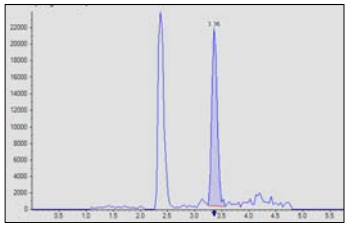
Targeted Metabolomics & Proteomics Laboratory

Metabolomics analysis by Targeted LC-MS

Taylor Berryhill

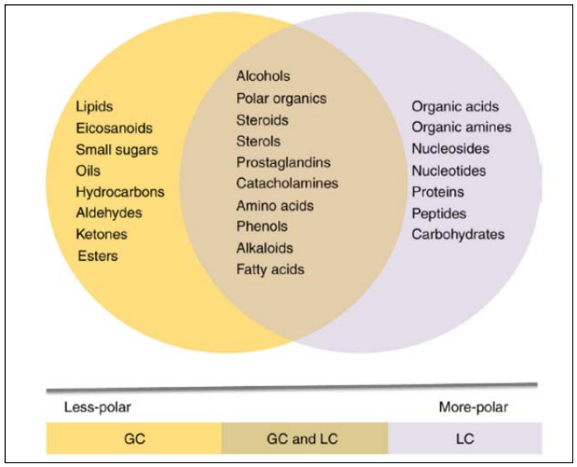
University of Alabama at Birmingham

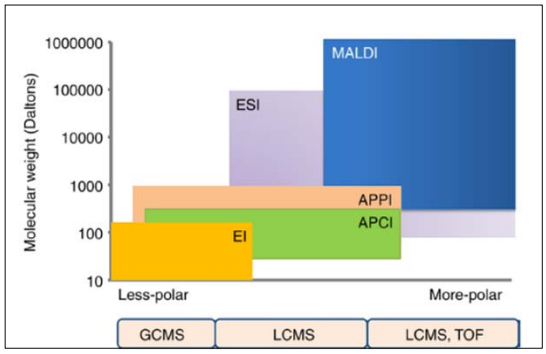
Tandem LC-MS

HPLC	HPLC Column	Mass Spectrometer	Response
			
Sample handling, introduction to system, & solvent delivery	Compound separation	Compound ionization, fragmentation, & detection.	Detection over time – Data output

Path of sample →

Why targeted LC-MS?





Targeted LC-MS pros and cons

- **Pros**
  - High specificity
  - High throughput
  - Cost-effective
  - Automated
  - High sensitivity
  - Variety of scan types
  - Variety of ionization methods
  - Absolute quantification
- **Cons**
  - Availability
  - Material costs
  - Time consuming
  - Equipment issues
  - Extensive sample prep

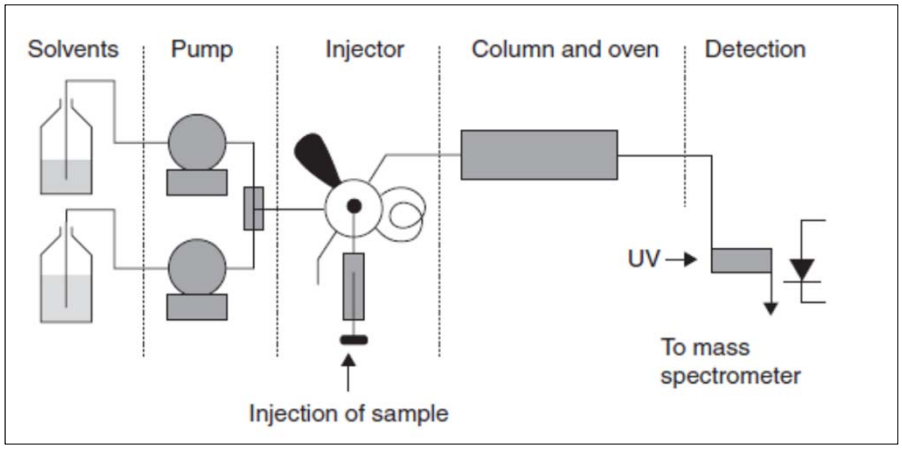
Relevant Vocabulary

- **LC-MS** – Tandem Liquid Chromatography Mass Spectrometry
- **Analyte** – A compound of interest
- **ESI** – Electrospray Ionization
- ***m/z*** – Mass-to-charge ratio.
- **Precursor Ion** – Ionic species *m/z* ratio
- **Product Ion** – Ionic species produced by fragmentation of precursor ion
- **Mass transition** – Precursor ion to product ion change after fragmentation
- **Stable Isotopically Labeled Standards** – Standards that contain known amounts of ^{13}C, ^{15}N, or Deuterium.

Liquid Chromatography

- Technique for separation of compounds by exploiting chemical or physical properties in the presence of a stationary phase and mobile phase over time
- Time from sample introduction, to elution & detection is termed **retention time** (RT)
- Analyte separation by HPLC is highly dependent on compound properties, column stationary phase, mobile phase composition, pH and more.
- LC separation paired with MS specificity provides confidence compound ID and quantification

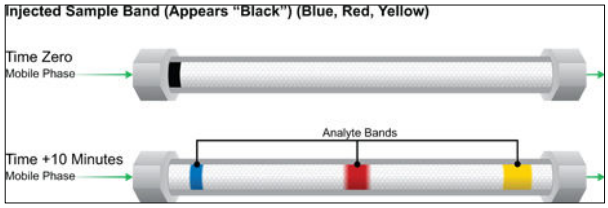
Diagram of HPLC



Liquid Chromatography – options and variety

- **HPLCs**
  - Pressure limit
  - Flow path internal diameter
- **LC techniques**
  - Isocratic
  - Normal Phase
  - Reversed Phase
  - HILIC
  - Ion Pairing
- **Columns**
  - Stationary phases
  - L x W
  - ID
  - Particle size
  - pH range
  - Pressure limit

Liquid Chromatography - columns



Analytical column: Top Right – Microflow, Top Left – Nanoflow, Bottom – Analytical flow

Mass Spectrometry

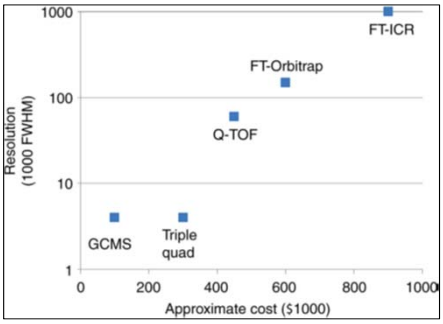
- **Mass spectrometry involves**:
  - Generation of gas phase ionic chemical species
  - Manipulation ions based on mass-to-charge ratio
  - Detection of ions
- **QQQ MS**
  - MS1 Scan – Ions pathed with mass filtering through instrument to detector – mass spectra
  - MS2 Scan - Ions pathed with mass filtering through instrument to detector
    - Fragments will be a finger print of parent ion and are also mass filtered – MS/MS spectra
  - MRM - Known precursor ion and resulting product ion(s) (a mass transition), are filtered twice before detection.

Mass Spectrometer characteristics

Table 2.1 The Analytical Performance Characteristics of the Different Mass Analyzers

Mass Analyzer Type	Resolving Power	Mass Range	Mass Accuracy	Sensitivity	Speed	Dynamic Range	Cost
FTICR	xxxxx	xxxx	xxxxx	xxx	x	xxx	$$$$$$
FT-Orbitrap	xxxx	xxx	xxxx	xxx	xx	xxx	$$$$$
TOF	xxx	xxxxx	xxx	xxxxx	xxx	xxxx	$$$
Quadrupole[a]	x	x	x	xxxxx	xxxxx	xxxxx	$
Quadrupole ion trap	x	x	x	xxx	xxx	xxx	$

FTICR, Fourier transform ion cyclotron resonance; SRM, single reaction monitoring; TOF, time of flight.
[a]In triple quadrupole configuration, SRM mode.



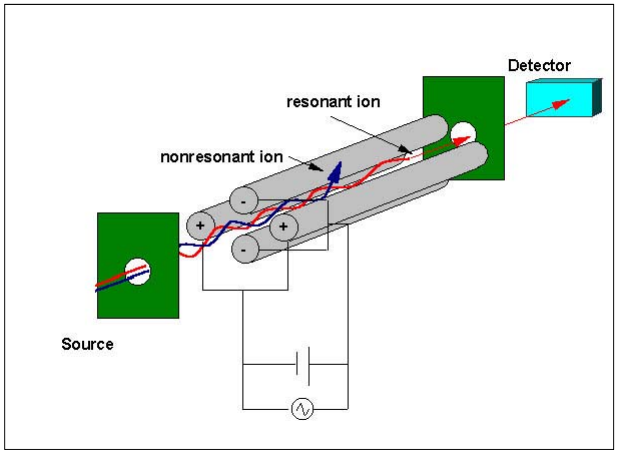
MS Ionization Techniques

Table 1.1 Overview of Three Ionization Techniques Used in Clinical Mass Spectrometry (MS)

Ionization Technique	Advantages	Limitations
ESI	• Sensitive ionization technique for polar analytes or ions generated in solution • Has broad applicability for relevant analytes in clinical MS • May yield multiply charged ions, which allows for analysis of larger molecules (i.e., >1000 Da)	• May be more sensitive to matrix effects compared to APCI
APCI	• Typically less sensitive to matrix effects than ESI • May provide better sensitivity for less polar analytes	• Typically only singly charged ions are formed, limiting the effective mass range, • May be unsuitable for thermally labile analytes • May yield less absolute signal relative to ESI
APPI	• Works well with nonpolar analytes • In some cases will ionize analytes that do not ionize by either ESI or APCI.	• Demonstrates limited applicability in clinical MS to date.

APCI, Atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; ESI, electrospray ionization.

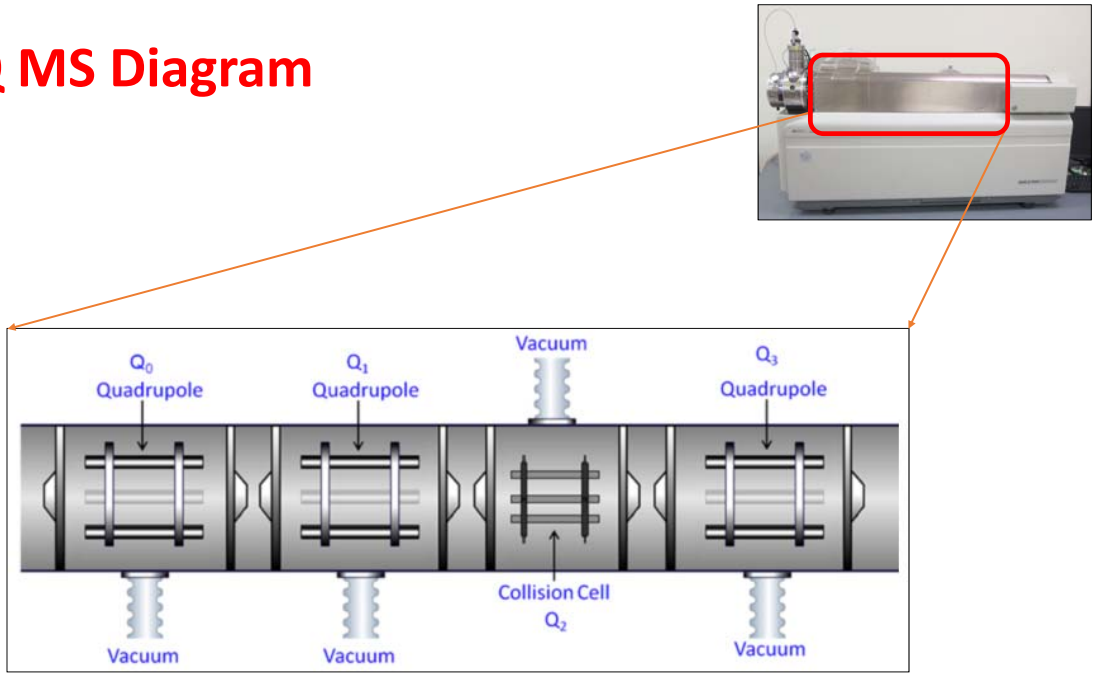
MS - Quadrupole diagram



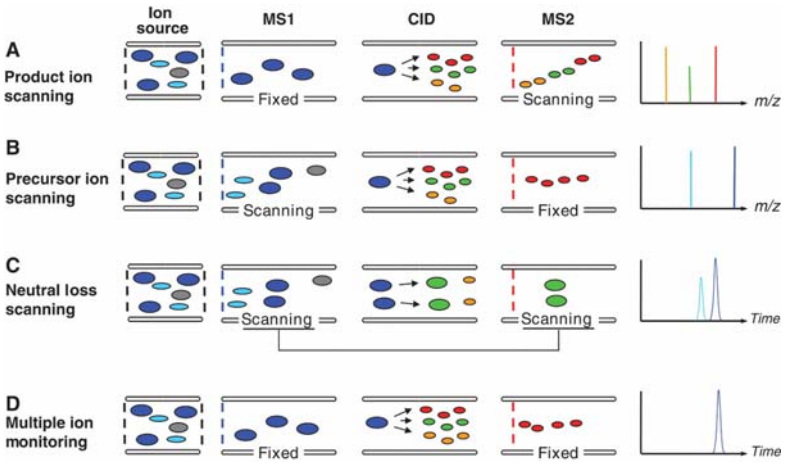
This is a mass filter. It uses a R/F combination to allow ions of a particular *m/z* value to pass through the rod. The bandwidth is typically 0.7 *m/z.* This process is like a filter on a spectrophotometer. A mass spectrum results from scanning the R/F to create a range of *m/z* values, e.g., 50-500.

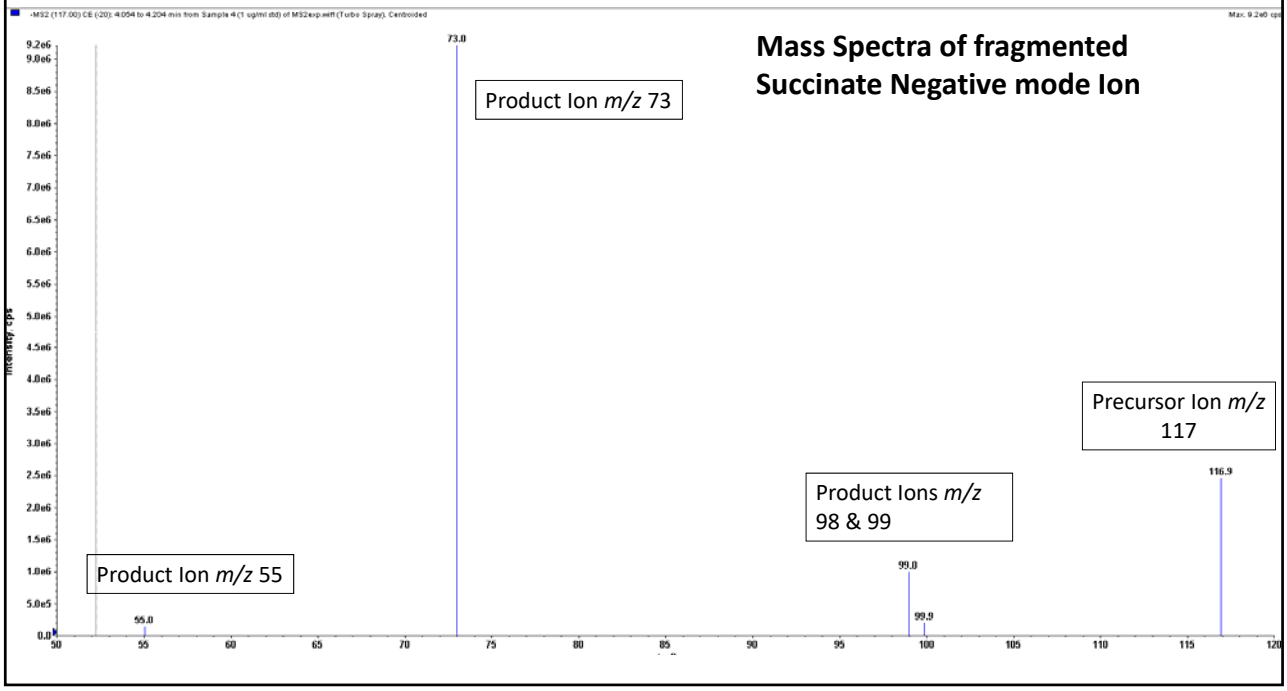
Pictured: Quadrupole and path of ions through

QQQ MS Diagram



QQQ MS Scan Types





Tandem LC-MS

HPLC

Sample handling, introduction to system, & solvent delivery

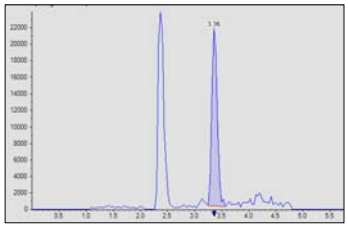
HPLC Column

Compound separation

Mass Spectrometer

Compound ionization, fragmentation, & detection.

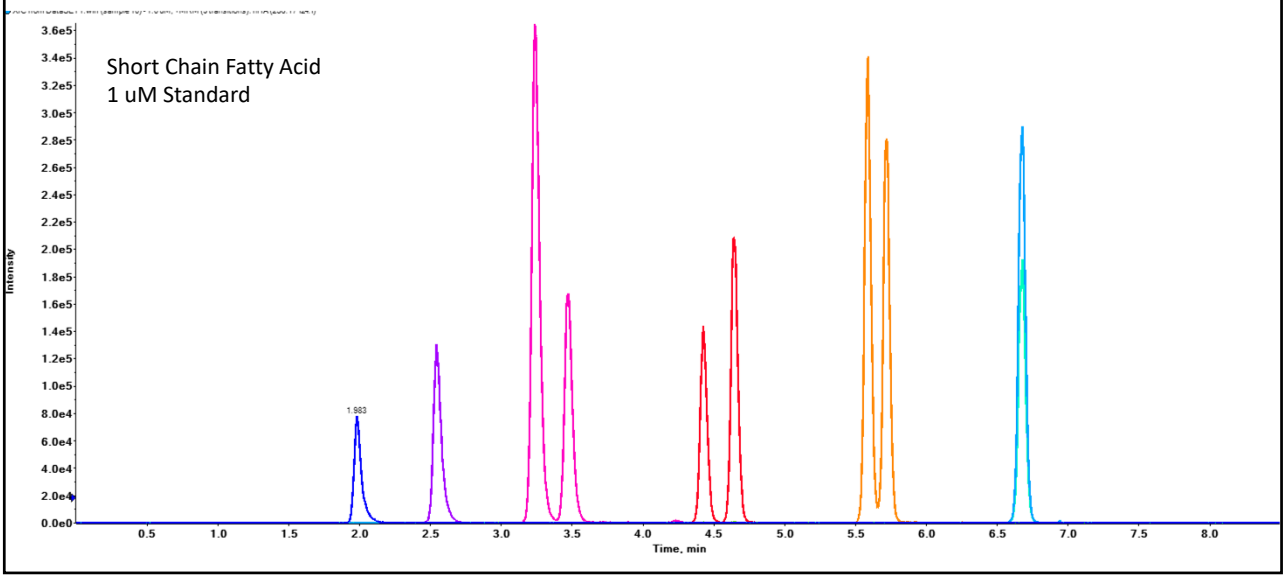
Response



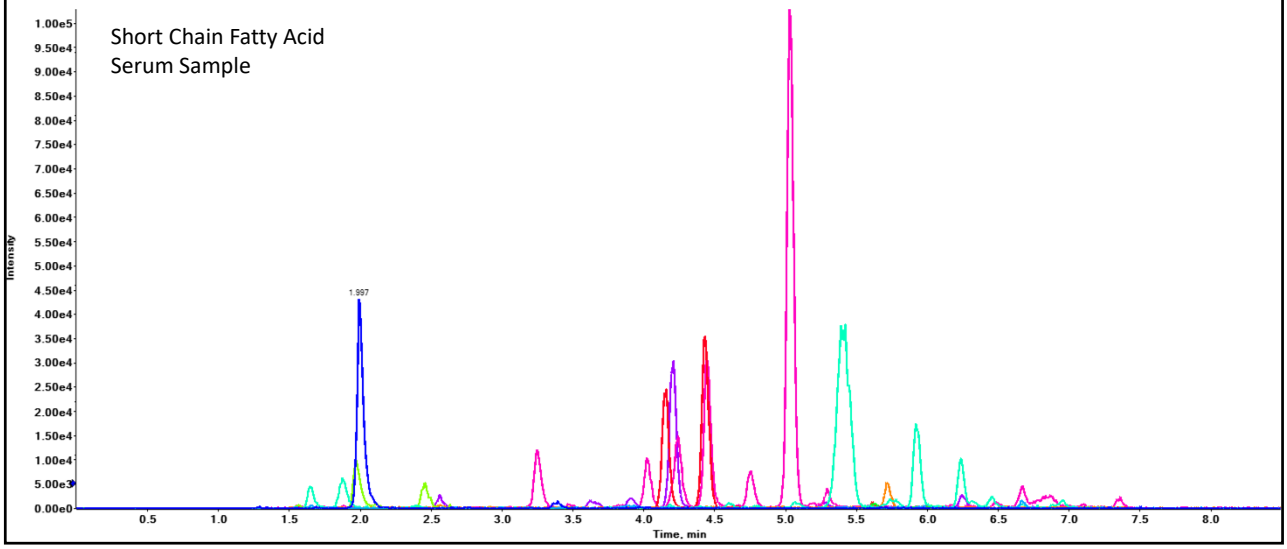
Detection over time – Data output

Path of sample

LC-MS MRM Chromatogram



LC-MS MRM Chromatogram



So you want to develop a targeted LC-MS method…

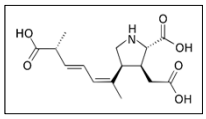
- Has it been published on before?
- What is the analyte of interest? Biomolecule? Drug?
- What matrixes is analyte in? How prevalent is it?
- How will analyte be extracted & isolated?
- Will the analyte ionize? Can it be made to ionize?
- Will it chromatographically separate?

Previous publications

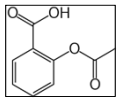
- **Previous publications on analytes of interest can save a lot of time & effort**
- **Analytical equipment companies publish application notes for demos of product**s
- **Important factors to resource**
  - Analytical Equipment
  - HPLC Separation technique & column choice
  - MS parameters of analytes
  - Extraction techniques
  - Matrix quantity
  - Complications or issues regarding analysis

Literature searches can help prevent waste of time, money, and this reaction

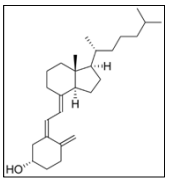
Analytes of interest



Domoic Acid



Aspirin



25-OH VD3

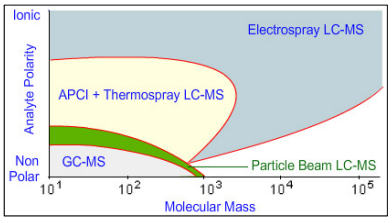
- **Analytes of interest can be**: Small molecules, lipids, peptides, proteins, drugs, biomarkers, etc.
  - Compound characteristics will determine sample processing, extraction & detection techniques.
- **Matrix of analyte is important!**
- **Distribution of analyte within matrix**
  - Whole tissue/lysate, specific cell population, subcellular fraction, etc.
- **Quantity of analyte**
  - Will determine amount of matrix required for future processing.

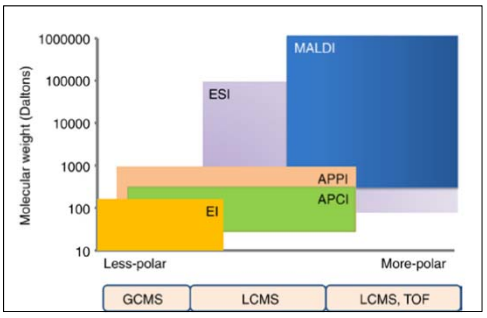
Complex Biologic Matrices

- Typically bio-fluid or tissue
- Contains analyte of interest as well as other macromolecular species
- Other species can enhance or suppress signal of analyte of interest
  - Generally suppresses signal
- Matrix 'contaminants' to be concerned with during prep:
  - Phospholipids – can clog column and will lend to matrix suppressing effects
  - Proteins – can clog column or LC lines
  - Salts – interfere with electrical conductivity
- Evaluation and consideration of matrix effects in LC-MS analysis is imperative
- Extraction technique strategies try to maximize analyte recovery and minimize contaminant recovery

Analytes of interest

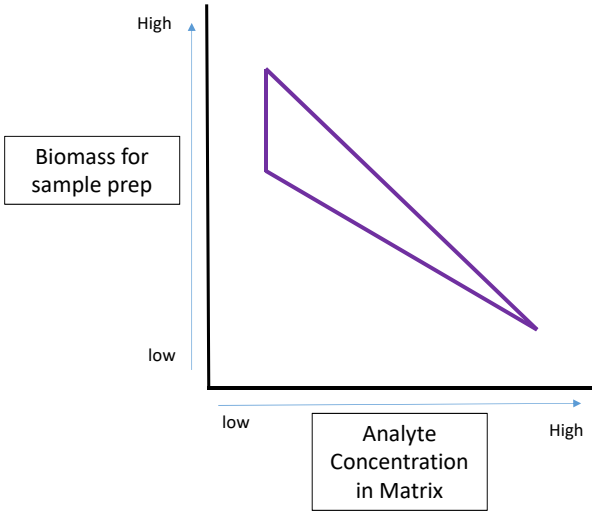
- **MS Detection**
  - Can the analyte be ionized? Depends on compound properties & functional groups.
  - If no, then perhaps the analyte can be derivatives/chemically modified to allow for ionization.
- **Reference Standards**
  - Resource a purified standard(>98%) for analyte of interest
  - If a standard cannot be found – could make one or find stand-in analyte





Extractions – how much sample?

- Dependent on the concentration of metabolite and tissue
- Metabolites within a single pathway can have extreme variance in concentrations
  - Circulating plasma concentrations Vitamin D3
    - 25-OH Vitamin D3: 5 – 100 ng/ml
    - 1,25-OH Vitamin D3: 22 – 85 pg/ml
- Based on above example to quantify 1,25-OH VD3 one would need to increase starting sample biomass or turn to unique sample prep techniques
- Background research along with empirical testing is the best means to hone down on 'how much?'



Sample preparation

- **Sample Pre-treatment**
  - Protein Precipitation
  - Acid or base adjustment
  - Filtering
- **Common Extraction Techniques**
  - Liquid-Liquid Extraction(LLE)
  - Solid Phase Extraction(SPE)
  - Supported Liquid Extraction(SLE)
  - Immunoextraction(IE)
  - Super Critical Fluid Extraction(SCFE)
- **All techniques have pros & cons associated**
- **Extraction techniques can be combined for specific needs**

Sample Preparation

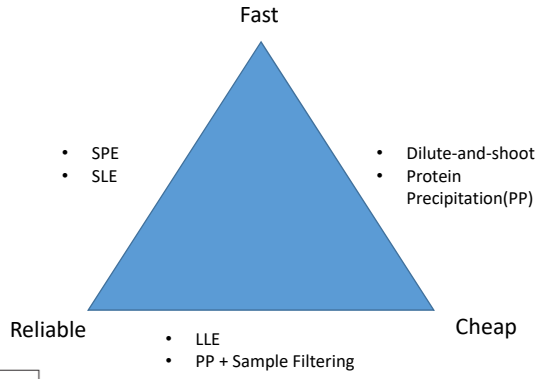


Table 3.1 Simplified Comparison of LC-MS/MS Sample Preparation Types

Sample Preparation Protocol	Analyte Dilution (D) or Concentration (C) Possible	Relative Cost	Relative Complexity	Relative Matrix Removal
Dilution (DIL)	D	Low	Simple	Less
Protein precipitation (PPT)	D	Low	Simple	Least
Liquid–liquid extraction (LLE)	D or C	Low	Complex	More
Phospholipid removal (LPR)	D	High	Moderately complex	More, selective*
Supported liquid extraction (SLE)	D or C (moderate)	High	Moderately complex	More
Solid phase extraction (SPE)	D or C	High	Complex	More
Online SPE/Turboflow	D or C	High	Complex	More

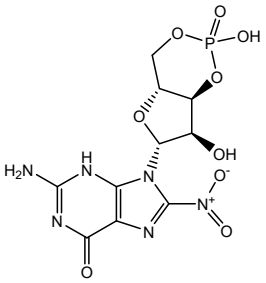
**Only phospholipids are removed, other matrix components are not depleted.*

Internal Standards

- **Internal standards are known analytes used during extraction and LC-MS quantification**
- **Composition**
  - Ideal – ^{13}C, ^{15}N, or ^{2}H labeled stable isotope of standard
  - Otherwise – chemically and/or physically similar, yet different compound of interest
- **Spiked into standards and samples at static, known amount**
  - Difference in recovered amount in samples will help more accurately back calculate
  - Can correct for matrix effects as well as extraction recovery
- **Gold standard for quantitative LC-MS**
- **Downside – limited and costly.**

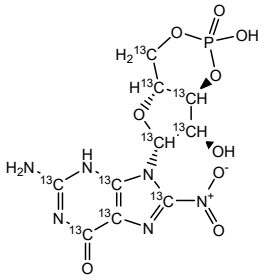
Internal standards – 8-nitro-cGMP

8-nitro-cGMP



Chemical Formula: $C_{10}H_{11}N_6O_9P$
Molecular Weight: 390.20

[13C10] 8-nitro-cGMP

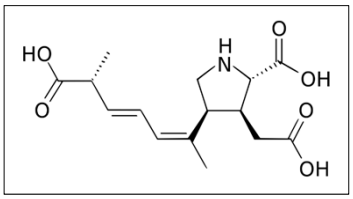


Chemical Formula: $^{13}C_{10}H_{11}N_6O_9P$
Molecular Weight: 400.13

Example project development – Domoic Acid

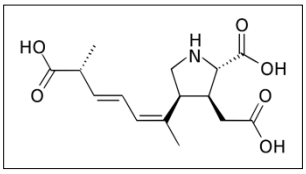
1. Project goals and background research
2. Obtain spectra & MS parameters
3. LC testing & validation
4. Standard curve range & limits of quantification
5. Extraction & Recovery with mock samples
6. Sample analysis for experimental data

1. DA – Project Research



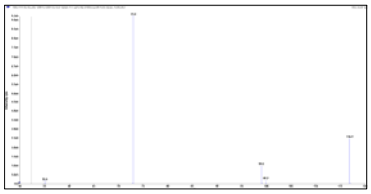
- **Factors to to consider**
  - **What are the analytes of interest?**
    - **Domoic Acid – Algal toxin that causes to foodborne illness.**
  - **Has anybody measured it & published a method?**
    - **Yes, allowed for quick start and reduced development time.**
  - **What matrixes are the analytes in? How prevalent is said analyte?**
    - **Fish oil products. Estimated low [ng/ml] amounts, if any. Empirically confirmed.**
  - **How will analyte be extracted & isolated?**
    - **Fish Oil samples. Bligh-Dyer LLE for delipidation. Water phase recovered with analyte.**
  - **Will the analyte ionize? Can it be made to ionize?**
    - **Yes. Can ionize in Positive or Negative polarity. Positive polarity chosen. Literature suggestion.**
  - **Will it chromatographically separate?**
    - **Yes. DA can be separated using C_{18} or Phenyl-Hexyl column. PH column chosen. Literature suggestion.**

2. DA – Obtaining Spectra & MS parameters



DA, MW = 311
$[M+H]^+$ = 312 *m/z*

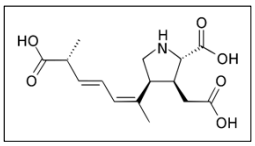
Fragment & obtain spectra



Major mass transitions:
312 → 266 & 161

Major mass transition of DA standard obtained. Next step LC separation

3. DA – HPLC Development & Validation

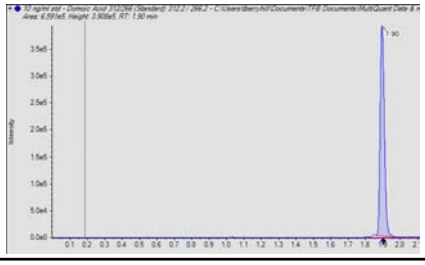


DA Solution 10 ng/ml

HPLC System

Phenyl Hexyl Column

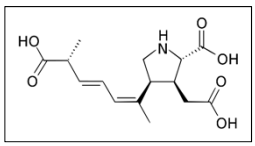
MS Filtering for 312/266 & 312/161 $[M+H]^+$ Ions



5 minute/injection separation. 1.90 min RT

Next Step: Need to determine standard curve & limits of range.

4. DA – Standard Curve Development



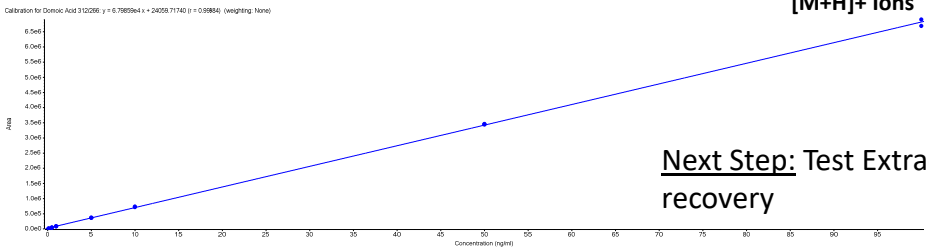
Serial diluted DA standard 100 – 0.1 ng/ml

HPLC System

Phenyl Hexyl Column

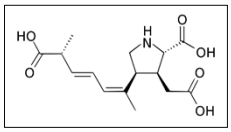
MS Filtering for 312/266 & 312/161 [M+H]+ Ions

Standard curve & linearity of response determined



Next Step: Test Extraction & recovery

5. DA – Extraction testing & validation



DA Solution 10 ng/ml

Clean or Stripped Sample

Spike at known Concentration

Extract Sample

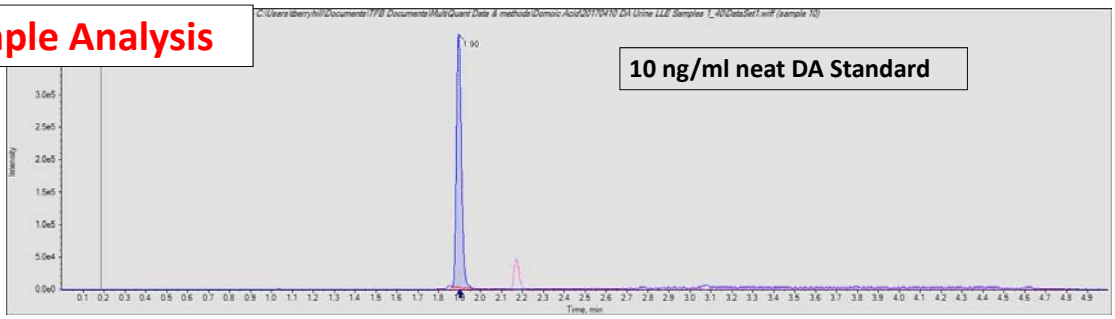
Recover Sample

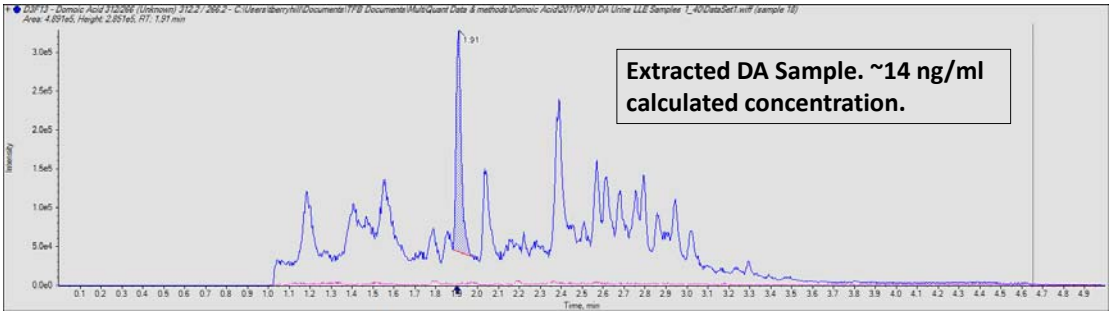
LC-MS Analysis

- Validate Extraction Method
- Extraction efficiency/Recovery
- Matrix Effects

<u>Next Step:</u> Extract & analyze samples for data

6. Sample Analysis





Resources - MS Manufacturers

- Sciex – www.sciex.com/
- Thermo-Fisher - www.thermofisher.com/
- Agilent - www.agilent.com/
- Waters - www.waters.com/
- Shimadzu - www.shimadzu.com/
- Perkin-Elmer - www.perkinelmer.com/
- Bruker - www.bruker.com/

Resources – Reference Standards

- Cerilliant/Sigma - www.cerilliant.com/
- Cambridge Isotope Labs - www.isotope.com/
- Cayman Chemical - www.caymanchem.com/
- Avanti Polar Lipids – www.avantilipids.com/
- Thermo-Fisher –www.thermofisher.com/
- Phenomenex –www.phenomenex.com/
- Steraloids - steraloids.com/
- Toronto Research Chemicals - www.trc-canada.com/
- Sigma/Millipore - www.sigmaaldrich.com

Resources – Column & Extraction Products

- Waters – www.waters.com/
- Phenomenex - www.phenomenex.com/
- Agilent – www.agilent.com/
- Thermo-Fisher – www.thermofisher.com/
- Restek - www.restek.com/
- Shodex - www.shodex.com/
- Sigma/Suppelco - www.sigmaaldrich.com/
- MAC-mod - mac-mod.com/

The End!

Any questions?