

GBS 748

Choosing the metabolomics platform

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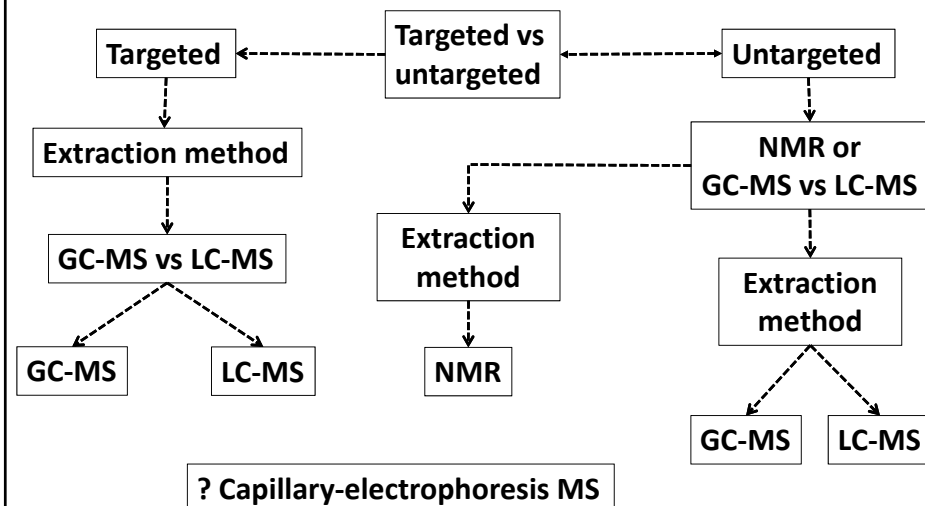
So, I have my samples – what's next?

- 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 16**
 - **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

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 23

LC-MS

- The principal advantage of this method is that doesn't 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 21

A mass spectrum

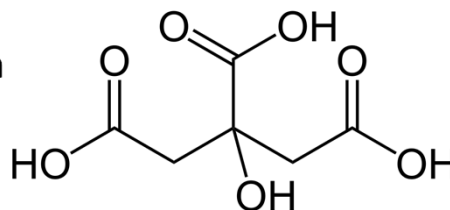
- ESI in LC-MS is a soft ionization process
 - Generates a molecular ion
 - $[M+H]^+$ or $[M-H]^-$
 - In metabolomics it can also be $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ or $[M+Cl]^-$
 - Both negative ion and positive ion data are typically collected

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 Proton mass = 1.007276
 - N = 14.00307 Electron mass = 0.000549
 - O = 15.99491
 - P = 30.97376
 - S = 31.97207

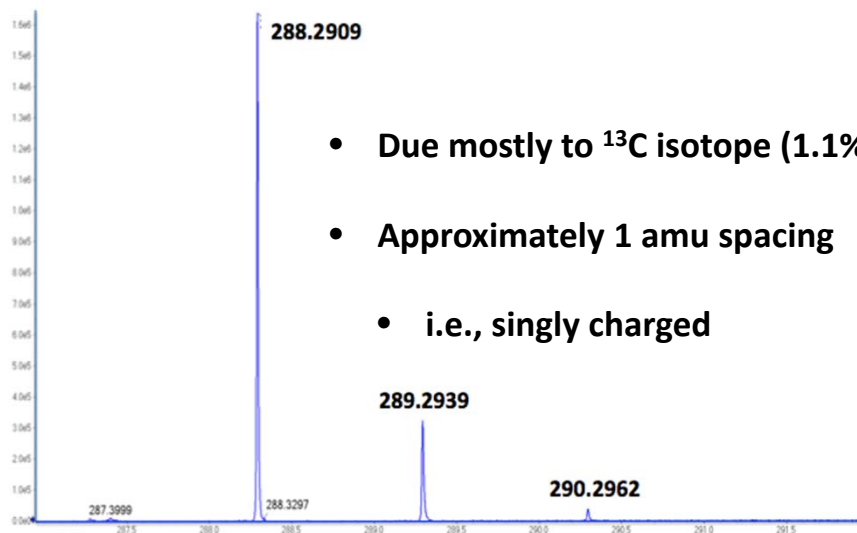
Citrate

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



- $m/z = 6 \times 12.00000 + 7 \times 1.00783 + 7 \times 15.99491 + 0.000549 = 191.01973$
- This is the **monoisotopic** mass of citrate

Naturally occurring isotope peaks



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 $7 \times 0.00783 = 0.05481$
 - The 7 O atoms will reduce the mass by $7 \times 0.00509 = 0.03563$
- 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**

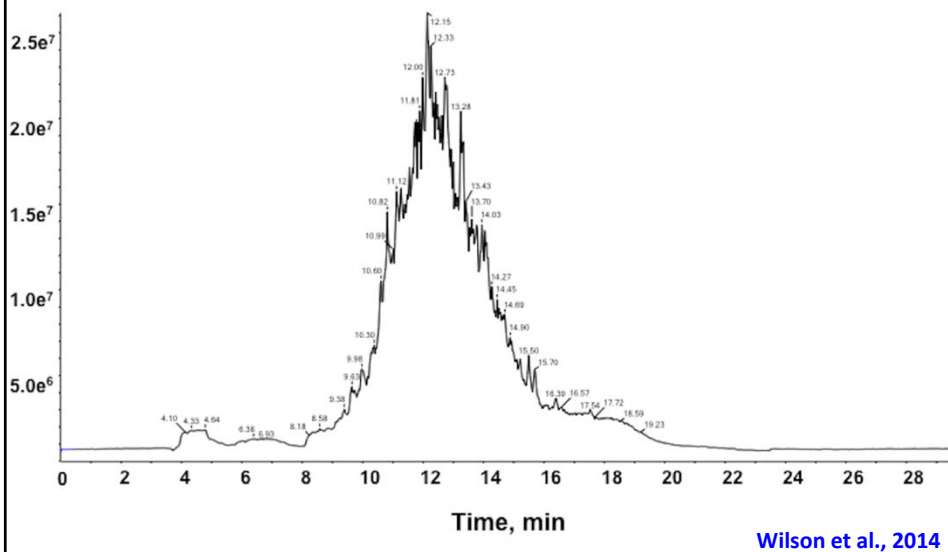
Selecting the mass spectrometer

- 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 26

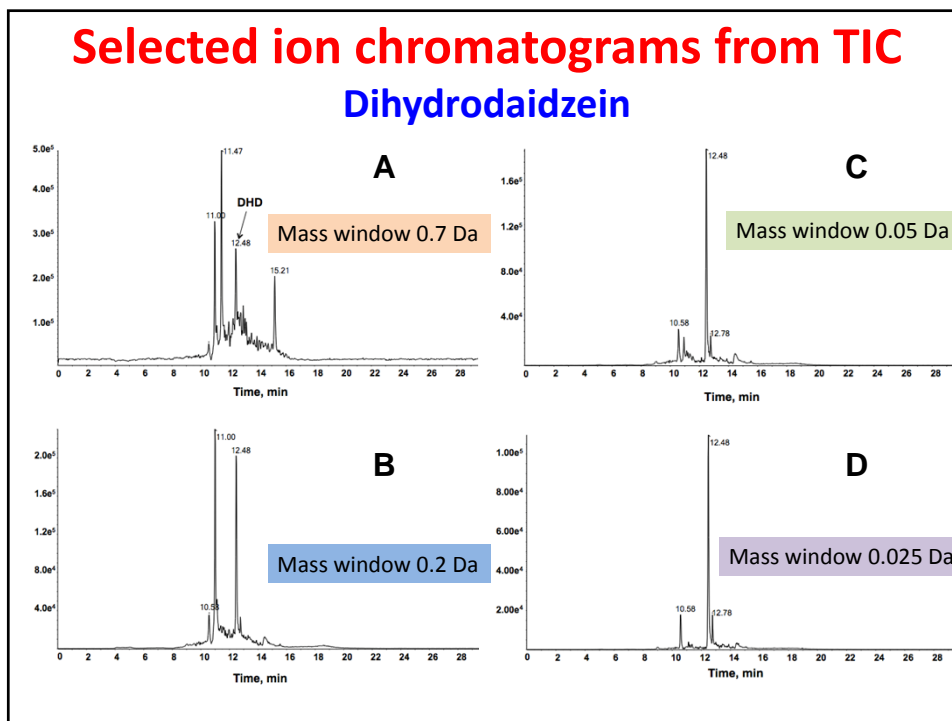
Selected ion monitoring

- 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

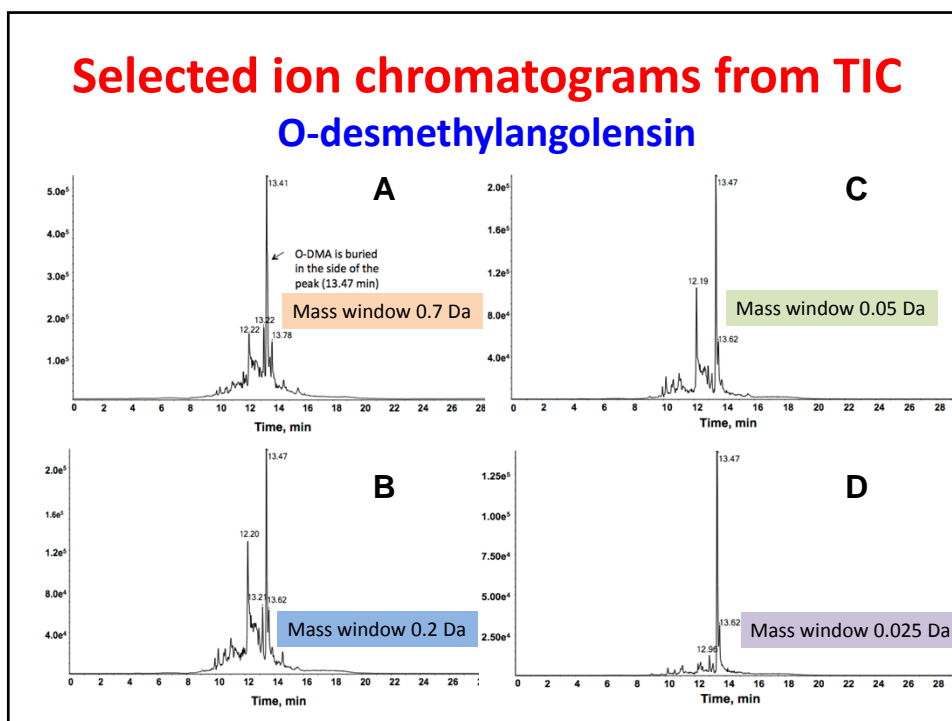
Example of a TIC of human urine



Selected ion chromatograms from TIC Dihydrodaidzein



Selected ion chromatograms from TIC O-desmethyngolensin



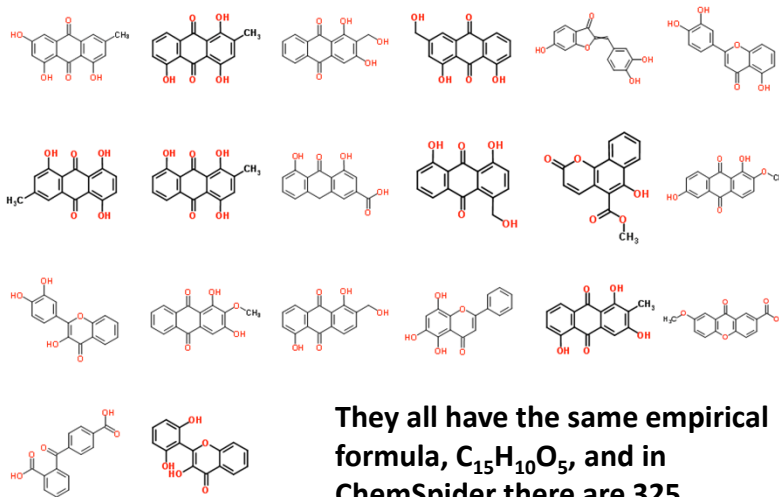
Uniqueness of molecular ions

- The molecular ion, even when measured with the highest 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?



Compounds in ChemSpider



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

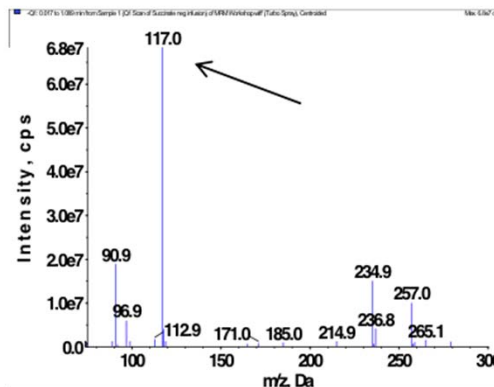
Measuring a mass transition

- 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**

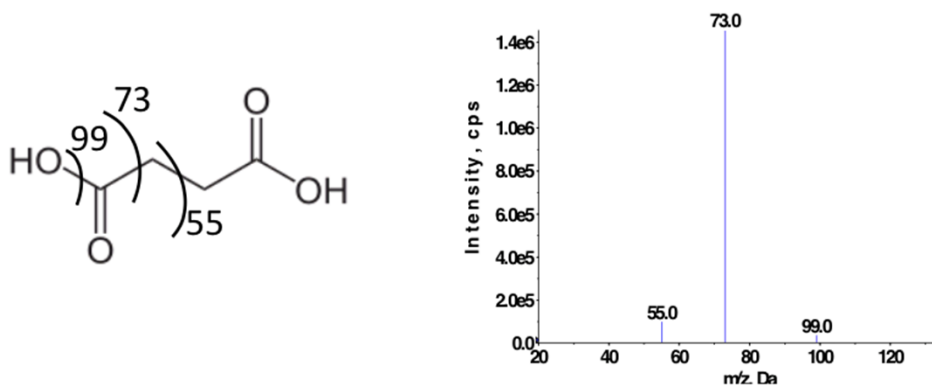
Analysis of succinate

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**.

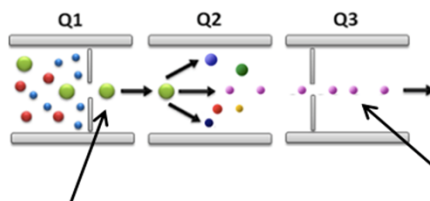


Fragmentation of succinate $[M-H]^-$



Multiple reaction ion monitoring (MRM)

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



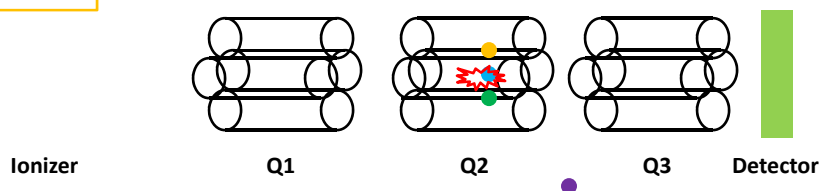
Selected precursor (parent) ion for succinate
 $m/z = 117$

Selected 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

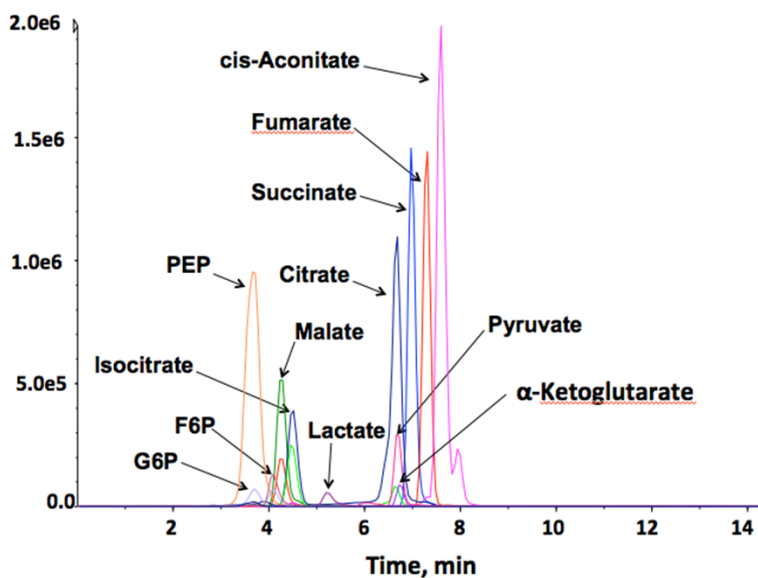
How many MRM transitions?

- 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.

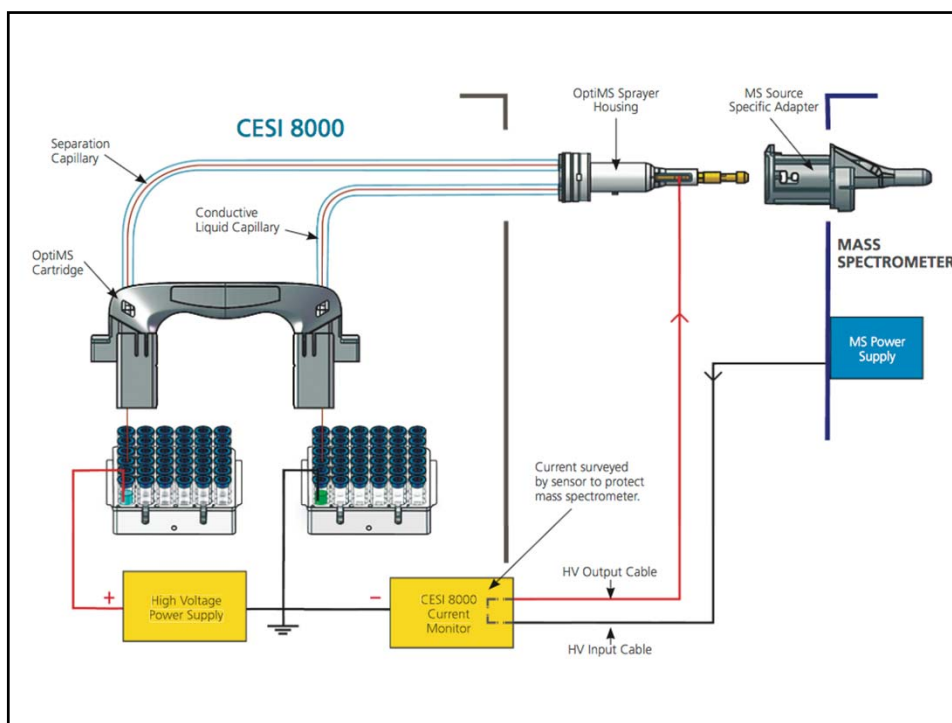
Combined channels for Krebs cycle



Future developments in platform analysis

Capillary-electrophoresis

Open tubular LC



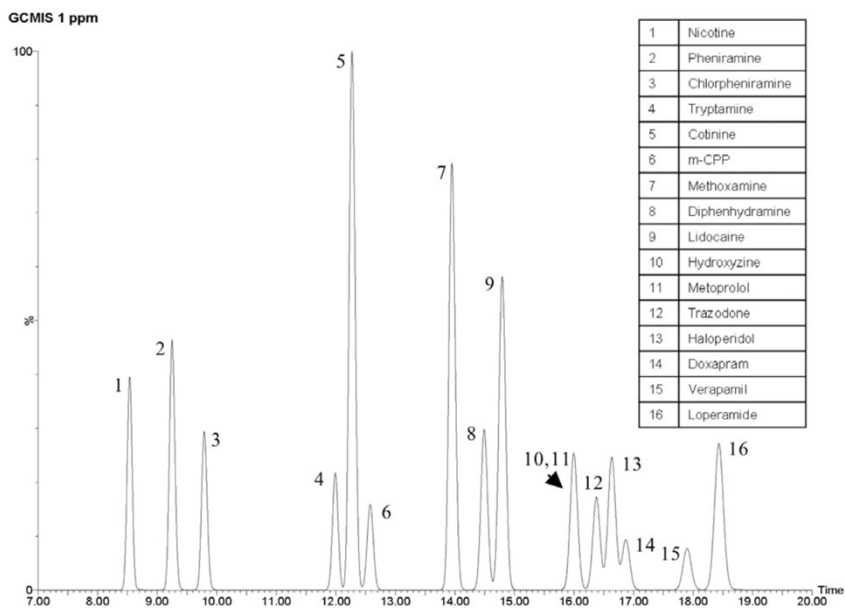
Features of CE-MS

- 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

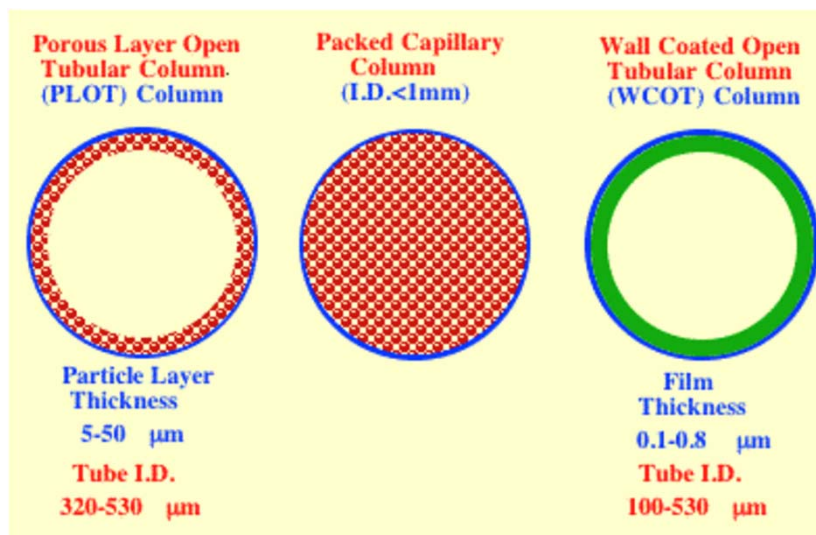
Advantages of CE-MS

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

CE-MS of basic drugs



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**