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Multi-platform Metabolomic Analyses of Rat Urine Following Exposure to Perfluorinated Chemicals (PFCs)

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This work was reviewed by EPA and approved for presentation but does not necessarily reflect official Agency policy.

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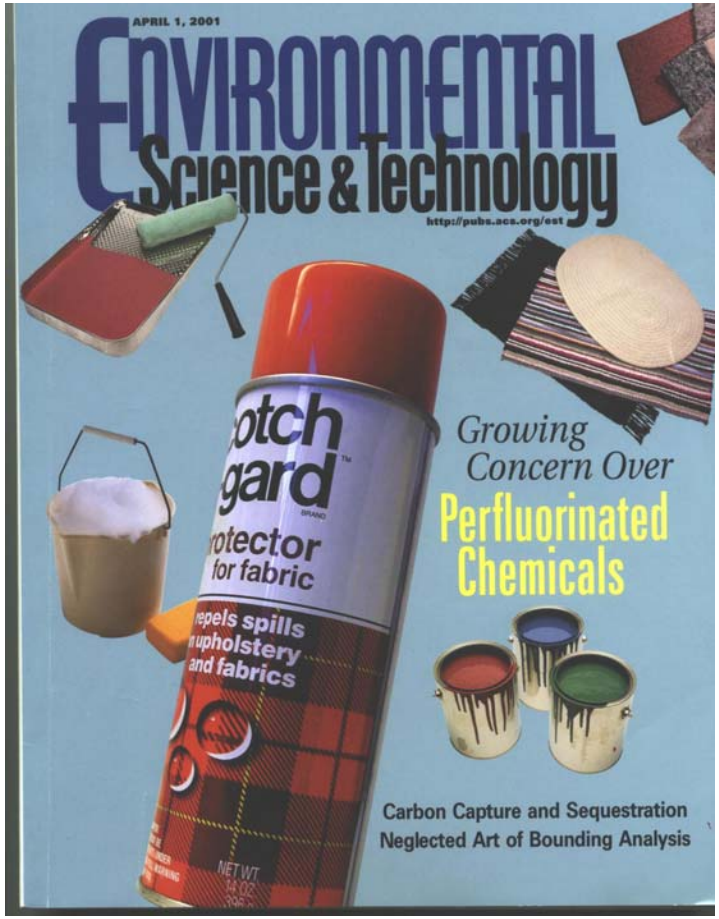
Perfluorinated Chemicals (PFCs)

- PFCs consist of a carbon backbone and a charged functional moiety
  - PFOA – carboxylate;
  - PFOS – sulfonate
- Anthropogenic, globally disseminated , non-biodegradable and persistent
- Uses and occurrences
  - Found in over 200 industrial and consumer formulations
  - PFOA is now the most common PFC in production
- Probable human exposure



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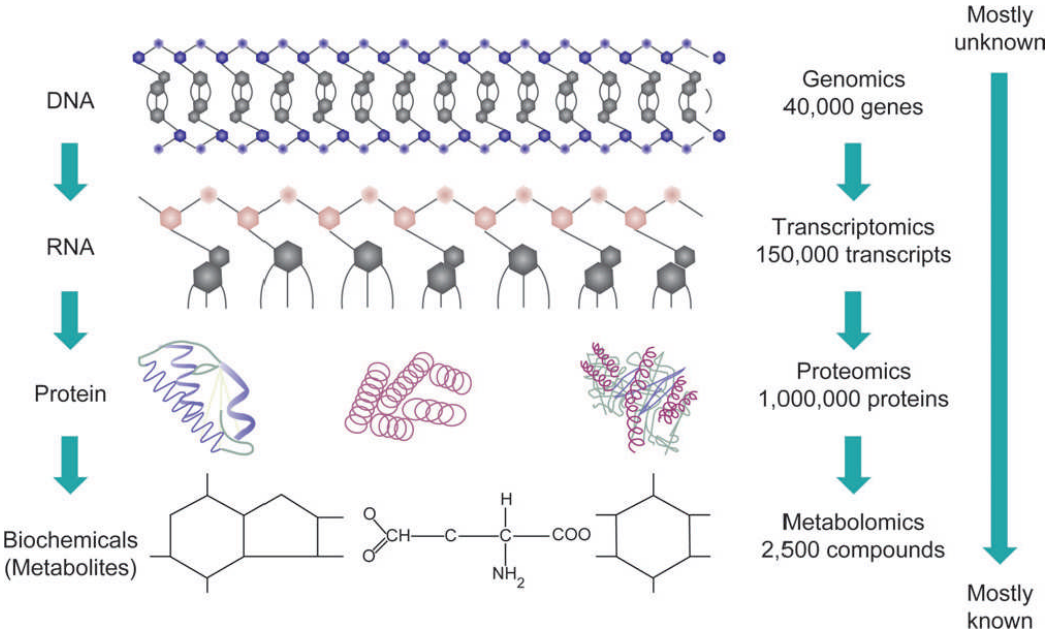
Toxicology of PFCs

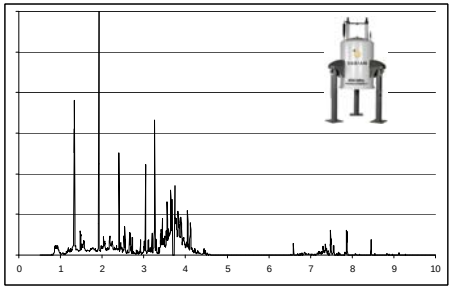
- General toxicology reviewed by Kennedy et al., 2004 and US EPA, 2005; developmental toxicology reviewed by Lau et al., 2004
- PFOA:
  - General hepatoxicity (increases liver weight and marker enzymes) and hypertrophy
  - Disruptions in fatty acid oxidation (mitochondrial and peroxisomal)
  - Reproductive and developmental toxicity
- PFOS:
  - Perturbations in cholesterol and lipid metabolism/transport
  - Hepatotoxicity and peroxisome proliferation
  - Interferences in cellular gap-communication
  - Developmental toxicity (delayed maturation, decreased PN survival)
- Gender and species-differences in toxicokinetics/pharmacokinetics

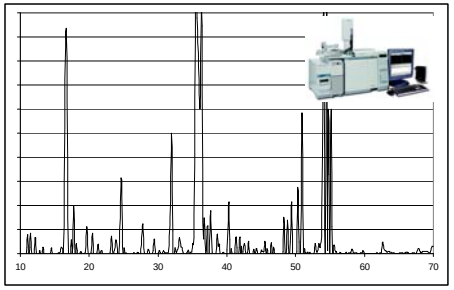
Introduction to Metabolomics

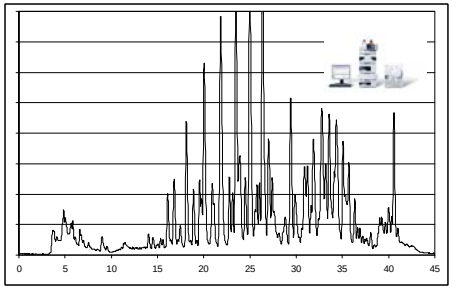


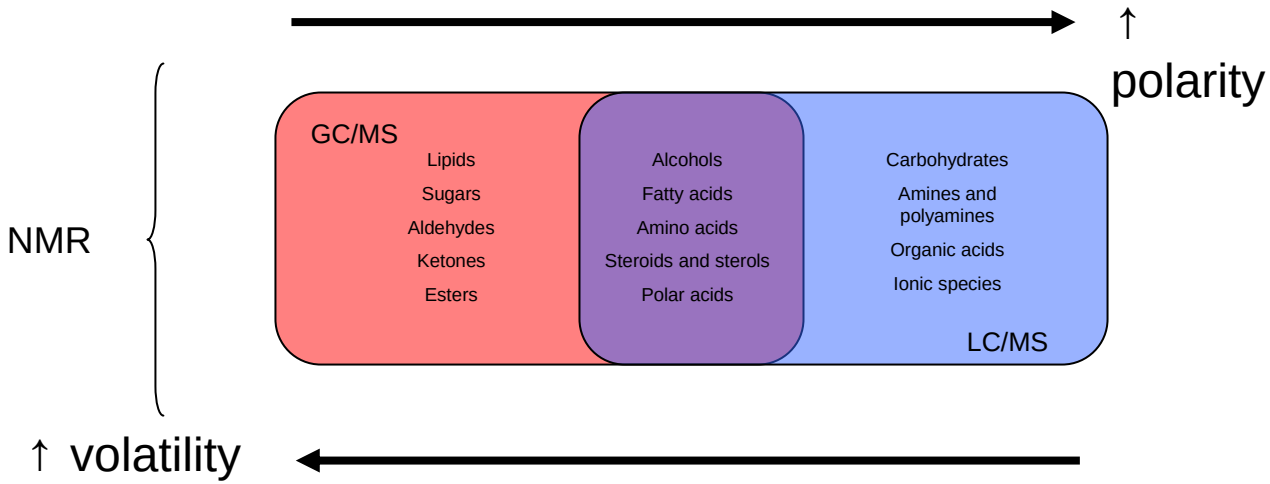
- Metabolomics investigates the metabolic status of the whole organism (metabolome).
- Provides a linkage between disruptions in genomics, transcriptomics, and proteomics to whole animal outcomes.

ERD's Metabolomic Platforms





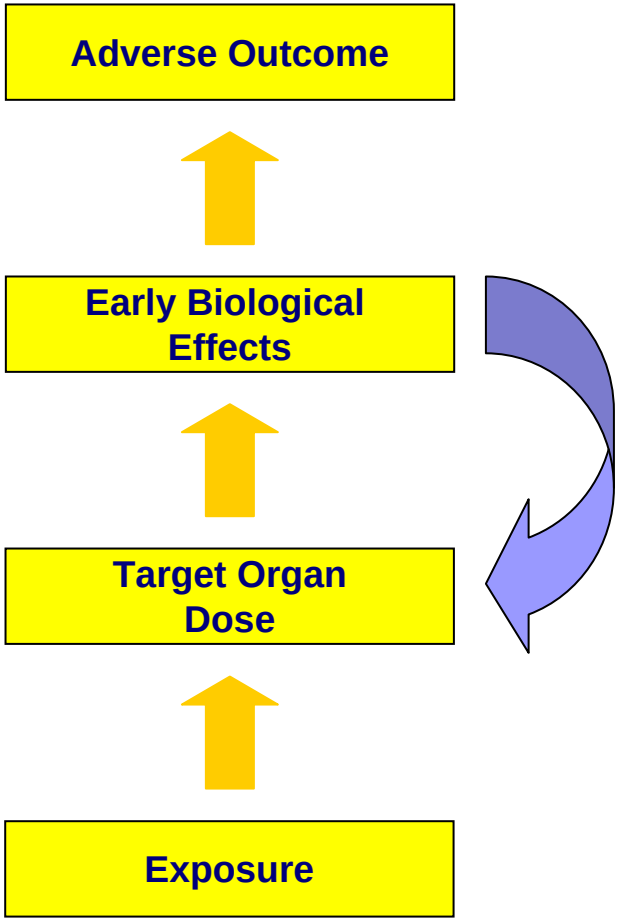




- Metabolites are chemically diverse (varying polarities, chemistries and sizes).
- Metabolites are present at a range of concentrations within a particular sample.

Purpose of Study

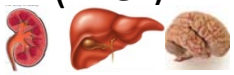
- To investigate the temporal, acute effects of PFC toxicity in the rat
- To identify potential 'markers' of PFC exposure/toxicity in the urine
- To compare urinary markers to organ-specific toxicity pathways
  - i.e. PFC induced hepatotoxicity
- To couple metabolomics and genomics data to better interpret the 'global' effects of PFC exposure in rodents



Methodology and Analysis

Biofluids (urine)

Tissues (liver)



Lyophilized → Reconstituted → NMR

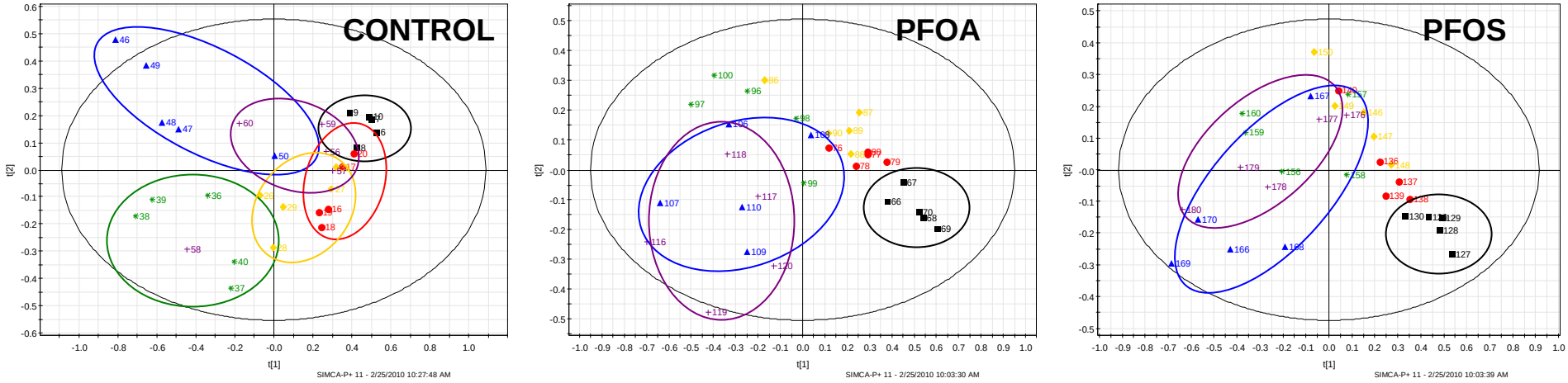
MeOH extracted → Derivatized → GC/MS

Diluted → LC/MS^n

$MeOH/CHCl_3$ extracted → Phase separated → Reconstituted / Derivatized

1D-^{1}H NMR Metabolomics (Urine)

Partial Least Squares Discriminant Analysis (PLS-DA)



Day -1 **Day 1** **Day 2** **Day 3** **Day 4** **Day 5**

- Vehicle effect discernable until day 3 when treatment groups begin to re-cluster with initial and pre-treatment groups
- PFC treatment related effects observed following 3-4 days of exposure when comparing imbedded controls (pre-dose collection)
- Understanding early effects of vehicle is critical for investigating acute PFC-related effects

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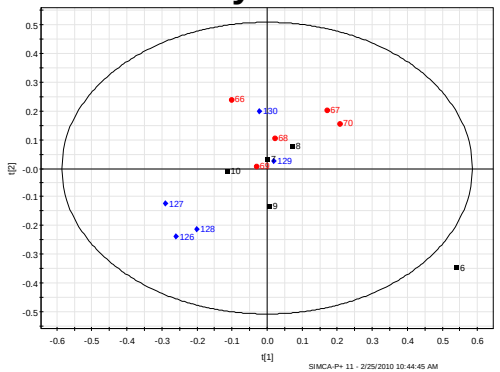
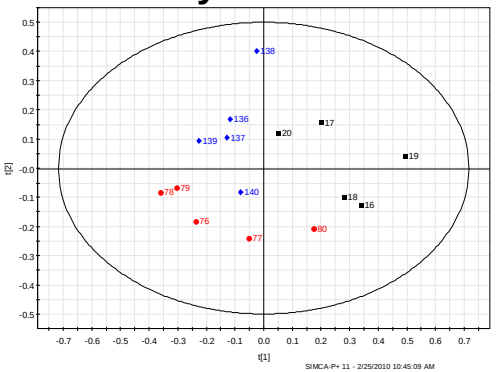
1D-^{1}H NMR Metabolomics (Urine)

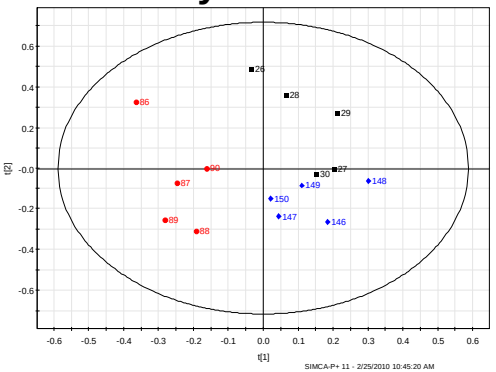
Day -1 to 1



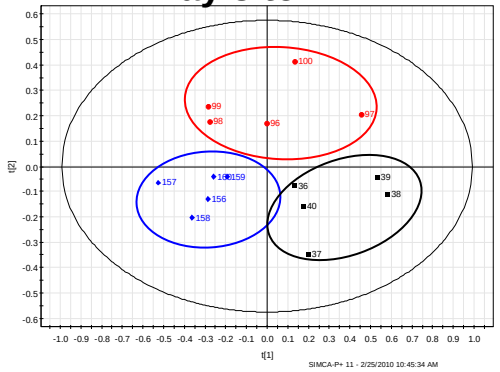
Day 1 to 2



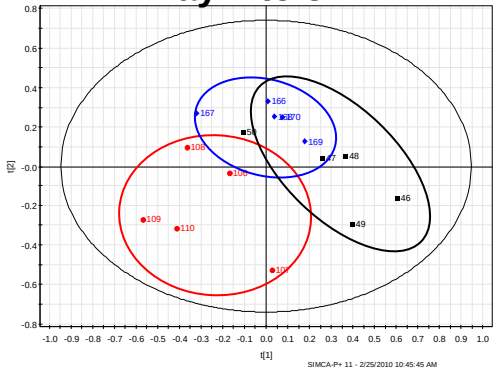
Day 2 to 3



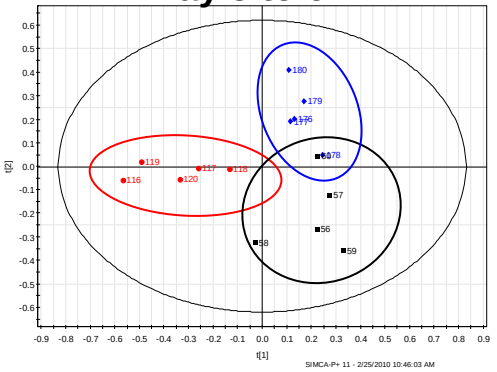
Day 3 to 4



Day 4 to 5

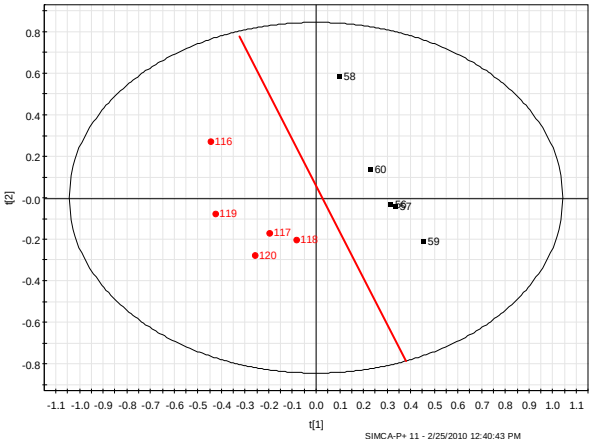


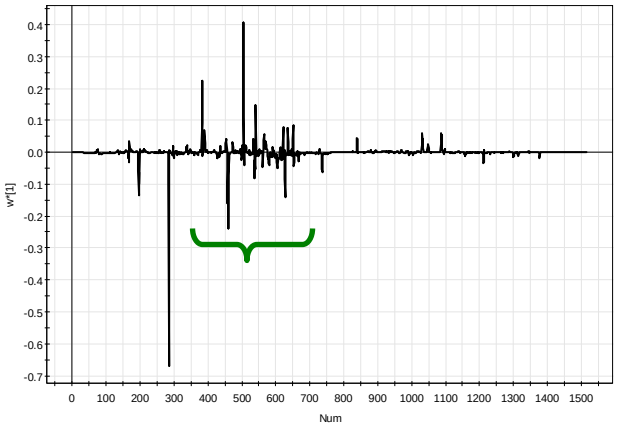
Day 5 to 6

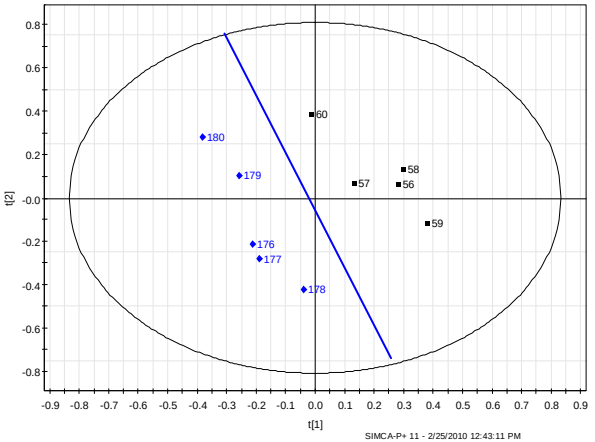


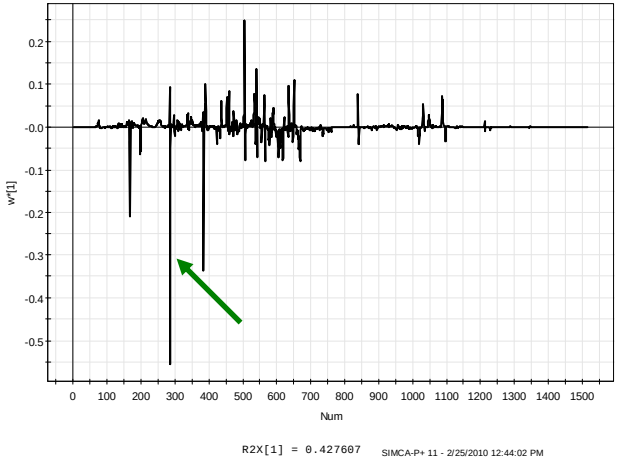
- PLS-DA models are valid 3 days post-exposure
- Class separation (Control, PFOA and PFOS) is evident following 24hr exposure; prominent after 3 days of PFC treatment

1D-^{1}H NMR Metabolomics (Urine)





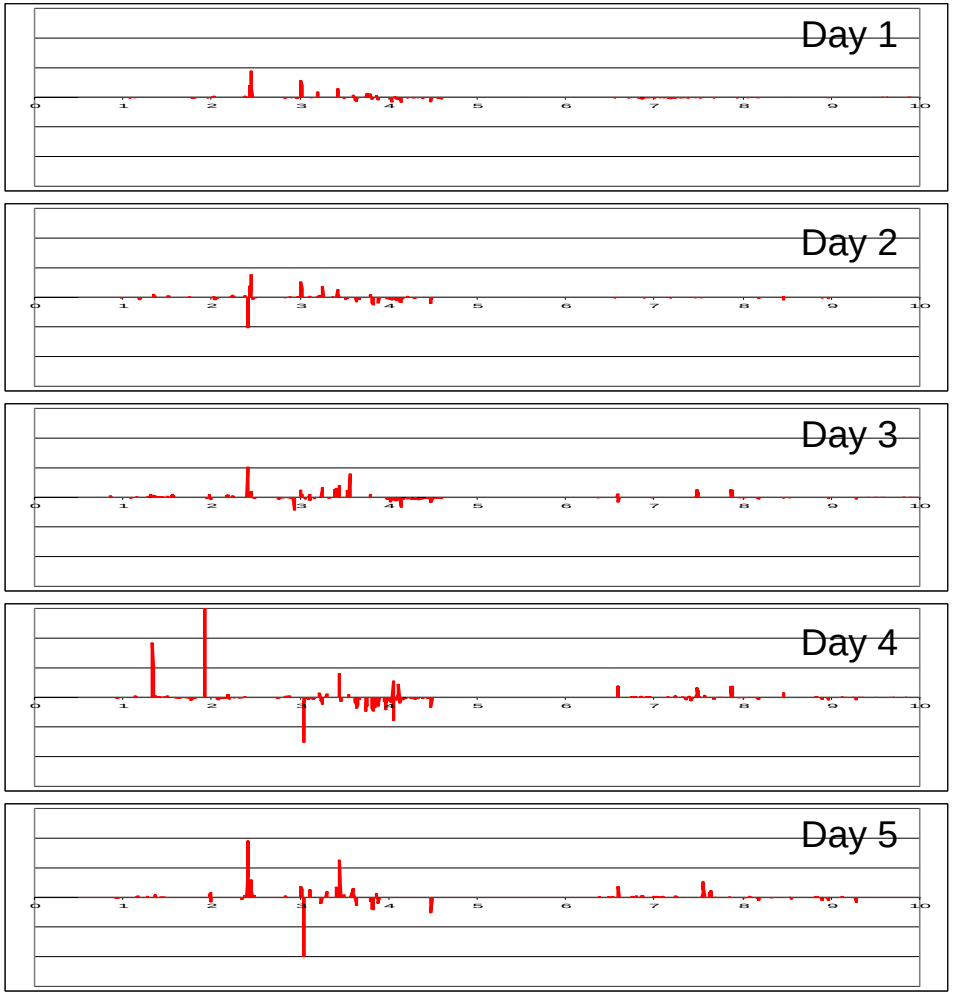




- PLS-DA loadings plot facilitates examining what metabolites are important in class separation.

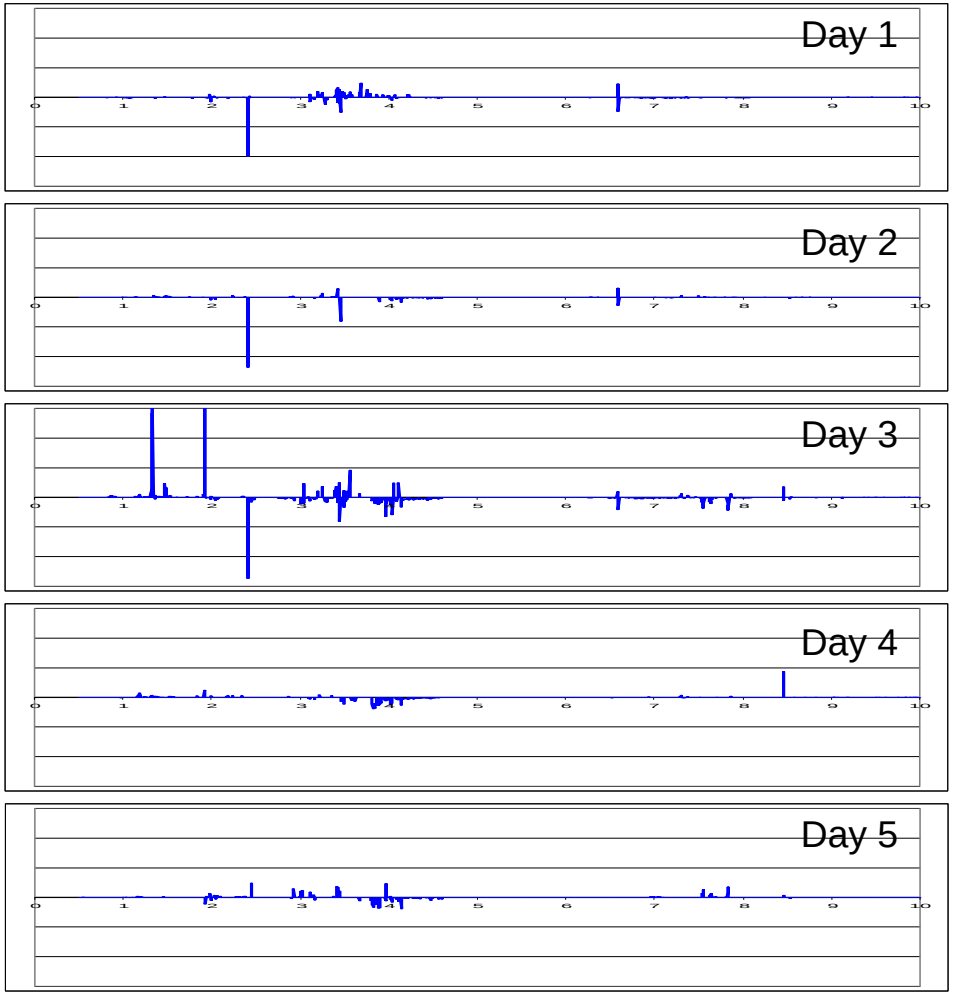
- Comparing both PFOA and PFOS to time-matched controls
- Structural similarities in up- and down-regulated metabolites

1D-^{1}H NMR Metabolomics (Urine)



- t-test filtered difference spectra ($p \leq 0.01$)
- PFOA
  - ↑ (up regulated)
    - N-methylnicotinamide
    - glutamate
  - ↓ (down regulated)
    - hippurate
    - α-ketoglutarate

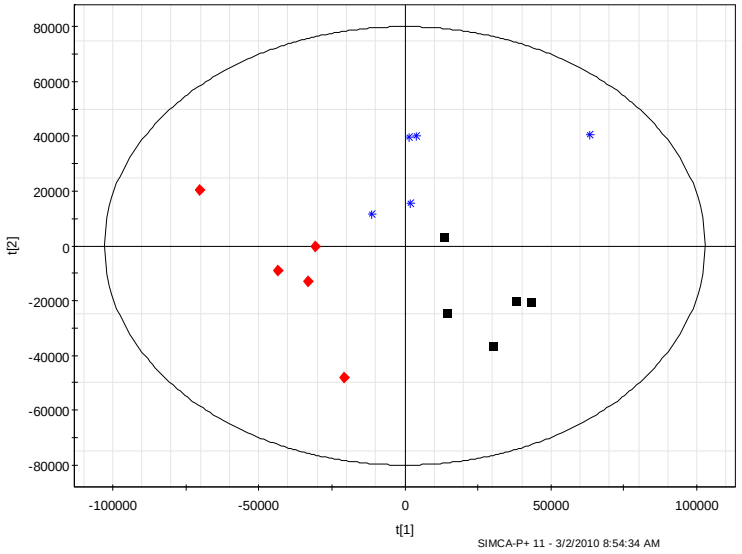
1D-^{1}H NMR Metabolomics (Urine)



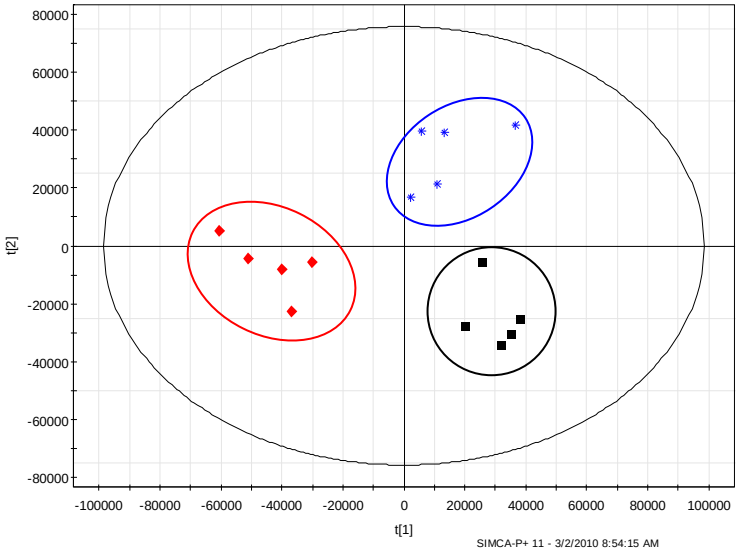
- t-test filtered difference spectra ($p \leq 0.01$)
- PFOS
  - ↑ (up regulated)
    - fructose
  - ↓ (down regulated)
    - taurine
    - α-ketoglutarate

GC/MS Metabolomics (Urine)

PCA Model



PLS-DA Model



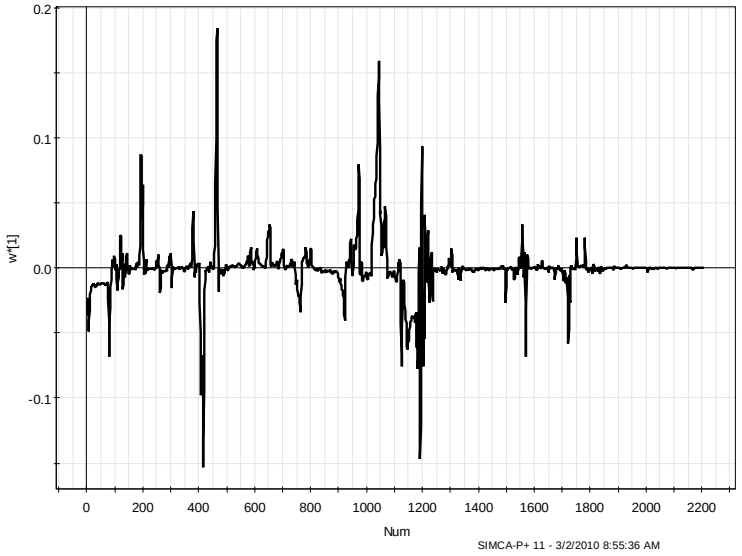
CONTROL **PFOA** **PFOS**

- Post-urease digestion and MeOX*/BSTFA derivatization
- Treatment groups cluster following 5 days of PFC exposure

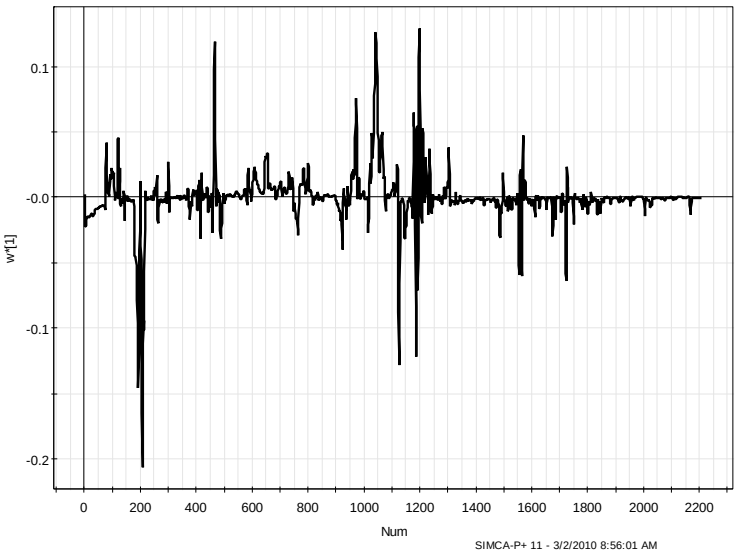
*methoxyamine hydrochloride

Metabolites of Interest – GC/MS

PFOA



PFOS



- PFOA and PFOS (common metabolites of interest)
  - ↑Control – acetic acid, butanedioic acid, fumaric acid, short chain fatty acids, glucopyranose, other complex sugars;
  - ↑Treated – succinic acid, galactose, glucose, fructose, and others.

- General disruption in short chain biological acids (metabolism of carbohydrates and lipids), citric acid cycle intermediates, glycolipid/protein synthesis precursors, and markers of metabolic syndrome as well as liver toxicity

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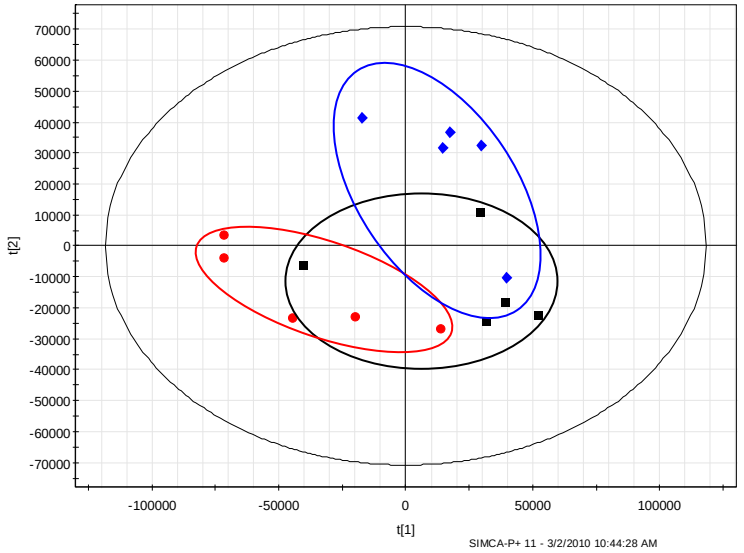
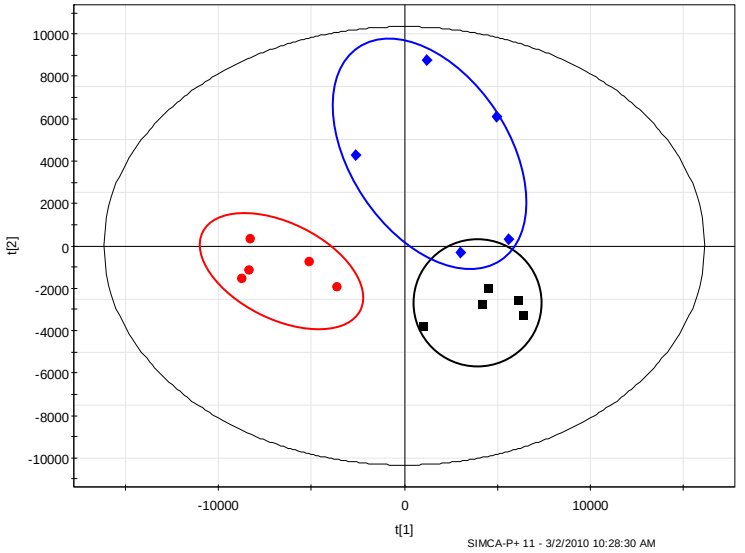
LC/MS-based Metabolomics (Urine)

+ESI

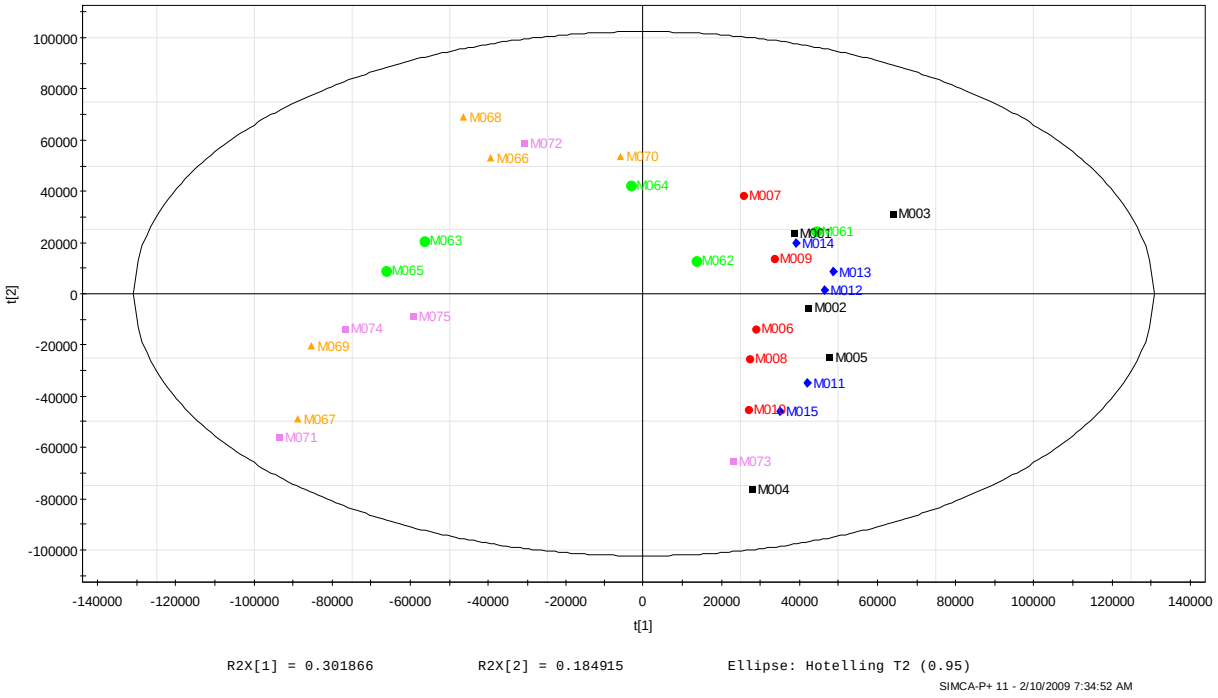


-ESI



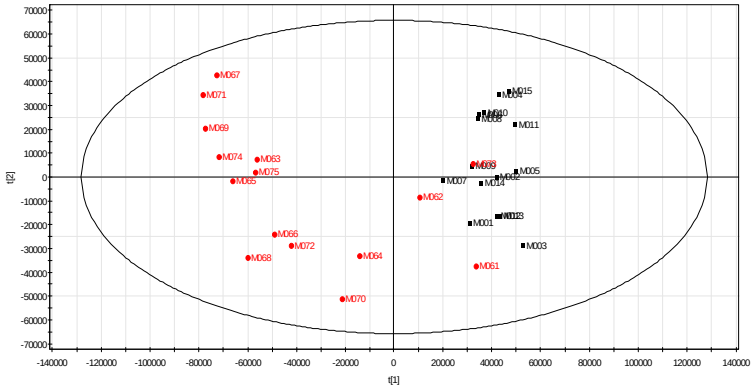
- Urine samples diluted with running buffer prior to LC/MS analysis
- LC/MS data collected in both ESI positive and negative modes
- Positive mode allows identification of M+H (metabolite id in progress)

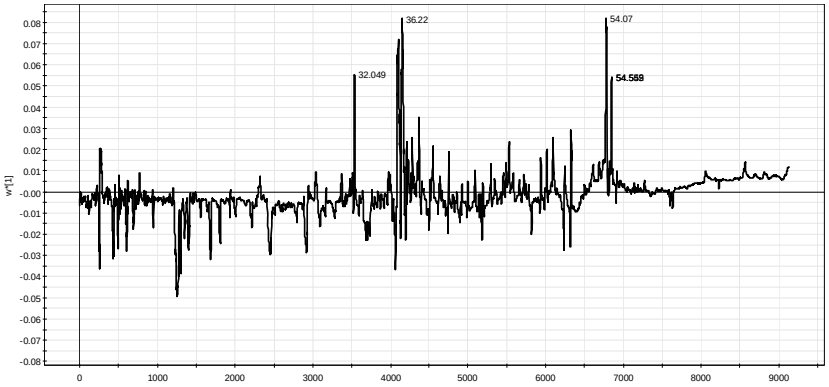
PFOA Liver Metabolomics – GC/MS



- Control (Red, Blue, Black) and 24 hr, 4 day and 6 day groups
- Differences in polar liver extracts are present at earliest time point (24 hrs PT)

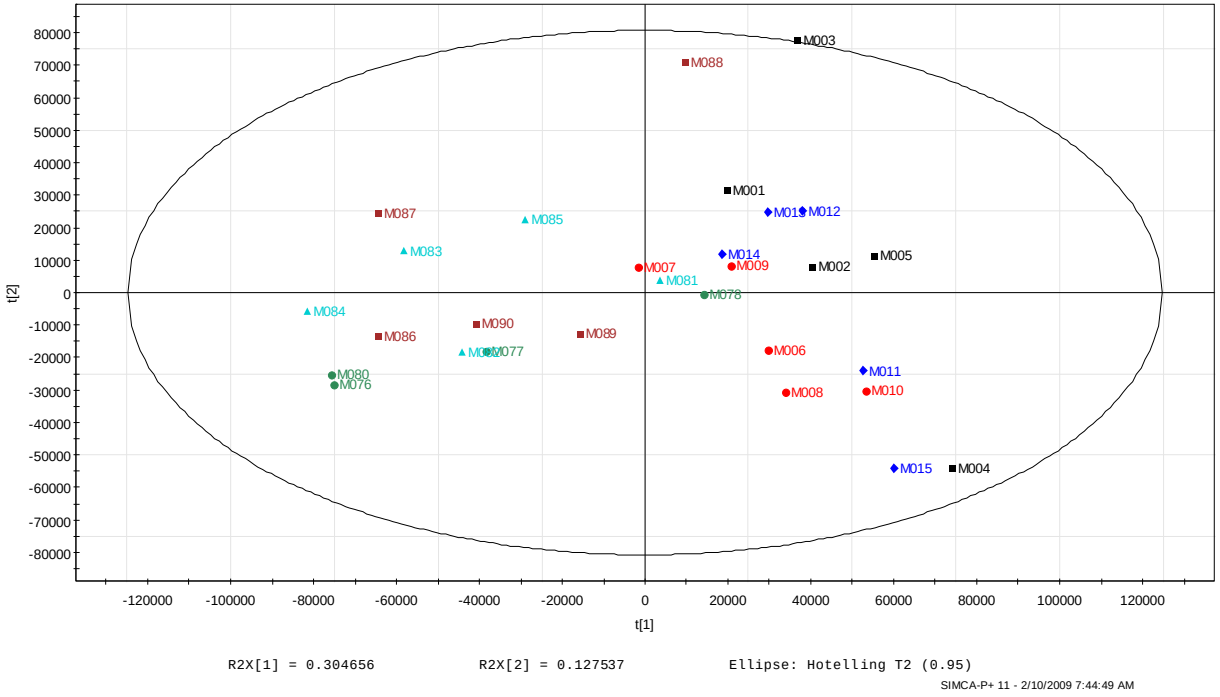
PFOA Liver Metabolomics – GC/MS





- PLS-DA model built with 2 classes (Control vs. Treated)
- Similar models built across dose day (data not shown)

PFOS Liver Metabolomics – GC/MS



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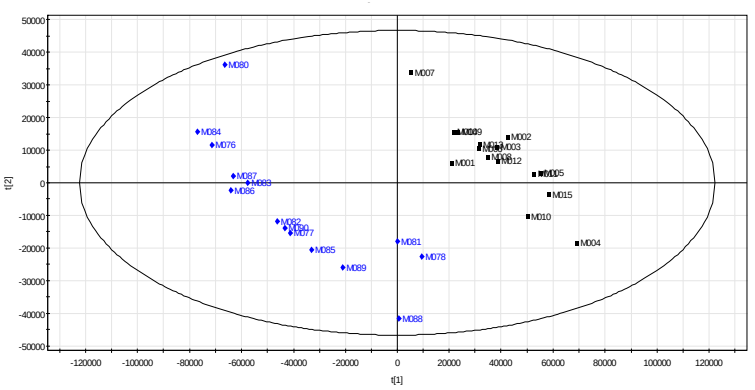
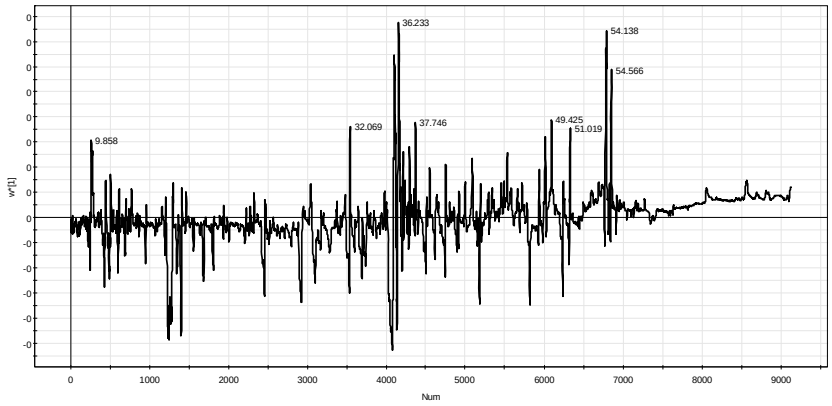
PFOS Liver Metabolomics – GC/MS





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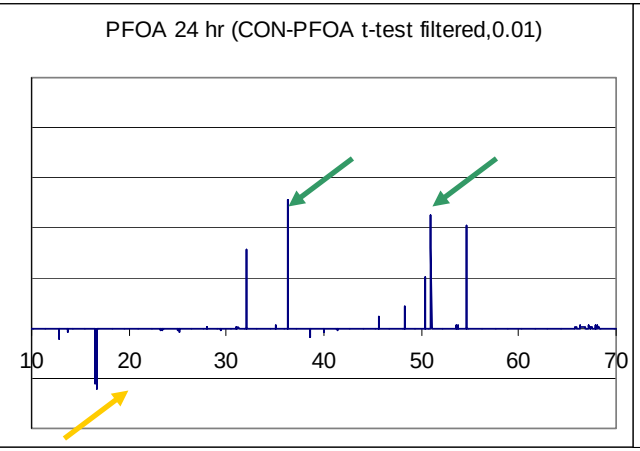
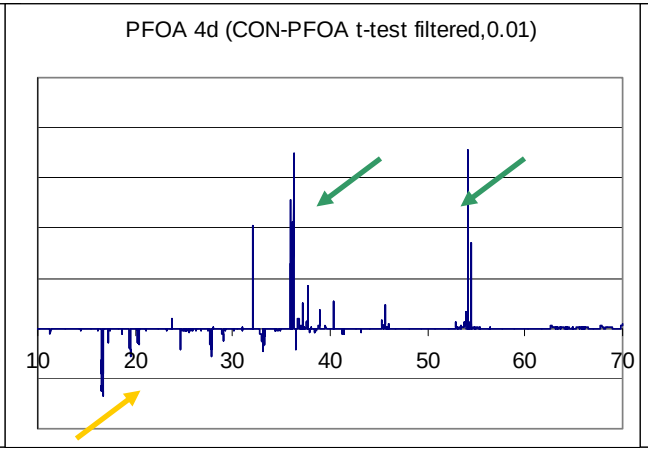
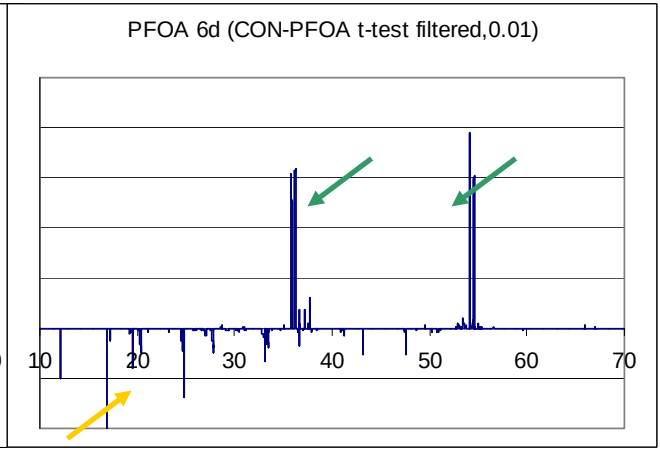
PFOA/PFOS Liver Metabolomics



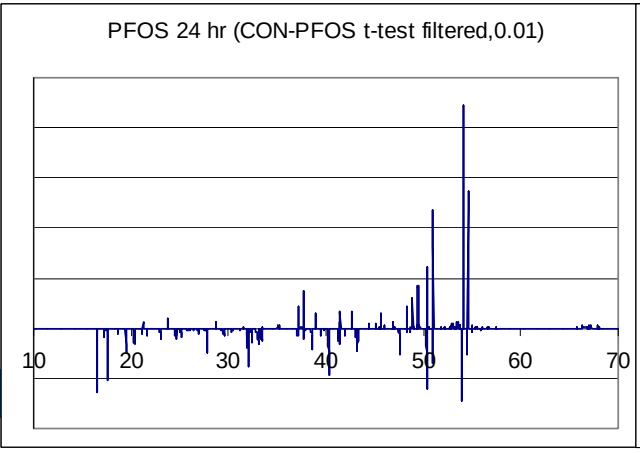


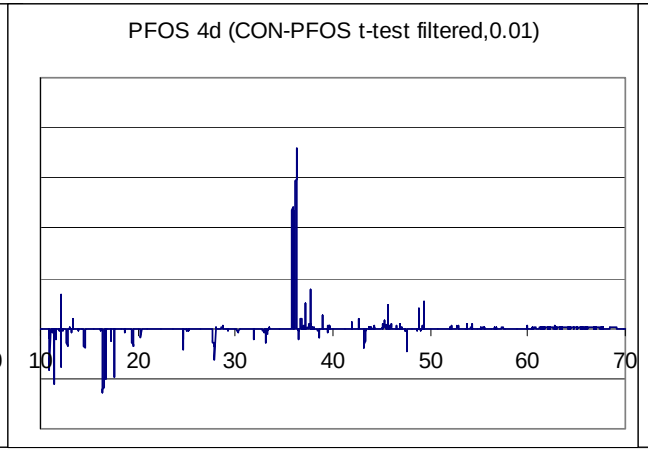


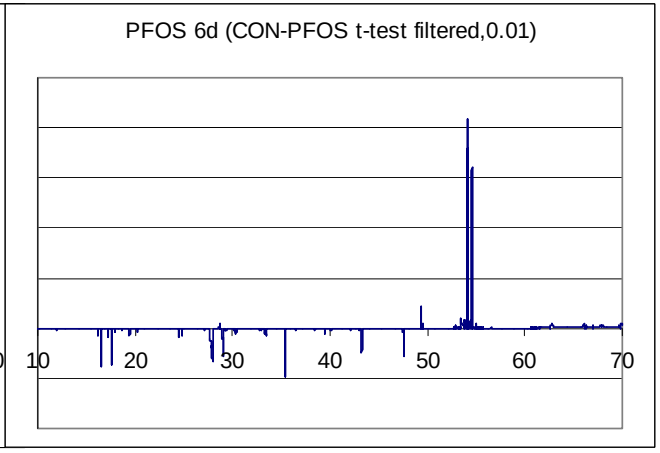
24hr PT: Initial disruption of sugar and carbohydrate synthesis

4d PT: Continued carbohydrate disruption, and increases in amino acids

6d PT: Sustained effects on both biological pathways.







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Global Metabolites of Interest

- Based on metabolomic analysis of urine and liver, affected biological pathways include:
  - Cellular metabolism, signaling and energetics (metabolic syndrome)
    - Glycolysis, electron-transport chain, and citric acid cycle intermediates
  - Lipid retention (cellular) and metabolism (steatosis)
  - Protein metabolism and amino acid synthesis
  - Complex carbohydrate and fatty acid metabolism

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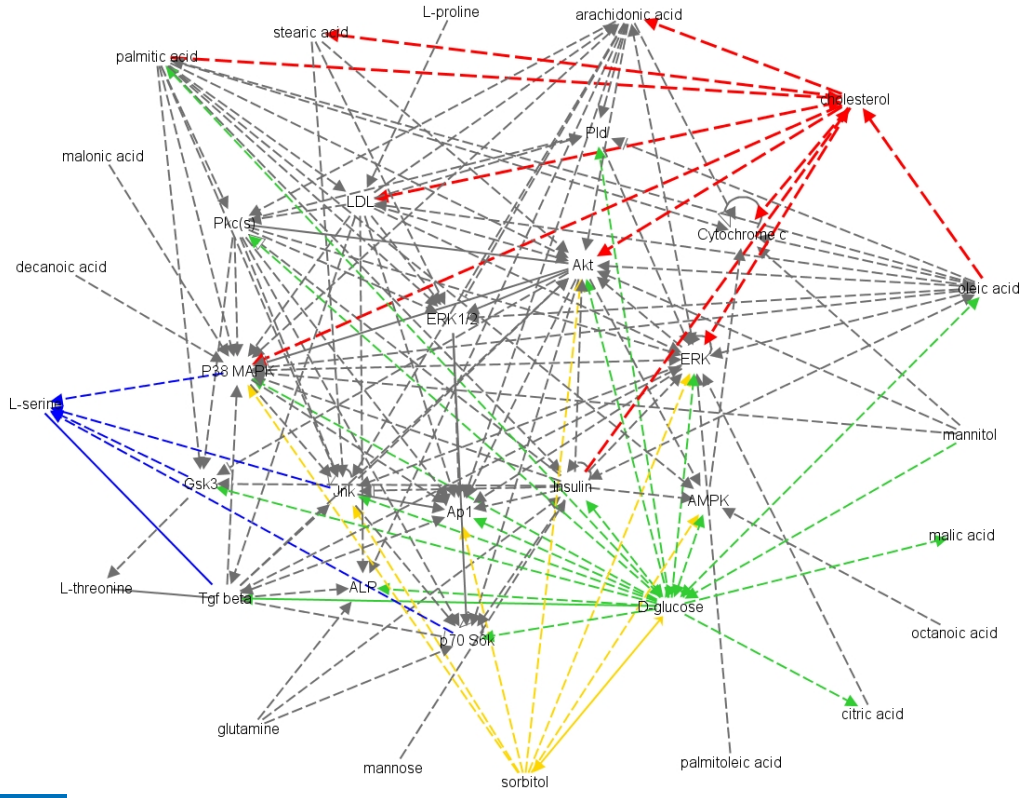
Interconnections through Pathway Analysis

Investigating the connectivity of both polar and lipophilic metabolites through genomic pathways

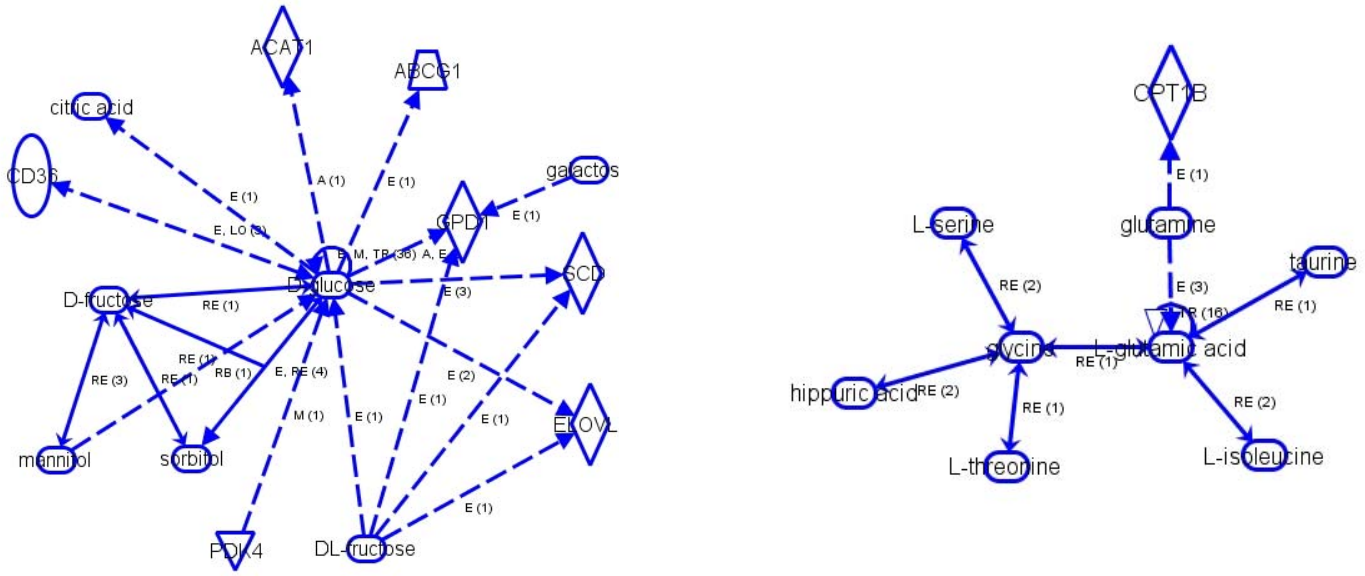


- Metabolites of importance:
  - glucose
  - serine
  - sorbitol
  - glutamine
  - cholesterol and fatty acids (not discussed in this presentation)
- Pathways of interest:
  - Insulin receptor signaling
  - Glucocorticoid receptor activation
  - PXR/RXR activation
  - IL signaling
  - AMPK signaling
  - Galactose metabolism
  - Cell cycle and cell death responses
  - … and others …

Using metabolomics to identify possible genomic targets

Interactions with Genes Involved in Toxicity

Up-regulated Genes Involved in Metabolism of Lipids, Fatty acids, and Carbohydrates

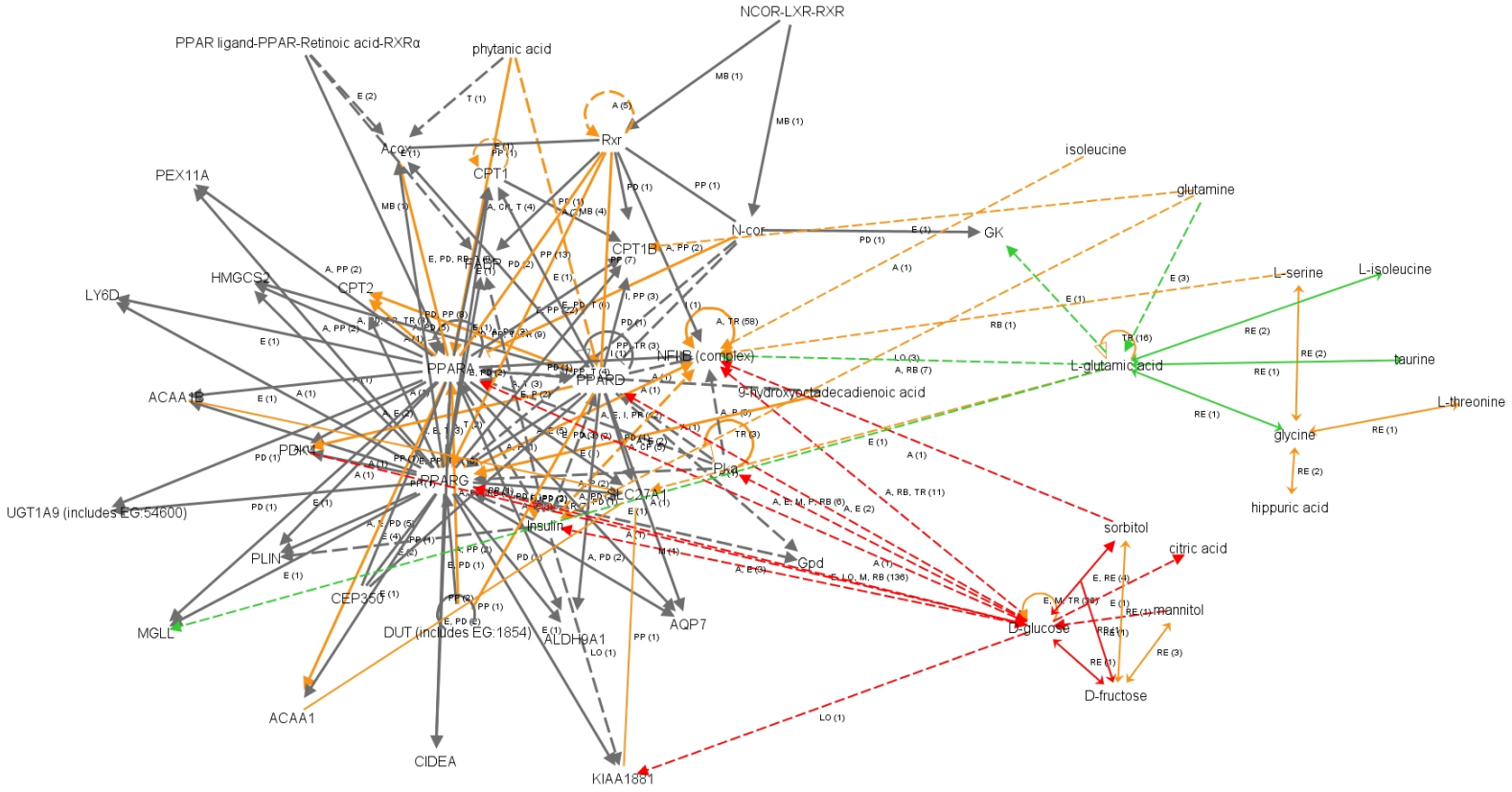


Using metabolomics to inform known genomic targets

- Identified metabolites play key roles in:
  - LXR/RXR activation; PPARα/RXRα activation
  - Fatty acid metabolism
  - Mitochondrial β-oxidation
  - Glycerophospholipid metabolism
  - Amino acid synthesis

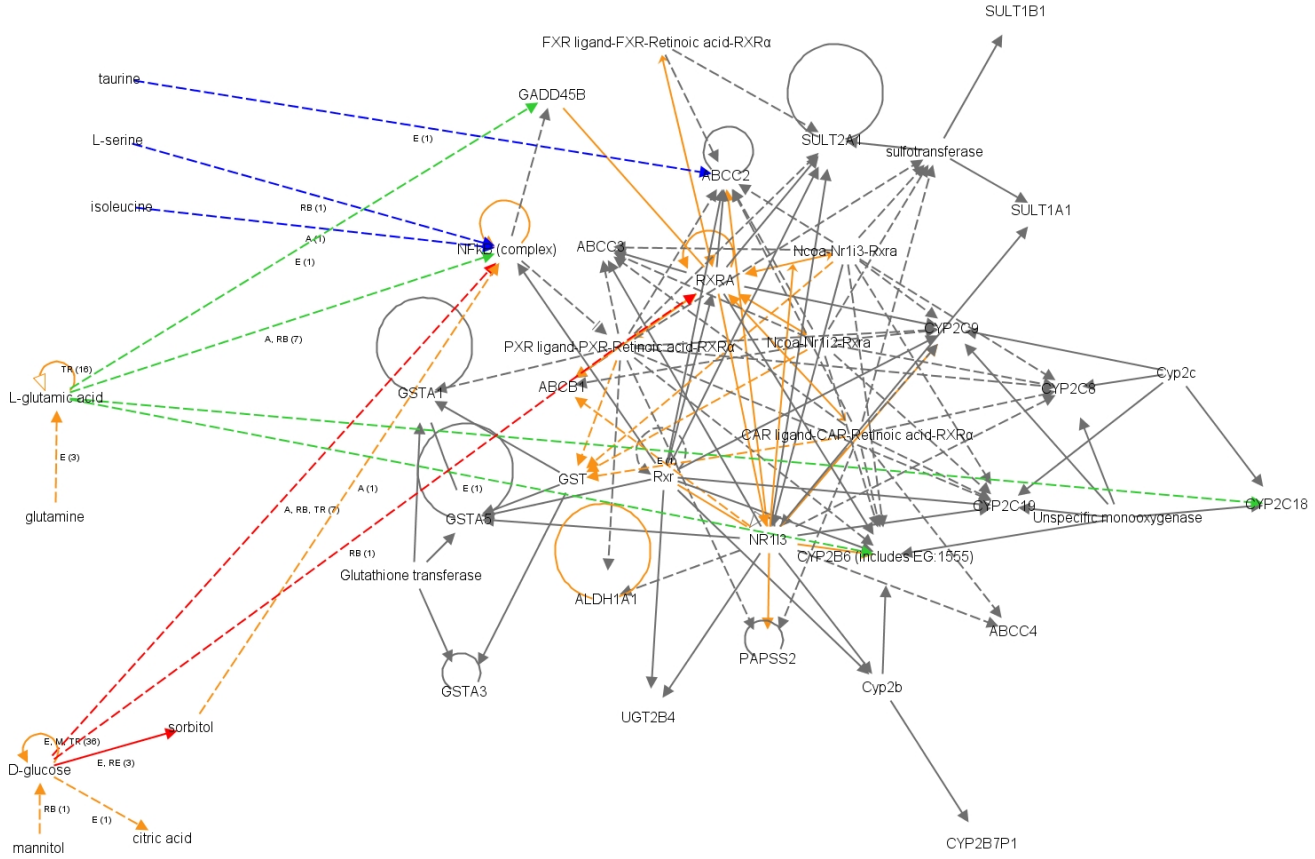
Gene data taken from Guruge et al., 2006

Metabolites and PPAR Gene Regulation



- Glutamate and glucose associate (directly and indirectly) with both receptors
- Understanding regulatory control will help inform mechanisms of toxicity

Metabolites and CAR/RXR Gene Activation



- Again, glutamate and glucose associate with both receptors
- Other metabolites of interest link with pro-apoptotic receptors as well as organic anion transporters and protein binding transporters

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Conclusions and Future Directions

1) Metabolomics is able to differentiate PFC-induced changes in the urinary metabolome as well as in target-organs
   a) PFCs disrupt pathways involved in small molecule metabolism and cellular integrity
2) Using multiple analytical platforms affords the ability to more accurately assess fluxes in the metabolome
3) Metabolomics can inform genomics as well as offer a direct complement to understanding changes in the genome

4) To use metabolomics data, coupled with other ‘omics data, to inform exposure and exposure pathways to these and other PFCs:

Acknowledgements

- NERL/ERD
  - Tim Collette
  - Drew Ekman
  - Quincy Teng
  - Kimberly Ralston-Hooper
- NCCT
  - David Dix
  - … and others

Questions?