Sweating the small stuff---the influence of metabolite extraction and separation on metabolomic studies

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Excellent References

- Mass spectrometry-based metabolomics
- Xenobiotic metabolomics: major impact on the metabolome
- The intestinal metabolome: an intersection between microbiota and host

Metabolomics

- Metabolomics is the systematic analysis of the unique chemical fingerprints left behind by specific cellular processes
  - These small molecule metabolite profiles provide insight into cellular status.
- All "-omics" based scientific disciplines aim at the collective characterization and measurement of their particular constituent molecules
  - A comprehensive approach to study complete pools of biological molecules
  - Defines the structure, function and dynamics of an organism.
- Vast chemical diversity among small molecule metabolites has made extended coverage of the metabolome challenging
  - Size (50 – 1500 Da)
  - Concentration (pM – mM)
  - Physicochemical properties (diverse log P values)
  - Stereochemistry (distinct biological activity)

Metabolite Extraction

- Currently no analytical technique exists that is capable of *in-situ* measurement of all classes of cellular metabolites
- Metabolite extraction therefore becomes a crucial step in any type of metabolomics study
  - Critical to both targeted and global based profiling strategies.
- Optimized extraction methodology should fulfill several criteria:
  - Extract the largest number of metabolites
  - Unbiased and non-selective - physical or chemical properties of a molecule
  - Non-destructive - no modification of metabolites

Separation of Metabolites

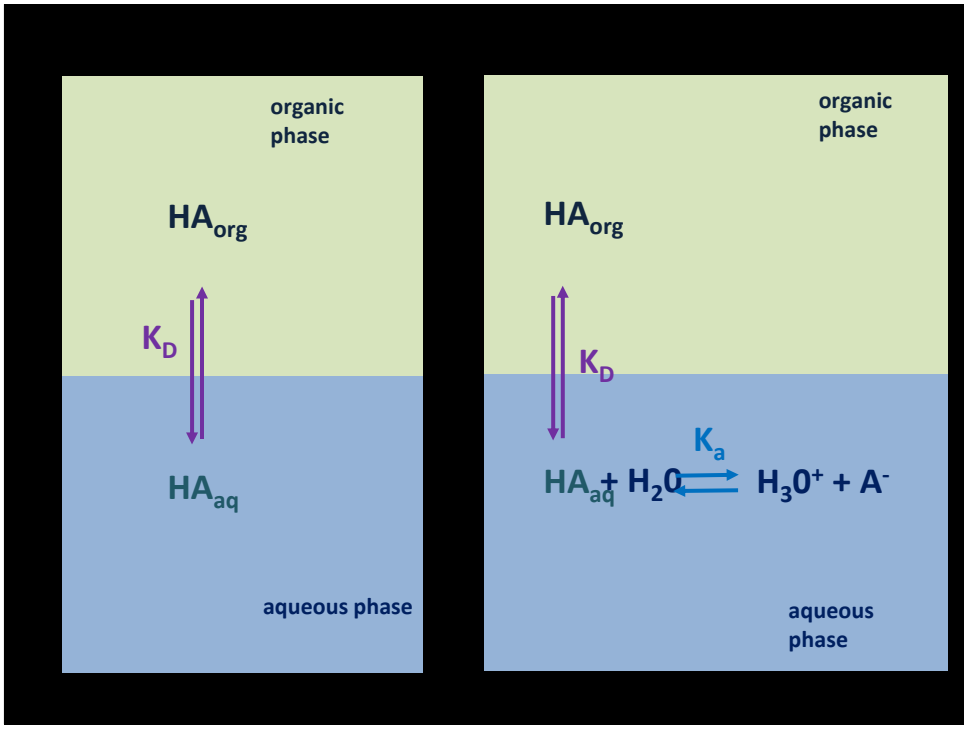
- Mass spectrometry usually requires some form of chromatographic separation
  - Most systems use either liquid or gas chromatography
  - CE-MS gaining popularity
- Fractionation of sample components simplifies the resulting mass spectra while ensuring more accurate compound identification
  - Capacity factor (k) is critical to optimizing resolution
  - Increased resolution allows longer MS dwell times resulting in better signal/noise ratios
- Inadequate chromatographic separation of metabolites results in:
  - signal suppression – ion suppression
  - compromised metabolite quantification
  - reduced metabolite coverage

Hypothesis

Extraction and separation of metabolites may influence metabolomic studies as much as the disease process being investigated

Rationale

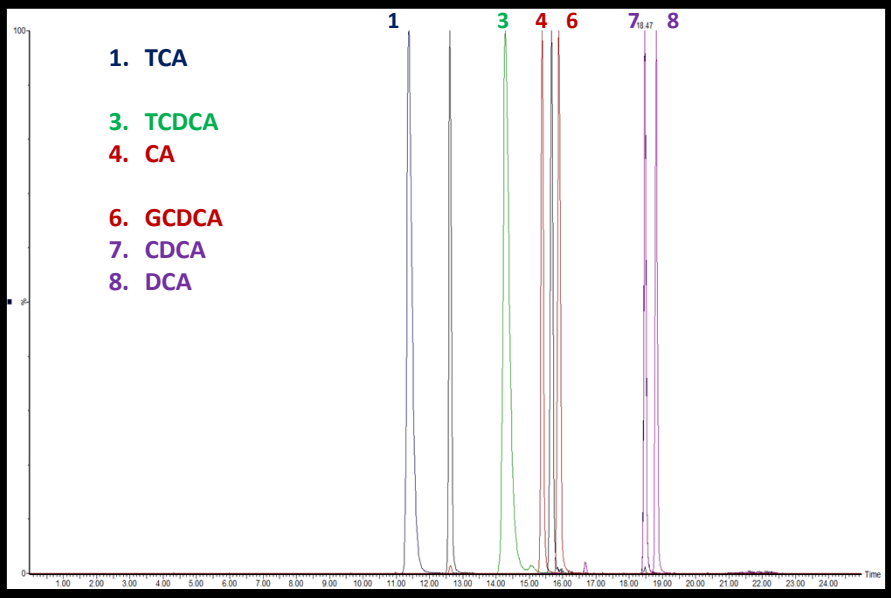
Developing optimized protocols for extraction efficiency and chromatographic resolution based on metabolite class and/or characteristics will dramatically improve accuracy and reproducibility of metabolomic data sets.



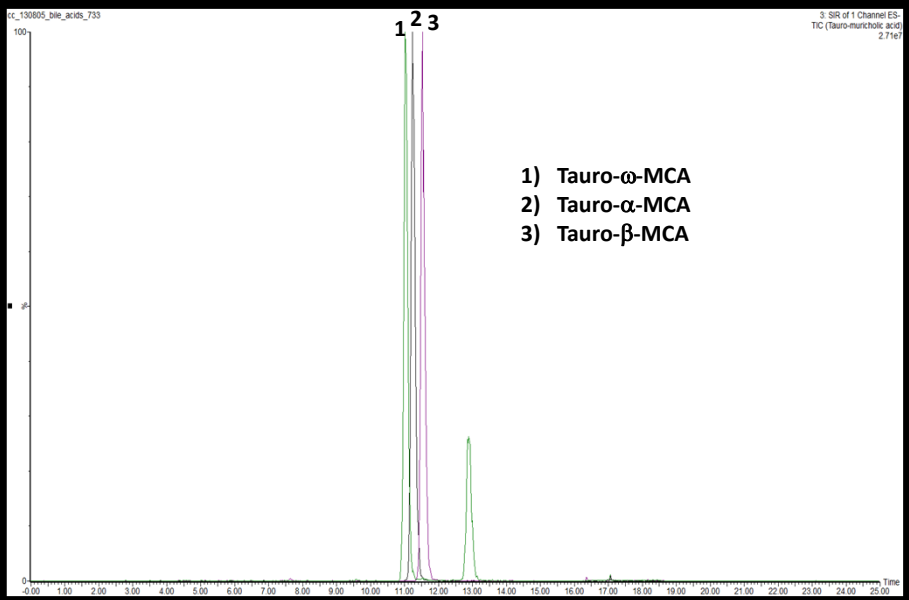
Definitions

- Isomer – same chemical formula, different chemical structure
- Stereoisomer – same chemical formula, same order/sequence of bonded atoms, different 3-dimensional orientation
- Isobar – same mass, but different chemical formula

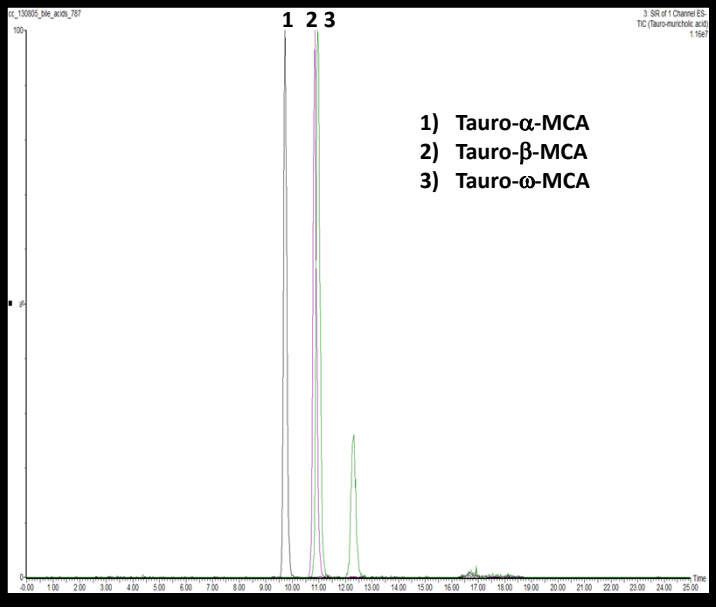
Resolution of Bile Acid Metabolites by RPLC using Waters BEH C18



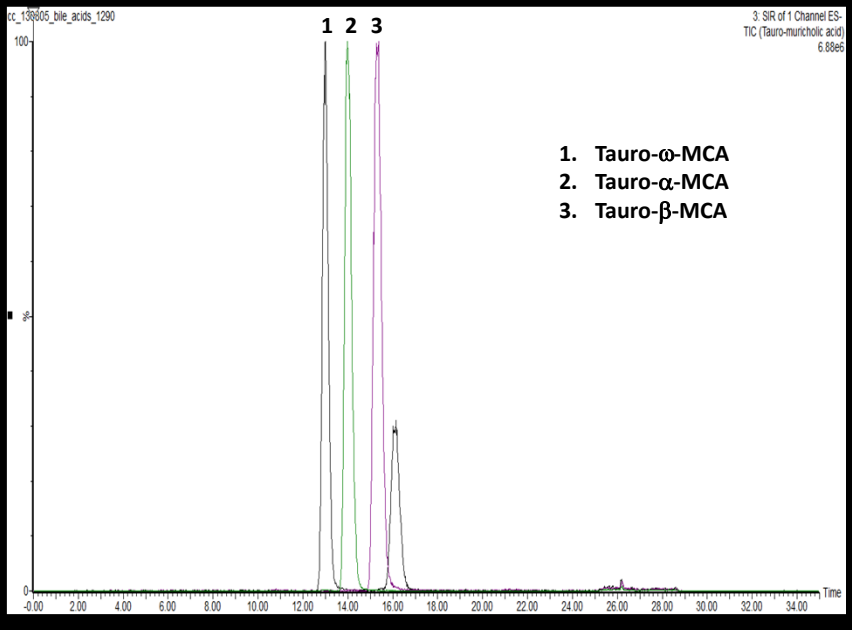
Resolution of Taurine Conjugated MCA Isomers by RPLC on WATERS BEH C18



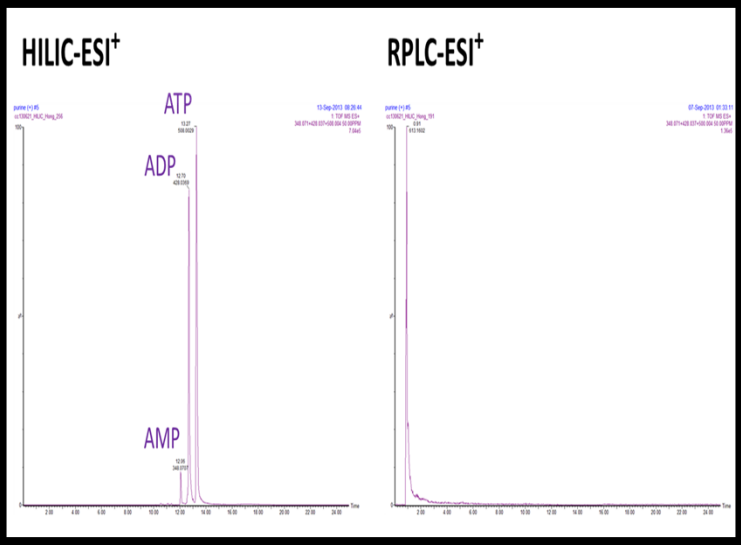
Resolution of Taurine Conjugated MCA Isomers by RPLC on Restek Rapture Biphenyl



Resolution of Taurine Conjugated MCA Isomers by RPLC on Restek Ultra AQ C18



Why should you care about chromatoraphy?



OUTLINE

- Extraction ---- Acetaminophen-Induced Hepatotoxicity
- Chromatography --- Search for Lung Cancer Biomarkers

METABOLOMICS FOR UNDERSTANDING DRUG TOXICITY---ACETAMINOPHEN

Bernard Brodie
1908-1989

ACETAMINOPHEN-INDUCED HEPATIC NECROSIS. I. ROLE OF DRUG METABOLISM[1]

J. R. MITCHELL, D. J. JOLLOW, W. Z. POTTER,[2,3] D. C. DAVIS,[2] J. R. GILLETTE AND B. B. BRODIE

Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland

ACETAMINOPHEN-INDUCED HEPATIC NECROSIS. II. ROLE OF COVALENT BINDING *IN VIVO*[1]

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ACETAMINOPHEN-INDUCED HEPATIC NECROSIS. III. CYTOCHROME P-450-MEDIATED COVALENT BINDING *IN VITRO*[1]

W. Z. POTTER,[2,3] D. C. DAVIS,[2] J. R. MITCHELL, D. J. JOLLOW, J. R. GILLETTE AND B. B. BRODIE

Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland

ACETAMINOPHEN-INDUCED HEPATIC NECROSIS. IV. PROTECTIVE ROLE OF GLUTATHIONE[1]

J. R. MITCHELL, D. J. JOLLOW, W. Z. POTTER,[2] J. R. GILLETTE AND B. B. BRODIE

Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland

APAP → NAPQI → Toxicity

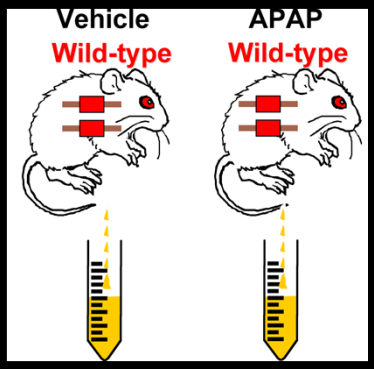
***N*-acetyl-*p*-benzoquinone imine**

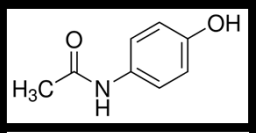
NORMAL MOUSE LIVER

NECROTIC MOUSE LIVER (400 mg/kg APAP 6 HOURS)

- Contained in 100s of products
- One of the most common pharmaceuticals associated with accidental and intentional poisoning **(>7 g per adult per day**)
- APAP overdose serves as a model for drug-induced liver toxicity
- Excess NAPQI (with reduced glutathione levels) leads to oxidative damage and inflammation leading to hepatocellular death/necrosis

Acetaminophen Metabolomics





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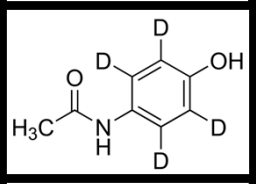
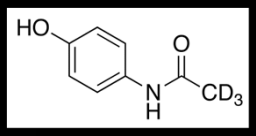


Ring - labeled



Acetyl - labeled

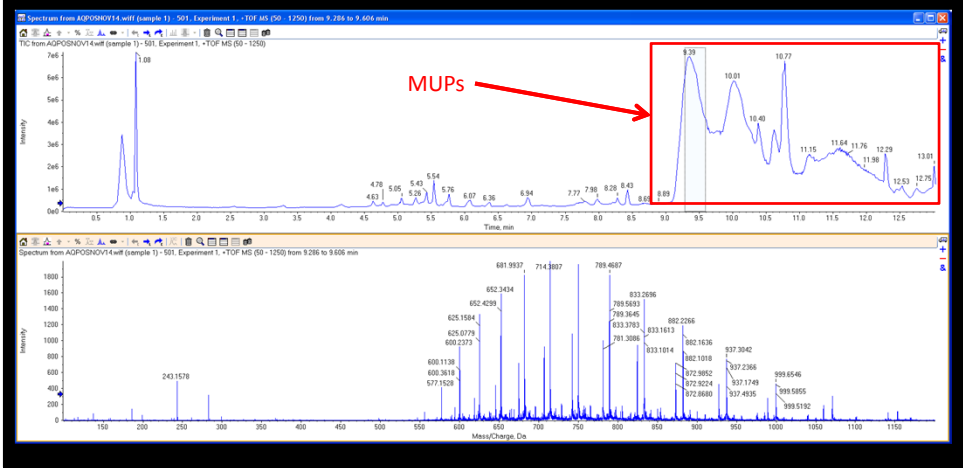
LC-MS

Data Analysis

Metabolite Identification

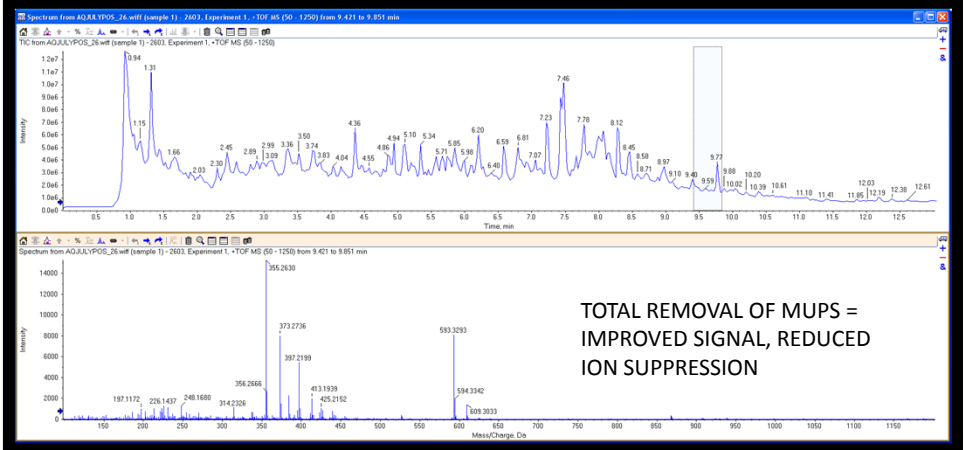
Mouse Urinary Proteins (MUPs)

- Dilute equal volume of mouse urine with an equal volume of 50% methanol



Mouse Urinary Proteins

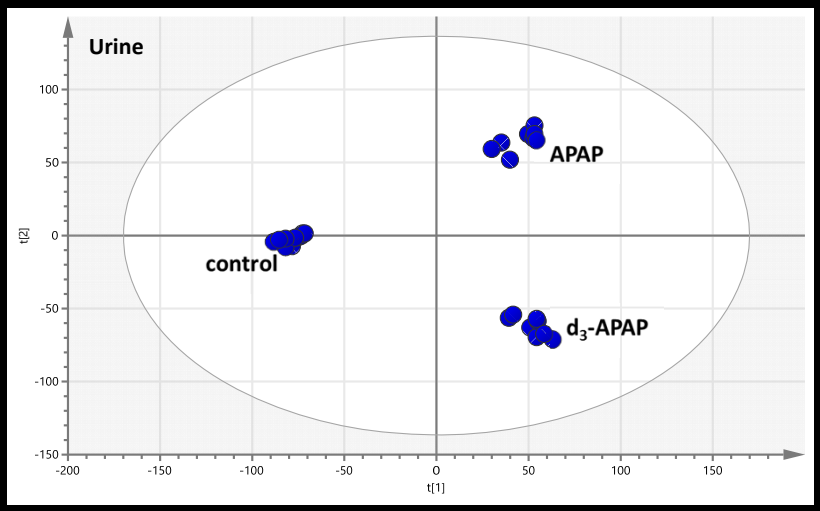
- Dilute equal volume of mouse urine with an equal volume of 100% methanol



TOTAL REMOVAL OF MUPS = IMPROVED SIGNAL, REDUCED ION SUPPRESSION

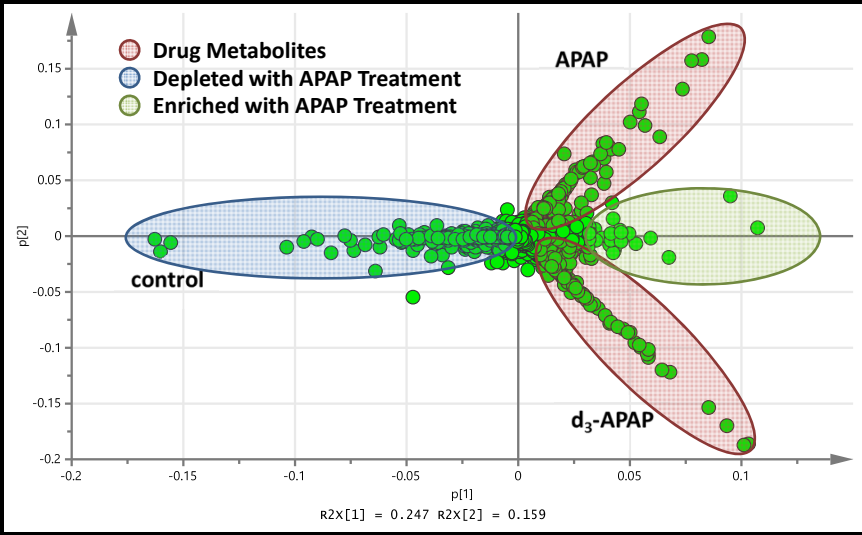
APAP Metabolism Study #4 Score Scatter Plot

PCA model

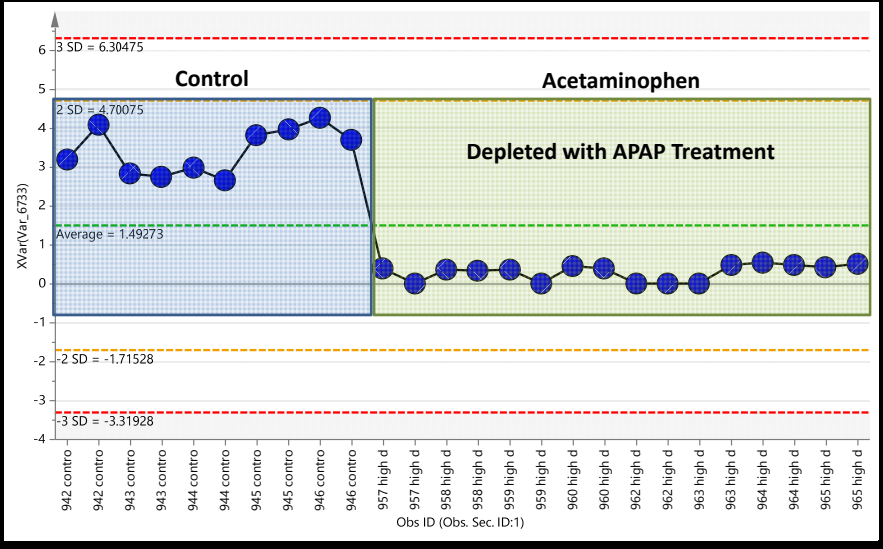


APAP Metabolism Loading Scatter Plot

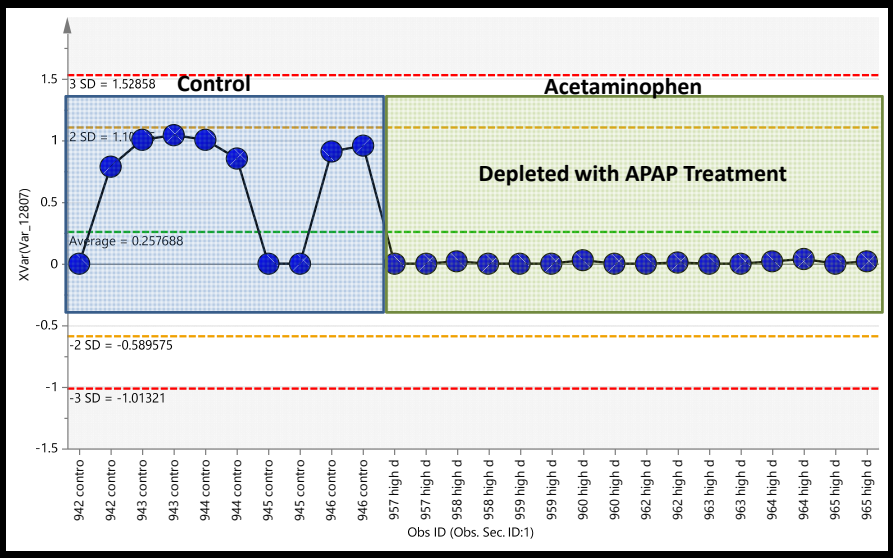
PCA Model



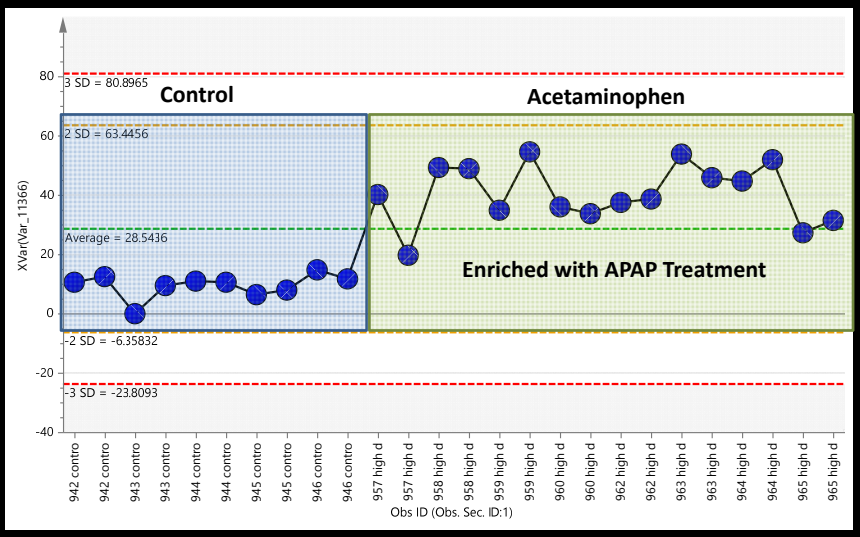
X-Variable Trend Plot for L-Carnitine (m/z=162.114+)



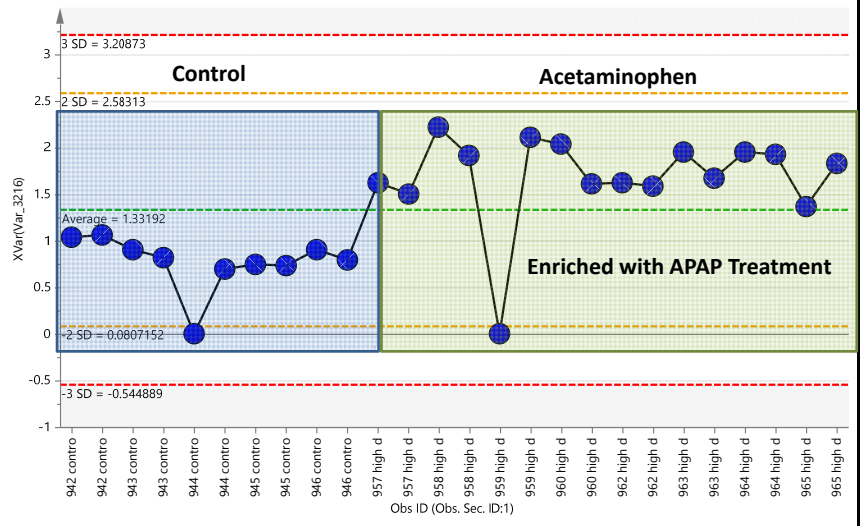
X-Variable Trend Plot for Propionylcarnitine (m/z=218.14+)



X-Variable Trend Plot for Acetylcarnitine (m/z=204.124+)

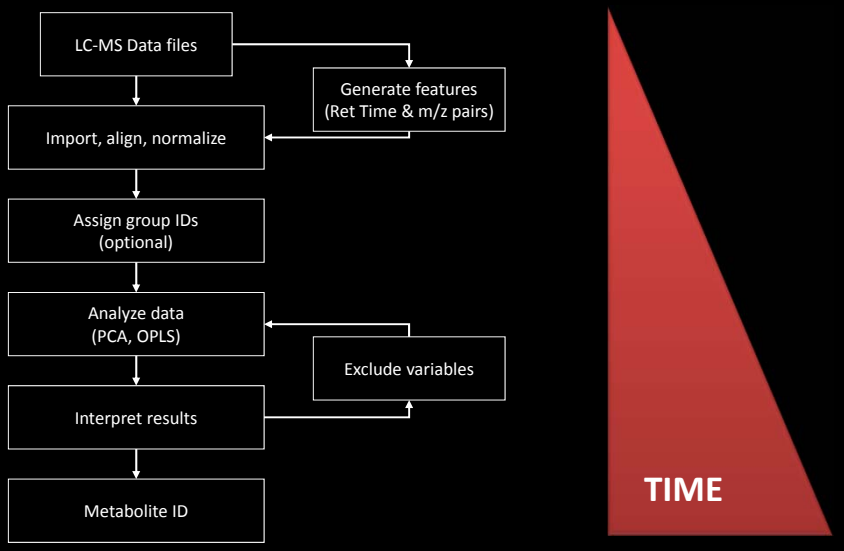


X-Variable Trend Plot for Decanoylcarnitine (m/z=316.247+)



STRUCTURAL ELUCIDATION USING INFORMATION DEPENDENT ACQUISITION

Typical Metabolomics Workflow



Metabolite ID is Time Consuming

1. Populate MS/MS product ion acquisition parameter list with m/z and RT values
2. Prepare new samples
3. Acquire MS/MS product ion spectra
4. Optimize collision energy and reacquire spectra
5. Align new chromatograms with the previously obtained ones
6. Compare product ion spectra with reference spectra

IDA Metabolite ID Workflow

1. Conventional LC-MS survey scan used to trigger LC-MS/MS product ion acquisition
2. Up to 50 MS/MS product ions for each survey scan, optimal number 5-20
3. Optimal values are dependent on chromatographic peak widths and sample complexity
4. Compromise between missing a compound and acquiring many noisy unusable spectra
5. Dynamic background subtract
6. Collision energy spread

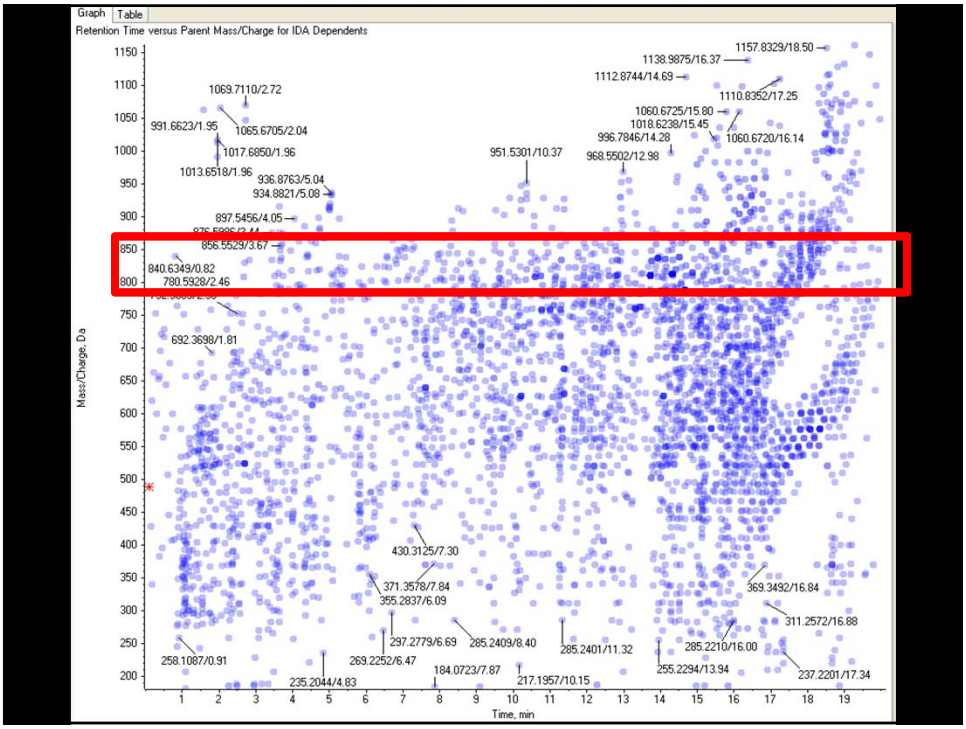
Philip Smith

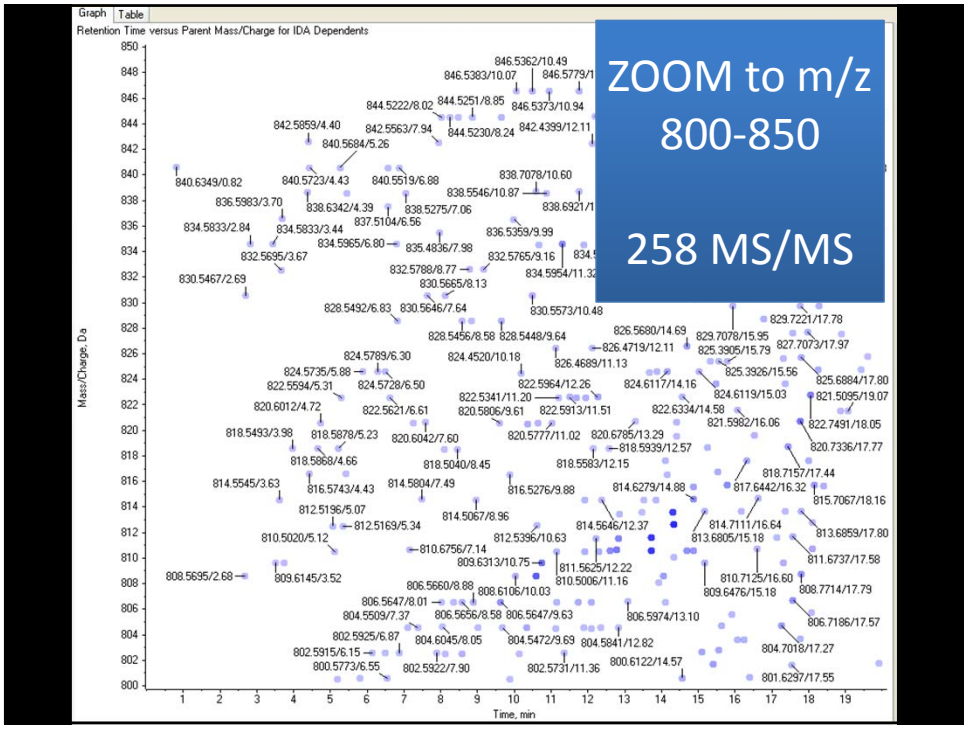
IDA Metabolite ID Workflow

- Autonomous acquisition of product ion spectra
- No product ion m/z list generation, sample re-preparation or chromatographic realignment required
- Ideally high quality MS/MS product ion spectra acquired for all components present in mixture
- If subsequent data analysis reveals previously unidentified features, have archived MS/MS product ion spectra

Lipid Extract IDA Workflow

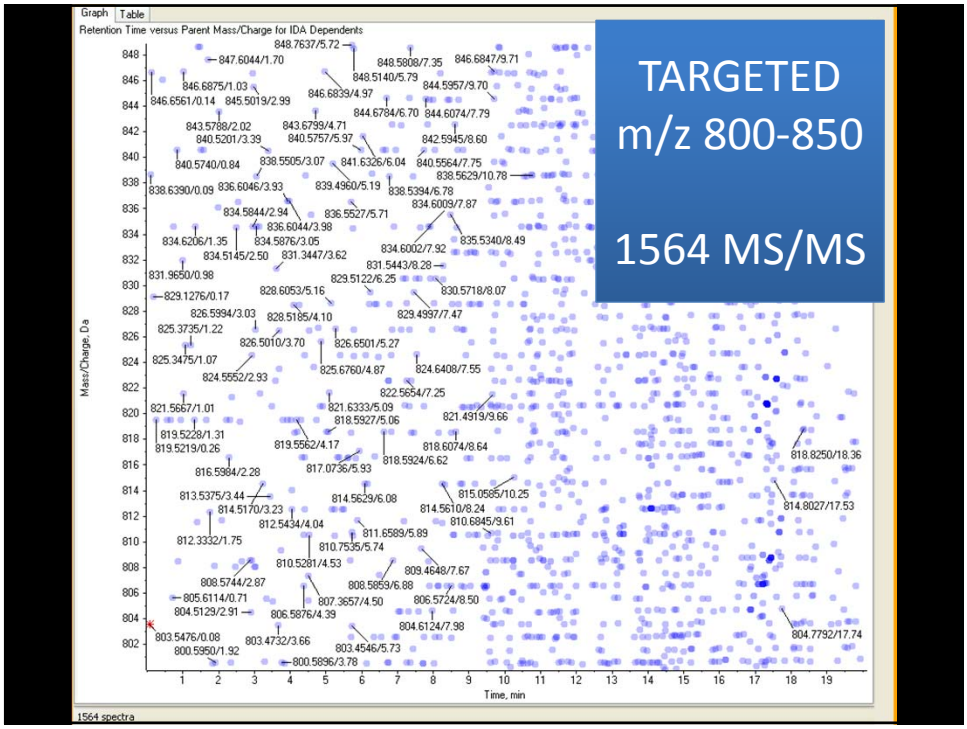
- Bovine liver total lipid extract 0.5 mg/ml (Avanti)
- Gradient elution aqueous isopropanol/acetonitrile w/ 10mM ammonium formate 0.1% formic acid
- Waters CSH® 2.1 x 100 mm C18 column
- Up to 20 IDA scans (100 ms)
- 2.1s duty cycle
- Exclude for 10 s after 1 occurrence
- Product ion m/z 150-1150
- Total 3408 product ion mass spectra

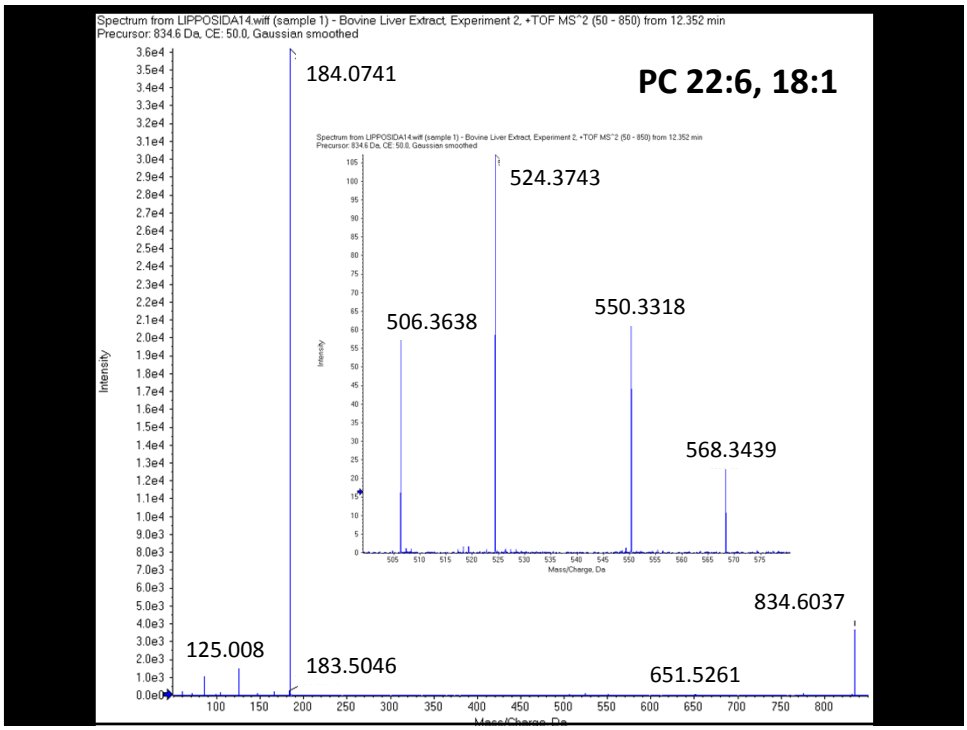




Lipid Extract IDA Workflow

- Limit m/z range to 50-200 Da increments
- Up to 10 IDA scans (200 ms)
- 2.1s duty cycle
- Exclude for 10 s after 2 occurrences
- Product ion m/z 800-850
- Total 1564 product ion mass spectra


Graph
Table
Retention Time versus Parent Mass/Charge for IDA Dependents
Mass/Charge, Da
Time, min
1564 spectra
TARGETED
m/z 800-850
1564 MS/MS


Spectrum from LIPPOSIDA14.wiff (sample 1) - Bovine Liver Extract, Experiment 2, +TOF MS^2 (50 - 850) from 12.352 min
Precursor: 834.6 Da, CE: 50.0, Gaussian smoothed
Intensity
Mass/Charge, Da
184.0741
PC 22:6, 18:1
524.3743
506.3638
550.3318
568.3439
834.6037
125.008
183.5046
651.5261

Chem Res Toxicol. 2009 Apr;22(4):699-707. doi: 10.1021/tx800464q.

Serum metabolomics reveals irreversible inhibition of fatty acid beta-oxidation through the suppression of PPARalpha activation as a contributing mechanism of acetaminophen-induced hepatotoxicity.

Chen C[1], Krausz KW, Shah YM, Idle JR, Gonzalez FJ.

J Biol Chem. 2008 Feb 22;283(8):4543-59. Epub 2007 Dec 19.

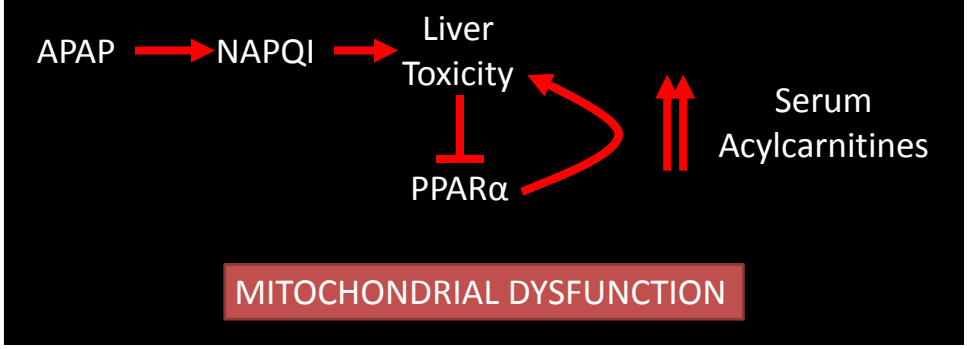
Identification of novel toxicity-associated metabolites by metabolomics and mass isotopomer analysis of acetaminophen metabolism in wild-type and Cyp2e1-null mice.

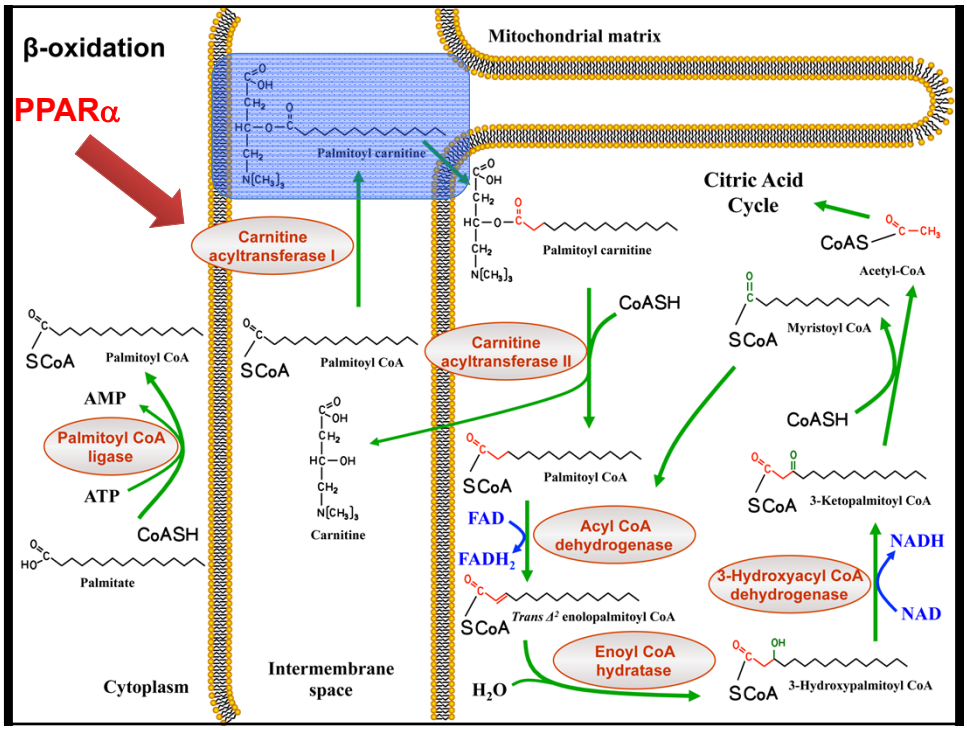
Chen C[1], Krausz KW, Idle JR, Gonzalez FJ.

Hepatology. 2012 Jul;56(1):281-90. doi: 10.1002/hep.25645. Epub 2012 Jun 6.

Peroxisome proliferator-activated receptor alpha induction of uncoupling protein 2 protects against acetaminophen-induced liver toxicity.

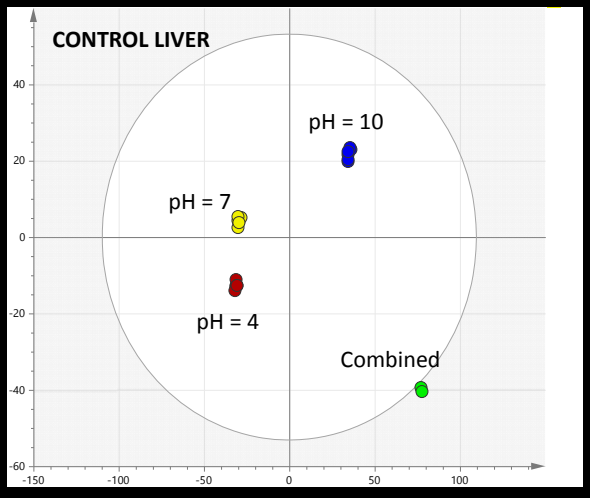
Patterson AD[1], Shah YM, Matsubara T, Krausz KW, Gonzalez FJ.





INFLUENCE OF EXTRACTION PROTOCOL

Influence of pH on Metabolite Extraction from Mouse Liver



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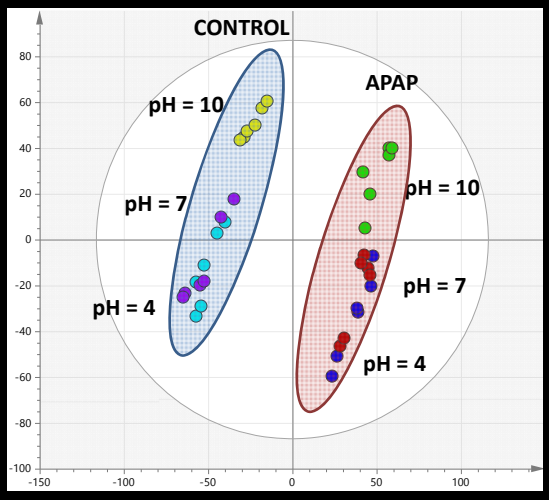

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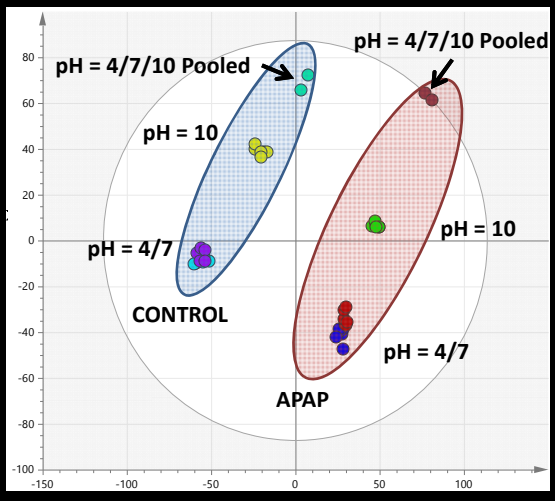
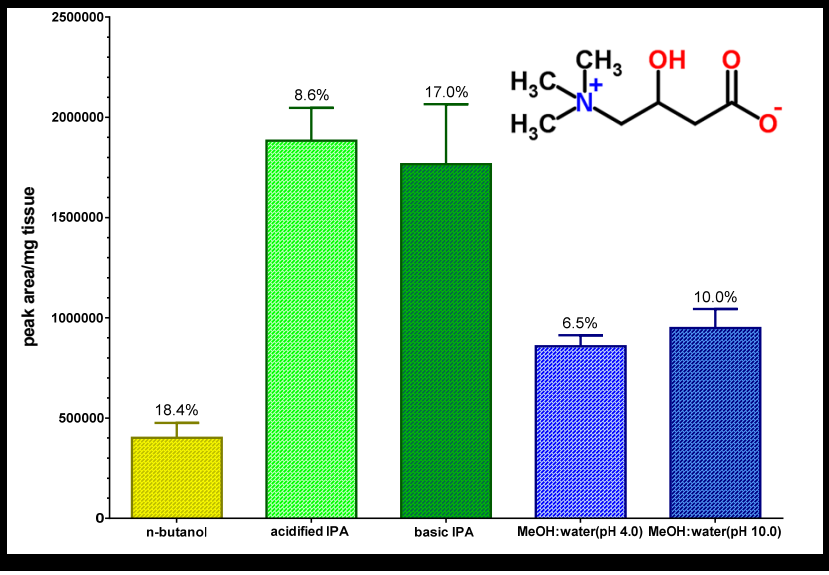
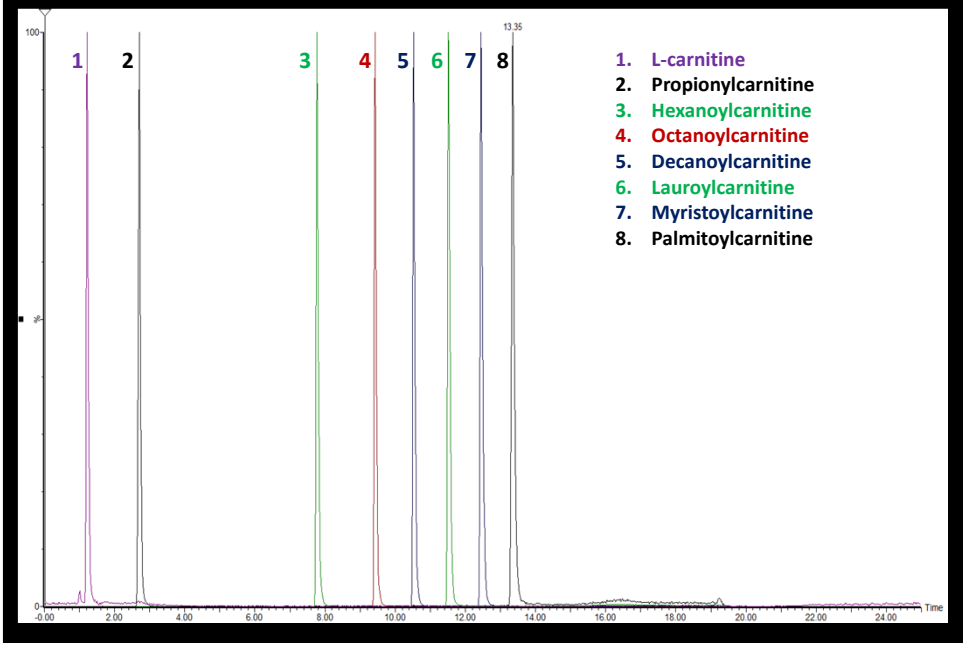


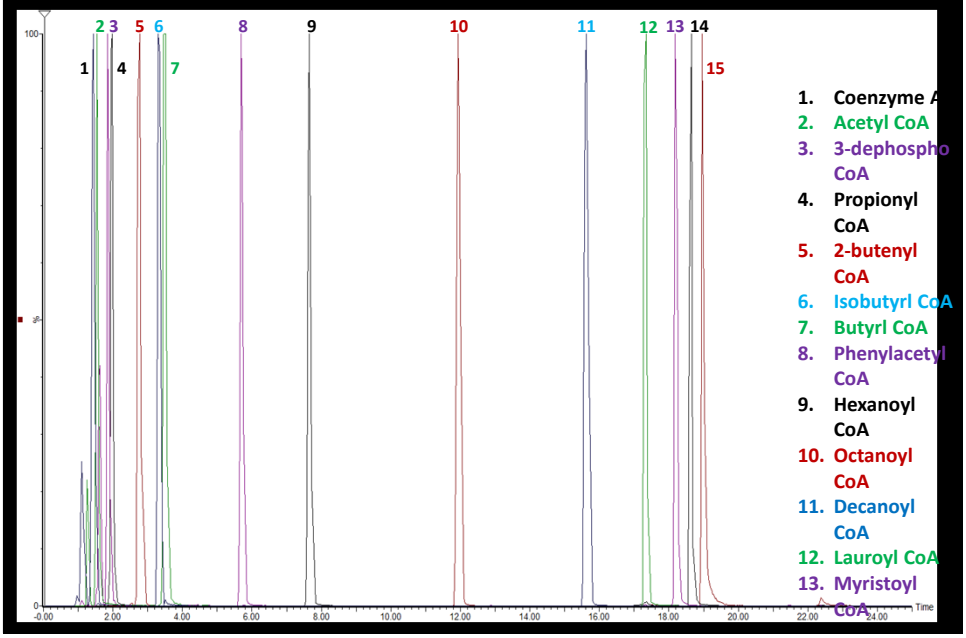
Extraction Efficiency of L-Carnitine from Mouse Liver



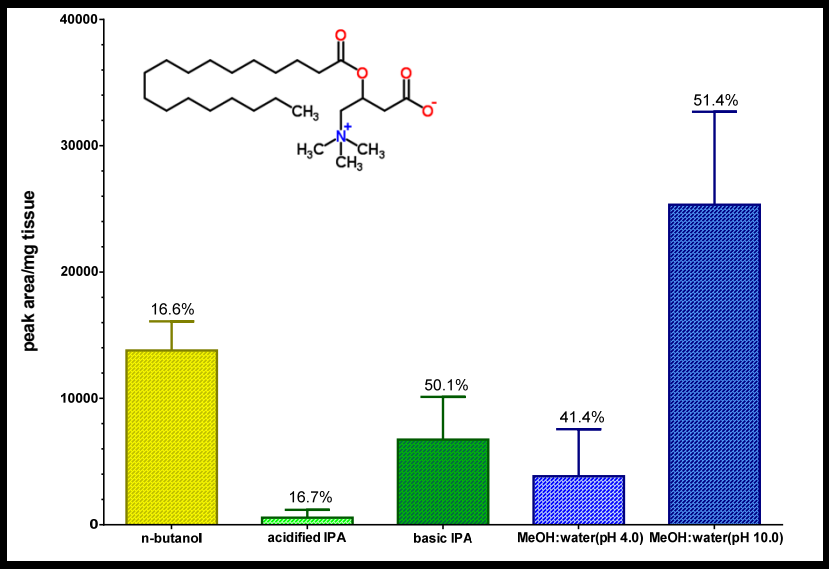
Resolution of Acyl Carnitine Standards by RPLC on Waters BEH C18



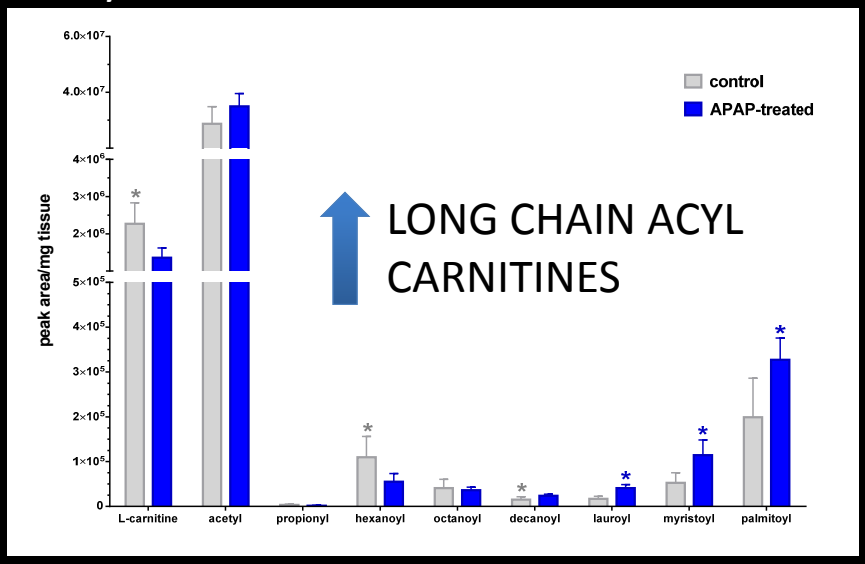
Resolution of Coenzyme A (CoA) Thioester Metabolites by RPLC using Waters BEH C18



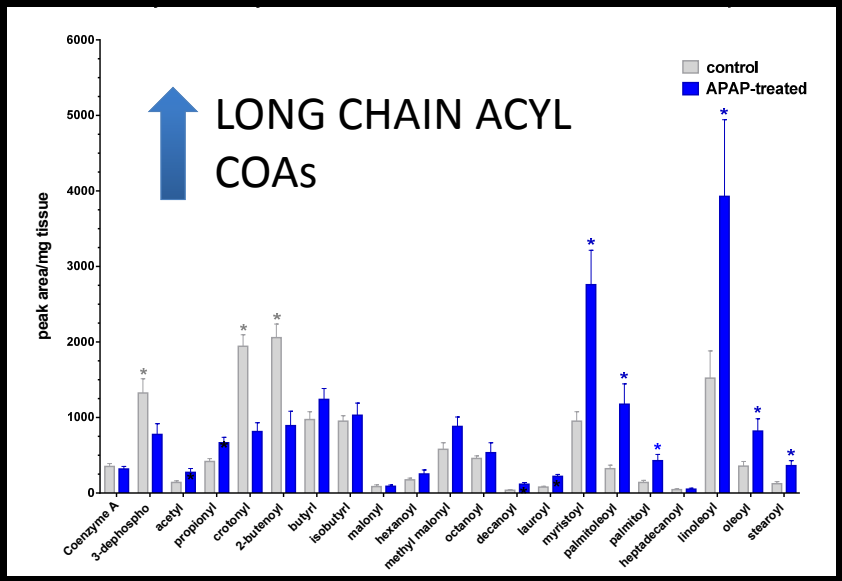
Extraction Efficiency of Stearoyl Carnitine from Mouse Liver

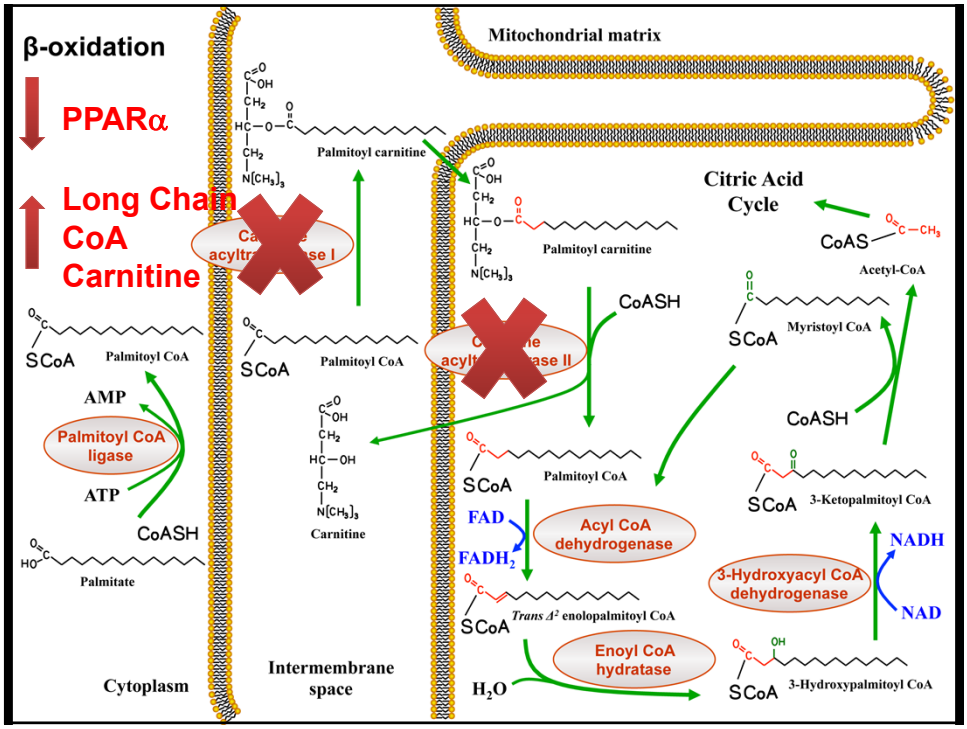


Acylcarnitine Extraction in Acidified IPA



AcylCoa Extraction via Modified Bligh/Dyer



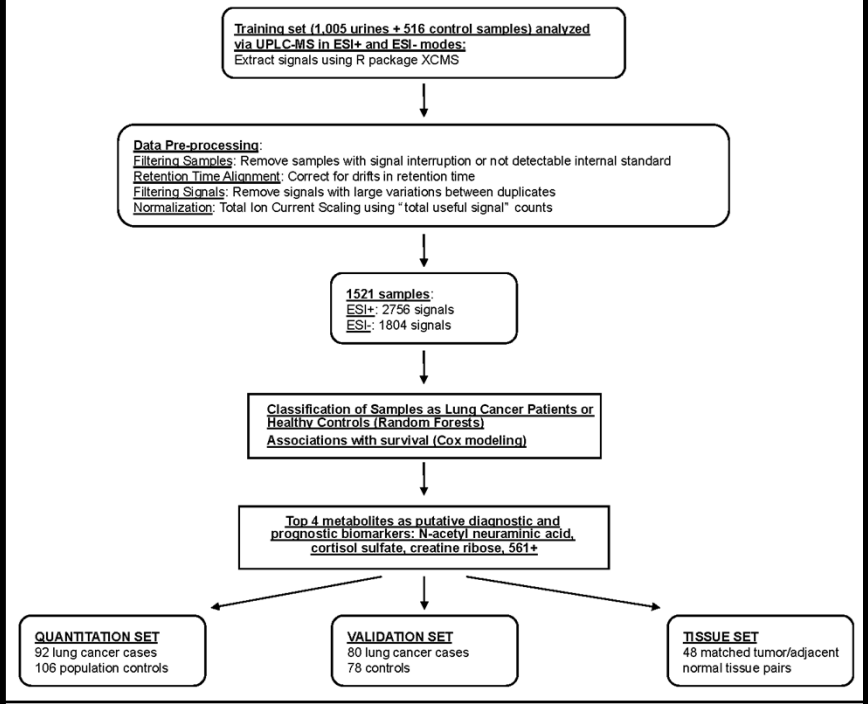


METABOLOMICS FOR CASE CONTROL STUDIES – LUNG CANCER BIOMARKERS

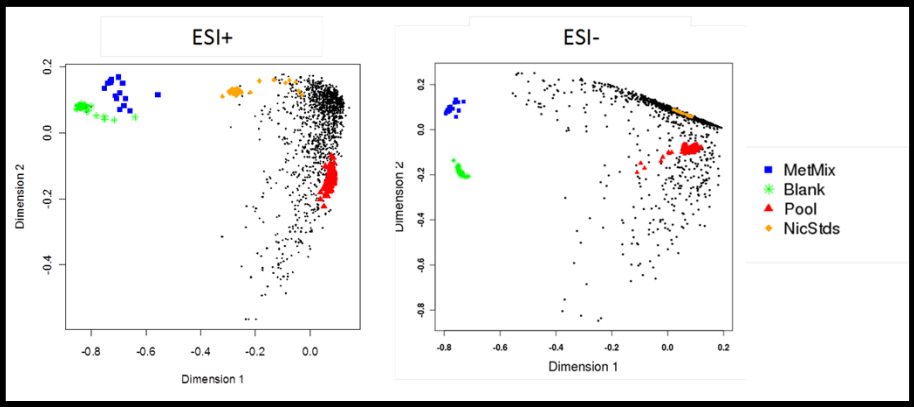
Lung Cancer Biomarkers

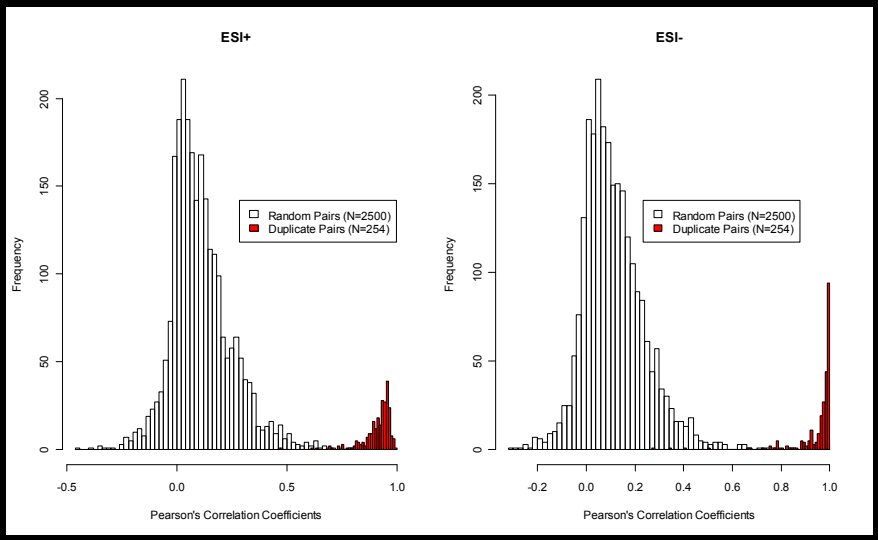
- Most common cause of cancer deaths worldwide

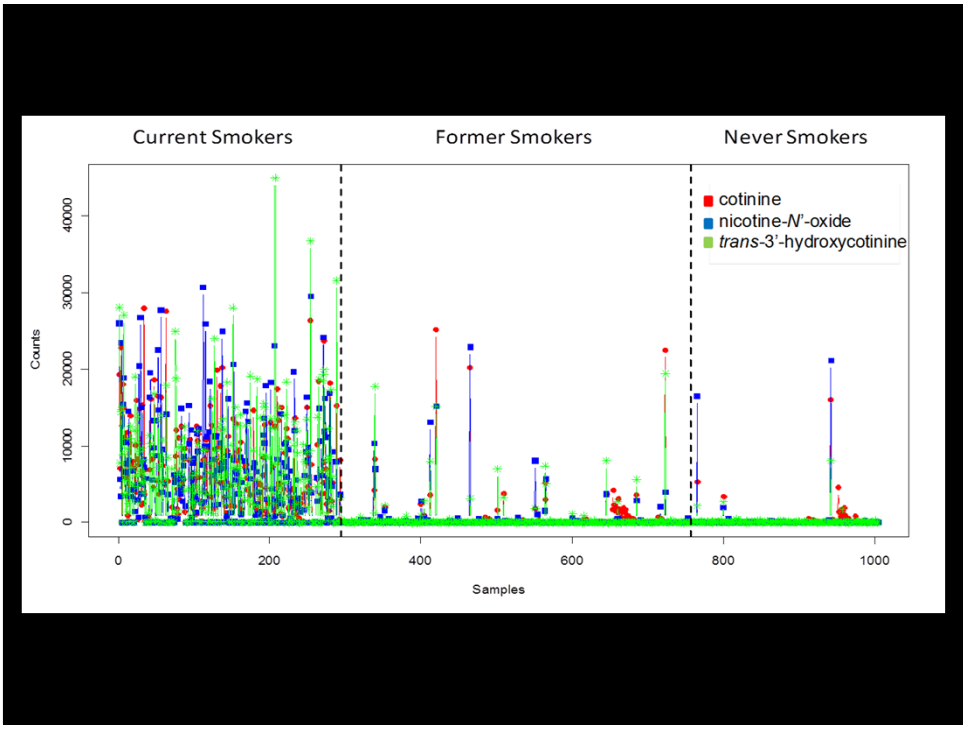
- Training Set
  - 536 population controls, 469 lung cancer patients (pre-treatment)
- Validation Set
  - 78 population controls, 80 lung cancer patients
- Quantitation Set
  - 106 population controls, 92 lung cancer patients
- Tumor Tissue Set
  - 48 non-tumor, 48 tumor

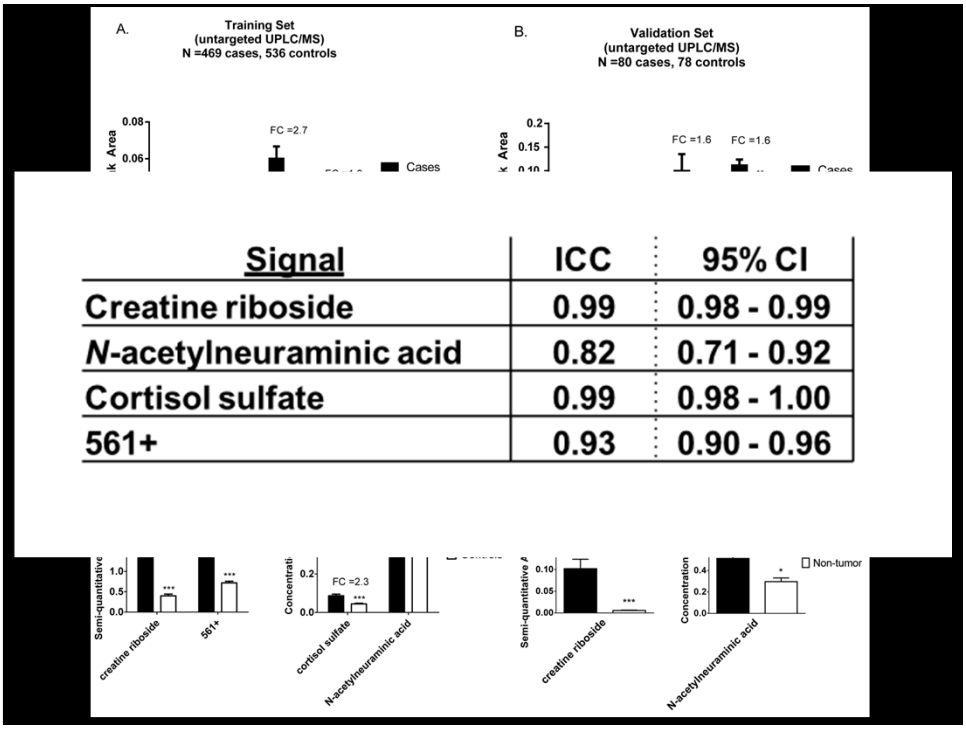


Quality Control

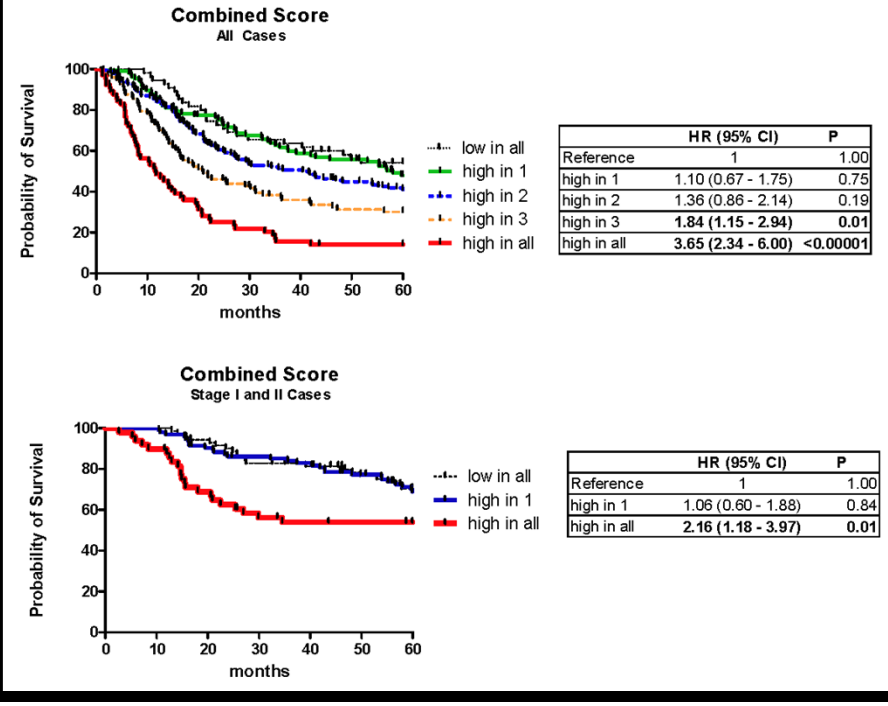








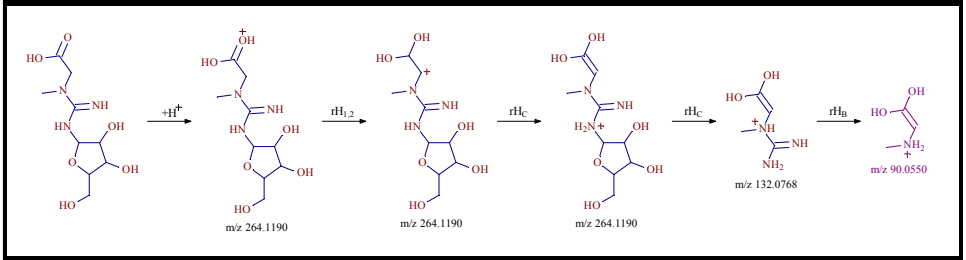
Signal	ICC	95% CI
Creatine riboside	0.99	0.98 - 0.99
N-acetylneuraminic acid	0.82	0.71 - 0.92
Cortisol sulfate	0.99	0.98 - 1.00
561+	0.93	0.90 - 0.96



	HR (95% CI)	P
Reference	1	1.00
high in 1	1.10 (0.67 - 1.75)	0.75
high in 2	1.36 (0.86 - 2.14)	0.19
high in 3	**1.84 (1.15 - 2.94)**	**0.01**
high in all	**3.65 (2.34 - 6.00)**	**<0.00001**

	HR (95% CI)	P
Reference	1	1.00
high in 1	1.06 (0.60 - 1.88)	0.84
high in all	**2.16 (1.18 - 3.97)**	**0.01**

Creatine Riboside (non IUPAC)



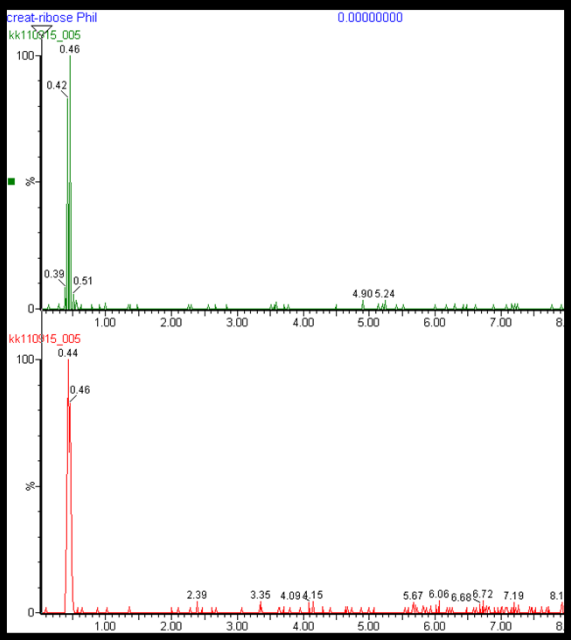
<u>Maillard Reaction</u> (makes grilled stuff tasty)

Creatine + D-ribose + heat = creatine riboside

Reverse Phase

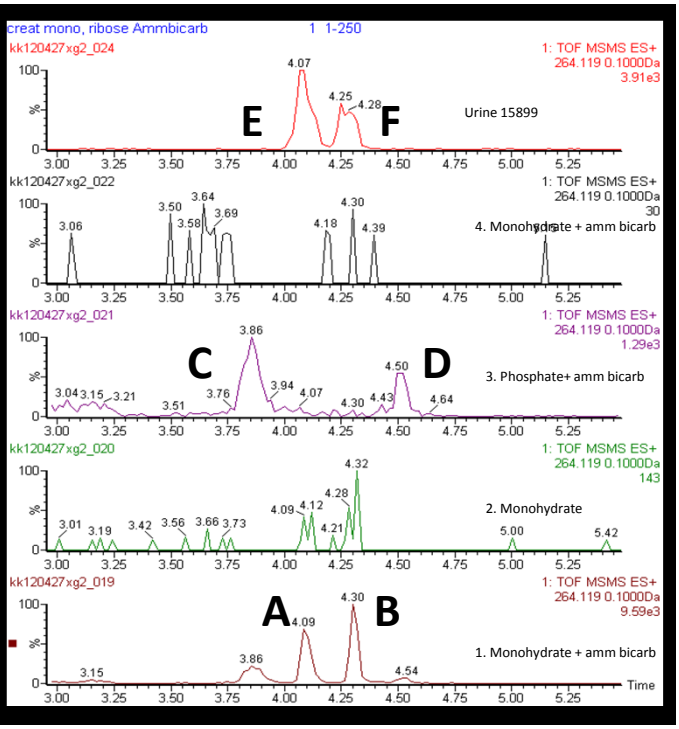
Waters BEH C18

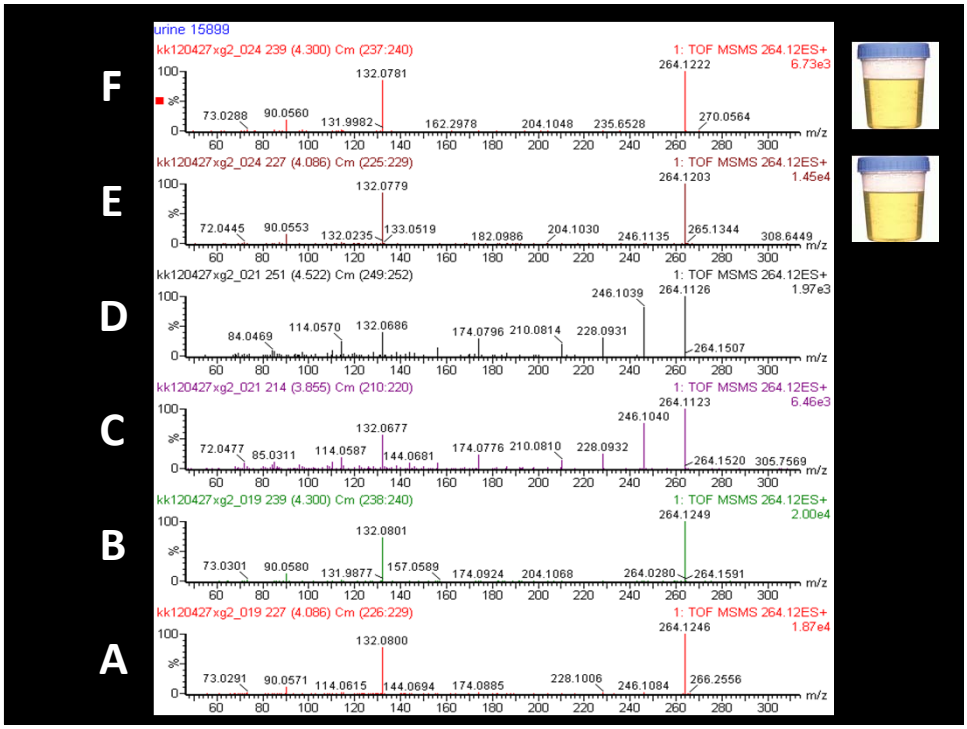
Poor retention of small, polar metabolites



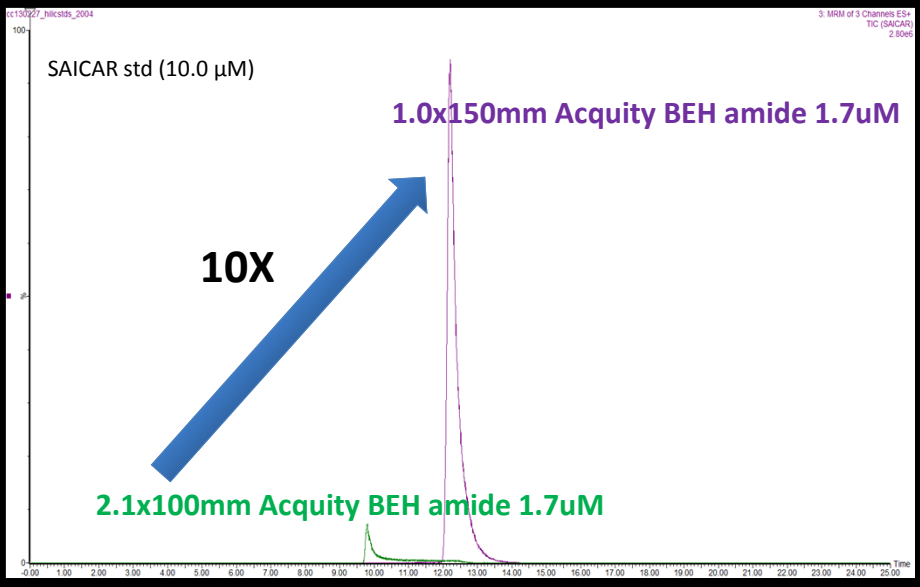
HILIC

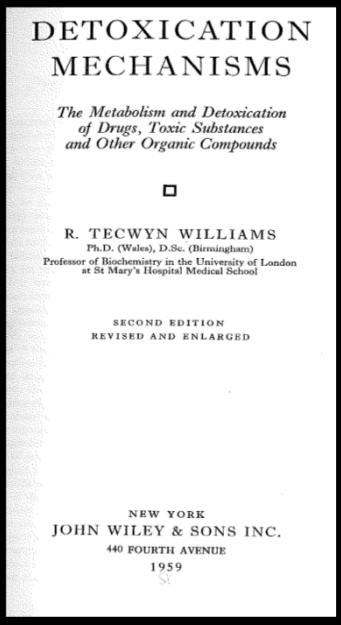
Waters BEH Amide





Effect of Column Bore Diameter on Sensitivity





DETOXICATION MECHANISMS

The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds

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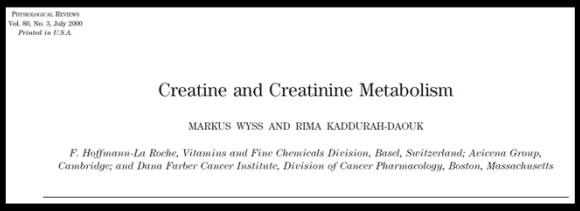

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Creatine and Creatinine Metabolism

MARKUS WYSS AND RIMA KADDURAH-DAOUK

F. Hoffmann-La Roche, Vitamins and Fine Chemicals Division, Basel, Switzerland; Aricena Group, Cambridge; and Dana Farber Cancer Institute, Division of Cancer Pharmacology, Boston, Massachusetts

- Histamine is metabolized to a similar riboside (Williams)
  - Similar mechanism for creatine?
- Creatine and ribose can form a mutagen similar to PhIP (Kaddurah-Daouk)
- Synthesis of creatine riboside with SRI as part of the NIH Common Fund

Summary

- Demonstrated the value of optimized metabolite extraction
- Emphasized study design (particularly for human studies) and the value of good chromatography
- Provided an improved means to simultaneously collect high quality MSMS spectra for later metabolite structural elucidation
- Annual meeting present findings from interlab comparisons

Conclusions

- Extraction protocols can impact metabolomic data sets considerably
- Solvent system composition and pH exhibit the most dramatic effects on metabolite recovery
  - The magnitude of these effects depend on metabolite class
  - Some classes of metabolites
- The number of extraction repetitions also plays a role in enhancing metabolite recovery
  - Tradeoff - longer sample prep time
  - Larger sample volumes to process (evaporate)

Conclusions

- Traditional RPLC methods can provide efficient separation of acyl-carnitine, bile acid and CoA thioester mixtures.
  - Advancements in hybrid particle technologies
  - Allowing for extremes in mobile phase pH and temperature – manipulate selectivity
  - Complex ligand stationary phase interactions
- HILIC methods are superior at separating highly polar metabolites.
  - Nucleotides and derivatives
  - Small polar metabolites – sugars, organic acids, amino acids, hydrophilic vitamins
- Advanced column chemistries (amide, aminopropyl, biphenyl, graphite, phenyl-hexyl) and alternative chromatographic methodologies (HILIC) can provide enhanced coverage of the metabolome.

Future Plans

- There's no one "perfect" extraction or LC method available capable of efficiently resolving all components or features in the metabolome
- Therefore, our goal is to continue to develop optimized extraction and chromatography protocols for various classes of liver metabolites

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