THE UNIVERSITY OF
ALABAMA AT BIRMINGHAM
Knowledge that will change your world

Choosing the metabolomics platform

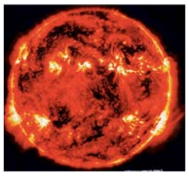
Stephen Barnes, PhD
Department of Pharmacology & Toxicology
University of Alabama at Birmingham
sbarnes@uab.edu

Targeted
Metabolomics &
Proteomics
Laboratory

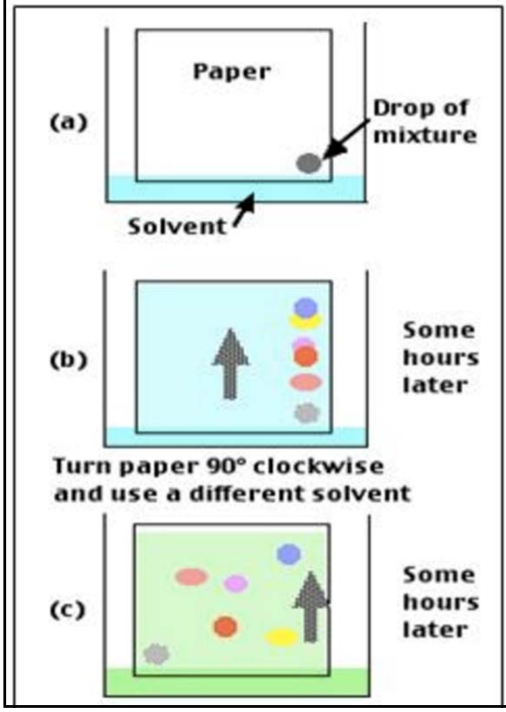
Challenges

- **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



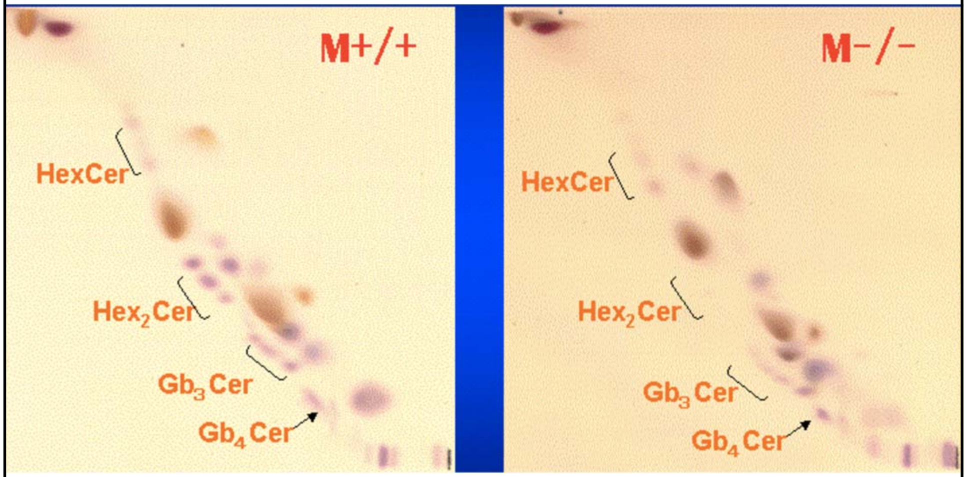
- **Earwax (long chain fatty acids, both saturated and unsaturated, alcohols, squalene, and cholesterol)**
- **No single method of analysis**



Early forms of metabolomics

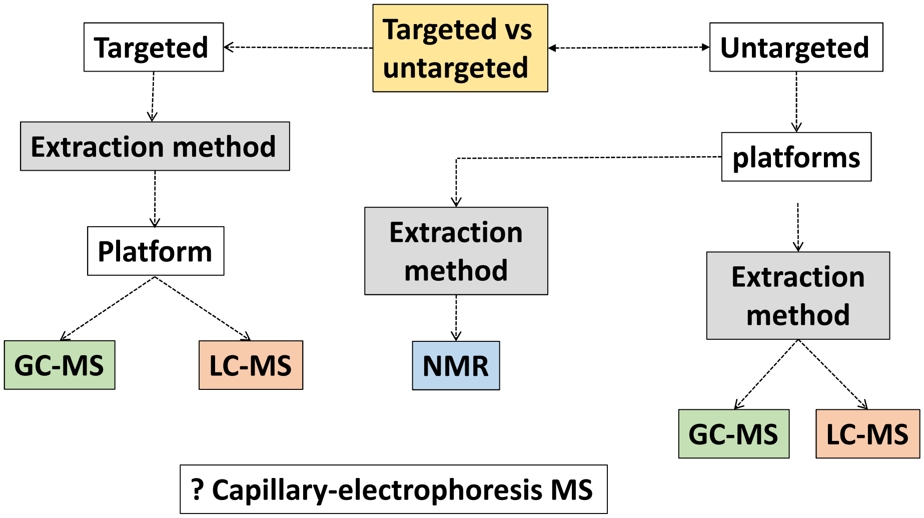
2D-paper chromatogram
low capacity

2D-Thin layer chromatography of lipids
KO of cerebroside sulfatase in kidney



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

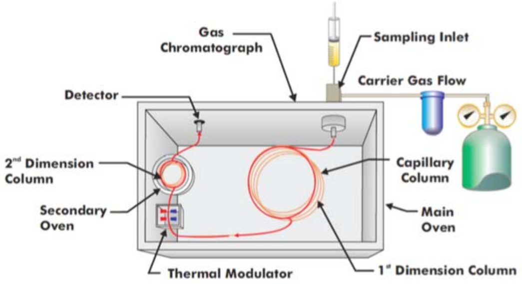
Decision tree

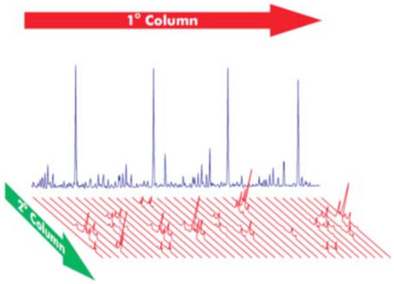


Metabolomics and GC-MS

- 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



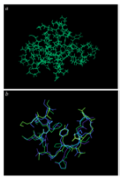


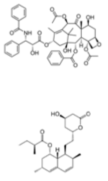
As compounds elute from column 1, they are passed to (cooler) column 2 where they condense. After a period of collection, column 2 is heated so as to separate and elute the compounds.

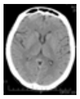
Leco Corp.

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

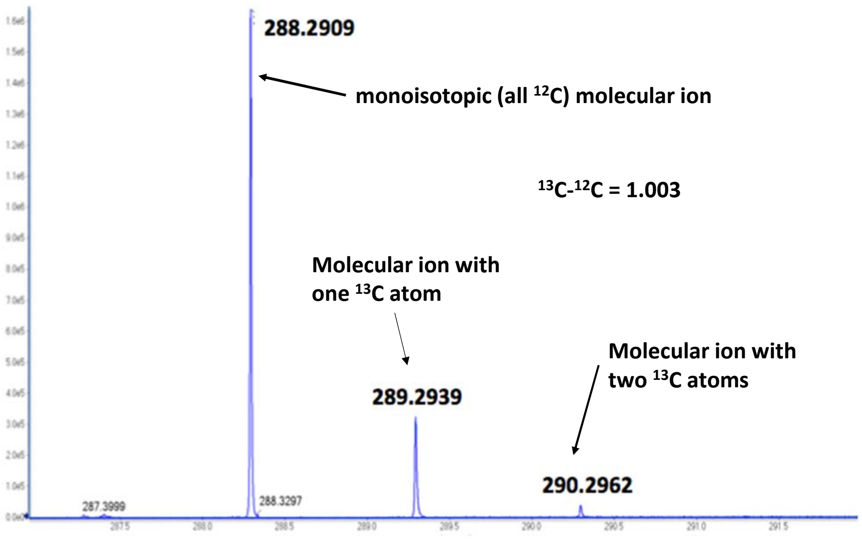
Hyperpolarization NMR

- **The NMR signal comes from non-equilibrium of the two or more energy states a nucleus experiences in a strong magnetic field**
  - However, the natural excess population of the higher energy states is no more than 0.01%
  - This accounts for the low sensitivity of NMR
- **By hyperpolarizing the compound, the excess population can be increased by 10^4-10^5.**
  - Much increased sensitivity
- **Carbonaceous materials (metabolites) can be hyperpolarized by cooling to 1^oK in a strong magnetic field (3 T or larger)**
  - However, the lifetime of the hyperpolarized state is quite short (10-30 s) making metabolomics experiments quite difficult

Liquid chromatography-Mass Spectrometry

- PROS
  - Almost all compounds can be analyzed by LC-MS
    - Exceptions - 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

Mass spectrum of a compound



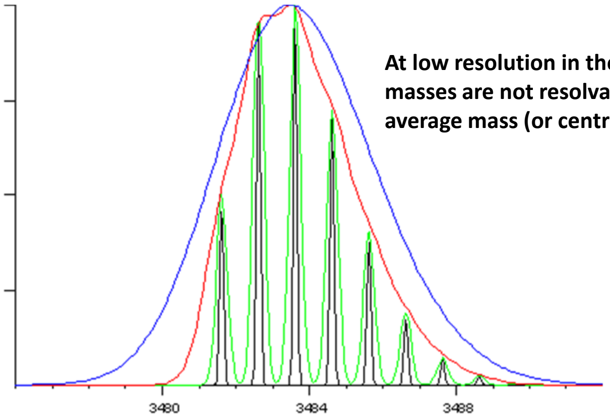
monoisotopic (all ^{12}C) molecular ion

^{13}C-^{12}C = 1.003

Molecular ion with one ^{13}C atom

Molecular ion with two ^{13}C atoms

Mass resolution



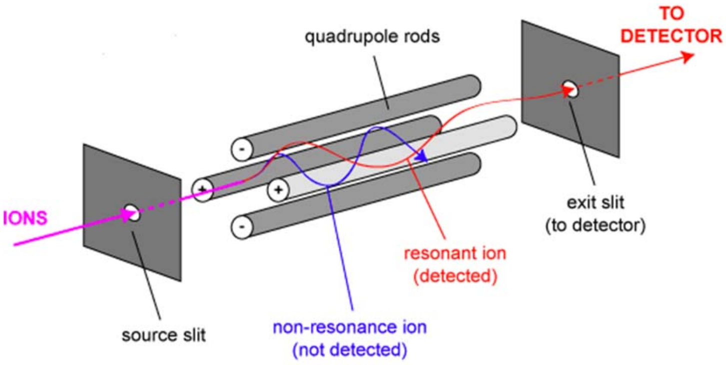
At low resolution in the blue and red, the masses are not resolvable. Instead, the average mass (or centroid) is reported

Blue = ion trap, Red = quadrupole, Green = Quadrupole TOF, Purple = FT-ICR

Selecting the mass spectrometer

- It is necessary to use an instrument to measure:
  - The mass of the metabolites accurately
  - To provide sufficient mass resolution to distinguish the isotopes associated with each metabolite
- There are several types of MS detectors
  - Quadrupole
  - ion trap
  - time-of-flight (TOF)
  - Orbitrap
  - Fourier Transform-Ion Cyclotron Resonance (FT-ICR)

Quadrupole mass filter

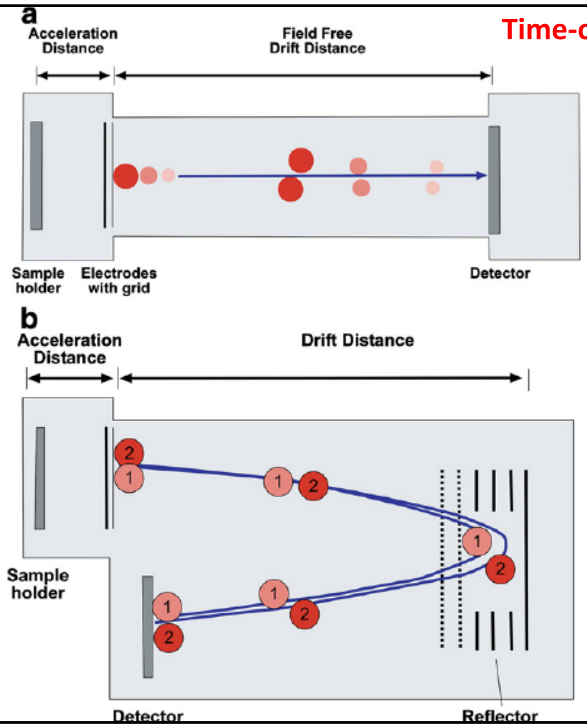


Consists of four parallel rods. Each opposing rod pair is connected together electrically, and a radio frequency (RF) voltage with a DC offset voltage is applied between one pair of rods and the other. This causes the ions to rotate in spirals as they go through the quadrupole. For a given voltage, only ions of a specific *m/z* can pass through. The voltage can be scanned to generate a mass spectrum or held constant to allow one ion to pass through.

The mass spectrometer

- **For untargeted analysis it is important to have high mass resolution, accuracy and speed**
  - 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 using short acquisition times (<100 ms)
  - They are best used for follow up experiments

Time-of-flight (TOF) analyzer



Ions can come from a static position (MALDI plate or frozen tissue section) or those passing through a quadrupole mass selector.

The ions pass down the flight tube in ~1 µs

Using the reflectron process, ions can be more effectively focused. Over-energized ions of the same mass dig deeper into the reflectron allowing the less energetic ions to catch up so they both arrive at the detector at the same time

TOF is the 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 30-80,000 mass resolution and 1 ppm or better mass accuracy

Links to the different Q-TOFs

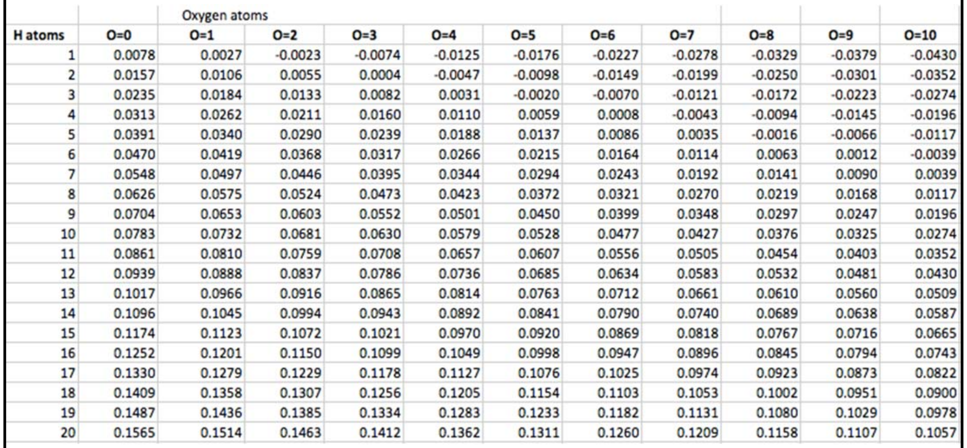
- **Agilent - 6560 Ion Mobility Q-TOF LC/MS**
  - **https://www.agilent.com/cs/library/brochures/5991-3640EN_6560_Ion_Mobility_QTOF_LCMS_Brochure_Final_singlepgs.pdf**
- **Bruker –**
  - https://www.bruker.com/products/mass-spectrometry-and-separations/lc-ms/o-tof/timstof/overview.html
- **SCIEX – 6600 TripleTOF/SelexION**
  - https://sciex.com/products/mass-spectrometers/qtof-systems/tripletof-systems/tripletof-6600-system
- **Waters – Synapt G2Si**
  - http://www.waters.com/waters/en_US/SYNAPT-G2-Si-High-Definition-Mass-Spectrometry/nav.htm?cid=134740622&locale=en_US

Masses of elements and their isotopes

- **Mass is defined using the mass of carbon-12 being 12.0000 (exactly) – the others have non-integer mass defects**
- **On this scale,**
  - **^{1}H is 1.007825 and ^{2}H is 2.014102 (extra neutron)**
  - **^{14}N is 14.003074 and ^{15}N is 15.000108 (extra neutron)**
  - **^{16}O is 15.994915, ^{17}O is 16.999132 and ^{18}O is 17.999161**
  - **^{31}P is 30.973761**
  - **^{32}S is 31.972071 and ^{34}S is 33.967867 (4%)**
- **You can find the mass of every element and its isotopes and their natural abundances at**

  http://www.nist.gov/pml/data/comp.cfm
- **The mass of a proton is 1.0072766 and that of an electron is 0.000548597**

Predicted mass defects for $C_xH_nO_m$



		Oxygen atoms									
H atoms	**O=0**	**O=1**	**O=2**	**O=3**	**O=4**	**O=5**	**O=6**	**O=7**	**O=8**	**O=9**	**O=10**
1	0.0078	0.0027	-0.0023	-0.0074	-0.0125	-0.0176	-0.0227	-0.0278	-0.0329	-0.0379	-0.0430
2	0.0157	0.0106	0.0055	0.0004	-0.0047	-0.0098	-0.0149	-0.0199	-0.0250	-0.0301	-0.0352
3	0.0235	0.0184	0.0133	0.0082	0.0031	-0.0020	-0.0070	-0.0121	-0.0172	-0.0223	-0.0274
4	0.0313	0.0262	0.0211	0.0160	0.0110	0.0059	0.0008	-0.0043	-0.0094	-0.0145	-0.0196
5	0.0391	0.0340	0.0290	0.0239	0.0188	0.0137	0.0086	0.0035	-0.0016	-0.0066	-0.0117
6	0.0470	0.0419	0.0368	0.0317	0.0266	0.0215	0.0164	0.0114	0.0063	0.0012	-0.0039
7	0.0548	0.0497	0.0446	0.0395	0.0344	0.0294	0.0243	0.0192	0.0141	0.0090	0.0039
8	0.0626	0.0575	0.0524	0.0473	0.0423	0.0372	0.0321	0.0270	0.0219	0.0168	0.0117
9	0.0704	0.0653	0.0603	0.0552	0.0501	0.0450	0.0399	0.0348	0.0297	0.0247	0.0196
10	0.0783	0.0732	0.0681	0.0630	0.0579	0.0528	0.0477	0.0427	0.0376	0.0325	0.0274
11	0.0861	0.0810	0.0759	0.0708	0.0657	0.0607	0.0556	0.0505	0.0454	0.0403	0.0352
12	0.0939	0.0888	0.0837	0.0786	0.0736	0.0685	0.0634	0.0583	0.0532	0.0481	0.0430
13	0.1017	0.0966	0.0916	0.0865	0.0814	0.0763	0.0712	0.0661	0.0610	0.0560	0.0509
14	0.1096	0.1045	0.0994	0.0943	0.0892	0.0841	0.0790	0.0740	0.0689	0.0638	0.0587
15	0.1174	0.1123	0.1072	0.1021	0.0970	0.0920	0.0869	0.0818	0.0767	0.0716	0.0665
16	0.1252	0.1201	0.1150	0.1099	0.1049	0.0998	0.0947	0.0896	0.0845	0.0794	0.0743
17	0.1330	0.1279	0.1229	0.1178	0.1127	0.1076	0.1025	0.0974	0.0923	0.0873	0.0822
18	0.1409	0.1358	0.1307	0.1256	0.1205	0.1154	0.1103	0.1053	0.1002	0.0951	0.0900
19	0.1487	0.1436	0.1385	0.1334	0.1283	0.1233	0.1182	0.1131	0.1080	0.1029	0.0978
20	0.1565	0.1514	0.1463	0.1412	0.1362	0.1311	0.1260	0.1209	0.1158	0.1107	0.1057

For positively charged ions, add **1.007276** to the overall *m/z* value
For negatively charged ions, subtract **1.007276** from the overall *m/z* value

Empirical formula

If the mass of an ion is known accurately enough, then it is possible to write down its empirical formula

What is the mass of a metabolite?

- **Hexanol**

$C_6H_{14}O$ = 6*12.0 + 14*1.007825 + 15.994915
= 102.1044651

- **Glucose**

$C_6H_{12}O_6$ = 6*12.0 + 12*1.007825 + 6*15.994915
= 180.063388

Masses of genistein's ions

- **Genistein, $C_{15}H_{10}O_5$**

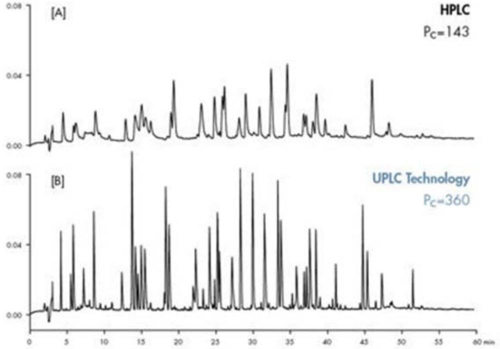
Mass = 15*12.0 + 10*1.007825 + 5*15.994915
$[M+H]^+$ = M + 1.00727638 = 271.060073
$[M-H]^-$ = M - 1.00727638 = 269.045547

- **If glucose is joined to genistein and water (H_2O) is eliminated, what are the values of the $[M+H]^+$ ion and the $[M-H]^-$ ion?**
- **Please send me the answer before class on Friday, Jan 26th.**

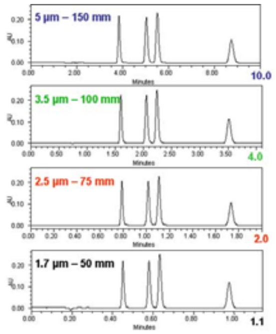
The LC

- **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

microflow/capillary (5-50 µl/min)

normal flow (0.2-0.4 ml/min)

nanoflow (0.3-5 µl/min)

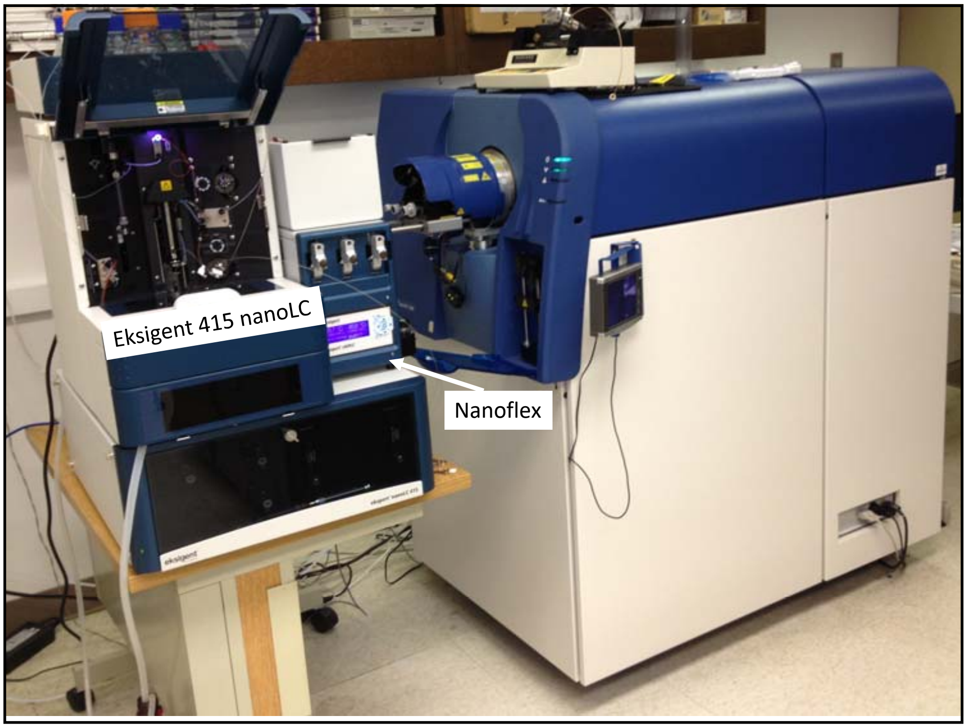
Optimizing nanoLC for metabolomics

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

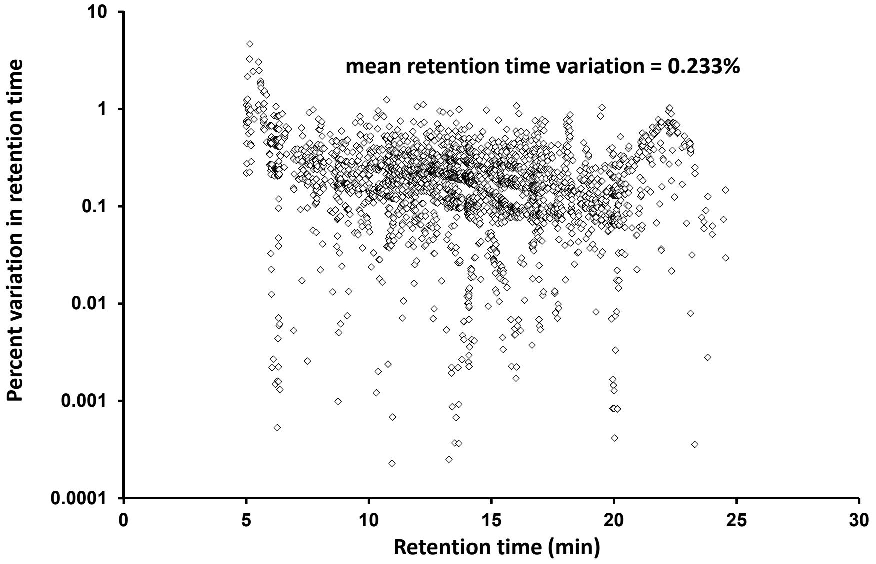
- A single zebrafish yields about 1 μl of plasma
- Need to move down to the nanoscale
- Important to maintain consistency and quantitation
  - Reproducible columns and temperature

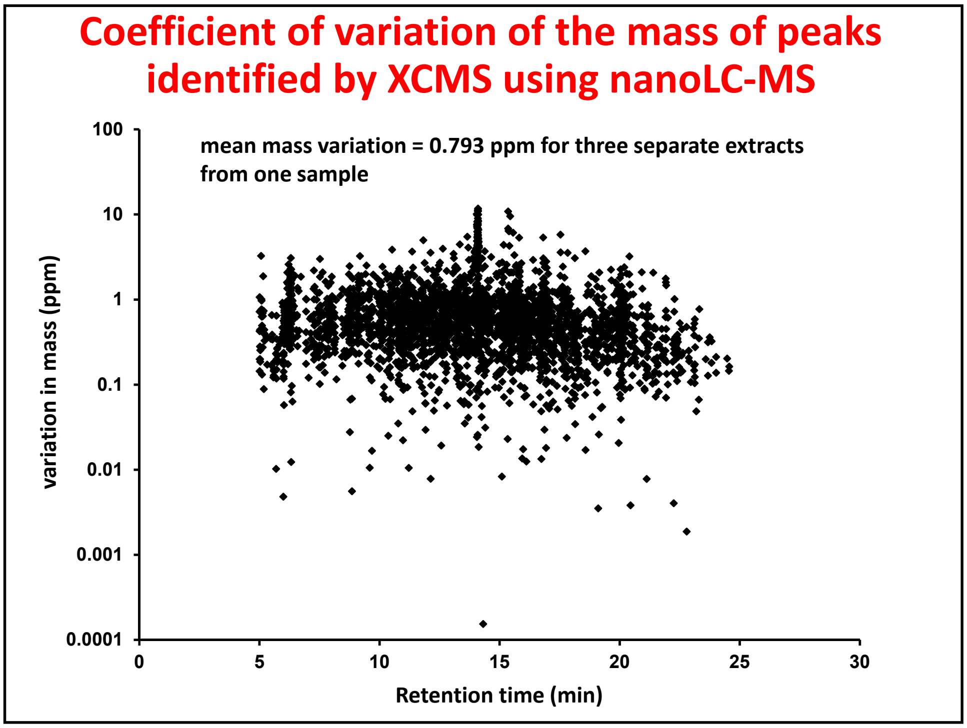
Close up of a nanochipLC cartridge (15 cm x 0.2 mm ID).

- 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



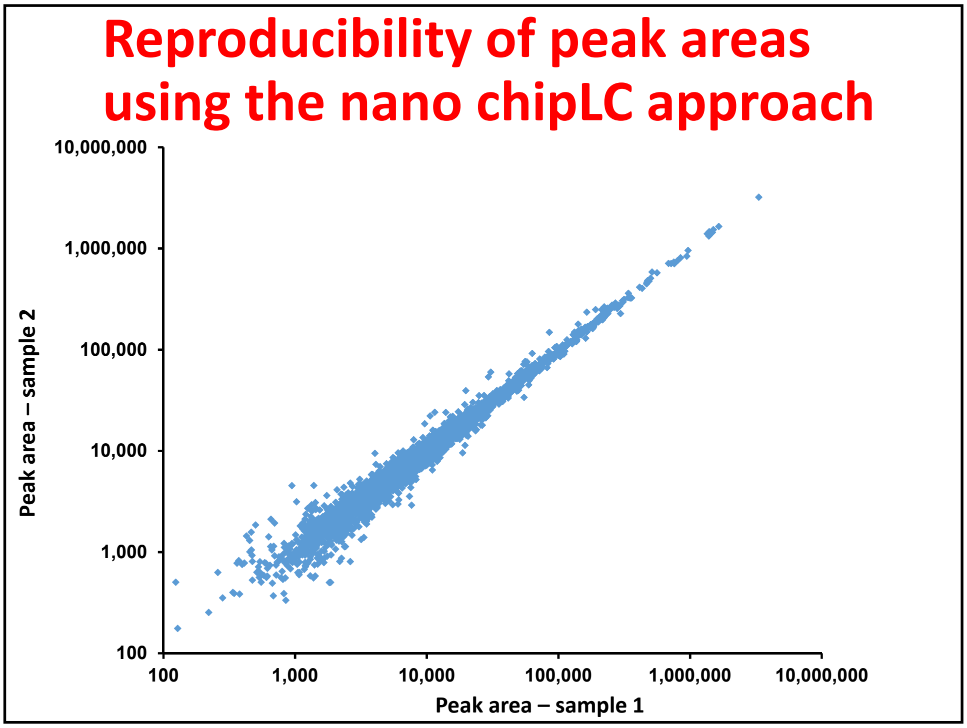
Coefficient of variation of retention time for the three separate extracts by nanoLC-MS





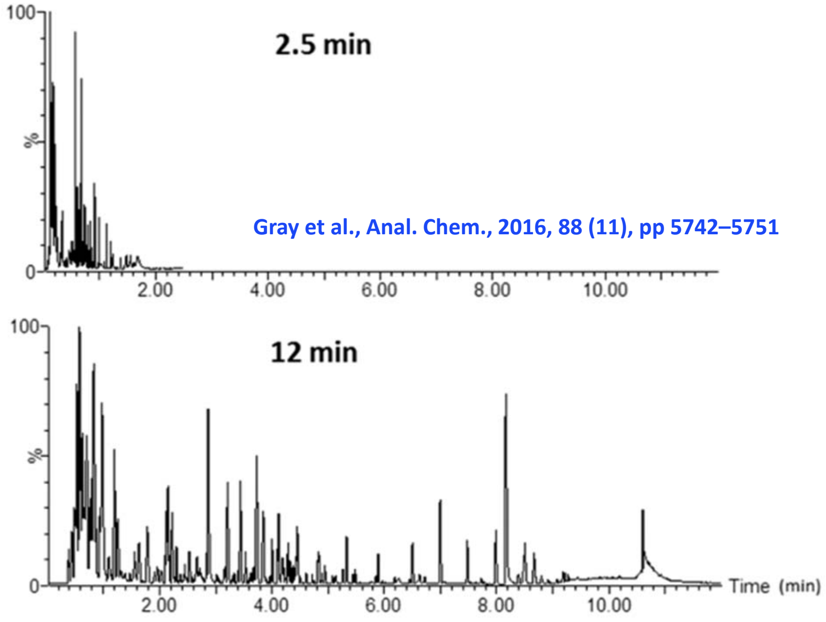
Coefficient of variation of the mass of peaks identified by XCMS using nanoLC-MS

mean mass variation = 0.793 ppm for three separate extracts from one sample



Reproducibility of peak areas using the nano chipLC approach

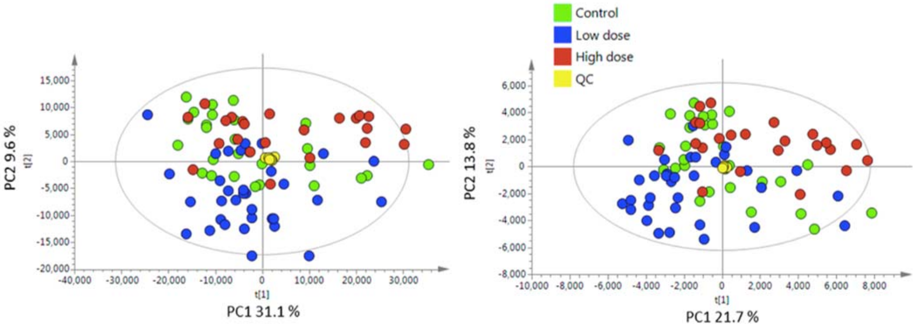
RAMMP, speeding up metabolomics



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

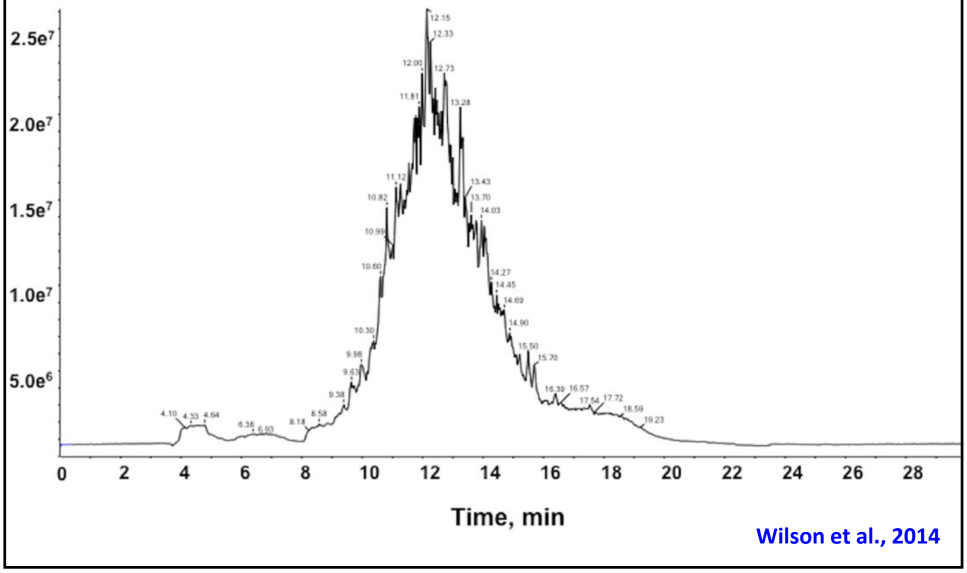
RAMMP

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

Selected ion monitoring

- **The summation of all the ions collected in a GC or LC analysis is called the total ion current (TIC) and produces a total ion chromatogram**
- **By selecting a particular mass-to-charge ratio (*m/z)* value, one can see where a metabolite's molecular ion elutes from the column**
  - **This produces a selected ion chromatogram (SIC or XIC)**
  - **The quality of the SIC depends on the mass accuracy and resolution of the collected data**

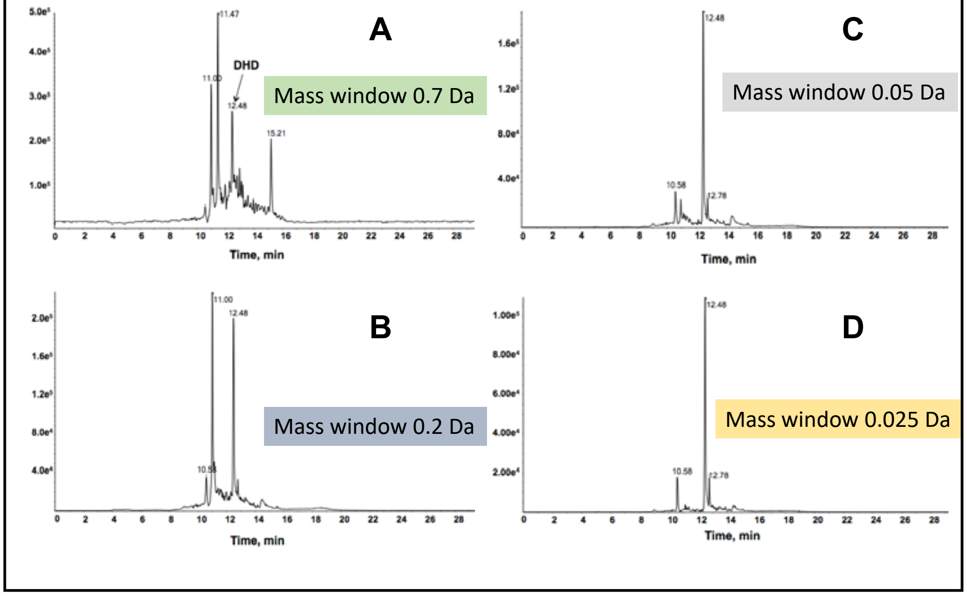
Example of a TIC of human urine



Wilson et al., 2014

Selected ion chromatograms from TIC

Dihydrodaidzein



Mass window 0.7 Da

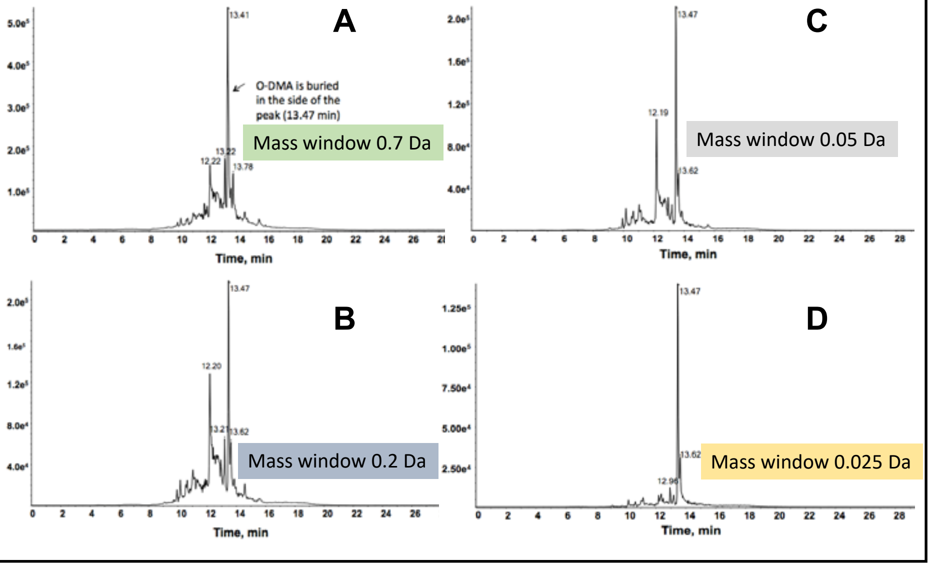
Mass window 0.05 Da

Mass window 0.2 Da

Mass window 0.025 Da

Selected ion chromatograms from TIC

O-desmethylangolensin



O-DMA is buried in the side of the peak (13.47 min)

Mass window 0.7 Da

Mass window 0.05 Da

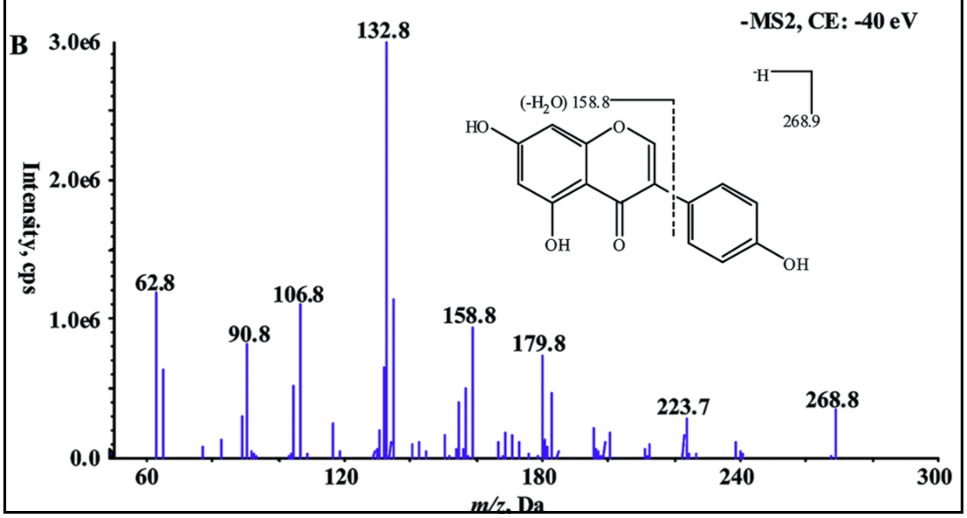
Mass window 0.2 Da

Mass window 0.025 Da

MS/MS

- A second mass spectrum (MSMS) that is informative arises from isolating the molecular ion
- The molecular ion is heated, either by collision with neutral gas (quadrupole, ion traps) or by using IR radiation (FT-ICR)
  - The extra energy increases the stretching of critical bonds, leading to dissociation of the molecular precursor ion into charged product ions
  - These generate the MS/MS spectrum for a metabolite
  - Ion traps can also isolate a product ion and create MS^n spectra

MS/MS spectrum of genistein



Measuring a mass transition

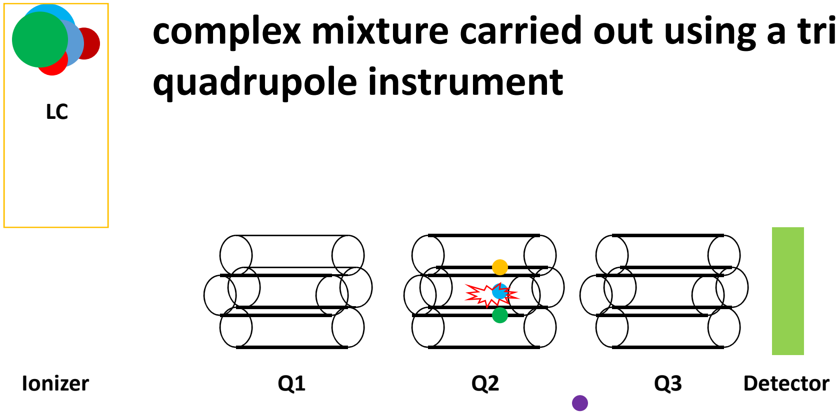
- Instead of measuring the full MS/MS spectrum, ions from the MS/MS can be individually measured
- This is referred to as a mass transition from the molecular or precursor ion to a specific product ion
- It is also known as reaction ion monitoring

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



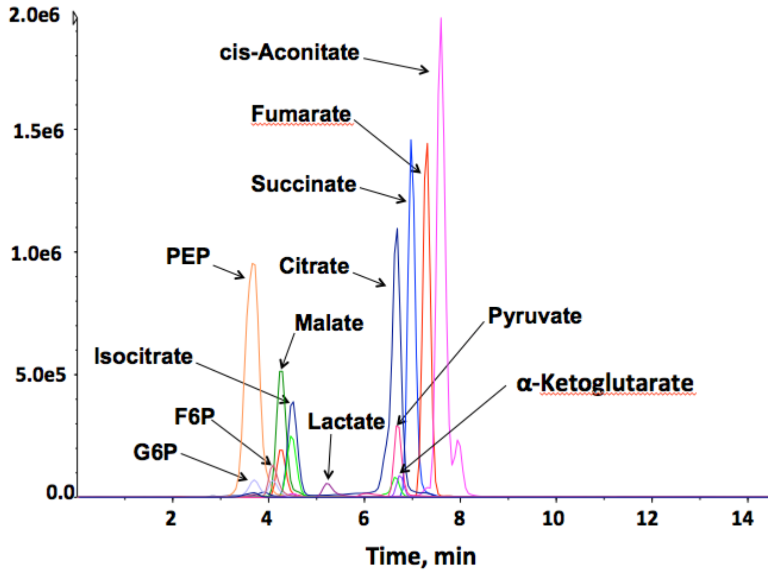
Based on precursor ion/product ion pair(s)

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 <u>different</u> 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

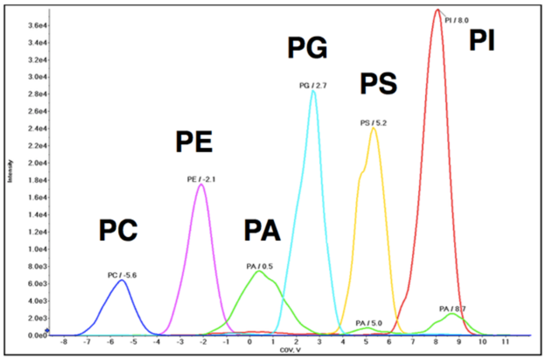
Combined channels for Krebs cycle



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



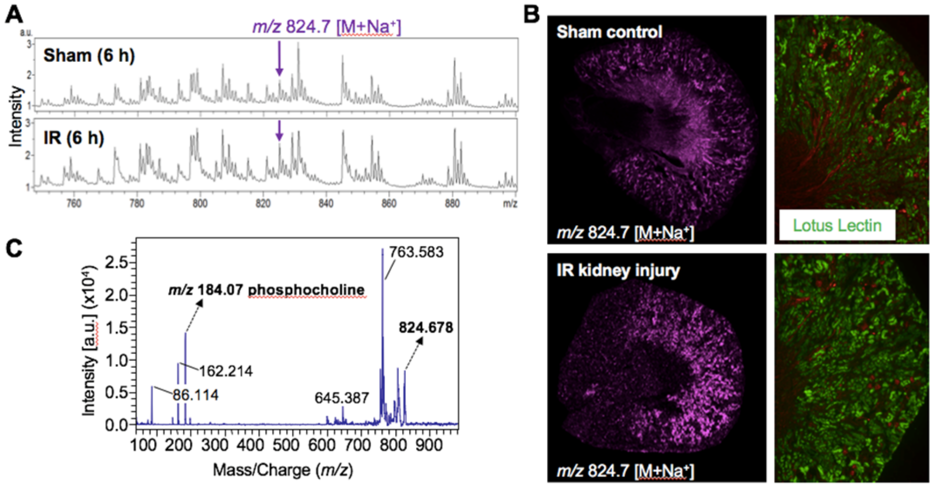
This is a gas-phase separation of these phospholipids, i.e., no chromatography

SCIEX use a differential mobility process outside of the mass spectrometer

Waters have a totally different approach to ion mobility

In their case, the ions are separated inside the mass spectrometer

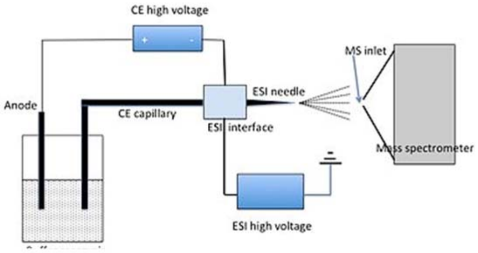
Imaging mass spectrometry

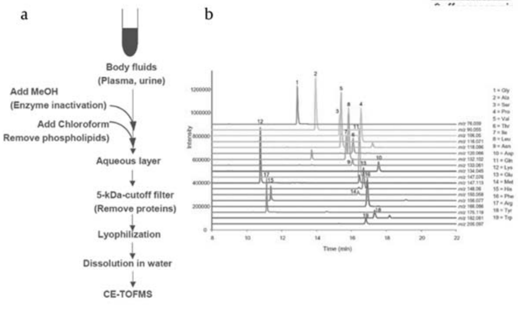


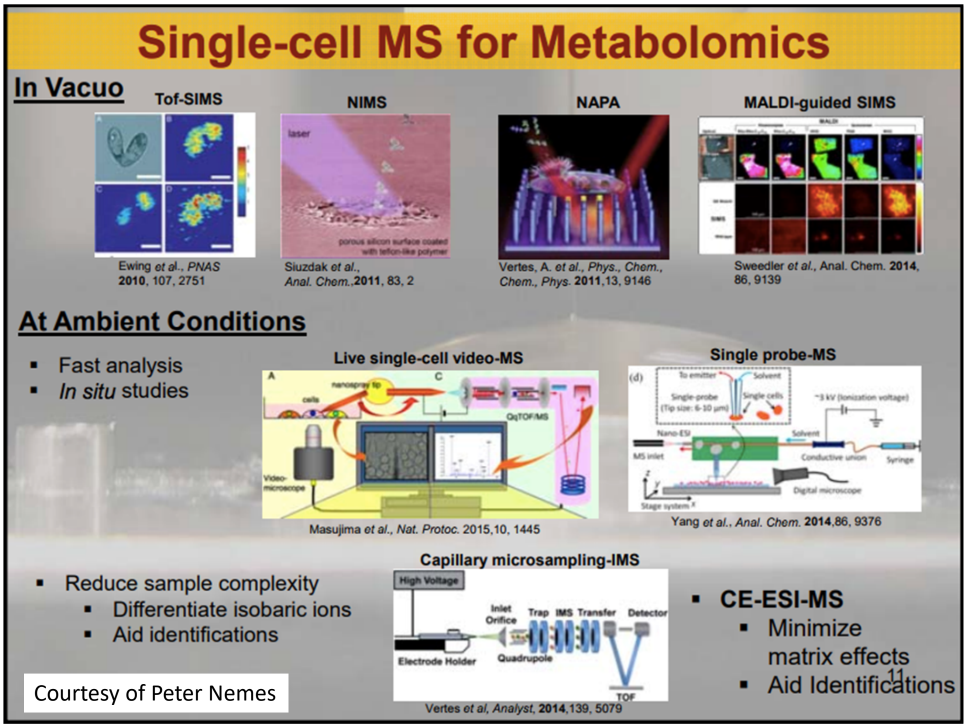
Generated by Janusz Kabarowski and Kelly Waters

Future methods in metabolomics

- Capillary electrophoresis (CE-MS)







Single-cell MS for Metabolomics

In Vacuo

Tof-SIMS

Ewing *et al.*, *PNAS* **2010**, 107, 2751

NIMS

Siuzdak *et al.*, *Anal. Chem.*,**2011**, 83, 2

NAPA

Vertes, A. *et al.*, *Phys., Chem., Chem., Phys.* **2011**,13, 9146

MALDI-guided SIMS

Sweedler *et al.*, Anal. Chem. **2014**, 86, 9139

At Ambient Conditions

- Fast analysis
- *In situ* studies

Live single-cell video-MS

Masujima *et al.*, *Nat. Protoc.* 2015,10, 1445

Single probe-MS

Yang *et al.*, *Anal. Chem.* **2014**,86, 9376

- Reduce sample complexity
  - Differentiate isobaric ions
  - Aid identifications

Capillary microsampling-IMS

Vertes *et al*, *Analyst*, **2014**,139, 5079

- **CE-ESI-MS**
  - Minimize matrix effects
  - Aid Identifications

11

Courtesy of Peter Nemes

Imaging metabolites in real time

- **In an ideal world, we want to measure metabolites without their degradation, spatially (preferably subcellularly) and with regard to time**
  - MS has high qualitative mass resolution and sensitivity, but it is destructive and not subcellular. Has poor time resolution
  - NMR is non-destructive and quantitative, but is not sensitive and not subcellular. Poor time resolution
- **Correlated anti-Stokes Raman Spectroscopy**
  - https://bernstein.harvard.edu/research/cars-why.htm
  - Is nondestructive, has high sensitivity and spatial and time resolution, but poor qualitative resolution (distinguishing metabolites)