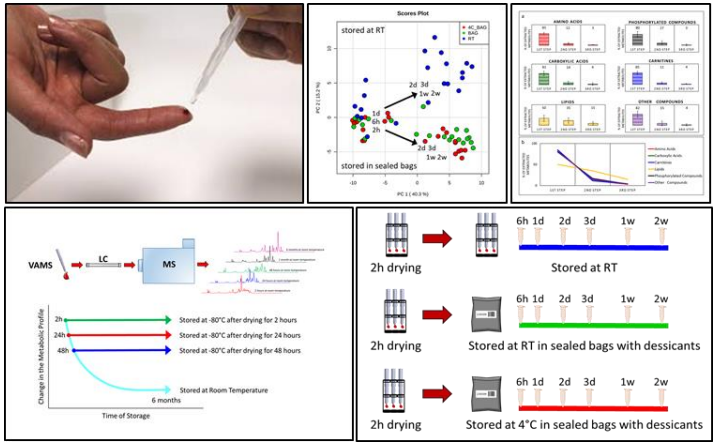
Pre-analytic evaluation of volumetric absorptive microsampling and integration in a mass spectrometry based metabolomics workflow



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Metabolomics

Metabolic Phenotype

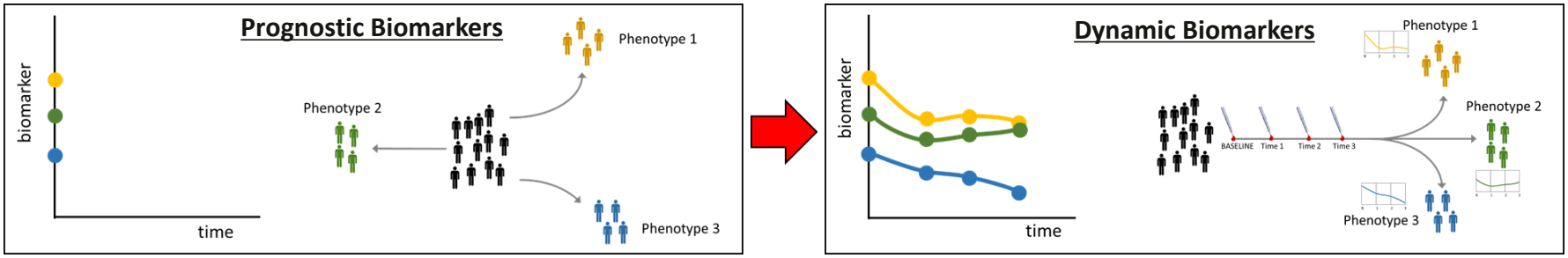
P = I + E + (E x I)

P = Metabolic Phenotype;

I = Intrinsic Factors; (Epi)genetic variation ;

E = Extrinsic Factors. Life Style, Diet, etc...

- **Prognostic biomarkers** predict a clinical response. Single metabolite profile predicts interindividual variation in clinical response
- **Dynamic biomarkers** describe the clinical response (disease progression, treatment response). Longitudinal metabolite profile describes dynamic and inter-individual variation in clinical response



Volumetric Absorptive Microsampling (VAMS)

Mitra, Neoteryx

Plastic pin

Absorbent polymeric tip

Reduced and defined blood volume (about 10 µL)

Homogeneous sample every time

No haematocrit problem

Integration into automated sample prep procedures

Metabolomics and VAMS

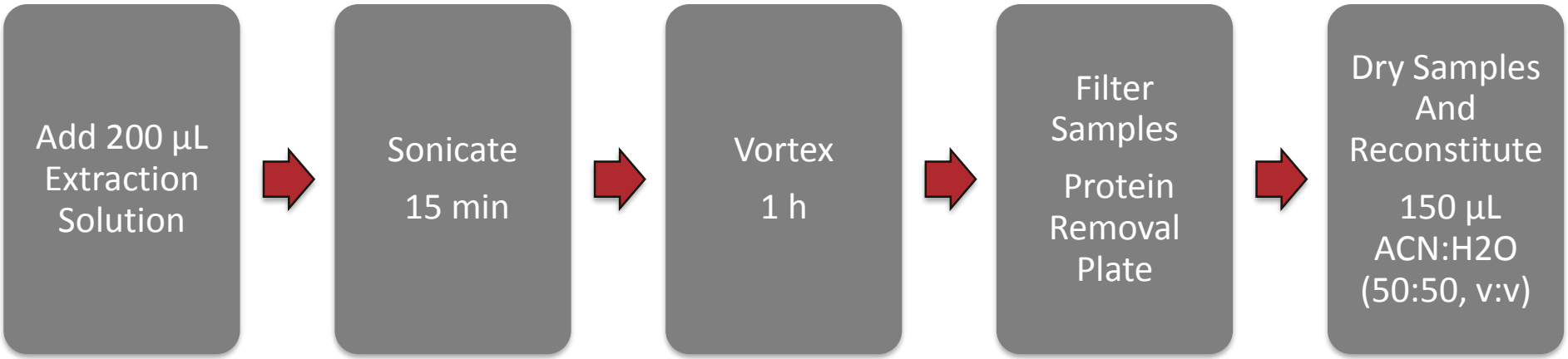
- Increase cohort size for biomarker discovery
- Collect repeated samples in challenge-response experimental designs or in longitudinal studies.
- Reduce the animal sample size and minimizing potential pain for the animal (3Rs principle).

Is VAMS suitable for untargeted metabolomics?

Evaluation of Extraction Procedures

VAMS tips were prepared by collecting blood from the same sample tube

1) **pH2:** methanol:water (7:3, v:v) mixture using water containing 1% of formic acid
2) **pH7:** methanol:water (7:3, v:v) mixture (un-buffered)
3) **pH9:** methanol:water (7:3, v.v) mixture using water containing 2% of sodium hydroxide.
4) **ACN:** 100% acetonitrile
5) **MeOH:** 100% methanol
6) **ACN-H2O:** acetonitrile:water (7:3) mixture (un-buffered).



Untargeted Metabolomics

MS-based untargeted metabolomics focused on polar metabolites.

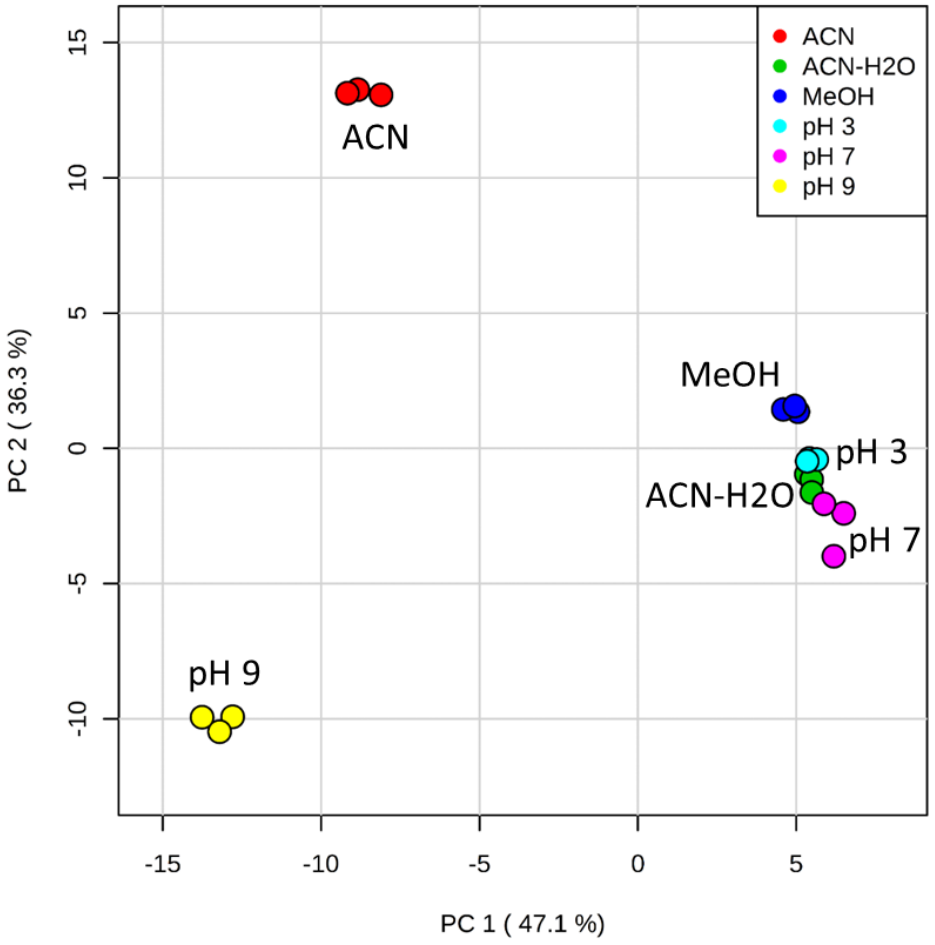
UHPLC-HRMS - Agilent 1290 (HILIC Amide column) + Triple TOF (AB Sciex)

- LC-MS maintenance at the beginning of each batch (source cleaning, calibration)
- System suitability mix (RT, m/z, signal intensity)
- 15 injections of pooled QC sample to equilibrate the LC-MS system before running
- Samples analyzed in randomized order
- Pooled QCs every 8 samples
- Data were processed with XCMS
- Total Ion Current (TIC) to correct intra-batch variability (robust linear regression)
- Features with RSD higher than 30% in pooled QCs were removed

Evaluation of Extraction Procedures

Principal component analysis (PCA)

- ACN and pH9 clustered separately
- All other extractions clustered very closely suggesting similar metabolome.



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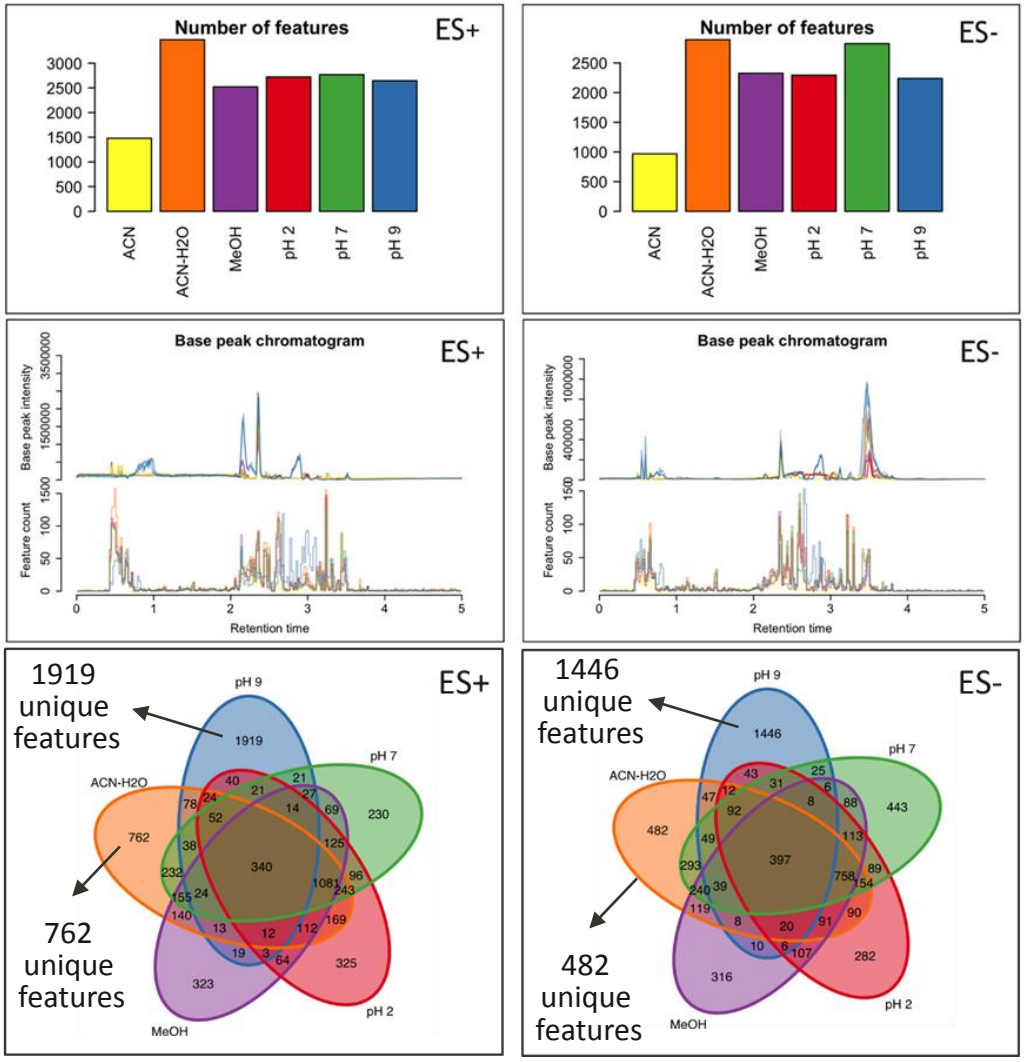
Evaluation of Extraction Procedures



- Highest number of feature in ACN-H2O
- Lowest number of features in ACN
- Different mass chromatograms at pH 9 (Blue)
- pH9: 1919 unique features in ES+
- pH9: 1446 unique features in ES-
- ACN-H2O: 762 unique features in ES+
- ACN-H2O: 482 unique features in ES-

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Evaluation of Extraction Procedures

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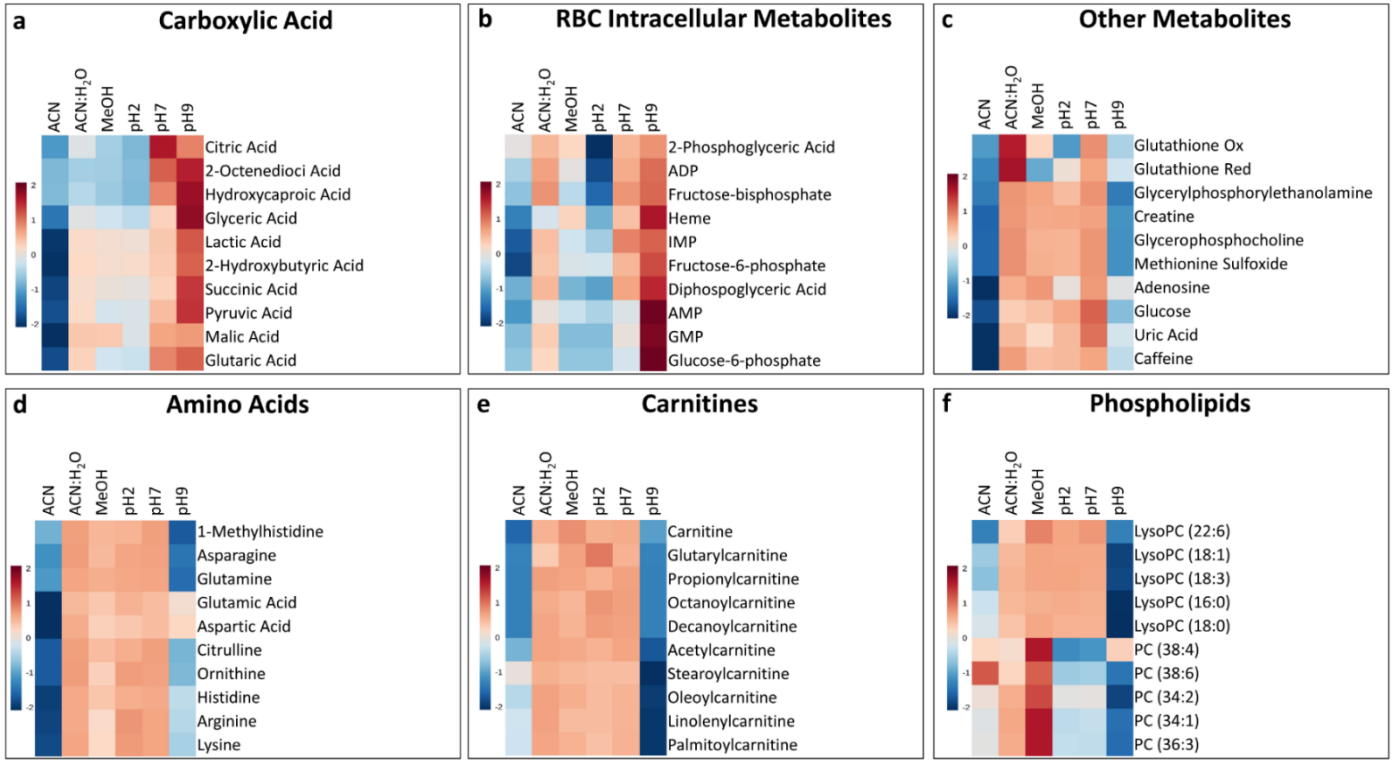

- 133 metabolites annotated
- ACN poor recovery
- MeOH better extracts lipids

- Carboxylic acids better extracted at pH 9
- Intracellular (RBC) compounds at pH9
- Only acidic amino acids recovered at pH9

ACN:H_2O was selected

- **Good coverage**
- **Good recovery**
- **HILIC-MS**

Evaluation of Extraction Procedures

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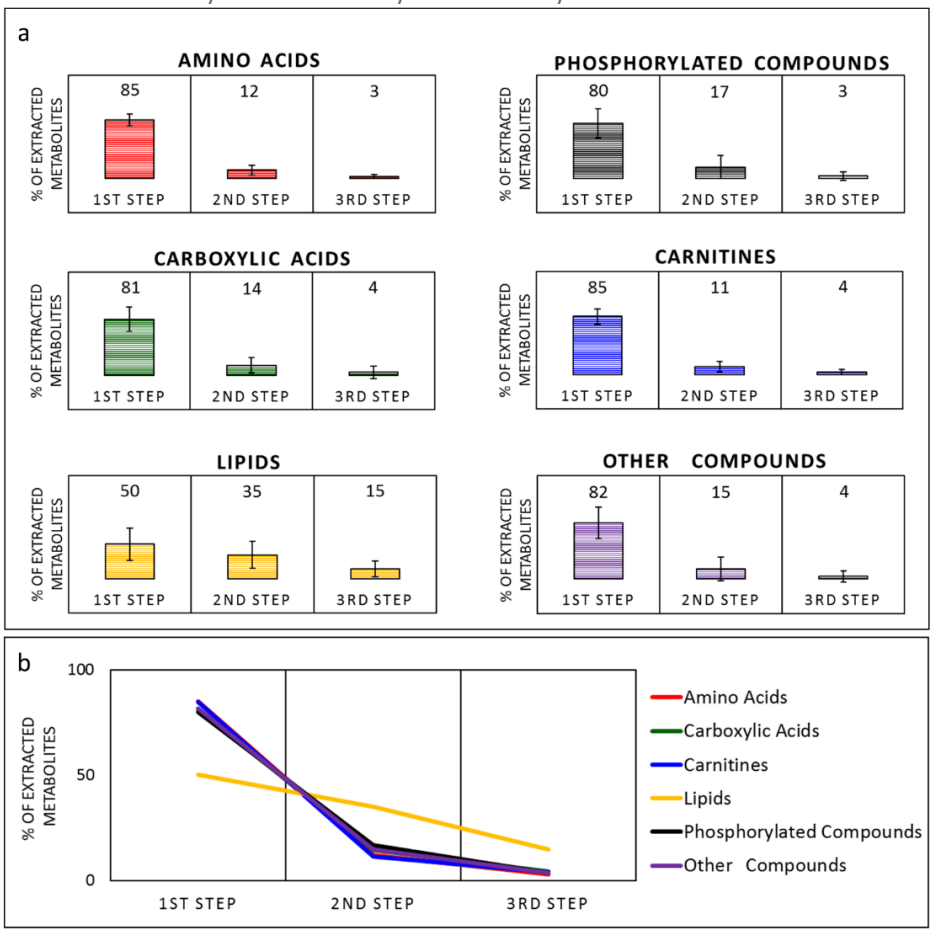


Three consecutive steps.
Extracts analyzed separately.

Amino Acids
1st STEP ≈85% total extractable content
2nd STEP ≈12% total extractable content
3rd STEP ≈ 3% total extractable content

Phosphorylated compounds
1st STEP ≈80% total extractable content
2nd STEP ≈17% total extractable content
3rd STEP ≈ 3% total extractable content

Carboxylic Acids
1st STEP ≈81% total extractable content
2nd STEP ≈14% total extractable content
3rd STEP ≈ 4% total extractable content

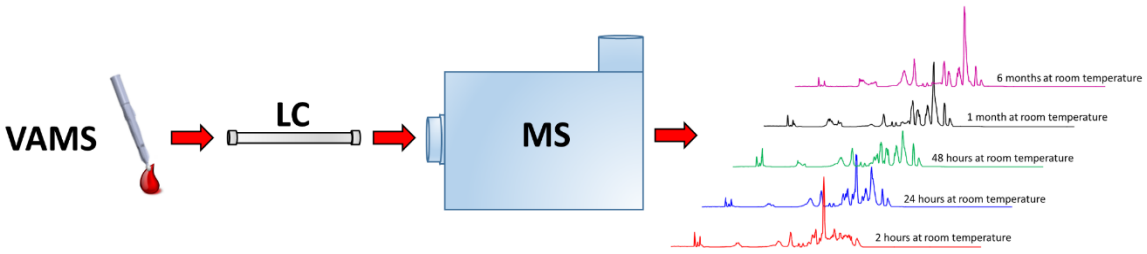
Acylcarnitines
1st STEP ≈85% total extractable content
2nd STEP ≈11% total extractable content
3rd STEP ≈ 4% total extractable content

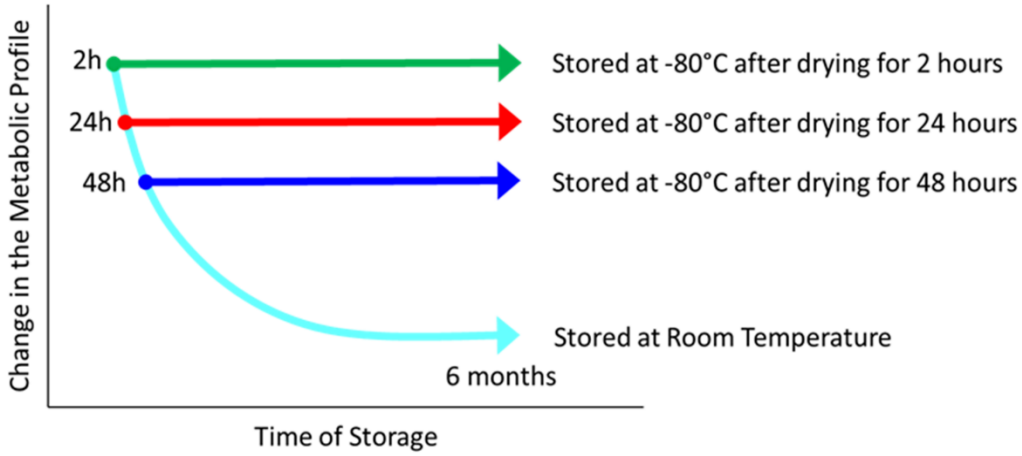
Lipids
1st STEP ≈50% total extractable content
2nd STEP ≈35% total extractable content
3rd STEP ≈15% total extractable content

1st step extracts ≈80% total blood polar metabolome

Evaluation of Sample Stability

A variable time frame might pass between the collection and the extraction of the metabolome





Long Term Stability

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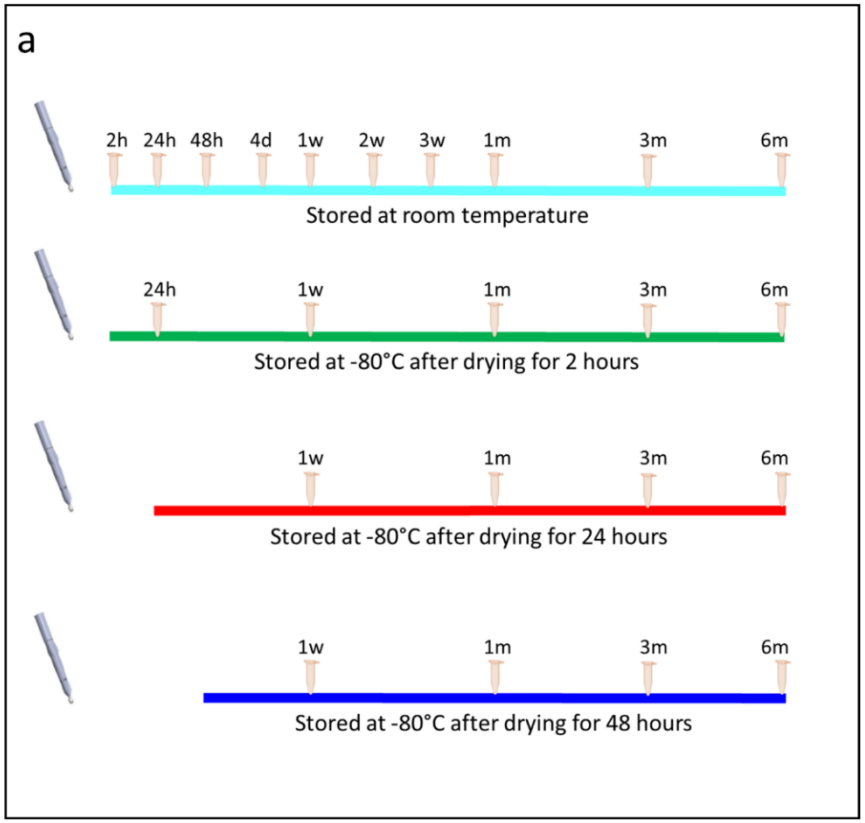
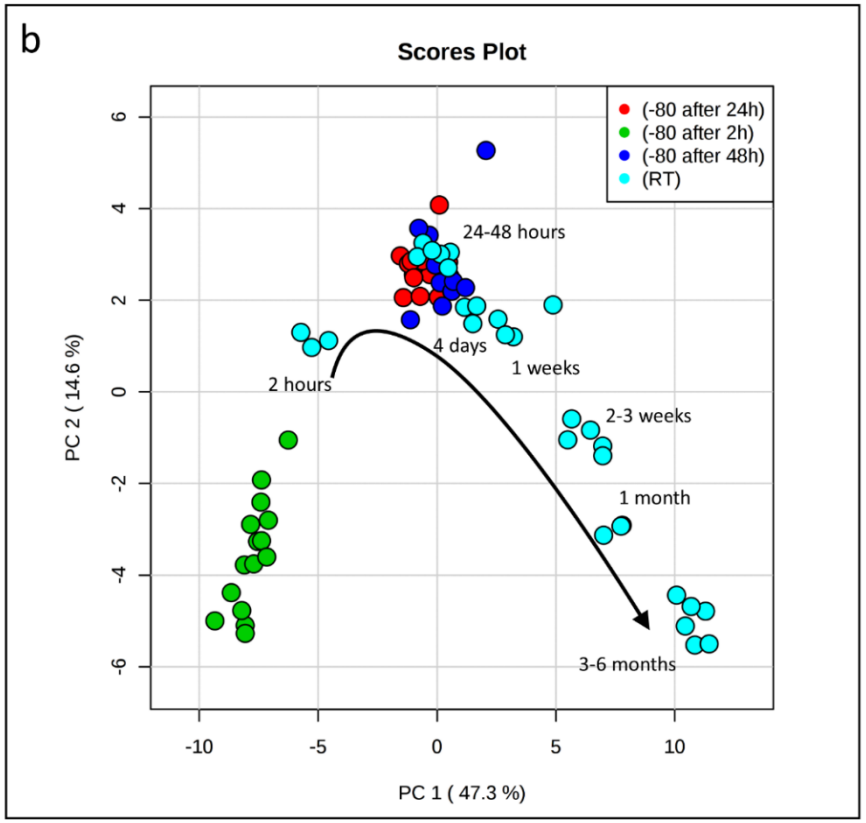
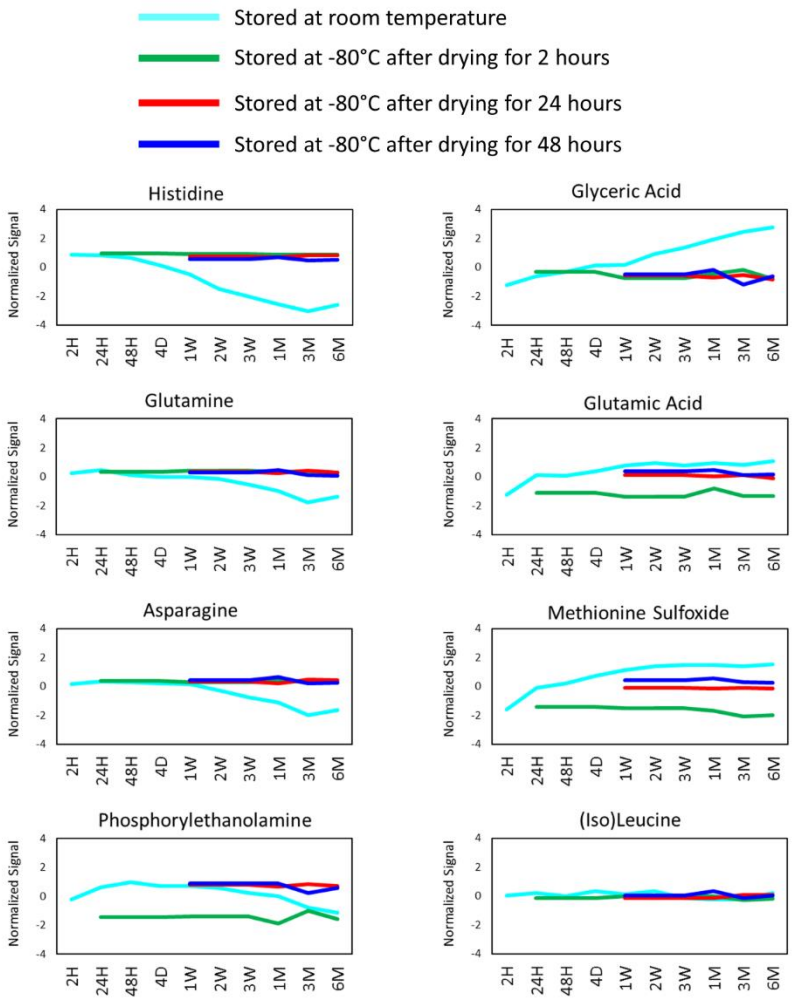




- Clear trend for the samples stored at room temperature that are clustering based on the time of storage
- Samples stored at -80°C did not show any trend related with the time of storage
- Samples stored at -80°C after drying for 24 or 48 hours clustered together
- Samples stored at -80°C after drying for 2 hours clustered separately

Long Term Stability



- 75% of the metabolites changed over time (Anova, $p<0.05$)
- 35% decreased in concentration over time of storage
- 31% increased in concentration over time of storage
- 9% increased (first 48h). Gradual degradation overtime

- Degradation of histidine started after 24 hours of storage
- Degradation of asparagine after 1 week of storage

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Conclusion

- In this study, we developed for the first time a workflow integrating VAMS with MS-based metabolomics
- Extraction with acetonitrile:water (70:30) yielded the best results for our scope
- Other solvents and extraction steps might be applied based on the scope and the experimental design
- For untargeted Metabolomics:
  - Sample stored at -80 Celisius degree are stable up to 6 months
  - Sample can be stored for 1 day in sealed bags with dessicants
- For targeted metabolomics we suggest to carefully evaluate the stability of the selected metabolites
- Further validation of VAMS for metabolomics in humans.

Thank you

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