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In the next step in our quest to explain what is life

The human genome project has largely been completed and many other genomes are surrendering to the gene sequencers. However, all this knowledge does not give us the information that is needed to explain how living cells work. To do that, we need to study proteins.

In 2002, mass spectrometry has developed to the point where it has the capacity to obtain the "exact" molecular weight of many macromolecules. At the present time, this includes proteins up to 150,000 Da. Proteins of higher molecular weights (up to 500,000 Da) can also be studied by mass spectrometry, but with less accuracy. The paradigm for sequencing of peptides and identification of proteins has changed – because of the availability of the human genome database, peptides can be identified merely by their masses or by partial sequence information, often in minutes, not hours. This new capacity is shifting the emphasis of biomedical research back to the functional aspects of cell biochemistry, the expression of particular sets of genes and their gene products, the proteins of the cell. These are the new goals of the biological scientist:

- to know which proteins are expressed in each cell, preferably one cell at a time
- to know how these proteins are modified, information that cannot necessarily be deduced from the nucleotide sequence of individual genes. Modification may take the form of
  - specific deletions (leader sequences),
  - enzymatically induced additions and subsequent deletions (e.g., phosphorylation and glycosylation),
  - intended chemical changes (e.g., alkylation of sulfhydryl groups),
  - and unwanted chemical changes (e.g., oxidation of sulfhydryl groups, nitration, etc.).
  - to determine how proteins assemble in solution and how they interact with each other

Mass spectrometry is enabling us to investigate these changes in the chemistry of proteins. New types of instruments have made mass spectrometry available to every biomedical researcher.

Students and postdocs should be aware that the change has been so sudden that there is a dearth of mass spectroscopists who have sufficient training in the biological sciences to take full advantage of the these new instruments. Mass spectrometry may also find a niche in the analysis of DNA – agarose gels may pass into history of the late 20th Century.

What a biomedical scientist needs to know about mass spectrometry

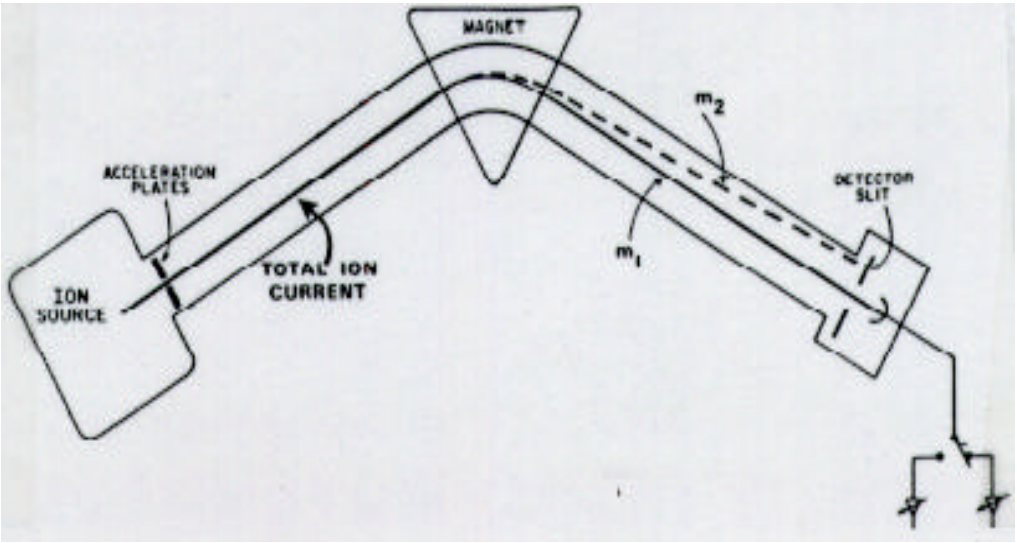
- Substances have to be ionized to be detected.
- The net charge can be either positive or negative.
- The mass-to-charge ratio of an ion (m/z) is the most important parameter.
- The mass spectrometer is a selective detector (based on mass differences), but all the substances that are present in a sample and can be ionized can be measured.
- Polyionic buffer salts, particularly phosphate, interfere with ion formation in the electrospray ionization interface.
- Matrix-assisted laser desorption time-of-flight mass spectrometry is very tolerant of the biomedical scientist.
- The mass spectrometer is always right.

Types of mass spectrometer analyzers

- Magnetic sector
- Quadrupole filter (with electrospray ionization or heated nebulizer atmospheric pressure chemical ionization)
  - single quadrupole
  - triple quadrupole (MS/MS experiments)
- Time-of-flight (TOF)
  - Matrix-assisted laser desorption ionization (MALDI)
  - Electrospray ionization interface
- Ion trap (MS^n experiments) with electrospray ionization interface
  - hybrid with TOF
  - in superconducting magnet using ion cyclotron radiation (FT-ICR-MS)
- Hybrid instruments
  - hybrid quadrupole/time-of-flight (Qtof/Q-Star)
  - TOF/TOF
  - MALDI-TOF/FT-ICR-MS

Magnetic sector analyzer

1 x 10^6 resolution

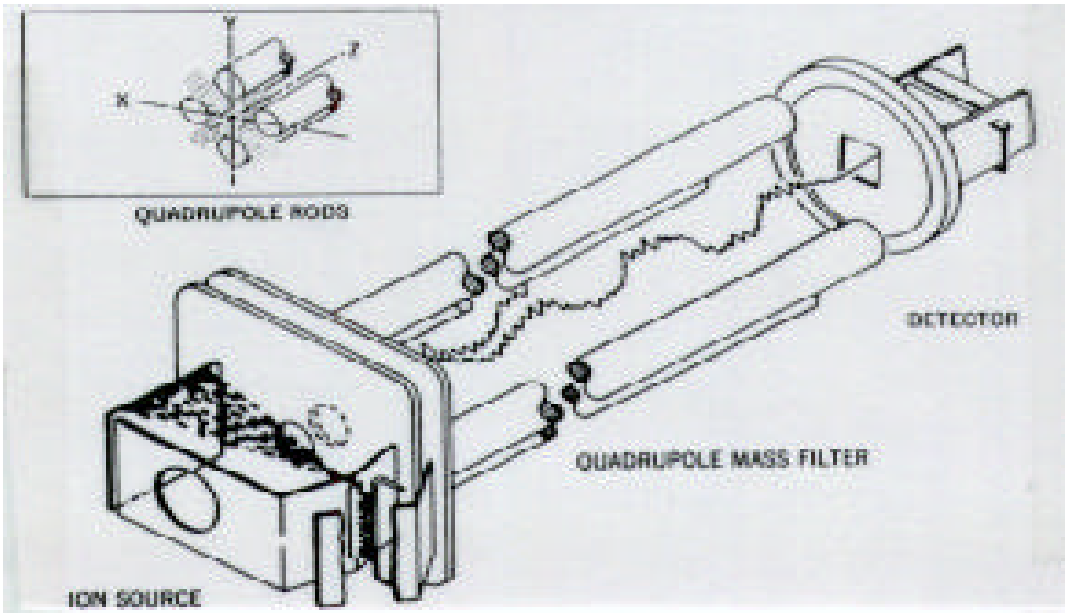


Generated ions are accelerated and are passed around a curved track (the sector) leading to a detector. By increasing the magnetic field applied to the ions, heavier ions with higher momentum can be induced to follow the curved track. A mass spectrum is obtained by applying a magnetic field gradient. Scanning is somewhat slower than in a quadrupole analyzer due to “magnetic reluctance”.

Quadrupole analyzer

Resolution 2 x 10^3 over mass range selected

Upper limit of mass range is m/z 2200-3000.



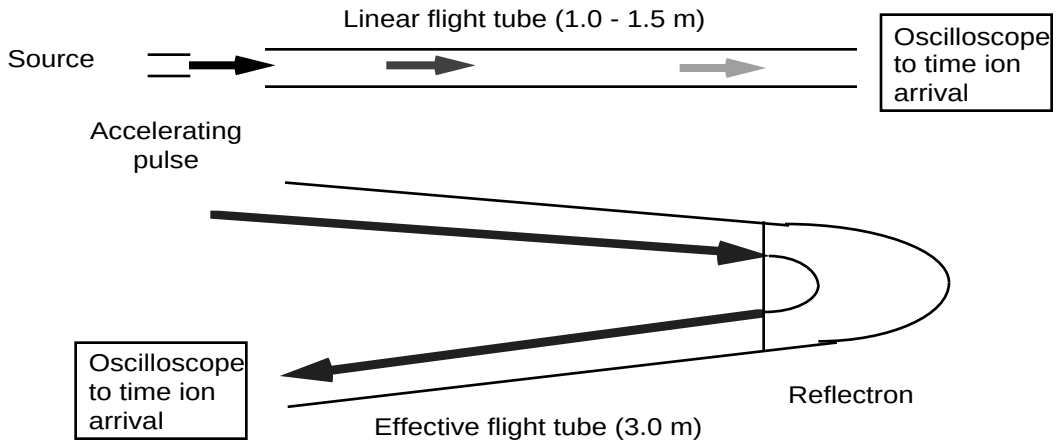
Generated ions are accelerated electrically (5-15V) and passed along the long central axis of four rods arranged symmetrically. By applying combined DC and oscillating RF potentials, the ions drift along irregular flight paths along the rod axis. The DC/RF ratio is held constant and the absolute values of DC and RF are varied. Only ions with a particular m/z value have stable trajectories for a given value of DC and RF. *A scan can be accomplished over a period of 10-1000 msec.* If DC is set to 0, then all ions have stable trajectories. Resolution can be enhanced but at the cost of lower sensitivity. The quadrupole analyzer is tolerant of relatively high pressure (10^{-4} torr).

Time-of-flight

Resolution 2.5 x 10^3, but now 2 x 10^4 with reflectron and statistical analysis, and higher with delayed extraction.

No upper limit of mass range.

Construction of a TOF instrument



- Generated ions are accelerated so that they have equal kinetic energy. They are allowed to "drift" down a 1 - 1.5 meter tube before striking a photomultiplier detector. The "time of flight" (t) depends on the mass of the ion (m), where

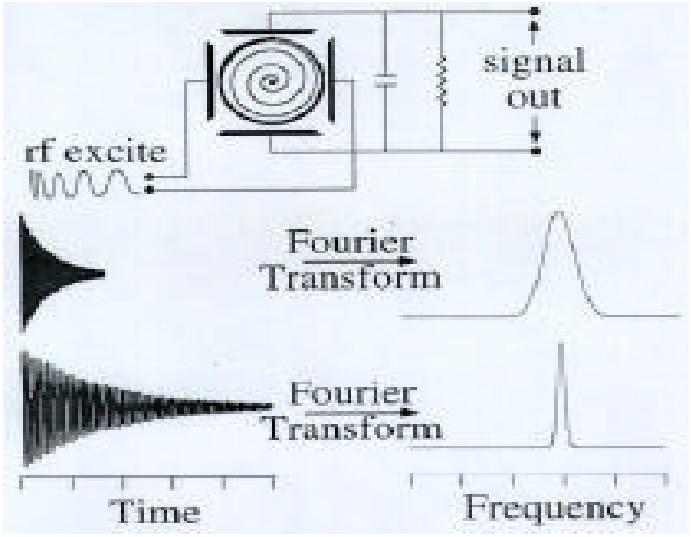
  $t = (m/2eV)^{1/2}.D$

  V is the applied potential and D is the flight tube distance. For a given instrument, the flight time varies as the square root of the mass of the ion.

- *Scan times are less than 1* μ*sec* - highly suited to on-line high speed capillary-based separations (see later).

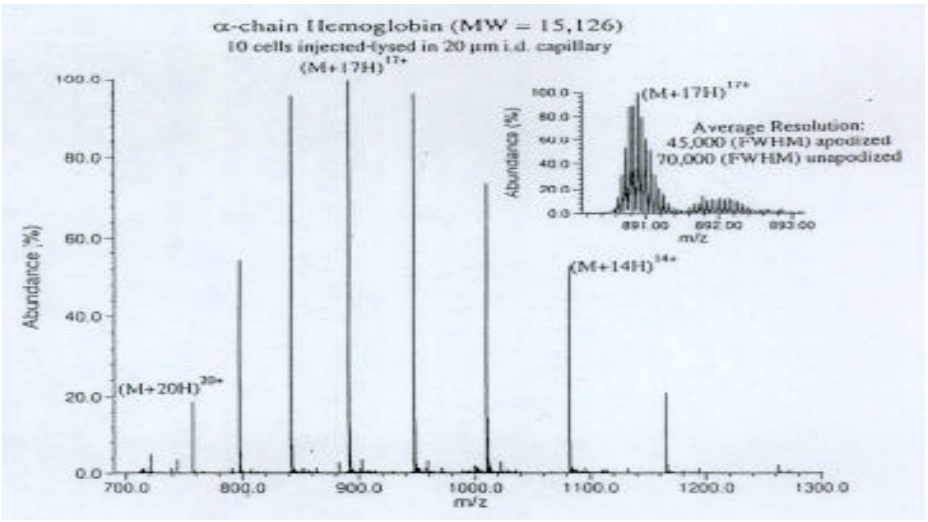
Ion traps

- The ion trap is an energy well - ions with sufficient energy to enter the trap are retained by an energy barrier on the exit side of the trap. The advantage of the ion trap is that it accumulates selected ions prior to their analysis giving it high initial sensitivity (detection limit of approx. 20 fmol). Ions are fragmented by collision with helium gas and their daughter ions analyzed within the trap. Selected daughter ions can undergo further fragmentation, thus allowing MS^n. This is important for structural experiments such as in peptide sequencing. The ion trap has a high efficiency of transfer of fragment ions to the next stage of fragmentation (unlike the triple quadrupole instrument).

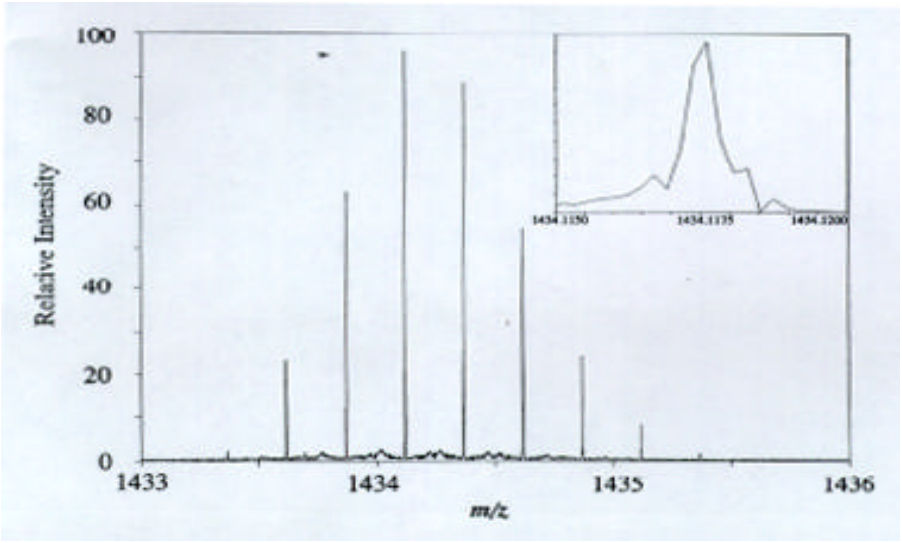


- By placing the ion trap within a superconducting magnet, the trapped ions undergo cyclotron gyration and are radially confined. The frequency of the cyclotron radiation is inversely proportional to the m/z ratio for an ion and directly proportional to the magnetic field. If an ion is excited at its natural cyclotron frequency, it moves to a higher energy level. A range of rf components are used to excite a sample. The ions clouds then induce an image current at two or more detection electrodes. The resulting signal when subjected to FT analysis yields an extremely precise measure of ion cyclotron frequencies, and hence m/z values, and molecular weights. The sensitivity is substantially enhanced and a 1 to 10^6 mass resolution can be achieved using a 9 tesla magnet.

Examples of FT-MS using an ion trap mass spectrometer

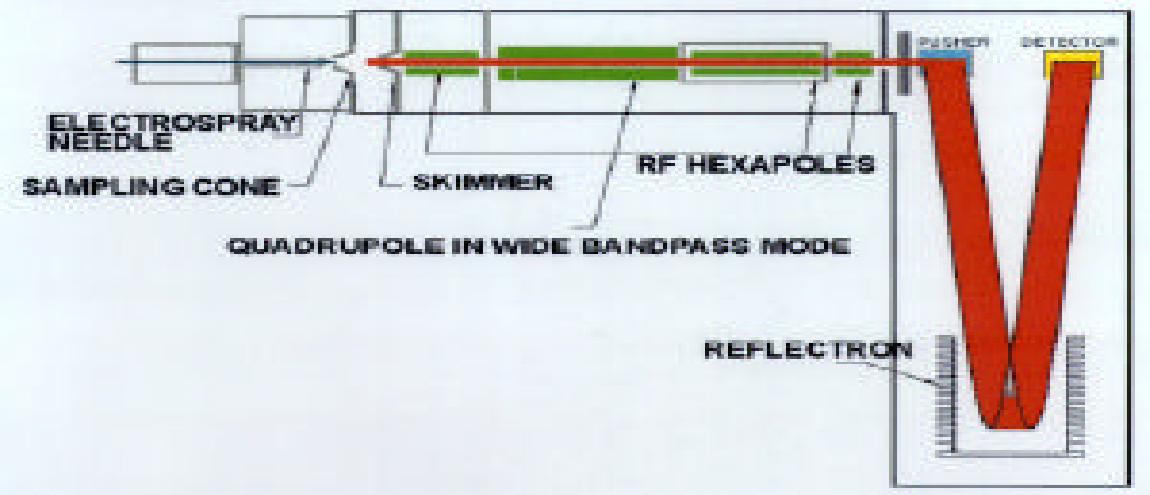


Mass spectrum of hemoglobin obtained by introducing 10 red blood cells into the capillary of a CE-MS instrument.



Mass spectrum of bovine insulin showing the separation of the naturally occurring isotopic forms. The mass resolution of the peak shown in the inset is > 2.5 x 10^6. Data were collected for 80 sec for this experiment.

Hybrid instruments



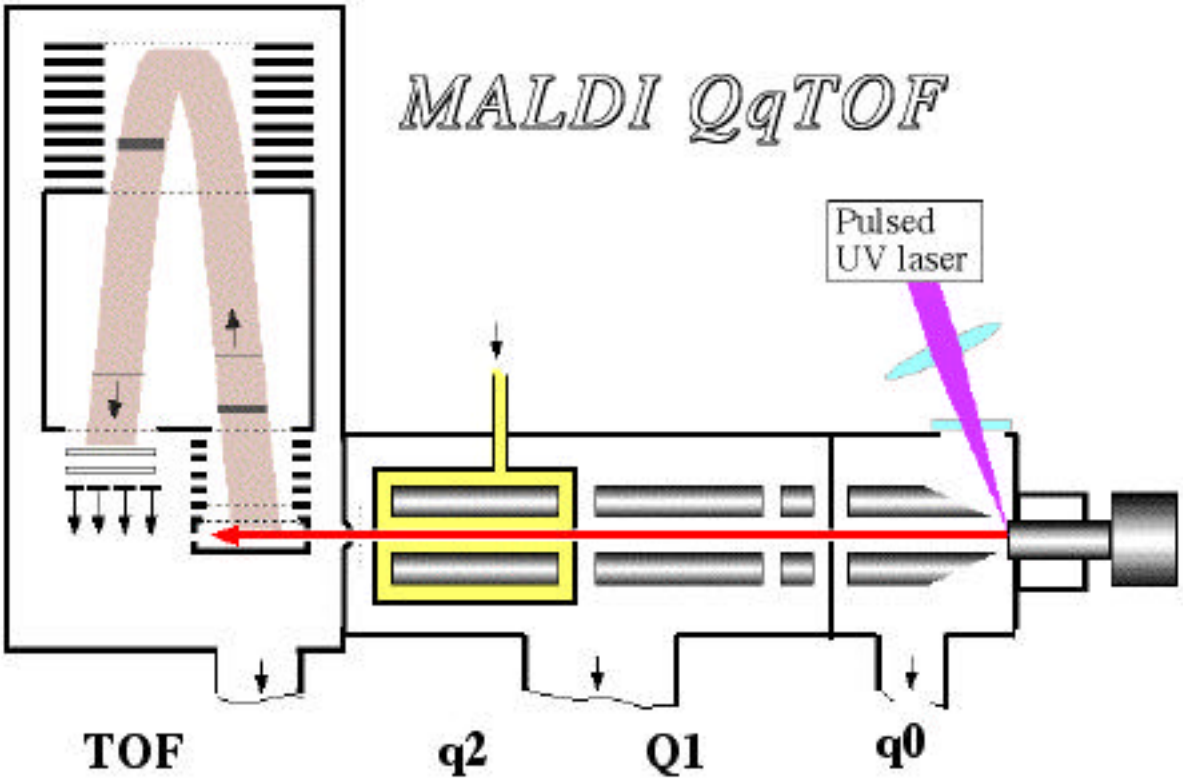
- A limitation of the magnetic sector and quadrupole analyzers is that only one ion is measured at a time. Thus while analyzing ions over a mass range of m/z 1-1000 at unit mass resolution, **at any one m/z value all the ions at other m/z values are ignored**. This results in discarding 99.9% of the available information. This limits the sensitivity of the conventional ESI-triple quadrupole analyzer for sequencing of peptides to 10-50 pmol.

- In contrast, the time-of-flight analyzer is far more efficient. All the ions created by the laser ionization pulse are measured - they are separated by their time of arrival at the detector. Since repetitive pulsing of the laser can be used to acquire data (the target stands still), sensitivities down to a few fmol are achievable.

- In 1995, a hybrid quadrupole/TOF analyzer was introduced (Q-tof). It has a similar construction to a triple quadrupole instrument. Ions selected in the first quadrupole are collided with N_2/Ar gas. The daughter ion fragments are distributed in the x-plane. By applying an accelerating voltage orthogonally, the ions travel in the z-direction and their m/z values can be determined by a TOF analyzer (this is analogous to two dimensional PAGE analysis). This allows for a much wider mass range (up to m/z 10,000) as well as the expected increased sensitivity.

- The speed of analysis (1 μsec) using the TOF analyzer is entirely suited to this approach, particularly when the width of the peak passing into the mass spectrometer is very narrow. This has lowered sensitivity for sequencing of peptides to the fmol range. When combined with nanospraying techniques, the sensitivity has been further lowered into the attomole range.

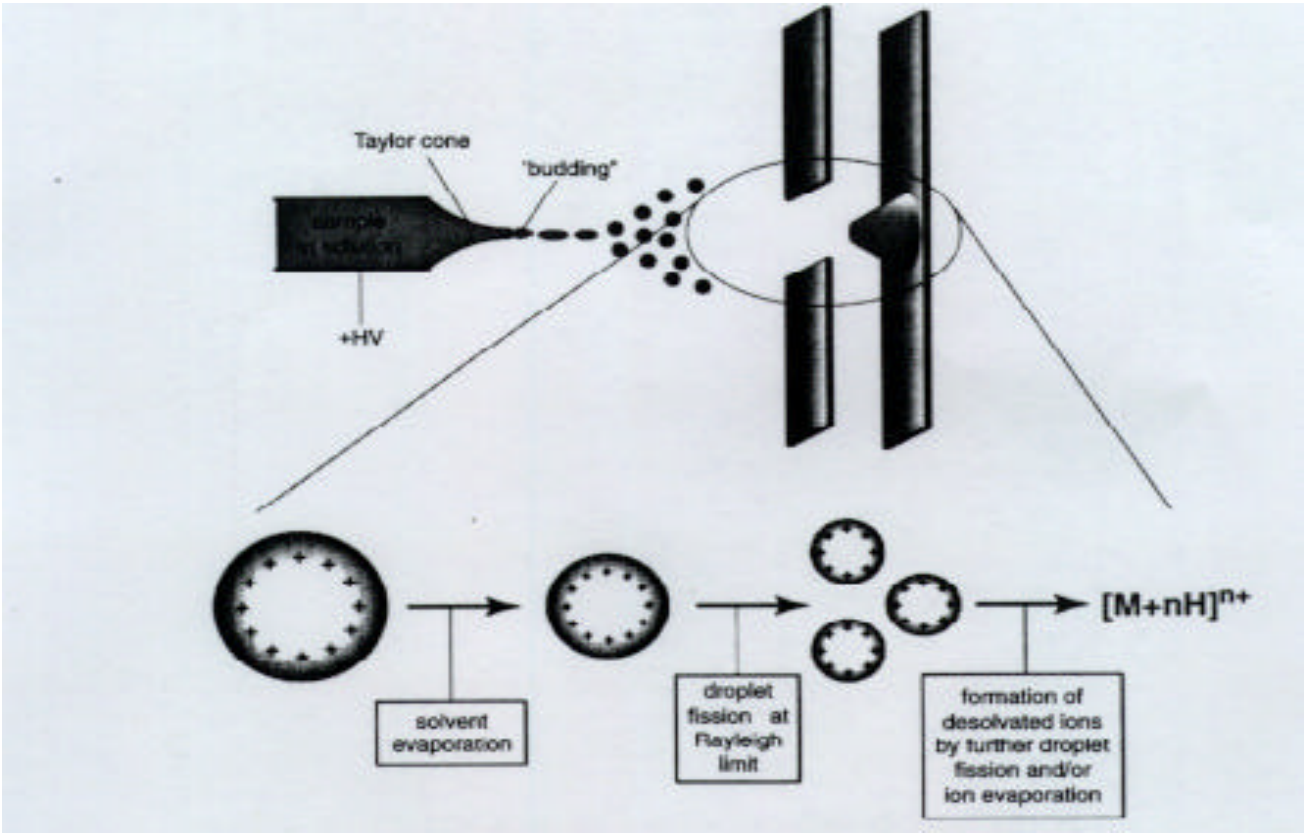
MALDI source and a Qtof hybrid
(Ken Standing, University of Manitoba)



It’s all about interfaces and ion sources

- Direct insertion probe - probe heated
- GC-MS (Ryhage, Biemann) volatile derivatives, thermal decomposition, not good for either peptides or proteins
- Field desorption - on carbon fibers
- Sputtering - glycerol matrix
    - fast atom bombardment (FAB)
    - laser desorption
- Spraying
    - Thermospray ionization
    - Heated nebulizer atmospheric pressure chemical ionization
    - Electrospray ionization

Electrospray ionization



- Peptides and proteins can be transferred from solution into the gas phase without degradation by forming a nebulized spray of droplets (a Taylor cone) which are subject to rapid evaporation by warm nitrogen “curtain” gas. Typically, the nebulizing solution contains 30% acetonitrile which lowers the surface tension (and decreases droplet size) and facilitates the evaporation. The solutes are ejected from the surface of the droplet probably by coulombic repulsion. This occurs at atmospheric pressure.

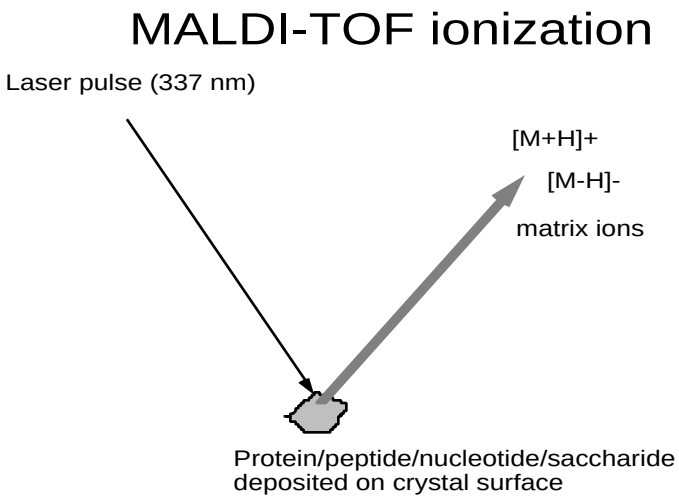
Electrospray ionization

- Positively charged ions are drawn into the mass spectrometer through a narrow orifice down a voltage gradient. Once in the mass spectrometer, they are accelerated - this serves to remove adduct molecules (solvent, Na^+, K^+, etc). Since the ions are not in a complete vacuum, it is possible to cause some fragmentation of the peptides due to collisions with the solvent molecules.

- Analysis of the masses of the ions occurs in a quadrupole analyzer. Parent peptide molecular ions can be selected in the first quadrupole and allowed to pass through into a second quadrupole where they collide with Argon-10% nitrogen gas. The resulting daughter ion fragments are analyzed in a third quadrupole, hence MS-MS.

- The flow rates that are suitable for ESI interfaces vary from 10 nl/min up to 100 µl/min. Samples can be introduced by flow injection (no chromatography) or following chromatographic separation.

MALDI-TOF mass spectrometry



- Sample mixed with a UV-absorbing matrix and is allowed to co-crystallize on the metal target.

  Peptides/proteins
  - 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid)
  - α-cyano-4-hydroxycinnamic acid (CHCA)
  - 2,5-dihydroxybenzoic acid (DHB)
  - 2-(4-hydroxyphenylazo)-benzoic acid (HABA)

  Oligonucleotides
  - 2-aminobenzoic acid
  - 3-hydroxypicolinic acid (3-HPA)
  - 2,4,6-trihydroxyacetophenone (THAP)

  The choice of matrix depends greatly on the solute to be analyzed.

- Focused, brief laser pulse (337 nm) strikes the surface of the target - the surface of the matrix is ablated, throwing the matrix and solutes in the surface layer into the gas phase. The ions formed are accelerated by an extraction pulse and allowed to “drift” to the detector.

- Infrared lasers now being introduced for TOF-MS directly from PVDF membranes or from glycerol solution

Peptide and protein analysis by electrospray ionization-MS

- In acid solution (0.1% formic acid), the peptide molecule is in the form $[M+H]^+$

  - i.e., $^+H_3NCHR_1CO(NHCHR_nCO)_nNHCHR_2COOH$

- If there are internal basic amino acid residues, then the ions will be of the form $[M+nH]^{n+}$, where n = 1, 2, etc.

- For tryptic peptides, the carboxy terminal amino acid is either arginine or lysine. Thus, for these peptides there are two positive charges. Therefore, two ions will be observed, $[M+H]^+$ and $[M+2H]^{2+}$. If the molecular weight of the peptide is 1000, the ions observed will be m/z 1001 ($[M+H]^+$) and 501 m/z ($[M+2H]^{2+}$).

- For synthetic peptides, higher charge states are possible if they contain more internal basic amino acids.

  - On the other hand, synthetic peptides without basic amino acids are studied with difficulty since for >20-mers the [M+H]+ ion will be out of the range from 1-2200 (the limit of the quadrupole detector). They are better analyzed by MALDI-TOF MS or by a ESI-TOF.

  - If they have acidic residues (aspartate/glutamate), negative ion spectra can be recorded, i.e, [M-H]-, $[M-2H]^{2-}$, etc. For a peptide with molecular weight 3003 and two interior acidic residues, the following ions will be observed:

    m/z 1500.5 $[M-2H]^{2-}$
    m/z 1000.0 $[M-3H]^{3-}$

- In the case of proteins, there are many basic amino acids and hence many charge states. For instance, for albumin the principal ions observed contain more than 50 +ve charges, giving a bell-shape distribution of m/z values centered around 1300.

Calculation of molecular weights and ion states

- For two ions in a series for a peptide of molecular weight M, the lower m/z value (x) will be for the n+1 ion state and the larger m/z value (y) will be for the n+ ion state.

  - (1) $(M+n)/n = y$
  - (2) $(M+n+1)/(n+1) = x$

  Hence

  - (3) $M+n = ny$ and $M = ny-n$
  - (4) $M+n+1 = (n+1)x$ and $M = (n+1)x-(n+1)$

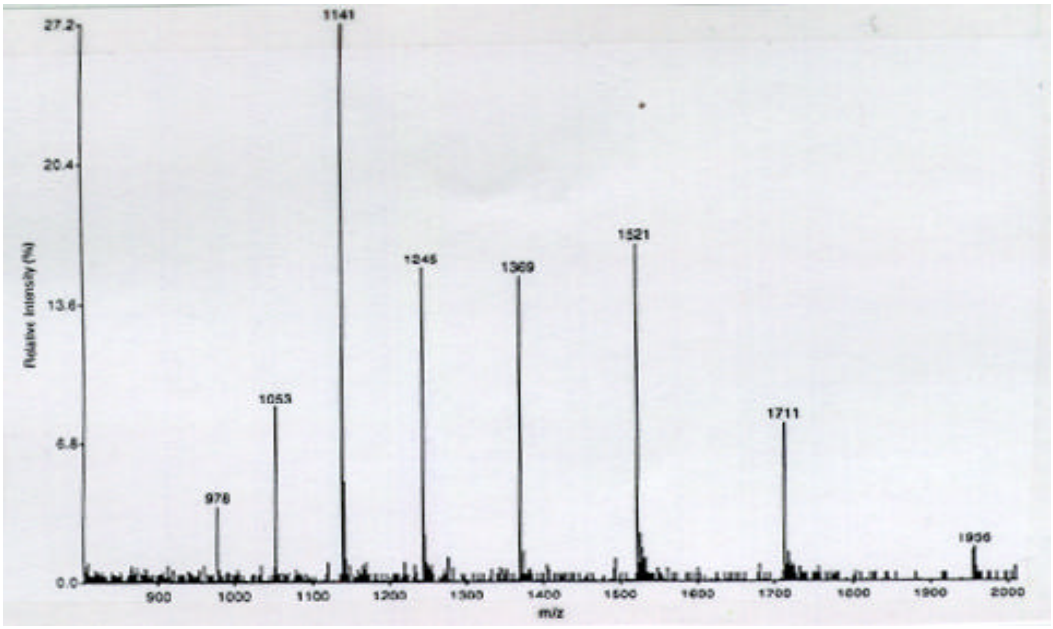
  Hence

  - $ny-n = (n+1)x - (n+1)$
  - $ny-n-xn+n = x-1$
  - $n(y-x) = x-1$
  - $n = (x-1)/(y-x)$

- The value of n can then be substituted in equation (1) to obtain the molecular weight of the peptide. For peptides giving rise to more than two molecular ions, adjacent pairs of ions in a series can be used to give several estimates of the molecular weight.

- Similar equations may be developed for negatively charged ions.

ESI mass spectrum of ribonuclease



Molecular weight calculations

Peak (m/z)	Intensity	Charge (est.)	Mol. Wt. (Est.)
978.00	7,778	14.00000	13,677.89
1,053.00	18,532	13.02656	13,675.90
1,141.00	59,087	11.95446	13,679.91
1,245.00	33,275	10.96146	13,683.91
1,369.00	32,390	10.03219	13,679.92
1,521.00	35,668	8.99995	13,679.93
1,711.00	16,624	7.99996	13,679.94
1,956.00	3,333	6.97955	13,684.94

Cumulative MW estimate = 13,680.29 SD = 2.94