Knowledge that will change your world

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GC, GC-MS and LC-MS in metabolomics

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Gas chromatography

- **Gas chromatography (GC) or sometimes referred to as gas-liquid chromatography (GLC)**
  - Can be adsorption or partition chromatography
- **Analytes:**
  - Gases and naturally volatile substances (H_2, acetone)
  - Compounds that require derivatization (glucose, amino acids, organic acids, sugar phosphates)

Basis of GC

Differential movement of compounds through a column

- ***Mobile phase*** **(H_2, N_2)**
- **Fixed (*stationary*) liquid phase**
  - Liquids are temperature stable polymers
    - Silicones
    - Esters
- **Originally 6 ft x ¼" glass or stainless steel columns**
  - Liquid phase coated onto an inert support and packed into the column

Capillary GC

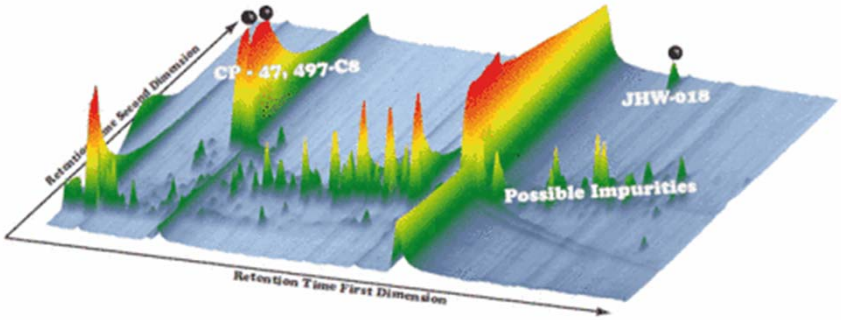
- **Packed columns had size limitations due to the pressure drop across the column (5000 plates)**
- **Van Deemter equation:**
  - Height of theoretical plate (HETP) (lower the better)
  - HETP = A + B/u + C*u where u = linear velocity
  - The compressibility of a gas means that the linear flow velocity is lower at the top of the column
- **By using open tubular capillary columns, the pressure drop is minimized and columns up to 100 meters are common (>100,000 plates)**

Coated capillary columns

- The liquid phase is either applied as a thin film on the inside wall of the quartz capillary, or is chemically bonded (more temperature stable)

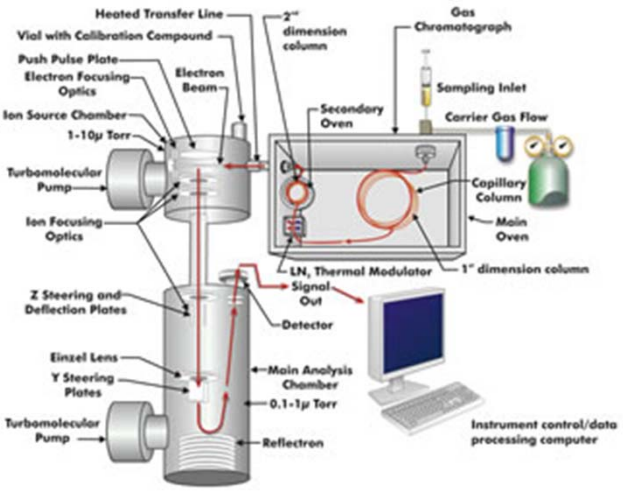
GC-GC

In this approach, fractions from the first GC column are orthogonally analyzed on a second, different GC column



2D-GC x GC reveals hidden peaks buried in major peaks in a 1D separation

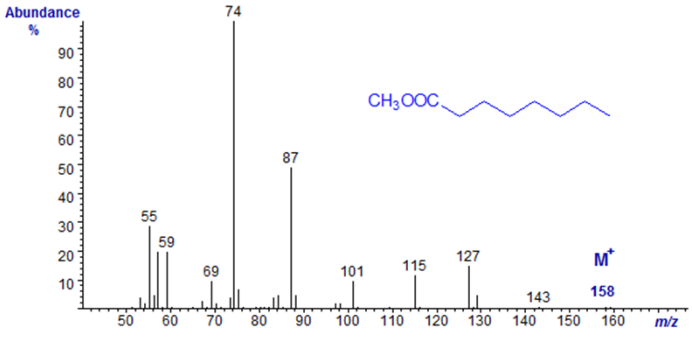
GC x GC-TOF-MS



Compounds emerging from the second column can be ionized and analyzed by time-of-flight MS analysis

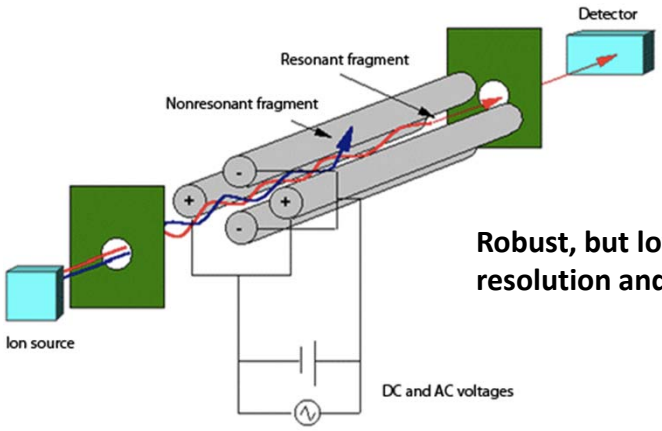
GC-MS

- **Compounds eluting from the capillary GC are ionized by an electron beam (70 eV)**
  - Ions have a mass-to-charge ratio (*m/z*)
  - Only rarely is the molecular ion observed, but many fragment ions are in the spectrum



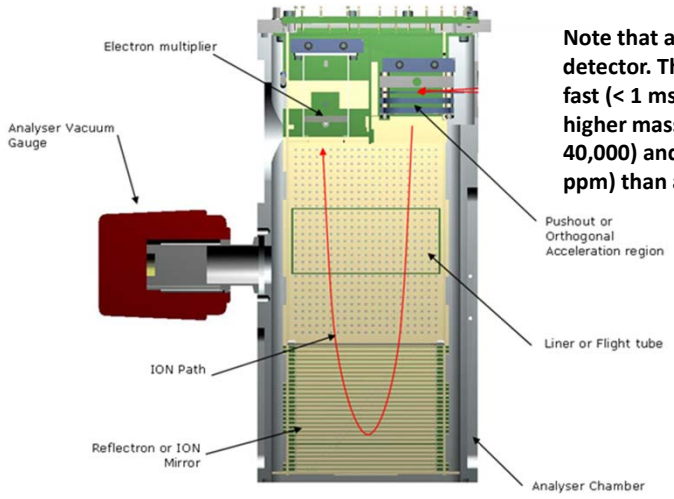
GC-MS mass analyzers

- **Ions are filtered one at a time (mass window of 0.2-0.7 Da) through a quadrupole mass filter**



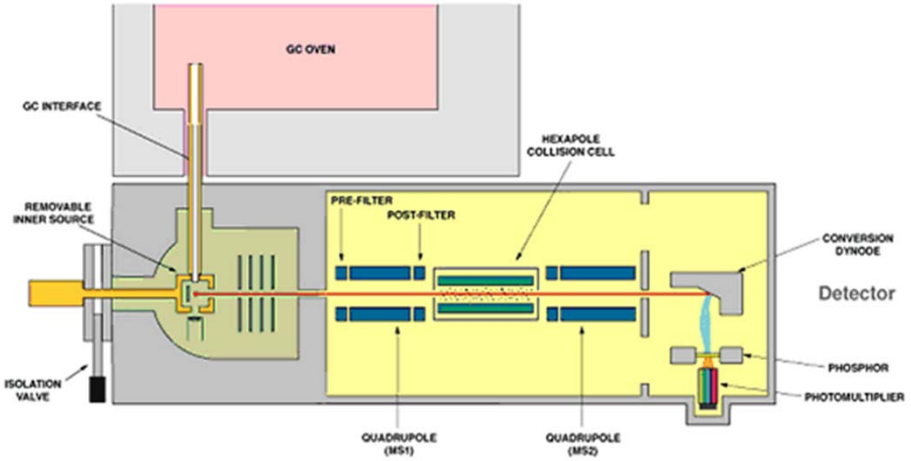
Robust, but low mass resolution and slow

GC-MS TOF analyzer



Note that all the ions reach the detector. The analysis is very fast (< 1 msec) and it has much higher mass resolution (up to 40,000) and mass accuracy (2-3 ppm) than a quadrupole

GC-triple quadrupole-MS/MS



Ions selected by the 1st quadrupole are collisionally dissociated and analyzed by the 3rd quadrupole.

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**

LC-MS

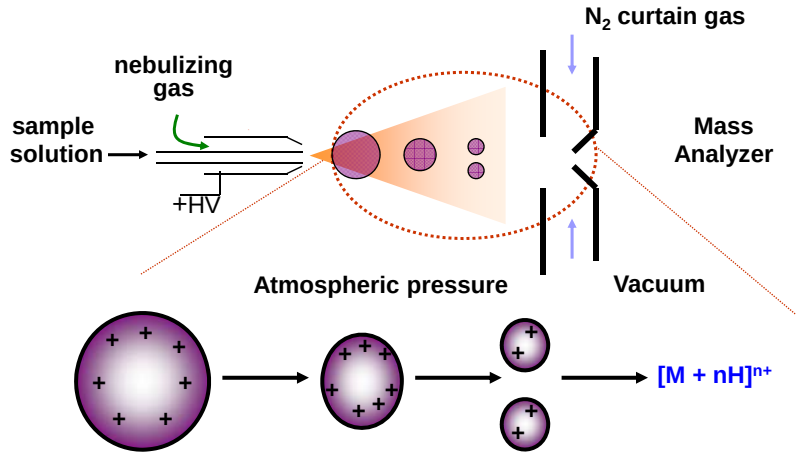
- **The advantage of an effective LC-MS system would be that it would allow thermally unstable compounds, even large ones (such as proteins), to go into the gas phase from liquid solution and into the mass spectrometer**
- **Importantly, the ionization methods used are soft in nature and molecular ions $[M+H]^+$ or $[M-H]^-$ are easily formed (see later re other molecular ions)**
- **However, there are some compounds that cannot be ionized by LC-MS**
  - polycyclic aromatic hydrocarbons, alkanes, waxes.

LC-MS interface

The key issue is how to transfer ions from the liquid phase into the gas phase while minimizing the transfer of solvent into the mass spectrometer

- **For compounds that can be charged, electrospray ionization (ESI) is the principal method of choice**
- **Nebulization of the electrical charged droplets more effectively decreases the size of droplets**
  - This allows all aqueous solvents to be processed by the interface
- **Heating the spray further increases sensitivity**
  - Not used in nanoelectrospray ionization

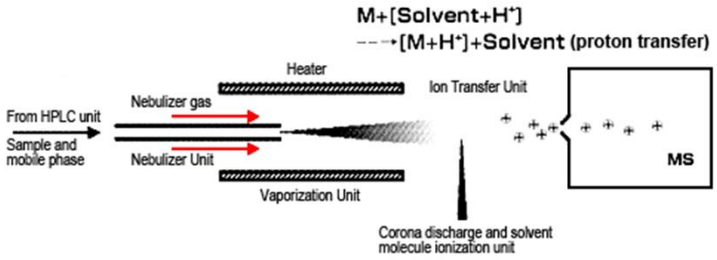
Electrospray Ionization (ESI)



1. Solvent evaporation
2. Coulombic repulsion

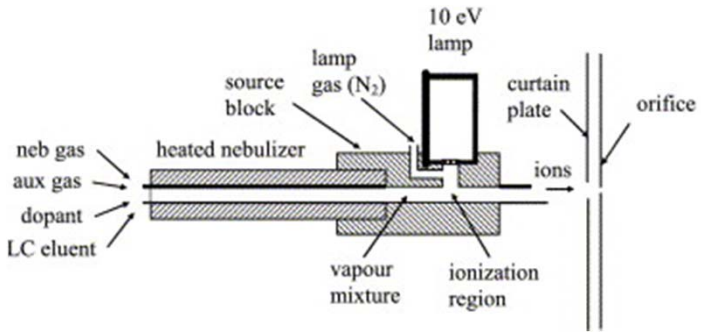
Atmospheric chemical ionization (APCI)

- Suitable for compounds that are neutral
- A corona discharge needle ionizes air molecules that transfer their energy to the solvent and hence the solutes



Atmospheric pressure photoionization (APPI)

- **In this ion source, ionization is created by a beam of ultraviolet photons**
- **A laser can also be used and it can ionize PAHs**



Guide to LC-MS flow rates

Type	Column ID	Flow rate	Solvent consumed*
Conventional	1.0-4.6 mm	0.050-1.00 ml/min	72-1440 ml
Capillary	0.3-1.0 mm	0.005-0.050 ml/min	7.2-72 ml
Nano	0.05-0.20 mm	100-1000 nl/min	0.144-1.44 ml

Sensitivity in LC-ESI-MS increases in proportion to the inverse of the flow rate. Therefore, there is value in going to lower flow rates – it also saves money on solvents.

ChipLC versus nanoLC

- A nanoLC column is so thin (75 μm i.d.) it has very little thermal capacity – this leads to variable retention times due to temperature fluctuations in the lab

A column etched in a block of silica can be engineered to have greater physical reproducibility and it has far greater thermal capacity. The CHIP can be placed in temperature-controlled chamber – we operate ours at 55°C – to recover more hydrophobic metabolites

Engineered microflow LC

Chromatography at flow rates of 5-50 μl/min using 0.3-0.5 mm ID columns

Very low dead volumes between the sample injection valve and the ESI interface despite the low flow rate

Enables short, reproducible gradients (1-2 min) or up to a 20 min gradient (for metabolomics) at 5 μl/min

Column phases

- **Reverse-phase**
  - C_4, C_5, C_8, C_{18}, phenyl-hexyl-bonded phases
- **Normal phase**
  - Bare silica, Cyano and amino-bonded phases
- **Hydrophilic interaction chromatography**
  - Bare silica, polyol-bonded phase
- **Particle sizes**
  - 5, 3, 2.5, 2.2 μm and 1.7 μm (for UPLC)

Mobile phases

- **Acidic media**
  - Typically 0.1% formic acid
- **Neutral media**
  - 1-10 mM ammonium acetate or formate
- **Alkaline media**
  - 0.1% ammonium hydroxide (but not with C_4-C_{18} phases)
- **Solvents (water-miscible)**
  - Methanol, acetonitrile, isopropanol (for hydrophobic metabolites)

Detector types

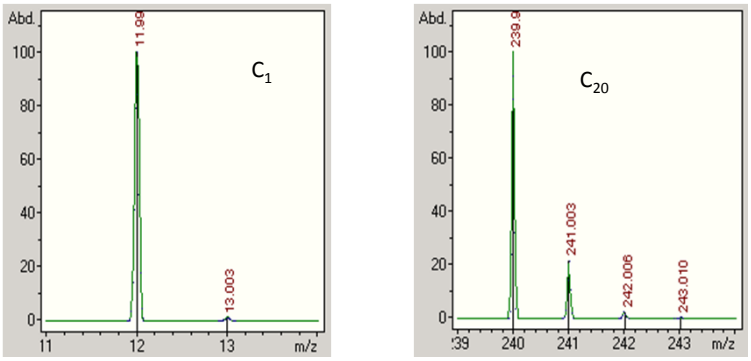
Type	Mass range (*m/z*)	Resolution	Accuracy (ppm)	Time for MSMS (msec)
Quadrupole	20-3000	2,000	50	1000
TOF	unlimited	30,00-40,000	2-3	50
Orbi-trap*		80,000-20,000	<1	200+
FT-ICR*	100-1,500	Up to 1,000,000	<1	1000

*These detectors depend on ion motion and therefore their performance declines as the acquisition time is shortened. Using a 50 msec MSMS acquisition, mass resolution on an Orbi-trap falls to 15,000. The TOF detector is the preferred one for untargeted analysis. The Orbi-trap and FT-ICR instruments are important for follow-up high mass accuracy experiments.

Detector combinations

- **Each detector can record a MS spectrum**
  - Not sufficient even with high mass accuracy to identify the metabolite
    - 100s of metabolites can have the same empirical formula (and identical mass)
- **Fragmentation of selected ions creates a MSMS spectrum to distinguish isobaric metabolites**
  - In IDA analysis, molecular ions detected in a quick Hi-Res MS, are “selected” by the quadrupole filter one at a time
  - The ion is fragmented and a MSMS spectrum recorded
    - TOF instruments can record 20 MSMS spectra per second

A mass spectrum of a compound



Many elements have stable isotopes. Although even for ^{13}C it is only 1.1% of ^{12}C, the chance of having one ^{13}C is cumulative. Other elements with stable isotopes add to the $[M+H+1]^+$ or $[M-H+1]^-$ peaks.

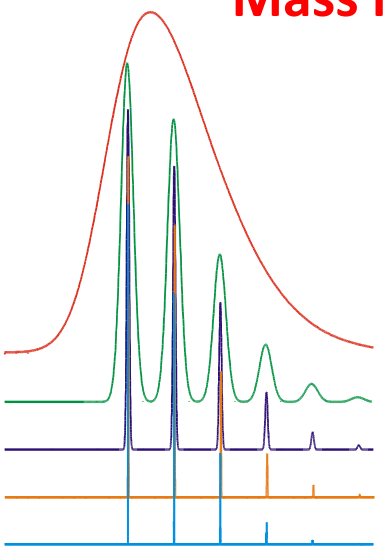
Naturally occurring isotopes

Isotope	Mass	% Abundance	Mass defect (mDa)
^{1}H	1.00783	99.985	7.83
^{2}H (Deuterium)	2.01400	0.015	14.00
^{12}C	12.00000	98.90	0.00
^{13}C	13.00336	1.10	3.36
^{14}N	14.00307	99.63	3.07
^{15}N	15.00011	0.37	0.11
^{16}O	15.99491	99.76	-5.09
^{18}O	17.99916	0.20	-0.84
^{19}F	18.99840	100	-1.60
^{23}Na	22.98977	100	-10.23
^{31}P	30.97376	100	-26.24
^{32}S	31.97207	95.03	-27.93
^{34}S	33.96787	4.22	-32.13
^{35}Cl	34.96885	76.77	-31.15
^{37}Cl	36.96590	31.98	-34.10
^{39}K	38.96371	93.26	-36.29
^{79}Br	78.91834	50.69	-81.66
^{81}Br	80.91629	49.31	-83.71

Adduct ions observed in LC-MS

- **Since the aqueous fraction used for metabolomics is not de-ionized first, molecular ions can form with cations and anions**
  - $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_3]^+$
    - Raising the orifice potential can break $[M+NH_3]^+$adducts
  - $[M-H]^-$, $[M-H+Cl]^-$, $[M-H+HCOO]^-$, $[M-H+CH_3COO]^-$
- **At high metabolite concentrations, they may form dimers**
  - $[2M+H]^+$, $[2M+Na]^+$
- **See remarks by Dr. Du on data processing about "collapsing" different ion states (isotopes and adducts)**

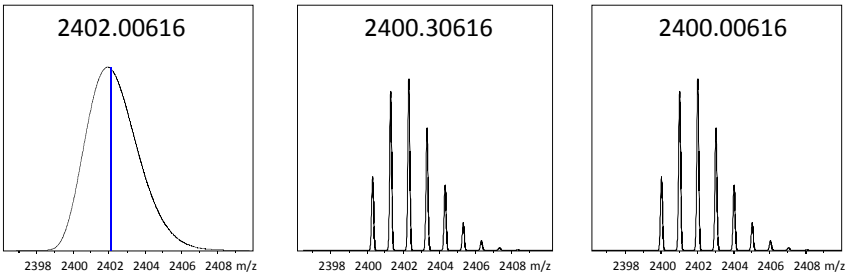
Mass resolution



1.000	linear TOF w/o DE
5.000	reflector TOF w/o DE
25.000	reflector TOF with DE
125.000	FTMS wideband mode
1.000.000	FTMS high-res mode

Courtesy of Bruker Daltonik GmbH

Mass accuracy



Poor resolution	High resolution	High resolution
High mass accuracy	Poor mass accuracy	High mass accuracy

- high mass accuracy does not necessarily require high resolution!
- high resolution is only mandatory to avoid overlapping peaks.

- mass accuracy is expressed in ppm
- 1 ppm: mass 1000 error 0.001 Da (1 mDa)

Courtesy of Bruker Daltonik GmbH

MS-MS of a metabolite

- **In GC-MS and LC-MS, molecular ions can be isolated selectively by a quadrupole filter or an ion trap**
  - Either pre-determined, or selected on-the-fly
  - Typically a 0.7 *m/z* or larger window
- **The selected ions can be heated**
  - By gas collision [He, N_2, Ar] in triple quad or Qtof
  - By an infra-red laser beam in a FT-ICR (to maintain a vacuum of 10^{-10} Torr to ensure no interference with ion motion)

Different types of MS-MS experiment

- **Product ion scan**
  - Select the precursor ion in Q1, fragment the selected ion, and scan the product ions in Q3
- **Precursor ion scan**
  - Scan the precursor ions in Q1, fragment the selected ion in Q2, and measure in Q3 the intensity of a pre-selected product ion
- **Neutral loss scan**
  - Scan the precursor ions one at a time in Q1, fragment the selected ion in Q2, and measure in Q3 the intensity of a product ion that has a specified mass loss
- **Reaction ion monitoring**
  - Select one precursor ion in Q1, fragment it in Q2, and measure in Q3 the intensity of one specific, pre-selected product ion
  - This is a precursor/product ion transition – multiple transitions can be studied every second – known as **MRM**

Types of metabolomics by LC-MS

- **Targeted LC-MS analysis allows a more hypothesis-driven analysis**
  - Based on triple quadrupole analyzer
  - Selects one compound at a time for analysis
  - Based on the transition from the molecular ion (precursor ion) to a specific product ion
- **Untargeted LC-MS analysis**
  - Many more analytes to measure
  - Needs a fast analyzer
  - TOF with (MSMS) and without quadrupole selection (MS)

Targeted LC-MS

Based on multiple reaction monitoring (MRM)

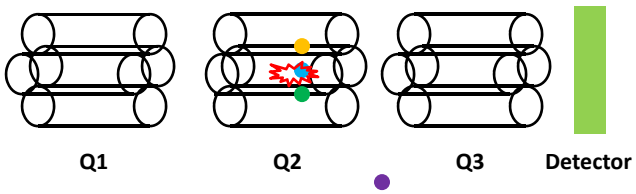
MRM is a type of MSMS where the *m/z* value in the 1st triple quad (precursor ion) and that in the 3rd quadrupole are fixed and analyte-specific

– To obtain MRM conditions for an analyte, if not already available (see hands on session)

1. MS spectrum by infusion
2. Perform MSMS experiment on $[M+H]^+$ or $[M-H]^-$ ion
3. Optimize the sensitivities of the product ions
4. Develop an LC method for the compound of interest
5. Select 3-4 precursor-product ion pairs

Multiple reaction ion monitoring

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



Based on precursor ion/product ion pair(s)

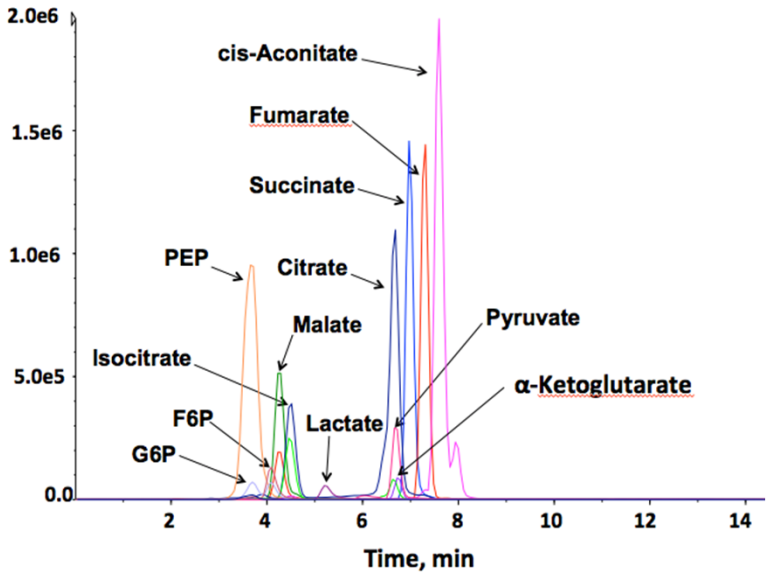
Courtesy, John Cutts

Primer for selecting ions for MRM

Peak width (sec)	Cycle time (sec)	Dwell time (msec)	Number of channels
5	0.5	20	25
10	1.0	20	50
5	0.5	10	50
10	1.0	10	100
5	0.5	5	100
10	1.0	5	200
5	0.5	2	250
10	1.0	2	500

The number of channels can be increased by using timed windows

Combined channels for Krebs cycle



Untargeted LC-MS

- **Need to analyze "every" ionized species that is eluted from the LC**
  - No pre-formed hypotheses
- **Since there may be >10,000 compounds that are eluted in a single run, the detector must be very fast**
  - Quadrupole is too slow (1 sec to scan 20-1000 *m/z*)
  - TOF is the principal choice – gives good mass resolution and mass accuracy
  - Suited to collect both MS and MSMS at the same time