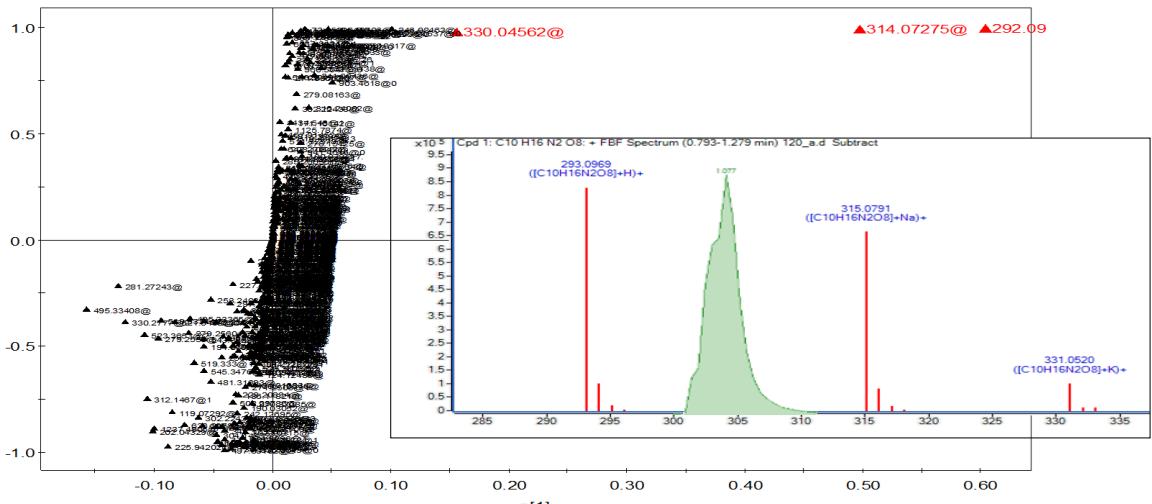
Comp No.	M9.R2Y(cum)	M9.Q2(cum)
Comp[1]P	0.989426	0.984756

Post-analytical



EDTA

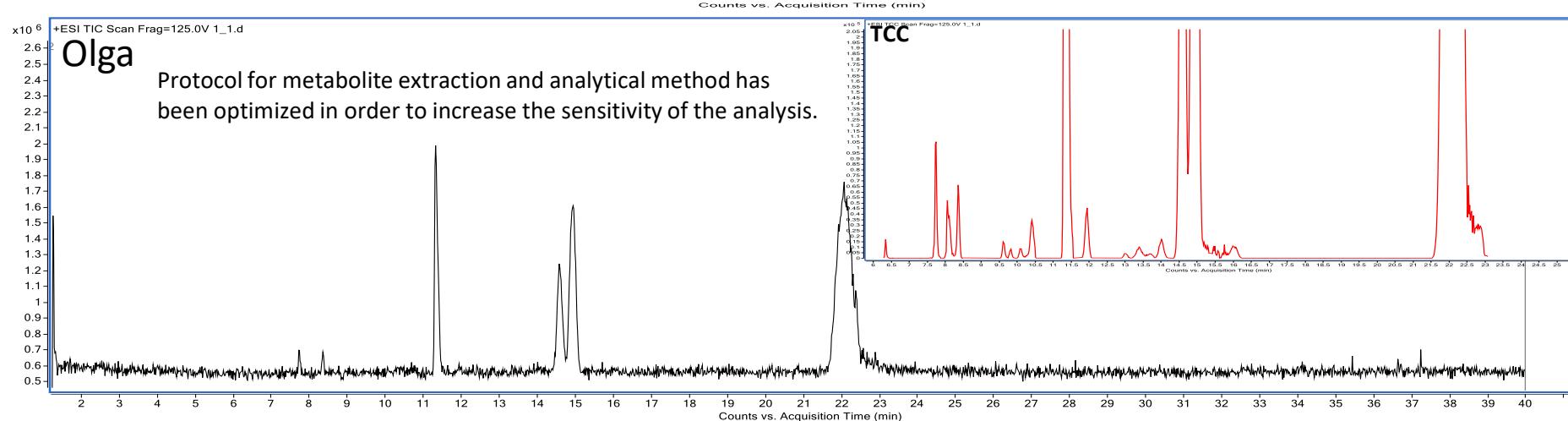
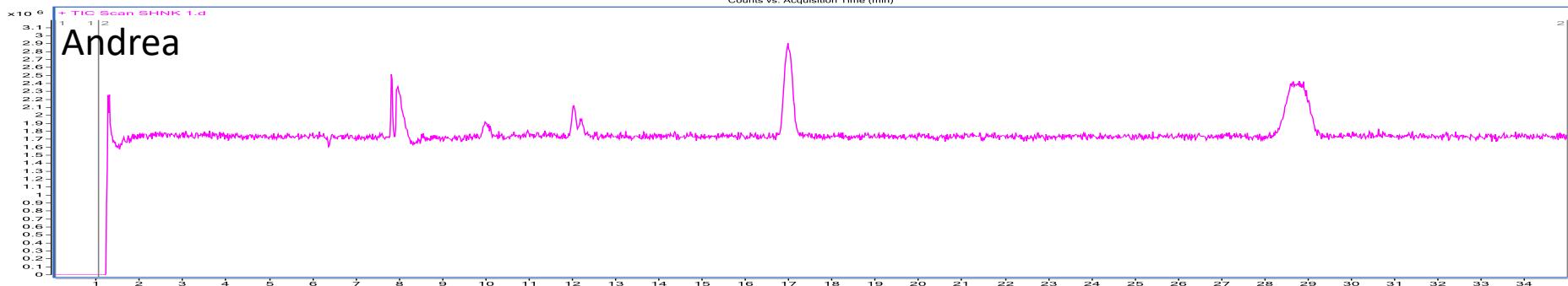
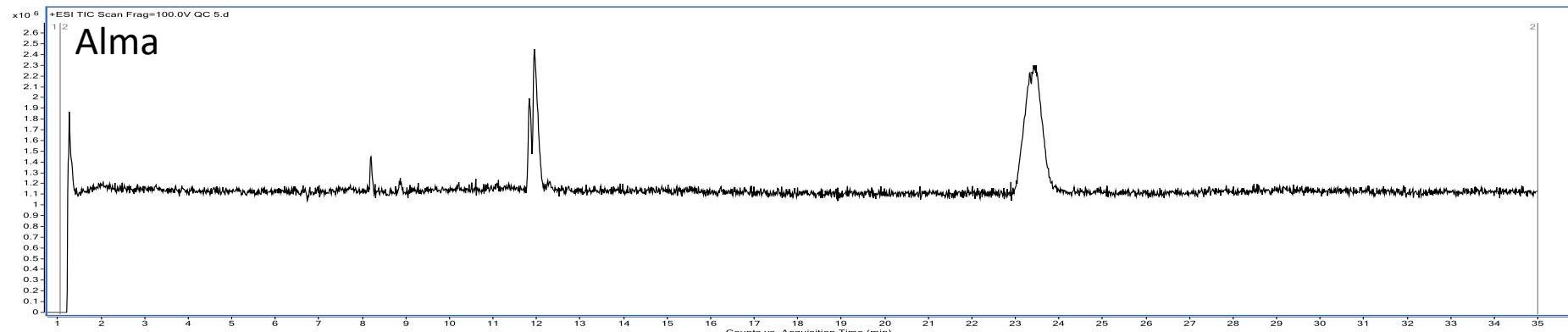
Ethylenediaminetetraacetic acid;
Exact mass = 292.0907
Formula: C₁₀H₁₆N₂O₈



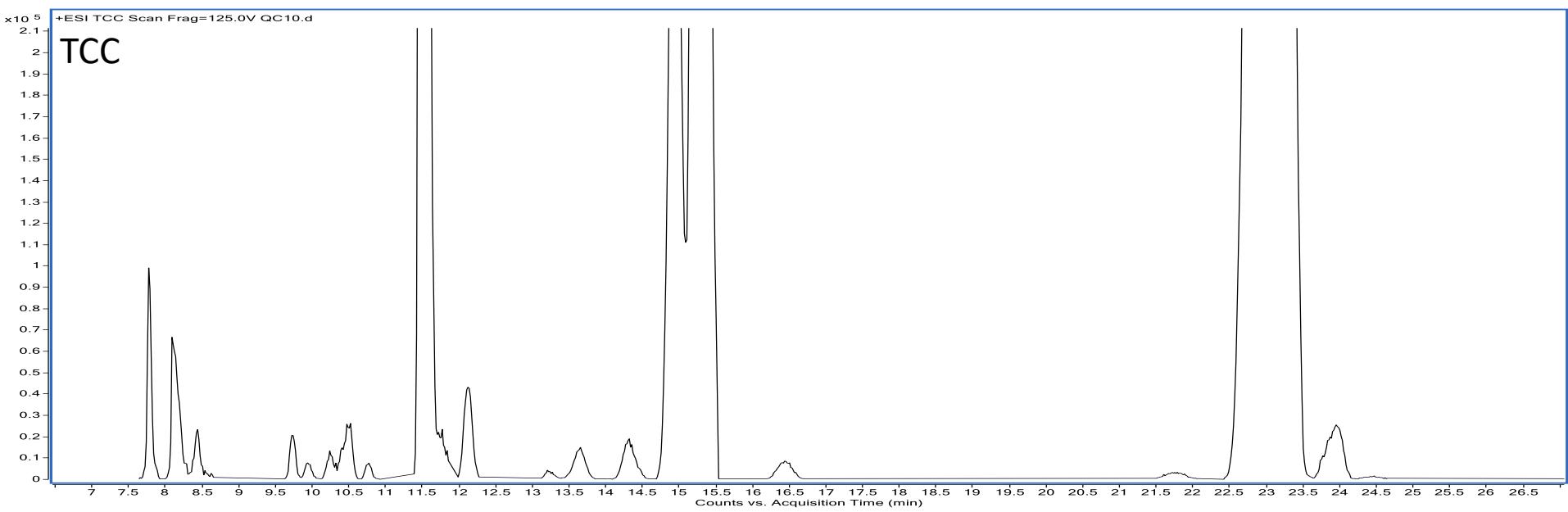
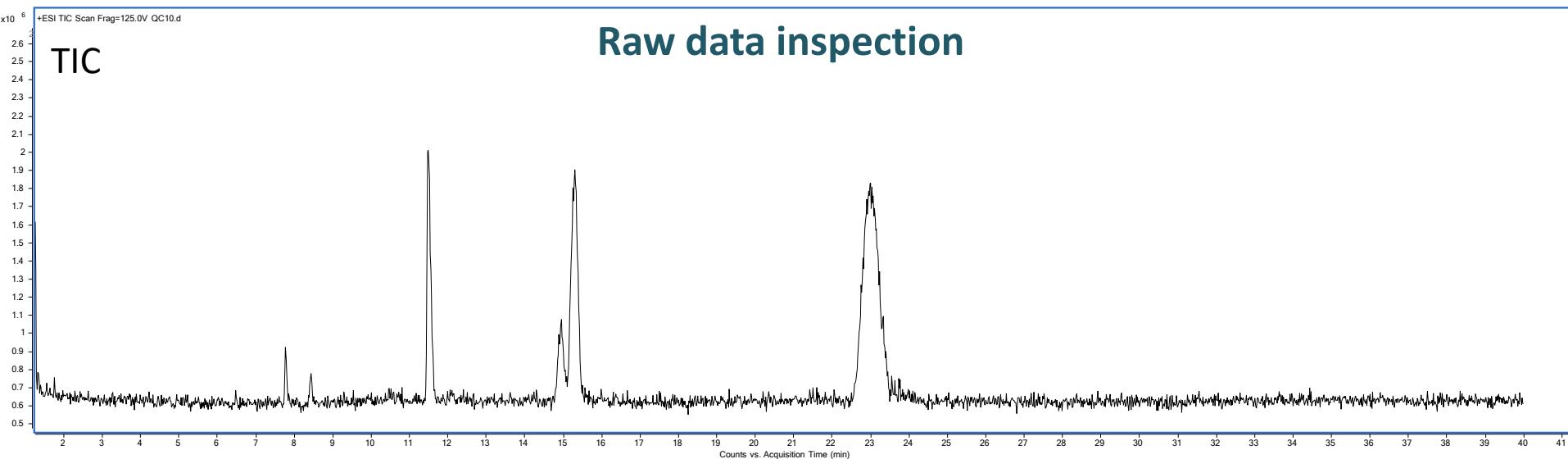


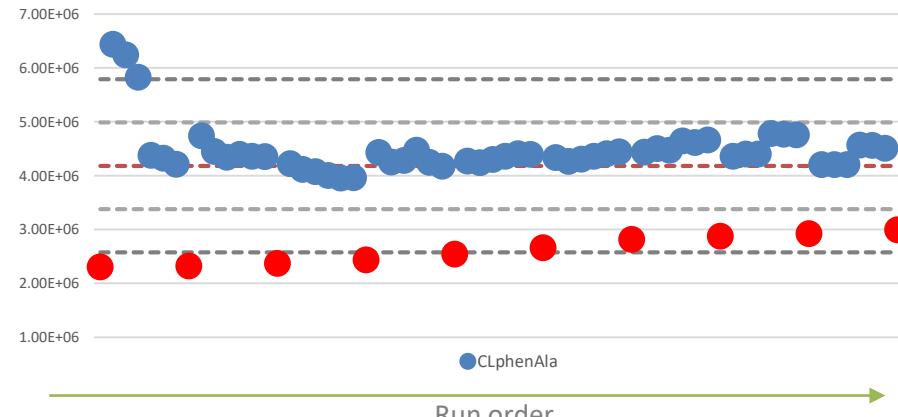
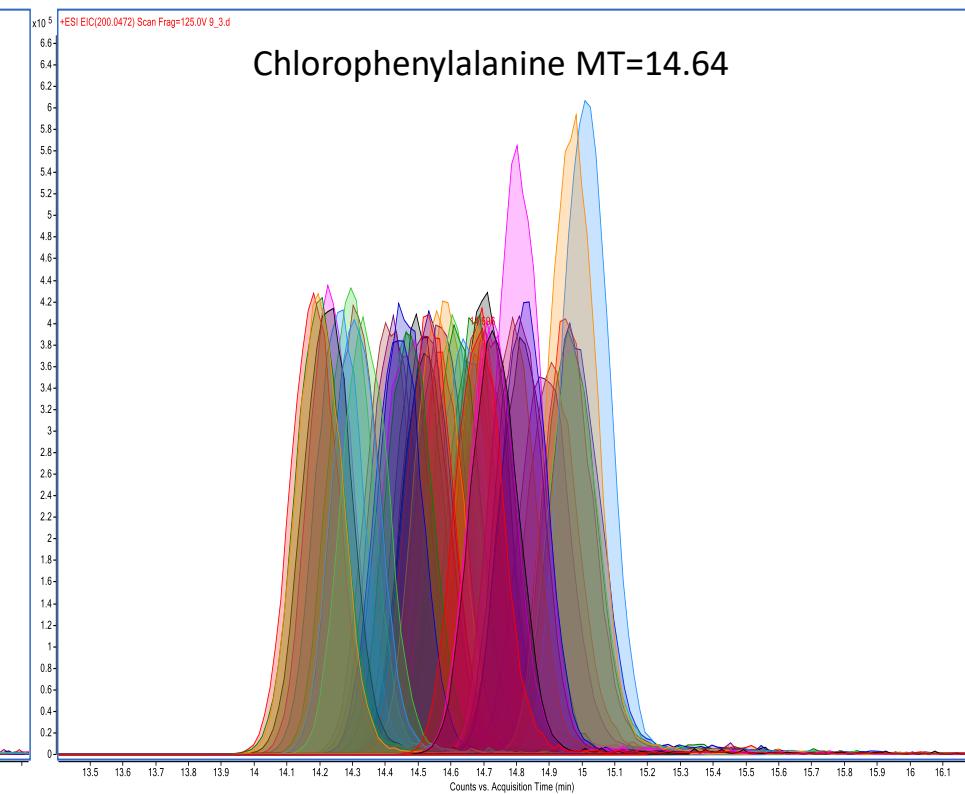
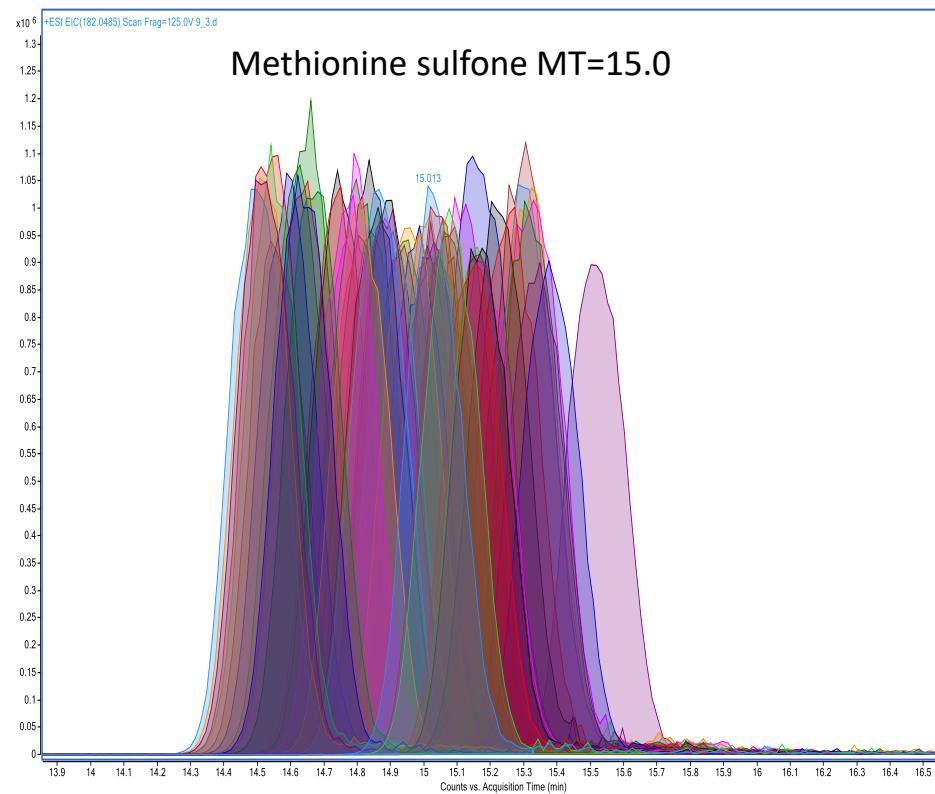
CE-MS - what we can expect?

Electrochromatograms from several projects in which mitochondria were analyzed have been reviewed prior to the analysis in order to have insights of the expected metabolite coverage that could be obtained in CE-MS analysis.



DATA QUALITY ASSESSMENT



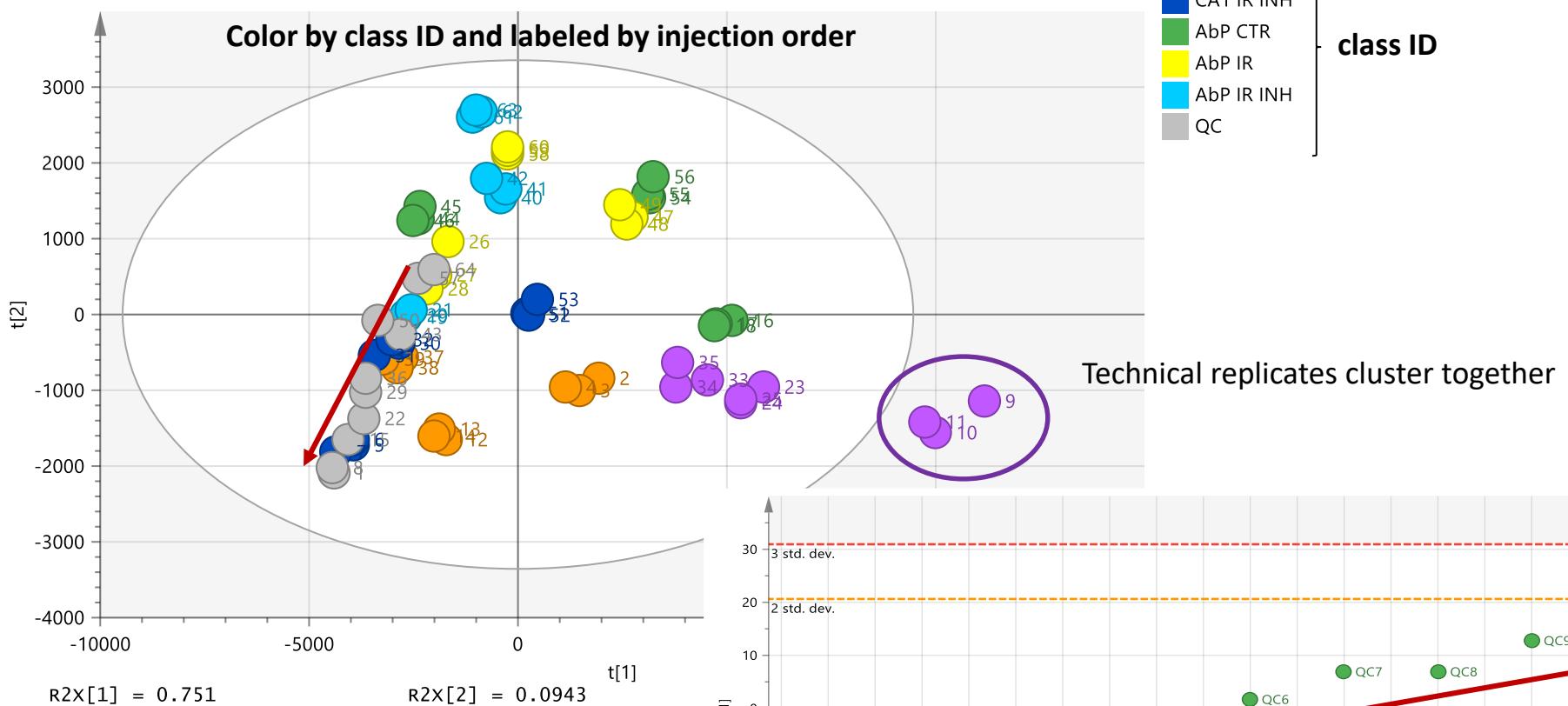


Evaluation of the quality of the signal acquired for internal standards

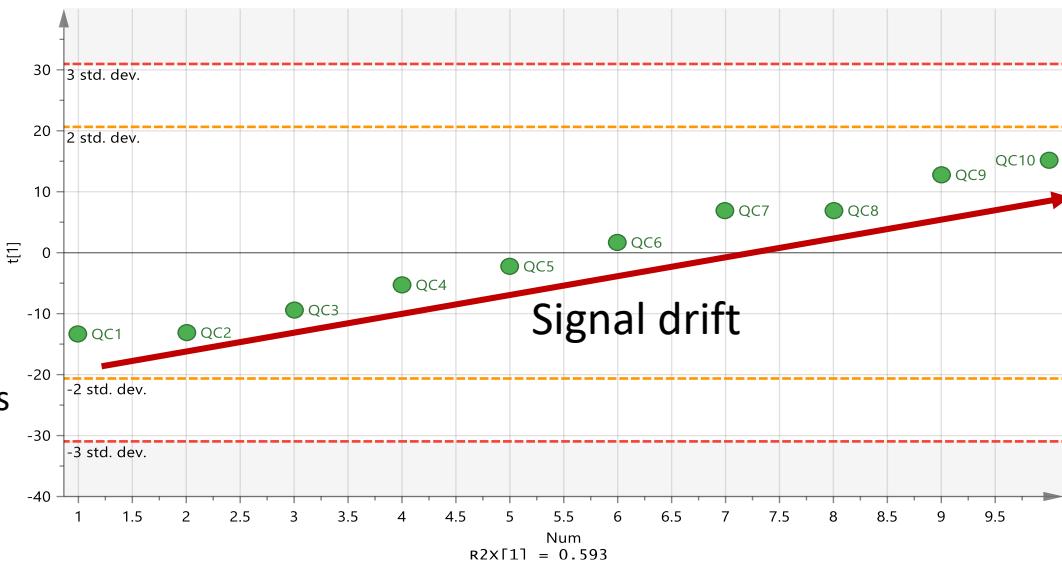
DATA QUALITY ASSESSMENT

PCA-X Analysis (Raw data)

Olga_Mit_CEMS_Rawdata.M9 (PCA-X), Pareto
Colored according to classes in M9



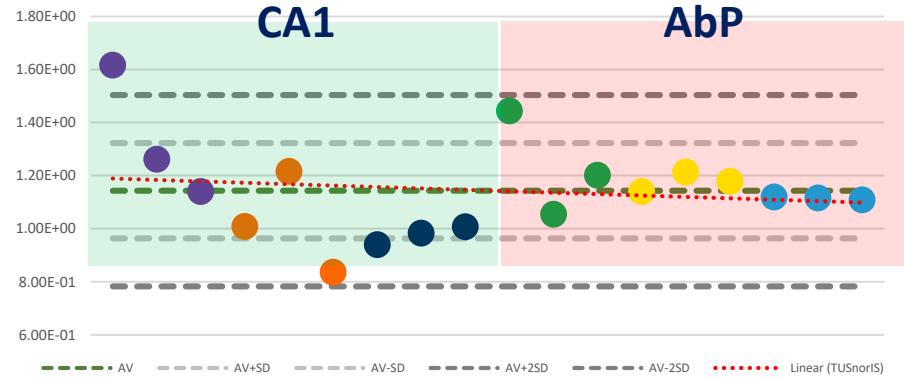
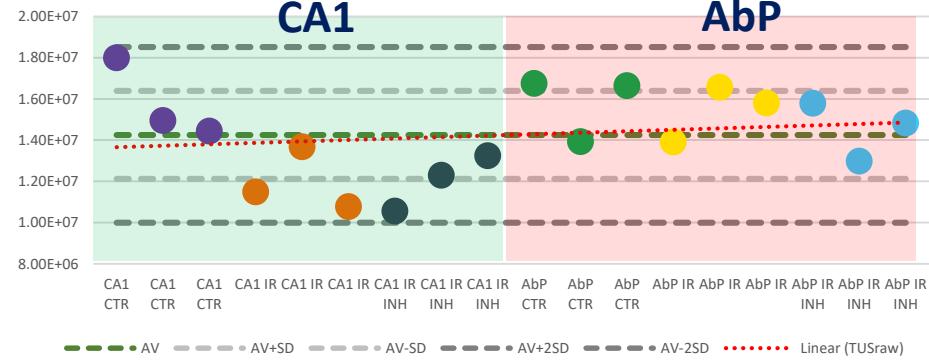
No outliers and any undesired trend observed.
Separation due to the structure of the hippocampus
(CA1 vs AbP) is observed.



DA Raw

CA1**AbP**

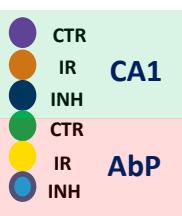
DA Norm by IS

CA1**AbP**

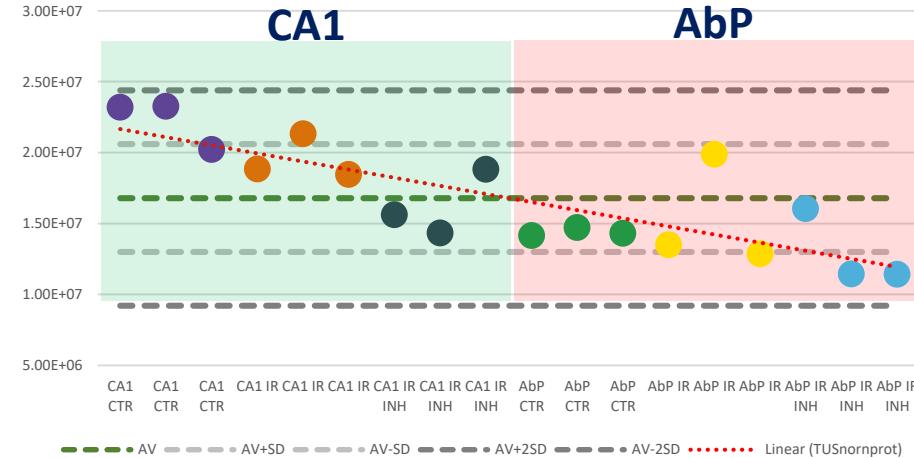
Protein content

13	15	17	16	18	14	Av CA1	AV AbP
CA1 CTR	CA1 CTR	CA1 CTR	AbP CTR	AbP CTR	AbP CTR	0.71	1.10
0.78	0.64	0.71	1.18	0.95	1.16	0.71	1.10
1	5	3	2	6	4		
CA1 IR	CA1 IR	CA1 IR	AbP IR	AbP IR	AbP IR	0.61	1.03
0.61	0.64	0.58	1.03	0.83	1.23	0.61	1.03
7	9	11	10	12	8		
CA1 IR	Av CA1	AV AbP					
INH	INH	INH	INH	INH	INH	0.68	1.14
0.68	0.86	0.70	0.98	1.14	1.30	0.75	1.14

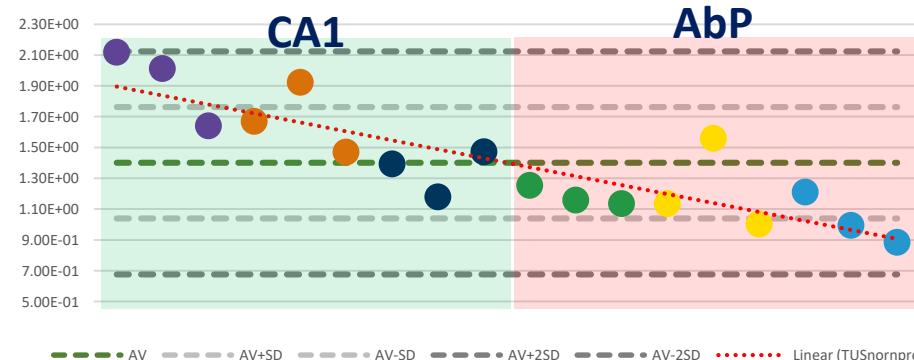
Protein content

CA1**AbP**

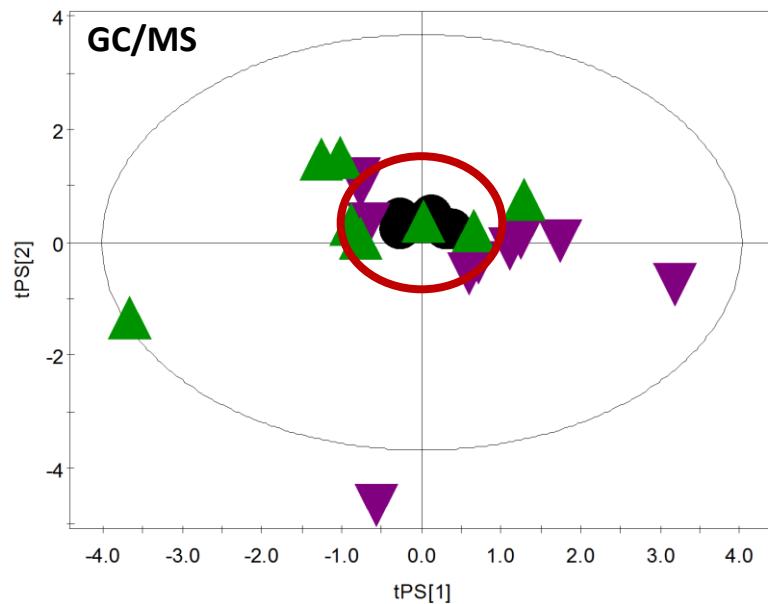
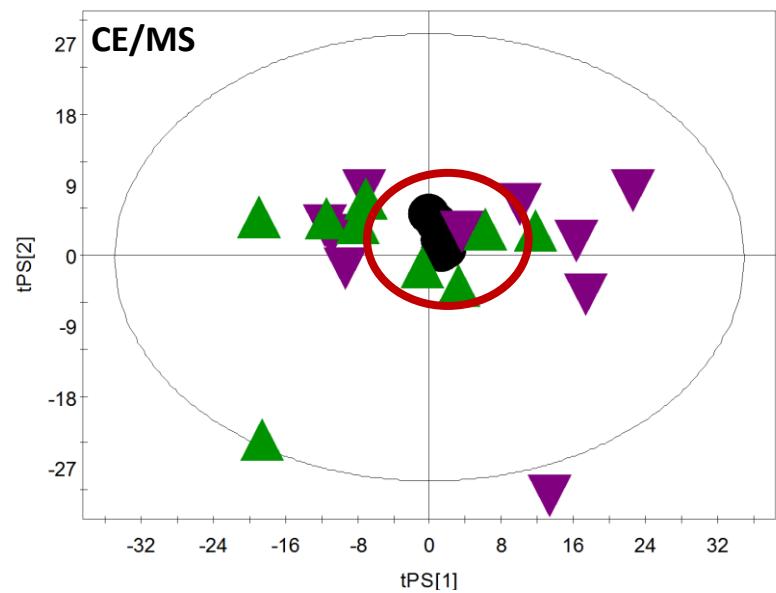
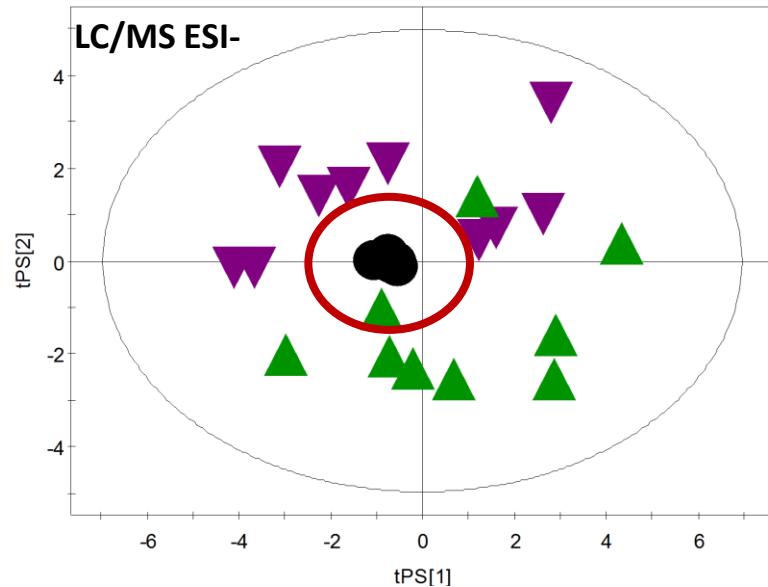
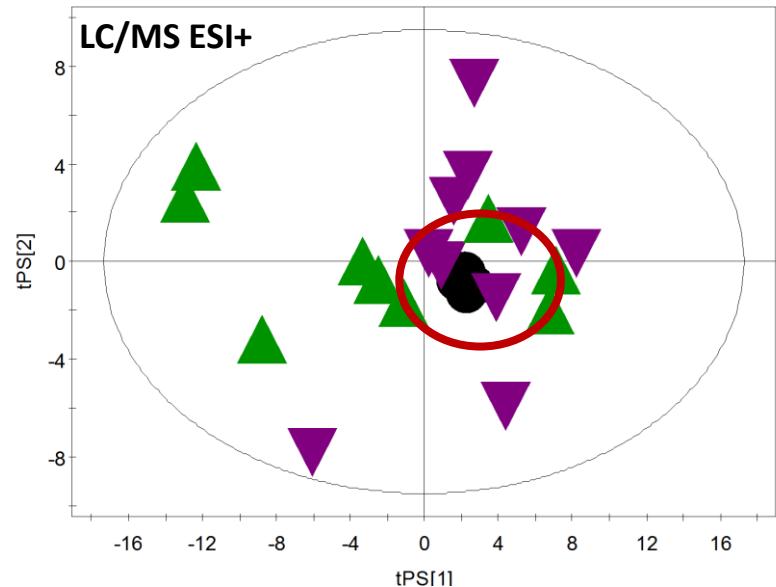
DA norm by protein

CA1**AbP**

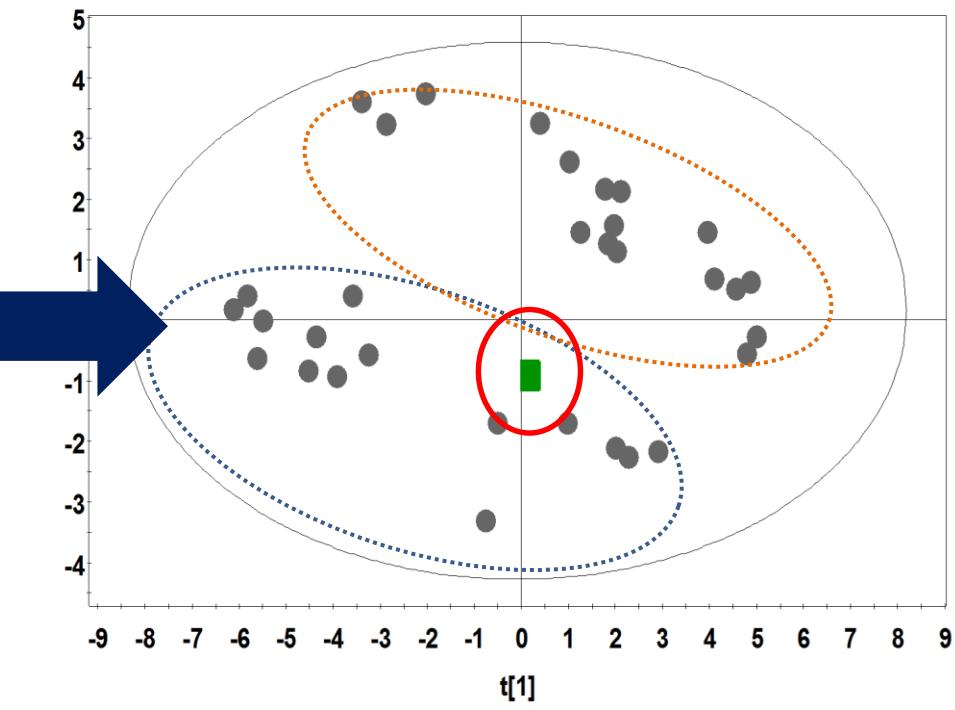
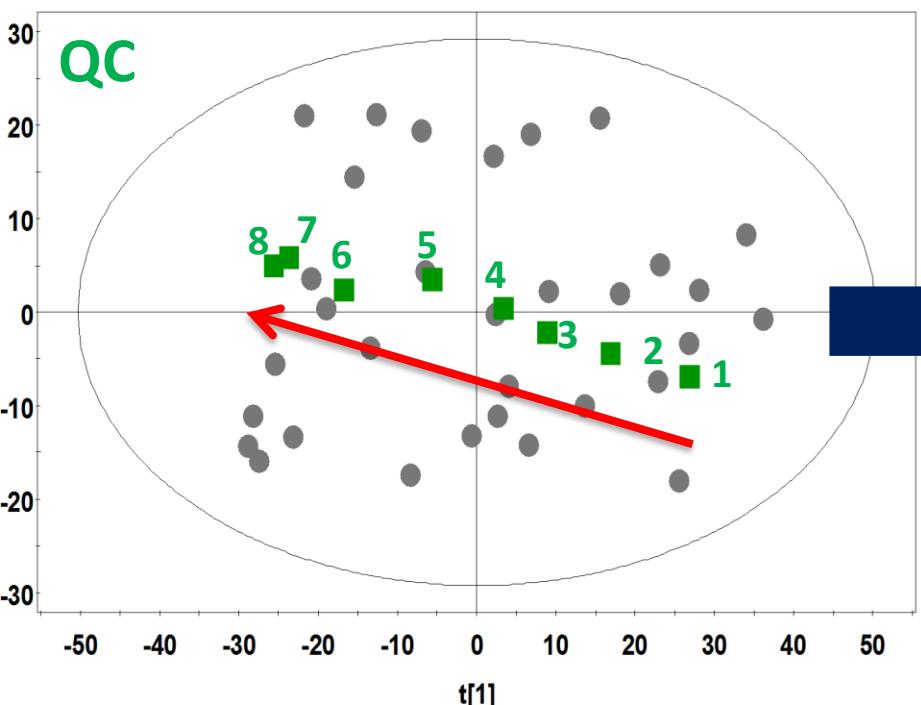
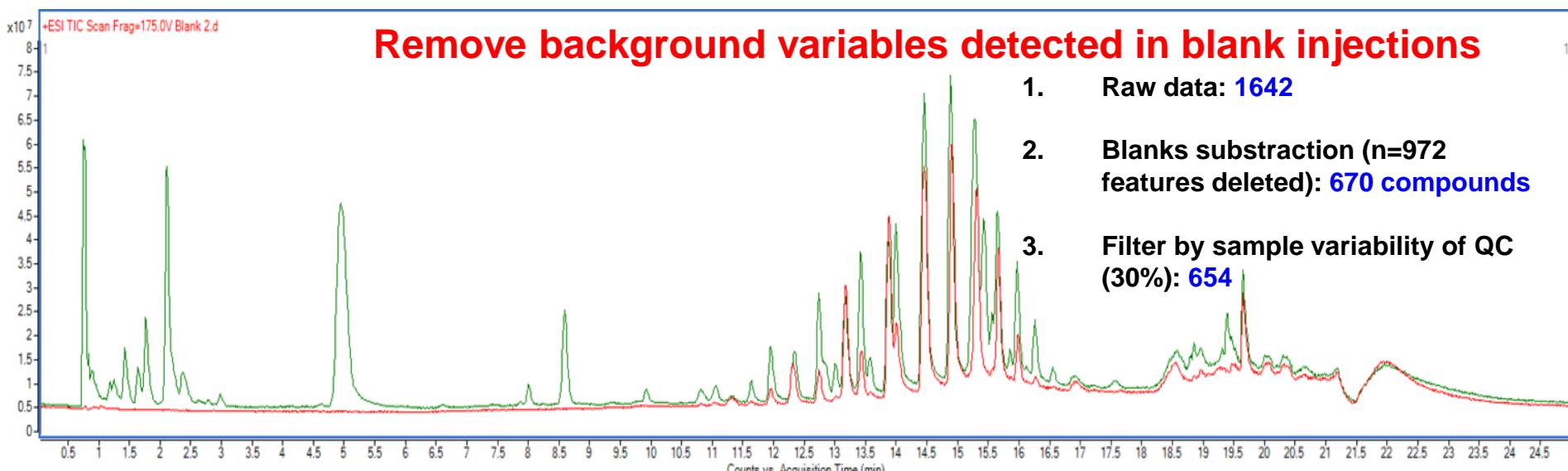
DA Norm by protein and IS

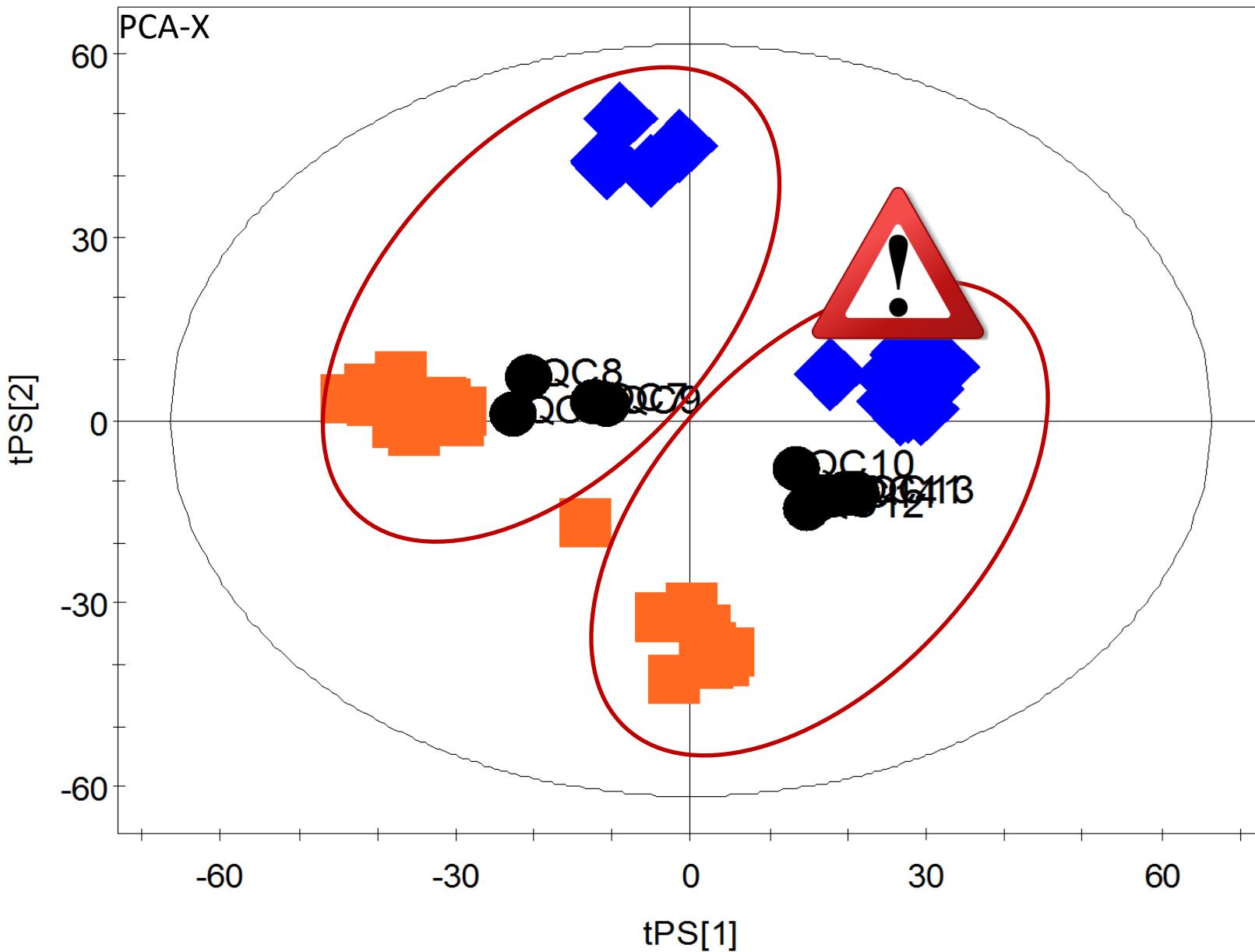
CA1**AbP**

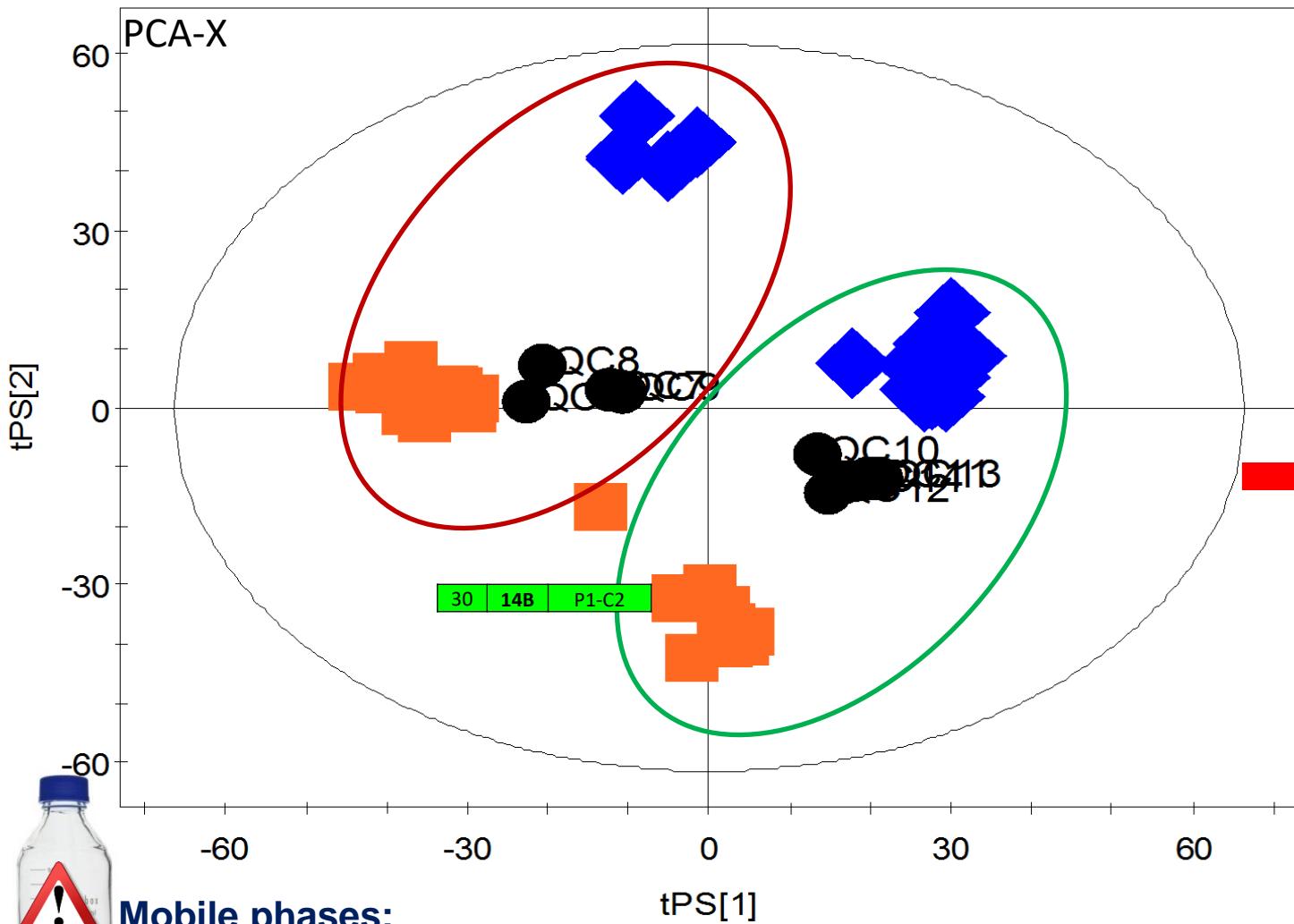
Blank
Blank
Blank_Etrx_1
Blank_Extr_2
QC1
QC2
QC3
QC4
QC5
QC6
QC7
QC8
QC9
QC10
1_84
7_32
1_18
7_33
1_7
QC11
7_85
1_45
7_87
1_60
7_8
QC12
1_27
7_91
7_44
1_39
7_15
QC13
1_52
1_92
7_37
1_93
7_9
QC14
Blank_Extr_3
Blank_Extr_4



Remove background variables detected in blank injections

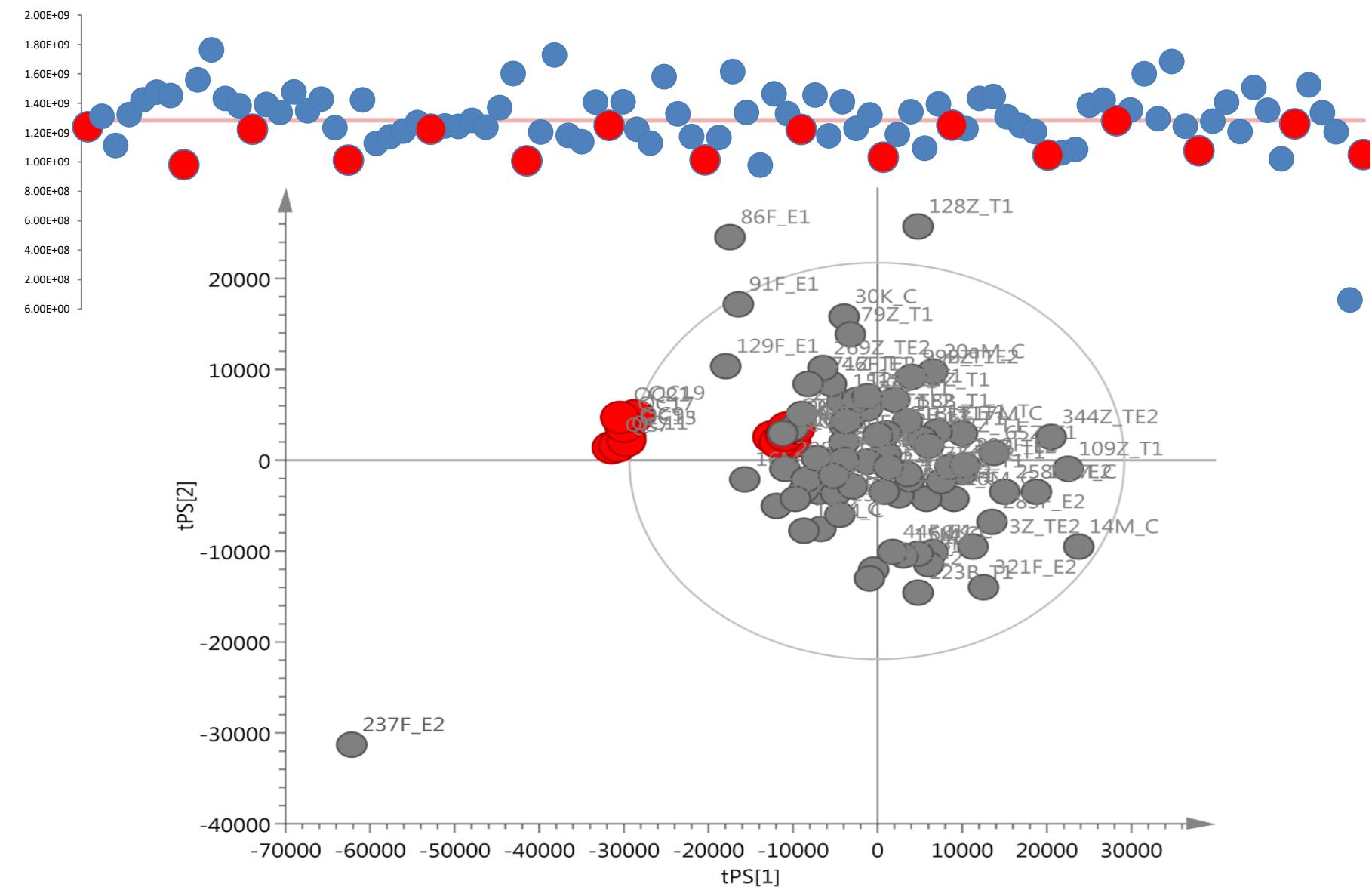






No.	Sample	Sample position
1	Blank1	Vial 1
2	Blank2	-1
5	QC1	P1-A2
6	QC2	P1-A3
7	QC3	P1-A2
8	QC4	P1-A3
9	QC5	P1-A2
10	QC6	P1-A3
11	QC7	P1-A2
12	17B	P1-A4
13	14A	P1-A5
14	9B	P1-A6
15	1A	P1-A7
16	6A	P1-A8
17	15B	P1-A9
18	QC8	P1-A3
19	8B	P1-B1
20	6B	P1-B2
21	12B	P1-B3
22	10A	P1-B4
23	2B	P1-B5
24	5B	P1-B6
25	QC9	P1-A2
26	21B	P1-B7
27	10B	P1-B8
28	20B	P1-B9
29	13A	P1-C1
30	14B	P1-C2
31	11A	P1-C3
32	QC10	P1-A3
33	7B	P1-C4
34	5A	P1-C5
35	12A	P1-C6
36	19B	P1-C7
37	4A	P1-C8
38	9A	P1-C9
39	QC11	P1-A2
40	3A	P1-D1
41	16B	P1-D2
42	16A	P1-D3
43	20A	P1-D4
44	3B	P1-D5
45	7A	P1-D6
46	QC12	P1-A3
47	21A	P1-D7
48	13B	P1-D8
49	19A	P1-D9
50	1B	P1-E1
51	15A	P1-E2
52	18B	P1-E3
53	QC13	P1-A2
54	2A	P1-E4
55	11B	P1-E5
56	8A	P1-E6
57	4B	P1-E7
58	18A	P1-E8
59	17A	P1-E9
60	QC14	P1-A3

QC6	QC7	QC8	QC9	QC10	QC11	QC12	QC13	QC14	QC15	QC16	QC17	QC18	QC19	QC20	QC21
2472612231	1.96E+09	2.44E+09	2.02E+09	2.44E+09	2.01E+09	2.49E+09	2.02E+09	2.44E+09	2.06E+09	2.5E+09	2.09E+09	2.56E+09	2.15E+09	2.51E+09	2.1E+09



Post-analytical

QA procedure	Controlled factor	General considerations*
Normalization	Unwanted variation Sample preparation Metabolite extraction Signal drift Within and between batch variation	<ul style="list-style-type: none"> • Method used to identify and remove sources of systematic variation between sample profiles due to the factors that are irrelevant with regard to biological processes. • Method-driven: <ul style="list-style-type: none"> ➢ Single internal standard (SIS). The method assumes that every metabolite in a sample is subject to the same amount of unwanted variation. That is often found to be inadequate in removing unwanted variation. ➢ Multiple internal standards (AIS). The AIS average is used for normalization. ➢ Locally weighted scatter plot smoothing (LOESS). Based on QC samples. It assumed that proportion of metabolic changes across biological samples is relatively small or that there is similar number of metabolites with increased and decreased signals across the peaks intensity range. Can robustly estimate the unwanted batch variation but also possess the risk to spuriously missing metabolites. • Data-driven: <ul style="list-style-type: none"> ➢ Scaling factors e.g., normalization by median or intensities; not applicable when the self-averaging property does not hold. ➢ Total intensity normalization. Forces all samples to have equal total intensity; assumes that the total concentration of all metabolites in the sample does not vary across samples in a data set. The changes in the peak intensities of a few high concentration metabolites can notably compromise the normalization performance because of their substantial contribution to the total peak intensity. ➢ Median fold change (FC). Adjust the median of log FC of peak intensities between samples to be approximately zero. Assumes that the metabolite peaks affected purely by dilution will exhibit the same FC between two sample profiles. The performance is robust against at least 50% peaks intensities exhibiting asymmetrical increase or decrease in response to biological factors. ➢ Quantile normalization. Enforces all samples to have identical peak intensity distribution. Assumes that the distribution of peak intensity across a data set is nearly the same for all samples and that can be problematic with high intensity values. It assumed that there is a similar number of metabolites with increased and decreased signals across the peak intensity range. ➢ Probabilistic quotient normalization (PQN). Transforms the metabolomics spectra according to an overall estimation on the most probable dilution. ➢ Variance-stabilizing transformation. Assumes that the noise is multiplicative in nature and the standard deviation of peak intensity is proportional to its expected value.

Feature	RT	13	15	17	1	5	3	7	9	11	16	18	14	2	6	4	10	12	8
x		CA1CTR	CA1CTR	CA1CTR	CA1IR	CA1IR	CA1IR	CA1IR	CA1IR	CA1IR	AbPCTR	AbPCTR	AbPCTR	AbPIR	AbPIR	AbPIR	AbPIR	AbPIR	AbPIR
100.0014	88.39	0.033501	0.024774	0.020904	0.011792	0.015706	0.010105	0.007772	0.010422	0.014065	0.029021	0.009603	0.022478	0.011698	0.021734	0.013756	0.011885	0.011247	0.014299
101.0485	140.04	0.001084	0.000796	0.000794	0.000688	0.000665	0.000719	0.000842	0.000994	0.000965	0.001483	0.001677	0.000969	0.001445	0.001137	0.002017	0.001756	0.001523	0.001657
101.1207	104.07	0.006043	0.006131	0.005864	0.008468	0.006545	0.004453	0.005124	0.003322	0.007559	0.00496	0.006646	0.004153	0.005003	0.007286	0.006058	0.003366	0.004195	0.004743
102.0323	92.022	0.002543	0.002041	0.001767	0.001197	0.001486	0.001092	0.000877	0.001082	0.001299	0.002046	0.001177	0.001825	0.001165	0.001592	0.001213	0.001273	0.001022	0.001326
103.0637	111.3	0.061895	0.048572	0.042332	0.047967	0.03666	0.022442	0.018768	0.024095	0.030491	0.05073	0.020312	0.014413	0.028611	0.040759	0.031318	0.031996	0.030866	0.03199
103.0638	106.5	0.003355	0.002479	0.002335	0.001823	0.00209	0.001955	0.002817	0.003546	0.00304	0.007197	0.008338	0.003599	0.00569	0.003546	0.006677	0.009286	0.007678	0.009146
103.0999	99.65	0.001852	0.001721	0.001602	0.006216	0.006263	0.007799	0.006592	0.008878	0.008104	0.011728	0.012037	0.011059	0.008945	0.009343	0.012637	0.013078	0.018018	0.012784
105.0434	102.32	0.001997	0.0014	0.001574	0.018382	0.018881	0.01681	0.001691	0.002396	0.001983	0.003673	0.009768	0.003255	0.003249	0.003215	0.003861	0.005022	0.005393	0.004629
106.4296	122.45	0.010144	0.010229	0.009434	0.006843	0.00765	0.00765	0.005715	0.007265	0.008474	0.009667	0.007631	0.009527	0.007217	0.008871	0.008513	0.007988	0.006765	0.008566
113.3982	88.38	0.003934	0.002863	0.002392	0.001296	0.001832	0.001127	0.000918	0.001242	0.001160	0.00332	0.001083	0.002553	0.001499	0.002303	0.001517	0.001344	0.001411	0.001666
114.031	22.17	0.02071	0.02129	0.019729	0.013455	0.017234	0.012219	0.01263	0.012634	0.015429	0.020486	0.012045	0.018544	0.013263	0.021638	0.01492	0.013462	0.012821	0.014012
115.0651	138.03	0.001257	0.000969	0.001081	0.000961	0.001821	0.000962	0.001139	0.001553	0.001083	0.001688	0.00185	0.001016	0.001539	0.001654	0.002413	0.002051	0.002020	0.001501
115.9782	77.75	0.006062	0.006944	0.00894	0.005569	0.006524	0.007046	0.005762	0.004406	0.007179	0.008547	0.006997	0.005627	0.003479	0.005671	0.005791	0.005193	0.005715	0.004049
116.04	22.22	0.003698	0.003749	0.002601	0.002244	0.002392	0.00259	0.001899	0.002292	0.002485	0.003018	0.002624	0.002965	0.002377	0.002767	0.002723	0.002367	0.002078	0.002843
117.0792	133.13	0.002112	0.013029	0.01052	0.01369	0.02104	0.01069	0.01516	0.002998	0.001894	0.004625	0.005268	0.003229	0.004624	0.003765	0.005008	0.006234	0.00456	0.005367
117.0793	133.13	0.002508	0.002281	0.002037	0.001731	0.001947	0.00155	0.001912	0.0161	0.00218	0.001607	0.00194	0.00206	0.001963	0.001816	0.001691	0.001604	0.001861	0.001604
117.0793	133.13	0.003073	0.002405	0.002233	0.003806	0.004458	0.003718	0.002716	0.003895	0.003071	0.006297	0.007097	0.004607	0.007345	0.006344	0.007671	0.009008	0.008518	0.007888
118.0114	88.38	0.0006	0.001524	0.003963	0.002522	0.003931	0.002085	0.001797	0.002058	0.000298	0.006323	0.002052	0.004155	0.002523	0.004342	0.004403	0.002706	0.002377	0.002771
119.0587	136.02	0.001044	0.001137	0.001118	0.001045	0.00138	0.001036	0.001313	0.001793	0.001344	0.002715	0.002993	0.002024	0.002662	0.002049	0.002865	0.003464	0.003833	0.002812
122.0486	104.02	0.010601	0.00906	0.007036	0.00855	0.010801	0.007352	0.007965	0.011091	0.008987	0.016737	0.011914	0.015707	0.017681	0.015124	0.014807	0.018598	0.015081	0.018869
123.3703	22.22	0.002557	0.003456	0.004043	0.003103	0.002595	0.004218	0.003098	0.004074	0.005155	0.003247	0.00498	0.005346	0.003812	0.004164	0.005416	0.005484	0.004089	0.004411
125.0178	22.11	0.00214	0.002207	0.002553	0.00041	0.00206	0.000412	0.000482	0.000862	0.000627	0.000827	0.001387	0.000682	0.002141	0.002722	0.001625	0.001615	0.001299	0.001294
126.0661	87.07	0.001221	0.001269	0.001149	0.000884	0.001013	0.001097	0.001138	0.001048	0.001055	0.001333	0.001091	0.001329	0.001122	0.001218	0.001205	0.001061	0.000991	0.000991
129.0784	99.85	0.000646	0.000478	0.000362	0.0004	0.000739	0.000566	0.000517	0.000578	0.000569	0.000854	0.001053	0.000724	0.001051	0.000911	0.001138	0.001007	0.000958	0.000997
129.9577	87.76	0.004777	0.005062	0.005693	0.004475	0.005024	0.005553	0.004819	0.003945	0.003574	0.006087	0.005659	0.004545	0.002888	0.004331	0.004047	0.003863	0.004592	0.003046
131.0695	119.04	0.003767	0.027958	0.025164	0.027446	0.02348	0.024819	0.026941	0.038084	0.030381	0.05577	0.04288	0.038882	0.052032	0.064826	0.063581	0.063391	0.059989	0.059977
131.0944	134.2	0.012194	0.017588	0.006764	0.007124	0.014397	0.008122	0.007827	0.011813	0.009647	0.02515	0.032781	0.019894	0.027013	0.020723	0.02815	0.032783	0.029976	0.026852
132.0273	88.39	0.040684	0.039214	0.025982	0.013941	0.019119	0.012001	0.00942	0.012985	0.016919	0.034303	0.010317	0.027229	0.014477	0.026065	0.017649	0.014836	0.013438	0.017514
133.0373	145.7	0.01111	0.008777	0.007694	0.008049	0.006442	0.006519	0.010164	0.007928	0.01726	0.013604	0.015494	0.01848	0.010703	0.017533	0.018903	0.013918	0.016255	

STATISTICAL ANALYSIS

MULTIVARIATE & UNIVARIATE