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UAB Metabolomics Workshop
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Developing a targeted metabolomics quantification method with focus on LC-MS

Targeted Metabolomics & Proteomics Laboratory

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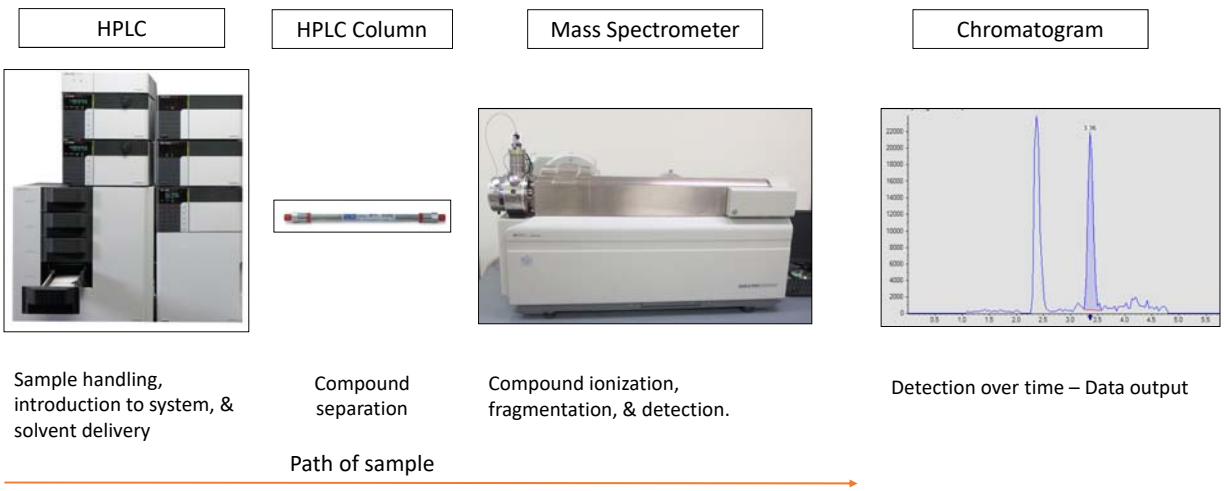
Introduction to LC-MS metabolomics

- Within biological experiments there is a need measure individual components, or metabolites, within complex biological matrices
- Liquid Chromatography-Mass Spectrometry provides a means to absolutely quantify analytes of interest
- Targeted LC-MS involves separation & detection of pre-determined ions species
- Challenges associated with LC-MS quantification include extraction, HPLC separation, and matrix interferences
- Targeted LC-MS is capable of automated high throughput analysis

Terms of interest

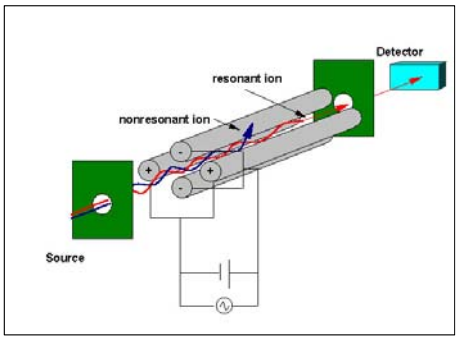
- **LC-MS** – Tandem Liquid Chromatography Mass Spectrometry
- **Analyte** – A compound of interest
- **ESI** – Electrospray Ionization
- **APCI** – Atmospheric Pressure Chemical Ionization
- ***m/z*** – Mass to charge ratio. Typically singly charged
- **Precursor Ion** – Ionic species with particular *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 ^{13}C, ^{15}N, or ^{2}H

Tandem LC-MS



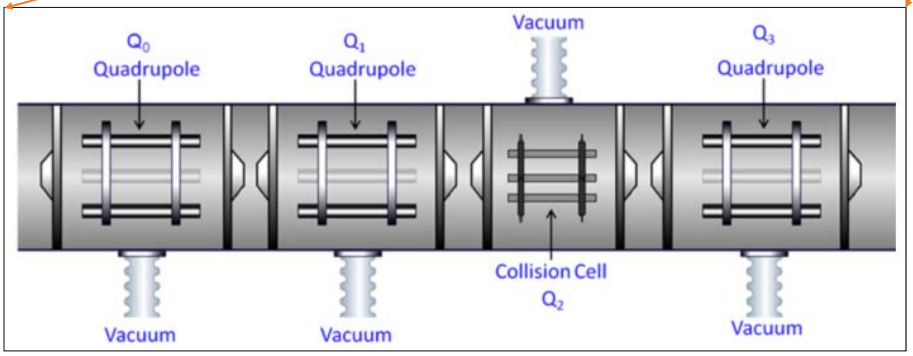
LC-MS Introduction continued

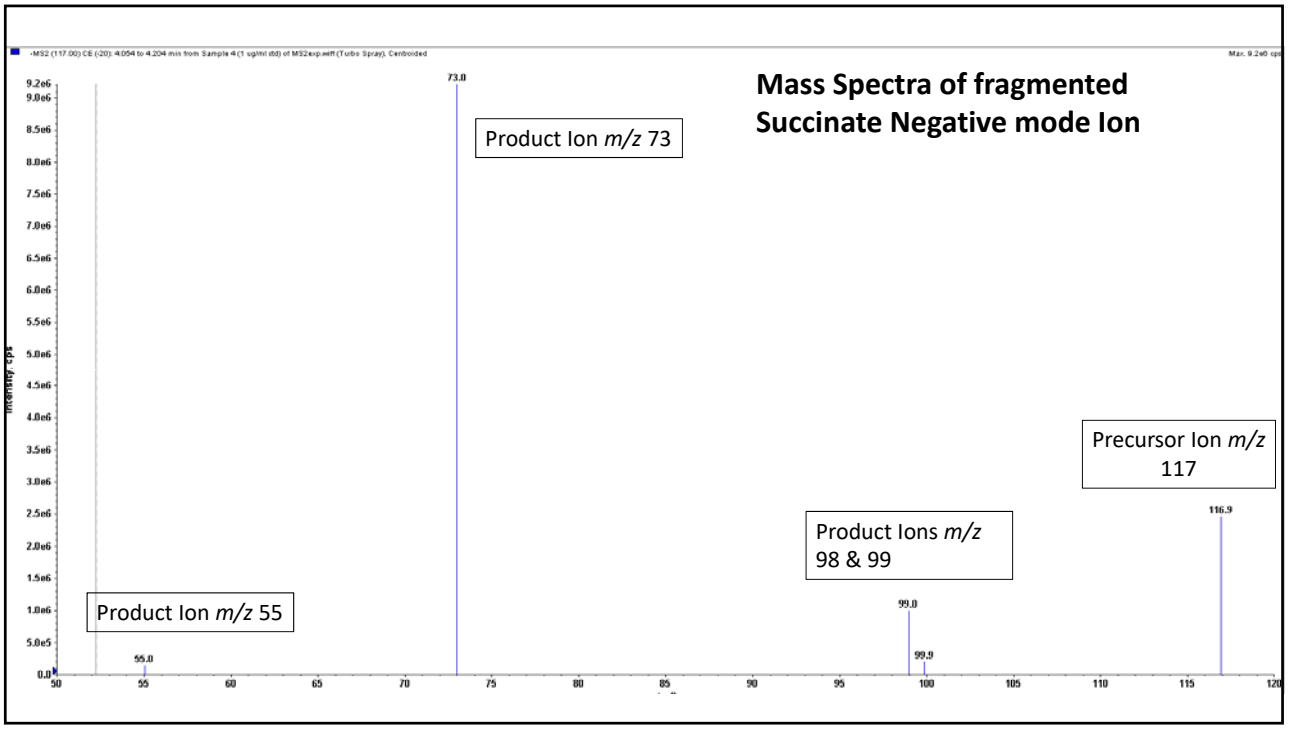
- Mass spectrometry involves the generation on ionic species, introduction ion to instrument, & manipulation ions to detector
- Many different configurations of mass spectrometers
  - Single Quad, **QQQ**, QToF, Orbitrap, ToF/ToF
- QQQ MS instruments contain a collision cell
- Ions can be collided to break apart or fragment
- Collided Ions have fingerprint fragment ions – Mass Spectra
- Characteristic primary & secondary ions can be used to measure specific ionic species – Mass transitions
- Pairing HPLC separation with MS mass filtering allows for analyte ID with confidence



Pictured: Quadrupole & mean free path of ions

Triple Quadrupole MS Diagram

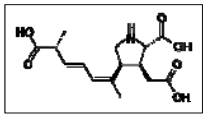


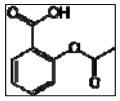


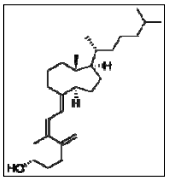
So you want to develop a targeted LC-MS method…

- **Factors to to consider**
  - What are the analytes of interest?
  - Has anybody measured it & published a method?
  - What matrixes are the analytes in? How prevalent is said analyte?
  - How will analyte be extracted & isolated?
  - Will the analyte ionize? Can it be made to ionize?
  - Will it chromatographically separate?

Analytes of interest


Domoic Acid

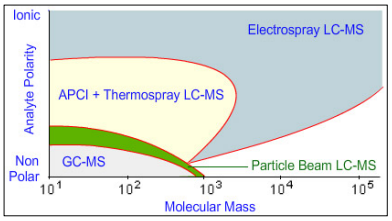

Aspirin


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.

Analytes of interest - continued

- **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



Stable Isotope dilution

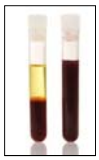
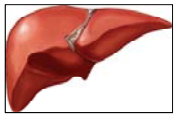
- Stable isotopically labeled standards added in a known amount to samples pre-extraction
- Will control for extraction efficiency & matrix effect during analysis
- Isotope standards will co-elute with analyte & provide greater degree of confidence in measure
- Standards will also have the same amount of stable labeled compounds – improves calculated concentration accuracy
- Isotope Dilution-MS is gold standard for absolute quantification
- Cons – Expensive & not all compounds have stable isotope standards

Previous publications

- Previous publications on analytes of interest can save a lot of time & effort
- Analytical equipment companies publish application notes for certain products
- Important factors to resource
  - Analytical Equipment
  - HPLC Separation technique & column
  - 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

Matrices & Extractions

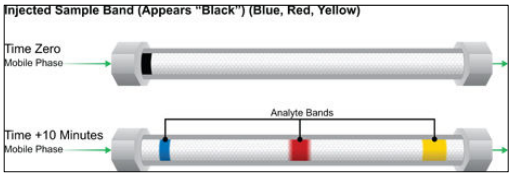

- Complex biological matrices will contain analyte of interest along with many other molecular species
- The ideal scenario for quantification analyte would be to extract & purify
- Requires knowledge of matrix, analyte & extraction techniques
- The cleanest sample you can generate the more accurate your results, however comes at a cost
- Realistic limits on extraction efficiency, cleanliness, effort & $$ cost
- Best practice requires empirical testing & validation

Matrices & Extractions - continued

- 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
- Must weigh techniques against a number of factors: effort, cost, reproducibility, sample throughput, etc.



Liquid Chromatography



- Analytical technique for separation of compounds by exploiting chemical or physical properties in the presence of a stationary phase over time
- LC separation of analytes are carried out using liquid solvents called mobile phases
- Time from sample introduction, to elution & detection termed retention time (RT)
- Analyte separation by HPLC is highly dependent on compound properties, column properties & mobile phases
- LC separation paired with MS specificity provides confidence compound ID

Liquid Chromatography - continued

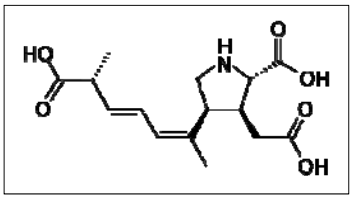
- HPLC variety
  - HPLC vs UHPLC
  - Macro, micro, & nanoflow systems
- Normal Phase vs Reversed Phase
- Many different stationary phases for RP
  - C_{18}, C_8, Phenyl, Phenyl Hexyl, Ion Exchange, size exclusion, & more
- Range in column dimensions, particle size, pore size, & more.
- HPLC is a topic in and of itself – scope is beyond this presentation.

Analytical column: Top Right – Microflow, Top Left – Nanoflow, Bottom - Macroflow

Example project development – Domoic Acid

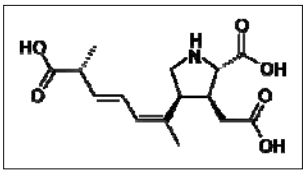
1. Project background research
2. Obtain spectra & MS parameters
3. HPLC testing & validation
4. Standard curve range & limits of quantification
5. Extraction & Recovery with mock samples
6. Sample analysis for experimental data

1. DA – Project Research



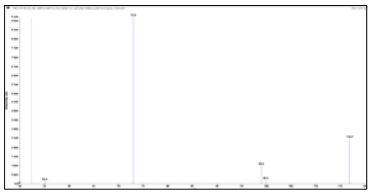
- **Factors to to consider**
  - What are the analytes of interest?
    - **Domoic Acid – Algal toxin that causes to foodborne illness.**
  - Has anybody measured it & published a method?
    - **Yes, allowed for quick start and reduced background research.**
  - What matrixes are the analytes in? How 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 C18 or Phenyl-Hexyl column. PH column chosen. Literature suggestion.**

2. DA – Obtaining Spectra & MS parameters



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

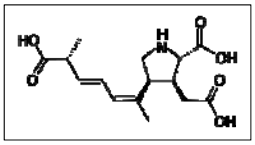
Fragment & obtain spectra



Major mass transitions:
312 → 266 & 161

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

3. DA – HPLC Development & Validation

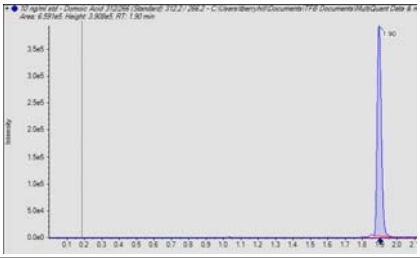


DA Solution 10 ng/ml

HPLC System

Phenyl Hexyl Column

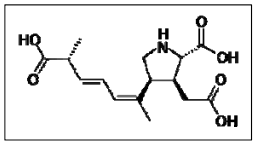
MS Filtering for 312/266 & 312/161 [M+H]+ Ions



5 minute/injection separation. 1.90 min RT

<u>Next Step</u>: Need to determine standard curve & limits of range.

4. DA – Standard Curve Development



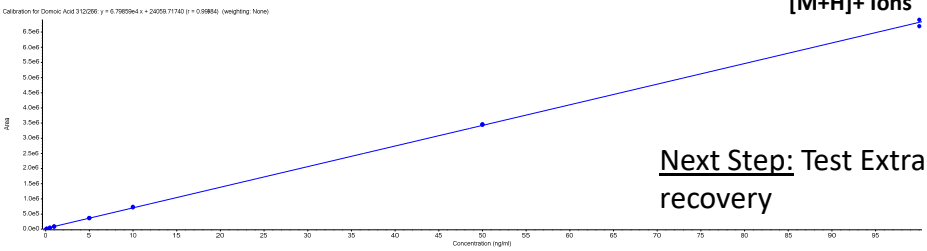
Serial diluted DA standard 100 – 0.1 ng/ml

HPLC System

Phenyl Hexyl Column

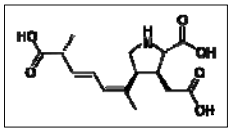
MS Filtering for 312/266 & 312/161 [M+H]+ Ions

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

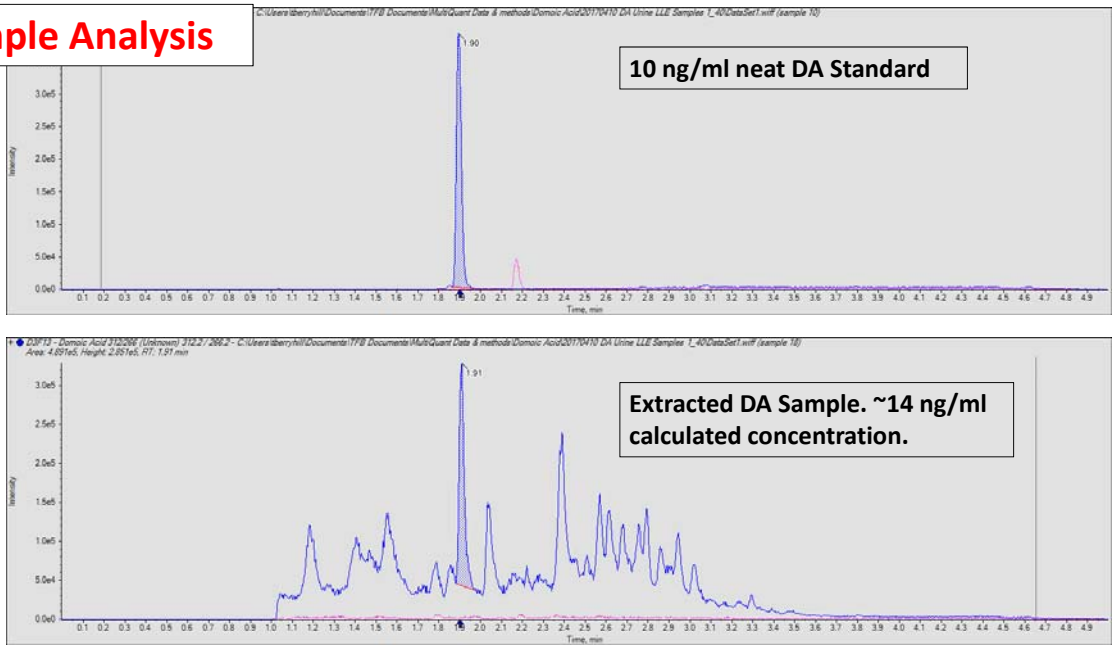
Recover Sample

LC-MS Analysis

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

Next Step: 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?