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

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Resources

- Metabolomics Workbench
  - www.metabolomicsworkbench.org
- XCMS Institute
  - Great tutorials on chromatography, platforms, databases
- Twitter

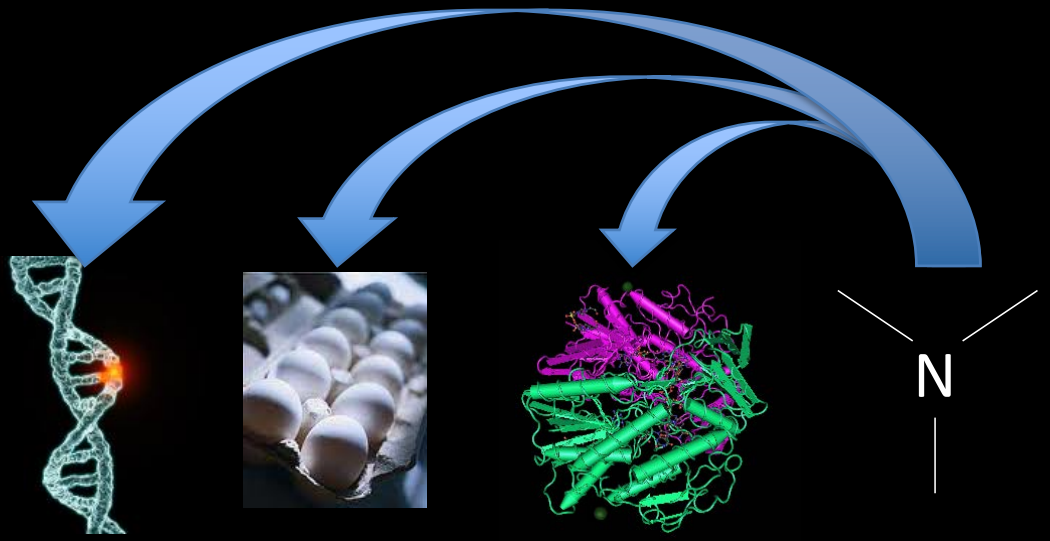
References

- Mass spectrometry-based metabolomics

- Xenobiotic metabolomics: major impact on the metabolome

- The intestinal metabolome: an intersection between microbiota and host

"What have we here? A man or a fish? Dead or alive? He smells like a fish; a very ancient and fish-like smell . . ." W. Shakespeare *The Tempest*



GENOME
FMO3 Mutation

FISHY
EGGS

PROTEOME

METABOLOME

USE METABOLITE CHANGES TO INFORM ABOUT MECHANISM

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

Metabolite Class	Separation Mode	Stationar	
Bile Acids - general	RPLC	Waters B	.01%)
Bile Acids - MCA isomers	RPLC	Waters B	.01%)
Bile			
		Restek Rapt	
		Waters B	.01%)
		Waters CSH	d (0.01%)
		Waters BEH	ate (pH=10.0)
		Waters C	e (pH=10.0)
		Waters BE	e (pH=10.0)
Eicosanoids	RPLC	Waters B	.01%)
Keto-prostaglandins	RPLC	Waters B	.01%)
Nucleotides	HILIC	Waters BE	e (pH=9.0)
Nucleotides	HILIC	Waters BE	te (pH=9.0)
Nucleotides - NAD(P)+/NAD(P)H	RPLC	Thermo - Hypercarb graphite	acetonitrile/water/ammonium acetate

Waters Amide column used for HILIC will rapidly and dramatically clog with salt over time unless flushed extensively at the end of each run with methanol/isopropanol/water.

Phenomenex Luna NH2 silica will break down over time at high pH thus reducing column life, reducing resolving power, and dirtying the source.

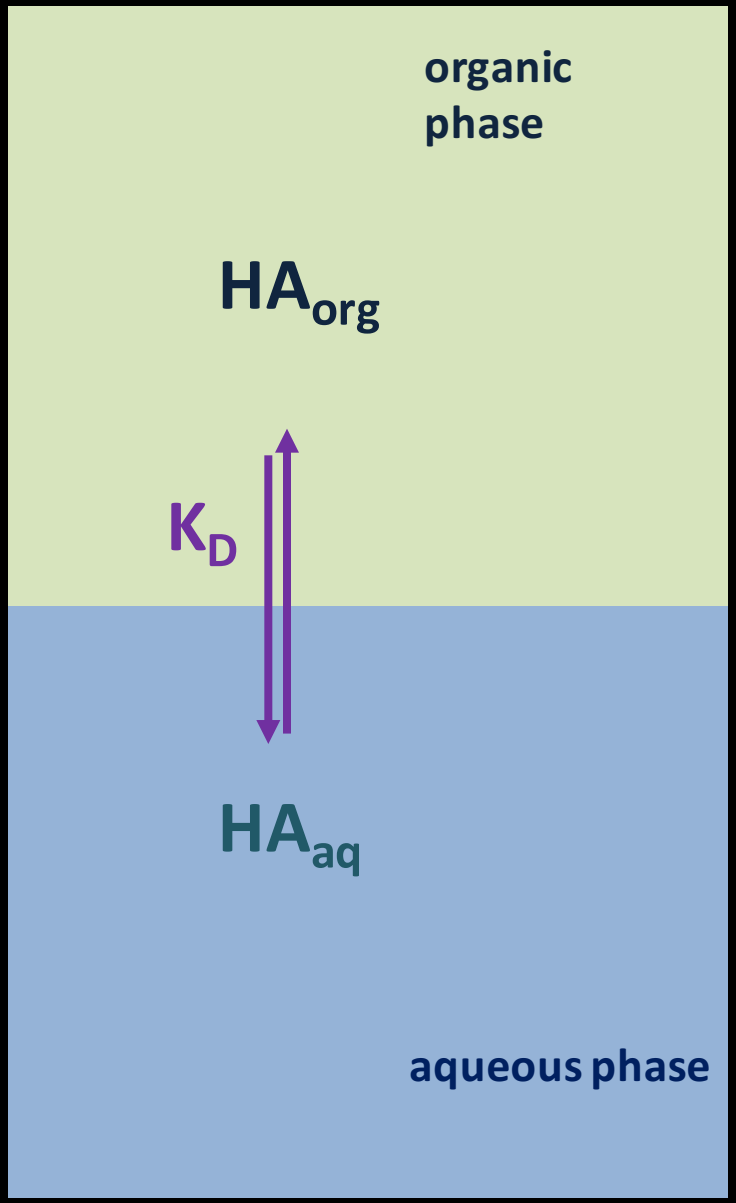
Thermo Hypercarb is robust, provides excellent separation, but is extremely sticky.

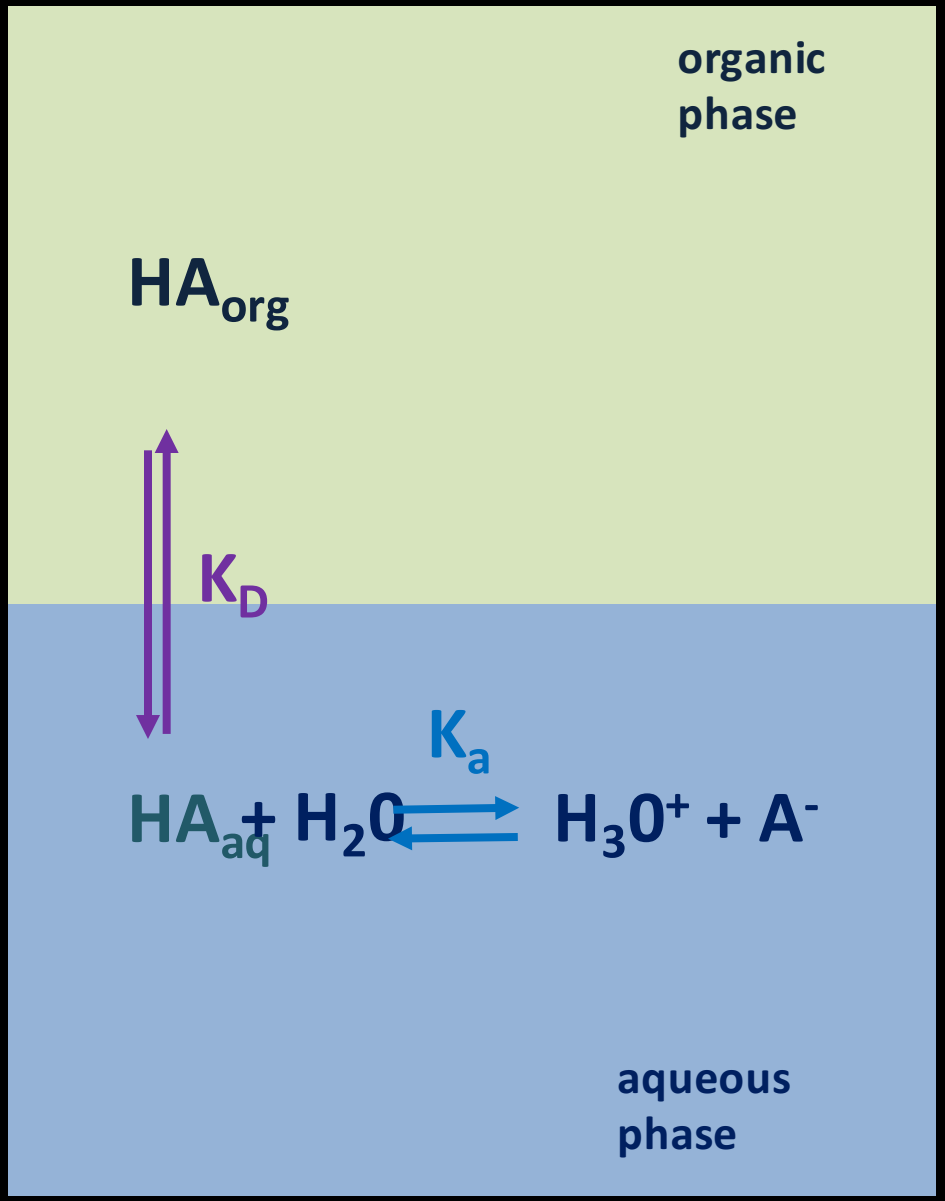
Hypothesis

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.

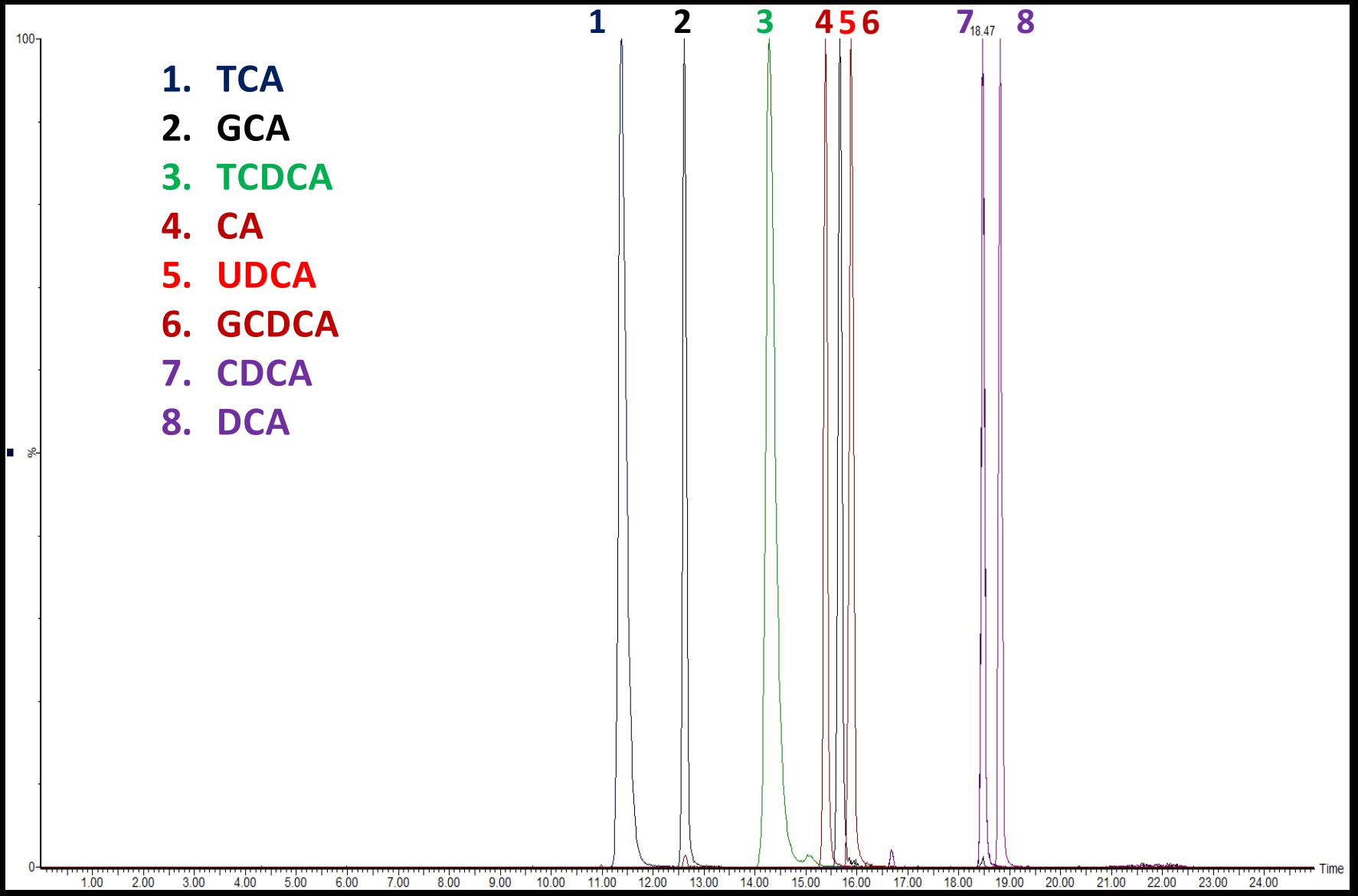

organic phase
HA_{org}
K_D
HA_{aq}
aqueous phase


organic phase
HA_{org}
K_D
K_a
$HA_{aq} + H_2O \rightleftharpoons H_3O^+ + A^-$
aqueous phase

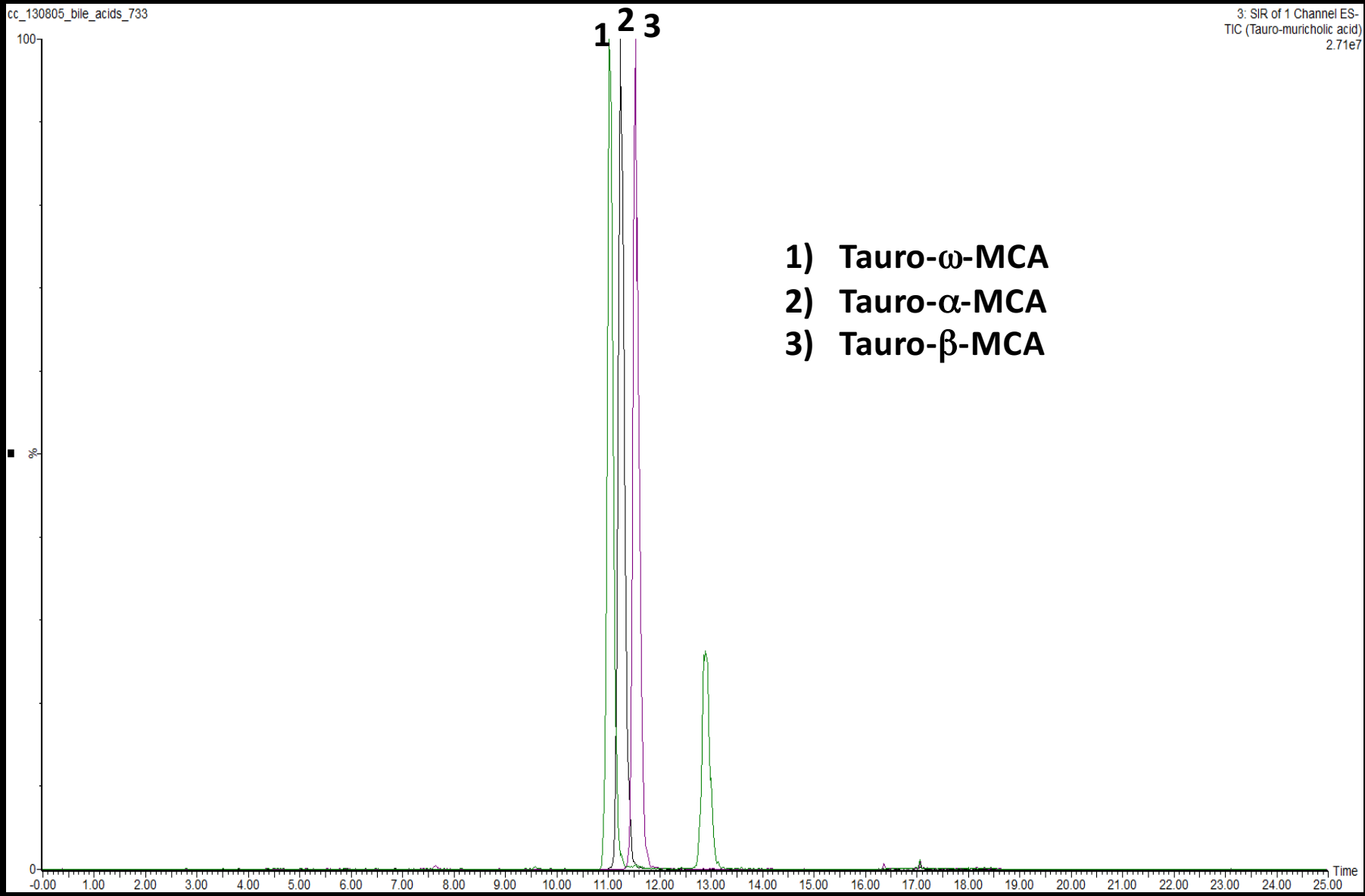
Definitions

- 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

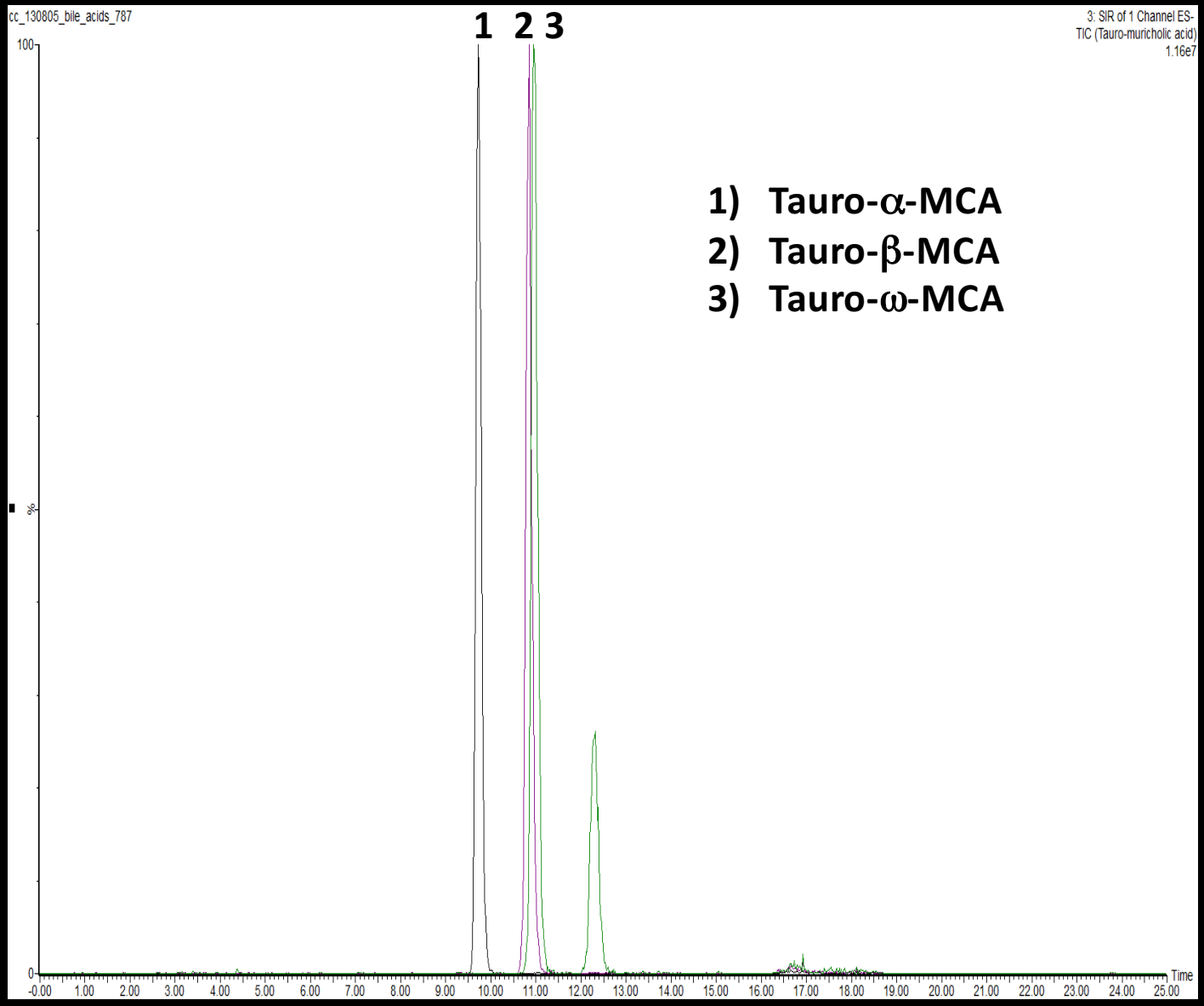
Resolution of Bile Acid Metabolites by RPLC using Waters BEH C18



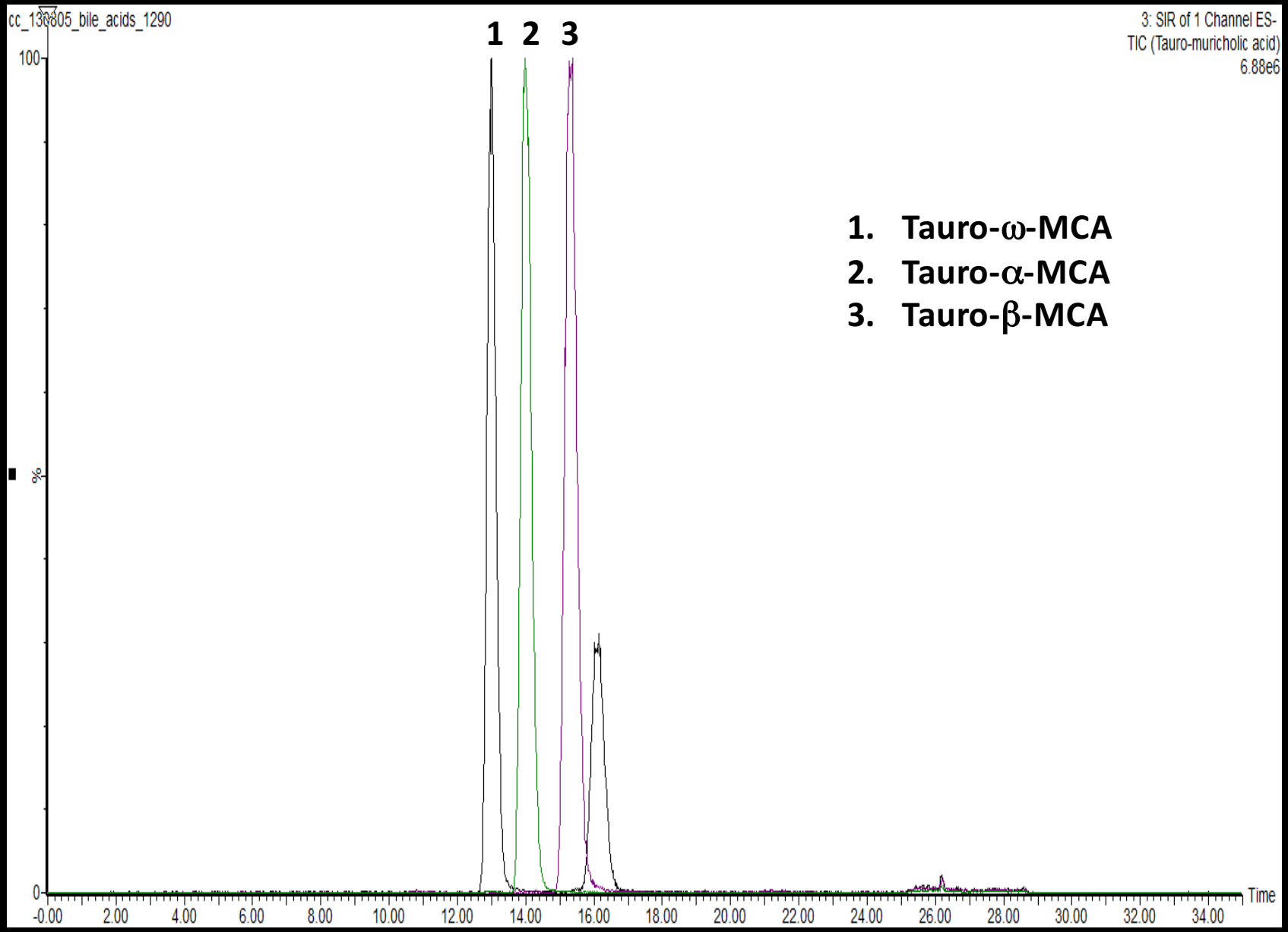
Resolution of Taurine Conjugated MCA Isomers by RPLC on WATERS BEH C18



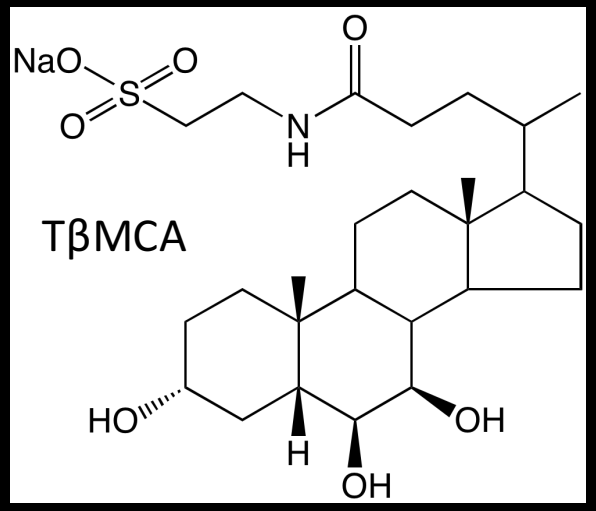
Resolution of Taurine Conjugated MCA Isomers by RPLC on Restek Rapture Biphenyl

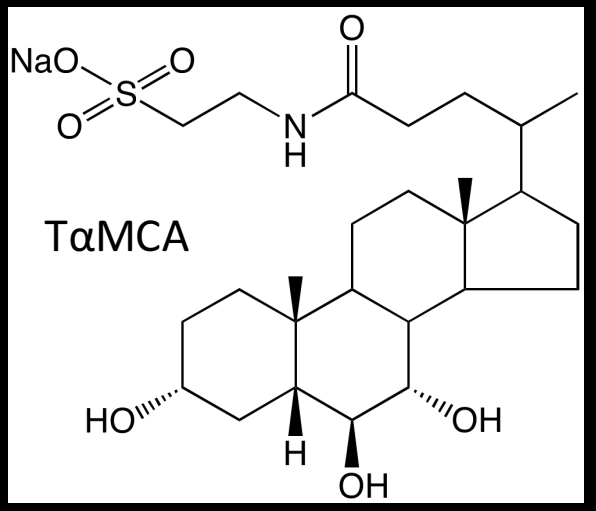


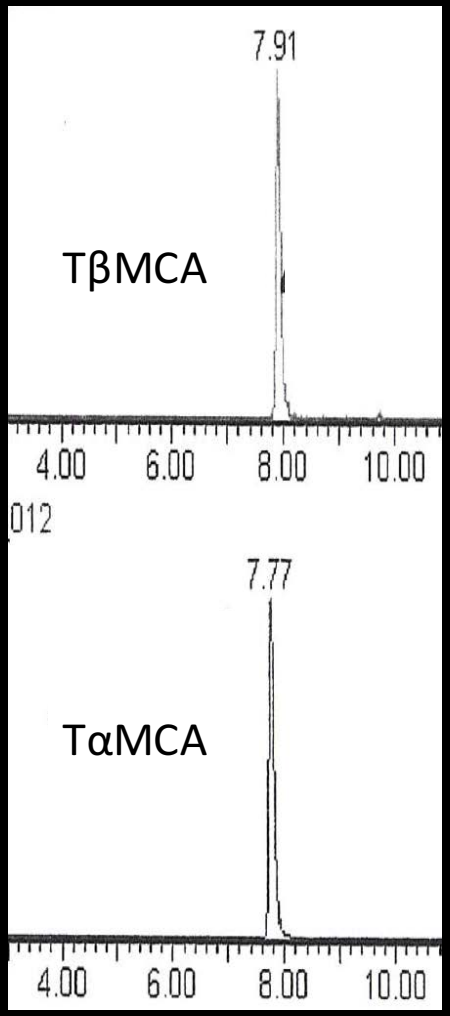
Resolution of Taurine Conjugated MCA Isomers by RPLC on Restek Ultra AQ C18

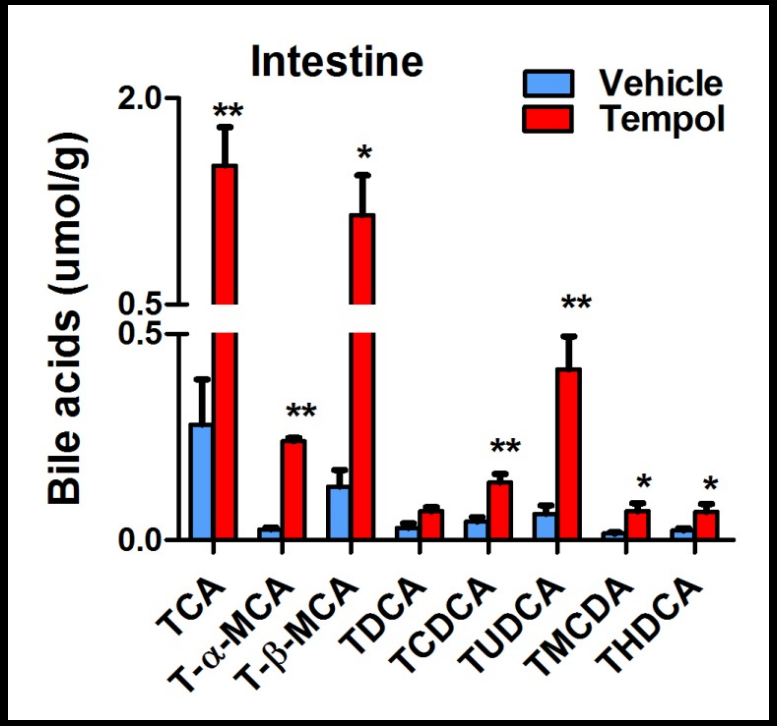


Tauro-β-Muricholic Acid
An Typical Isomer Example in Metabolomics



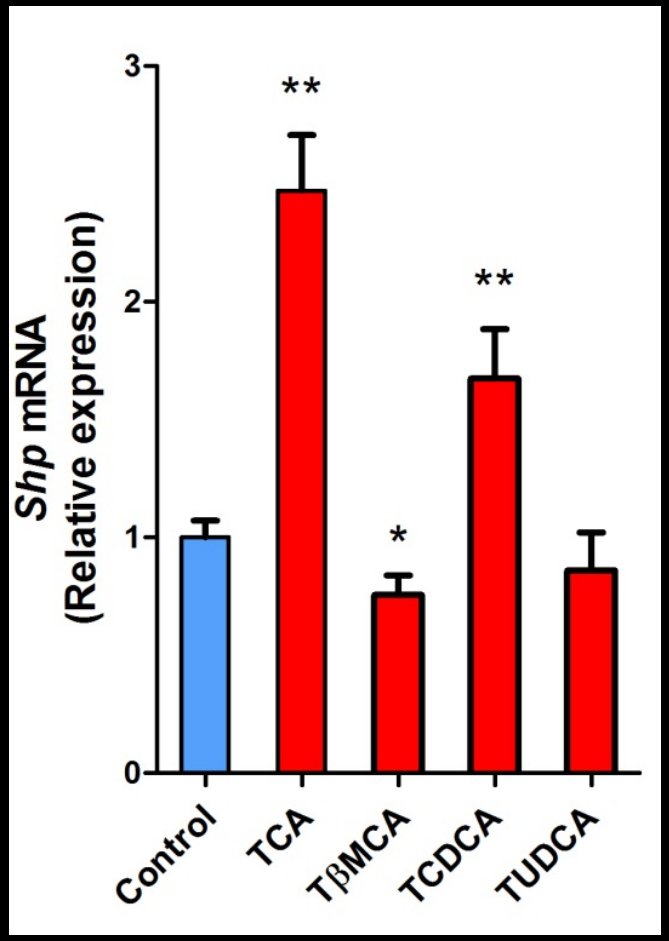






TβMCA is an Farnesoid X Receptor Antagonist

Shp (FXR target gene) induction in hepatocytes



Sayin et al *Cell Metabolism* 2013

Li F et al *Nature Communications* 2013

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

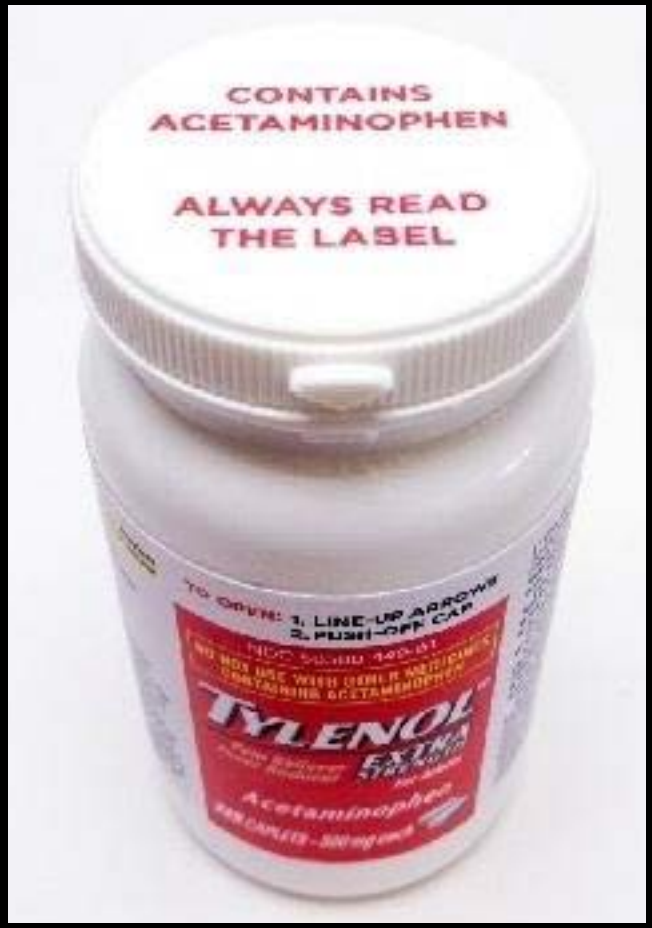
APAP → NAPQI → Toxicity

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

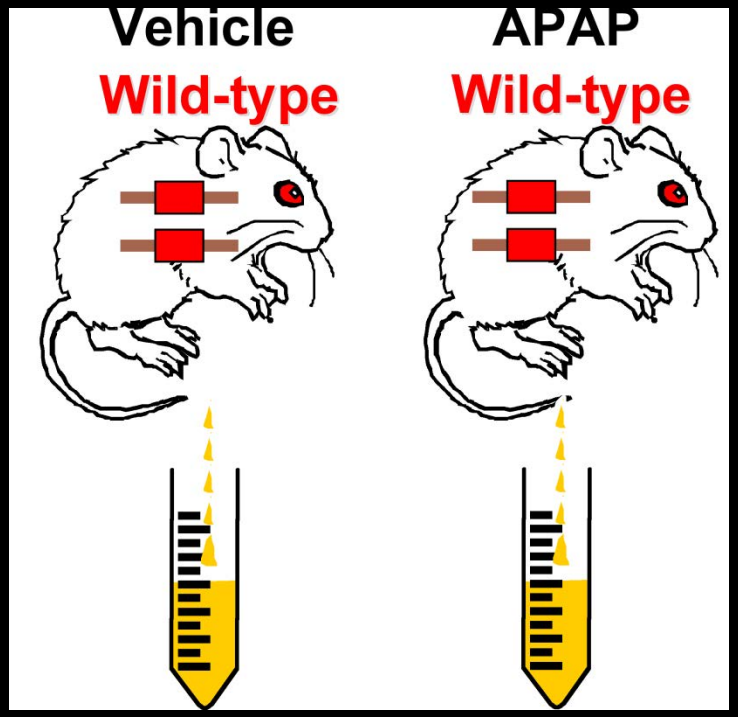
NORMAL MOUSE LIVER

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 (<u>with reduced glutathione levels</u>) leads to oxidative damage and inflammation leading to hepatocellular death/necrosis



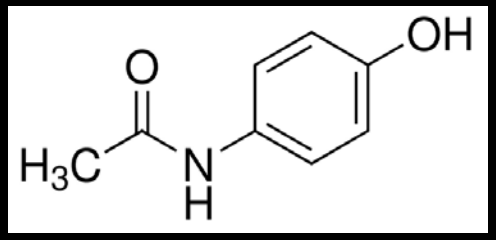
Acetaminophen Metabolomics



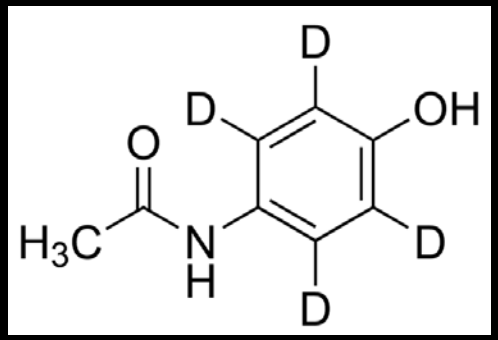
LC-MS

Data Analysis

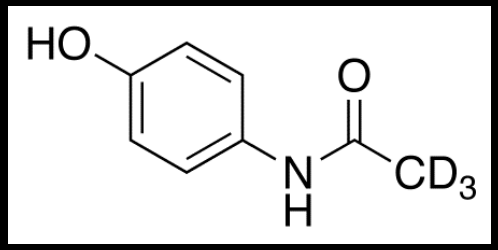
Metabolite Identification



Unlabeled



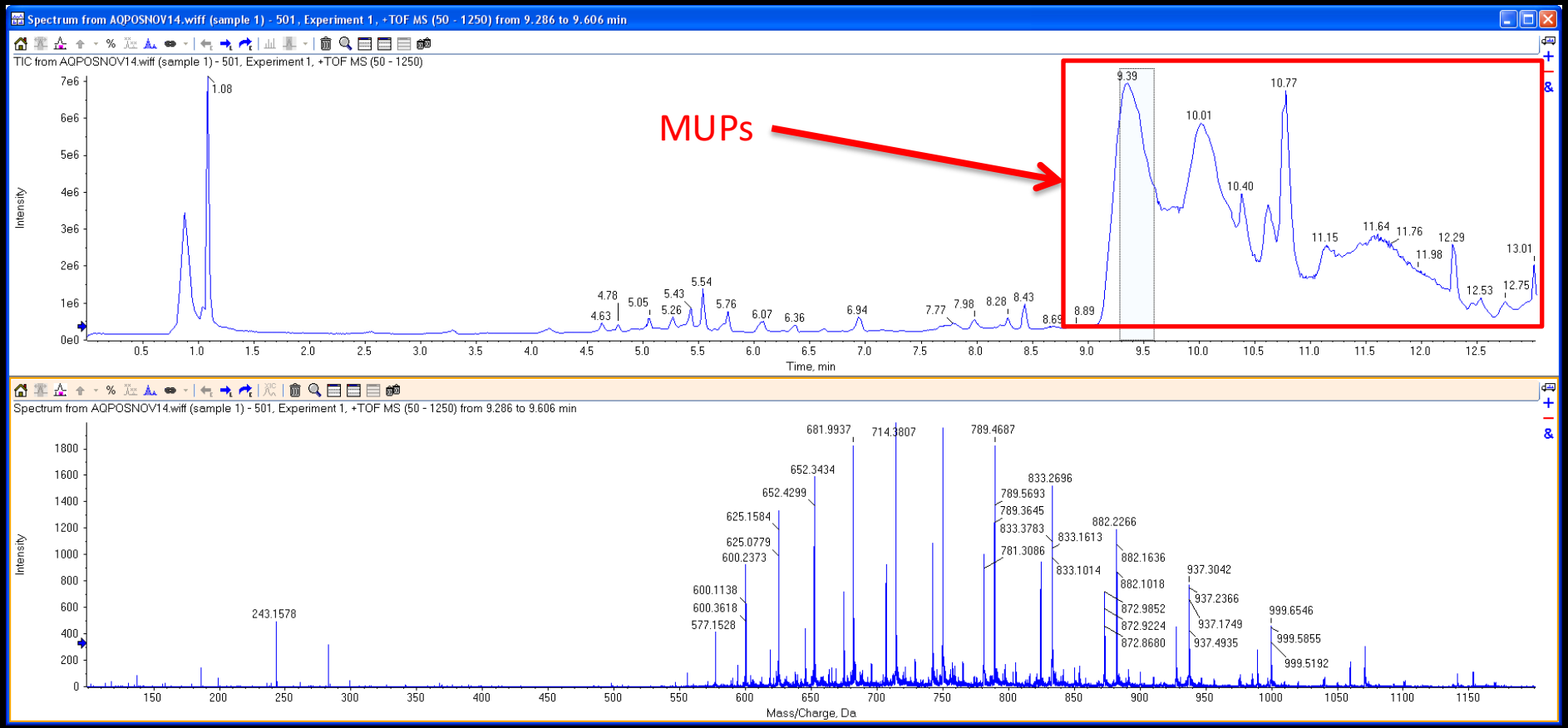
Ring - labeled



Acetyl - labeled

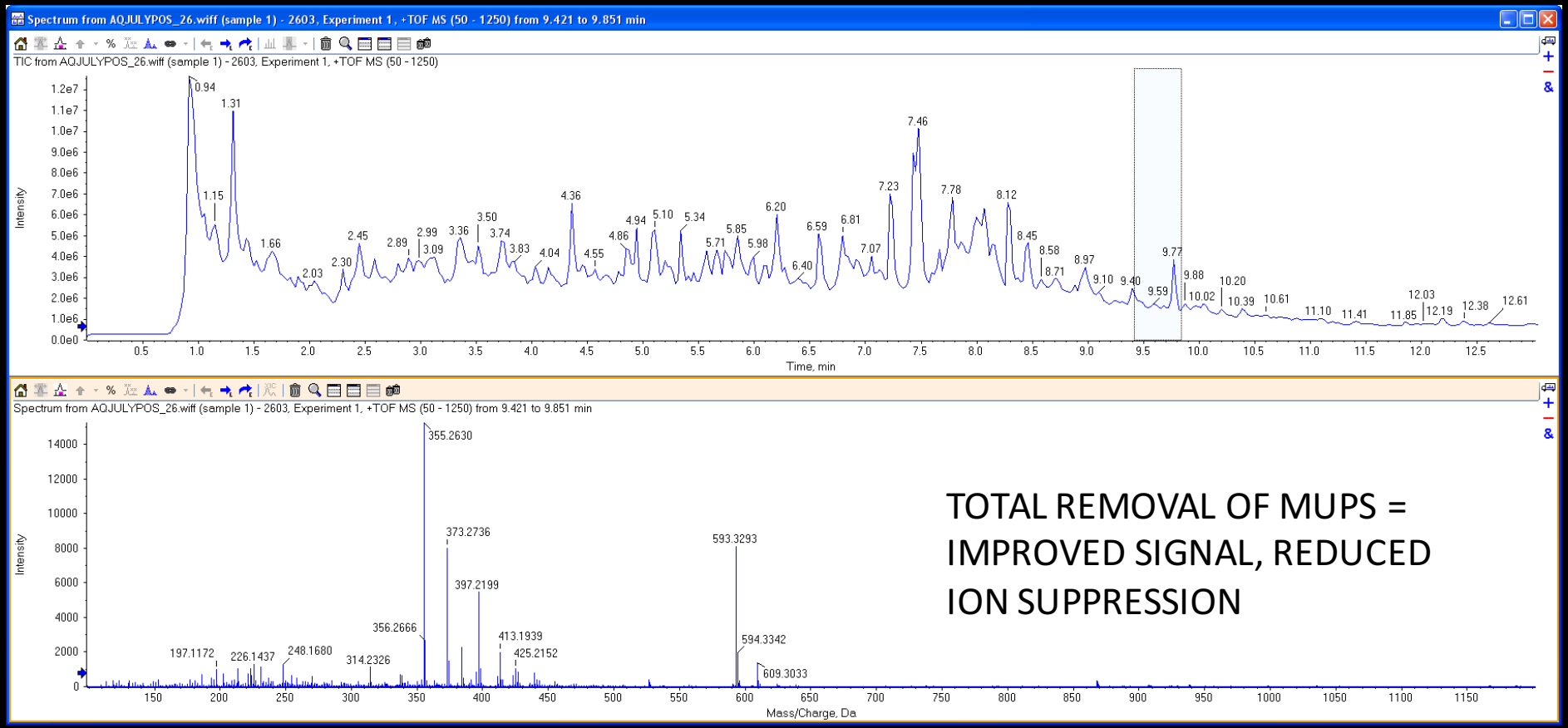
Mouse Urinary Proteins (MUPs)

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



Mouse Urinary Proteins

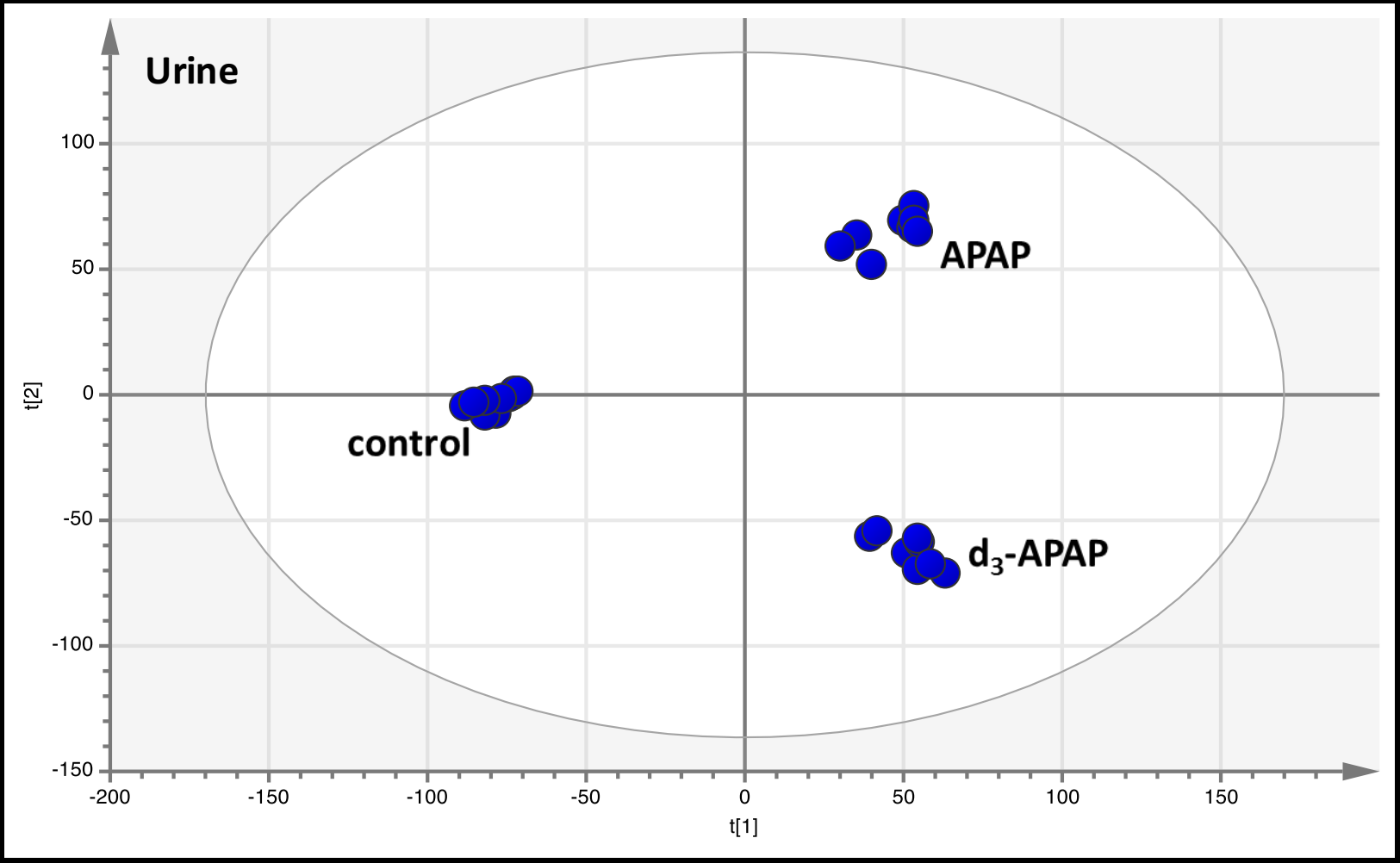
- Dilute equal volume of mouse urine with an equal volume of 100% methanol



TOTAL REMOVAL OF MUPS = IMPROVED SIGNAL, REDUCED ION SUPPRESSION

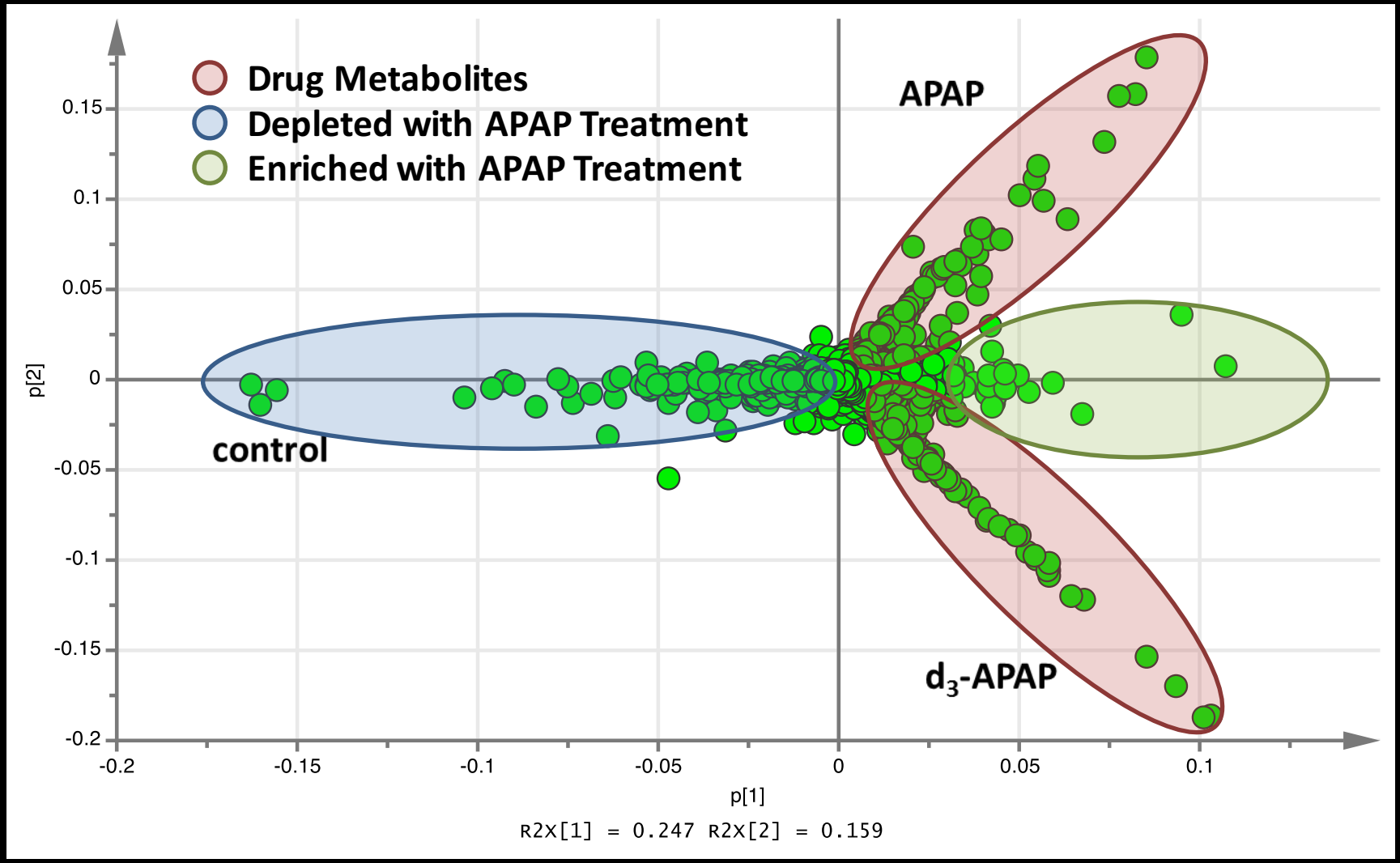
APAP Metabolism Study #4 Score Scatter Plot

PCA model

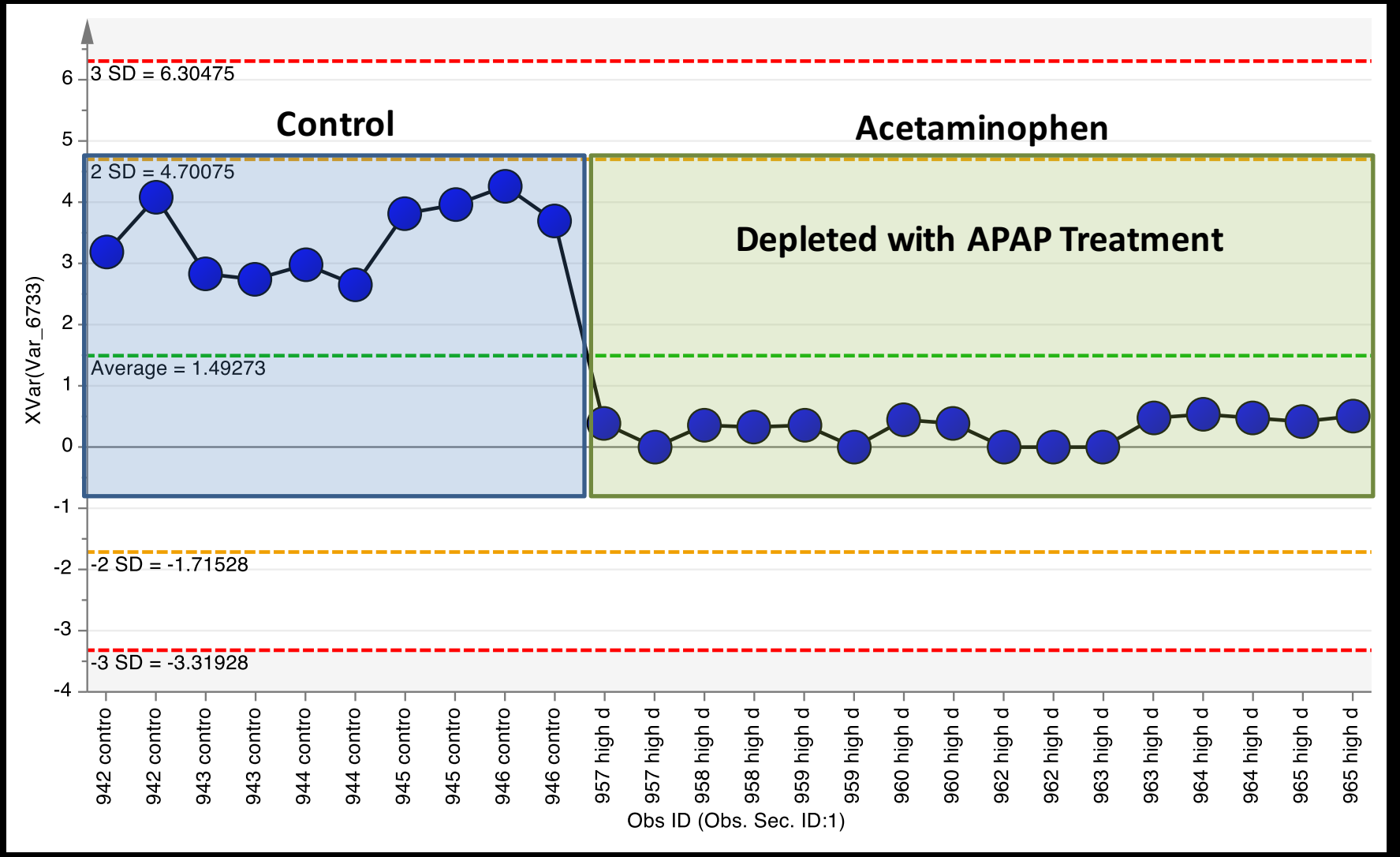


APAP Metabolism Loading Scatter Plot

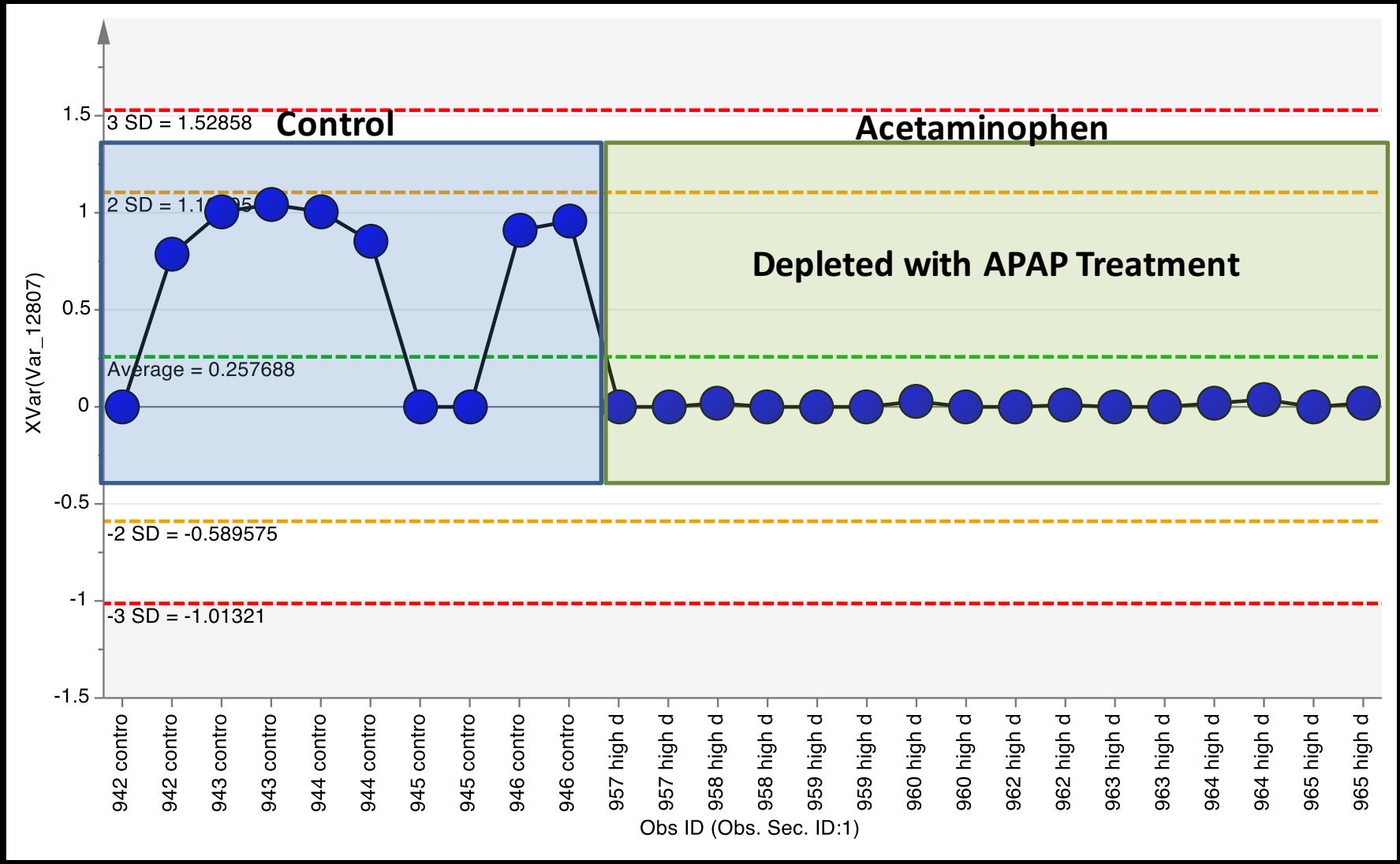
PCA Model



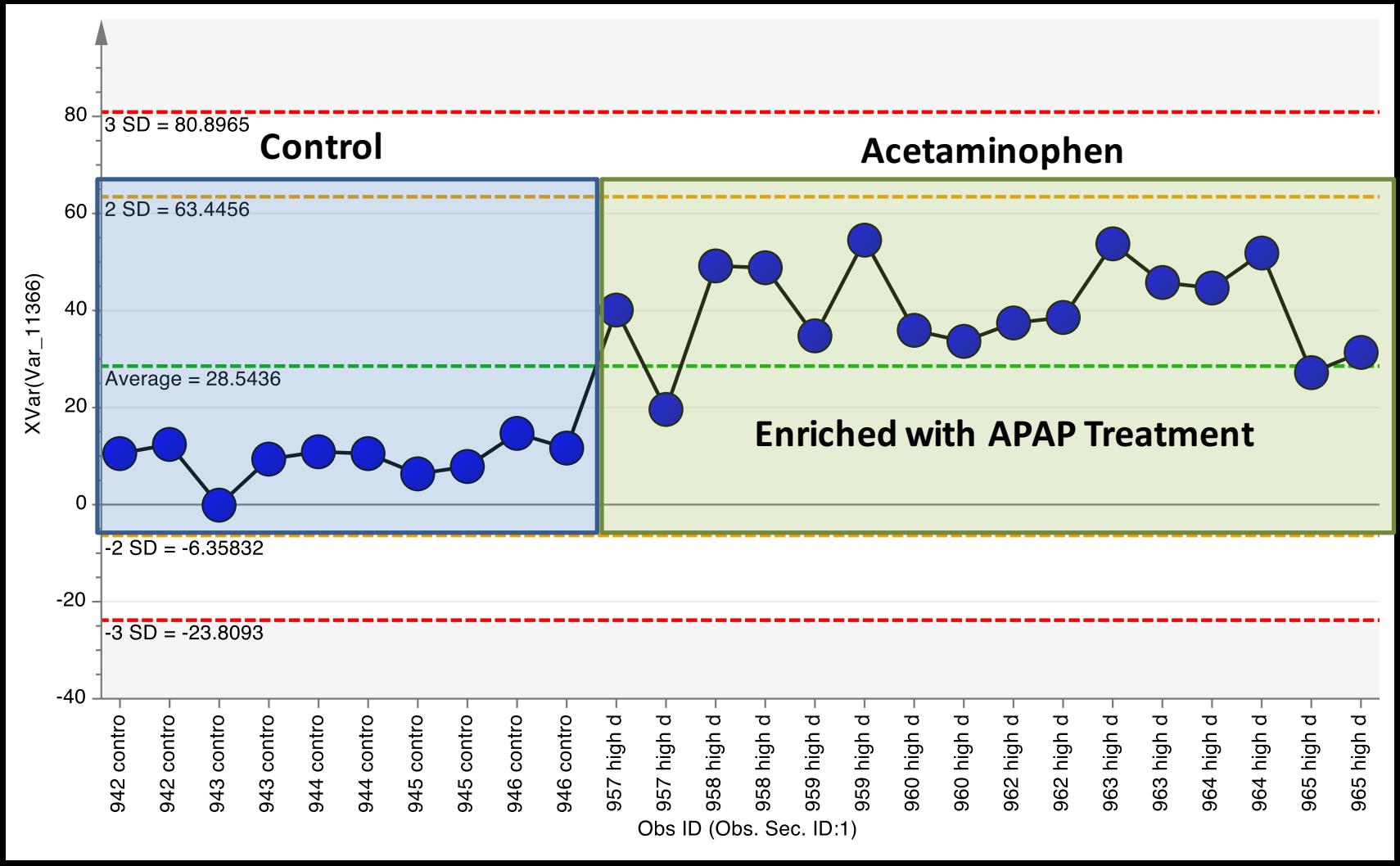
X-Variable Trend Plot for L-Carnitine (m/z=162.114+)



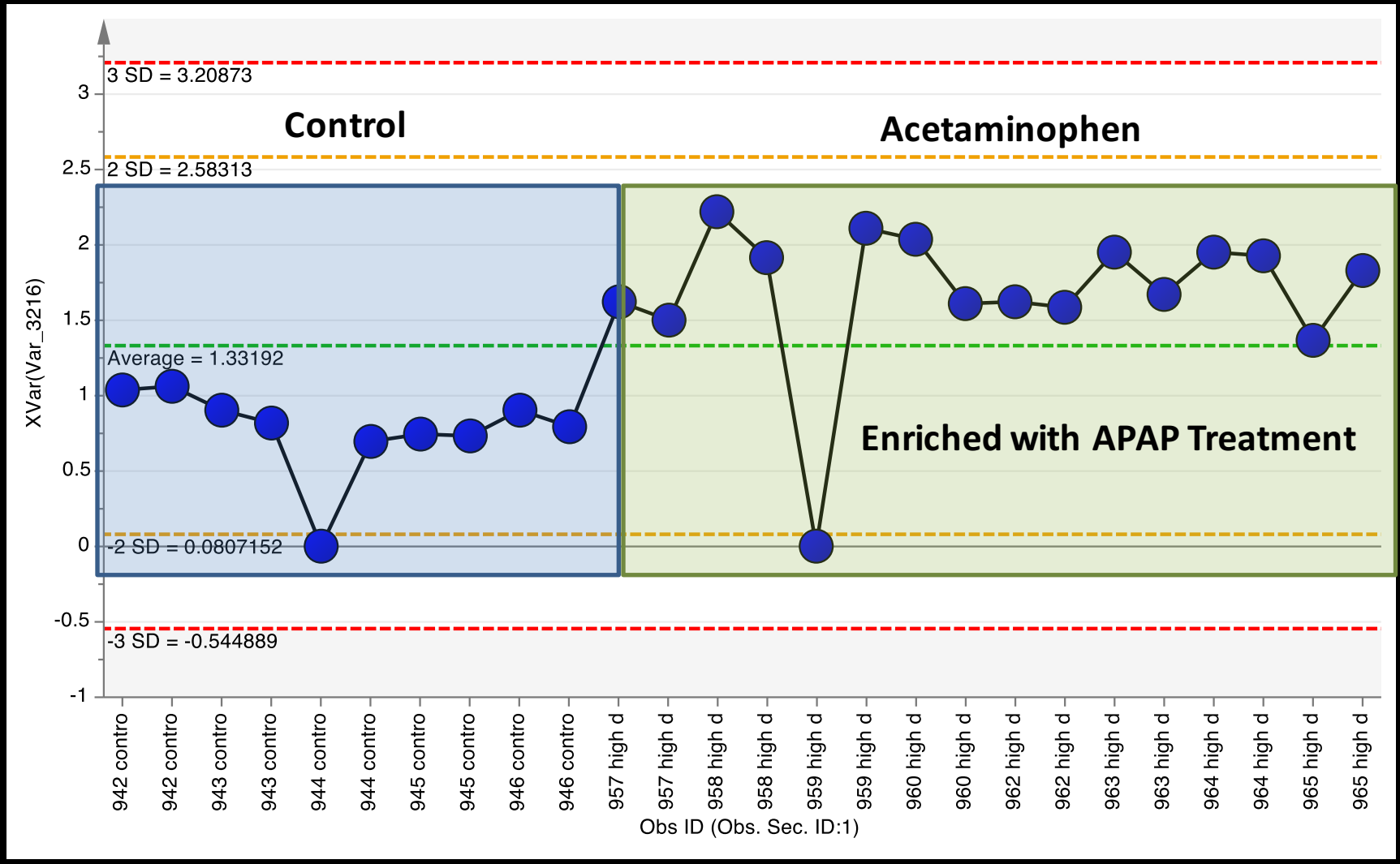
X-Variable Trend Plot for Propionylcarnitine (m/z=218.14+)



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



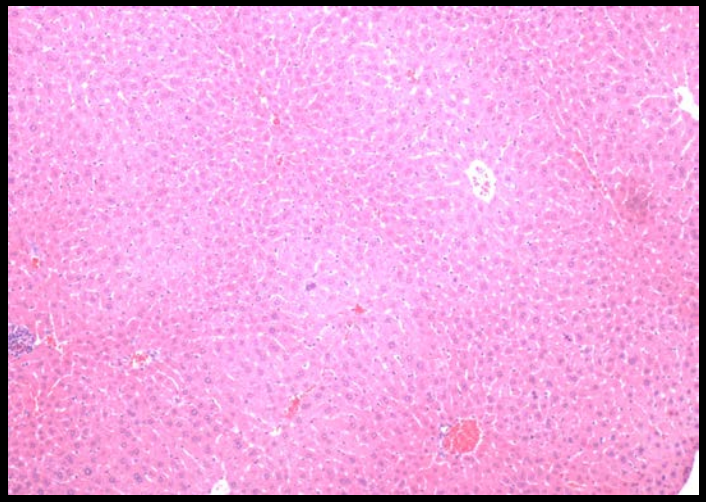
X-Variable Trend Plot for Decanoylcarnitine (m/z=316.247+)

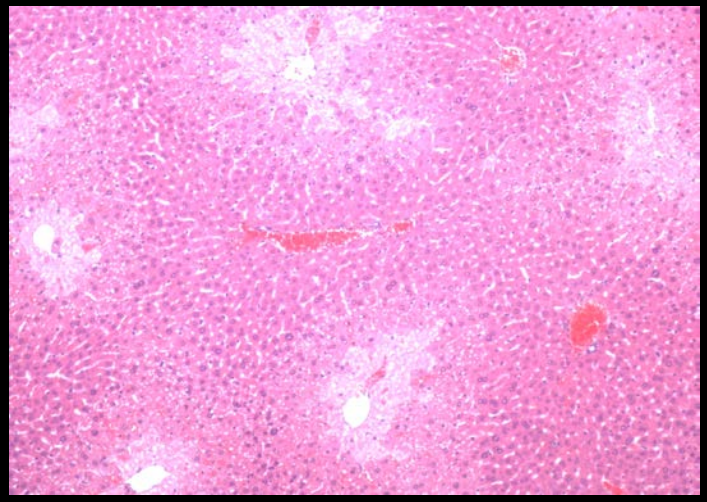


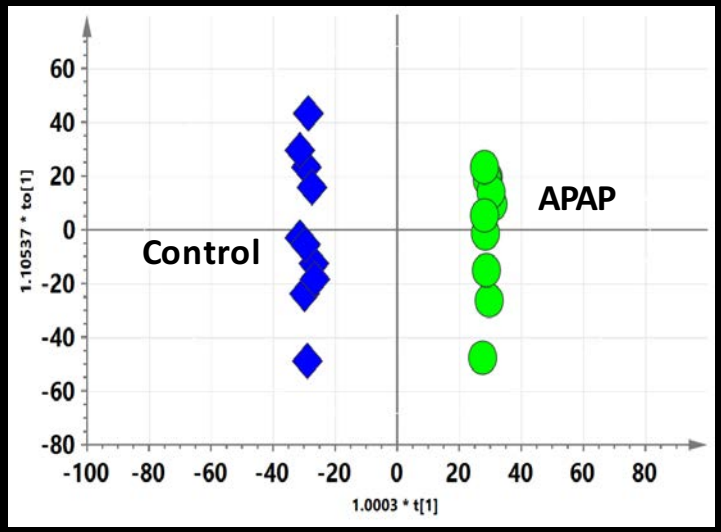
INFLUENCE OF EXTRACTION PROTOCOL – Carnitines and CoAs

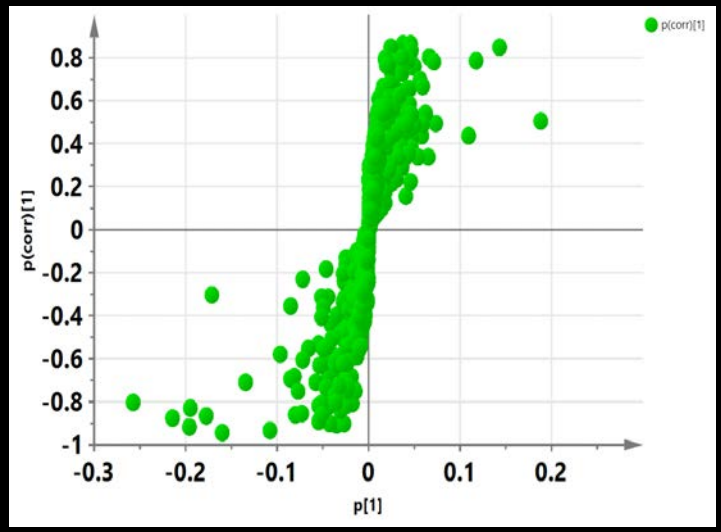
CONTROL

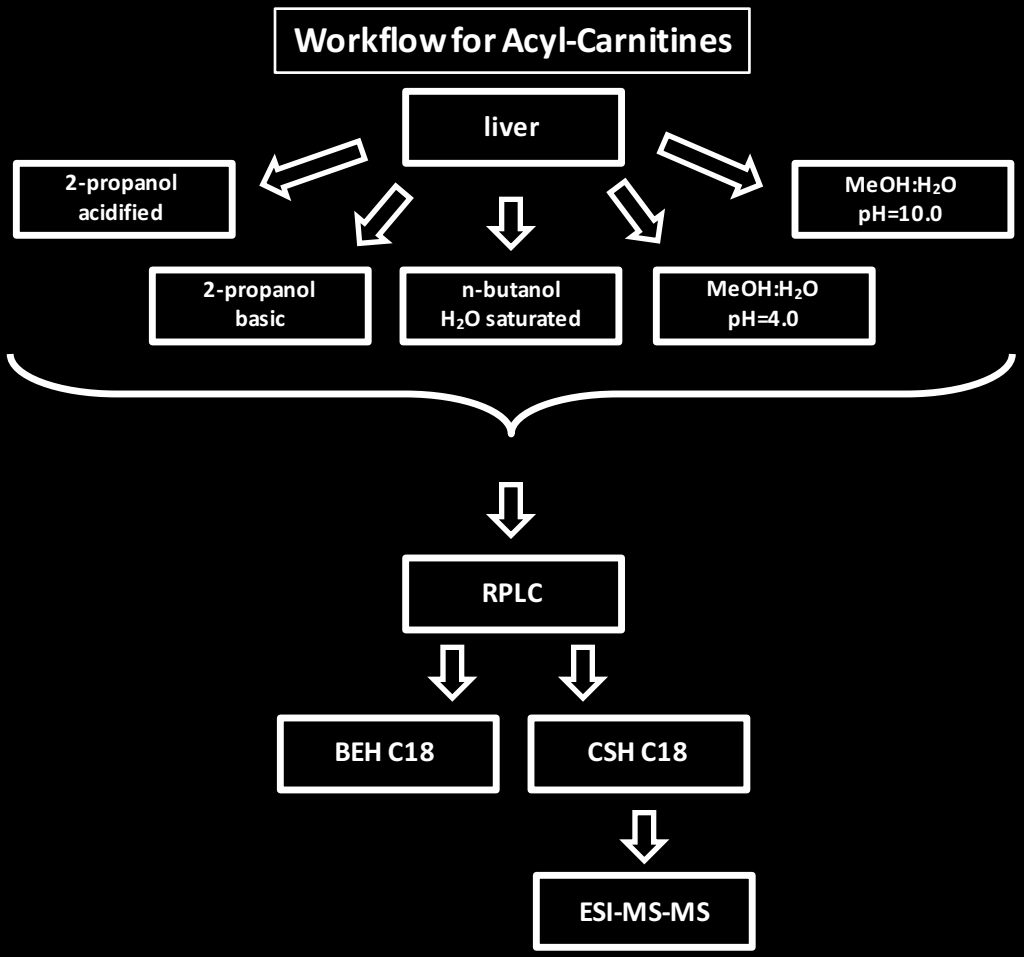
APAP



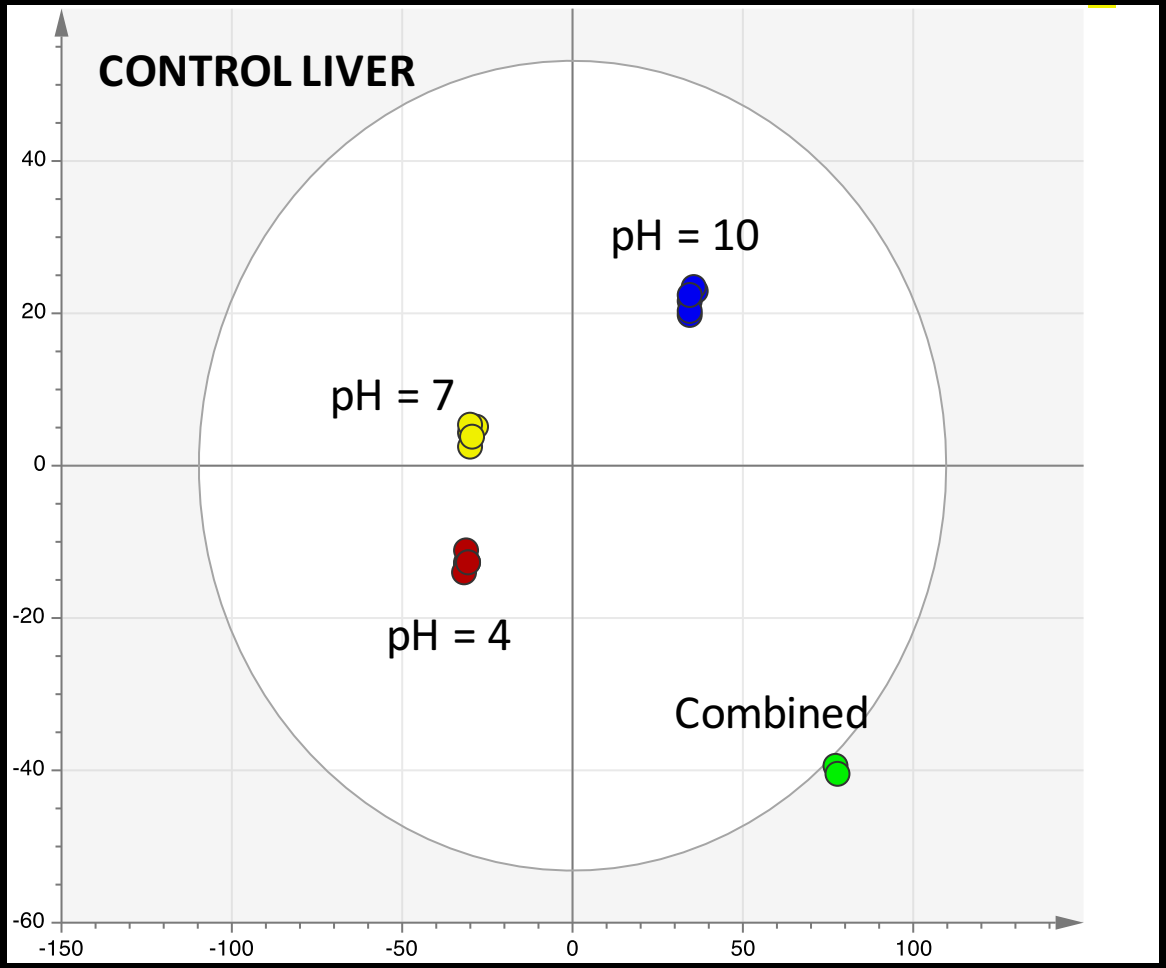







Workflow for Acyl-Carnitines
liver
2-propanol acidified
MeOH:H2O pH=10.0
2-propanol basic
n-butanol H2O saturated
MeOH:H2O pH=4.0
RPLC
BEH C18
CSH C18
ESI-MS-MS

Influence of pH on Metabolite Extraction from Mouse Liver



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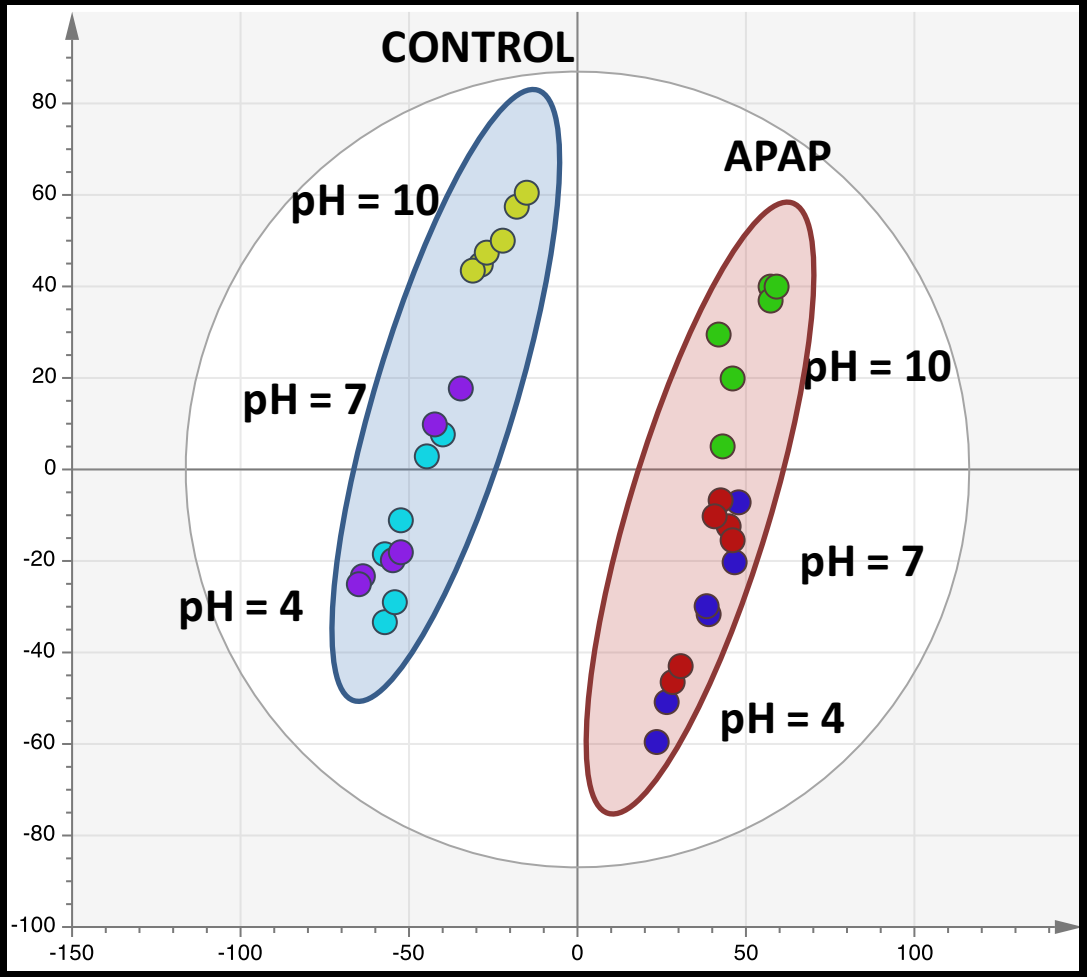
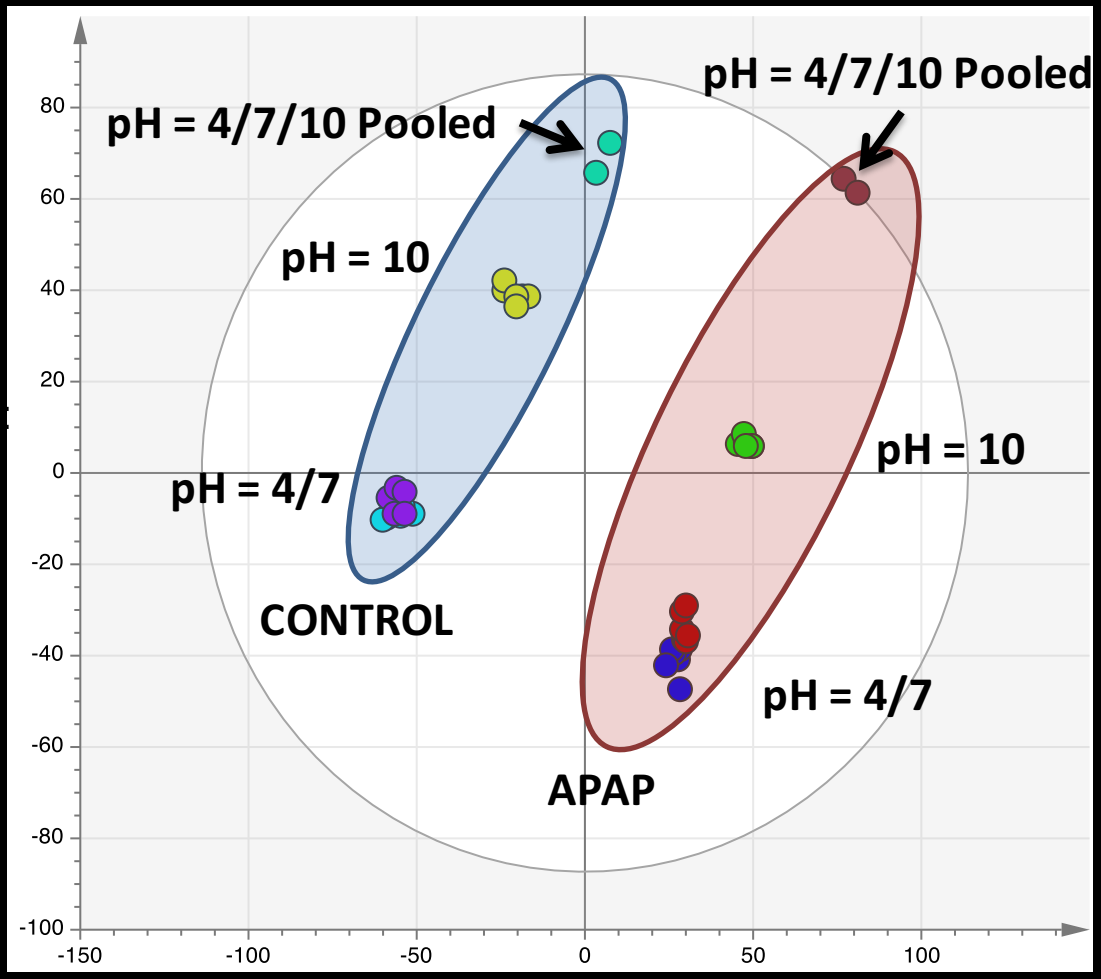
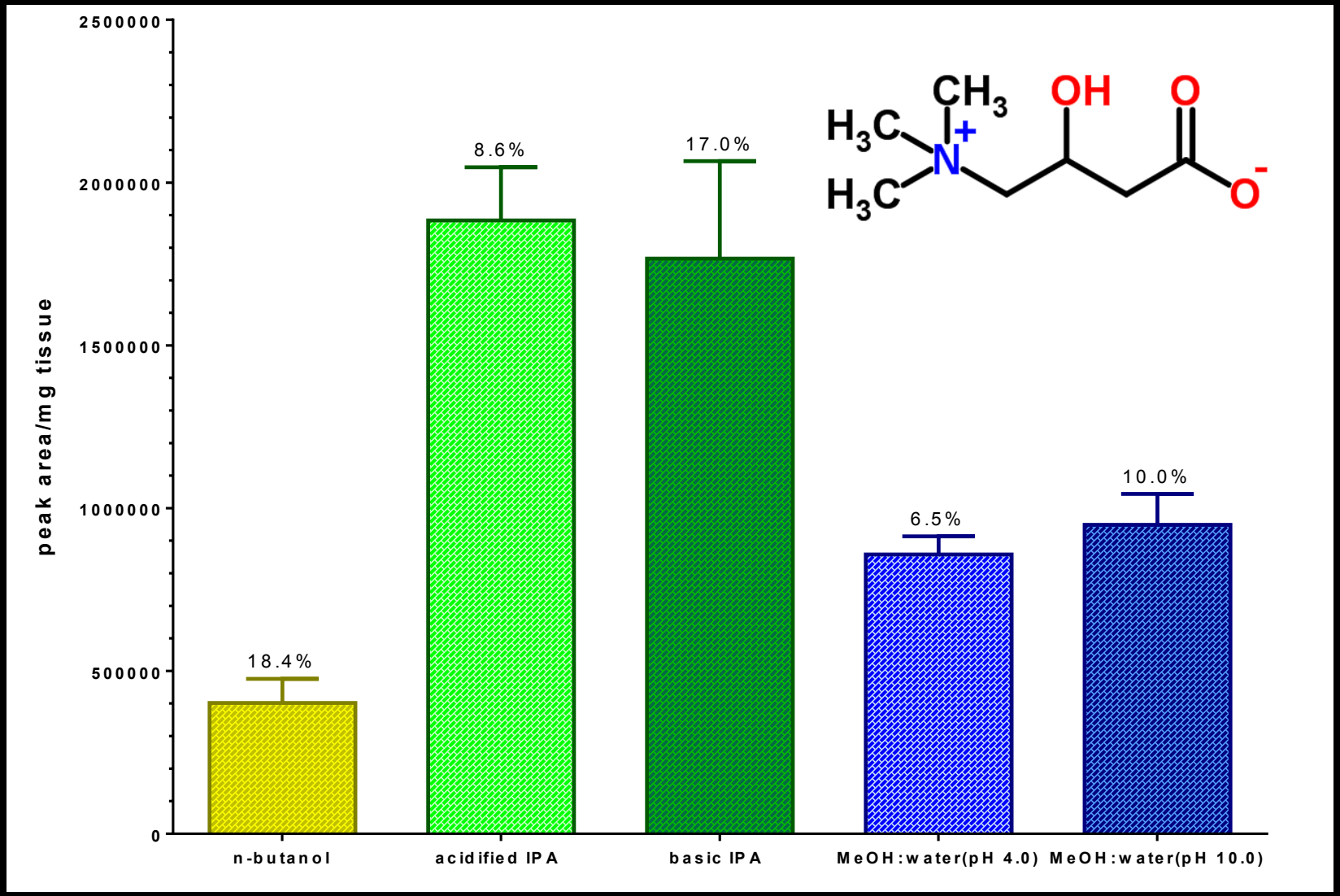


Influence of pH on Metabolite Extraction from Mouse Liver

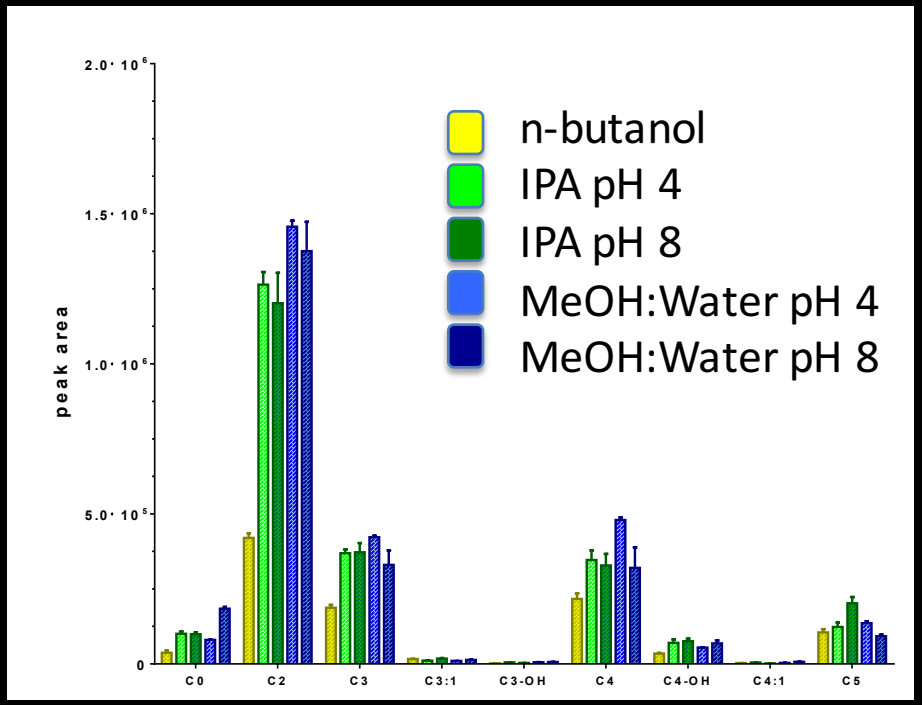


Extraction Efficiency of L-Carnitine from Mouse Liver

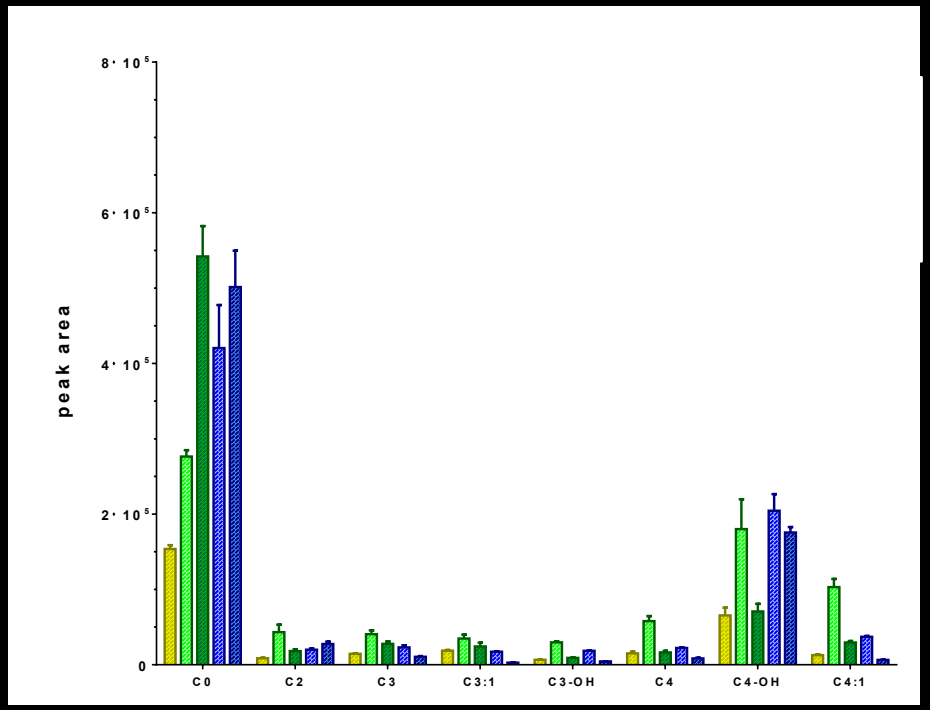


Matrix Effects and Extraction

LIVER



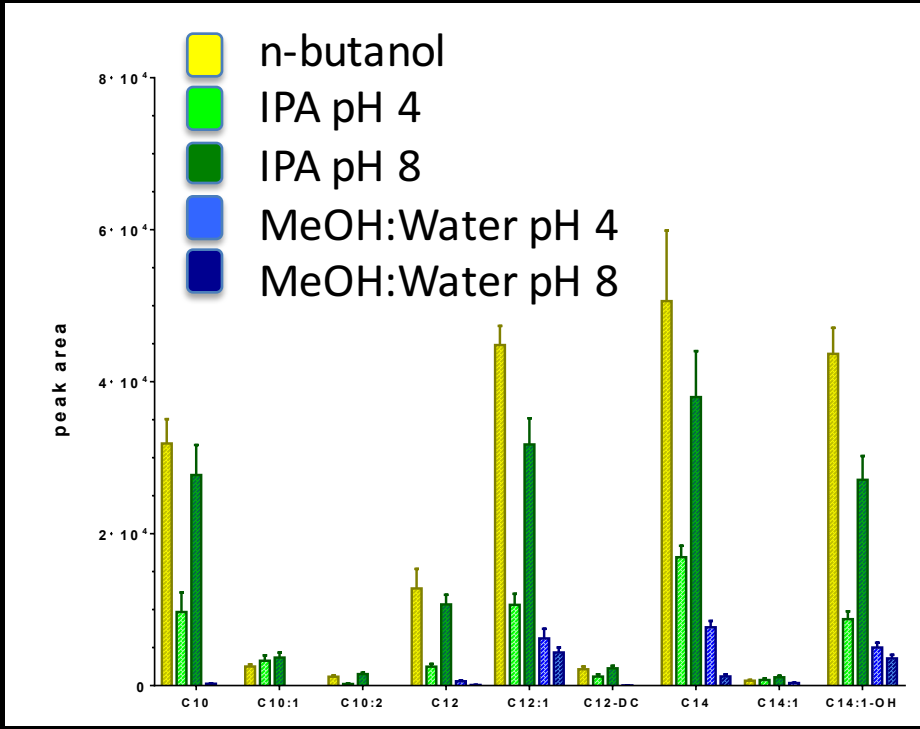
SERUM



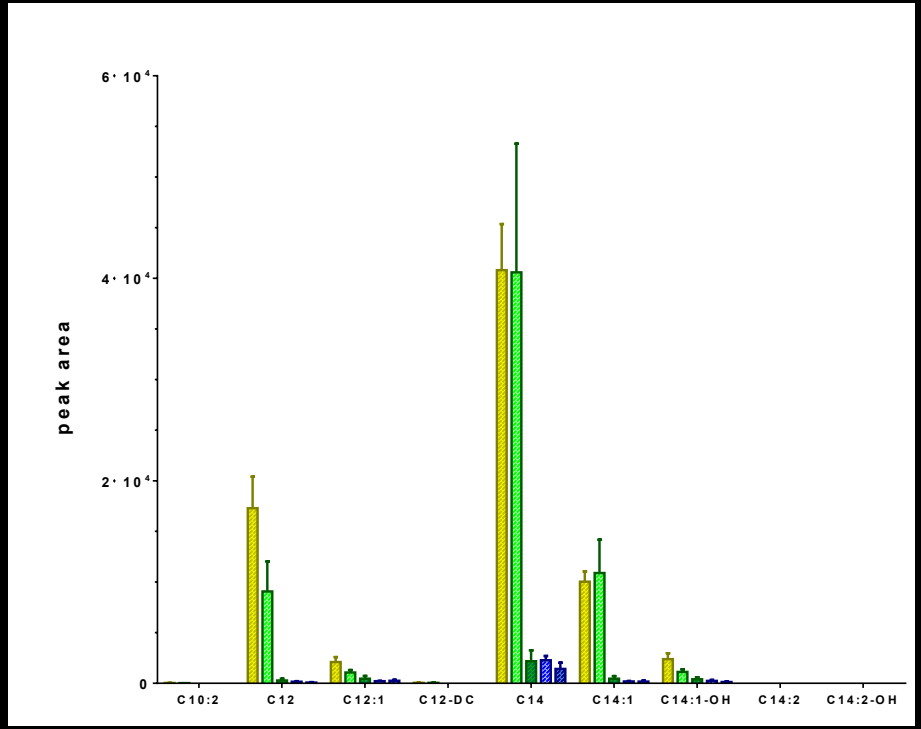
CARNITINES C0 – C5

Matrix Effects and Extraction

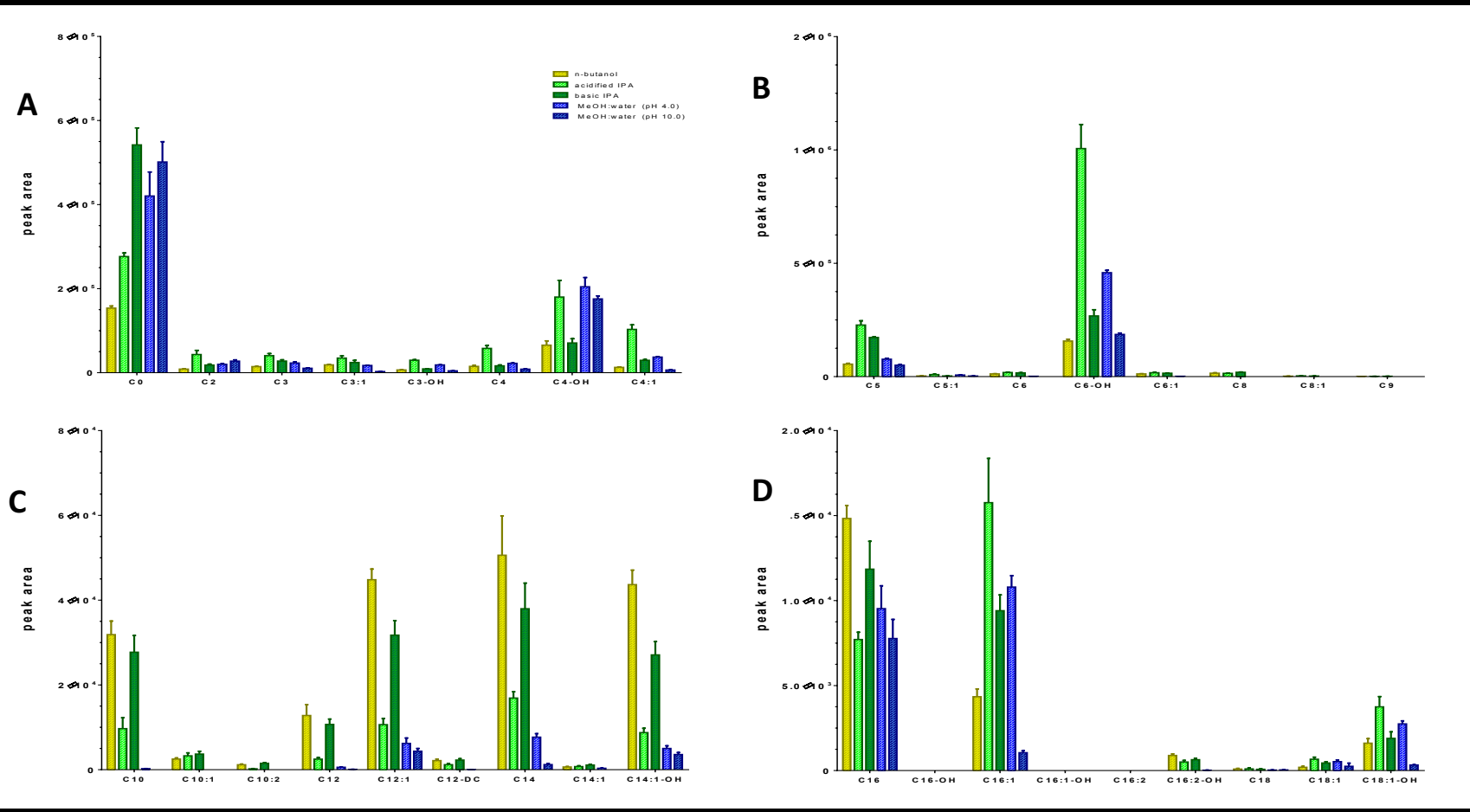
LIVER



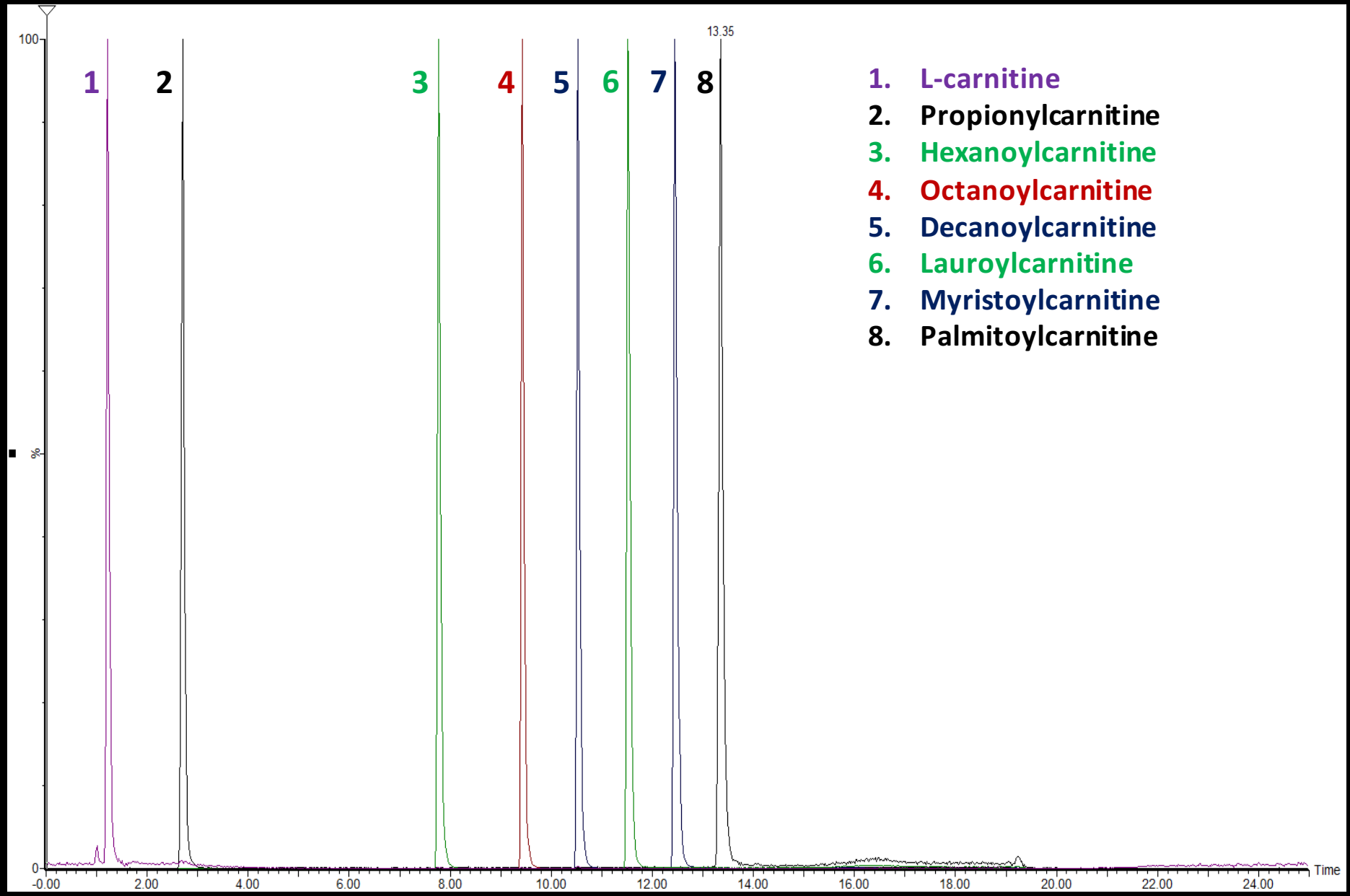
SERUM



CARNITINES C10 – C14

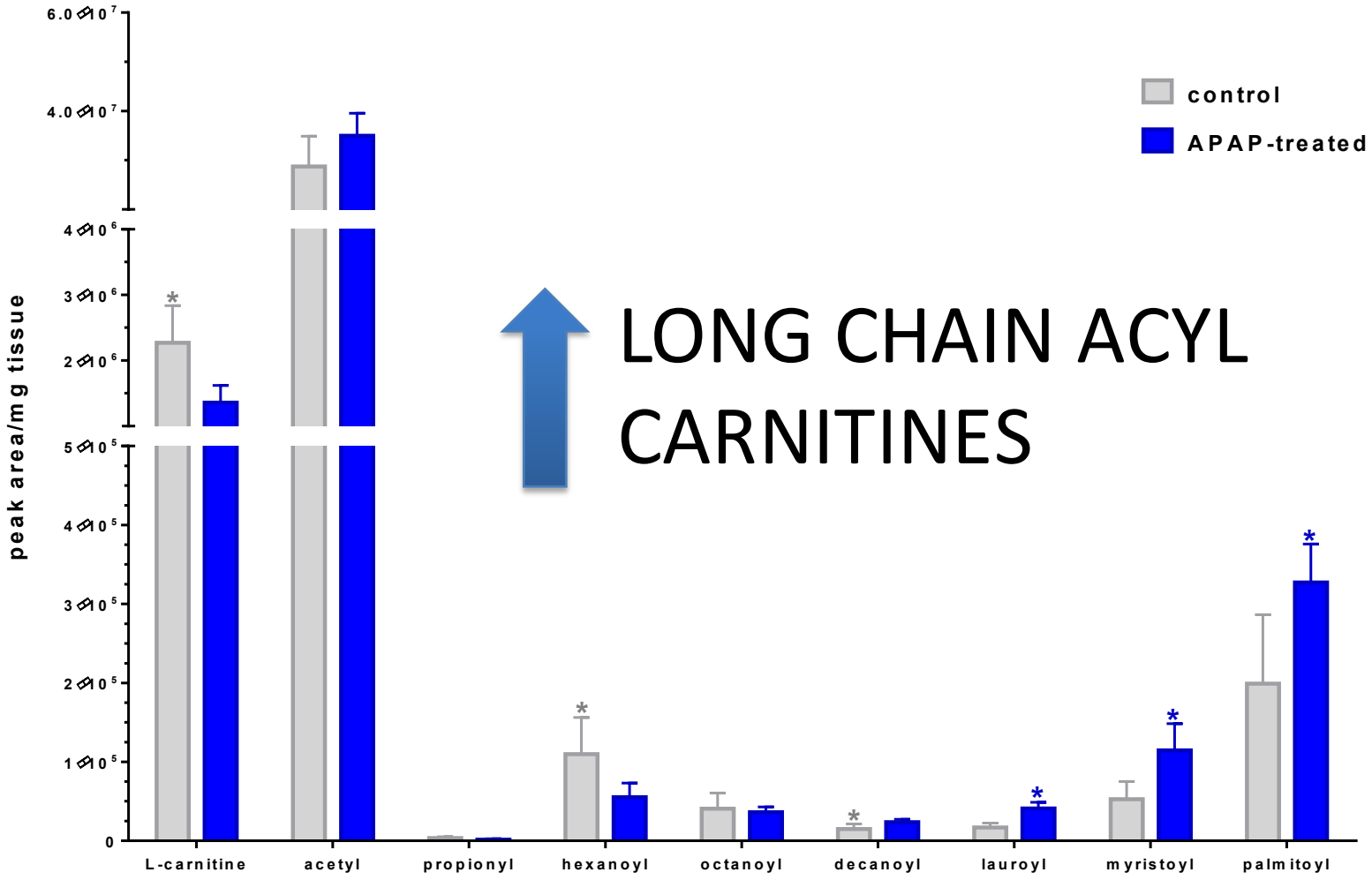

A
B
C
D
peak area
n-butanol
acidified IPA
basic IPA
MeOH:water (pH 4.0)
MeOH:water (pH 10.0)
C0
C2
C3
C3:1
C3-OH
C4
C4-OH
C4:1
C5
C5:1
C6
C6-OH
C6:1
C8
C8:1
C9
C10
C10:1
C10:2
C12
C12:1
C12-DC
C14
C14:1
C14:1-OH
C16
C16-OH
C16:1
C16:1-OH
C16:2
C16:2-OH
C18
C18:1
C18:1-OH

Resolution of Acyl Carnitine Standards by RPLC on Waters BEH C18

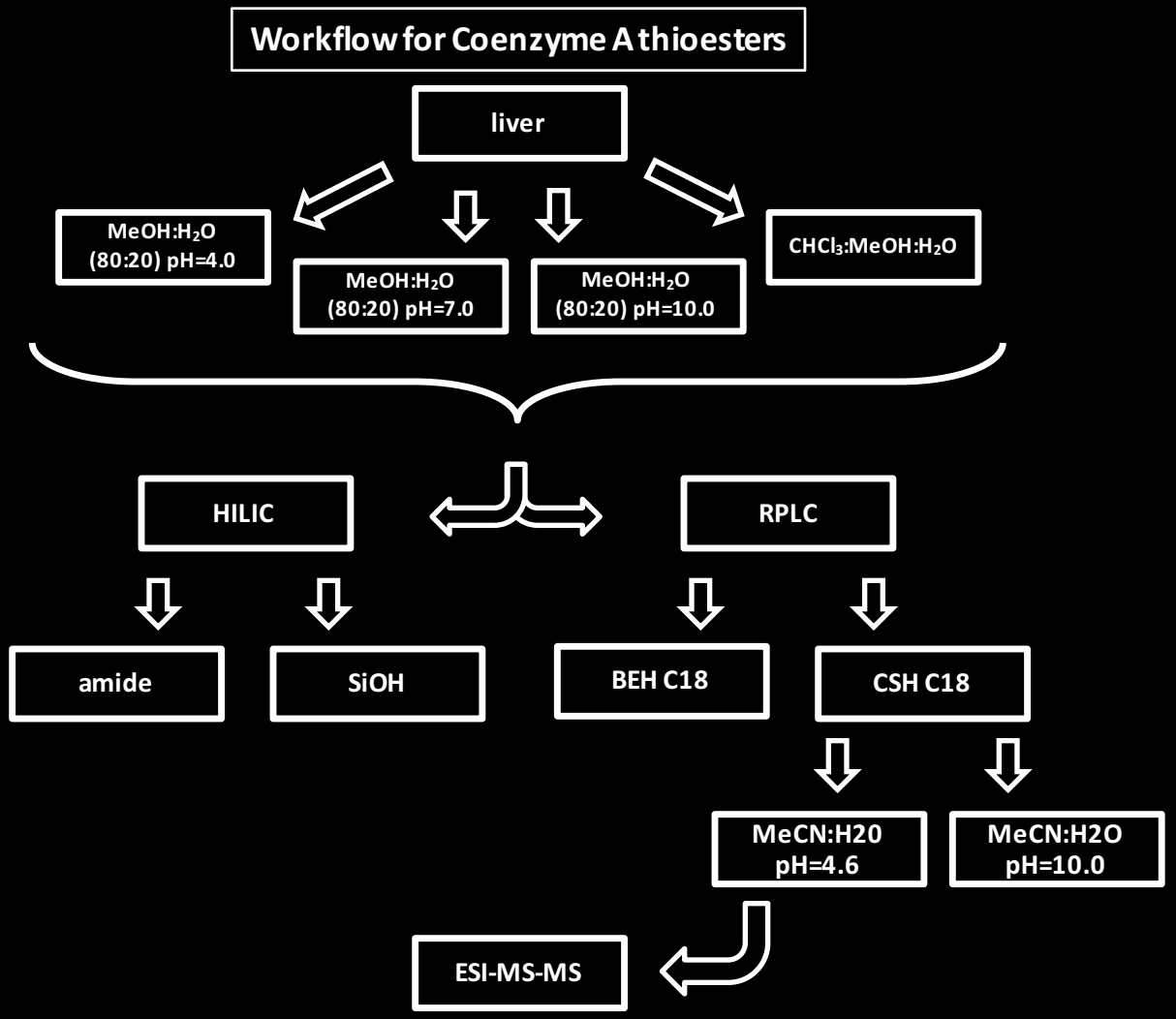


1. L-carnitine
2. Propionylcarnitine
3. Hexanoylcarnitine
4. Octanoylcarnitine
5. Decanoylcarnitine
6. Lauroylcarnitine
7. Myristoylcarnitine
8. Palmitoylcarnitine

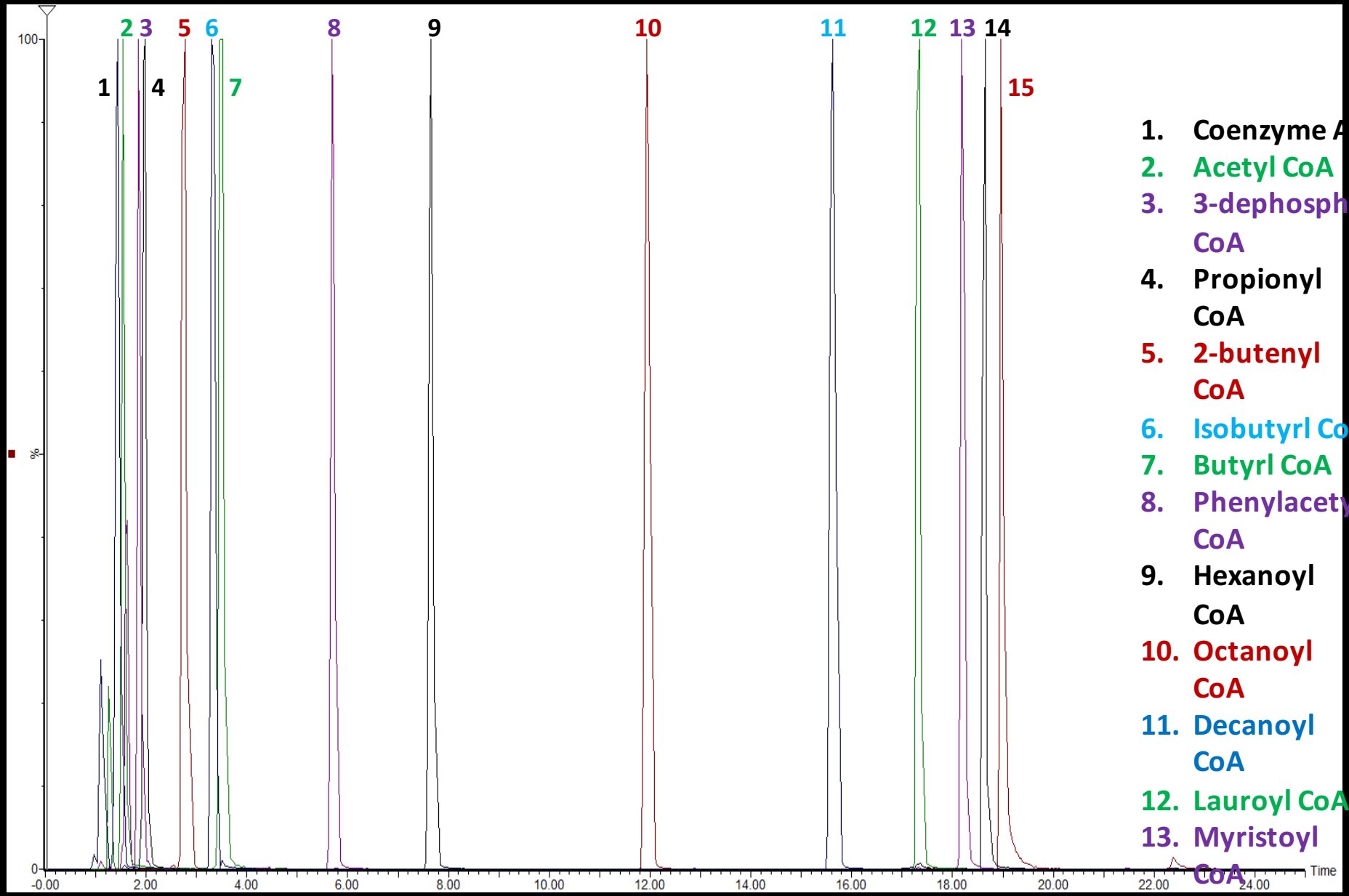
Acylcarnitine Extraction in Acidified IPA

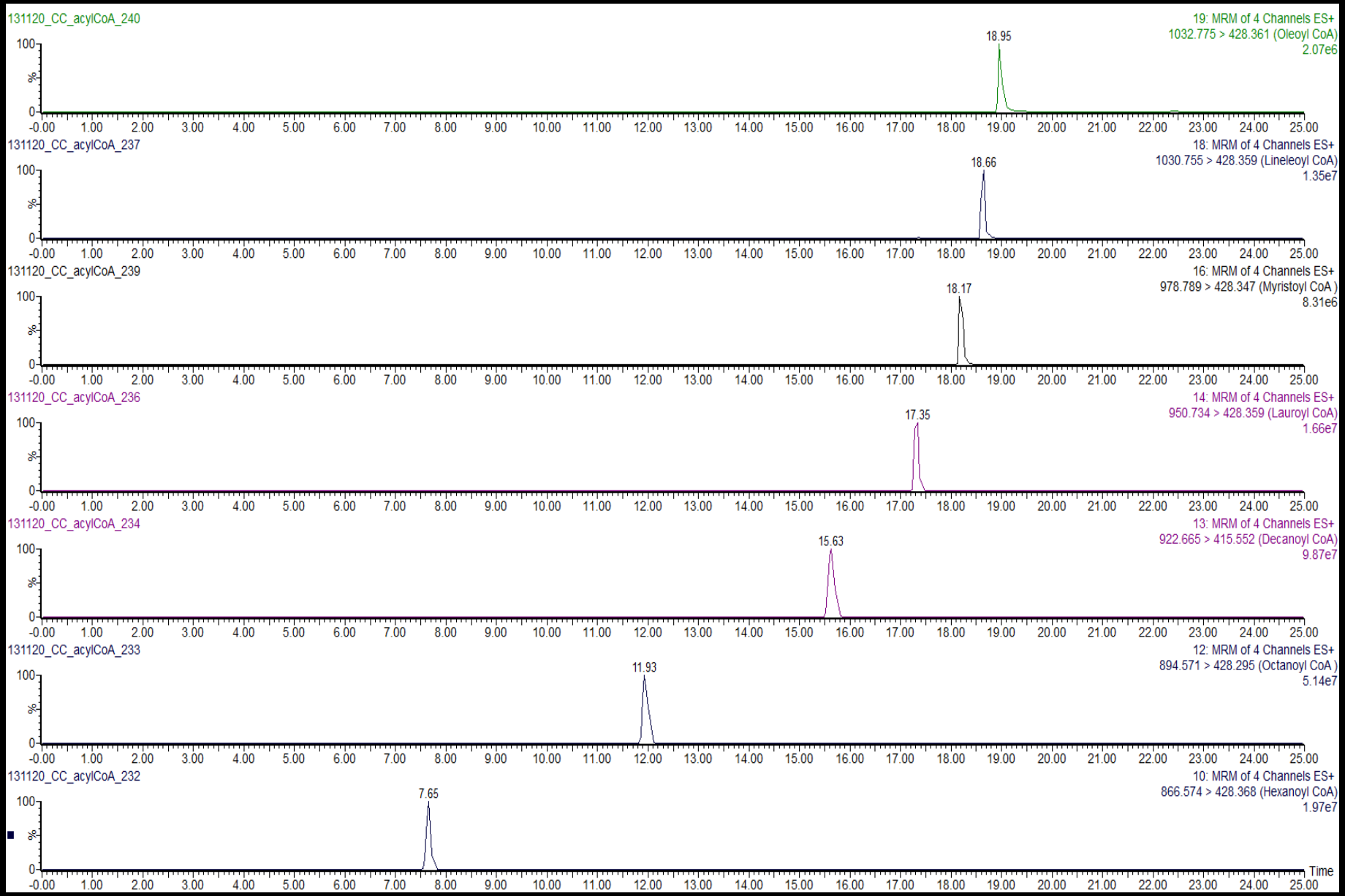


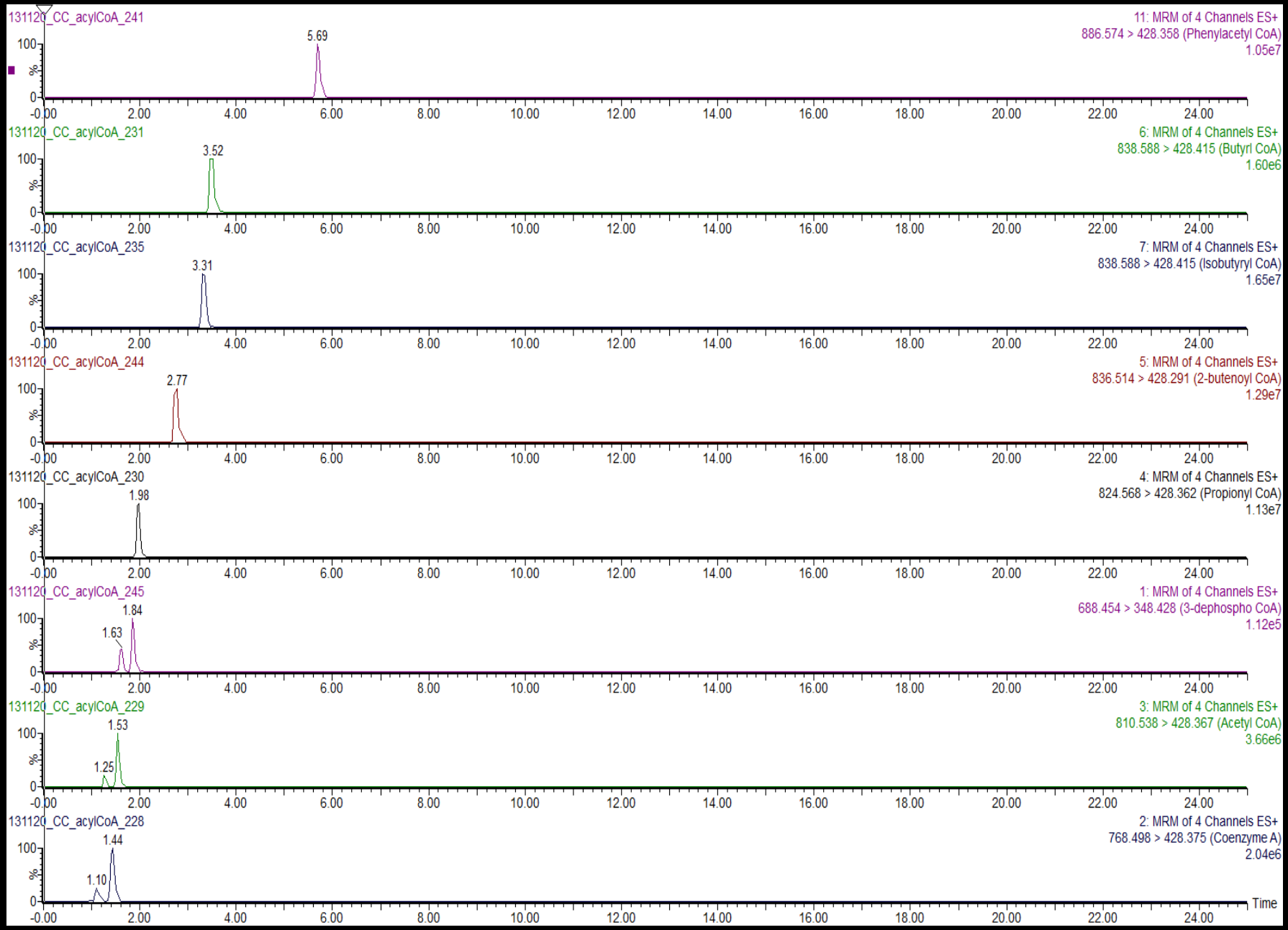
Increasing Chain Length

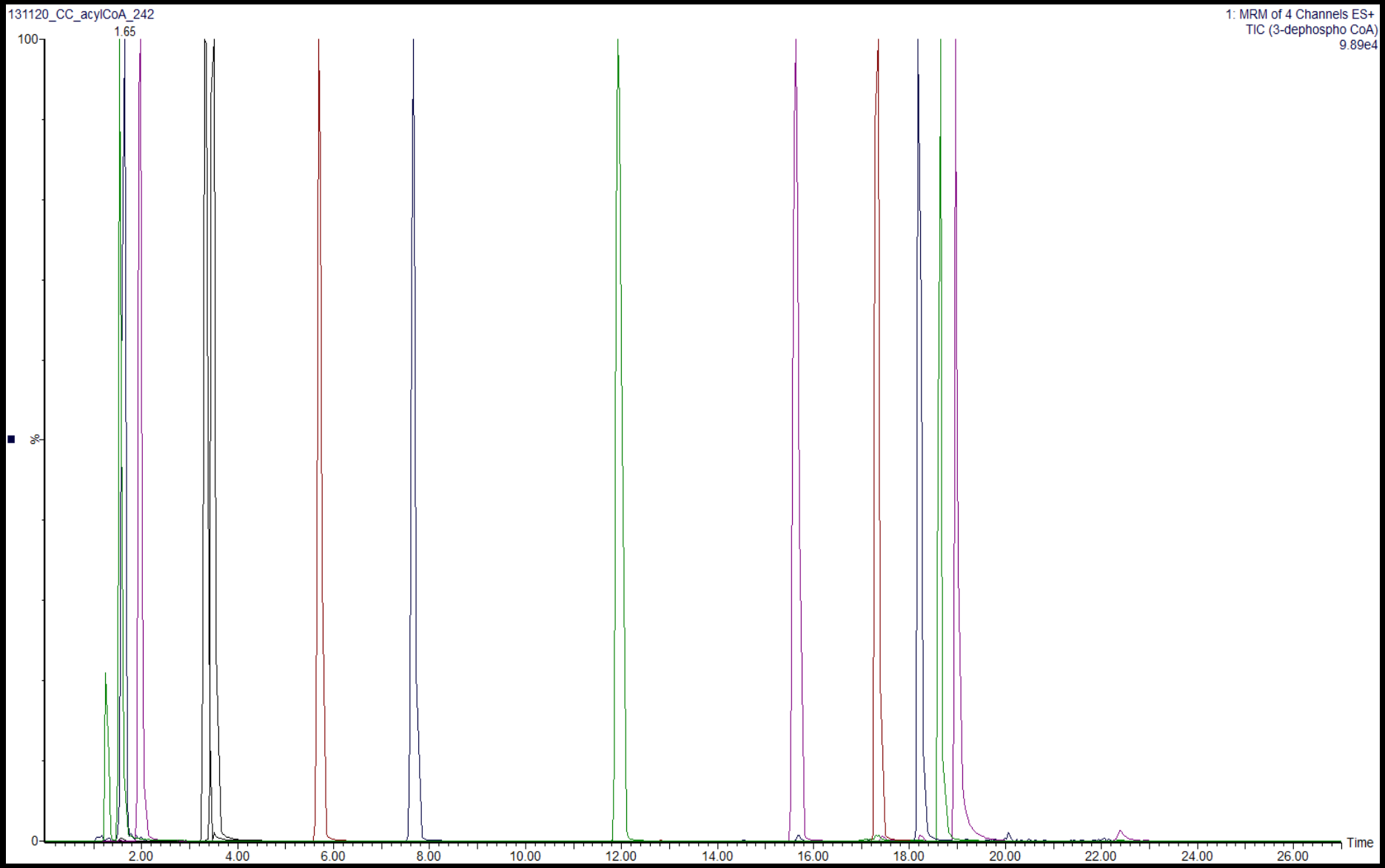

Workflow for Coenzyme A thioesters
liver
MeOH:H2O (80:20) pH=4.0
MeOH:H2O (80:20) pH=7.0
MeOH:H2O (80:20) pH=10.0
CHCl3:MeOH:H2O
HILIC
RPLC
amide
SiOH
BEH C18
CSH C18
MeCN:H20 pH=4.6
MeCN:H2O pH=10.0
ESI-MS-MS

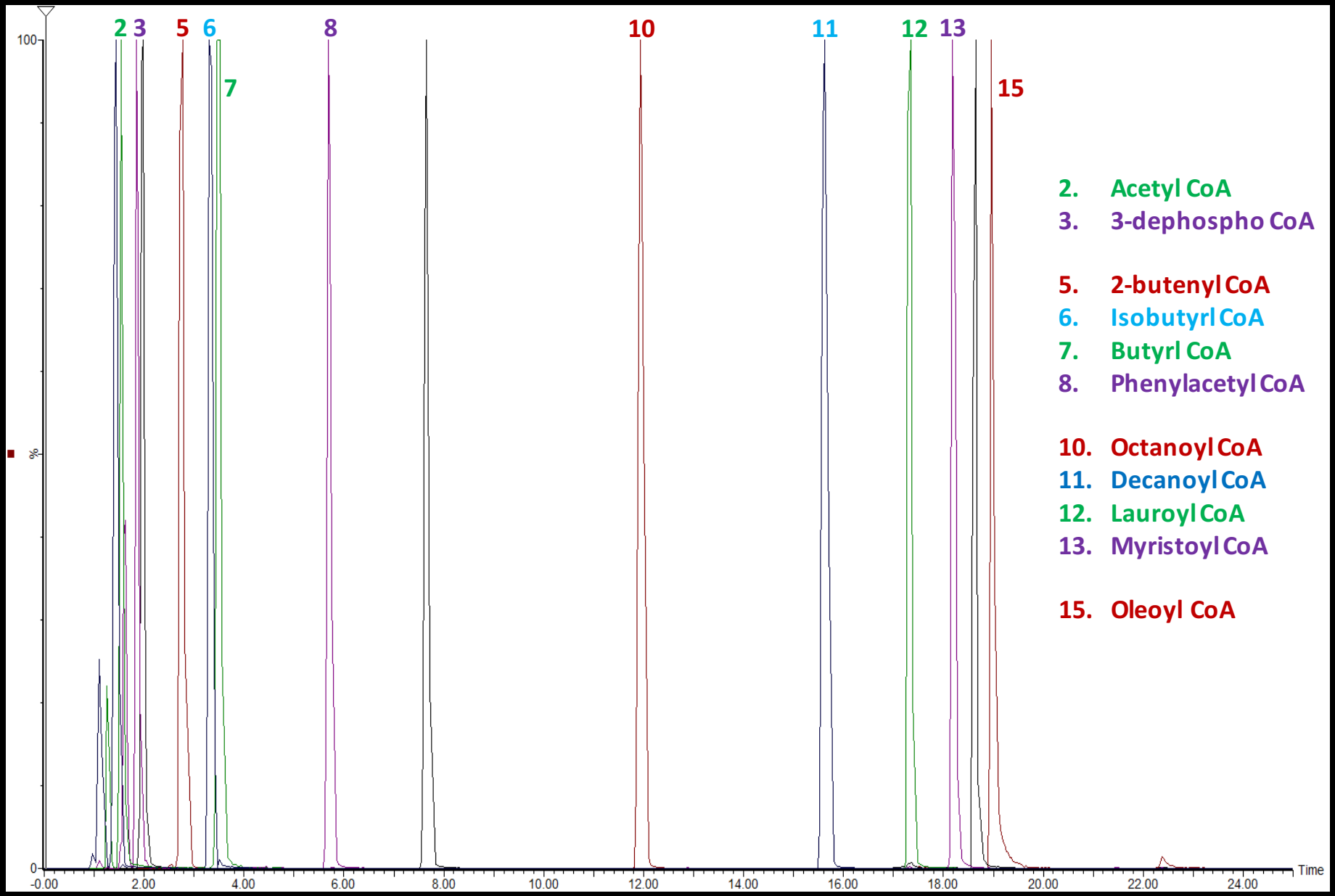
Resolution of Coenzyme A (CoA) Thioester Metabolites by RPLC using Waters BEH C18



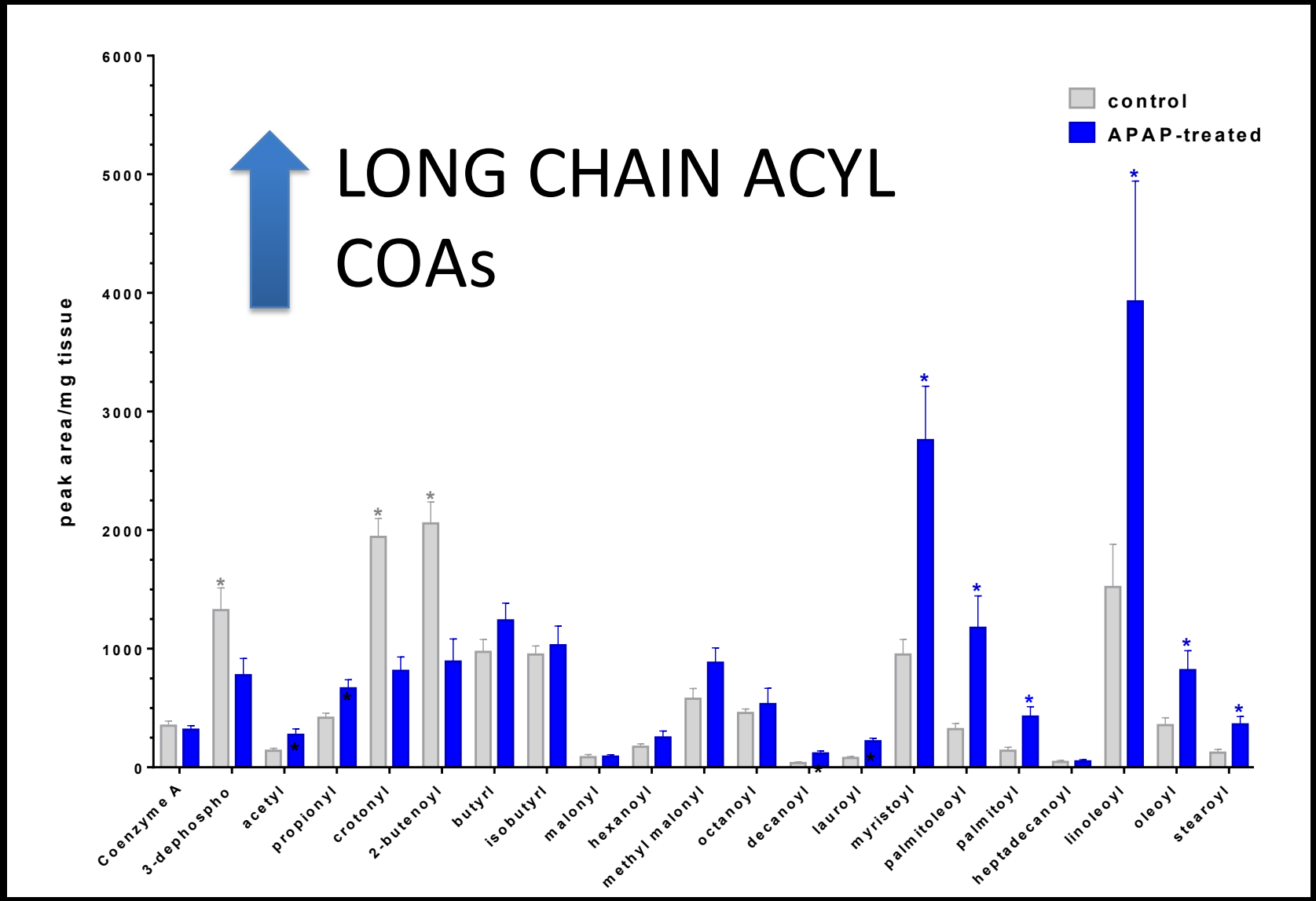

131120_CC_acylCoA_240
19: MRM of 4 Channels ES+
1032.775 > 428.361 (Oleoyl CoA)
2.07e6
18.95
131120_CC_acylCoA_237
18: MRM of 4 Channels ES+
1030.755 > 428.359 (Lineleoyl CoA)
1.35e7
18.66
131120_CC_acylCoA_239
16: MRM of 4 Channels ES+
978.789 > 428.347 (Myristoyl CoA)
8.31e6
18.17
131120_CC_acylCoA_236
14: MRM of 4 Channels ES+
950.734 > 428.359 (Lauroyl CoA)
1.66e7
17.35
131120_CC_acylCoA_234
13: MRM of 4 Channels ES+
922.665 > 415.552 (Decanoyl CoA)
9.87e7
15.63
131120_CC_acylCoA_233
12: MRM of 4 Channels ES+
894.571 > 428.295 (Octanoyl CoA)
5.14e7
11.93
131120_CC_acylCoA_232
10: MRM of 4 Channels ES+
866.574 > 428.368 (Hexanoyl CoA)
1.97e7
7.65
100
%
0
-0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.00 20.00 21.00 22.00 23.00 24.00 25.00
Time


131120_CC_acylCoA_241
11: MRM of 4 Channels ES+
886.574 > 428.358 (Phenylacetyl CoA)
1.05e7
5.69
131120_CC_acylCoA_231
6: MRM of 4 Channels ES+
838.588 > 428.415 (Butyrl CoA)
1.60e6
3.52
131120_CC_acylCoA_235
7: MRM of 4 Channels ES+
838.588 > 428.415 (Isobutyryl CoA)
1.65e7
3.31
131120_CC_acylCoA_244
5: MRM of 4 Channels ES+
836.514 > 428.291 (2-butenoyl CoA)
1.29e7
2.77
131120_CC_acylCoA_230
4: MRM of 4 Channels ES+
824.568 > 428.362 (Propionyl CoA)
1.13e7
1.98
131120_CC_acylCoA_245
1: MRM of 4 Channels ES+
688.454 > 348.428 (3-dephospho CoA)
1.12e5
1.84
1.63
131120_CC_acylCoA_229
3: MRM of 4 Channels ES+
810.538 > 428.367 (Acetyl CoA)
3.66e6
1.53
1.25
131120_CC_acylCoA_228
2: MRM of 4 Channels ES+
768.498 > 428.375 (Coenzyme A)
2.04e6
1.44
1.10
%
100
0
-0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
Time


131120_CC_acylCoA_242
1.65
1: MRM of 4 Channels ES+
TIC (3-dephospho CoA)
9.89e4
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
Time


2 3 5 6 8 10 11 12 13
7
15
100
%
0
-0.00 2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00
Time
2. Acetyl CoA
3. 3-dephospho CoA
5. 2-butenyl CoA
6. Isobutyrl CoA
7. Butyrl CoA
8. Phenylacetyl CoA
10. Octanoyl CoA
11. Decanoyl CoA
12. Lauroyl CoA
13. Myristoyl CoA
15. Oleoyl CoA

AcylCoa Extraction via Modified Bligh/Dyer



Increasing Chain Length

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.

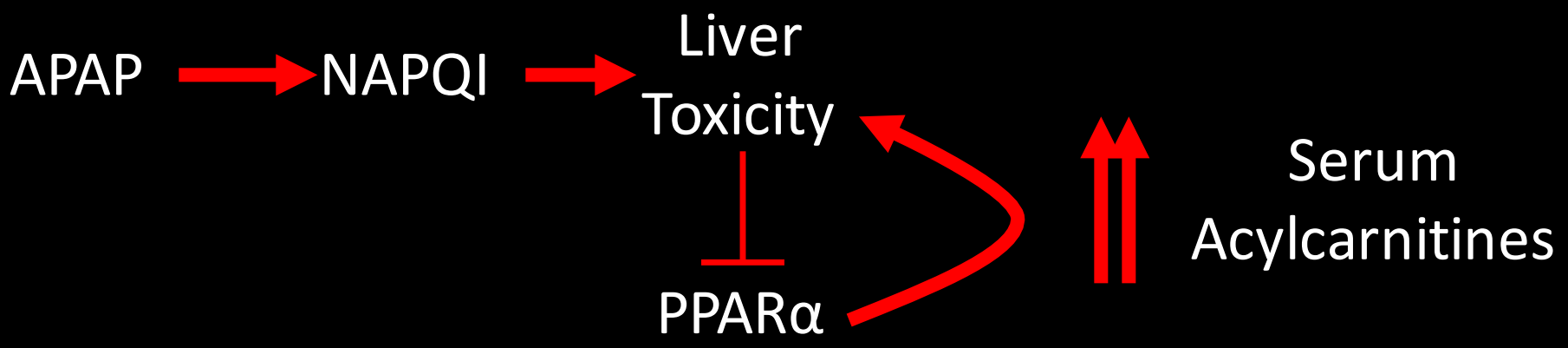
Identification of novel toxicity-associated metabolites by metabolomics and mass isotopomer analysis of acetaminophen metabolism in wild-type and Cyp2e1-null mice.

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

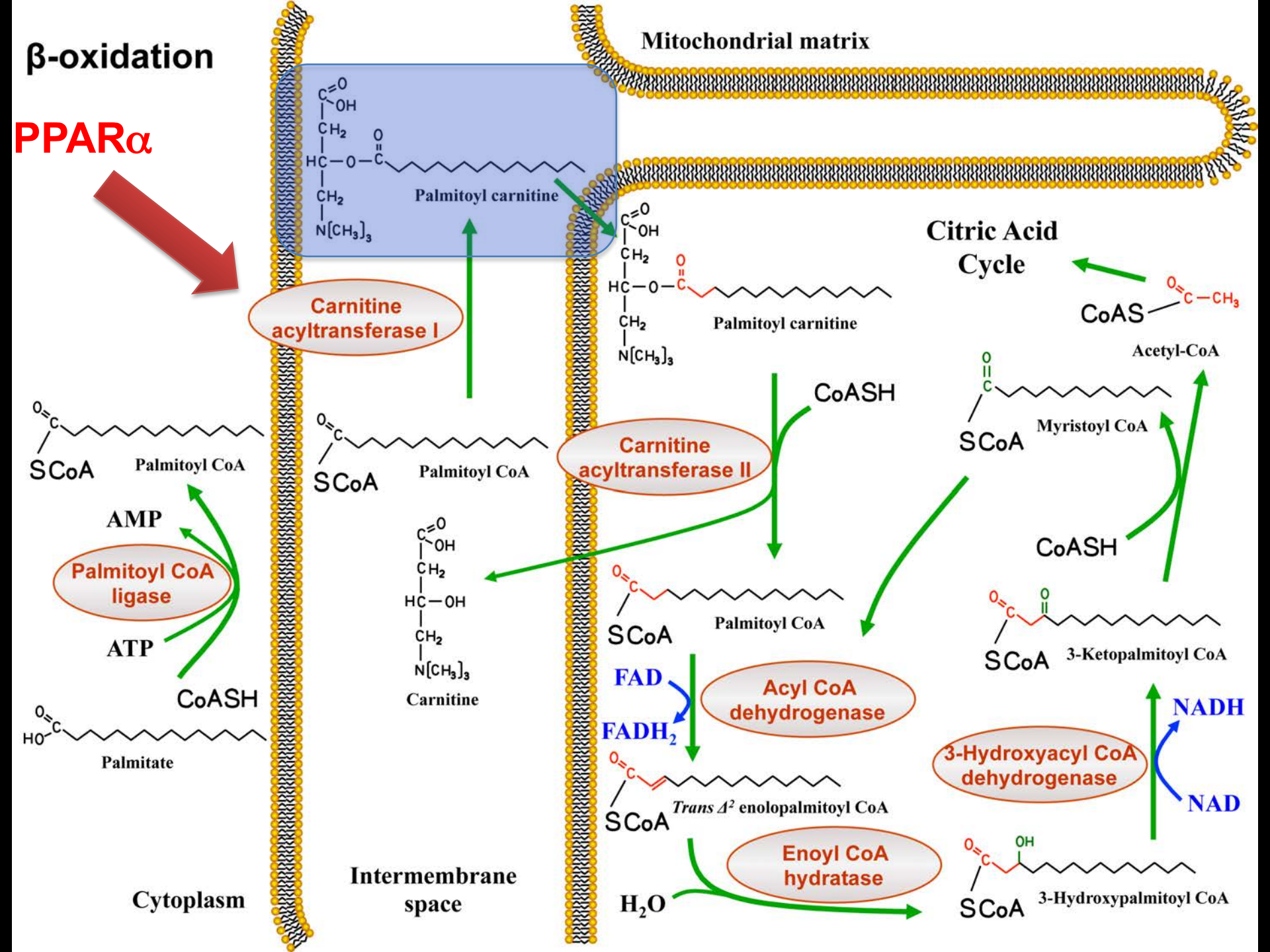
Hepatology. 2012 Jul;56(1):281-90. doi: 10.1002/hep.25645. Epub 2012 Jun 6.

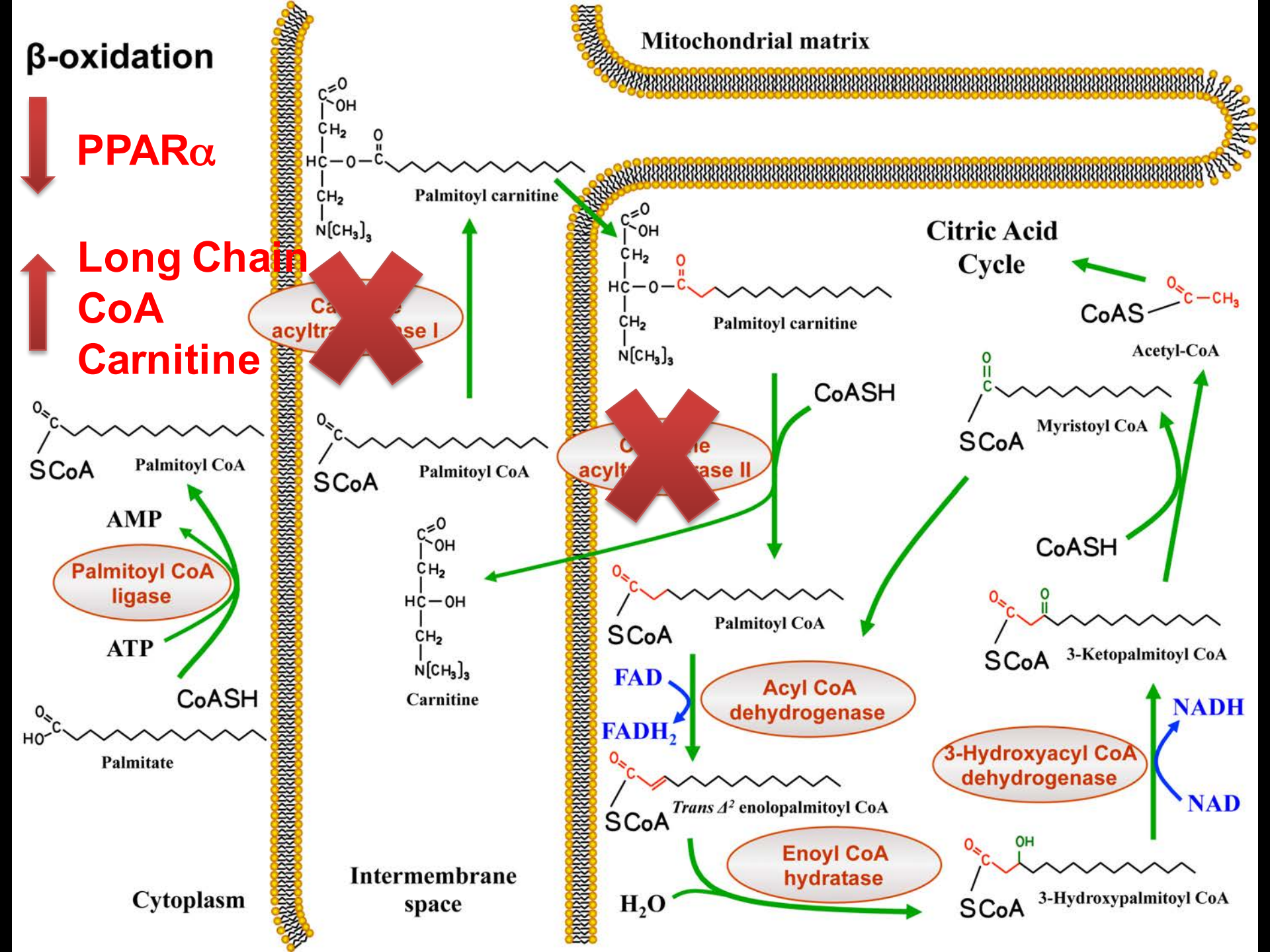
Peroxisome proliferator-activated receptor alpha induction of uncoupling protein 2 protects against acetaminophen-induced liver toxicity.

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



MITOCHONDRIAL DYSFUNCTION


β-oxidation
PPARα
Mitochondrial matrix
Palmitoyl carnitine
Carnitine acyltransferase I
Palmitoyl carnitine
Citric Acid Cycle
CoAS
Acetyl-CoA
CoASH
Carnitine acyltransferase II
Myristoyl CoA
SCoA
Palmitoyl CoA
AMP
Palmitoyl CoA ligase
ATP
CoASH
Palmitate
Carnitine
Palmitoyl CoA
FAD
FADH2
Acyl CoA dehydrogenase
Trans Δ2 enolopalmitoyl CoA
Enoyl CoA hydratase
H2O
3-Ketopalmitoyl CoA
NADH
NAD
3-Hydroxyacyl CoA dehydrogenase
3-Hydroxypalmitoyl CoA
Cytoplasm
Intermembrane space

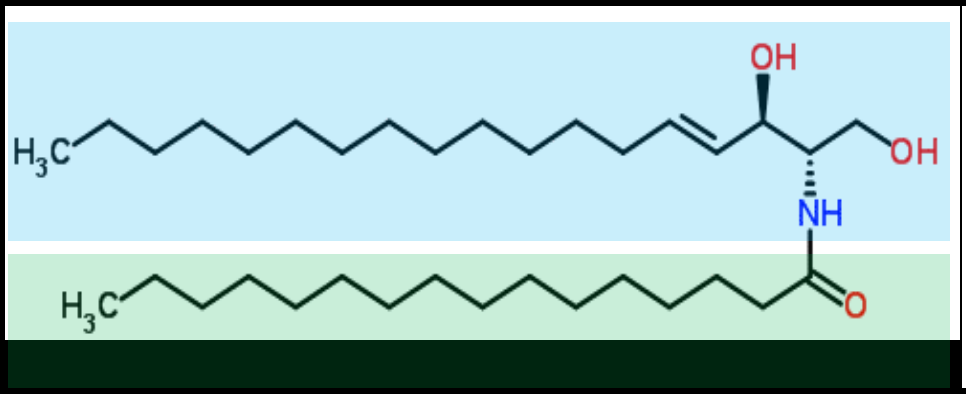

β-oxidation
PPARα
Long Chain CoA Carnitine
Mitochondrial matrix
Palmitoyl carnitine
Palmitoyl carnitine
Citric Acid Cycle
CoAS
Acetyl-CoA
Myristoyl CoA
SCoA
CoASH
Palmitoyl CoA
SCoA
Palmitoyl CoA
SCoA
Palmitoyl CoA
AMP
Palmitoyl CoA ligase
ATP
CoASH
Palmitate
Carnitine
CoASH
SCoA
3-Ketopalmitoyl CoA
FAD
FADH2
Acyl CoA dehydrogenase
NADH
3-Hydroxyacyl CoA dehydrogenase
NAD
SCoA
Trans Δ2 enolopalmitoyl CoA
Enoyl CoA hydratase
H2O
OH
SCoA
3-Hydroxypalmitoyl CoA
Cytoplasm
Intermembrane space

INFLUENCE OF EXTRACTION PROTOCOL – Ceramides

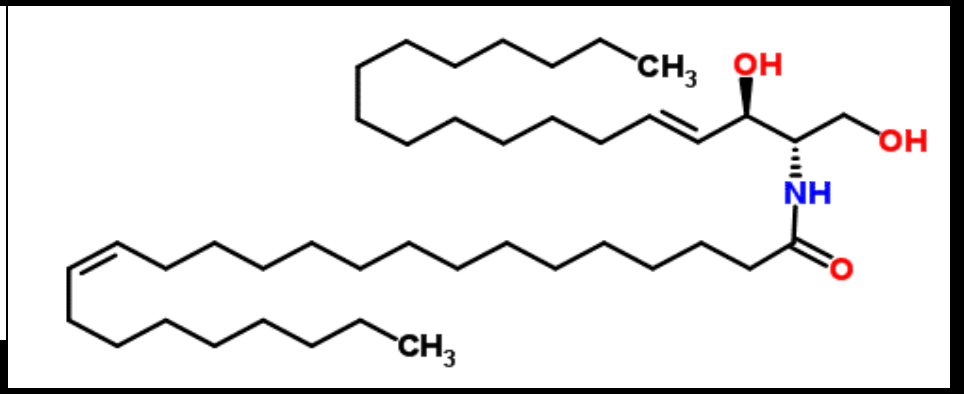
Ceramide Physicochemical Properties

- Ceramides are a family of waxy lipid molecules.
  - Name derived from the latin word: cera = waxy + amide
- Ceramides are comprised of:
  - sphingosine: 18 carbon unsaturated amino alcohol
  - fatty acid moiety – amide bond
- Ceramides are not water soluble:
  - Very hydrophobic
  - Confined to cellular membranes
  - Participate in lipid raft formation
  - >200 structurally distinct species have been identified in mammalian cells

Ceramide General Structure



- **Ceramide (d18:1/16:0)**
- 2-amino-1,3-octadec-4-ene-diol
  - *Amino alcohol (sphingoid) backbone*
- Palmitic acid
  - *Fatty acyl group*



- **Ceramide (d18:1/24:1(15Z))**
- 2-amino-1,3-octadec-4-ene-diol
  - *Amino alcohol (sphingoid) backbone*
- 15-tetracosenoic acid
  - *Fatty acyl group*

Ceramide Biochemistry

- Ceramides are found in high concentration in the membrane of cells.
    - Structural component of the lipid bilayer
    - Bioactive lipid - implicated in a variety of physiological functions including:
        - ✓ Apoptosis and cell growth arrest
        - ✓ differentiation and cell senescence
        - ✓ cell migration and adhesion
- Ceramides are converted rapidly to more complex sphingolipids:
    - Sphingomyelin
    - Glycosylceramides
    - Little accumulation observed
    - Except for the skin (50% of total lipids can be ceramides)

Biosynthesis of Ceramides

1. *De novo* biosynthesis:
    - **Ceramide synthases couple sphinganine + long chain fatty acid to form dihydroceramide.**
    - Double bond introduced into position 4 of the sphingoid base
        - ceramide synthases 5 and 6 generate are specific for palmitic acid → C16 ceramide
        - ceramide synthases 1 (brain and skeletal muscle) specific for stearic aid → (C18:0 & C18:1 ceramides)
        - ceramide synthases 2 specific for very long chain CoA-thioesters (C_{20}-C_{26}) → (C20:0, C22:0, C24:0, C24:1 etc)
        - ceramide synthases 3 unusual ceramides of skin & testes

2. Catabolism of complex sphingolipids:
    - **Sphingomyelinases/phospholipase C breakdown sphingomyelin in animal tissues**
    - Many factors can stimulate the hydrolysis of sphingomyelin to produce ceramide:
        - Cytokines :TNF-α, IFN-γ & various interleukins
        - 1,25-dihydroxy-vitamin D_3
        - endotoxin
        - nerve growth factor
        - ionizing radiation & heat

Ceramides and Disease

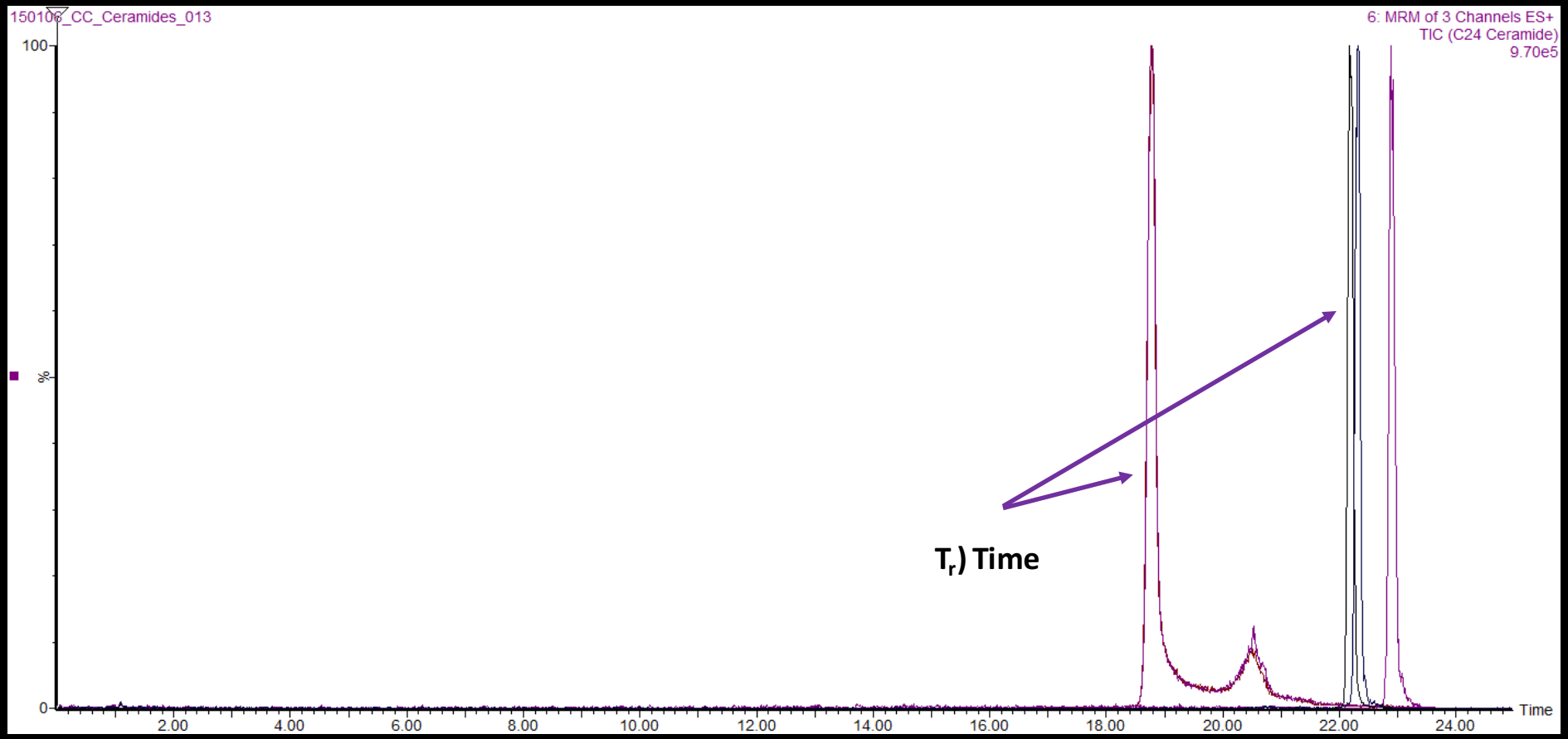
- Ceramide metabolites have been implicated in various pathological conditions including:
  - Cancer
  - Diabetes
  - Obesity
  - Inflammation
  - Neurodegeneration
- Although not understood, the structure of individual ceramides aids in defining their physiological function.
  - Ceramides containing specific fatty acids are generated in response to particular stimuli.

LC Method Development: Where to Start?

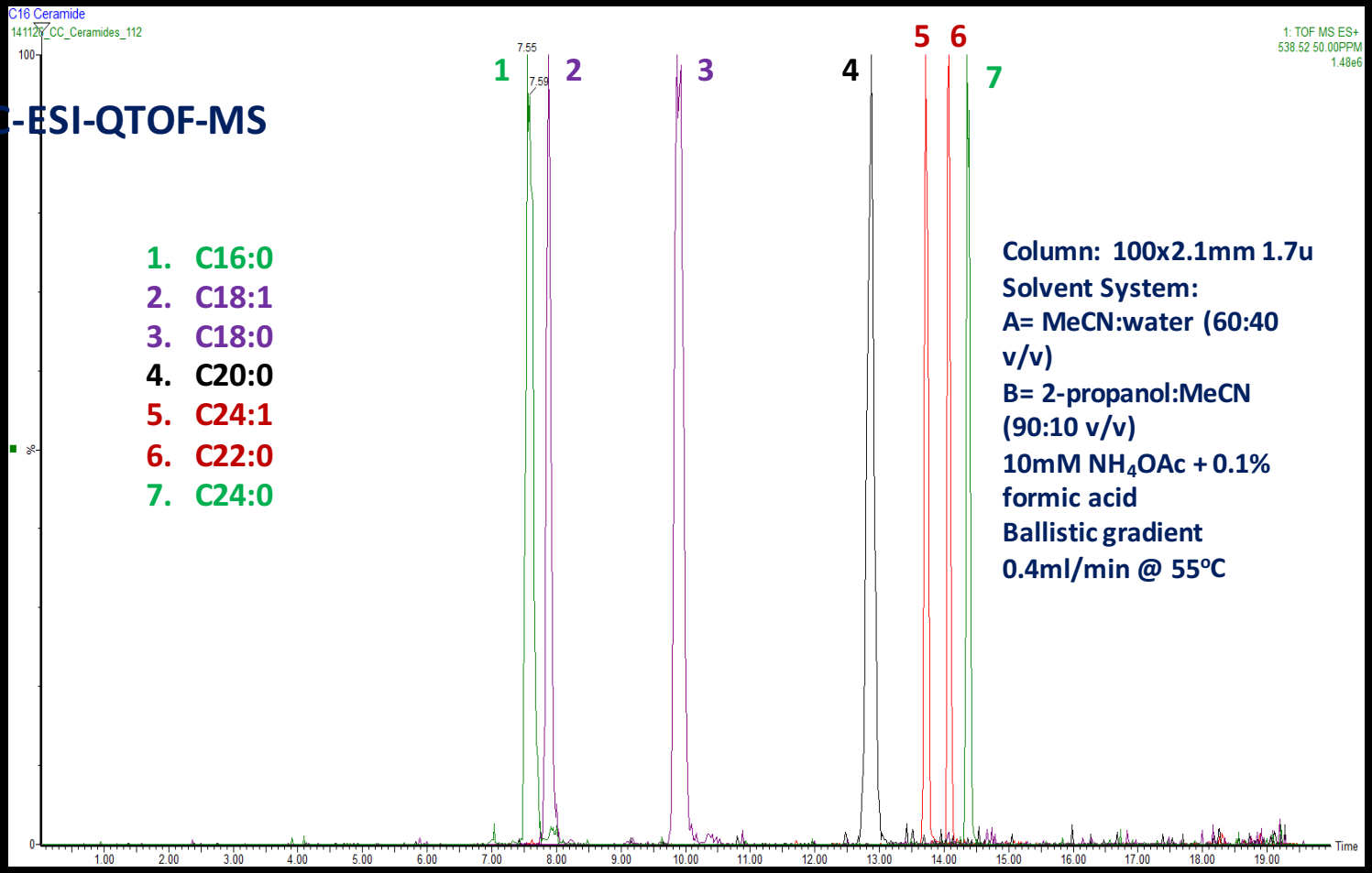
- Designing and optimizing an LC method involves choosing appropriate:
    1. Separation mechanism: NPC, RPLC, HILIC, size exclusion ion, exchange etc
    2. Column chemistry: C2, C4, C8, C18, cyanopropyl, phenyl, biphenyl, amide, SiOH etc
    3. Column properties: pore size, particle size & column dimensions
    4. Stationary and mobile phase combinations

- Critical to optimizing the chromatographic efficiency, retention, resolution & selectivity of analytes.

Ceramide Scouting Gradients on Waters BEH C18



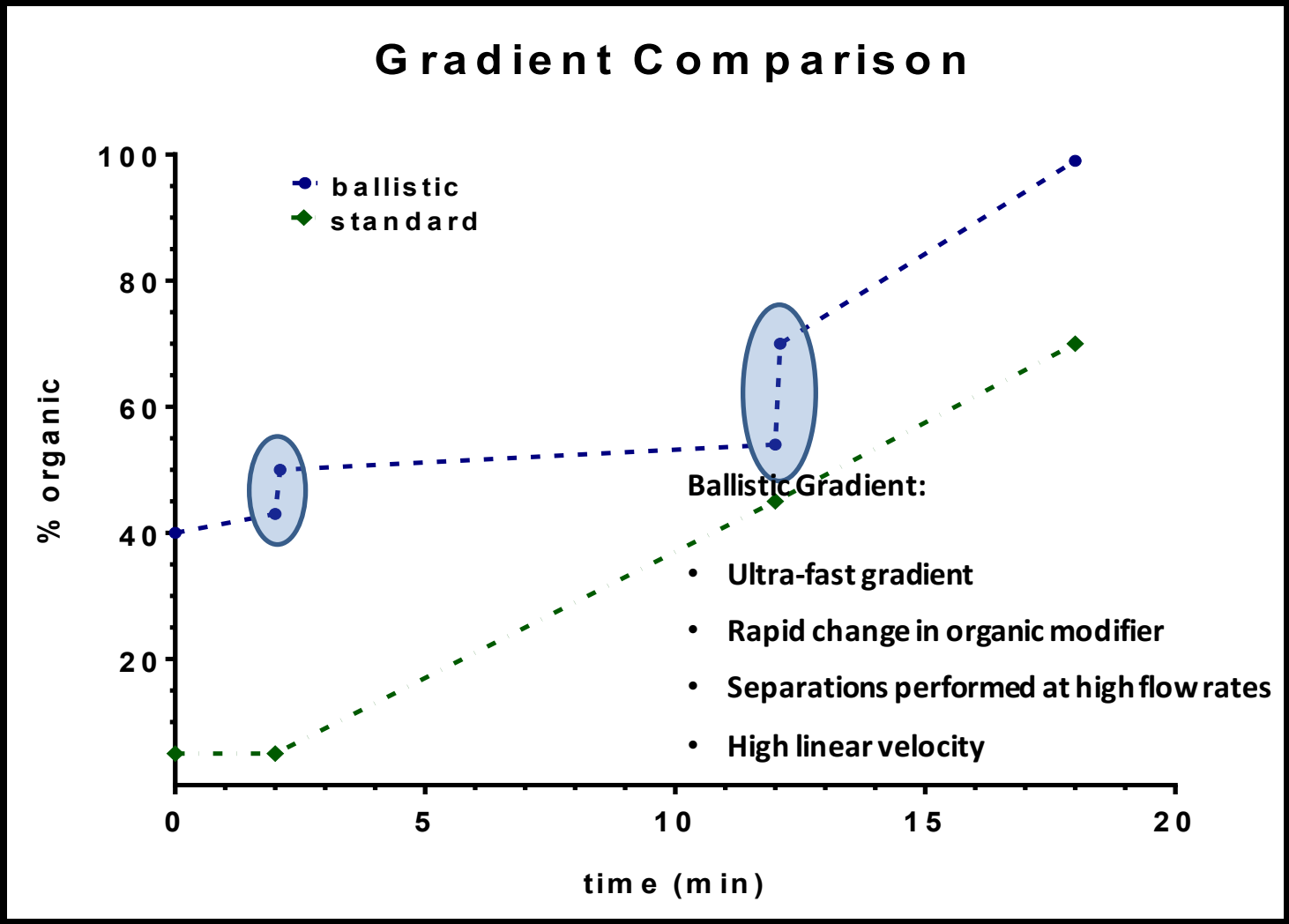
Fractionation of Ceramide Metabolites on Waters CSH C18 Column



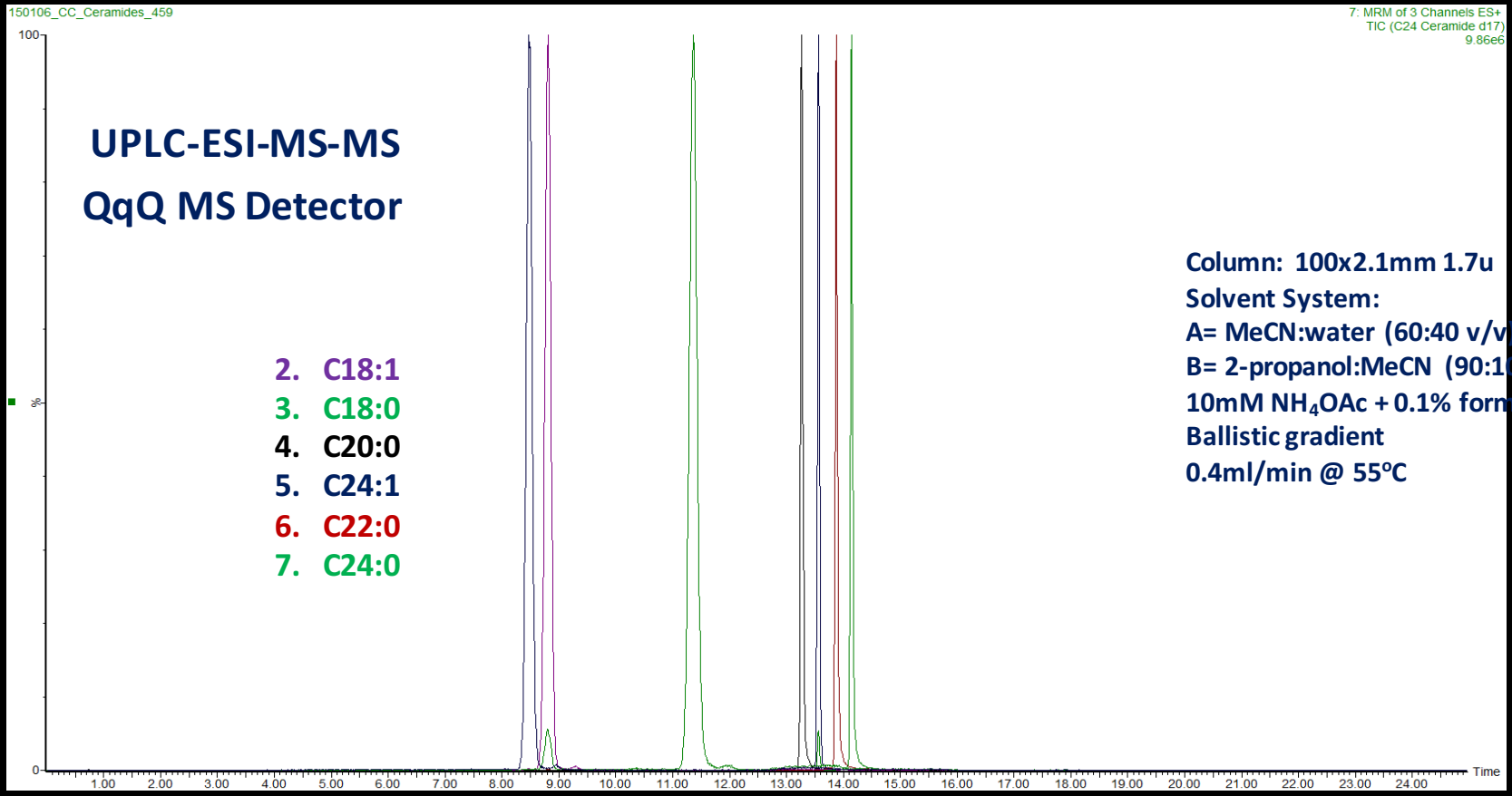
UPLC-ESI-QTOF-MS

1. C16:0
2. C18:1
3. C18:0
4. C20:0
5. C24:1
6. C22:0
7. C24:0

Column: 100x2.1mm 1.7u
Solvent System:
A= MeCN:water (60:40 v/v)
B= 2-propanol:MeCN (90:10 v/v)
10mM NH_4OAc + 0.1% formic acid
Ballistic gradient
0.4ml/min @ 55°C

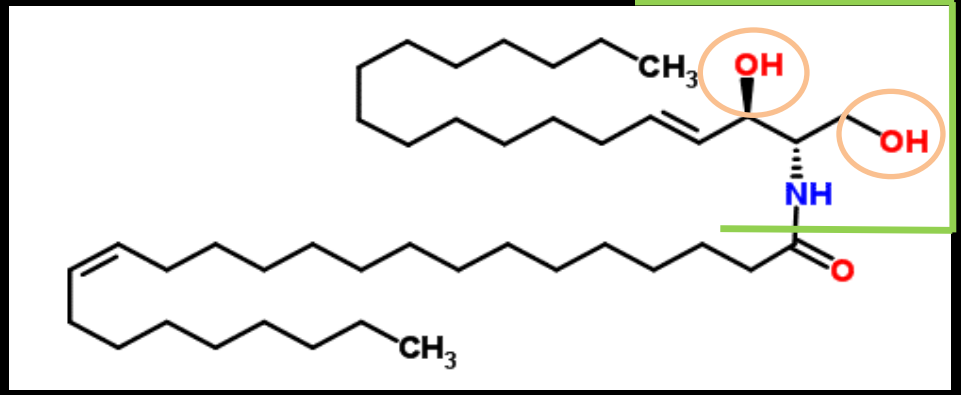

Gradient Comparison
100
80
60
40
20
% organic
ballistic
standard
0
5
10
15
20
time (min)
Ballistic Gradient:
• Ultra-fast gradient
• Rapid change in organic modifier
• Separations performed at high flow rates
• High linear velocity

Fractionation of Ceramide Metabolites on Waters CSH C18 Column

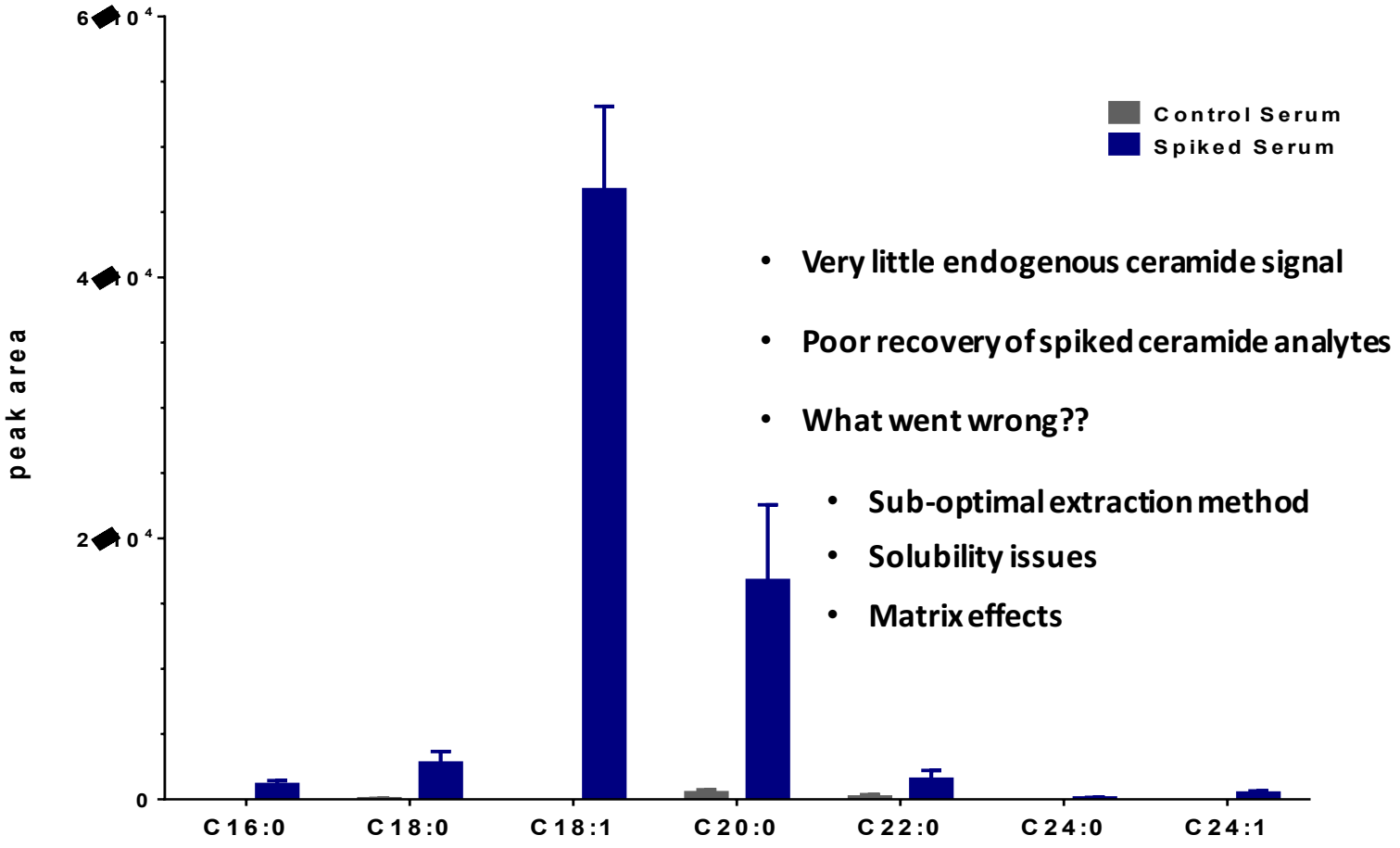


Ceramide Fragmentation Patterns

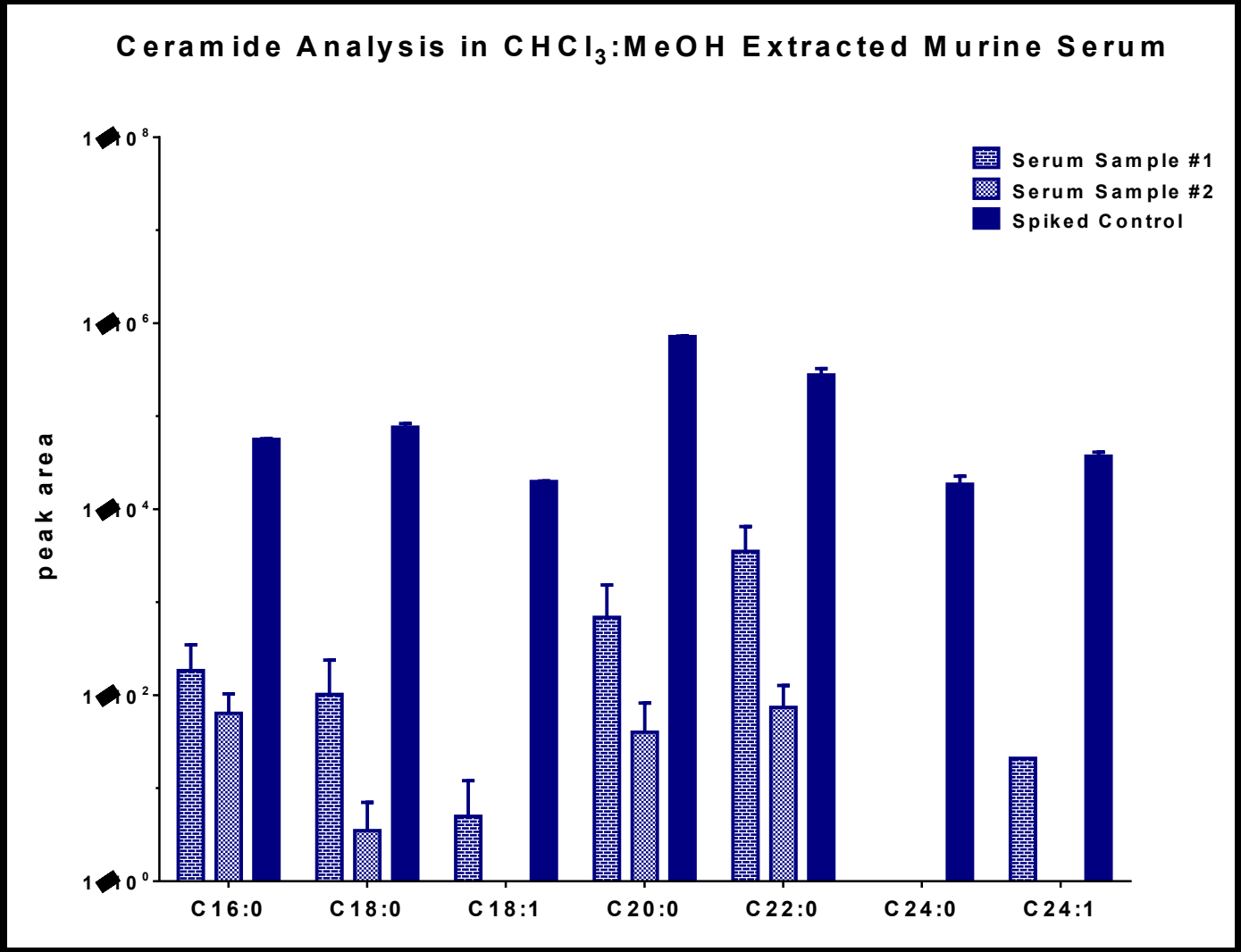
m/z = 262 or 284

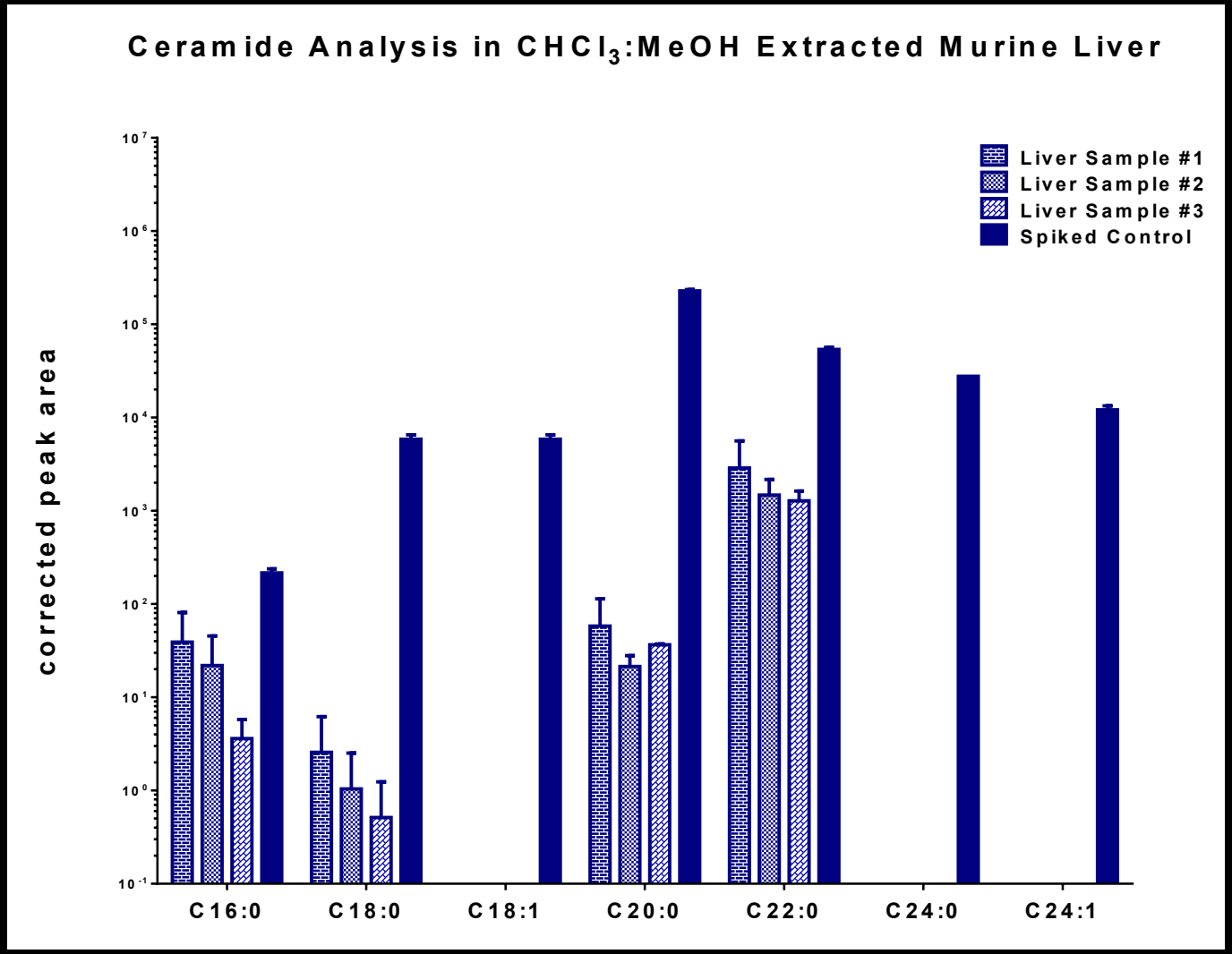


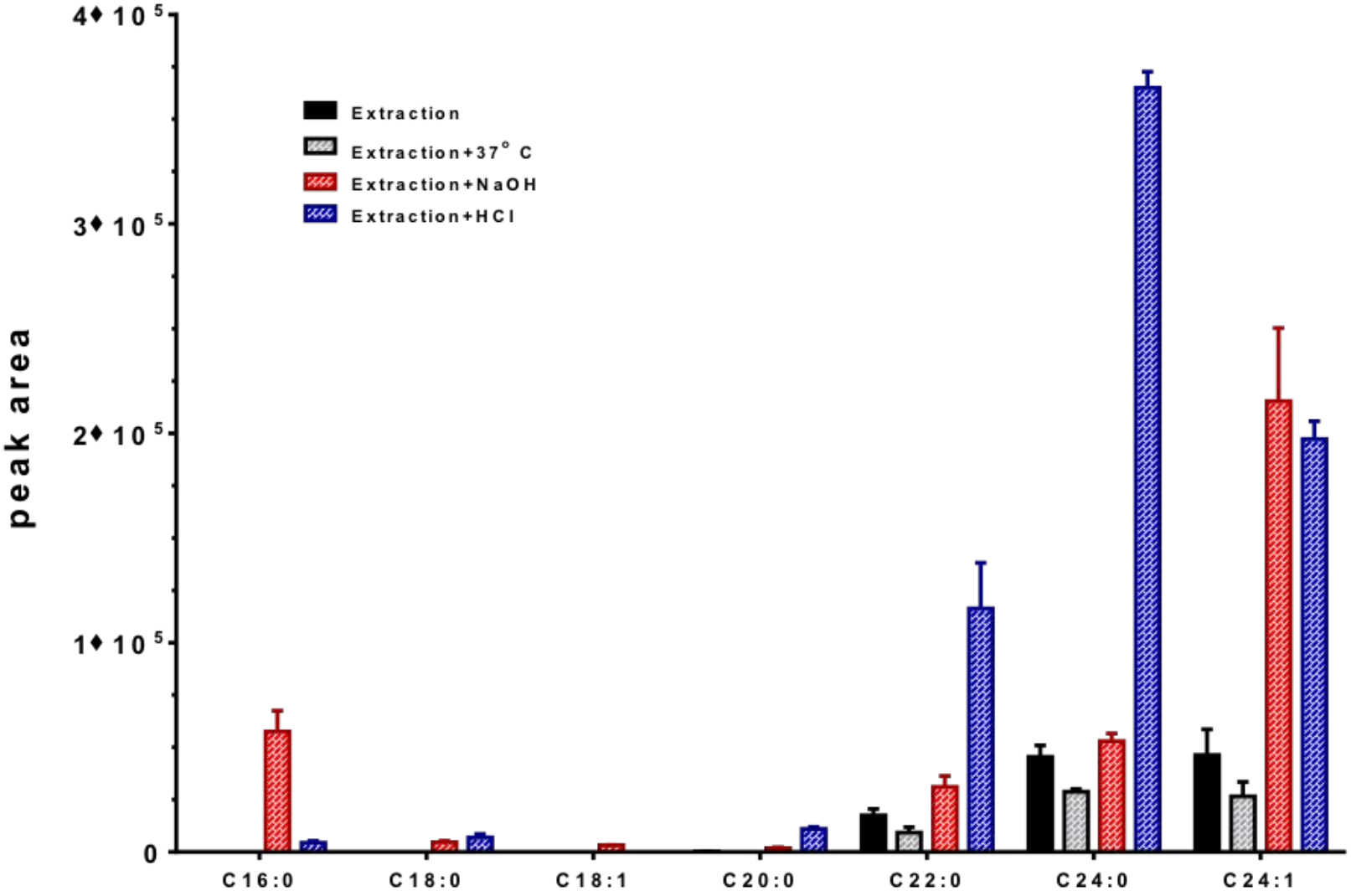
Ceramide Analysis in $CHCl_3$:MeOH Extracted Murine Serum



- Very little endogenous ceramide signal
- Poor recovery of spiked ceramide analytes
- What went wrong??
  - Sub-optimal extraction method
  - Solubility issues
  - Matrix effects


Ceramide Analysis in $CHCl_3$:MeOH Extracted Murine Serum
peak area
10^8
10^6
10^4
10^2
10^0
Serum Sample #1
Serum Sample #2
Spiked Control
C16:0
C18:0
C18:1
C20:0
C22:0
C24:0
C24:1


Ceramide Analysis in CHCl3:MeOH Extracted Murine Liver
corrected peak area
10 7
10 6
10 5
10 4
10 3
10 2
10 1
10 0
10 -1
Liver Sample #1
Liver Sample #2
Liver Sample #3
Spiked Control
C16:0
C18:0
C18:1
C20:0
C22:0
C24:0
C24:1


4♦ 10 5
3♦ 10 5
2♦ 10 5
1♦ 10 5
0
peak area
Extraction
Extraction+37° C
Extraction+NaOH
Extraction+HCl
C16:0
C18:0
C18:1
C20:0
C22:0
C24:0
C24:1

Conclusions

- Ceramides can be effectively resolved using reverse-phase liquid chromatography (RPLC) methodologies:
    - C18 column chemistry sufficient; but particle properties important (BEH vs CSH)
    - Stronger eluotropic series needed; MeOH or MeCN & water no good
    - Higher column temp required to compensate for increased backpressure (solvent viscosity & increased flow rates)
- Various ceramide metabolites can be detected using multiple UPLC-MS platforms:
    - Global metabolite profiling approach - UPLC-ESI-QTOF-MS
    - Targeted metabolite approach - UPLC-ESI-MS-MRM
- Poor detection of ceramides from bio-fluids and serum.
    - Low levels of endogenous ceramides?
    - Rapidly converted to more complex sphingolipids?
    - Ineffective extraction method?
    - Matrix effects - Ion suppression?

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

Acknowledgments

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- Chris Chiaro
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- Curt Harris
- Ewy Mathe
- Majda Haznadar

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Metastars
www.metastars.org