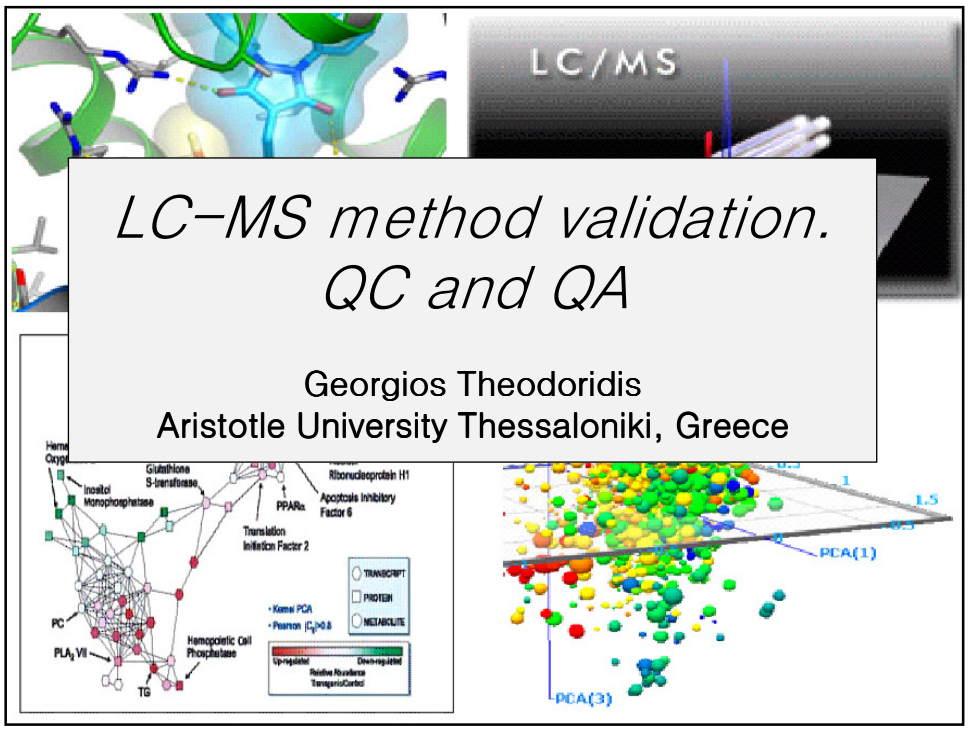


1

Acknowledgment

AUTh Group
Assist. Prof. Helen Gika
C. Virgiliou, PhD
O. Deda, PhD
A. Pechlivanis, PhD
A. Orfanidis, PhD
C. Zisi, PhD

PhD students
O. Begou
D. Diamantidou
A. Lioupi
M. Marinaki
A. Pesiridou
A. Krokos

All MSc students

Collaborators
Prof. Ian Wilson, ICL
Assist. Prof. M. Witting, Helmholtz
Assist. Prof. A. Agapiou, Cyprus Univ.

Biomic-AUTh
Prof. V. Mougios
Prof. G. Arsenos, Vet.
Assoc. Prof. N. Raikos, MD
Assoc. Prof. K. Sarafidis, MD
Assoc. Prof. G. Sianos MD
Assist. Prof. G. Giannakoulas, MD
Prof. M. Roilidis, MD

FoodOmics GR
Prof. N. Thomaidis, Athens Univ
Prof. M. Mikros, Athens Univ
Prof. A. Spyros, Crete Univ.
G. Tsangaris, Athens Academy and the FoodOmicsGR_RI Consortium

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ
Υπουργείο Αγροτικής Ανάπτυξης και Τροφίμων
ΕΛΙΔΕΚ
ΕΣΠΑ 2007-2013
ΕΣΠΑ 2014-2020
Altria
eatwalk functional food from greece
ανάλυση
MERCK
Horizon 2020 European Union Funding for Research & Innovation
ΙΚΥ
SEVENTH FRAMEWORK PROGRAMME

2

BIOMIC_AUTh

- Interdisciplinary Effort AUTh
- 17 Staff from 8 schools
- > 25 young researchers
- Omics with emphasis on metabolomics
- New laboratory facility 250 m^2
- http://biomic.web.auth.gr/

LC-MS/MS
GC-MS/MS
And NMR
Databases
Spectra libraries
Statistical software
Pathway analysis
>100 publications in metabolomics

3

FoodOmicsGR-RI

- Open Research Infrastructure
- 2019-2021
- 7 Greek Universities and Institutes
- AUTh, Athens Univ., Crete Univ, Ioannina Univ.,

Aegean Univ., Agric. Univ. Athens, Intern. Univ., Academy Athens

Scope

Map regional foods: comprehensive characterization of foods
Support nutritional studies, studies on food authenticity and control of geographical origin.
Provide hard molecular data on nutritional value
>450 man months for young researchers
> 70 Staff from > 25 Disciplines (plant growth, animal husbandry, milk Science, pharmacognosy, toxicology, analytical chemistry, food chem. Food technology, informatics, biochemistry…proteomics, NGS, Elemental metabolomics

4

The elements…

What is metabonomics?

"the quantitative measurement of the dynamic multiparametric response of living systems to pathophysiological stimuli or genetic modification"

J. Nicohlson et al. 1999

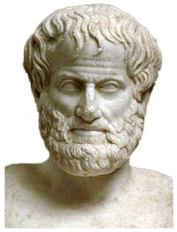
Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" - specifically, the study of their small-molecule metabolite profiles"

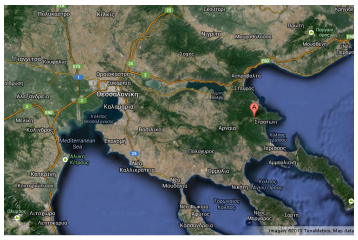
B. Daviss, 2005

5

Metabonomics/Metabolomics

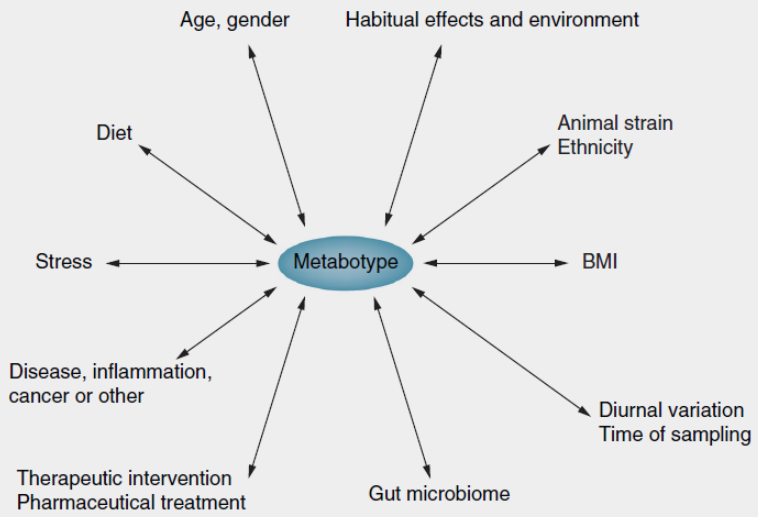
- The determination of the whole metabolic complement of a cell, tissue, organism, sample
- Correlation with physiology, stress, origin, other factors
- It is a holistic approach
- Why?
- "The whole is greater than the sum of its parts"





6

Figure 1.1. The interplay between different factors and the metabolic content revealed by metabolomics.



Source: *Considerations in the design of clinical and epidemiological metabolic phenotyping studies*
G Theodoridis et al 2013, ebook Metabolic profiling in clinical applications. doi:10.4155/EBO.13.487

7

Targeted Metabolomics

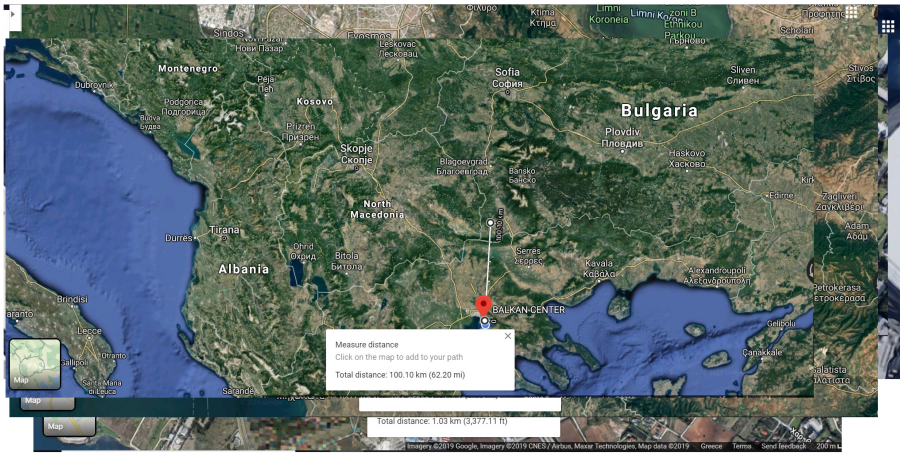
Key challenges

- Ion suppression issues
- No blanks available
- No agreed strategy for validation
- Metabolites are there! Unlike in pesticide analysis: Urine >140 determined, cell culture >100 metabolites …
- How to quantify ? Surrogate matrix, standard addition method, external calibration ?

Sample Preparation Strategies for the Effective Quantitation of Hydrophilic Metabolites in Serum by Multi-Targeted HILIC-MS/MS.
Tsakelidou et al Metabolites, 2017

8

Signal spans to several orders of magnitude



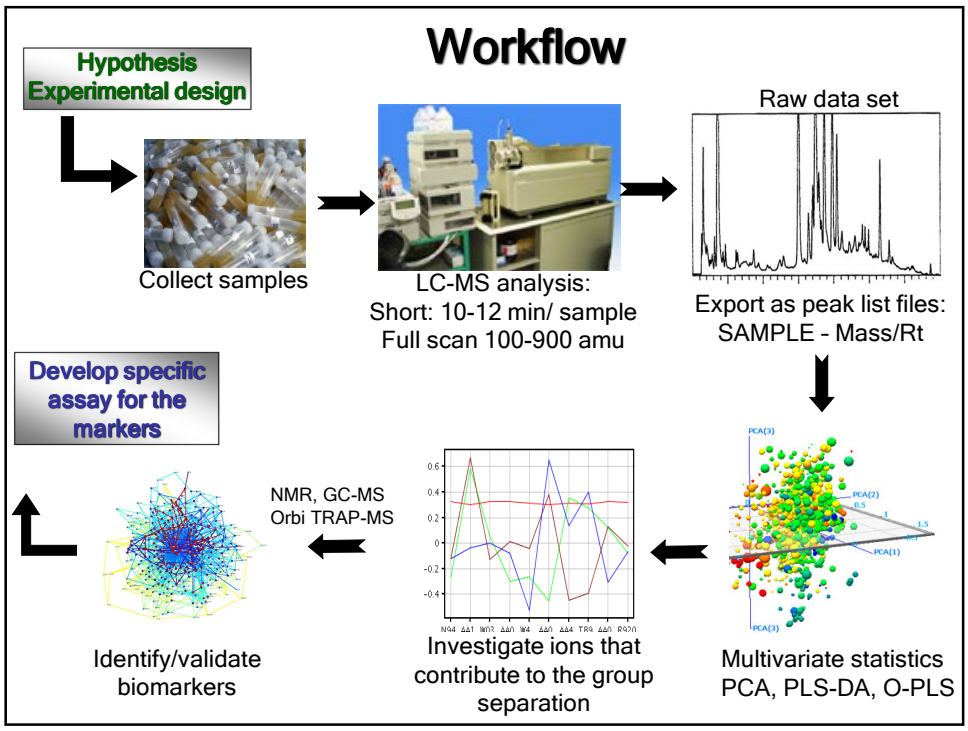
9

More issues

- Labelled internal standards not always available
- If available, $$$
- Not easy to handle
- Not easy to organise the calibrants
- Wide range of linear ranges depending on the matrix and condition

- Whatever the cost: **worth the effort**

10



11

Pipeline for untargeted LC–MS based Metabolomics

- Biological question
  - Study protocol
  - Sample collection– storage– treatment
  - LC–MS Analysis
  - Data preprocessing
    Deconvolution, normalisation
  - Multivariate statistics
  - Data interpretation

12

Obvious sources of error

- Changes in chromatographic properties (retention, peak shape, resolution, selectivity).
- Changes in mass accuracy
- Changes in sensitivity (e.g. source contamination)

Danger if instrument variation is misinterpreted as being due to biological process

- Extraction, inappropriate. Extraction edits the sample and thus the metabolic profile

13

Control Methods

- Internal standards?
- Test Mixtures (external standards ca. 40 metabolites of different classes).
- Quality controls (QCs)?
- Replicates?

Intervention

- How long can a batch be? 24h–48h–72h?
- When to stop and clean the source?
- When to change pre or analytical column?
- How can I put data together ? Extract all samples together?

14

Quality controls/internal standards

- For LC–MS in metabon/lomics internal standards (IS) are not as useful as in conventional quantitative methods. Suggestion: 5 or more Deuterated IS representative of important classes
- Test Mixture. Data (XIC) used to control the stability of the LC and MS (rt and intensity). This is used to chop bad data sets not to prove that a dataset is useful. Data **NOT** to be used in PCA.
- QCs – use of a standard/representative BIOLOGICAL sample:
  –an aliquot of each test sample to make a" master mix" or pooled sample.

For very large sample sets QCs can be made by aliquoting a number of test samples only (not all).

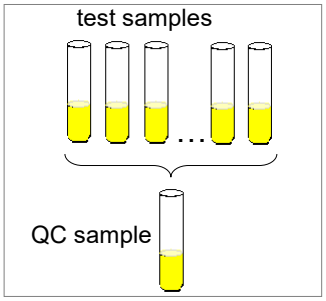
QC should be used for its own batch only for PCA and XIC. Peak Tables from QCs stored and used for comparison.

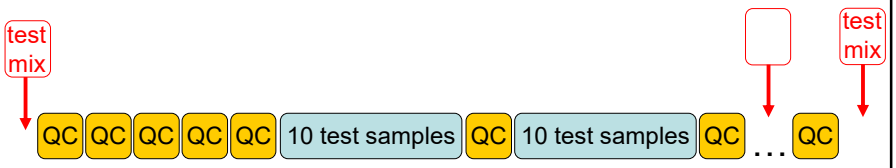
  –QC samples run before, during and after analysis to asses system suitability

- Replicate injections, All ? Selected samples? For large datasets 1 inj–2inj– or 3inj? Suggestion: First screen one injection of all samples and repeats (1x) of up to 20%. Repetitions of the whole batch in second stage to verify the finding (including the data processing).

15

QC approach





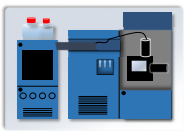
Data scrutiny: Conventional XIC and MVA.

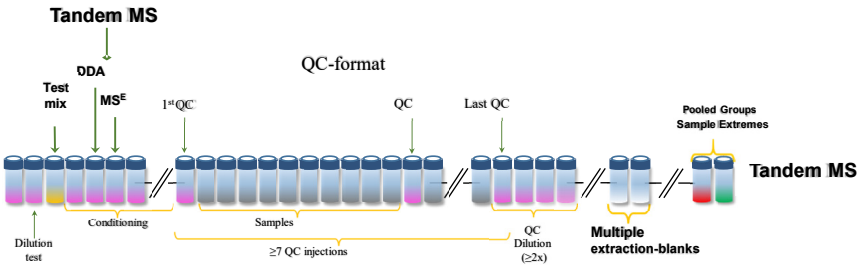
Gika, JPR 2007, 2012, Guy 2008, Kamleh 2012…

16

Sample Analysis

UPLC-MS





Data scrutiny: Conventional XIC and MVA.

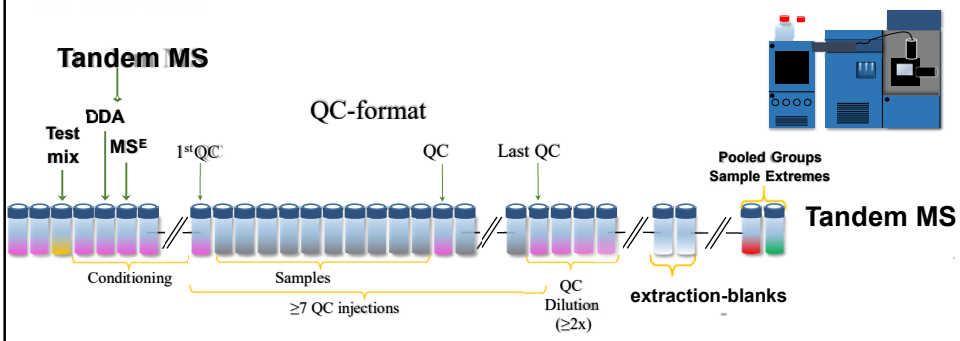
Gika, JPR 2007, 2012, Guy 2008, Kamleh 2012…

[Vorkas PA et al, Anal Chem, 2015]

[Vorkas PA et al, Anal Chem, 2015]

17

Sample Analysis UPLC-MS

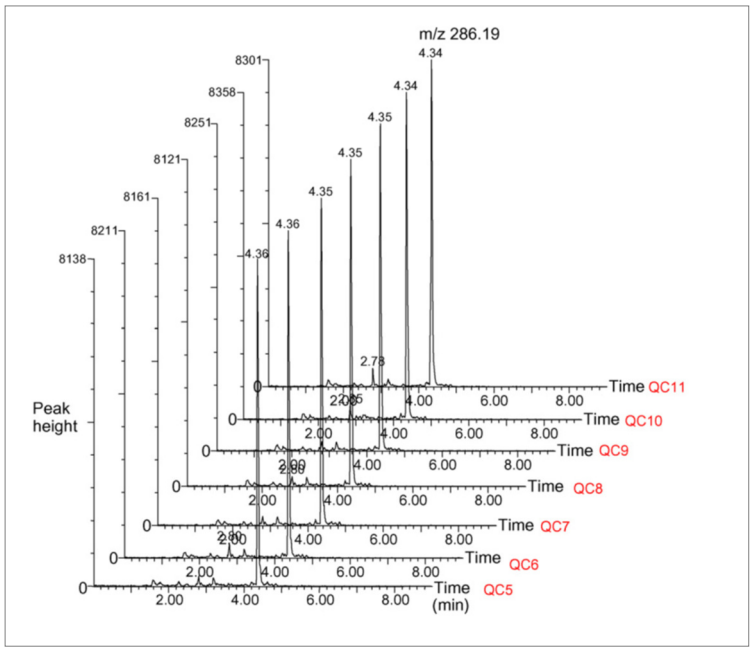


- **Know your method**
  - **Use methods from literature**
  - **Run authentic standards**
- **Run a test mix**
  - **Check RT**
  - **Accurate mass**
  - **Frequent adducts**
- **Unbiased tandem MS acquisition**
  - **Run Data Dependent Acquisition (DDA)**
  - **MSE**

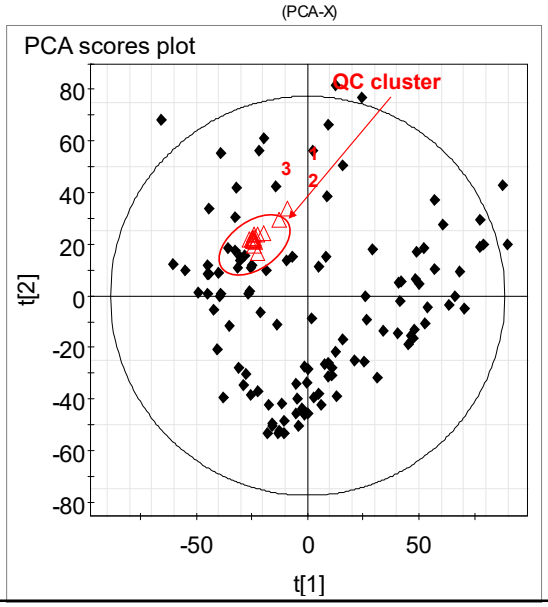
  (Use pooled/QC samples – pooled groups/extremes)
- **Run sample preparation blanks**
- **Run Pos and Neg modes with the same gradient**

18

Retention time

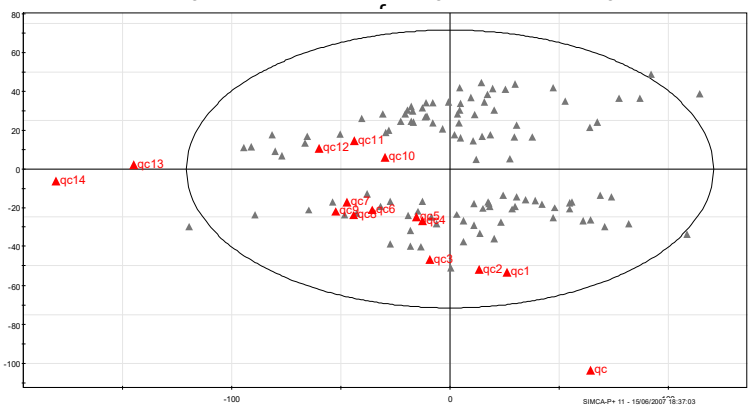


19



20

PCA plot :
Analytical variability – poor system

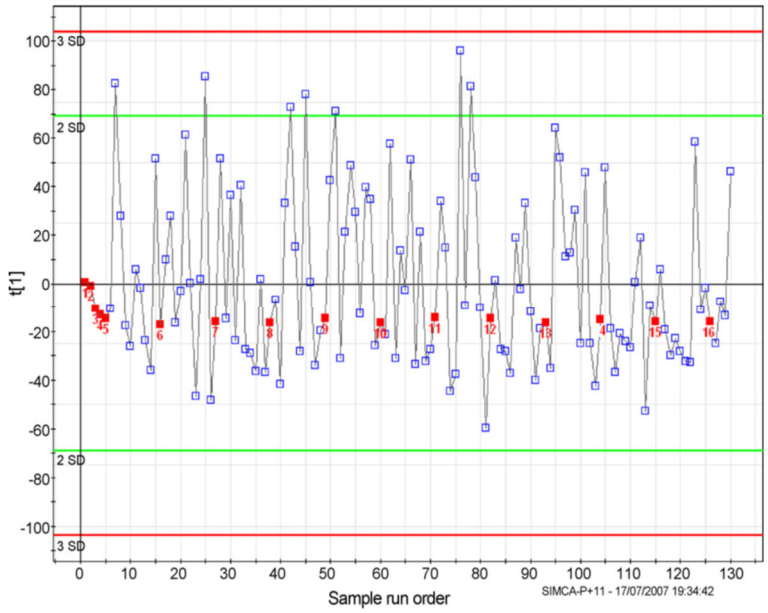


Note: PCA plot can be misleading

In case where test samples have small differences principal components represent small proportion of the variability in the data and QCs appear more scattered.

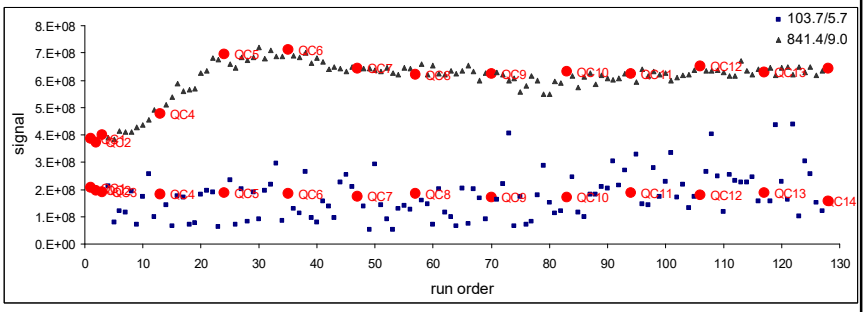
21

Time series plot



22

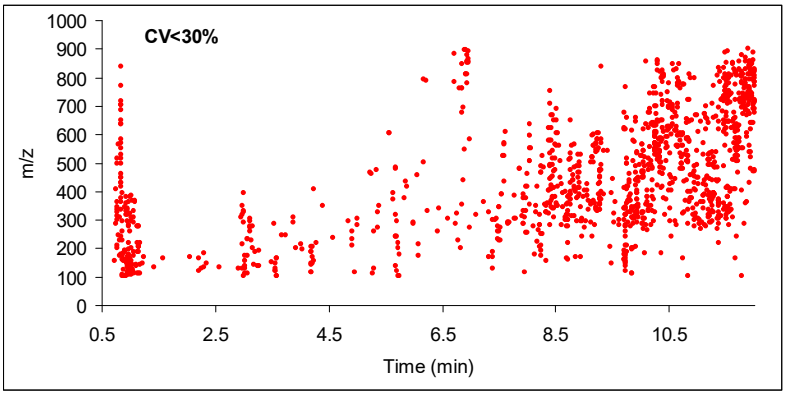
Plot profile of ions



23

QC Peak Table: Check ions with high CV% values

	QC6	QC7	QC8	⋯	QCx	CV%
m1_t1	1045	1280	1147	⋯	1321	8
m2_t2	876	757	120	⋯	850	35
⋮						⋮
mx_tx	2378	2395	2547	⋯	2367	6

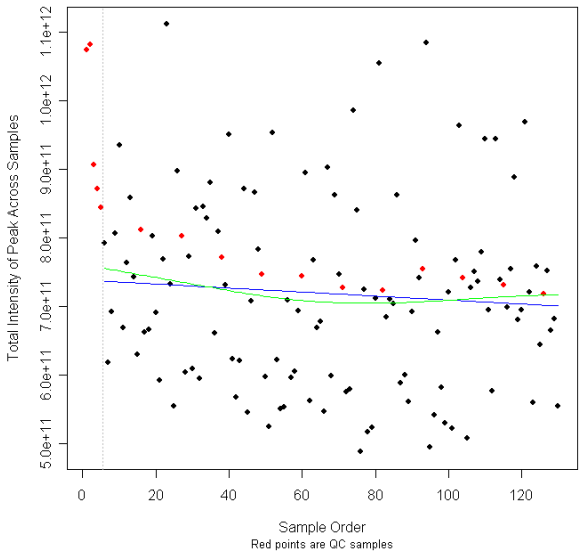


24

Metabonomic Study Diseased vs Control population

Raw data, TIC across all samples

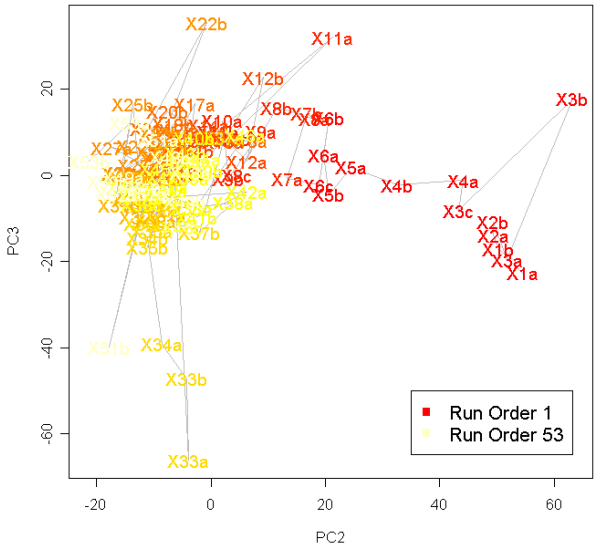
- QC samples



25

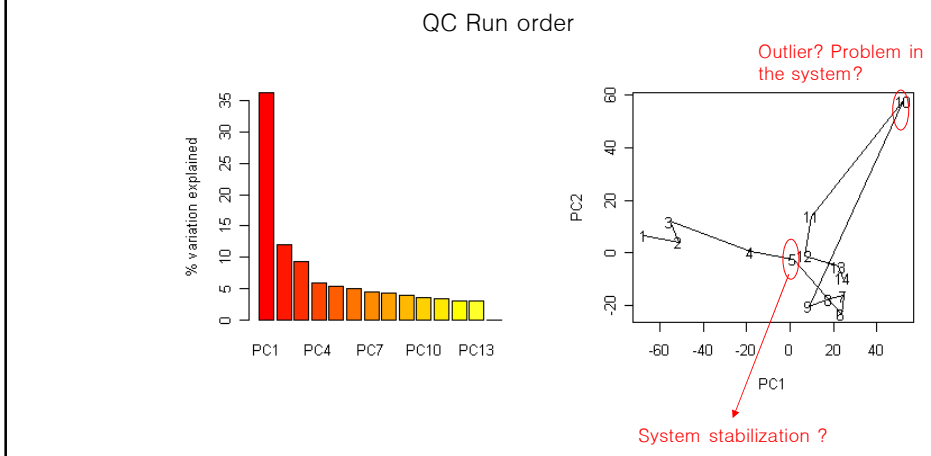
PCA

Check for run order effects



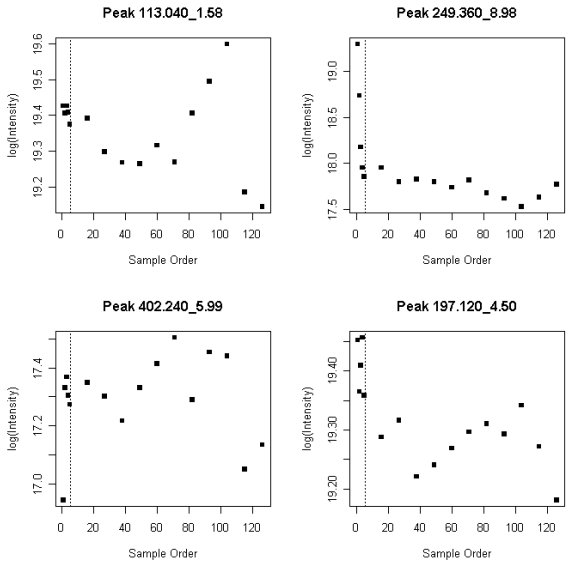
26

Metabonomic Study Diseased vs Control population



27

Raw data, Log (intensity), 4 randomly selected peaks

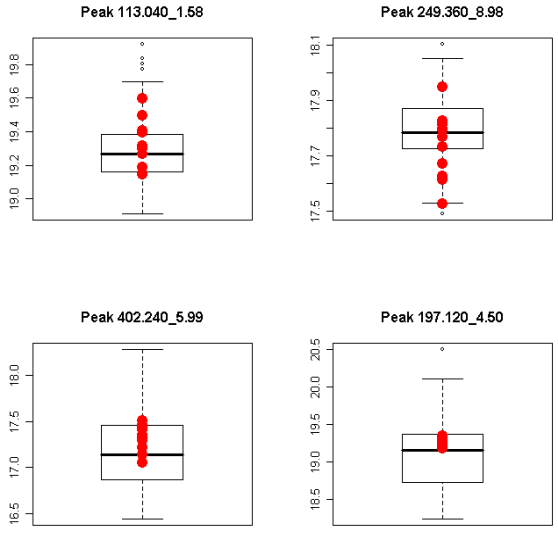


28

Log (Intensity) 4 randomly selected peaks

Boxplot shows test sample intensities

- QC samples



29

Other proposed filters

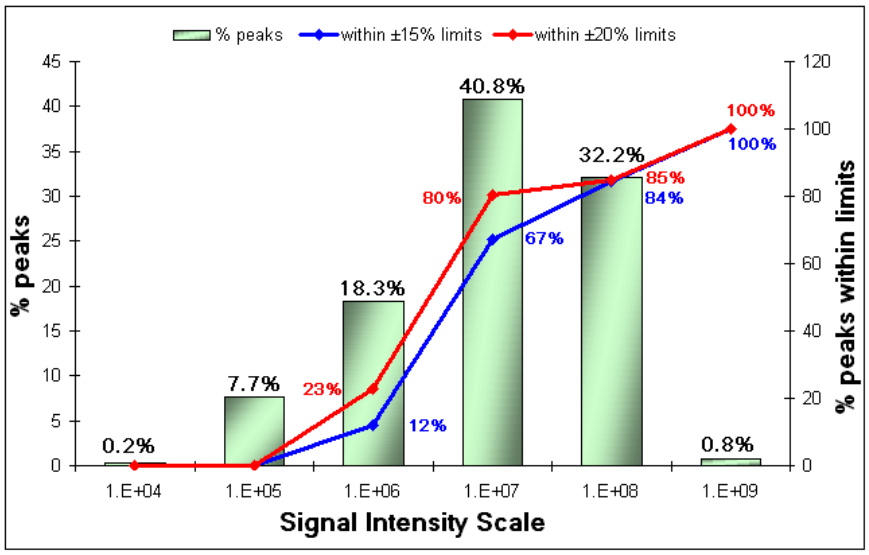
- 80% rule Not all researchers agree
- 50% rule
- System suitability testing

(i) m/z error of 5 ppm compared to theoretical mass, (ii) retention time error of < 2% compared to the defined retention time, (iii) peak area equal to a predefined acceptable peak area ± 10% and (iv) symmetrical peak shape with no evidence of peak splitting

- System suitability blank and process blank samples

30

Intensity vs reproducibility

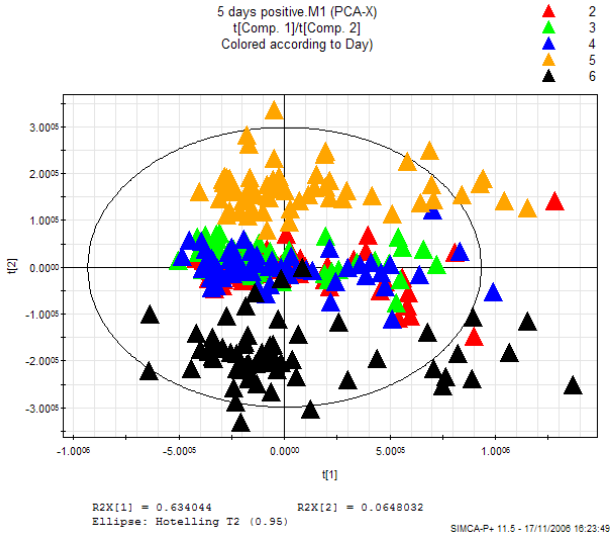


31

Day to day variation

Over 20h run (60 human urine samples) repeated for 5 days.

How reproducible the data are?



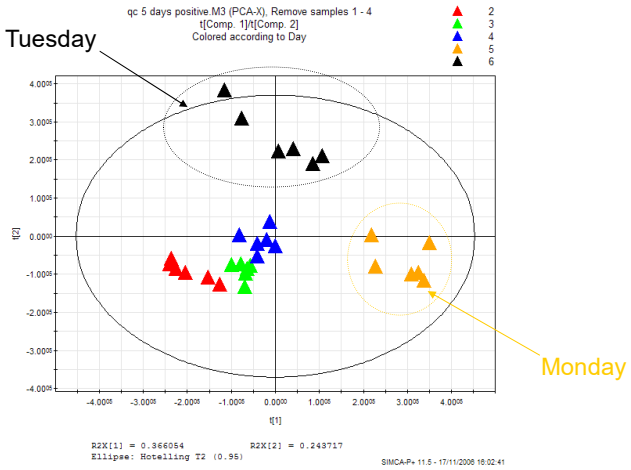
Obvious day-to day effect appears vertically in t2 although only 6% of total variance in data

t1 shows sample to sample variation horizontally

32

Day to day variation

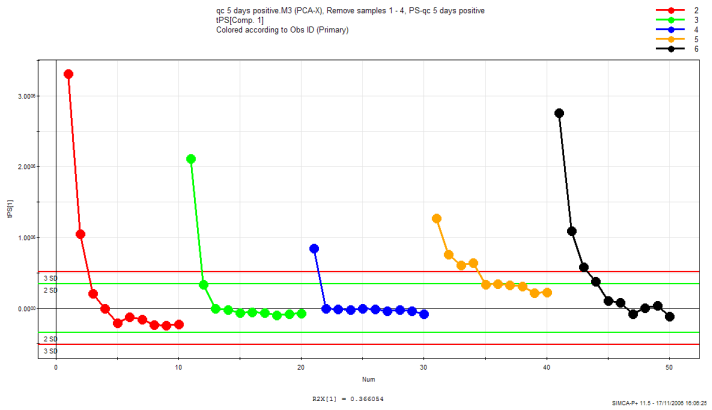
PCA “control model”



–both day–to–day and within day variation

33

Day–to–day variation

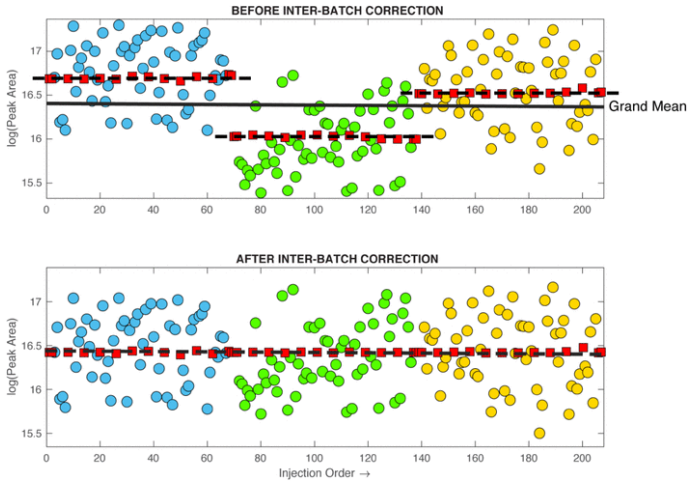


–Start up variation each day

–4th and 5th batch higher reproducibility

34

QC data offers ways to correct batch effect

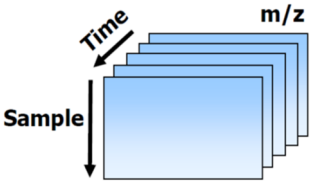


Broadhurst et al Metabolomics 2018

35

Pre-processing of data may have a profound effect on the results

LC-MS raw data, software parameters
- Unfolding and alignment issues
- Mass matching and time windowing



-Normalisation (to total signal, to constant peak to baseline noise etc): danger, may reverse the direction of trends!
Eg. If major peak absent then other peaks increase in apparent importance
-Scaling

-Improve reproducibility in the analytical method

When we cannot repeat experiment (ethical issues, small sample volume cost) we may 'rescue' the data by
- OPLS-DA to "See through" unwanted variation
- spectral filters

BUT overall it can be GIGO

36

MQACC

https://epi.grants.cancer.gov/Consortia/mQACC/

- The consortium's mission is to engage the metabolomics community to communicate and promote the development, dissemination and harmonization of best QA/QC practices in untargeted metabolomics through the following objectives:
- To identify, catalog, harmonize and disseminate QA/QC best practices for untargeted metabolomics.
- To establish mechanisms to enable the metabolomics community to adopt QA/QC best practices.
- To promote and support systematic training in QA/QC best practices for the metabolomics community.
- To encourage the prioritization and development of reference materials applicable to metabolomics research.

37

MQACC

https://epi.grants.cancer.gov/Consortia/mQACC/

Reference and Test Material Working Group

Dissemination of Current QA/QC Practices Working Group

- Think Tank on Quality Assurance and Quality Control for Untargeted Metabolomics Studies (October 2017)
- Workshops: Metabolomics 2018 and 2019 Workshop: QA and QC in Untargeted Metabolomics
- Think Tank on Quality Assurance & Quality Control for Untargeted Metabolomics Studies
- Quality Control in Untargeted Metabolomics
- Quality Assurance Panel
- Conferences
- Webex meetings

38

MQACC

https://epi.grants.cancer.gov/Consortia/mQACC/

Built 1300

Built 49000

39

Synopsis

- Metabolomics is coming of age for the regulators (best practice guidelines, discussions, few papers)
- LC–MS is going to be a major part of the solution (and the problem!)
- We need to be very cautious with data (instrument variation dependant)
- Always validate data before statistical analysis (Easy to produce statistical artifacts based on bad quality data!)

40

Reading

Gika, H.G., Theodoridis, G.A., Wingate, J.E. and Wilson, I.D. (2007) Within-Day Reproducibility of an HPLC-MS-Based Method for Metabonomic Analysis: Application to Human Urine. *J. Proteome Res.* 6, 3291-3303.

Wagner, S., et al (2007) Tools in metabonomics: An integrated validation approach for LC-MS metabolic profiling of mercapturic acids in human urine. *Analytical Chemistry* 79, 2918-2926.

Waybright, T.J., Van, Q.N., Muschik, G.M., Conrads, T.P., Veenstra, T.D. and Issaq, H.J. (2006) LC-MS in metabonomics: Optimization of experimental conditions for the analysis of metabolites in human urine. *Journal of Liquid Chromatography & Related Technologies* 29, 2475-2497.

Sangster, T., Major, H., Plumb, R., Wilson, A.J. and Wilson, I.D. (2006) A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. *Analyst* 131, 1075-1078.

Shen, Y.F., Zhang, R., Moore, R.J., Kim, J., Metz, T.O., Hixson, K.K., Zhao, R., Livesay, E.A., Udseth, H.R. and Smith, R.D. (2005) Automated 20 kpsi RPLC-MS and MS/MS with chromatographic peak capacities of 1000-1500 and capabilities in proteomics and metabolomics. *Analytical Chemistry* 77, 3090-3100.

Zelena E., Dunn W., Broadhurst D., Francis-McIntyre S., Carroll K., Begley P., O'Hagan S., Knowles J. D., Halsall A., HUSERMET Consortium, Wilson I. and Kell D. B. (2009). Development of a robust and repeatable UPLC-MS method for the long-term metabolomic study of human serum. *Anal. Chem.*, 81, 1357-64

Fiehn O, Wohlgemuth G, Scholz M, Kind T, Lee do Y, Lu Y, Moon S, Nikolau B. Plant J. 2008 Feb;53(4):691-704. Quality control for plant metabolomics: reporting MSI-compliant studies.

An LC-MS-Based Metabolomics Approach for Exploring Urinary. Metabolome Modifications after Cocoa Consumption. Rafael Llorach, Mireia Urpi-Sarda,, Olga Jauregui, Maria Monagas, and Cristina Andres-Lacueva, Journal of Proteome Research 2009, 8, 5060–5068

Large-Scale Human Metabolomics Studies: A Strategy for Data (Pre-) Processing and Validation Sabina Bijlsma et al Anal Chem 2006, 78 (2), pp 567–574.

Controlling the quality of metabolomics data: new strategies to get the best out of the QC sample, Godzien et al Metabolomics 2015

Lewis, M. R., et al. (2016). Development and application of ultra-performance liquid chromatography-TOF MS for precision large scale urinary metabolic phenotyping. Analytical Chemistry, 88(18), 9004–9013.

Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies, D. Broadhurst et al 2018

41