

# LC-MS fingerprinting of human urine:

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sample stability, storage and handling

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# Specimens for Metabon/Iomics

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- Biological: blood plasma/serum, urine, bile, CSF, faeces, faecal water, tissues
  - Plants: plant tissue, cell culture, cell culture medium etc.
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# Plasma vs Urine

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Plasma and urine samples give complementary "global" representations of the metabolic state of an organism,

Plasma gives an "instantaneous" readout representing the metabolic content at the exact time of collection

Urine gives a "time-averaged" pattern for polar metabolites.

*Drug metabolites appear in plasma for very limited time; e.g. 6-MAM the major heroin metabolite is found in plasma for a few min, in urine it is detected for several hours.*

Concentrations in urine are greatly dependent on urine volume which varies between individuals (normalisation??)

Stability:

Instable/volatile substances in urine have already "decayed" after 30-60 min in room temperature.

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# Confounding Factors in Metabon/Iomics

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- **Strain, sex, diurnal variation, stress, diet/feeding, age, disease, pharmacology, gut microflora.**
  - **LC-MS can be very sensitive, data can be governed/masked by unwanted variation (noise)**
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## **“Guidelines”:**

### **Sample collection for metabonomic studies**

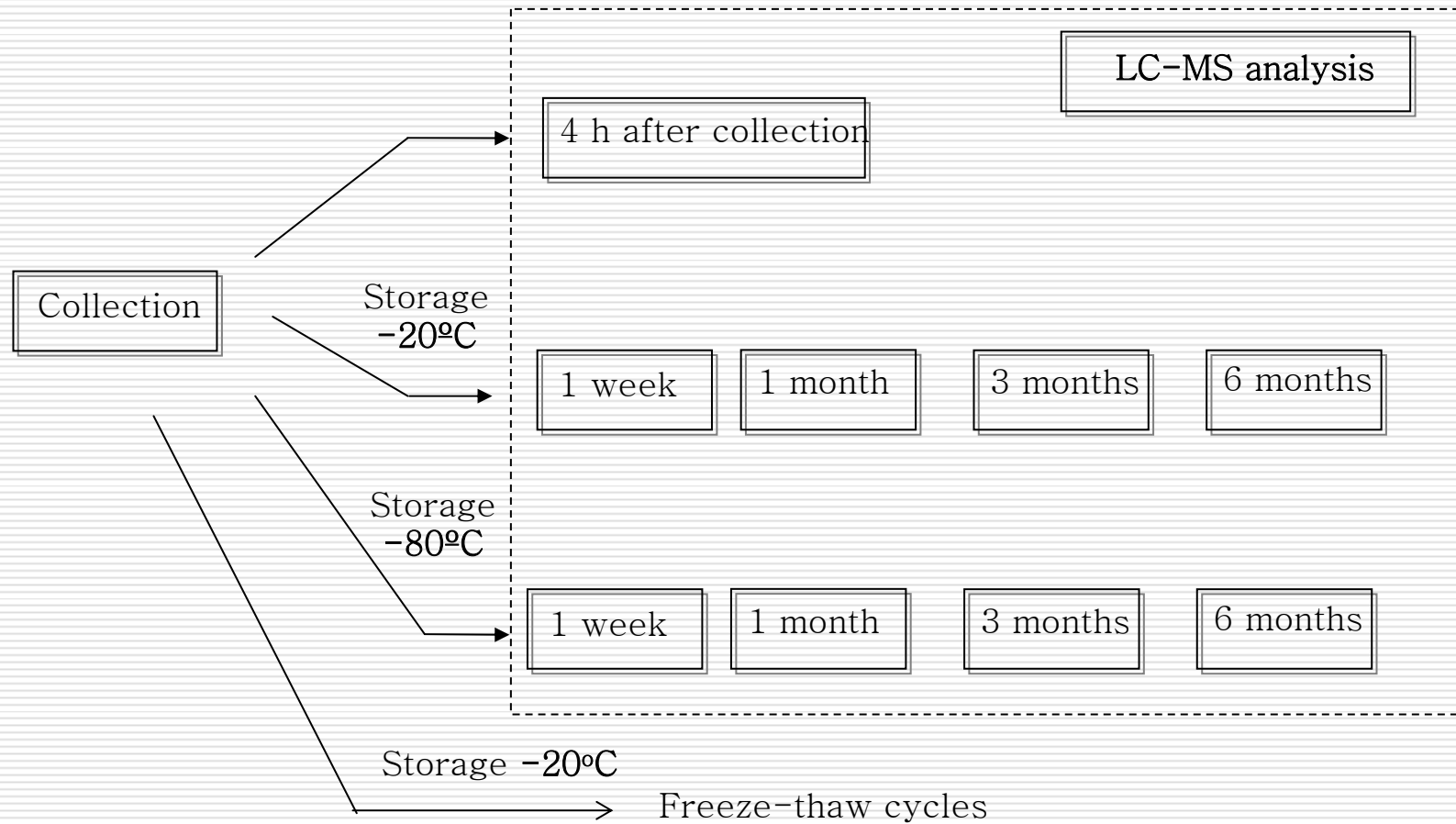
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- For animal experiments weekly collection preferably
  - Collection at the same time of day to minimise diurnal effects
  - Collection directly into sterile vessels to minimise microbial contamination.
  - The use of glass and Teflon vials prevent artifacts generated from plastics-leaching components into the samples
  - Samples must be frozen as quickly as possible after collection, preferably into dry ice.
  - Aliquoting to 3-4 vials before freezing in order to avoid freezing-thaw cycles, which can affect the sample
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# Experimental Design

Urine 6 male healthy volunteers, age range 20-54

*Gika et al J Chrom A in press*



# Experimental conditions

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□ **Symmetry C18 (Waters) column 3.5  $\mu\text{m}$ , 100x 2.1 mm at 40 °C**  
gradient elution program

Mass spectrometric data were collected by EMS ( $m/z$ : 80 – 850) in +ve and -ve ionisation mode with a **Q-Trap 4000 (AB Sciex)**.

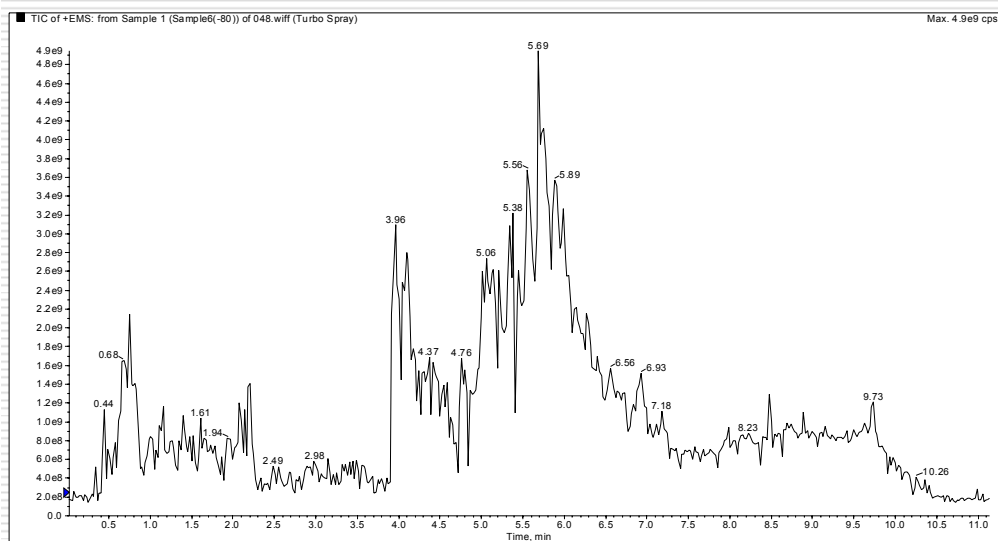
□ **Waters Acquity UPLC system, Acquity C18 BEH 1.7 $\mu\text{m}$ , 100mmX2.1mm**  
at 40 °C. gradient elution

Mass spectrometry: ( $m/z$ : 80 – 850) in +ve and -ve ionisation mode with a **Waters-Micromass Q-TOF Micro**.

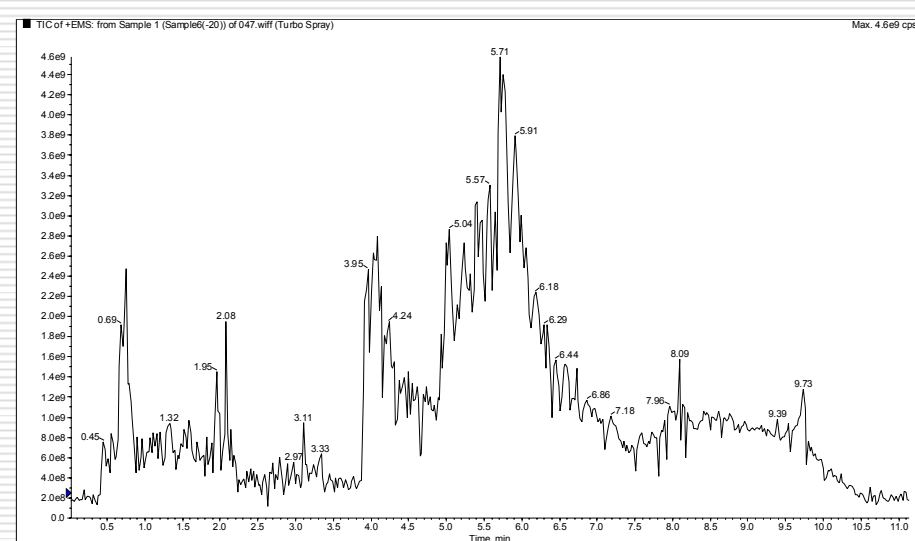
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## +EMS TIC urine profiles In QTRAP 4000 after 6 months storage

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-20°C

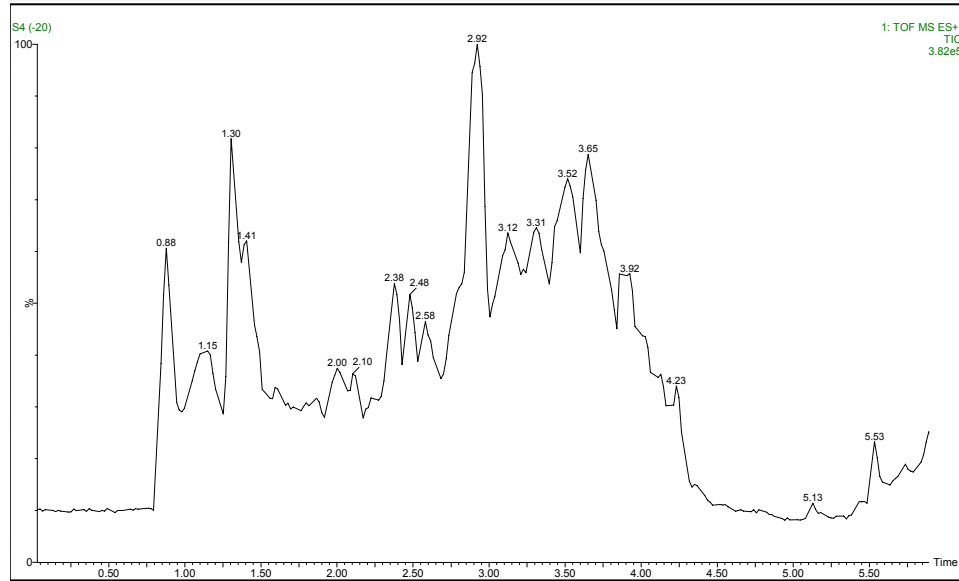


-80°C

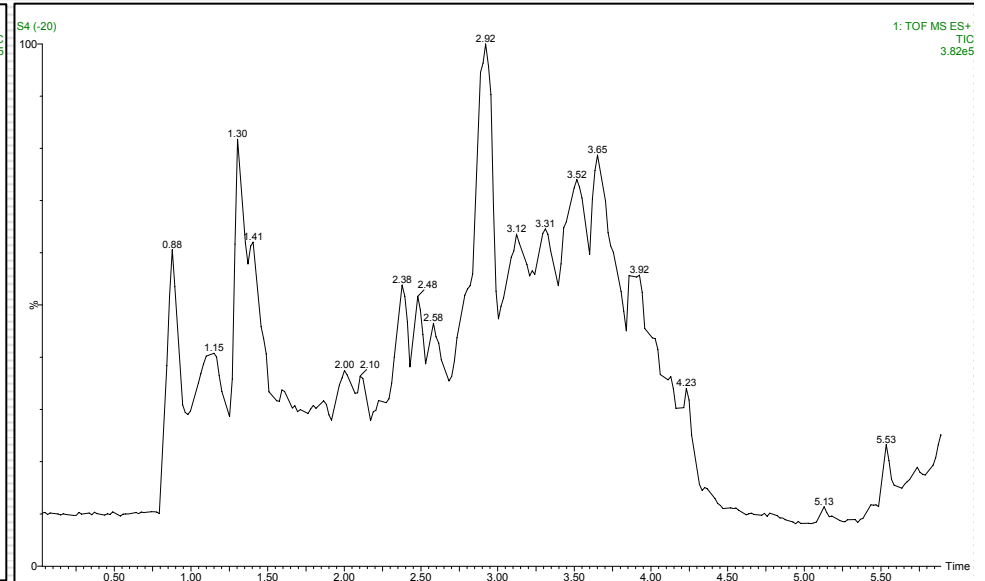
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# UPLC TOF MS profiles after 6 months storage

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-20°C

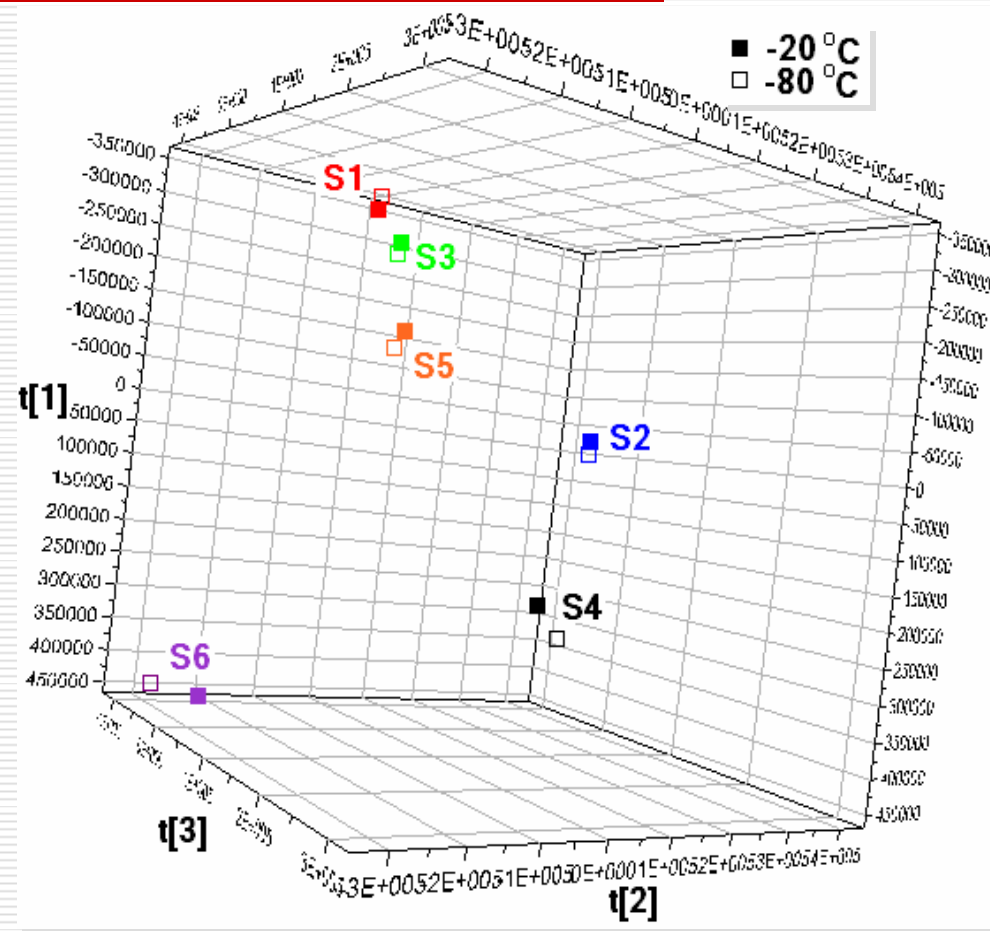


-80°C

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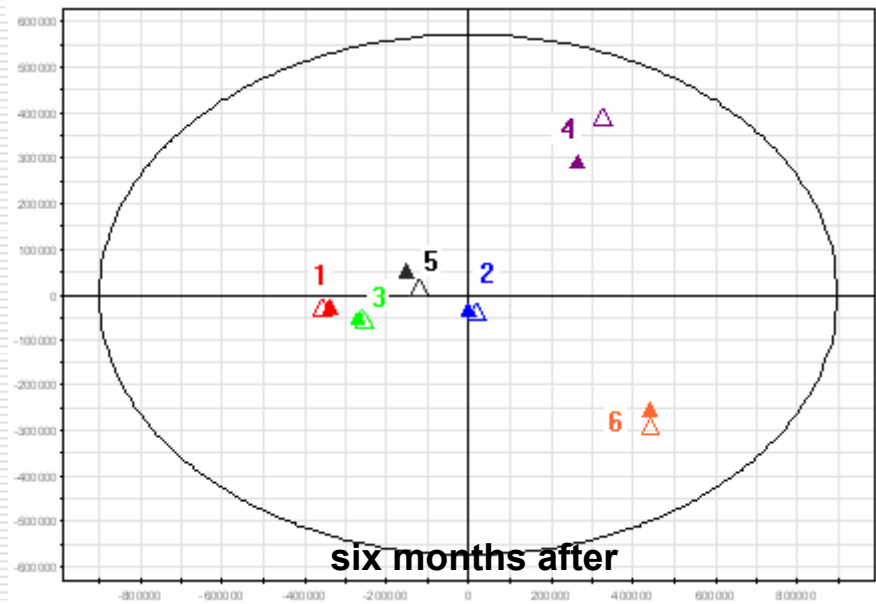
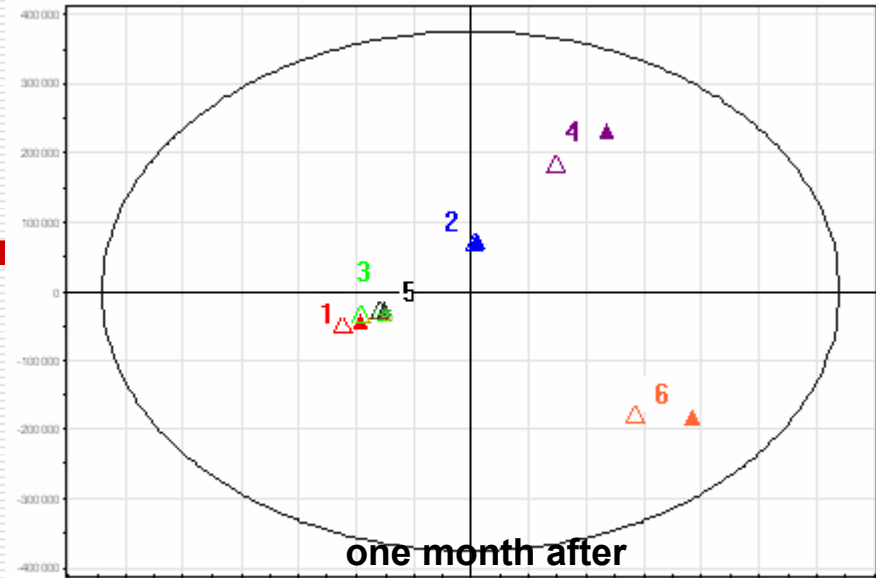
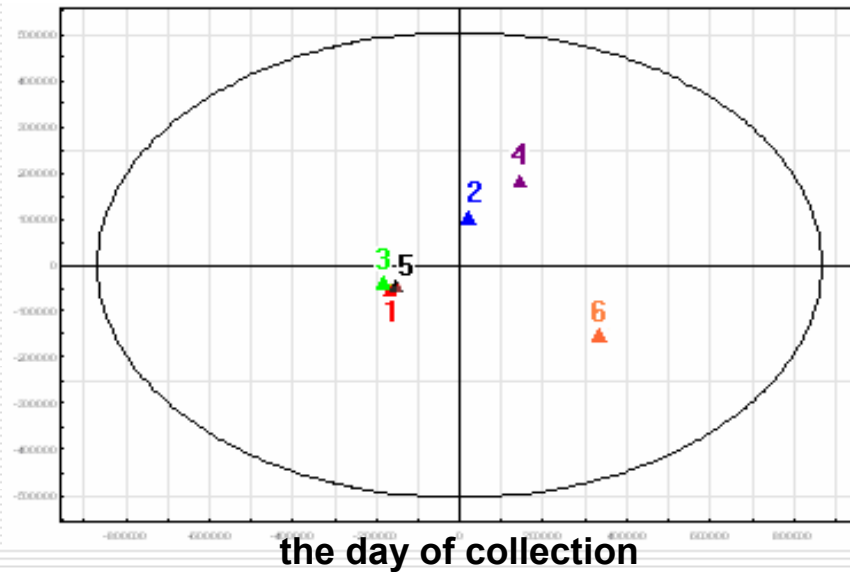
# Long term freezing stability

6 months storage



# Long term sample stability

(+ve) LC-QTRAP data. Samples coloured & numbered according to origin; solid triangles represent samples stored at -20°C; open triangles represent samples stored at -80°C.



PCA scores (explained variability of the sampleset) very similar in all 5 datasets

# Should we rely only on MVS ?

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In addition to multivariate statistical analysis; examination of the raw data should always be performed (e.g. XIC) to evaluate-verify the findings.

## **Examination of variability of Certain peaks/ions in the QC samples**

Selection criteria:

- ions spread along the time scale,
- masses representing the whole mass range
- ions commonly present in urine.

e.g., hippurate ( $m/z$  180, 5.3 min), creatinine ( $m/z$ : 114, 0.6 min)

unknowns, e.g. ( $m/z$  319, 4.5 min), ( $m/z$  571.4, 7.99 min), ( $m/z$  225, 7.04 min), ( $m/z$  175, 4.48 min), ( $m/z$  328 6.04 min)

only analytical variability was observed, no time-dependant change

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## Conclusion 1

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- profiles of the samples on the collection day were very similar to those obtained after 6 months of storage at either  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$
  - Urine sample variability robust (similar PCA scores plots)
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## What about the stability of samples laying in the autosampler?

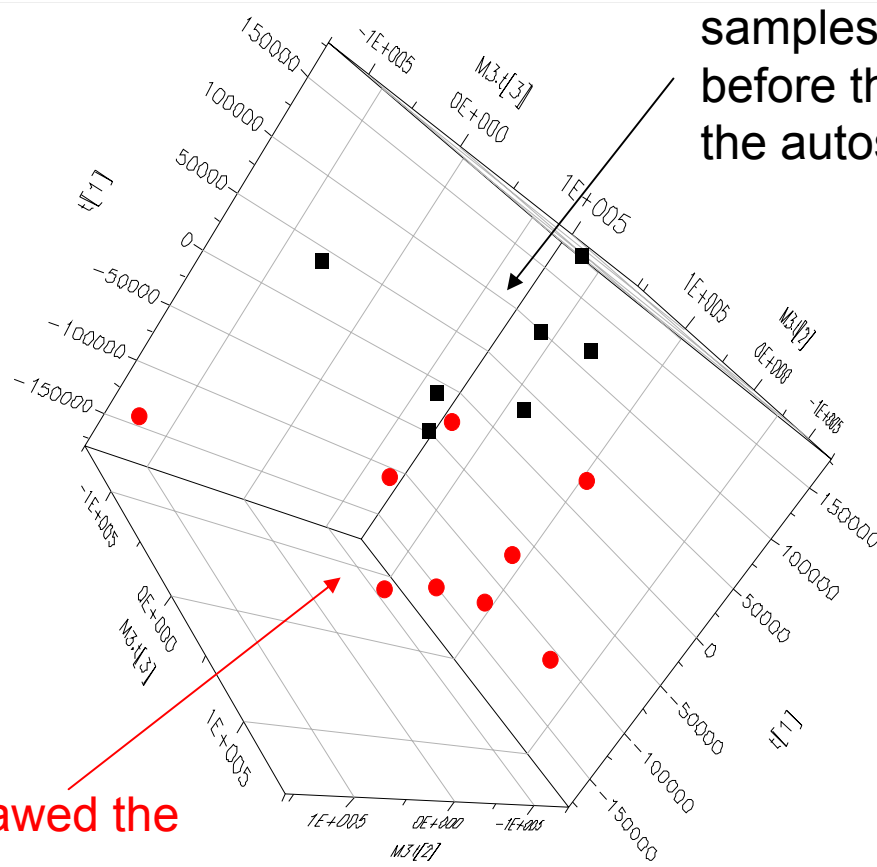
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This was evaluated by daily reanalysis of urine QC samples waiting in the autosampler (4 °C) for up to 6 days.

Each day the acquired LC–MS fingerprints of the “old” aliquots were compared against those of freshly thawed QC samples.

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# PCA scores plot of (+ve) LC-QTRAP analysis of "fresh" and "old" QCs.

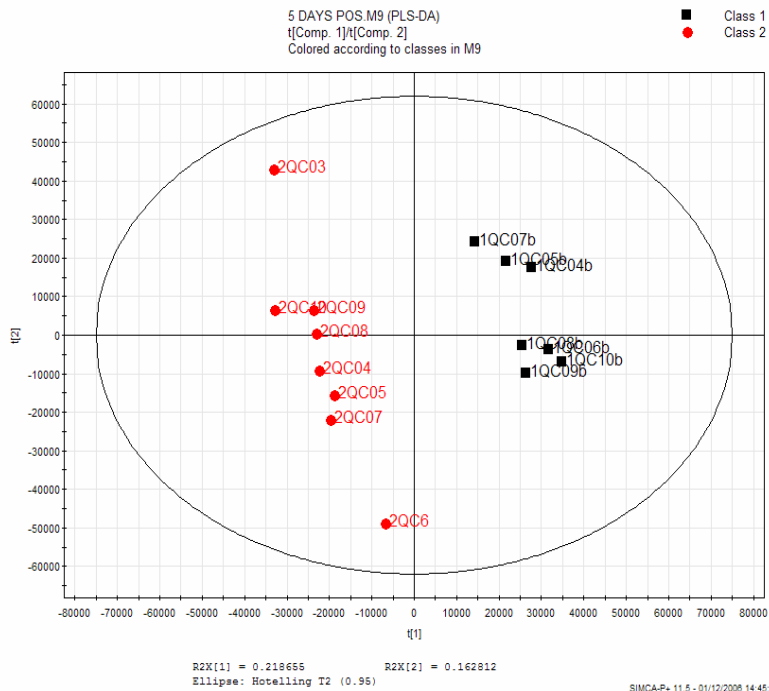


samples thawed 4 days before the analysis kept in the autosampler (4°C).

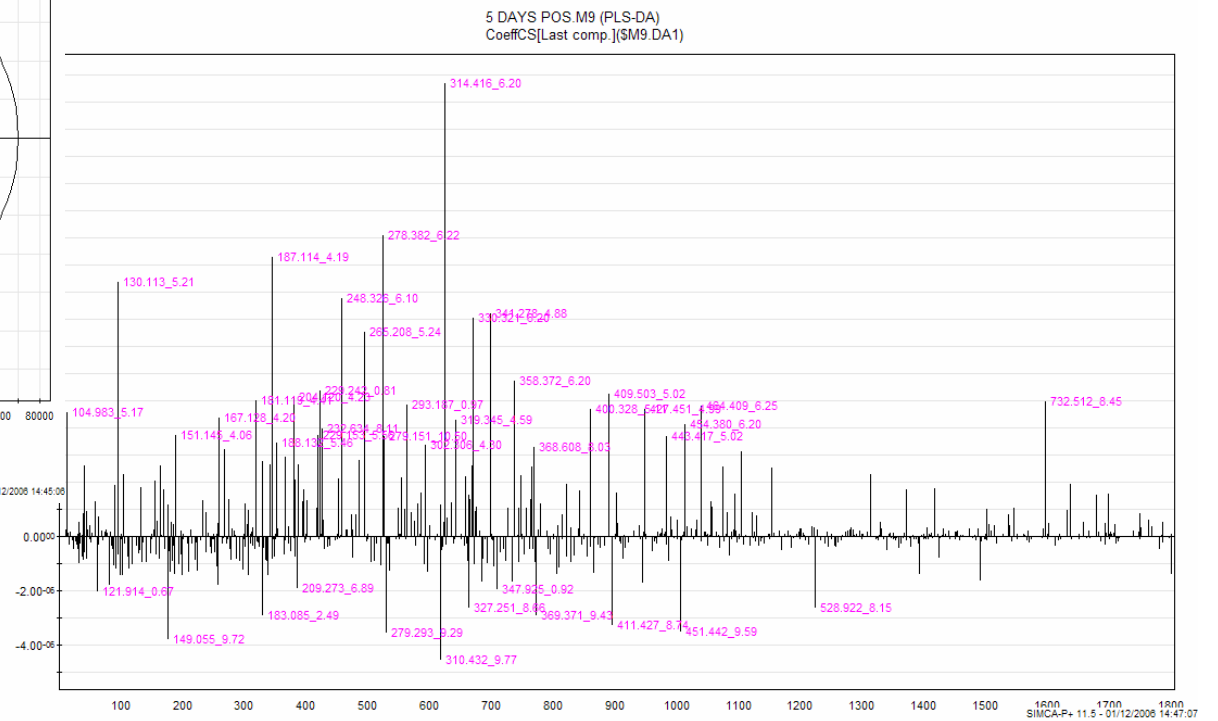
fresh samples, thawed the day of analysis.



# QC ageing effect : PLS-DA



Coefficients plot shows difference between old and freshly thawed QCs



## Conclusion 2

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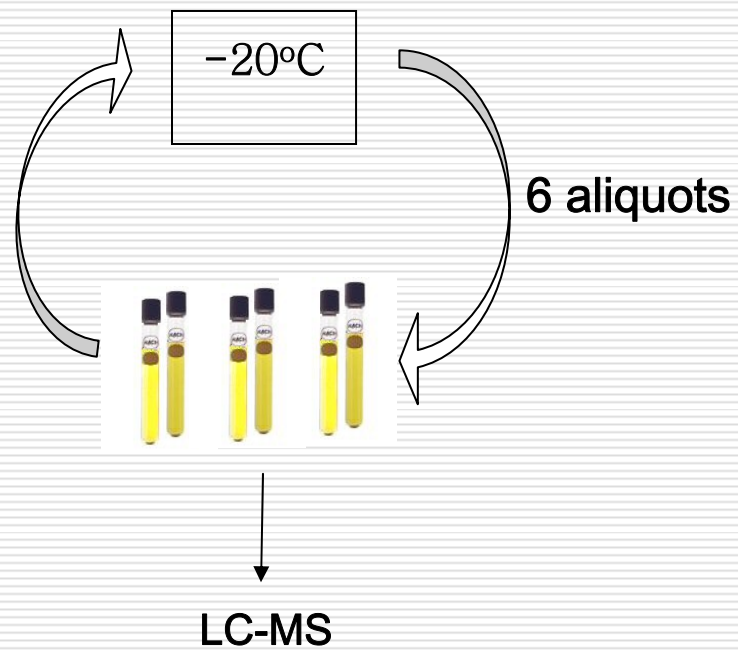
- Samples in the autosampler (4°C) can not be trusted after 48 hours
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# Freeze - Thaw Stability

*Can I trust my samples after one or more freeze-thaw processes?*

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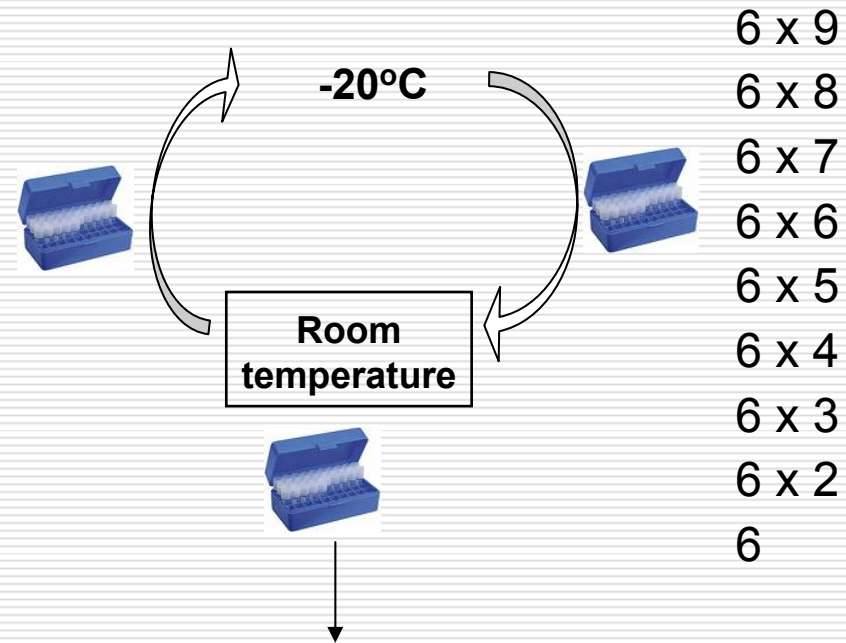
1-day freeze-thaw experiment:  
7 cycles in 1 day



# Freeze Thaw Stability 2

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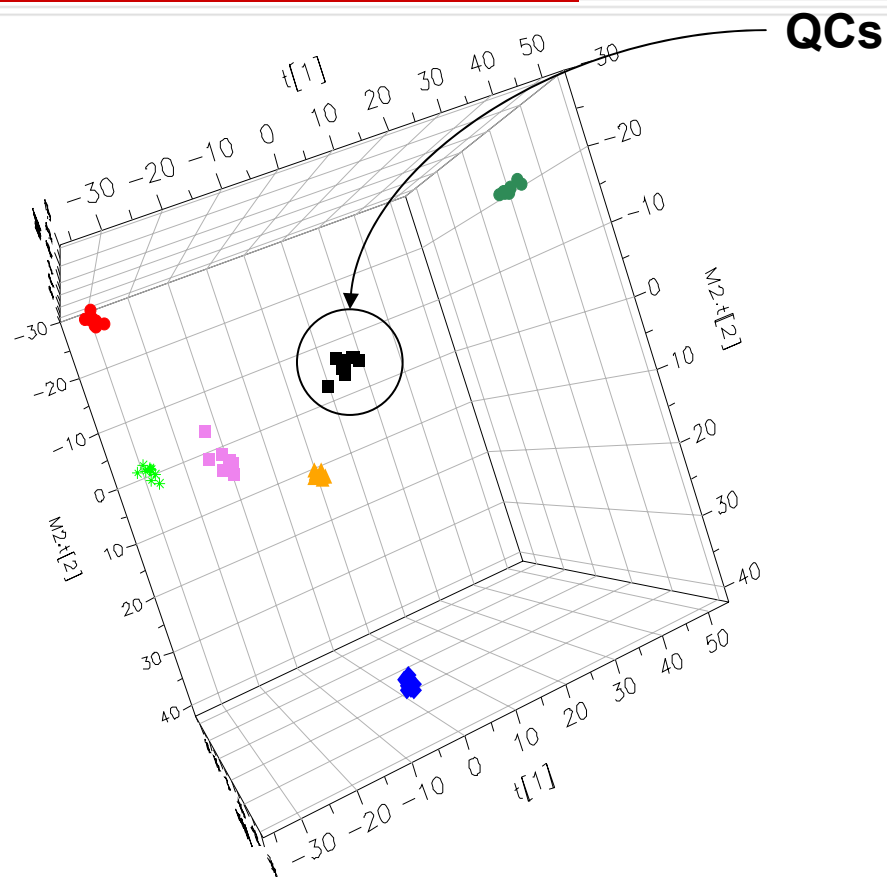
9 freeze-thaw cycles: 1 cycle per day



Single batch LC-MS  
analysis of all aliquots

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# Freeze thaw stability



SIMCA-P 11 - 01/02/2007 23:05:01

PCA of the (+ve) UPLC-QTOF data, urine of 6 individuals after 9 freeze-thaw cycles, with storage at  $-20^{\circ}\text{C}$  in between thawing.

# Mice blood plasma

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Blood plasma from 24 mice

- 1) fed with two types of diet: high fat (HF, 60% kcal) and low fat (LF, 10% kcal)
- 2) Two ages groups (12 & 15 months)

Body weight, Glucose level, metabolomics data etc.

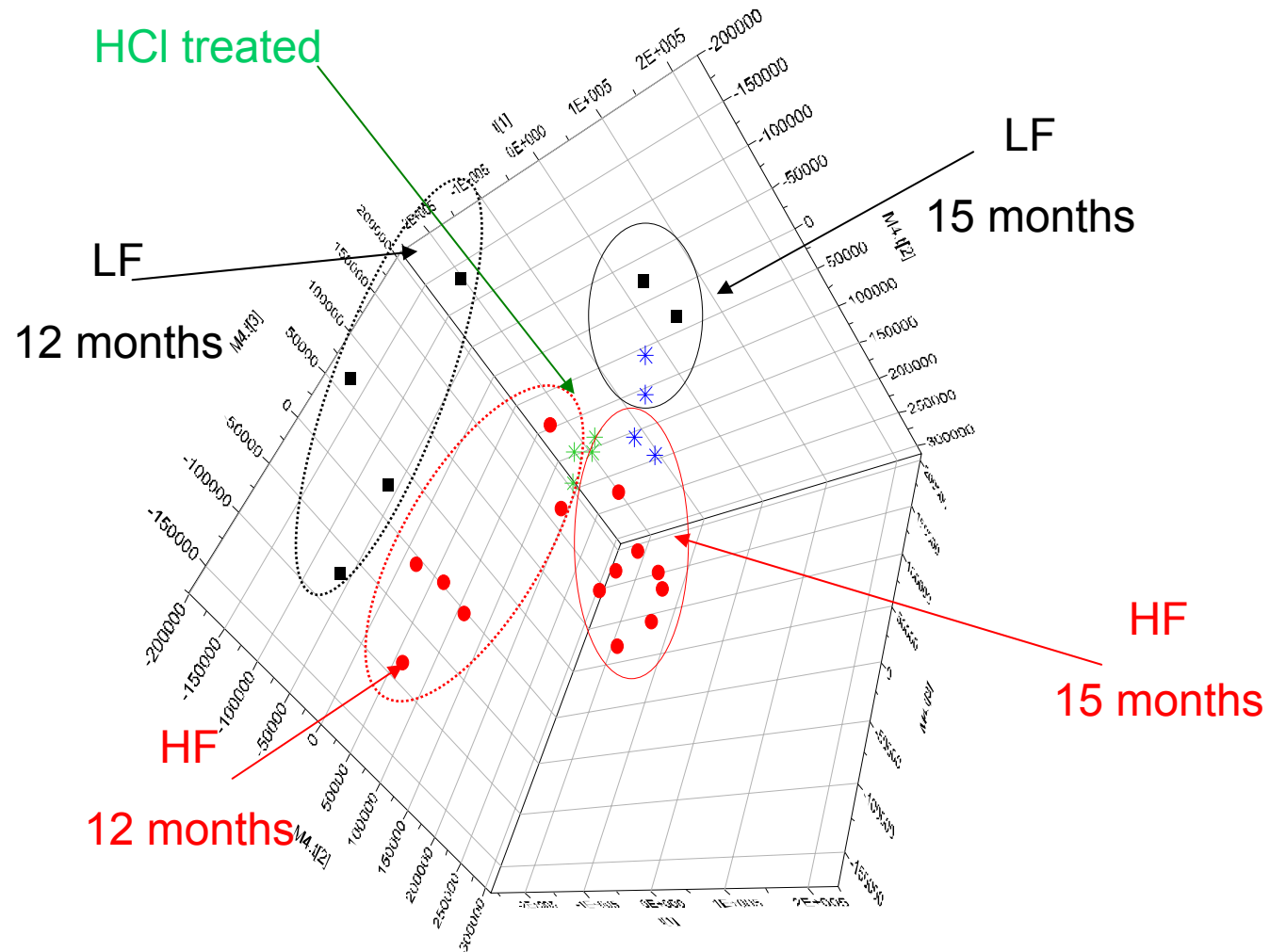
## SAMPE TREATMENT

50  $\mu$ L plasma mixed with 150  $\mu$ L acetonitrile,  
vortex and 14000 rpm

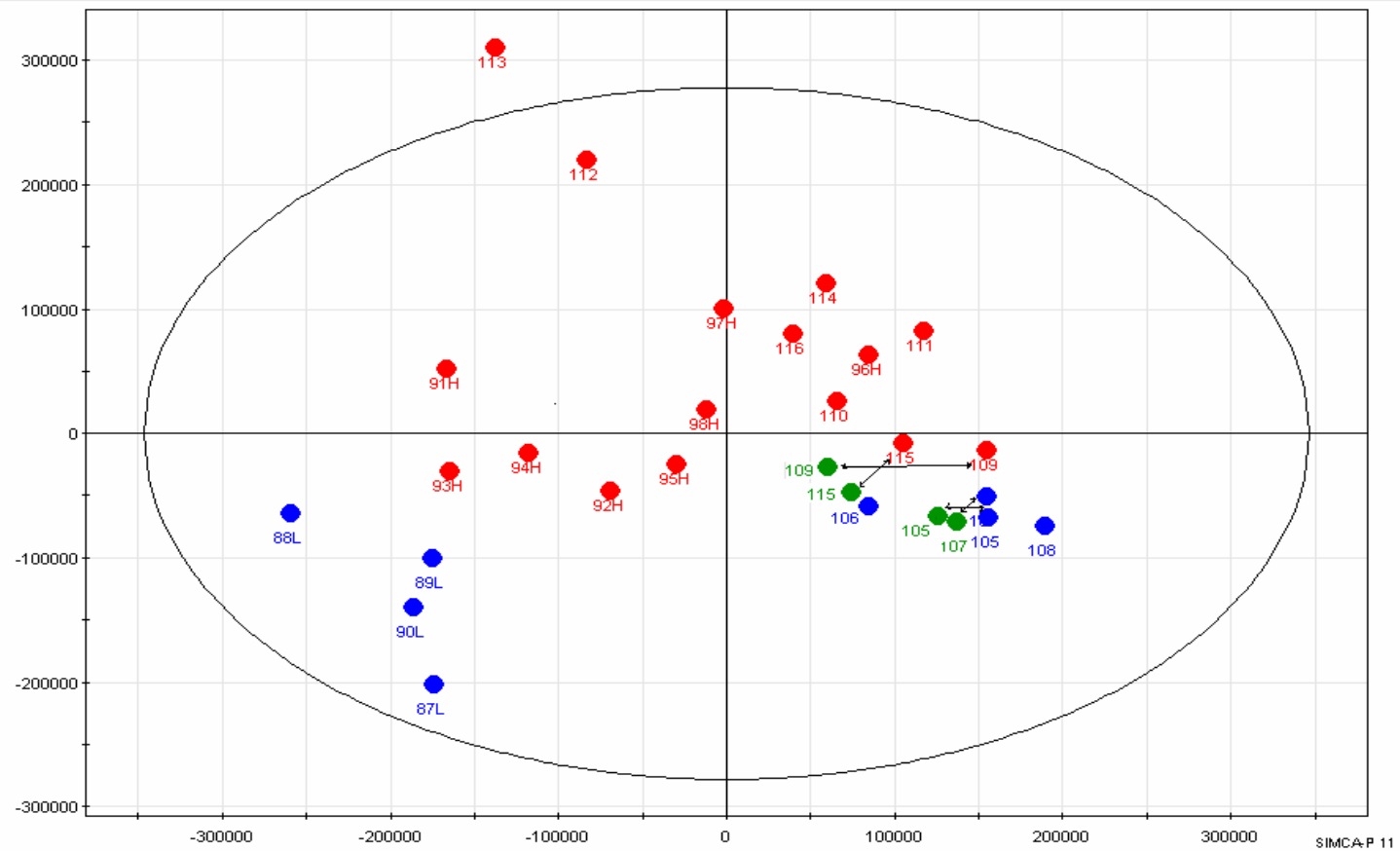
4 samples 2 LF, 2 HF treated with 1  $\mu$ L HCl 0.5 M for 30 min  
prior to protein precipitation (in order to "hydrolyse" the  
bonds to proteins and liberate potential biomarkers).

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# Blood plasma sample treatment



# Blood plasma sample treatment



## Remarks

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- ❑ Treatment with HCl prior to protein precipitation did not greatly alter the profile (especially for LF animals)  
>>> Treatment not adapted
  - ❑ LC-MS of Blood plasma samples requires thorough washing of the LC column (equilibration step) to eliminate carry over effects and maximise life time
  - ❑ Detrimental contamination with PEG evident in certain sample sets of human origin, dominating the MS spectrum >>> **sampling, vial selection critical**
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# Journal of Chromatography B special issue

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- ❑ Thematic “Hyphenated Mass Spectrometry technologies for global metabolite profiling”
  - ❑ Biological, plant, environmental perspective
  - ❑ Submission within January 2008
  - ❑ [www.ees.elsevier.com/chromb](http://www.ees.elsevier.com/chromb)
  - ❑ [gtheodor@chem.auth.gr](mailto:gtheodor@chem.auth.gr)
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# Acknowledgements

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