THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Knowledge that will change your world

Choosing the metabolomics platform

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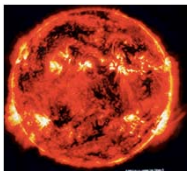
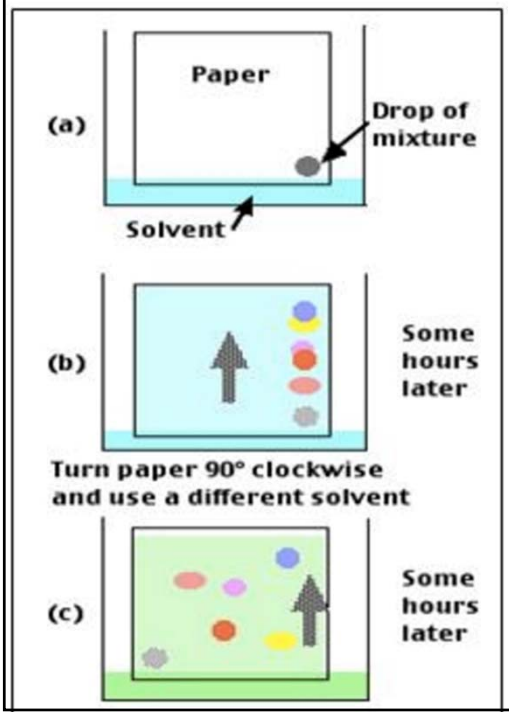
Targeted Metabolomics & Proteomics Laboratory

Challenges

- **Unlike DNA, RNA and proteins, the metabolome is phenomenally chemically diverse**
- **Ranges from a gas (H_2) that prevades the universe and is the principal component of the Sun**

to  

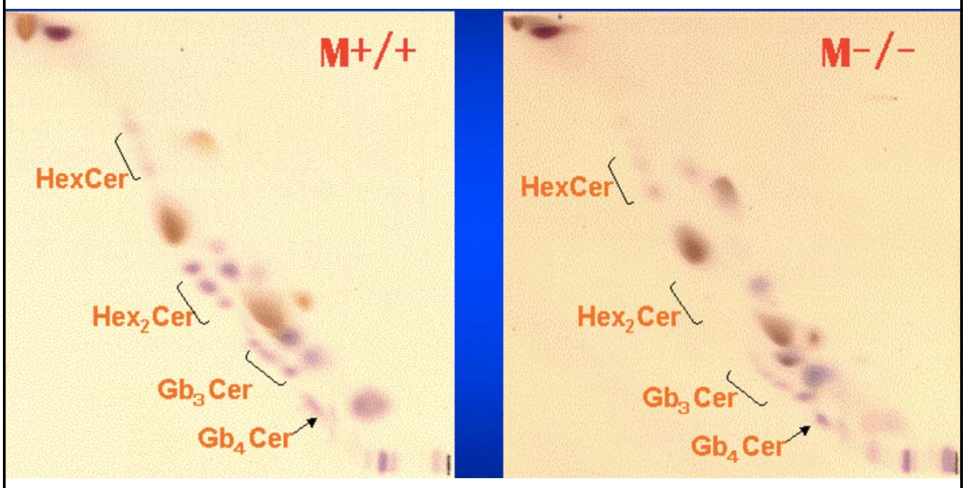
- **Earwax (long chain fatty acids, both saturated and unsaturated, alcohols, squalene, and cholesterol)**
- **No single method of analysis**



Early forms of metabolomics

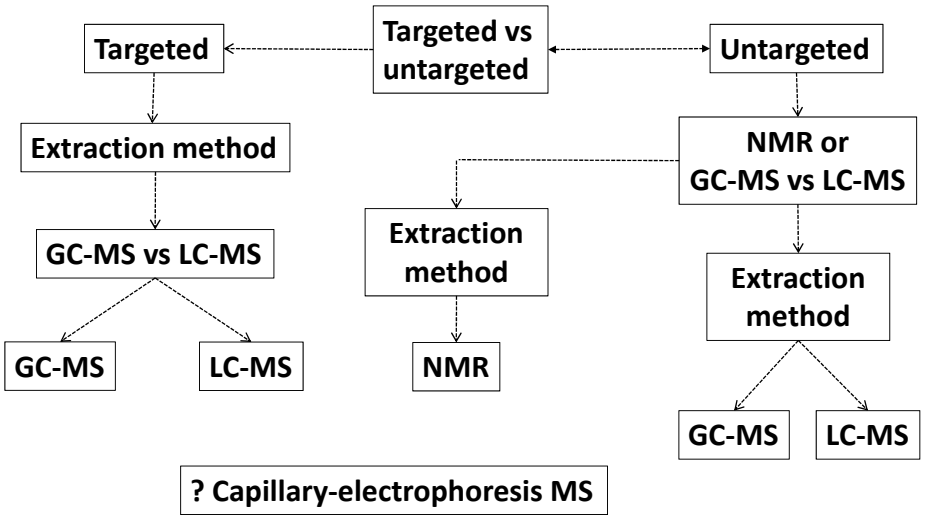
2D-paper chromatogram
low capacity

2D-Thin layer chromatography of lipids
KO of cerebrosidesulfatase in kidney



These days can be studied by direct electrospray ionization (DESI)

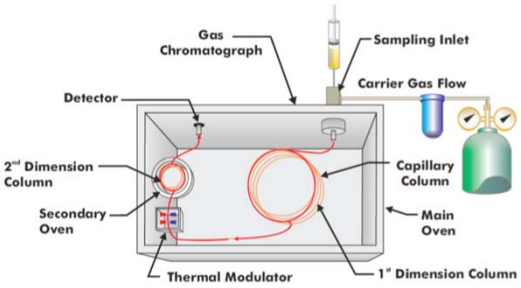
Decision tree

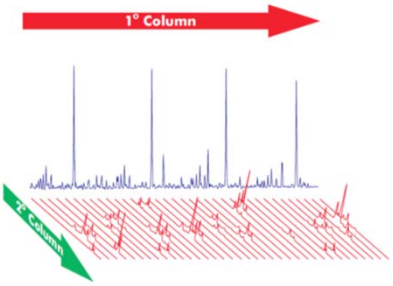


Metabolomics and GC-MS

- **PROS**
  - Capillary columns can achieve very high chromatographic resolution
  - Retention times are reproducible
  - Mass spectral libraries are well developed
- **CONS**
  - Not all compounds can be analyzed by GC-MS
  - Although amino acids, sugars, fatty acids, amines and organic acids can be derivatized, complex polyphenol glycosides and polar lipids are too unstable, even when derivatized, at the temperatures used to elute them
  - Approximate mass limit of 400 Da

Two dimensional GC to resolve metabolites



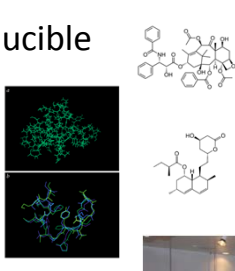


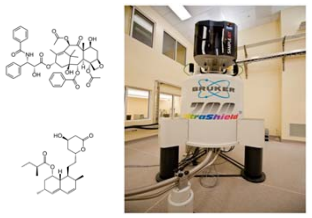
As compounds elute from column 1, they are passed to (cooler) column 2 where they condense. After a period of collection, column 2 is heated so as to separate and elute the compounds.

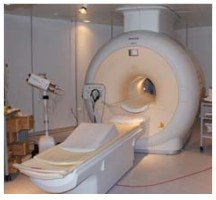
Leco Corp.

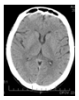
Nuclear Magnetic Resonance (NMR) Spectroscopy

- Detects NMR active nuclei
- Robust and highly reproducible
- Non-destructive
- Quantitative
- Used in
  - Structure elucidation
    - Small molecules
    - Macromolecules (DNA, RNA, Proteins)
  - A number of techniques
    - 1D , 2D, 3D
  - Molecular motion and dynamics
- Similar method used in medical Imaging (MRI, fMRI)









from Wimal Pathmasiri

NMR considerations

- **Sample amount:**
  - Typical 600 MHz instrument requires 0.5 ml plasma/serum
  - Higher field instrument and micro coil detector allows use of 0.1 ml
- **Quality control:**
  - In the UK Phenome Center, all samples are analyzed by NMR
    - This allows for detection of outliers
    - Also found that there is a correlation between the NMR spectrum and whether problems occur in LC-MS analysis
    - NMR analysis used to filter out these samples

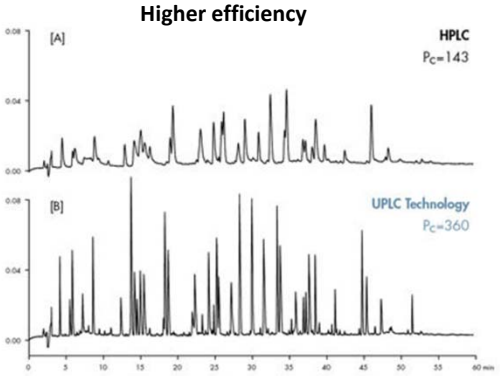
Liquid chromatography-Mass Spectrometry

- **PROS**
  - **Almost all compounds can be analyzed by LC-MS**
    - **hydrocarbons do not ionize**
  - **Several orders of magnitude increased sensitivity compared to NMR**
  - **Can collect MS, MSMS and ion mobility data**
- **CONS**
  - **Not uniformally quantitative**
  - **Mass spectral libraries are not well enough developed**
  - **Chromatographic separation not adequate**
  - **Retention time reproducibility not as good as GC-MS**

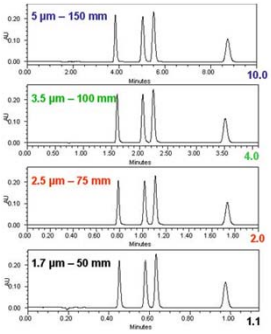
The LC

- **1D-approach**
  - Use of reverse-phase, normal phase and HILIC phase
  - particle size – smaller is more efficient, but back pressure is a problem

Higher efficiency



Same efficiency, but shorter run times



LC flow rate

- **Sensitivity is inversely related to flow rate**
  - Slower flow gives more sensitivity

normal flow (0.2-0.4 ml/min)

microflow/capillary (5-50 µl/min)

nanoflow (0.3-5 µl/min)

Optimizing nanoLC for metabolomics

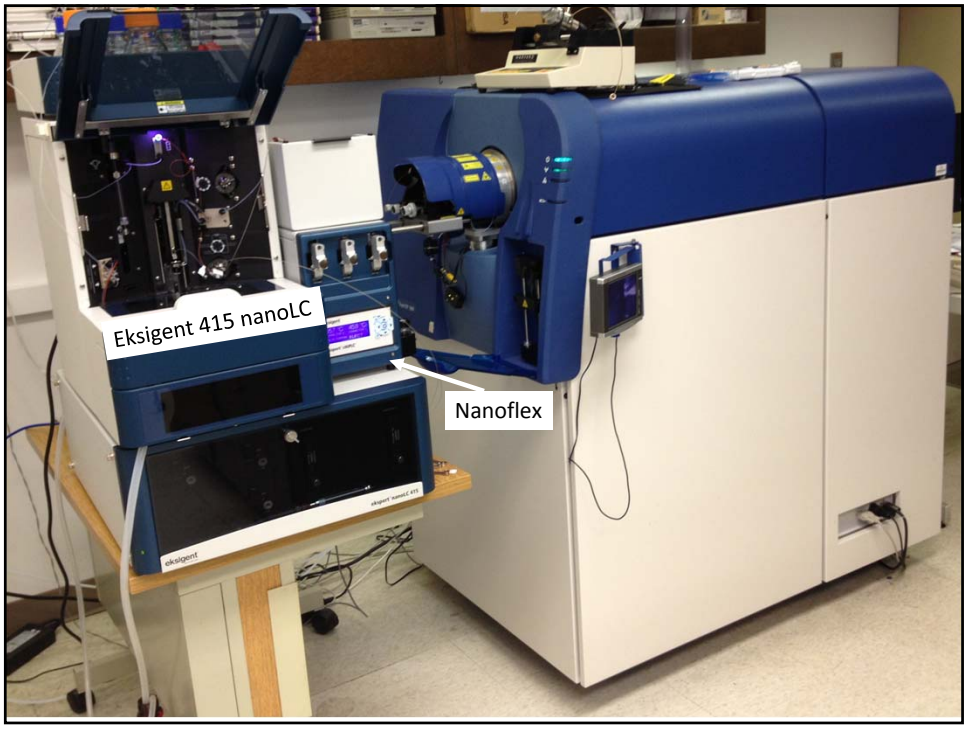
- Objective is to develop metabolomics for small animal model systems
  - *D. melangaster*
  - *C. elegans*
  - *D. rerio*

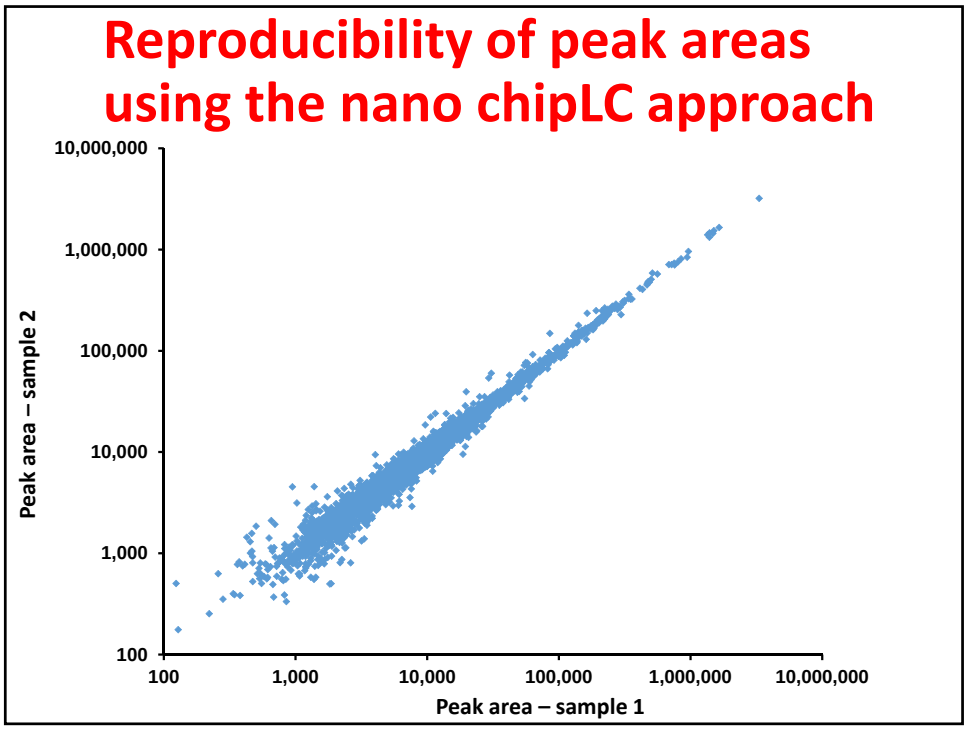


- A single zebrafish yields about 1 μl of plasma
- Need to move down to the nanoscale
- Important to maintain consistency and quantitation
  - Reproducible columns and temperature

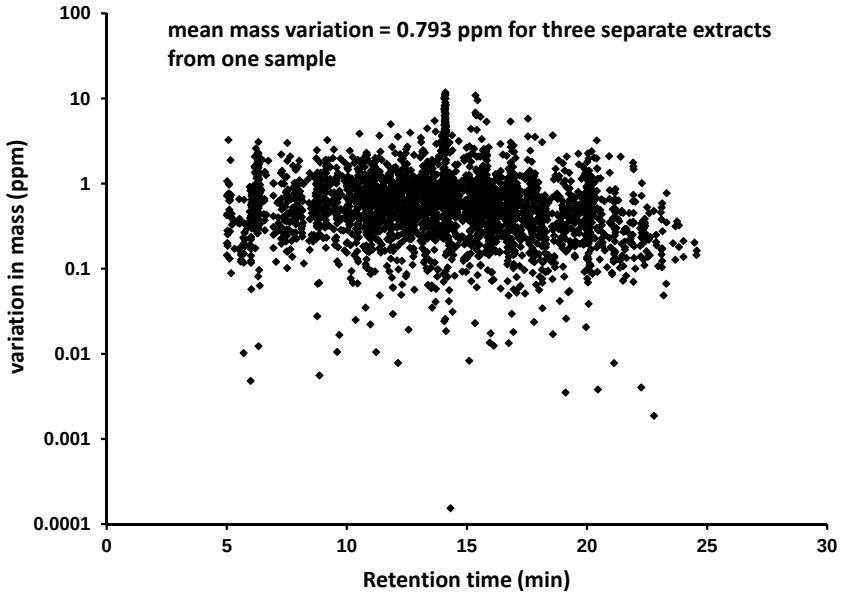
Close up of a nanochipLC cartridge (15 cm x 0.2 mm ID).

- Each long section of the column is ~2.5 cm (1 inch).
- Can be machined to a better tolerance.
- Simpler connections to the liquid stream.
- Can be placed in a temperature-controlled environment

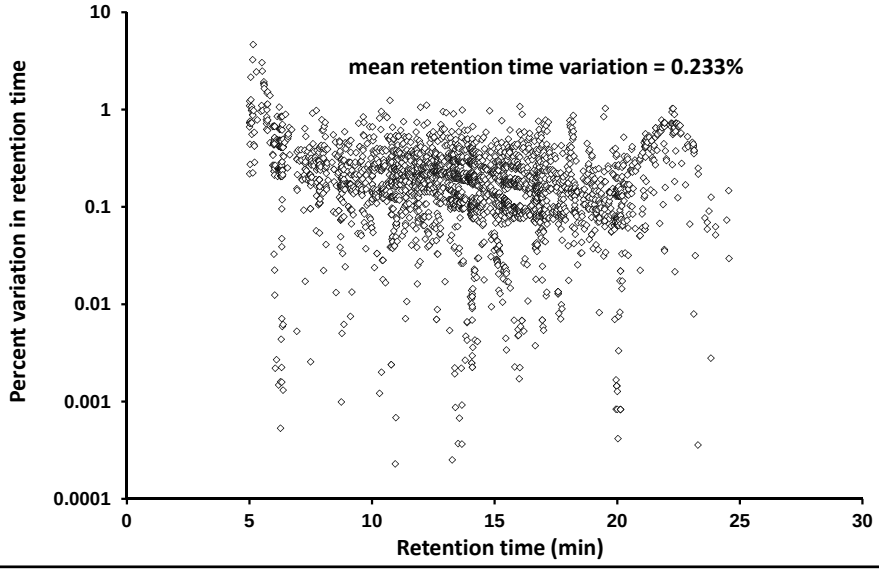

Eksigent 415 nanoLC
Nanoflex


Reproducibility of peak areas using the nano chipLC approach
10,000,000
1,000,000
100,000
10,000
1,000
100
Peak area – sample 2
100
1,000
10,000
100,000
1,000,000
10,000,000
Peak area – sample 1

Coefficient of variation of the mass of peaks identified by XCMS using nanoLC-MS



Coefficient of variation of retention time for the three separate extracts by nanoLC-MS



The mass spectrometer

- **For untargeted analysis it is important to have high mass resolution and accuracy**
  - Initial data analysis is performed on the molecular ions
  - Each metabolite has a unique mass (*m/z*)
  - Nonetheless, a particular mass, however exact, is not necessarily a unique metabolite
- **Fourier transform-ion cyclotron resonance and Orbitrap instruments have the greatest mass accuracy**
  - However, their performance is time-dependent and is degraded significantly by short acquisition times (<100 ms)
  - They are best used for follow up experiments

Mass analyzer of choice for untargeted metabolomics

- **Quadrupole-orthogonal time-of-flight (Q-TOF)**

Agilent 6500

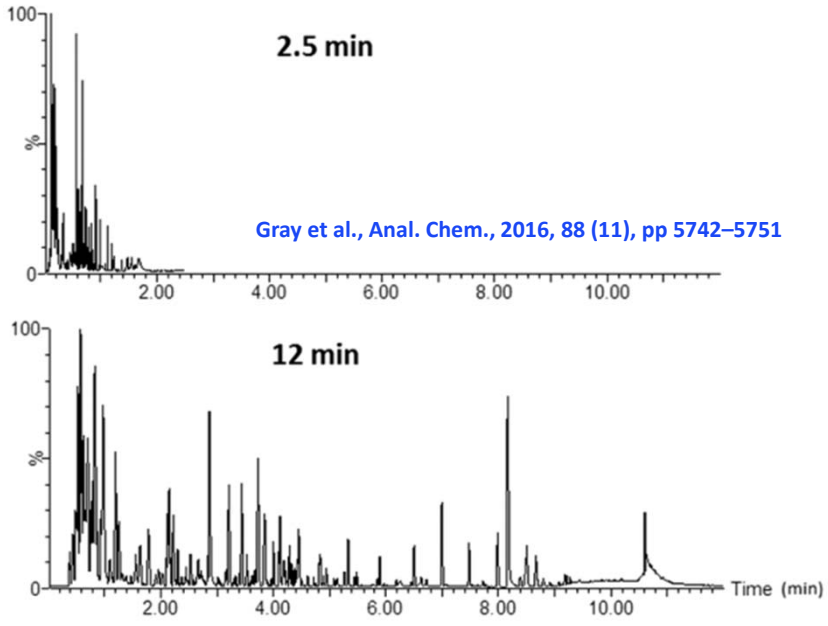
Waters Synapt G2/HMDS

Bruker

Sciex TripleTOF 6600

Current models have ~100,000 mass resolution and 1-2 ppm mass accuracy

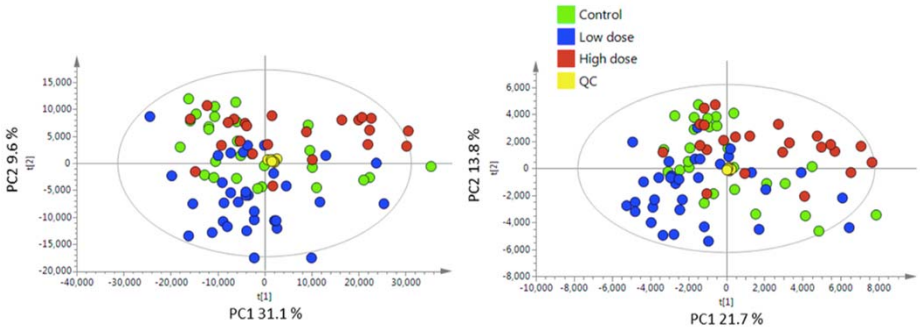
RAMMP, speeding up metabolomics



Gray et al., Anal. Chem., 2016, 88 (11), pp 5742–5751

RAMMP

- **There was a reduction in independent features**
  - 19,000 by conventional method
  - 6,000 by RAMMP



Conventional method **RAMMP**

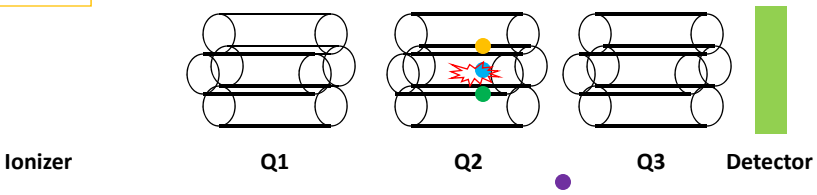
Gray et al., Anal. Chem., 2016, 88 (11), pp 5742–5751

Targeted vs untargeted methods

- If we know what the metabolites to be measured are (from previous untargeted analyses, or prior knowledge), then a multiple reaction monitoring (MRM) approach is the best way to go since allows quantitative analysis of possibly 100s of metabolites
- If there is no hypothesis, but instead you want to generate hypotheses, then the untargeted approach is better.

Multiple reaction ion monitoring

Quantitative analysis of metabolites in a complex mixture carried out using a triple quadrupole instrument



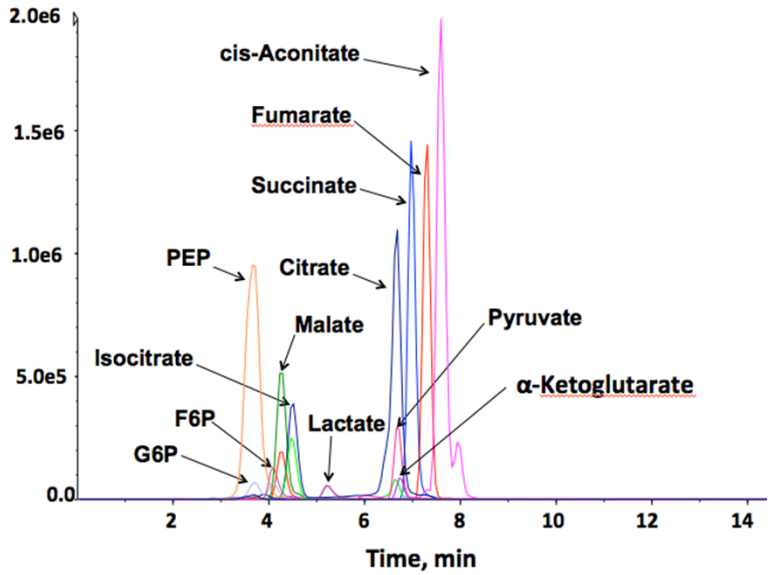
Based on precursor ion/product ion pair(s)

Courtesy, John Cutts

How many MRM transitions?

- Acquisition can be as little as 2 msec, but acquisition time determines sensitivity
- Fast switching electronics can measure as many as 500 different transitions per second
- Since measuring the area under a peak requires 10 data points, the number of transitions measured has to be matched against the shape and width of the chromatographic peaks – to be discussed in more detail later

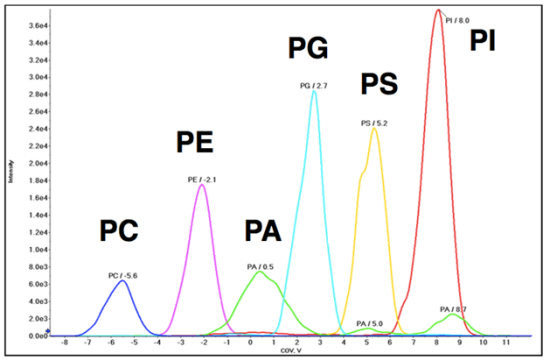
Combined channels for Krebs cycle



Ion mobility mass spectrometry

- Another method of separating classes of compounds as well as compounds with the same molecular mass

Experiment: MRM scan of 6 phospholipid standards with COV ramp

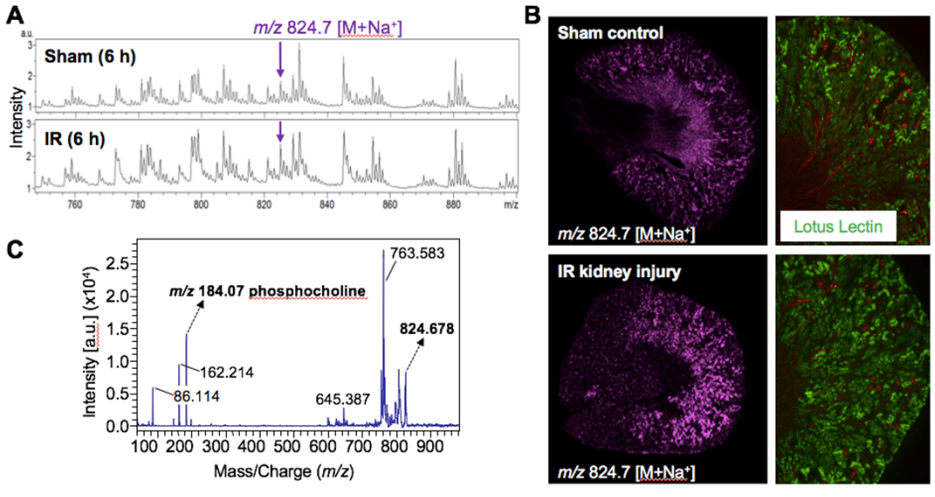


This is a gas-phase separation of these phospholipids, i.e., no chromatography

A hands-on session on this will be available as elective on Wednesday afternoon

Waters have a totally different approach to ion mobility – this will be discussed on Thursday by Tom Beaty

Imaging mass spectrometry



Generated by Janusz Kabarowski – a hands-on elective for 5 people on Wednesday

Questions?