



THE UNIVERSITY OF
ALABAMA AT BIRMINGHAM

Knowledge that will change your world

Choosing the metabolomics platform

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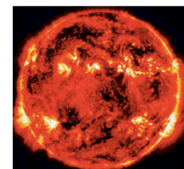
sbarnes@uab.edu

Targeted
Metabolomics &
Proteomics
Laboratory

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Challenges

- Unlike DNA, RNA and proteins, the metabolome is phenomenally chemically diverse
- Ranges from a gas (H_2) that pervades 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



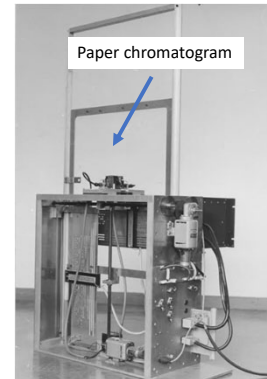
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Early beginnings of metabolomics in London

- Sir Ernst Chain (1945 Nobel Laureate – the biochemist who characterized penicillin)
 - Also renowned for his work on microanalysis
- Used 2D-paper chromatography to resolve glycolytic, Krebs cycle and amino acids derived from ^{14}C -glucose
 - Geiger counter mounted on a typewriter frame
 - Digitized the collected data and prepared computer-generated figures



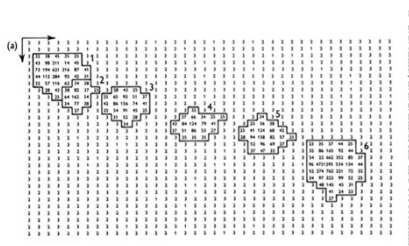
Keith Mansford



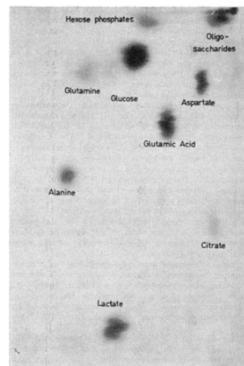
METABOLOMICS

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Radiochromatography examples



J Physiol (1960) 154:39
E.B. Chain, K.R.L. Mansford and F. Pocchiari



Biochem. J. (1969) 115, 537
E.B. Chain, K.R.L. Mansford and L.H. Opie

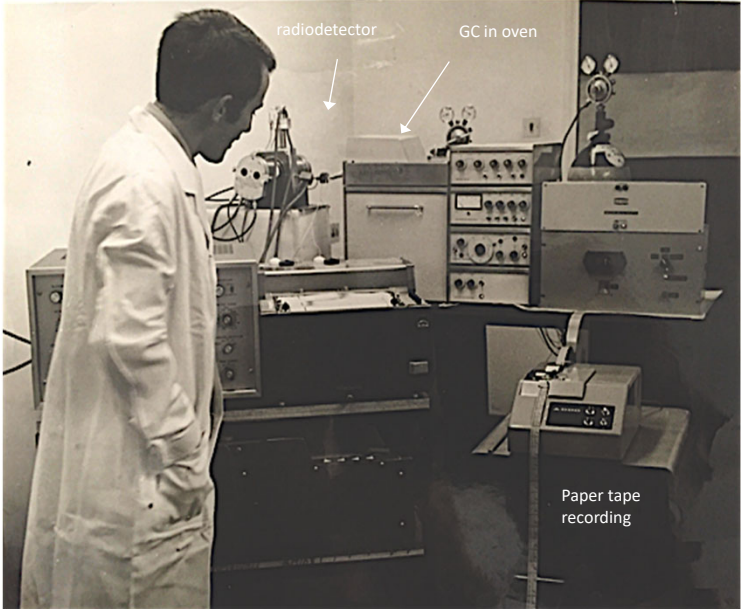
Autoradiogram of ^{14}C -glucose metabolites from an isolated perfused Langendorff rat heart preparation. The metabolites were separated by 2D-paper chromatography.

The conditions were:

1st dimension: butan-1-ol-acetic acid-water (40:11:25, by vol.) for 16hr;

2nd dimension: (-) phenol-aq. NH_3 (sp.gr. 0.88)-water (80:1:20, by vol.) for 24hr.

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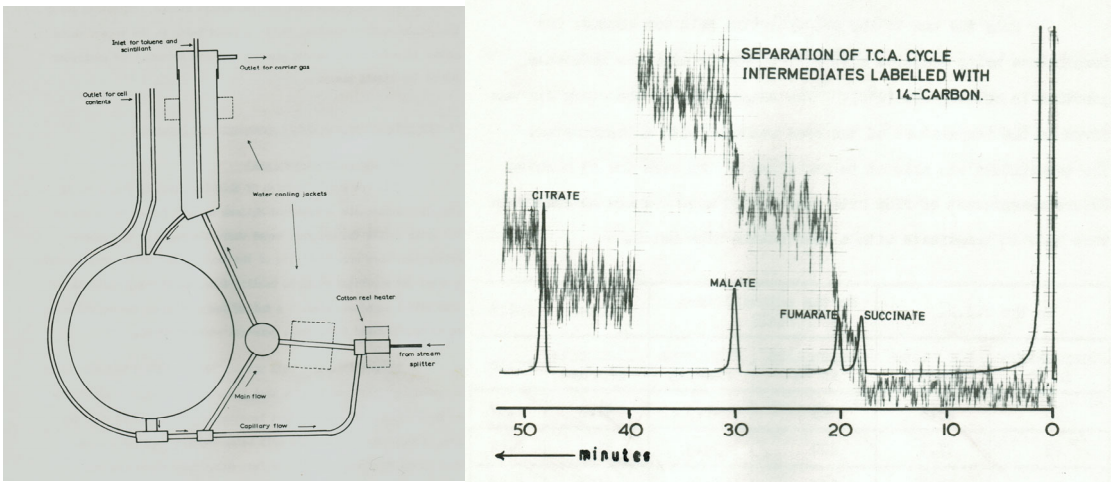
Radio-GC analysis
metabolomics in its infancy

Radio gas-liquid chromatography with digitization of collected data

Developed this for my PhD work (1967-1970) to study glucose metabolism in acellular slime mold, *Physarum polycephalum*

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Radio-GC of Krebs cycle intermediates



Popjak scintillation cell

Stephen Barnes, PhD thesis

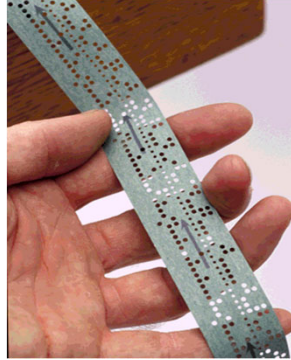
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Software for data analysis on a PDP9 computer

```

• DIMENSION Ibuff(1000), IDATA(725,2)
• COMMON Ibuff, IDATA, ITIME, INT, ISIG
• 5      ITIME=0
• 7      CALL TAPE(ISIG, INTA)
• 8      IWRITE(1, 1001) INTA
•      INT=INTA/100
• 10     NPOINT=1
• 11     CALL TAPE(ISG, IDATA(NPOINT, 1))
•      IF (ISIG.EQ.1) GO TO 16
•      IF (ISIG.EQ.2) GO TO 13
•      WRITE(1, 1001) NPOINT
• GO TO 11
• 13     IF (IDATA(1, 1).GT.940) GO TO 11
•      IF (IDATA(NPOINT, 1).EQ.0) GO TO 11
•      IF (NPOINT.EQ.725) GO TO 16
• 15     NPOINT=NPOINT+1
•      GO TO 11

```



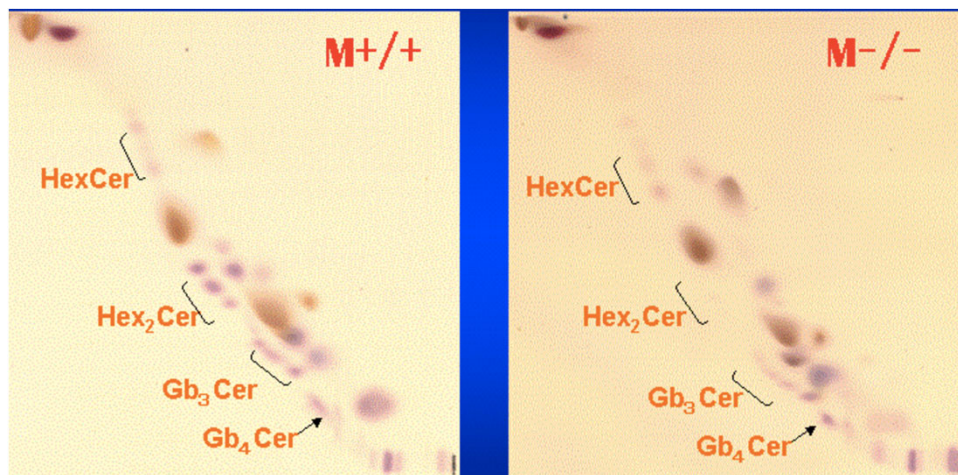
Punched tape data
1 data point/sec



Digital PDP computer
Had a screen

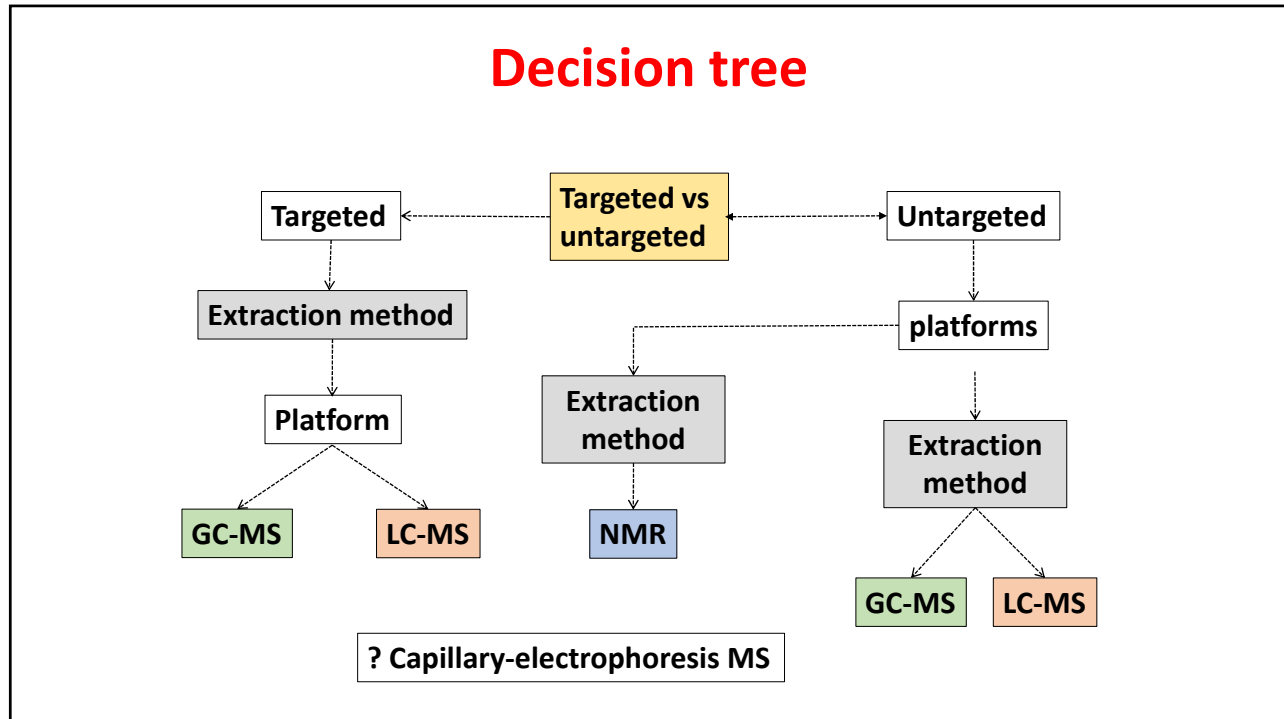
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2D-Thin layer chromatography of lipids KO of cerebroside sulfatase in kidney

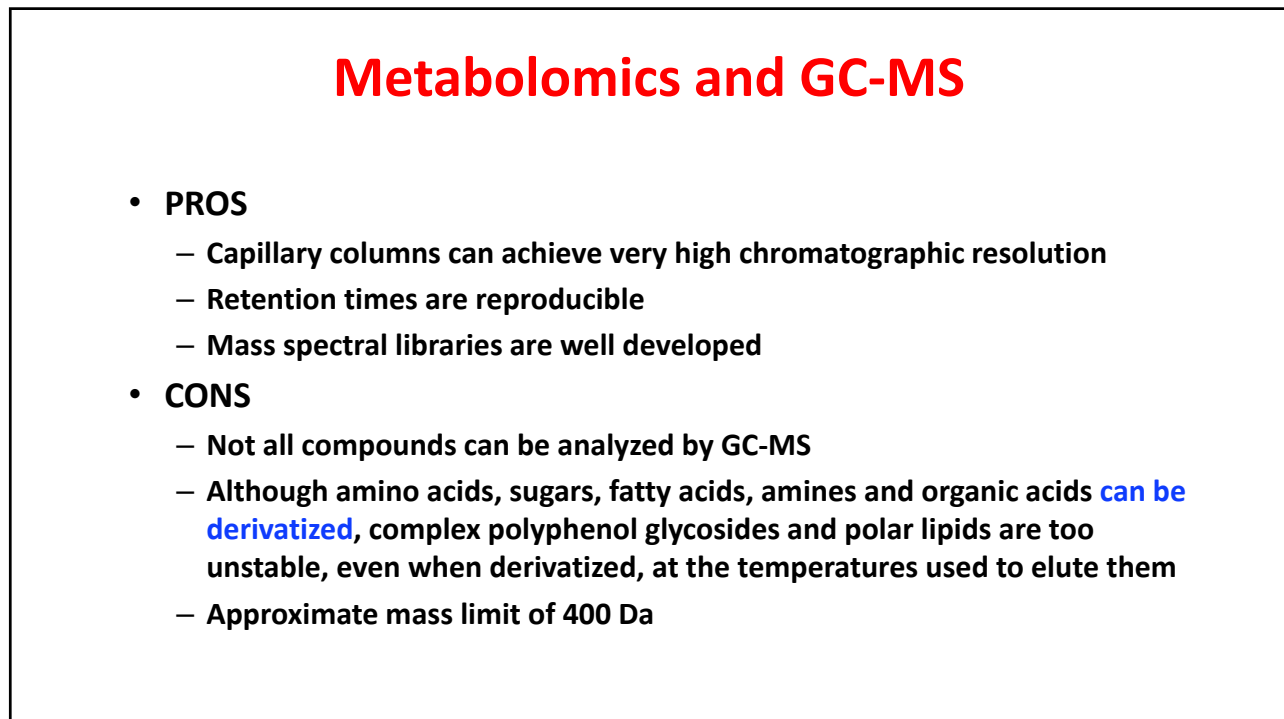


These days, TLC plates can be studied by direct electrospray ionization (DESI)

8

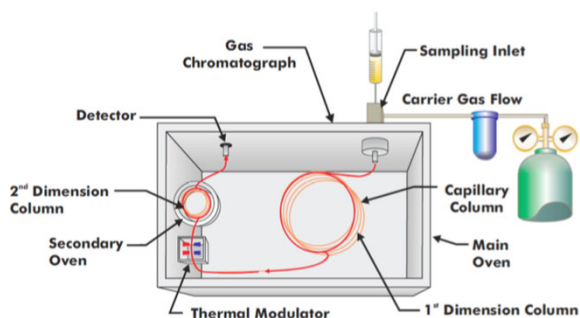


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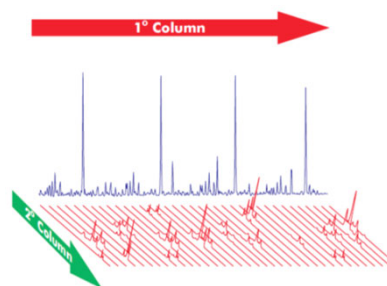
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Two dimensional GC to resolve metabolites



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.

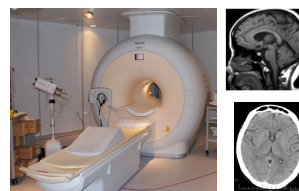
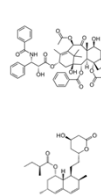
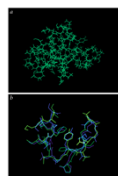
Leco Corp.



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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

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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

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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°K 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

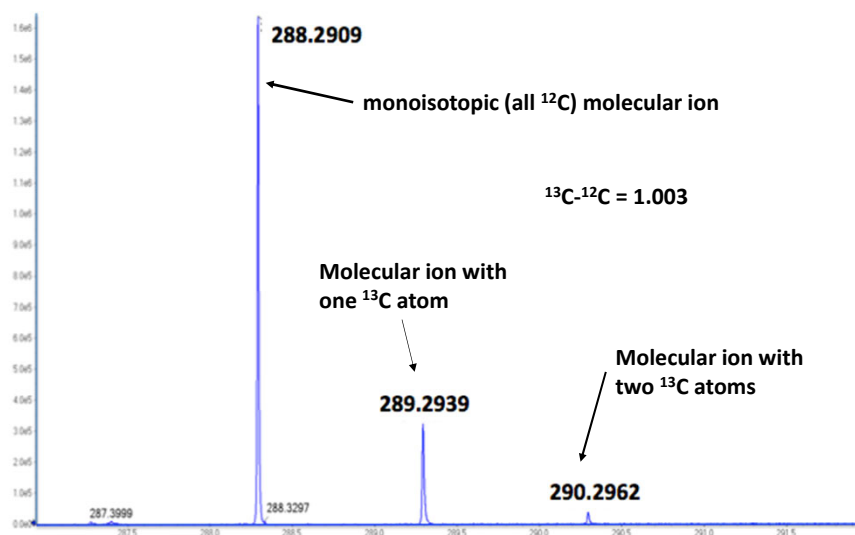
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Liquid chromatography-Mass Spectrometry

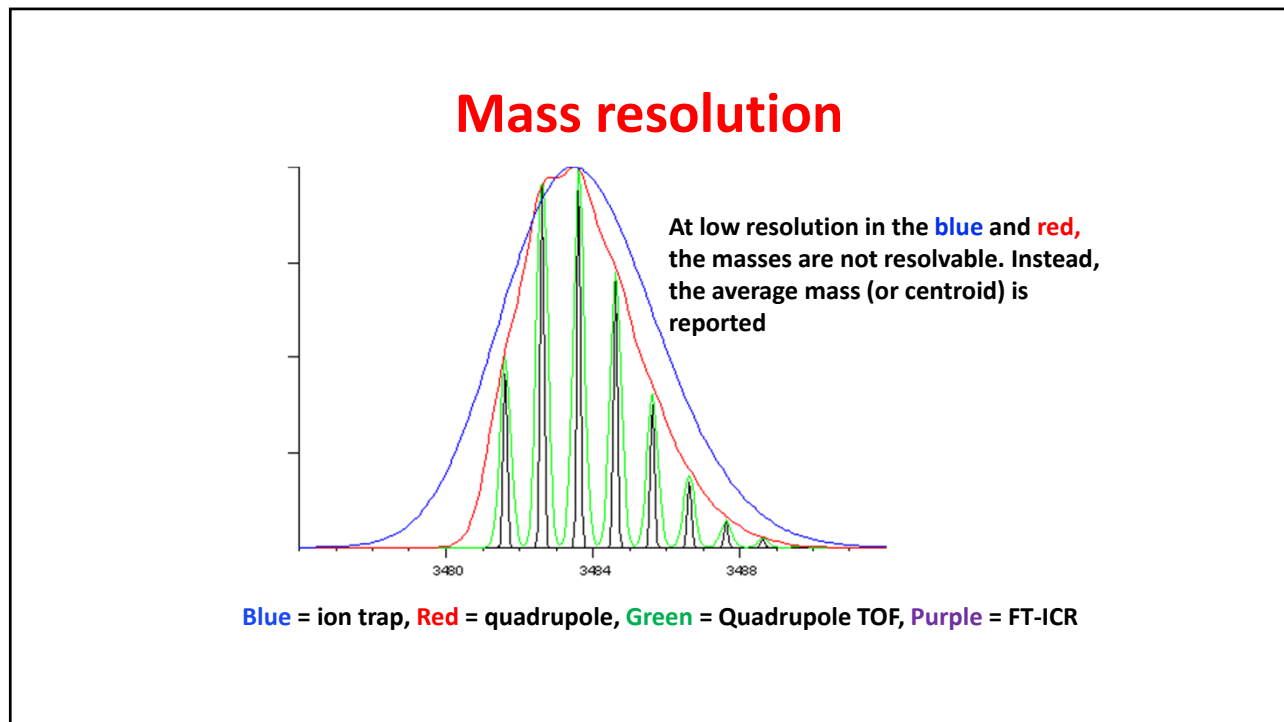
- **PROS**
 - Almost all compounds can be analyzed by LC-MS
 - Exceptions - hydrocarbons do not ionize
 - Several orders of magnitude increased sensitivity compared to NMR
 - Can collect MS, MSMS and ion mobility data
- **CONS**
 - Not uniformly quantitative
 - Mass spectral libraries are not well enough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

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Mass spectrum of a compound



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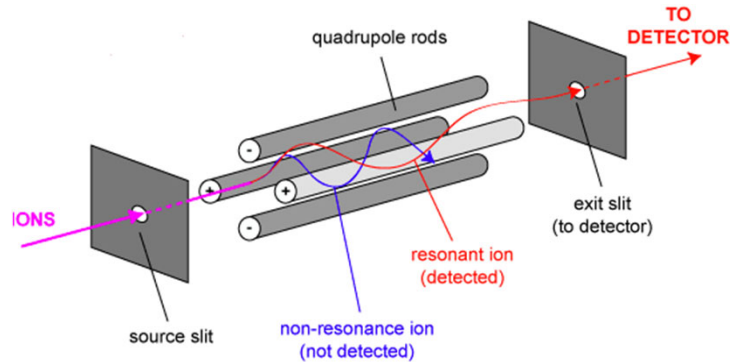
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Selecting the mass spectrometer

- 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)

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Quadrupole mass filter



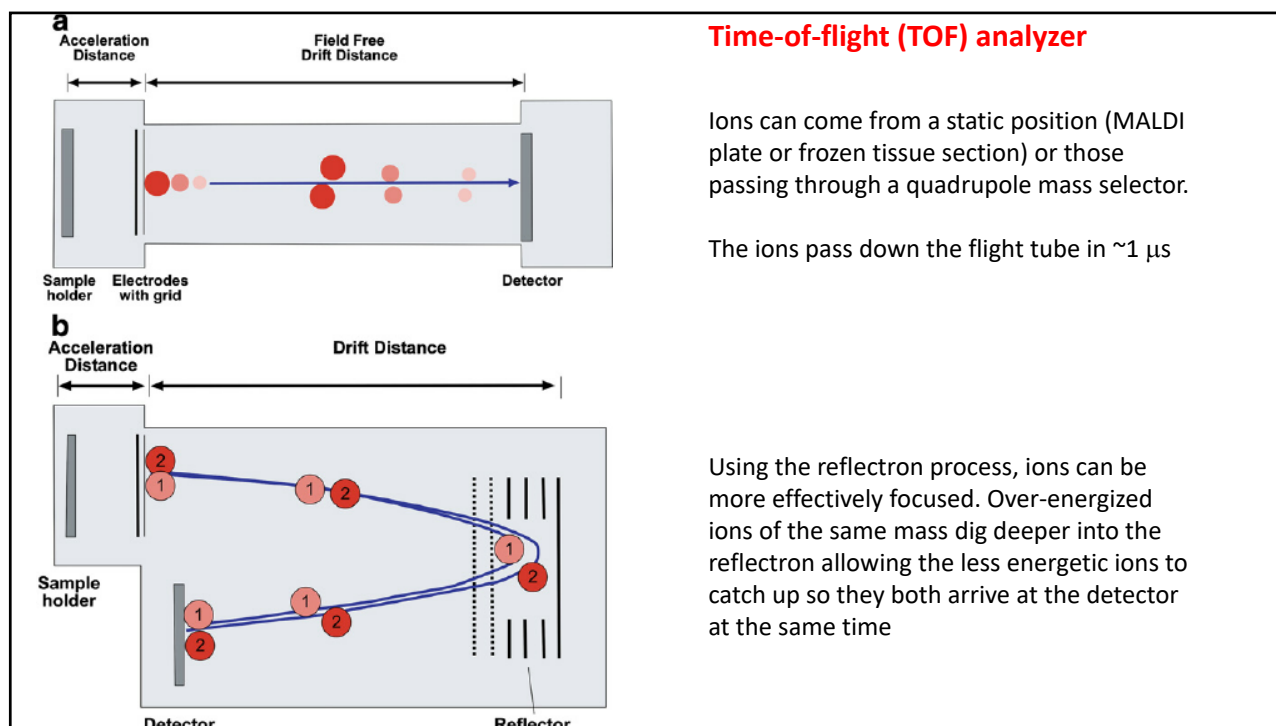
Consists of four parallel rods. Each opposing rod pair is connected together electrically, and a radio frequency (RF) voltage with a DC offset voltage is applied between one pair of rods and the other. This causes the ions to rotate in spirals as they go through the quadrupole. For a given voltage, only 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.

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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

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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

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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/en/products-and-solutions/mass-spectrometry/timstof/timstof-pro.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

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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,
 - ^1H is 1.007825 and ^2H 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

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Predicted mass defects for $C_xH_nO_m$

H atoms	Oxygen 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

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Empirical formula

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

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What is the mass of a metabolite?

- Hexanol

$$\begin{aligned} \text{C}_6\text{H}_{14}\text{O} &= 6*12.0 + 14*1.007825 + 15.994915 \\ &= 102.1044651 \end{aligned}$$

- Glucose

$$\begin{aligned} \text{C}_6\text{H}_{12}\text{O}_6 &= 6*12.0 + 12*1.007825 + 6*15.994915 \\ &= 180.063388 \end{aligned}$$

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Masses of genistein's ions

- Genistein, $\text{C}_{15}\text{H}_{10}\text{O}_5$

$$\text{Mass} = 15*12.0 + 10*1.007825 + 5*15.994915$$

$$[\text{M}+\text{H}]^+ = \text{M} + 1.00727638 = 271.060073$$

$$[\text{M}-\text{H}]^- = \text{M} - 1.00727638 = 269.045547$$

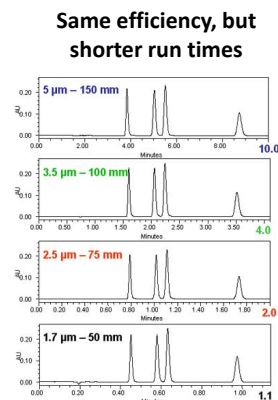
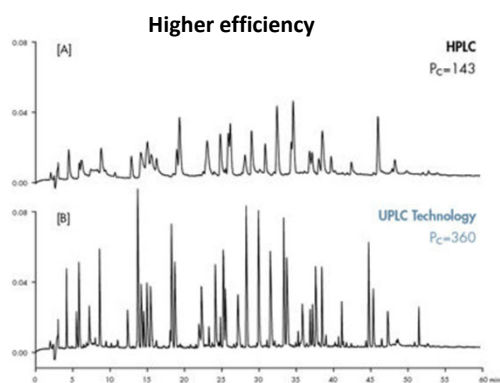
- If glucose is joined to genistein and water (H_2O) is eliminated, what are the values of the $[\text{M}+\text{H}]^+$ ion and the $[\text{M}-\text{H}]^-$ ion?
- Please send me the answer before class on Wednesday, Jan 27th.

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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



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LC flow rate

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



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Optimizing nanoLC for metabolomics

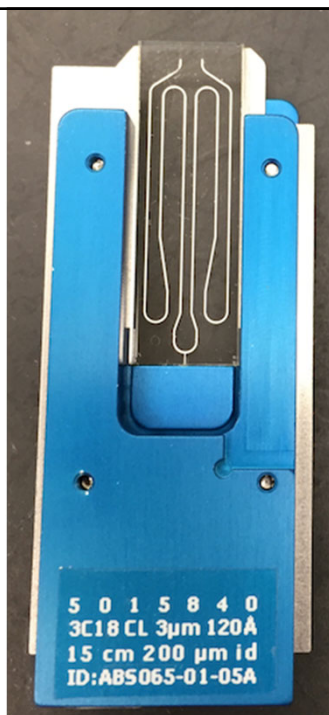
- Objective is to develop metabolomics for small animal model systems

- *D. melangaster*
- *C. elegans*
- *D. rerio*



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

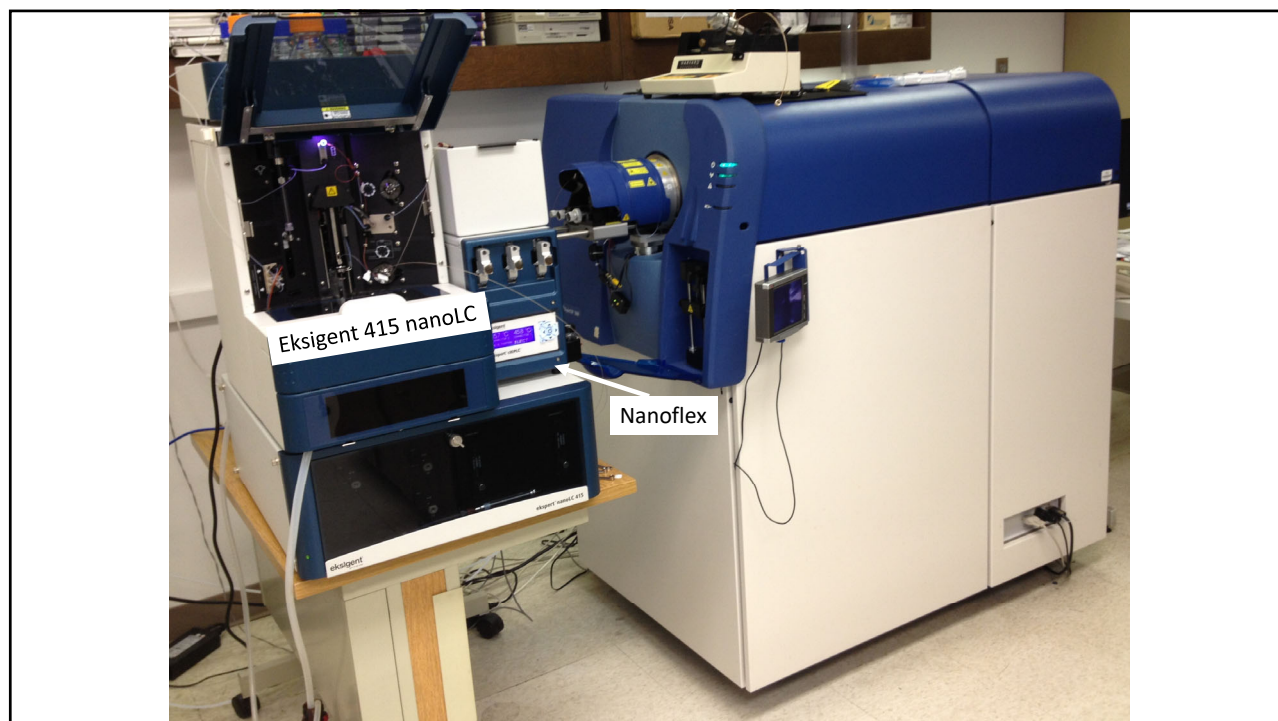
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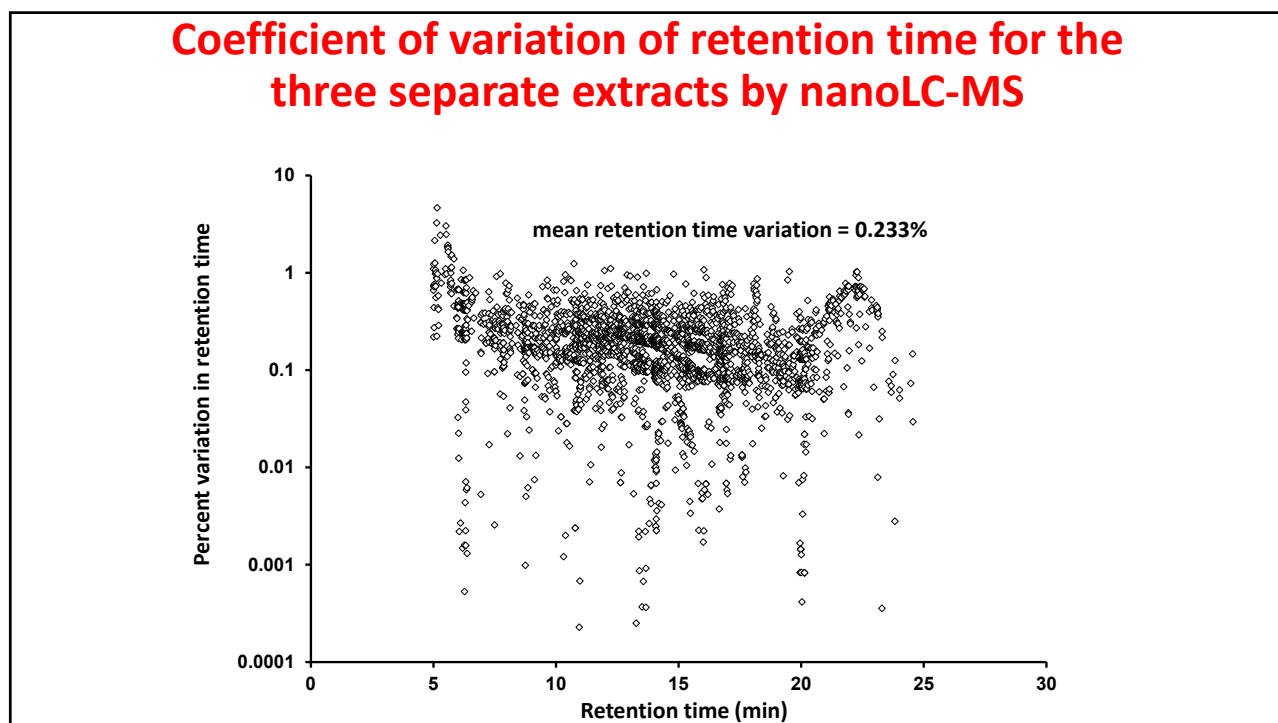
Close up of a nanochipLC cartridge (15 cm x 0.2 mm ID).

- 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

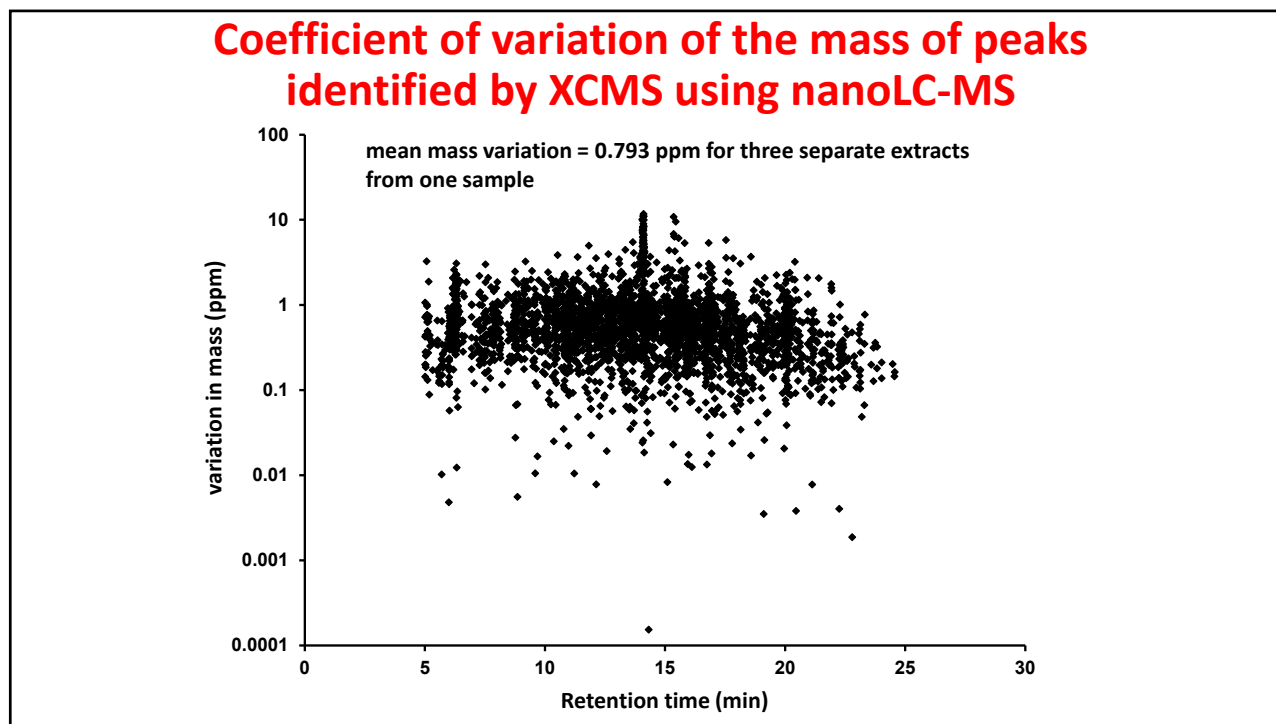
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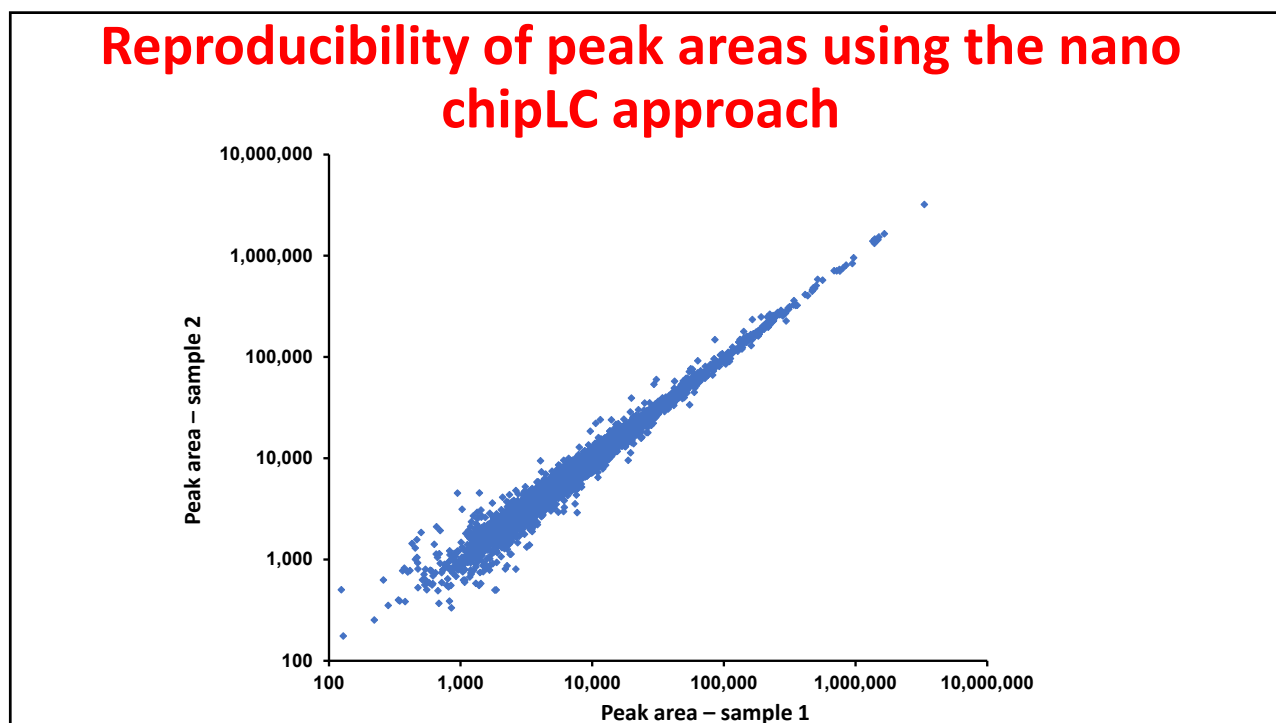
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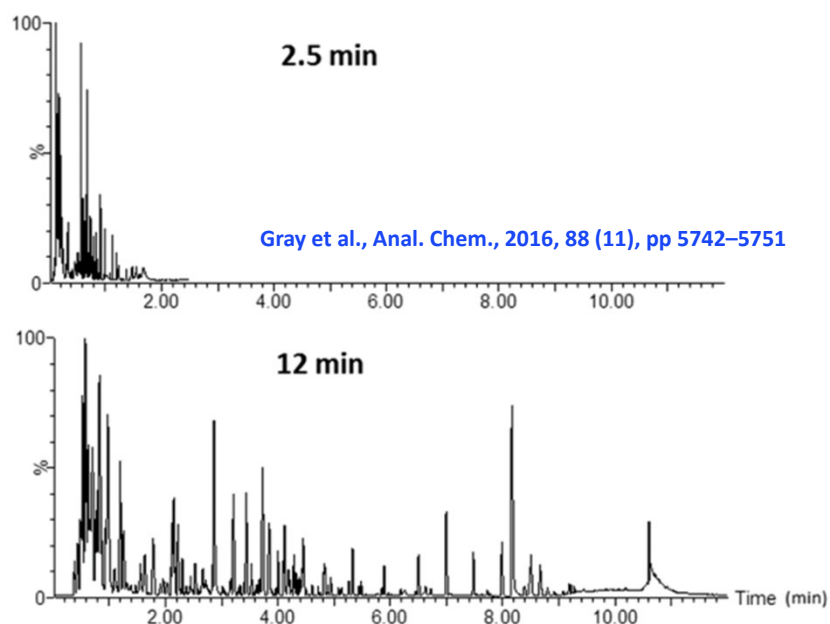


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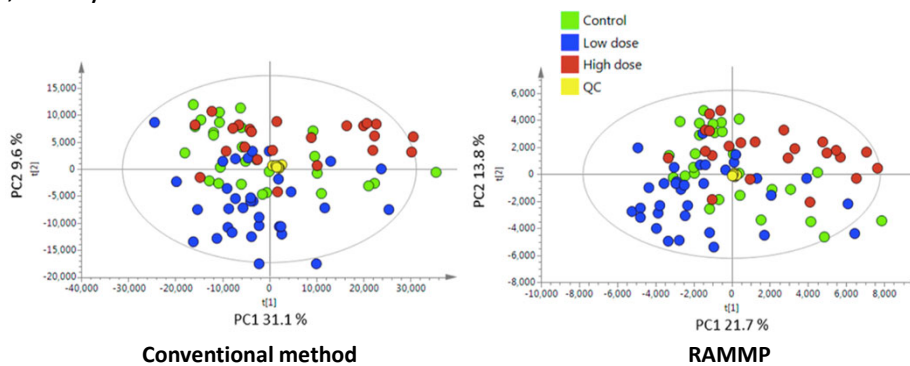
RAMMP, speeding up metabolomics



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RAMMP

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



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

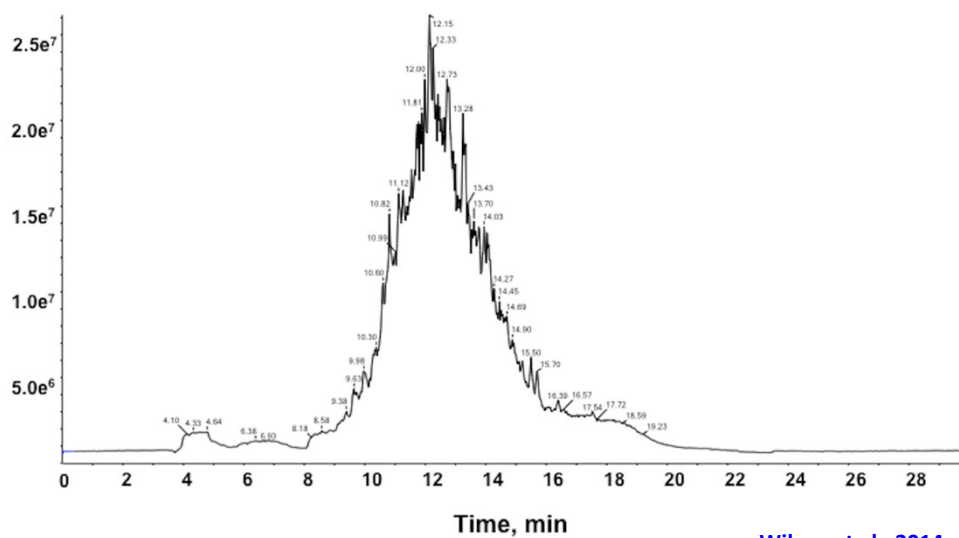
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Selected ion monitoring

- 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

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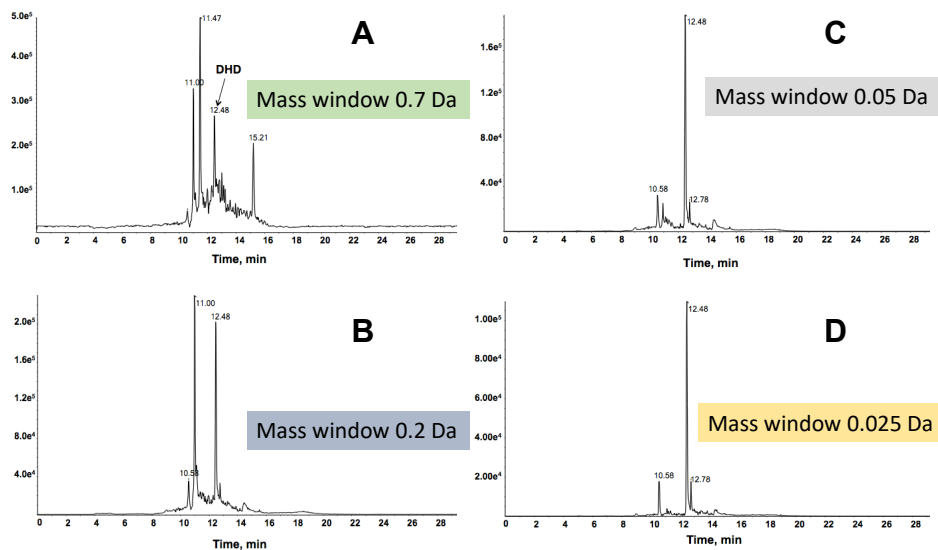
Example of a TIC of human urine



Wilson et al., 2014

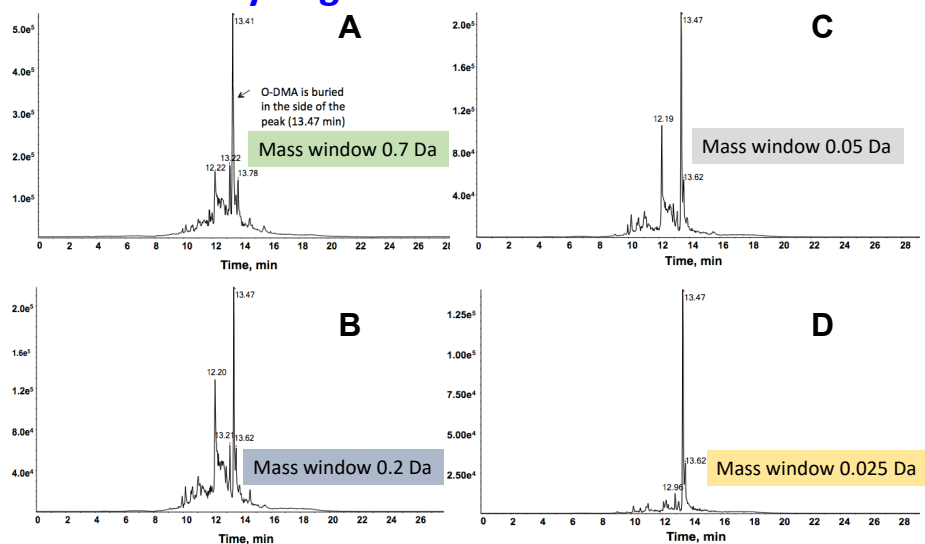
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Selected ion chromatograms from TIC Dihydroaidzein



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Selected ion chromatograms from TIC O-desmethylangolensin



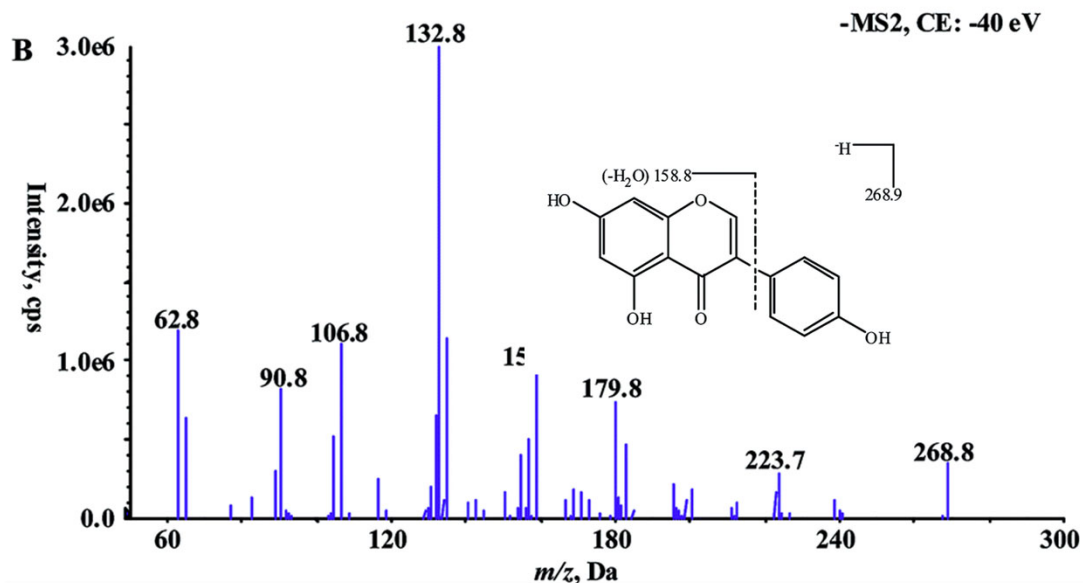
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MS/MS

- 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 MSⁿ spectra

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MS/MS spectrum of genistein



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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**

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Targeted vs untargeted methods

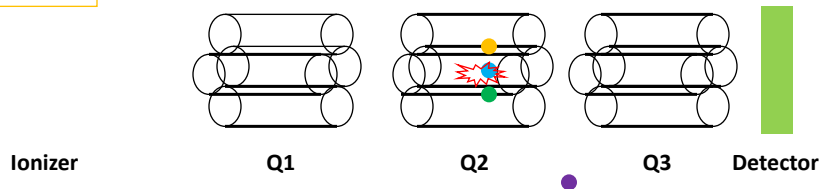
- If we know what the metabolites to be measured are (from previous untargeted analyses, or prior knowledge), then a **multiple reaction monitoring (MRM)** approach is the best way to go since allows quantitative analysis of possibly 100s of metabolites
- If there is no hypothesis, but instead you want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

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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

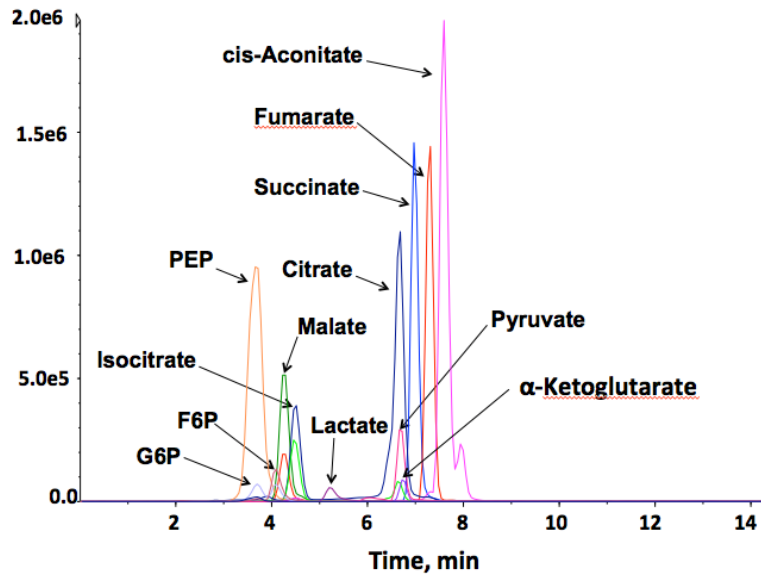
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How many MRM transitions?

- 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

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Combined channels for Krebs cycle

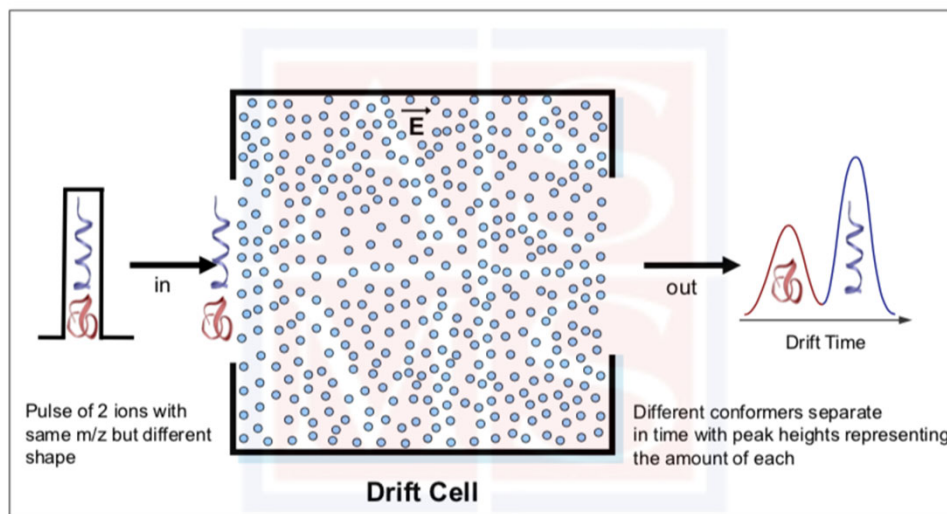


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Ion mobility – another parameter to characterize a component

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Ion Mobility Concept

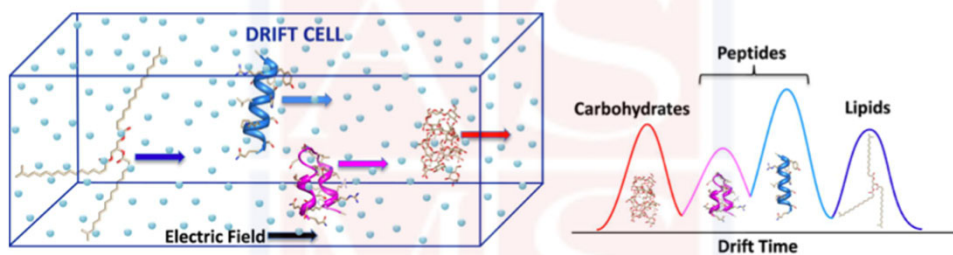


velocity is constant

Erin Baker – ASMS short course 2018

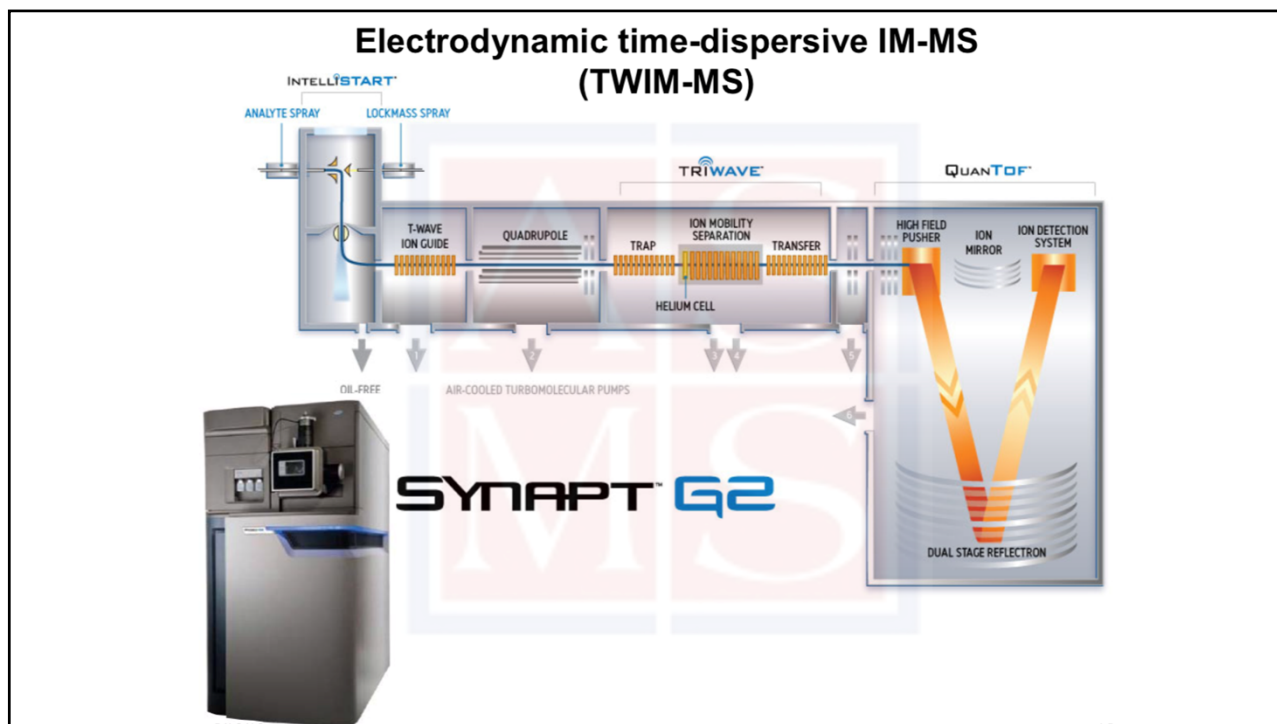
51

Ion Mobility Concept

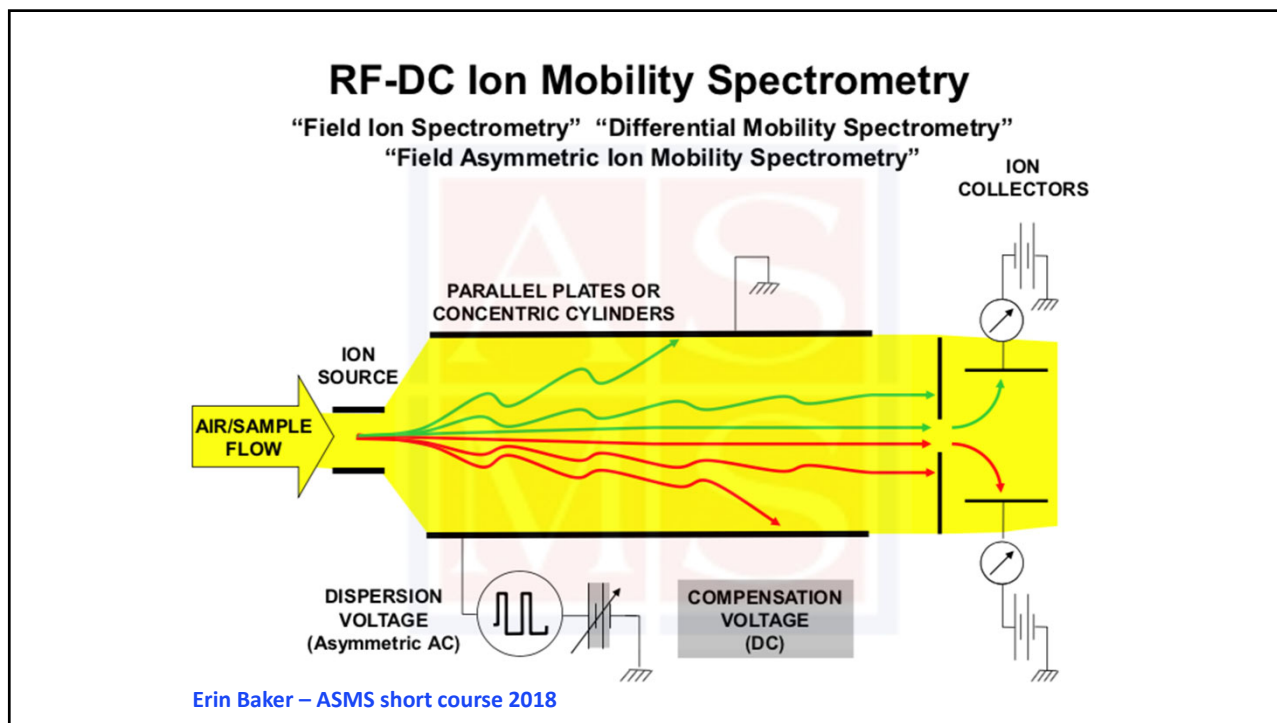


Erin Baker – ASMS short course 2018

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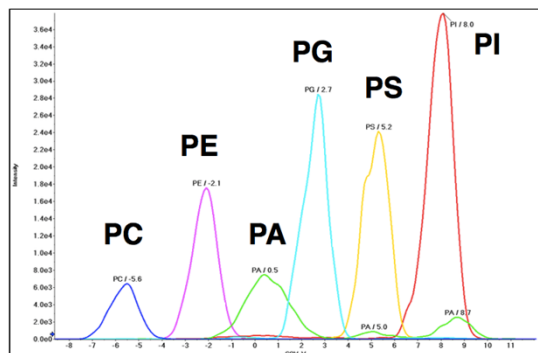


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Ion mobility mass spectrometry

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

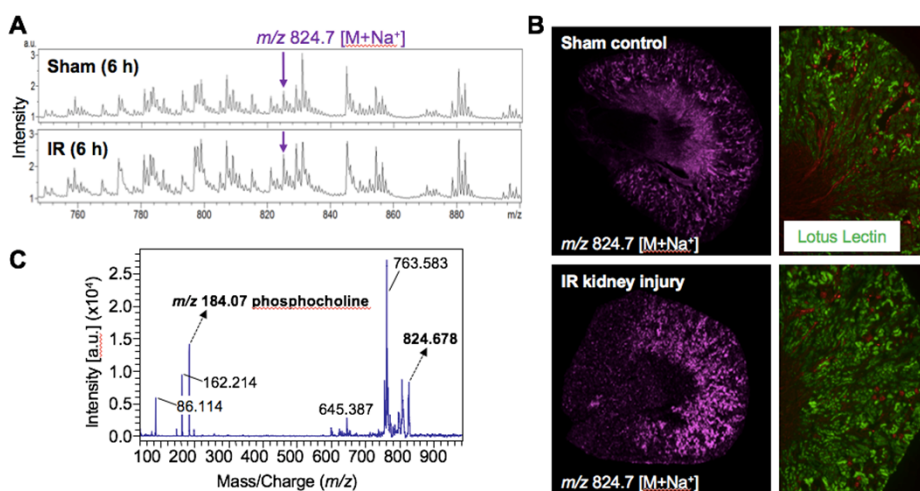


This is a gas-phase separation of these phospholipids, i.e., no chromatography.

SCIEX use a differential mobility process.

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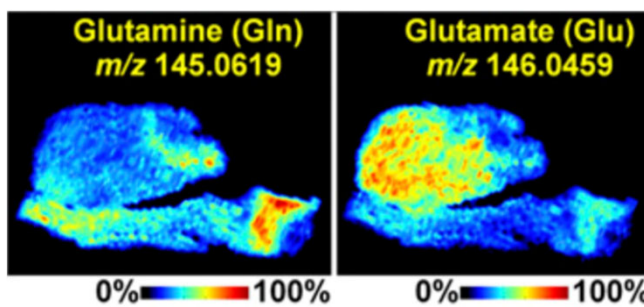
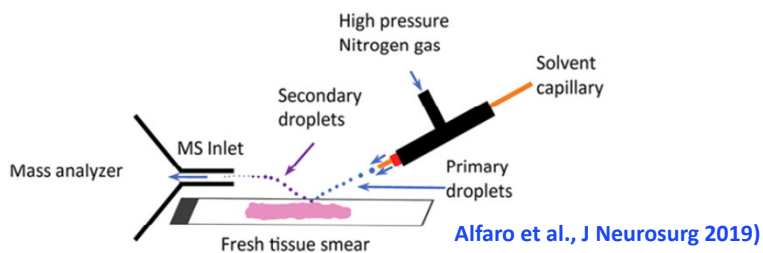
Imaging mass spectrometry



Generated by Janusz Kabarowski and Kelly Waters using MALDI-MS

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Desorption electrospray ionization (DESI)

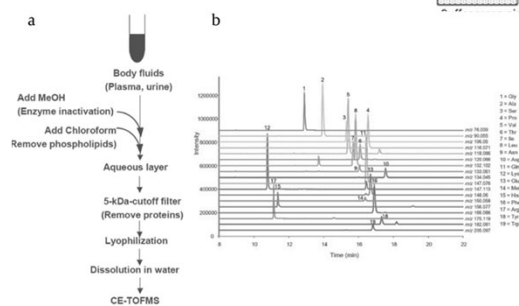
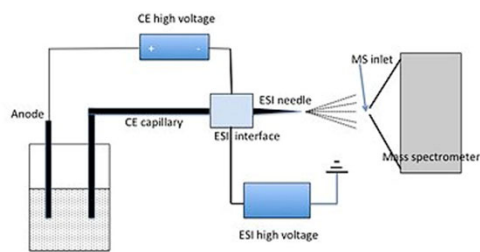


Sun et al., PNAS US 116:52, 2019

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Future methods in metabolomics

• Capillary electrophoresis (CE-MS)

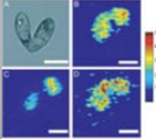


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Single-cell MS for Metabolomics

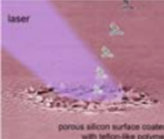
In Vacuo

Tof-SIMS



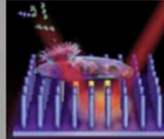
Ewing et al., *PNAS* 2010, 107, 2751

NIMS



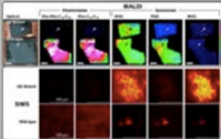
Sluzdak et al., *Anal. Chem.* 2011, 83, 2

NAPA



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

MALDI-guided SIMS

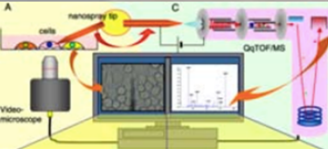


Sweedler et al., *Anal. Chem.* 2014, 86, 9139

At Ambient Conditions

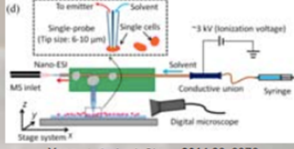
- Fast analysis
- *In situ* studies

Live single-cell video-MS



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

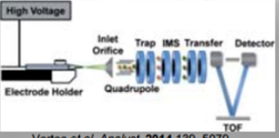
Single probe-MS



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

Courtesy of Peter Nemes

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Imaging metabolites in real time

- In an ideal world, we want to measure metabolites without their degradation, spatially (preferably sub-cellularly) 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)

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Where is metabolomics headed?

- **Better LC chromatography**
 - Micropillar columns allow the use of much longer, highly reproducible columns
 - Suitable for Precision Medicine samples
 - Often single samples to be mapped against pooled “normal” samples
 - Can take time and get the best resolution
- **Perhaps no chromatography?**

**Acoustic ejection
mass spectrometry**

SCIEX

Samples at 3/sec



Lossless ion mobility

Path length extended
from 24 cm to >10 m

<https://availabletechnologies.pnnl.gov/technology.asp?id=396>

<https://sciex.com/content/SCIEX/na/us/en/products/integrated-solutions/Echo-ms.html>