Metabolite Extraction and Platforms for Metabolomic Studies

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Resources

- Metabolomics Workbench
  - www.metabolomicsworkbench.org
  - Large resource of experimental protocols, datasets, and other resources
- XCMS Institute
  - https://xcmsonline.scripps.edu/institute
  - Great tutorials on chromatography, platforms, databases
- Metabolomics Society Forums
  - http://www.metabolomics-forum.com/
- Twitter
  - #metabolomics

Objectives

At the conclusion of this lesson, students will be able to:

- Define factors that influence metabolite extraction and describe their impact on metabolomic studies
- Explain the value of orthogonal approaches for improved metabolite identification and quantitation

Which parts of the metabolomic process might influence your data?

Other Classes of Metabolites

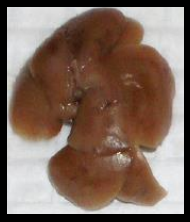
- Where might you have trouble extracting everything from a particular class of metabolites?
- Example: Are all bile acids the same in terms of general solubility in aqueous or organic solvents?

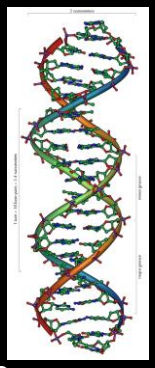
Matrix Effects

- Challenges with urine
  -
  -
- Challenges with blood, serum, or plasma
  -
  -
- Challenges with tissue
  -
  -

Extraction of Metabolites

Nucleic Acid Extraction

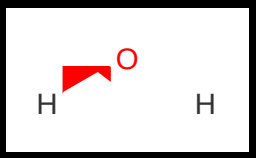


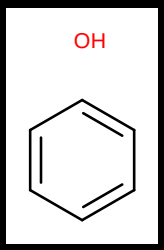


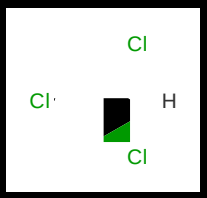
What do we know about our target analyte?

- Negatively charged phosphate backbone (polar)
- Need to remove proteins, lipids, etc

About Solvents





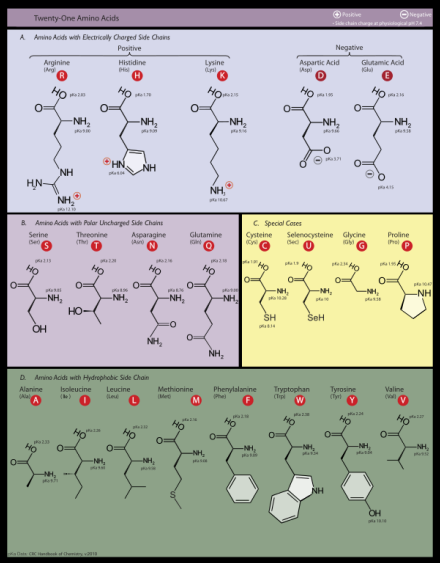


WATER PHENOL CHLOROFORM

1.00 g/cm^3 1.07 g/cm^3 1.49 g/cm^3

POLARITY

Protein Chemistry

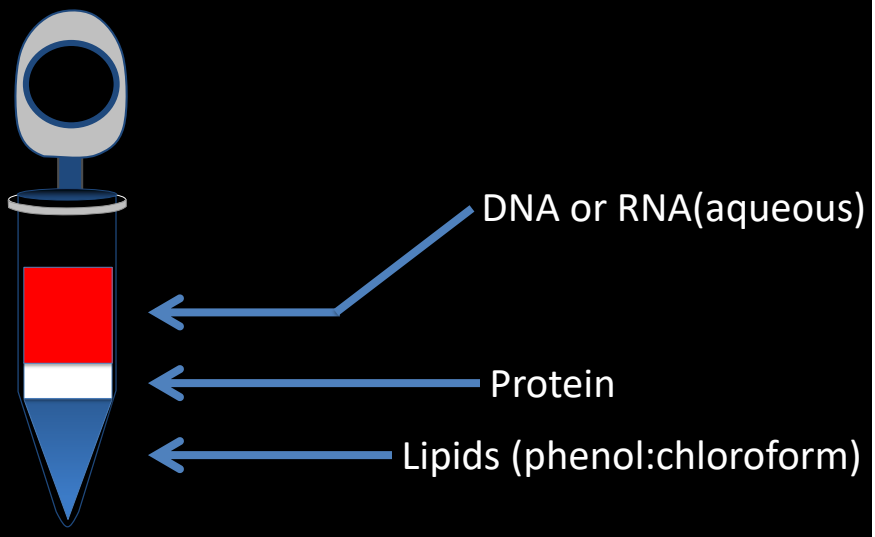


Denature proteins

- Hydrophobic amino acids face phenol:chloroform
- Phe, Tyr, Leu

https://en.wikipedia.org/wiki/Proteinogenic_amino_acid

Liquid Liquid Extraction



DNA Extraction Protocol

- Disrupt tissue in phenol:chloroform

**chloroform prevents small amounts of water in phenol from dissolving mRNA
**adjust pH to favor DNA (basic) or RNA (acidic) isolation

- Centrifuge to separate layers
- Dehydrate with alcohol

What could go wrong?

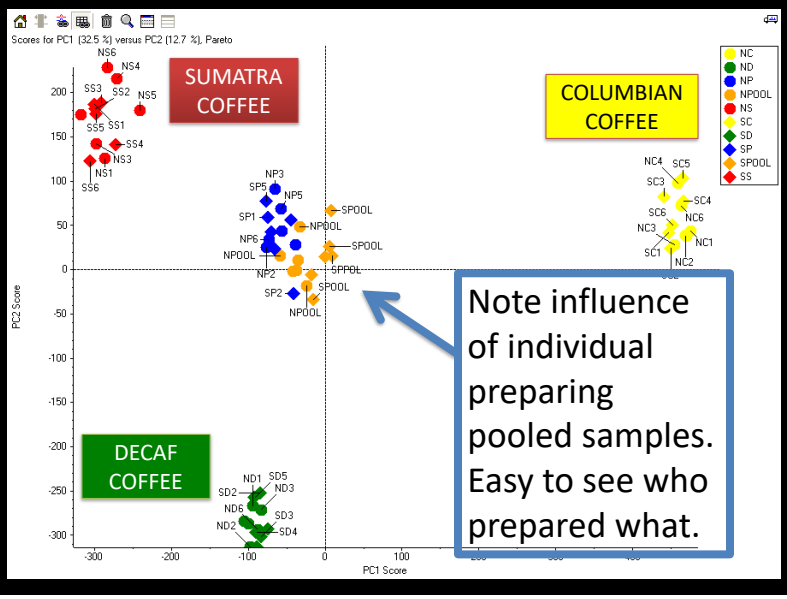
- Solvents not appropriate or prepared incorrectly
  - pH incorrect
  - Ratios incorrect
- Contamination of solvents or buffers
- What else can you think of?

Standard Operating Procedures

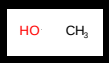
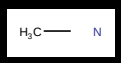
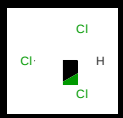
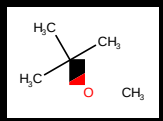
- Saves time and prevents mistakes
- Consistent results

- Checking in samples (sample lists, location)
- Labeling and storing samples (aliquot)
- Metabolite extraction (targeted or global)
- Acquiring data on various platforms (MS, NMR)

Even with SOPs…



Common Solvents for Metabolomics

- Methanol 
- Acetonitrile 
- Chloroform 
- Methyl tert-butyl ether (MTBE) 
- Water 

List is not inclusive

Methanol

- Relatively inexpensive compared to acetonitrile
- Not regulated like ethanol
- Easy to evaporate

- Extracts polar and (some) non-polar molecules – why?

Acetonitrile

- Advantages mostly for chromatography
  - Reduced absorbance for UV based methods
  - Reduced pressure compared to methanol
  - Greater elution strength (generally)
  - HILIC applications

- Expensive
  - Isolated as a byproduct not produced directly
  - Shortages can influence price and availability

Chloroform vs MTBE

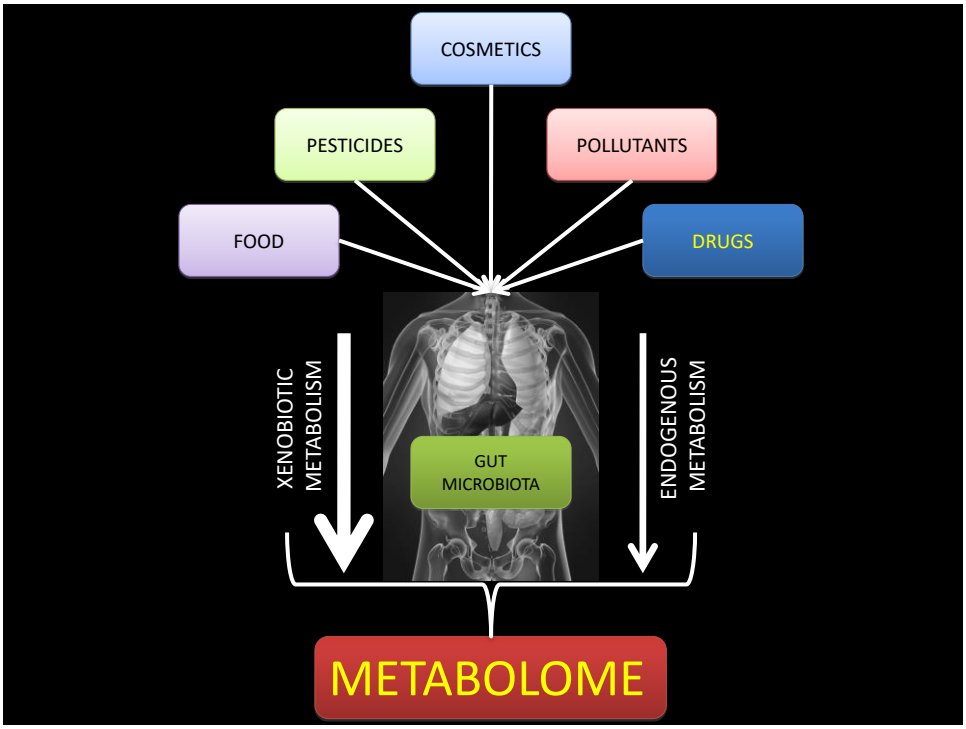
- Chloroform densitiy – 1.49 g/cm^3
- MTBE density – 0.740 g/cm^3

] SO WHAT?

- Toxicity of chloroform (check out ATSDR.CDC.GOV)
- Still made need to tailor specific extractions for lipid classes (hexane for TAGs or MTBE for Cer)
  - More detail checkout cyberlipid.org or lipidmaps.org

Metabolomics

Metabolomics is the systematic analysis of the unique chemical fingerprints left behind by specific cellular processes



Metabolomics

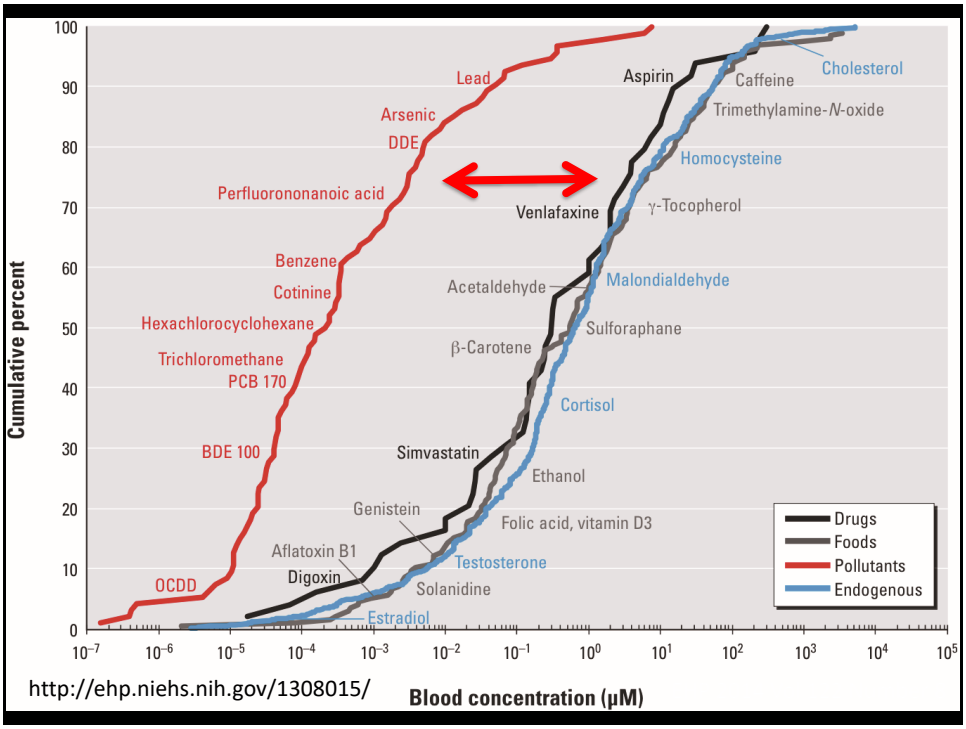
All "-omics" based scientific disciplines aim at the collective characterization and measurement of their particular constituent molecules

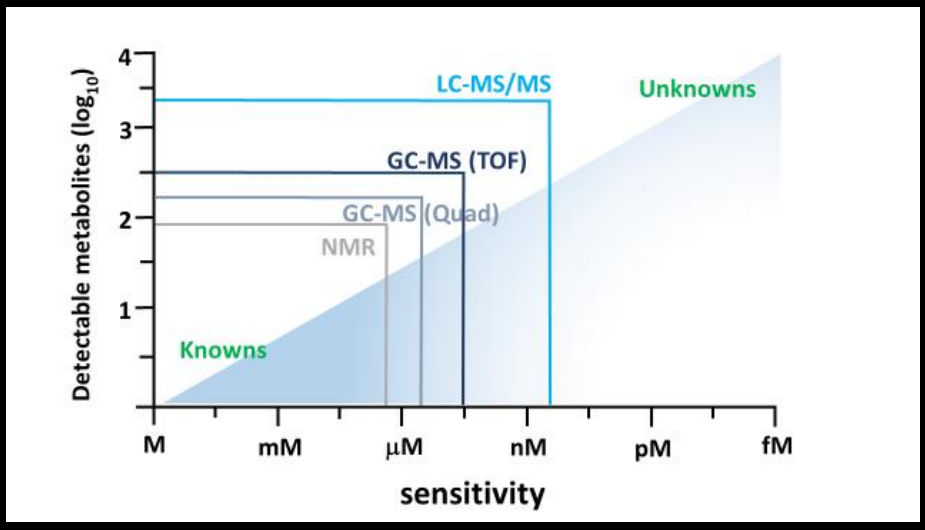
- A comprehensive approach to study complete pools of biological molecules
- Defines the structure, function and dynamics of an organism

Metabolomics

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)

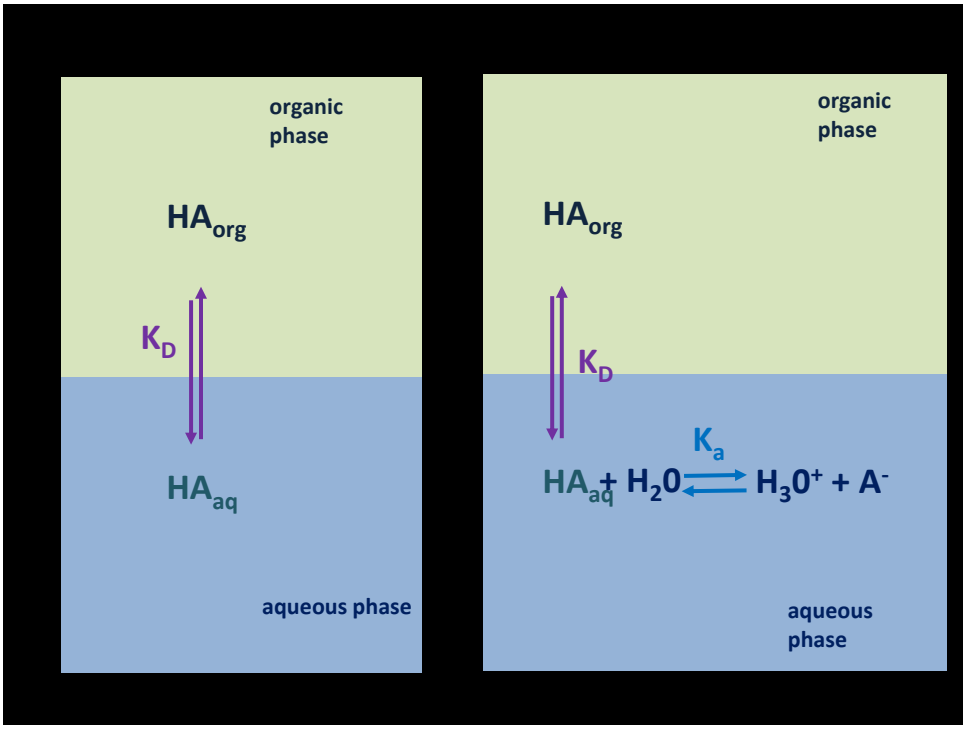




http://journal.frontiersin.org/article/10.3389/fimmu.2016.00044/full

Metabolite Extraction

- Currently no analytical technique exists that is capable of measurement of all classes of cellular metabolites
- Metabolite extraction is a crucial step in any 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



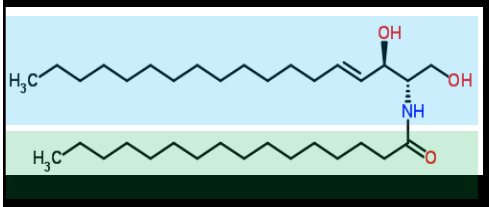
Separation of Metabolites

- Mass spectrometry usually requires some form of chromatographic separation
  - Most systems use either liquid or gas chromatography
- 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

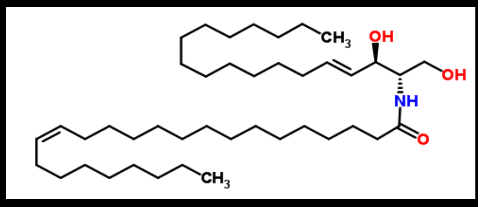
Ceramide Physicochemical Properties

- 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

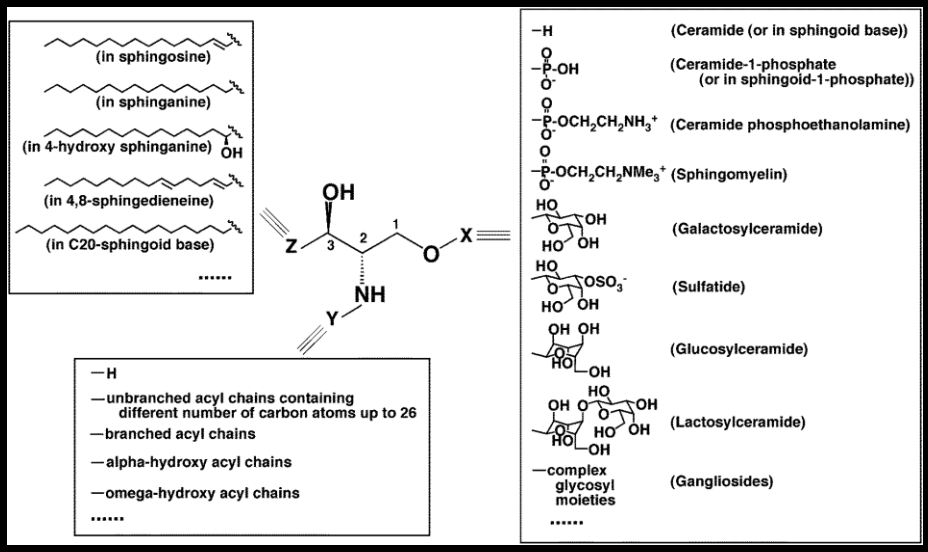


- Ceramide (d18:1/16:0)
- 2-amino-1,3-octadec-4-ene-diol
  - Amino alcohol (sphingoid) backbone
- Palmitic acid
  - Fatty acyl group



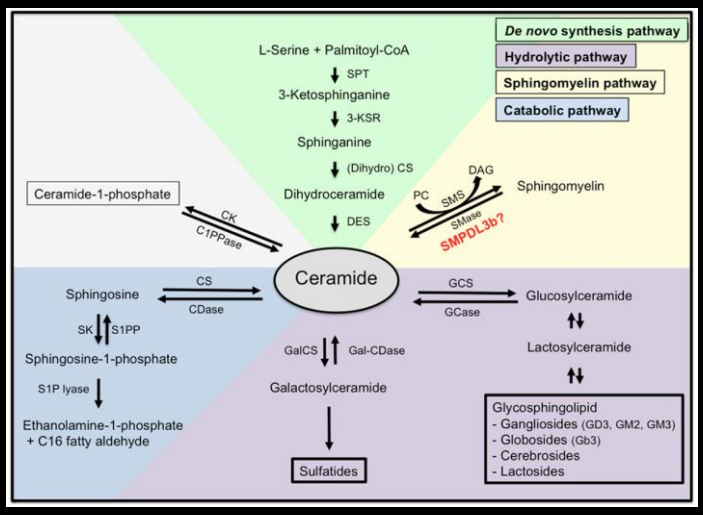
- **Ceramide (d18:1/24:1(15Z))**
- 2-amino-1,3-octadec-4-ene-diol
  - *Amino alcohol (sphingoid) backbone*
- 15-tetracosenoic acid
  - *Fatty acyl group*

Structures and Nomenclature



Mol. BioSyst., 2015, 11, 698--713 | 699

Ceramide Biosynthesis



Front. Endocrinol., 30 July 2014 | https://doi.org/10.3389/fendo.2014.00127

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

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
  - ceramide synthases 1 (brain and skeletal muscle) specific for stearic acid
  - ceramide synthases 2 specific for very long chain CoA-thioesters (C_{20}-C_{26})
  - ceramide synthases 3 unusual ceramides of skin & testes

Biosynthesis of Ceramides

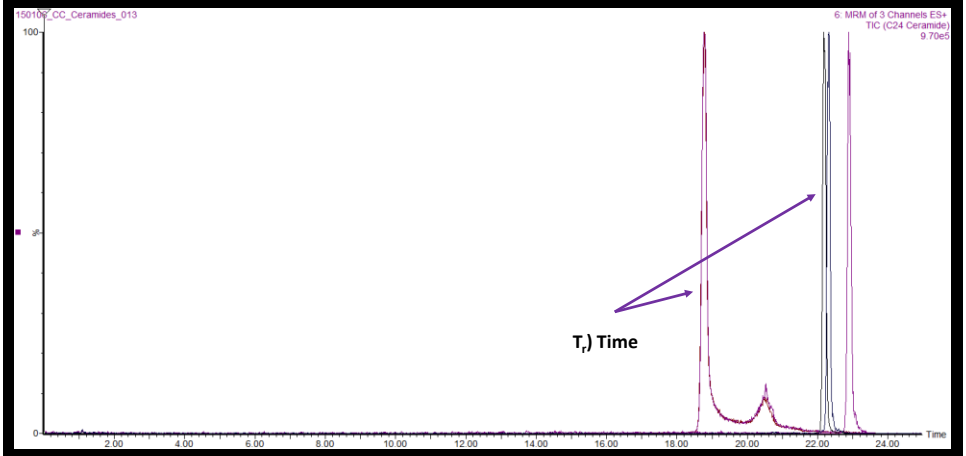
Catabolism of complex sphingolipids:

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

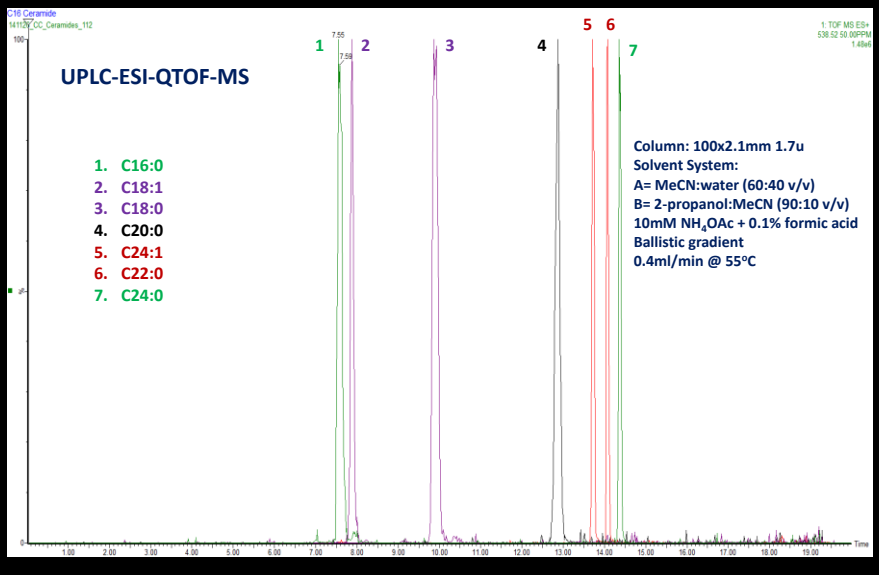
LC Method Development Where to Start?

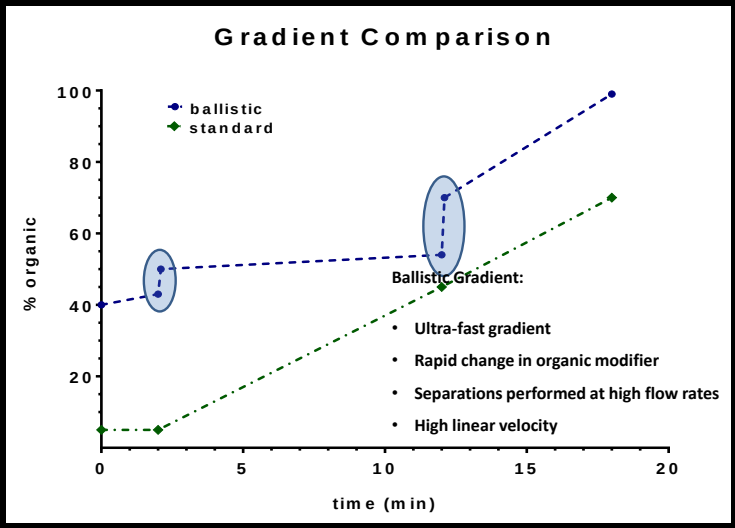
- Designing and optimizing an LC method involves choosing appropriate:
  1. Separation mechanism: NPC, RPLC, HILIC, size exclusion ion, exchange etc
  2. Column chemistry: C2, C4, C8, C18, cyanopropyl, phenyl, biphenyl, amide, SiOH etc
  3. Column properties: pore size, particle size & column dimensions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





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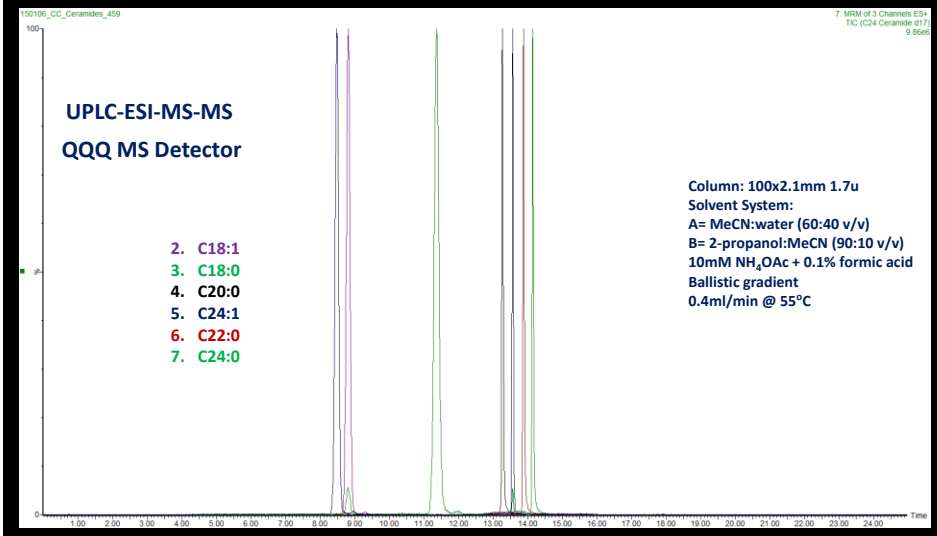


Ceramide Extraction

Extraction protocols and LC-MS methods adapted from Shaner RL et al *JLR* 2009

- Add 50 mg of liver tissue to 50% aqueous methanol
  - Why not chloroform directly?
- Homogenize in Bertin Precellys at 6500 rpm with ~10 zirconium beads for 30 seconds

Ceramide Extraction – cont'd

- Add 1 mL of $CHCl_3$:MeOH (2:1, v/v) containing 20 µl of C17:0 internal standard solution (use 1 mM stock solution)
  - Why internal standard at this point?
  - Should we use glass or plastic? Does it matter?
  - What if you swap chloroform for hexane or isopropanol?
- Homogenize again and centrifuge at 18,000xg for 10 min to separate phases
- Transfer organic phase to a new tube (#2) and repeat extraction of left over material
  - Why repeat?

Ceramide Extraction – cont'd

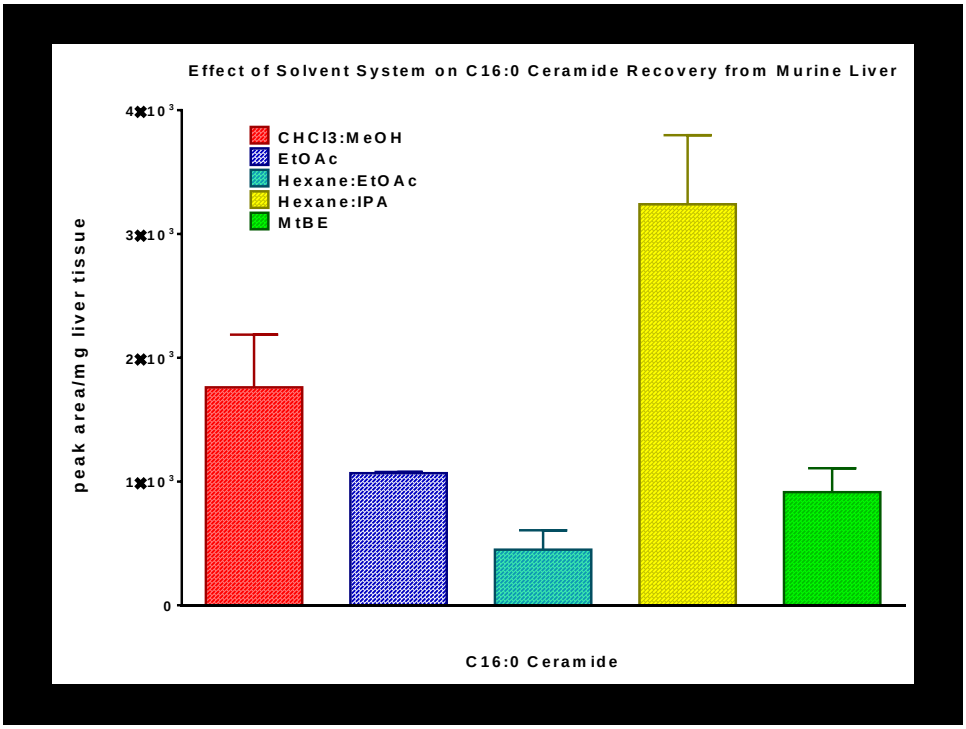
- Combine organic phases and dry down in a vacuum centrifuge
- Solubilize residuals in 50 µl of $CHCl_3$:MeOH (2:1, v/v)
  - Why chloroform here?
- Saponification or acid hydrolysis of residuals to release ceramides

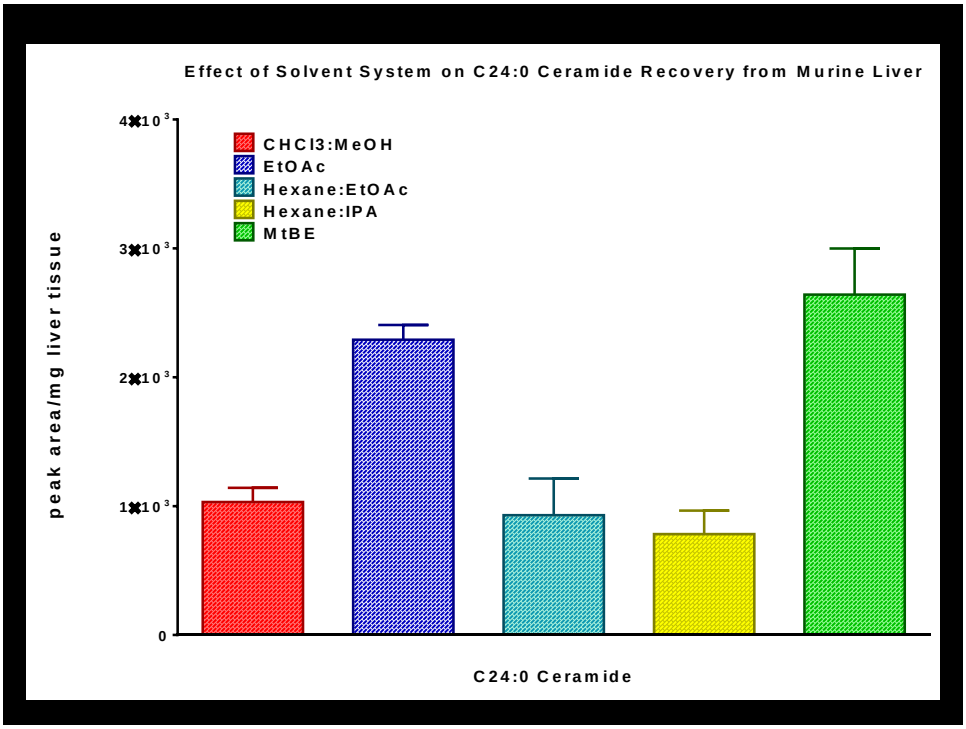
Ceramide Extraction – cont'd

- Incubate residuals with 0.5 mL of 1M HCl in MeOH @ 50°C for 1 hr (or base for sapn)
- Cool samples and re-extract
- Solubilize with in 30 µl of $CHCl_3$:MeOH (2:1, v/v), sonicate for 5 minutes in sonicating water bath
  - Why sonicate?

Ceramide Extraction – cont'd

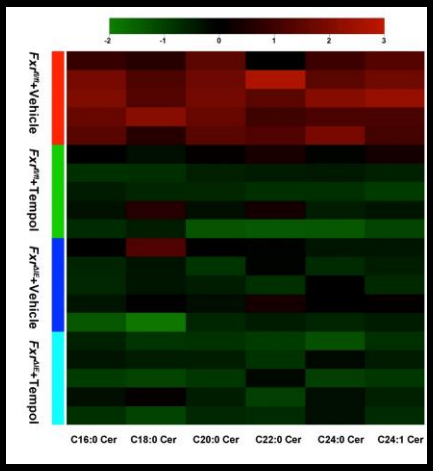
- Dilute 10 fold with acetonitrile:isopropanol:water (1:1:1, v/v)
- Centrifuge to remove any particulates and transfer to autosampler tube


Effect of Solvent System on C16:0 Ceramide Recovery from Murine Liver
peak area/mg liver tissue
4✖10 3
3✖10 3
2✖10 3
1✖10 3
0
CHCl3:MeOH
EtOAc
Hexane:EtOAc
Hexane:IPA
MtBE
C16:0 Ceramide

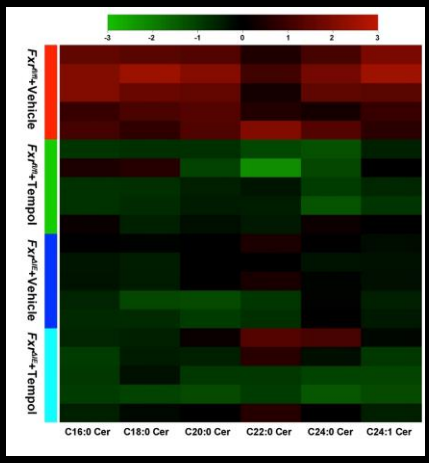

Effect of Solvent System on C24:0 Ceramide Recovery from Murine Liver
peak area/mg liver tissue
4✖10 3
3✖10 3
2✖10 3
1✖10 3
0
CHCl3:MeOH
EtOAc
Hexane:EtOAc
Hexane:IPA
MtBE
C24:0 Ceramide

Lipidomics Reveals Ceramides are Decreased in FXR Intestine-null Mice

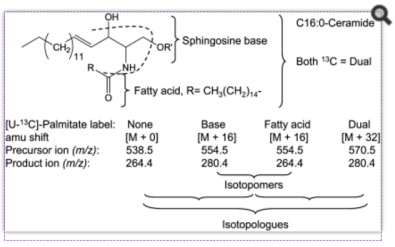
Ileum



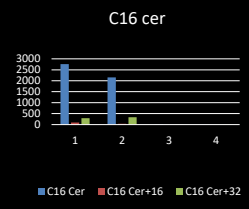
Serum

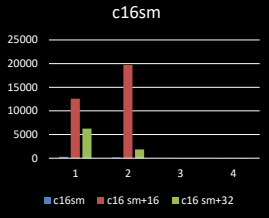


Ceramide Labeling



Nomenclature of C16:0-ceramide isotopologues and isotopomers with sites of fragmentation of most sphingolipids in positive ion mode using an API3000 triple quadrupole mass spectrometer. Also shown are the *m/z* for the precursor and product ions for C16-Cer with no ^{13}C (None), [U-^{13}C]palmitate in the sphingoid base alone (Base), [U-^{13}C]palmitate in the N-acyl chain alone (Fatty acid), or [U-^{13}C]palmitate in both the sphingoid base and fatty acid (Dual).

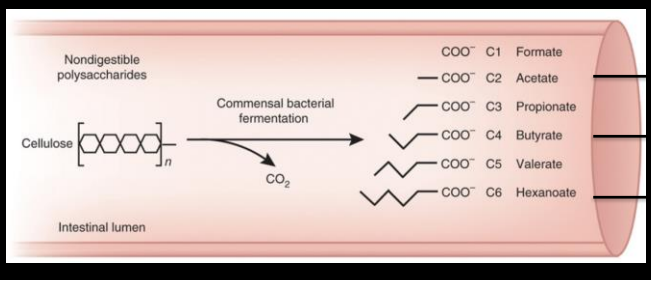




J Lipid Res. 2011 Aug; 52(8): 1583–1594. doi: 10.1194/jlr.D015586

Short Chain Fatty Acids

- SCFAs are those carboxylic acids that contain aliphatic tails less than 6 carbon atoms
- In humans, SCFAs are derived in large part from fermentation of carbohydrates and proteins in the colon
- By this process, the host is able to salvage energy from foods that cannot be processed normally in the upper parts of the gastrointestinal tract
- SCFAs serve as direct energy source, anabolic substrates and signaling molecules involve metabolic and immunological regulation.



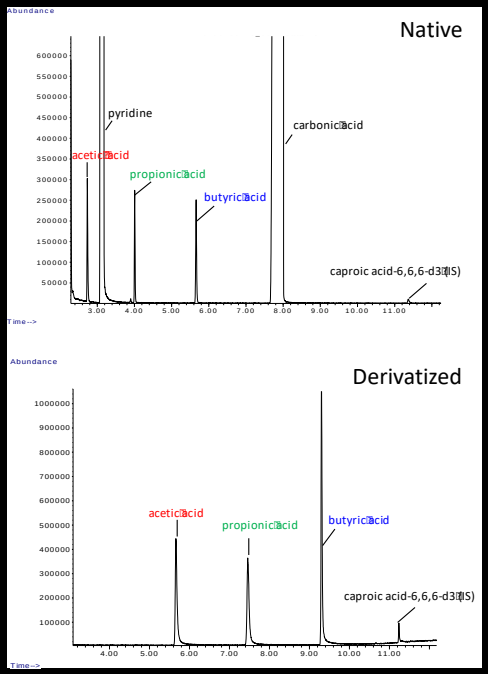
Direct energy source

Regulatory roles

Anabolic substrates

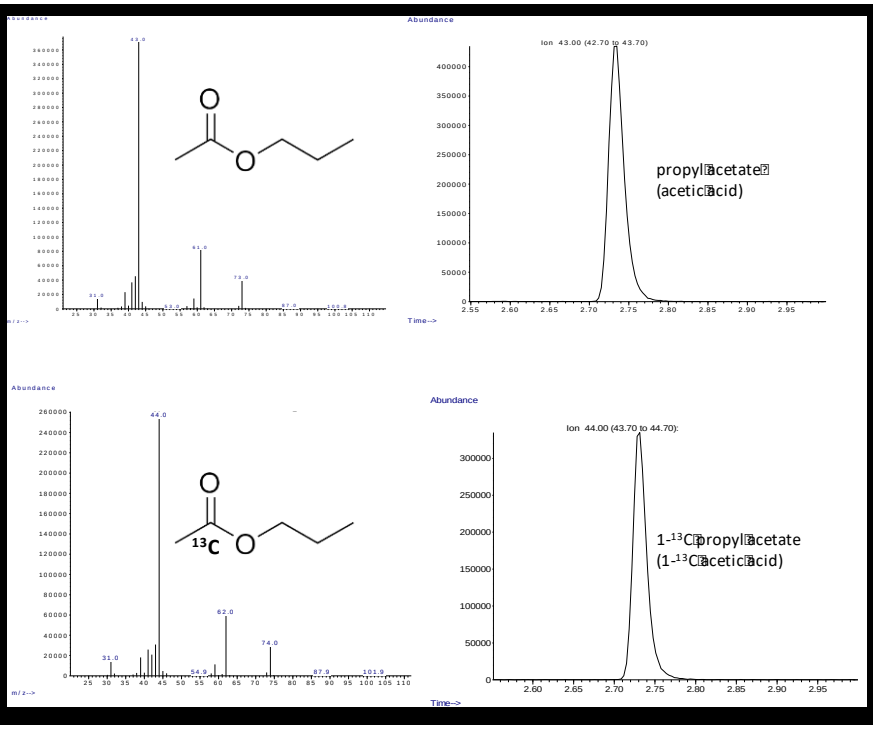
SCFA Extraction and Detection

- What do we know about SCFAs?
- How do we detect them?
- What problems might influence SFCA measurement?

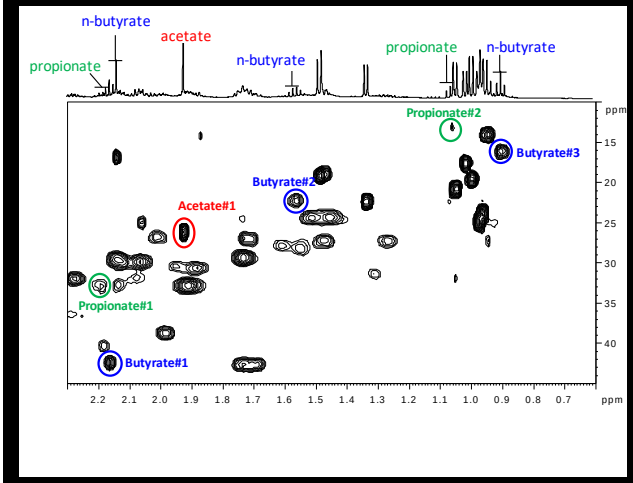


Total ion chromatogram of 100 µg/ml SCFAs standard mixture with 10 µg/ml caproic acid-6,6,6-d3 as internal standard (IS) by (top) propyl esterification method and (bottom) acidified water method.

Notice change in retention time between the two methods.



SCFA by ^{1}H NMR

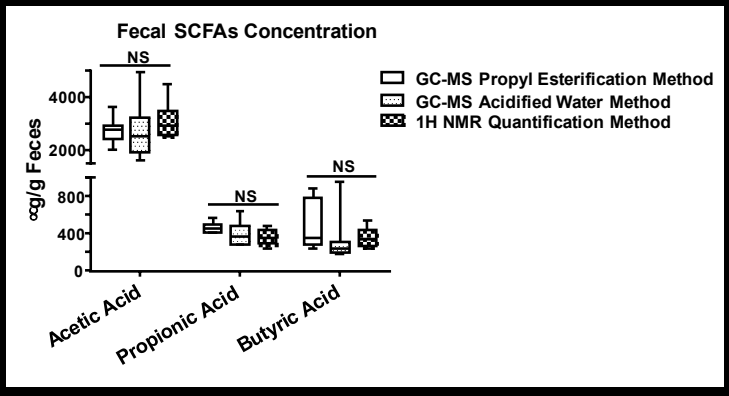


^{1}H-^{13}C HSQC NMR spectroscopy of mice feces. Reference spetra were obtained from Human Metabolome Database (HMDB). Acetate (HMDB00042), Propionate (HMDB00237) and Butyrate (HMDB00039)

GC-MS Propyl Esterification Method					
Compounds	Spiked amount (µg/ml)	Amount recovered (µg/ml)	%Recovery	%RSD	%Matrix effect
Acetic Acid	10	6.7±1.0	66.9	14.9	76.3
	100	108.3±15.0	108.3	13.9	94.4
	250	254.5±16.3	101.8	5.9	92.7
Propionic Acid	10	7.2±1.2	72.3	16.9	74.2
	100	100.2±17.6	100.2	17.5	84.2
	250	244.5±36.6	97.8	15.0	92.7
Butytic Acid	10	6.7±1.1	67.0	11.2	77.7
	100	99.4±20.4	99.4	20.6	88.2
	250	249.7±38.1	99.8	15.3	97.4
GC-MS Acidified Water Method					
Compounds	Spiked amount (µg/ml)	Amount recovered (µg/ml)	%Recovery	%RSD	%Matrix effect
Acetic Acid	10	7.5±1.9	74.8	25.1	116.8
	100	108.7±14.9	108.7	13.7	95.7
	250	226.1±19.2	90.4	8.5	76.2
Propionic Acid	10	7.5±1.2	75.4	16.0	95.1
	100	106.3±10.7	106.3	10.1	80.3
	250	297.2±23.1	118.9	7.8	76.2
Butyric Acid	10	5.2±0.4	52.2	6.8	89.6
	100	87.6±17.0	87.6	20.6	76.2
	250	291.7±24.3	116.7	8.3	79.7
^{1}H NMR Quantification Relative to TSP Method					
Compounds	Spiked amount (µg/ml)	Amount recovered (µg/ml)	%Recovery	%RSD	%Matrix effect
Acetic Acid	10	8.4±0.4	83.7	4.2	90.6
	100	97.7±4.3	97.7	4.4	118.5
	250	271.7±16.0	108.7	5.9	111.7
Propionic Acid	10	10.1±0.6	101.4	6.0	107.2
	100	116.6±5.5	116.6	4.7	116.1
	250	332.1±15.3	132.8	4.6	104.8
Butyric Acid	10	5.4±0.8	54.2	14.7	64.1
	100	92.9±4.6	92.9	5.0	118.1
	250	263.2±14.7	105.3	5.6	106.8
^{1}H NMR Quantification with Calibration Curve Method					
Compounds	Spiked amount (µg/ml)	Amount recovered (µg/ml)	%Recovery	%RSD	
Acetic Acid	10	8.6±0.4	85.6	4.2	
	100	98.7±5.1	98.7	5.2	
	250	277.9±16.3	111.2	5.9	
Propionic Acid	10	8.0±0.48	80.2	6.0	
	100	91.4±4.6	91.4	5.0	
	250	262.7±12.1	105.1	4.6	
Butyric Acid	10	5.4±0.8	54.1	14.7	
	100	92.0±4.7	92.0	5.1	
	250	263.0±14.7	105.2	5.6	

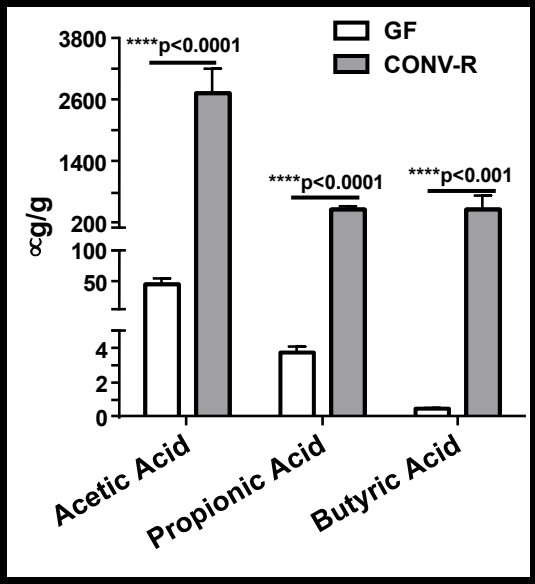
GC-MS Propyl Esterification Method								
Compounds	Intraday (%RSD)				Interday (%RSD)			
	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml
Acetic Acid	4.3	2.7	6.1	3.0	4.5	3.5	10.0	3.4
Propionic Acid	12.2	5.7	7.7	5.6	21.3	6.5	11.2	8.4
Butyric Acid	1.2	3.5	5.8	3.6	7.7	5.1	4.6	6.9
GC-MS Acidified Water Method								
Compounds	Intraday (%RSD)				Interday (%RSD)			
	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml
Acetic Acid	12.6	6.8	5.1	4.5	9.8	5.6	5.7	5.3
Propionic Acid	6.4	7.2	4.9	5.4	8.9	7.9	7.8	7.9
Butyric Acid	2.4	4.2	5.0	5.5	4.7	6.4	6.0	7.7
^{1}H NMR Quantification Relative to TSP								
Compounds	Intraday (%RSD)				Interday (%RSD)			
	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml
Acetic Acid	1.7	4.6	0.3	1.9	2.6	5.3	0.3	0.6
Propionic Acid	3.8	0.9	2.4	2.8	1.4	2.4	1.4	2.0
Butyric Acid	4.3	4.5	1.4	2.1	4.5	2.6	0.7	4.0
^{1}H NMR Quantification with Calibration Curve								
Compounds	Intraday (%RSD)				Interday (%RSD)			
	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml	10 µg/ml	25 µg/ml	100 µg/ml	250 µg/ml
Acetic Acid	1.1	6.7	1.7	1.8	1.6	4.3	0.3	0.6
Propionic Acid	2.0	0.7	2.3	2.7	0.7	1.7	1.3	1.9
Butyric Acid	2.3	3.2	1.3	2.1	2.4	1.9	0.7	3.9

How do the methods compare?



Biological concentrations of SCFAs in mice fecal samples measured by GC-MS propyl esterification method, GC-MS acidified water method and ^{1}H NMR quantification method. Values are expressed as the mean $\pm$ 95% CI. (n=10). One-way ANOVA. Biological Concentrations measured by different methods were not significantly different.

SCFA Measurement in the Real World



Biological concentrations of SCFAs in germ free(GF) and conventionally raised (CONV-R) fecal samples measured by GC-MS propyl esterification method. Values are expressed as the mean ± sd. (n=5). Two-tailed t-test.

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

Conclusions – cont'd

- There's no one "perfect" extraction or LC method available capable of efficiently extracting or resolving, respectively, all components or features in the metabolome
- Advanced column chemistries (amide, aminopropyl, biphenyl, graphite, phenyl-hexyl) and alternative chromatographic methodologies (HILIC) can provide enhanced coverage of the metabolome
- Different platforms can provide greater confidence in metabolite measurement

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