GBS 724

Choosing the metabolomics platform

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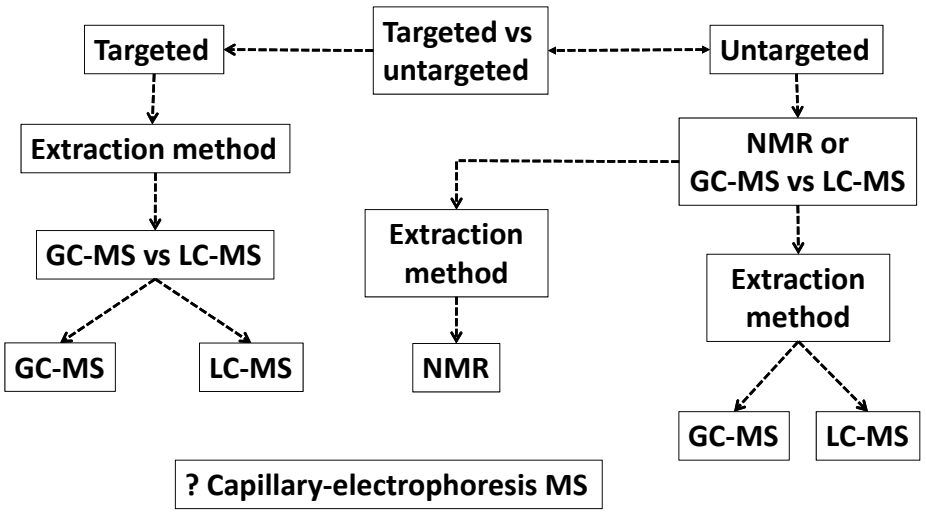
So, I have my samples – what's next?

- You've collected your samples and you may have extracted them
  - Protein precipitation with 4 volumes of methanol
  - OR partition across a chloroform-methanol-water two-phase system
    - Aqueous fraction has the hydrophilic metabolites
    - Chloroform fraction has the lipids
- Now it's time to select how to measure the metabolites

Choosing targeted or untargeted approaches

- A targeted approach requires that you know what you want to measure
  - It can be hypothesis-driven
  - Depends on the way you phrase the question and interpret the data
    - If you asked which of member(s) of Krebs cycle was(were) changed by a treatment, you may have to adjust the probabilities for multiple comparisons
    - On the other hand, if you were testing how much of the carbon atoms entering the Krebs cycle are converted to glutamate in response to a particular treatment, you may be able to model this from multiple metabolite levels

Decision tree



Targeted methods

- These are principally based on MS-MS methods – see later

NMR vs MS-based methods

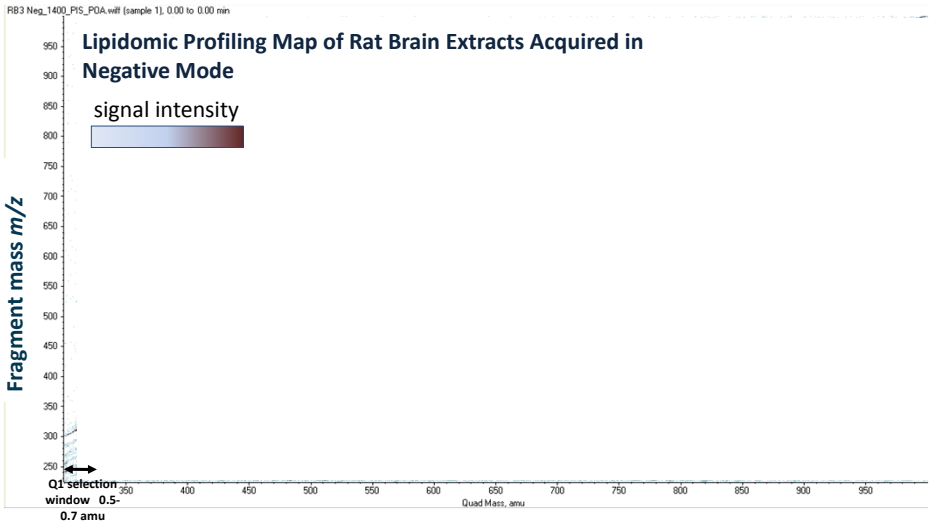
- NMR (Nuclear Magnetic Resonance)
  - It's an untargeted approach
  - Will be described in detail by Dr. Krishna on Jan 15
  - ADVANTAGE: does not consume the sample
  - DISADVANTAGE: Has limits on the metabolites that can be observed
  - Alone it measures all metabolites at the same time
    - However, it can be combined with LC

Mass spectrometry methods

- **Direct infusion (SWATH lipidomics)**
  - **We will discuss this later**
  - **Based on the chloroform-methanol extracts**
  - **Untargeted**
  - **Used with electrospray interface**

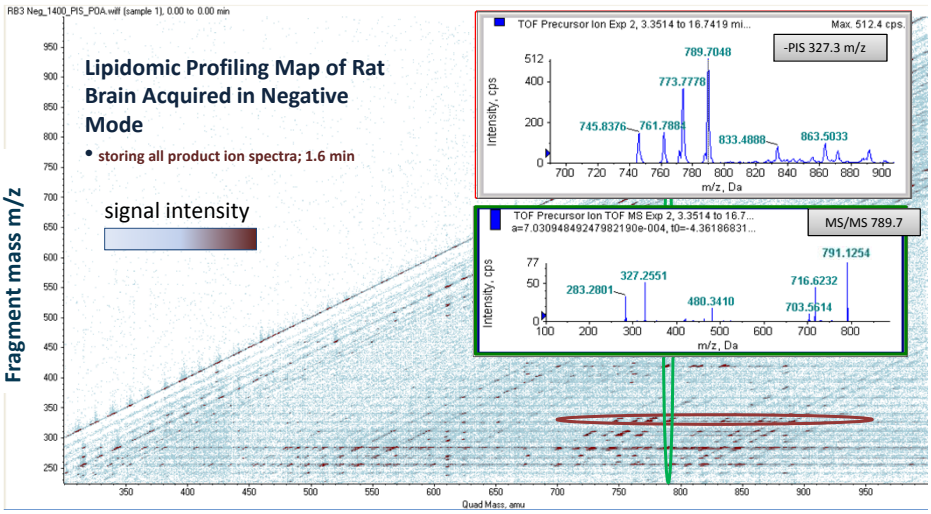
Complete and Comprehensive Data Collection

TOF MS Scanning Storing all Product Ions



MS/MS of Every Precursor

Product Ion Scan of Every Lipid



Precursor mass m/z

Gas chromatography

- For naturally volatile metabolites
- Or those that can be derivatized to form stable volatile forms
- Generally limited to compounds with MW <400
- There are extensive GC-MS compound databases
- Since mid-70s, capillary open tubular GC
- Will be discussed in more detail by Sara Cooper (Hudson Alpha) on Jan 20

LC-MS

- The principal advantage of this method is that doesn't necessarily require derivatization
  - There are circumstances where reagents can convert compounds that isomerize into a stable form
  - Can resolve 1,000s of metabolites
- There are several disadvantages
  - Lower chromatographic resolution than GC (packed as opposed to open tubular columns)
  - Less extensive metabolite libraries
  - I will discuss LC-MS in more detail on Jan 22

A mass spectrum

- ESI in LC-MS is a soft ionization process
  - Generates a molecular ion
    - $[M+H]^+$ or $[M-H]^-$
    - In metabolomics it can also be $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ or $[M+Cl]^-$
  - Both negative ion and positive ion data are typically collected

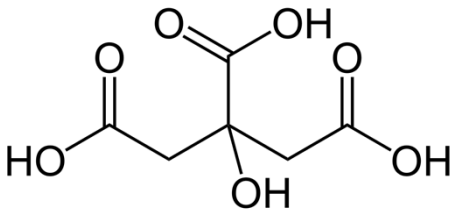
What is the mass of an ion?

- It's the sum of the atomic weights of the atoms comprising the molecule +/- the mass of a proton (if the ion is generated by the gain or loss of a proton)
  - C = 12.00000
  - H = 1.00782
  - N = 14.00307
  - O = 15.99491
  - P = 30.97376
  - S = 31.97207

Proton mass = 1.007276
Electron mass = 0.000549

Citrate

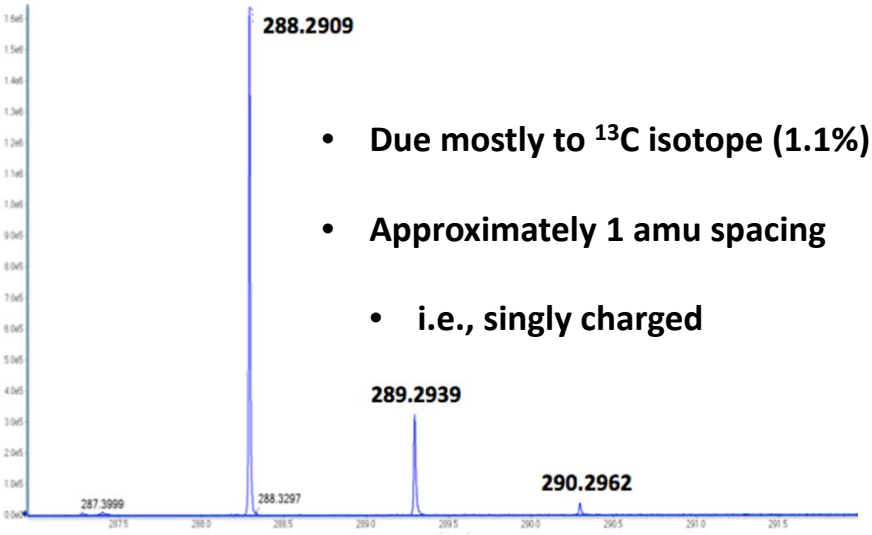
- $C_6H_7O_7$ is the $[M-H]^-$ ion



- *m/z* = 6 x 12.00000 + 7 x 1.00783 + 7 x 15.99491 + 0.000549 = 191.01973

- This is the monoisotopic mass of citrate

Naturally occurring isotope peaks



- Due mostly to ^{13}C isotope (1.1%)
- Approximately 1 amu spacing
  - i.e., singly charged

Mass defect

- On the scale when ^{12}C is 12.00000, the other elements have non-integer masses.
- The integer mass is called the nominal mass (the total number of protons and neutrons)
- The difference between the actual and nominal mass is called the mass defect.
  - For H, 1.00783 – 1.00000 = +0.00783
  - For O, 15.99491 – 16.00000 = -0.00509

Mass defect for citrate

- For the formula of $C_6H_7O_7$, the nominal mass is 72+7+112 = 191
  - The 7 H atoms will lead to an increase in mass of 7 x 0.00783 = 0.05481
  - The 7 O atoms will reduce the mass by 7 x 0.00509 = 0.03563
- The mass difference is +0.01918
- The observed mass also has to include the electron (+0.00055), giving the observed mass defect of +0.01973

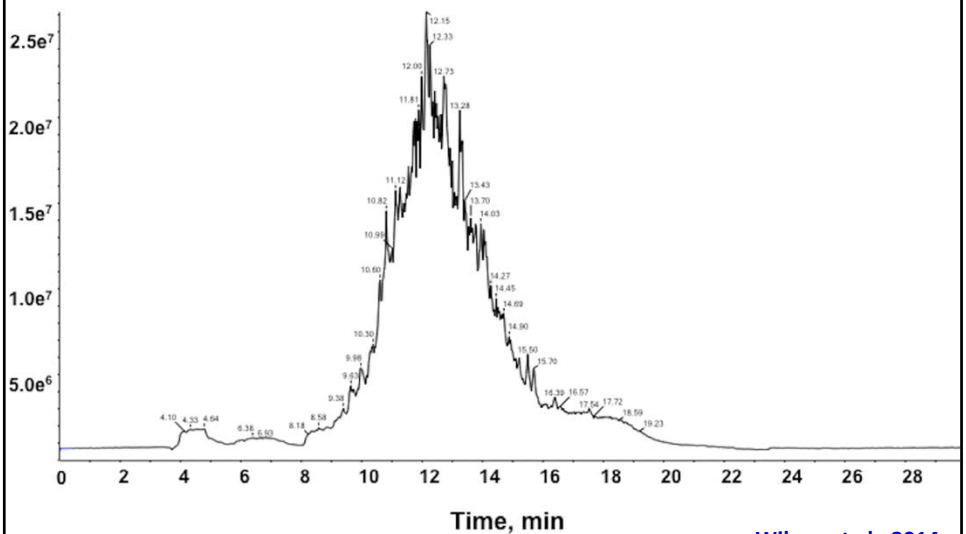
Selecting the mass spectrometer

- As you can see, 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 and Fourier Transform-Ion Cyclotron Resonance (FT-ICR)
  - These will be discussed in more detail by Dr. Renfrow on Jan 25

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 *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

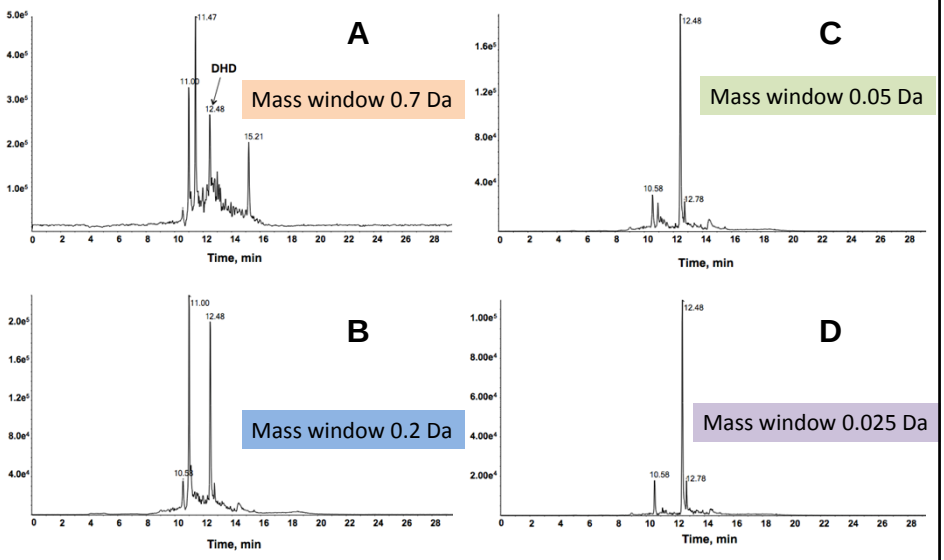
Example of a TIC of human urine



Wilson et al., 2014

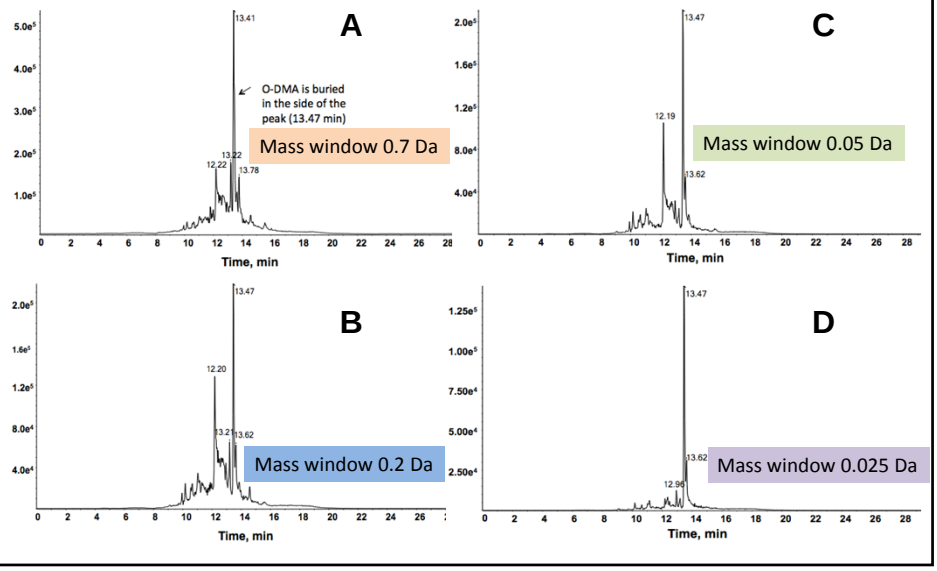
Selected ion chromatograms from TIC

Dihydrodaidzein



Selected ion chromatograms from TIC

O-desmethylangolensin



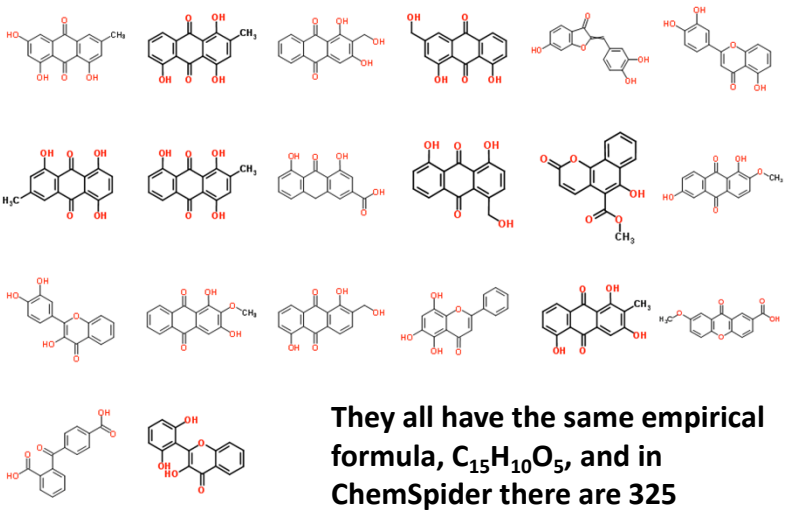
Uniqueness of molecular ions

- The molecular ion, even when measured with the highest possible accuracy, is not a unique descriptor
- There are many, many theoretical possible structures for a given mass and hence empirical formula
  - We'll discuss this more later once we have data to analyze

Have I just measured genistein?

$[M+H]^+ = 271.060\ m/z$

Compounds in ChemSpider



They all have the same empirical formula, $C_{15}H_{10}O_5$, and in ChemSpider there are 325

MSMS

- 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 MSMS spectrum for a metabolite
  - Ion traps can also isolate a product ion and create MS^n spectra

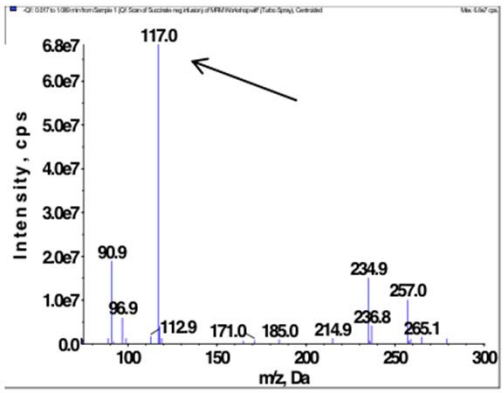
Measuring a mass transition

- **Instead of measuring the full MSMS spectrum, ions from the MSMS 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**

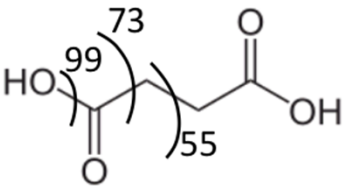
Analysis of succinate

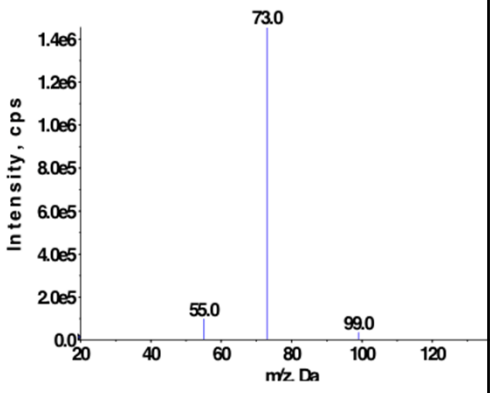
Succinate is displayed in the mass spectrum at m/z = 117

This type of scan will verify the **precursor ion**, also known as the **parent ion**.



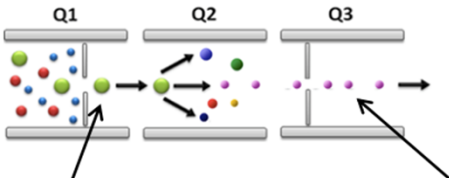
Fragmentation of succinate $[M-H]^-$





Multiple reaction ion monitoring (MRM)

A mass spectrometry technique in which quadrupoles one and three are locked onto a specific mass transition.

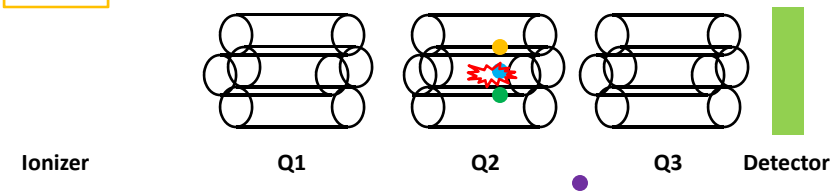


Selected precursor (parent) ion for succinate m/z = 117

Secected fragment ion for succinate m/z = 73

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

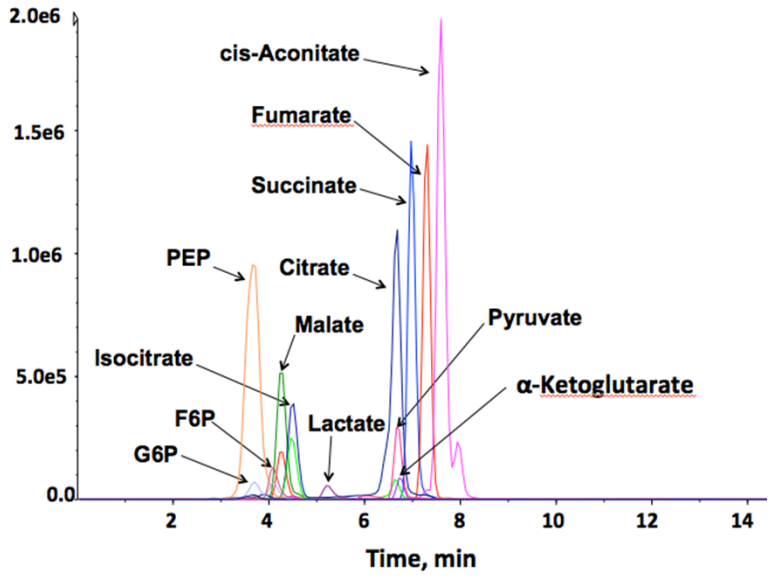
How many MRM transitions?

- 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 have to be matched against the shape and width of the chromatographic peaks – to be discussed in more detail later

Targeted vs untargeted methods

- If we know what the metabolites to be measured are (from previous untargeted analyses, or prior knowledge), then a 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.

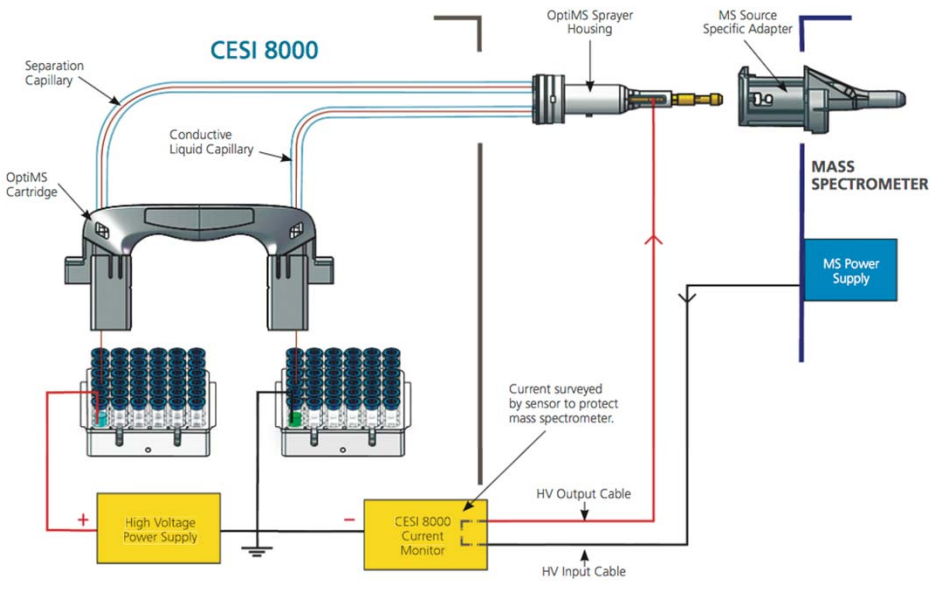
Combined channels for Krebs cycle



Future developments in platform analysis

Capillary-electrophoresis

Open tubular LC



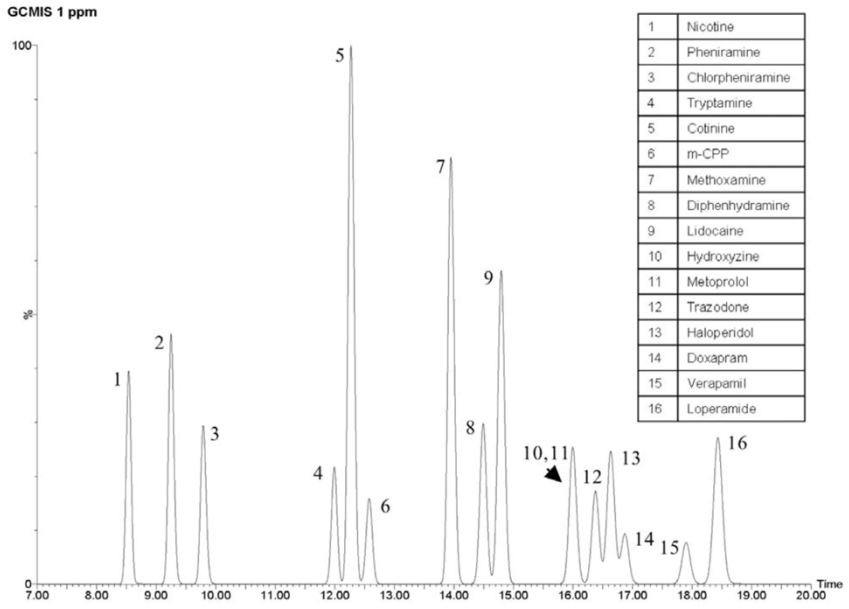
Features of CE-MS

- Capillary's distal end is porous to allow ion flow
- Electrical contact for the CE is achieved through an ESI needle filled with conductive fluid
- ESI's electrical contact is achieved through the protruding capillary tip
- Low flow at the tip terminus instantly generates a fine spray when ESI voltage is applied
- All electrochemistry associated with electrolysis of water is hereby decoupled from the spray
- Capillary inlet and exit are of the same dimension, reducing the clogging often associated with nanoscale techniques
- ESI is generated at lower voltages, which reduces the risks of oxidation artifacts

Advantages of CE-MS

- It is a nanosystem with no particles in its path
- The low flow rate (<10 nl/min) is conducive to very high sensitivity
- High resolving power

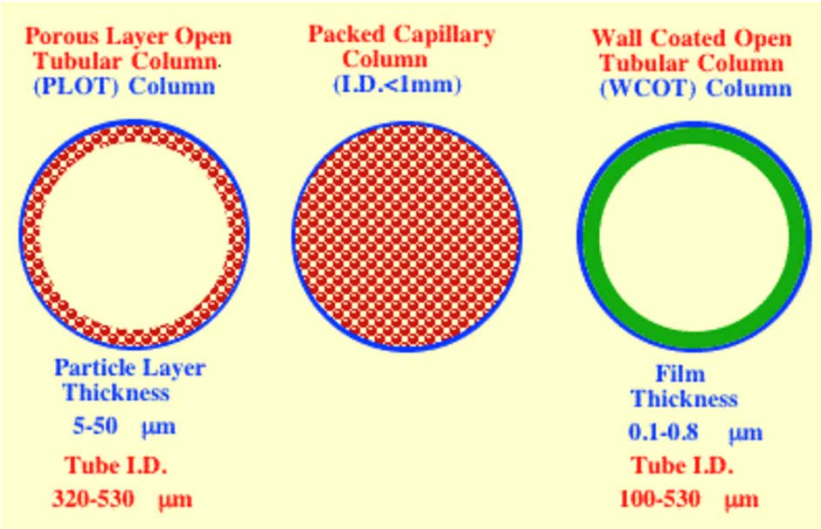
CE-MS of basic drugs



Advantages of increased peak capacity

- LC in LC-MS has been described as 'lousy' chromatography by the GC community
- If the chromatographic run used in metabolomics is 20 min long, and there are 4,000 metabolites, that means 200 are eluted each minute, or 3-4 per second
- If there are more metabolites (and there are), we need better chromatography
  - Open tubular columns, not smaller particles, is the answer

Open tubular LC columns



Features of open tubular columns

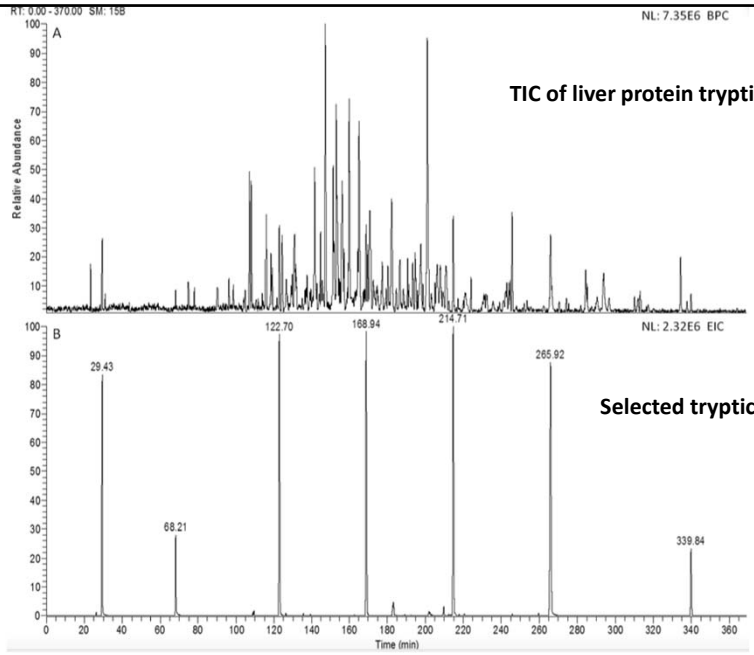
- Very low back pressure
- Columns can be very long (open tubular GC columns are often up to 100 meters)
- Low capacity since there is very little liquid phase on the top of the column for the sample components to stick to
  - Compensated for by the MUCH increased peak resolution, i.e., one can load a smaller amount of the sample

Open tubular columns - preparation

Use pressure bomb to load fluids into 10 or 50 μm ID capillary

- Treat with 1 M NaOH
  - Seal and place in oven at 100°C for 1 h
  - Flush with water and acetonitrile to completely dry
- Treat with 5 mg 2,2-diphenyl-1-picrylhydrazyl dissolved in a mixture of 300 μL γ-MAPS and 700 μL anhydrous DMF
  - Seal and heat to 110°C for 6 h
  - Flush and wash with acetonitrile to completely dry
- Treat with 5 mg azobisisobutyronitrile, 942 μL ethanol, 200 μL styrene, and 200 μL divinylbenzene
  - Seal and heat at 74°C for 16 h

Rogeberg M, et al J Sep Sci. 2013 Sep;36(17):2838-47.



TIC of liver protein tryptic peptides

Selected tryptic peptides

Rogeberg M, et al J Sep Sci. 2013 Sep;36(17):2838-47.