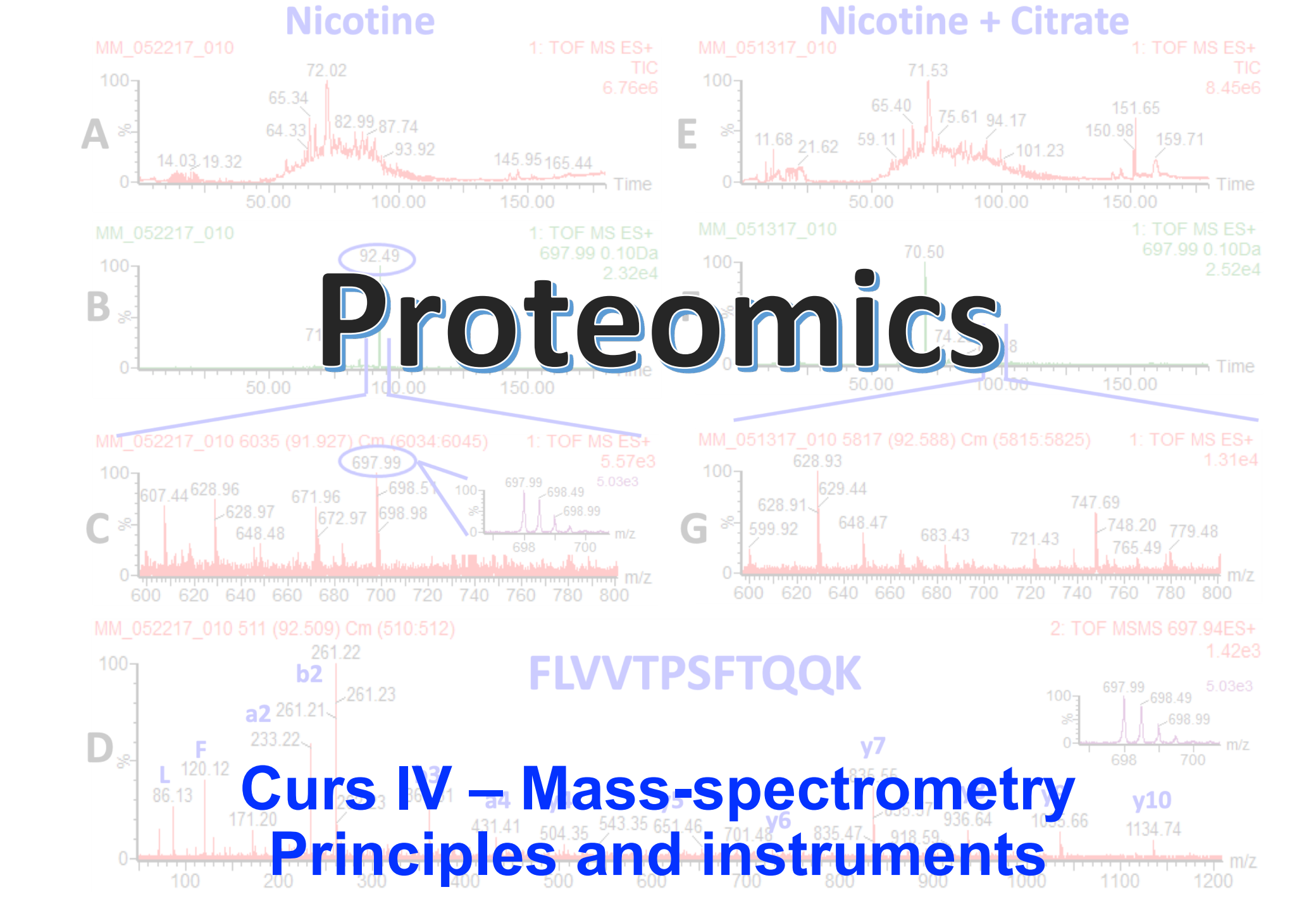
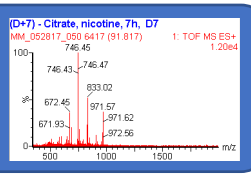


Proteomics

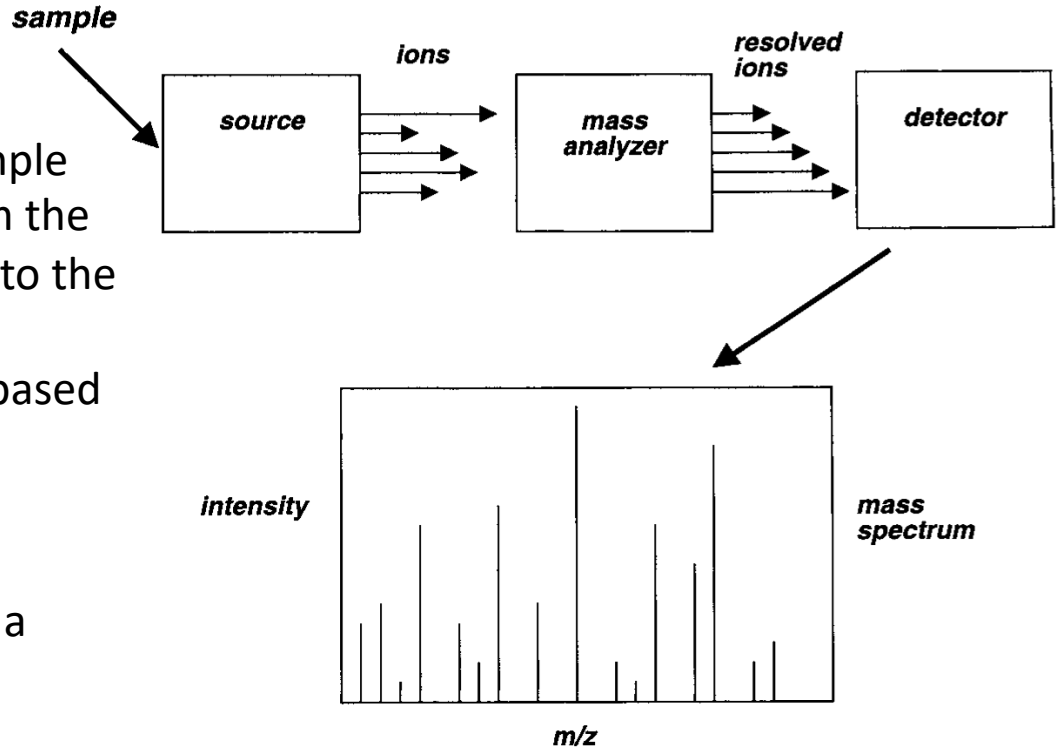
Curs IV – Mass-spectrometry
Principles and instruments

II. Mass-spectrometry



II. 1. Determining the mass of proteins/peptides – basic principles

Mass spectrometry is an analytical technique used to measure the mass-to-charge ratio (m/z) of electrically charged molecules. The m/z ratio can then be used to accurately identify the molecular weight (Mw) of neutral molecules. The result of a mass spectrometry analysis is a **mass spectrum – a graph with the m/z value on the X-axis in Da (Daltons) of the analyzed ions, and the intensity of the corresponding signal on the Y-axis**. The instrument that records the mass spectrum is called a mass spectrometer and is composed of three main components:

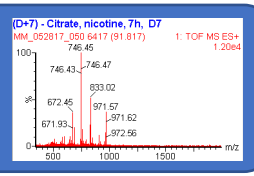


A. **Ion source** facilitates the transition of the sample into the gas phase, ionizes the molecules from the sample under analysis, and transfers the ions to the

B. **mass analyzer** - where the ions are separated based on m/z and are transferred to

C. a **detector** sensitive to the presence of ions separated by the analyzer, which sends signals to a computer that records the mass spectrum.

II. 1. Mass-spectrometry – basic principles

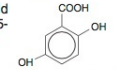
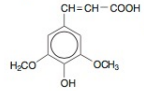
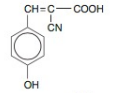
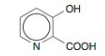
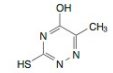
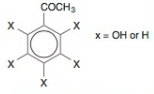
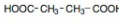
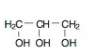


A. Ions Sources used in proteomics

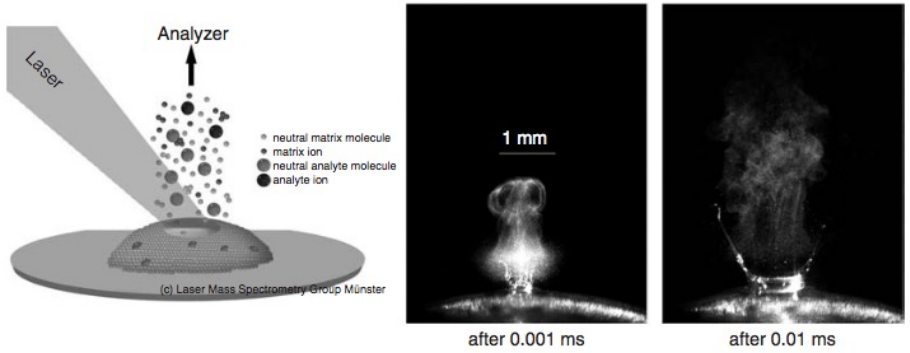
Neutral molecules can be converted into ions through various chemical or physical mechanisms. In order to be taken up by the mass analyzer, the ions generated in the source must be in the gas phase. In the case of biological molecules, ionization methods need to be highly efficient but should not destroy/fragment the analyte. For these reasons, two main types of ionization have predominated in proteomic studies:

1. Matrix-assisted laser desorption ionization - MALDI - The sample to be analyzed, placed in an acidic matrix, undergoes repeated laser pulses ranging from 50 to 200 times. These pulses typically occur in a vacuum and generate small explosions of the sample being analyzed, ionizing the peptides and proteins within the cloud formed after the explosion. **Since the environment is acidic, the ions formed are predominantly positive**. The matrix used in MALDI serves to generate protons, but also predominantly absorbs the energy of the laser pulses. The molecules in the matrix also ionize, but their molecular mass (100-200 Da) is significantly smaller than that of peptides or proteins (over 1000 Da), making them easily ignorable in the mass analyzer.

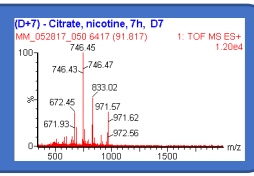
Commonly used compounds as matrices in MALDI

Matrix	Structure	Wavelength	Major applications
Nicotinic acid		UV 266 nm	Proteins, peptides, adduct formation
2,5-Dihydroxybenzoic acid (plus 10% 2-hydroxy-5-methoxybenzoic acid)		UV 337 nm, 353 nm	Proteins, peptides, carbohydrates, synthetic polymers
Sinapinic acid		UV 337 nm, 353 nm	Proteins, peptides
α-Cyano-4-hydroxycinnamic acid		UV 337 nm, 353 nm	Peptides, fragmentation
3-Hydroxy-picolinic acid		UV 337 nm, 353 nm	Best for nucleic acids
6-Aza-2-thiothymine		UV 337 nm, 353 nm	Proteins, peptides, non-covalent complexes; near-neutral pH
k,m,n-Di(tri)hydroxy-acetophenone		UV 337 nm, 353 nm	Protein, peptides, non-covalent complexes; near-neutral pH
Succinic acid		IR 2.94 µm, 2.79 µm	Proteins, peptides
Glycerol		IR 2.94 µm, 2.79 µm	Proteins, peptides, liquid matrix

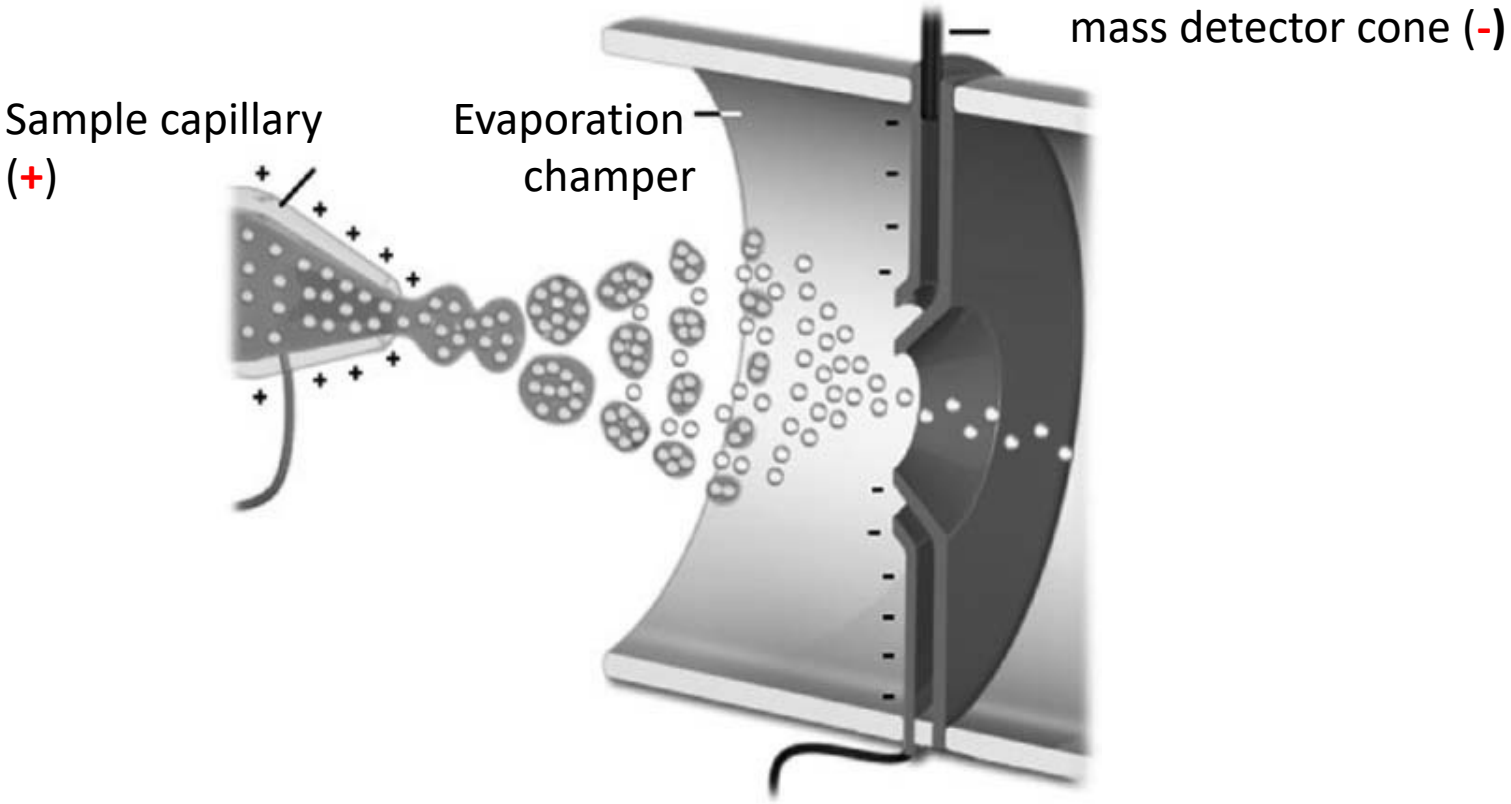
IR = infrared; UV = ultraviolet.



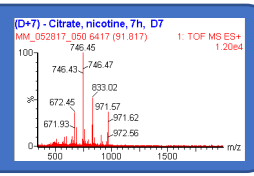
II. 1. Mass-spectrometry – basic principles



2. **Electrospray ionization - ESI -** In contrast to MALDI, where the sample to be analyzed needs to be solid, in ESI, it is in solution. The sample dissolved in a volatile and acidic solvent is finely sprayed through a capillary into an evaporation chamber connected to the mass analyzer cone. The solvent is gradually removed from the droplets formed in the evaporation chamber, causing the analyte to become gas. The evaporation of the acidic solvent generates an excess of protons, resulting in the ionization of the analyte. There is a significant voltage difference between the capillary spraying the sample and the mass analyzer cone, directing the ions towards the mass detector cone.



II. 1. Mass-spectrometry – basic principles



For peptides, their net charge is variable, as fallows:

1. In solution, the **degree of ionization of peptides depends on pH**:

- The side chains of dicarboxylic amino acids are un-ionized at pH values below 3.0 and ionized (negatively charged) at pH values above 7.0.
- The N-terminal end and di-aminic amino acids are ionized at pH values below 8.5.
- Depending on the pH of the environment, peptides can therefore be positively charged (pH < 3.5) or negatively charged (at basic pH values). Most often, peptide ionization occurs in an acidic environment, resulting in ions through the protonation of NH_2 residues and thus acquiring a positive charge.

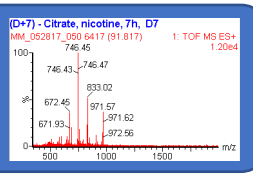
2. In ESI, peptides do not ionize uniformly. Any peptide obtained using trypsin during the hydrolysis phase will have at least 2 ionizable radicals: the N-terminal end and the Lys/Arg residue where hydrolysis occurred at the C-terminal end. Through ESI, the peptide will thus produce at least 3 types of molecules:

- peptide not ionized- cannot be picked up by the mass analyzer and therefore will not appear in the mass spectrum;
- **Peptide ionized at one end only** - has a positive charge, will appear in the mass spectrum with $m/z = (M_w+1A_H)/1 = M_w+1$ Da;
- **Peptide ionized at both ends** - has two positive charges, will appear in the mass spectrum with $m/z = (M_w+2A_H)/2$ Da.

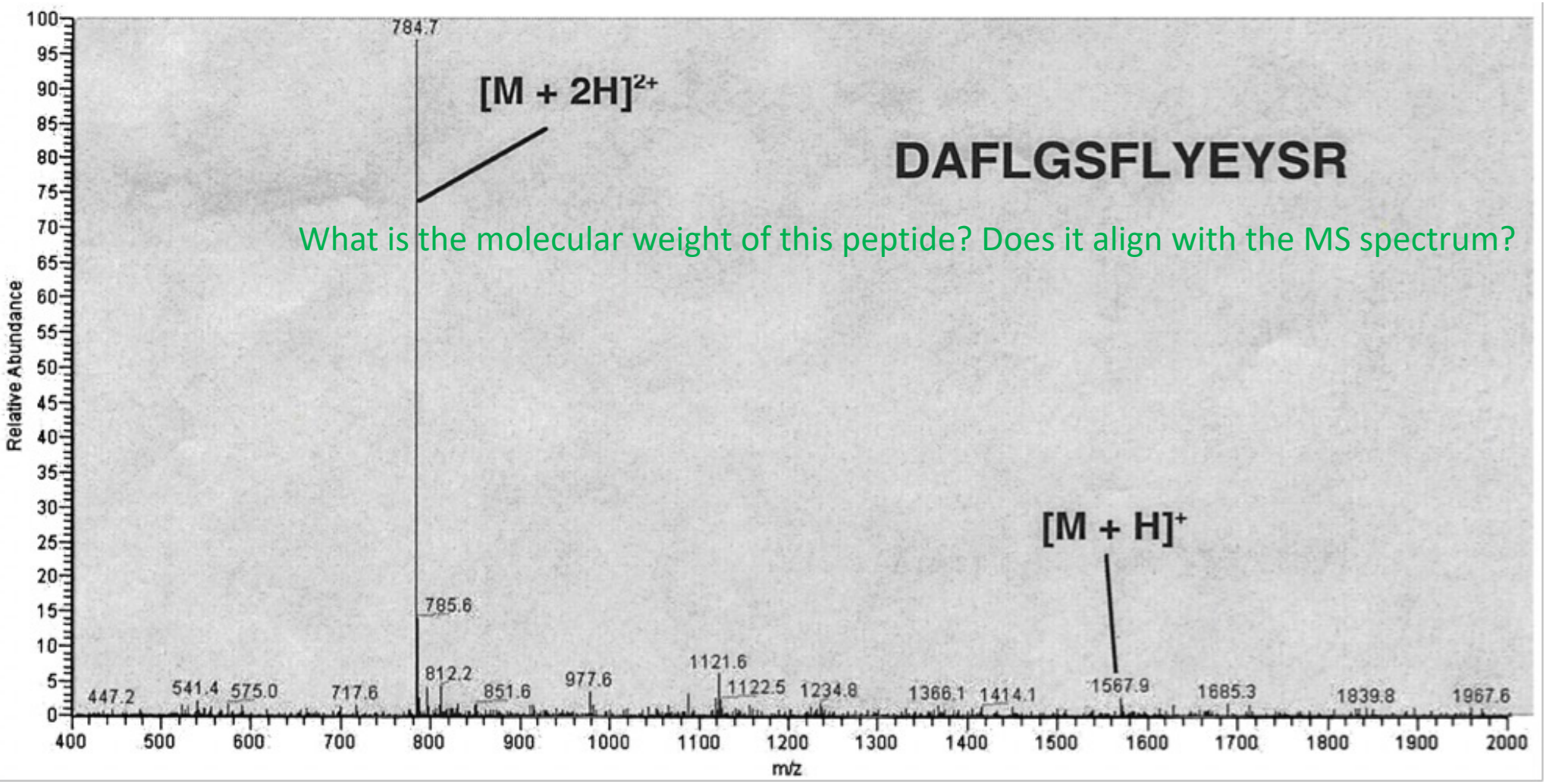
Native proteins can also be ionized through ESI too. Proteins, through the exposed side chains of the amino acids on their exterior, **can accept 10-30 protons**. Similar to a peptide, **a protein will generate a population of ions with m/z values ranging from (M+10)/10 to (M+30)/30**.

The distribution of ion populations originating from the same molecule but with different m/z values is called a multi-charge envelope. In the case of peptides, **the multi-charge envelope typically contains 2 signals in most cases**, or **3 signals if there are basic amino acids in the sequence**. **The multi-charge envelope of proteins contains numerous signals** and can be used for the automated identification of the molecular mass of a protein of interest through a signal deconvolution process.

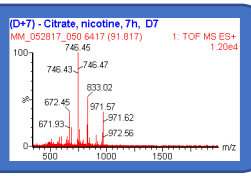
II. 1. Mass-spectrometry – basic principles

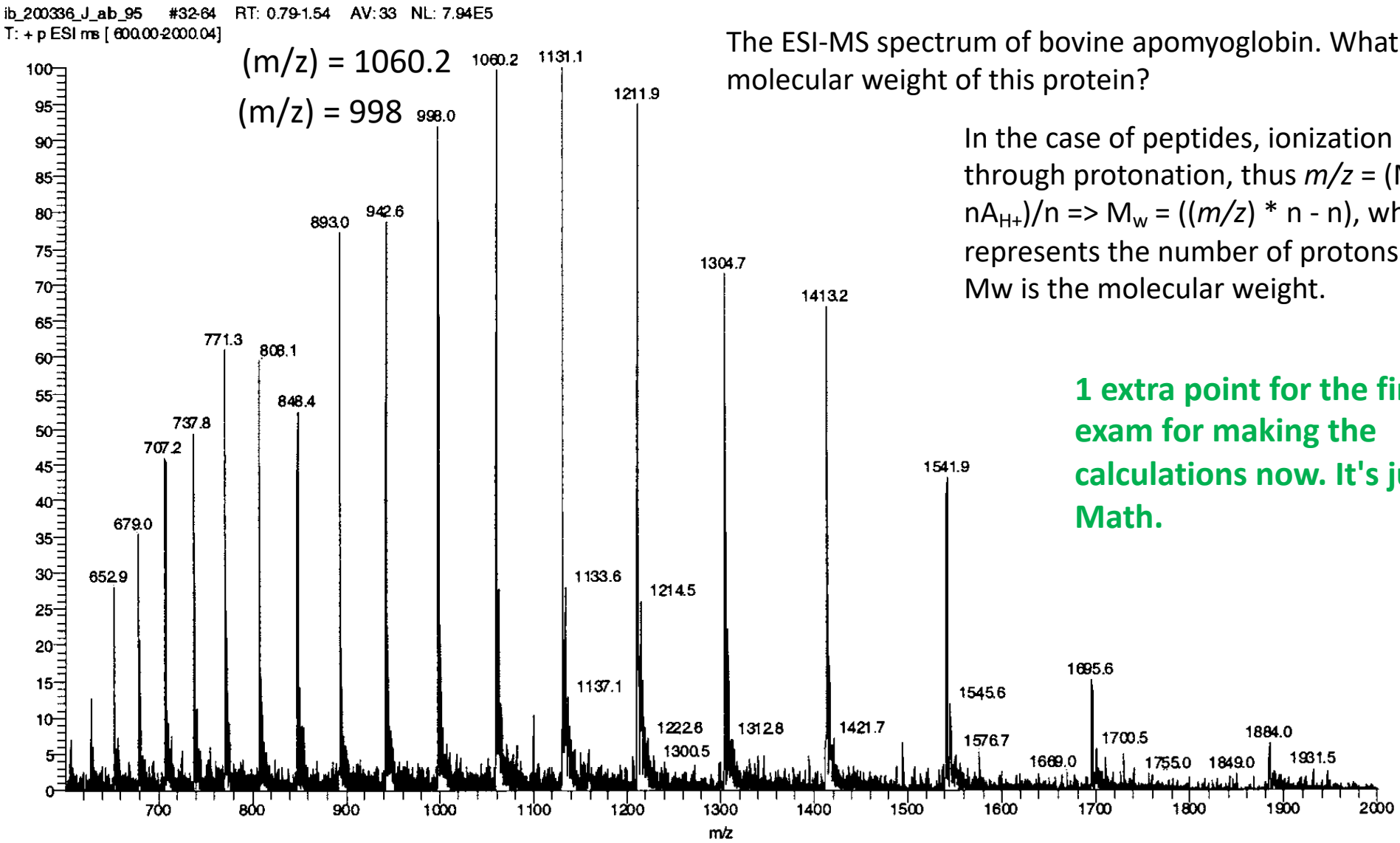


The ESI-MS spectrum of a peptide with the provided sequence is depicted. The two signals representing the two ionization states are emphasized.



II. 1. Mass-spectrometry – basic principles



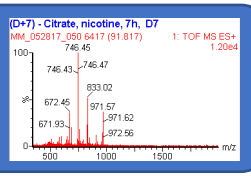


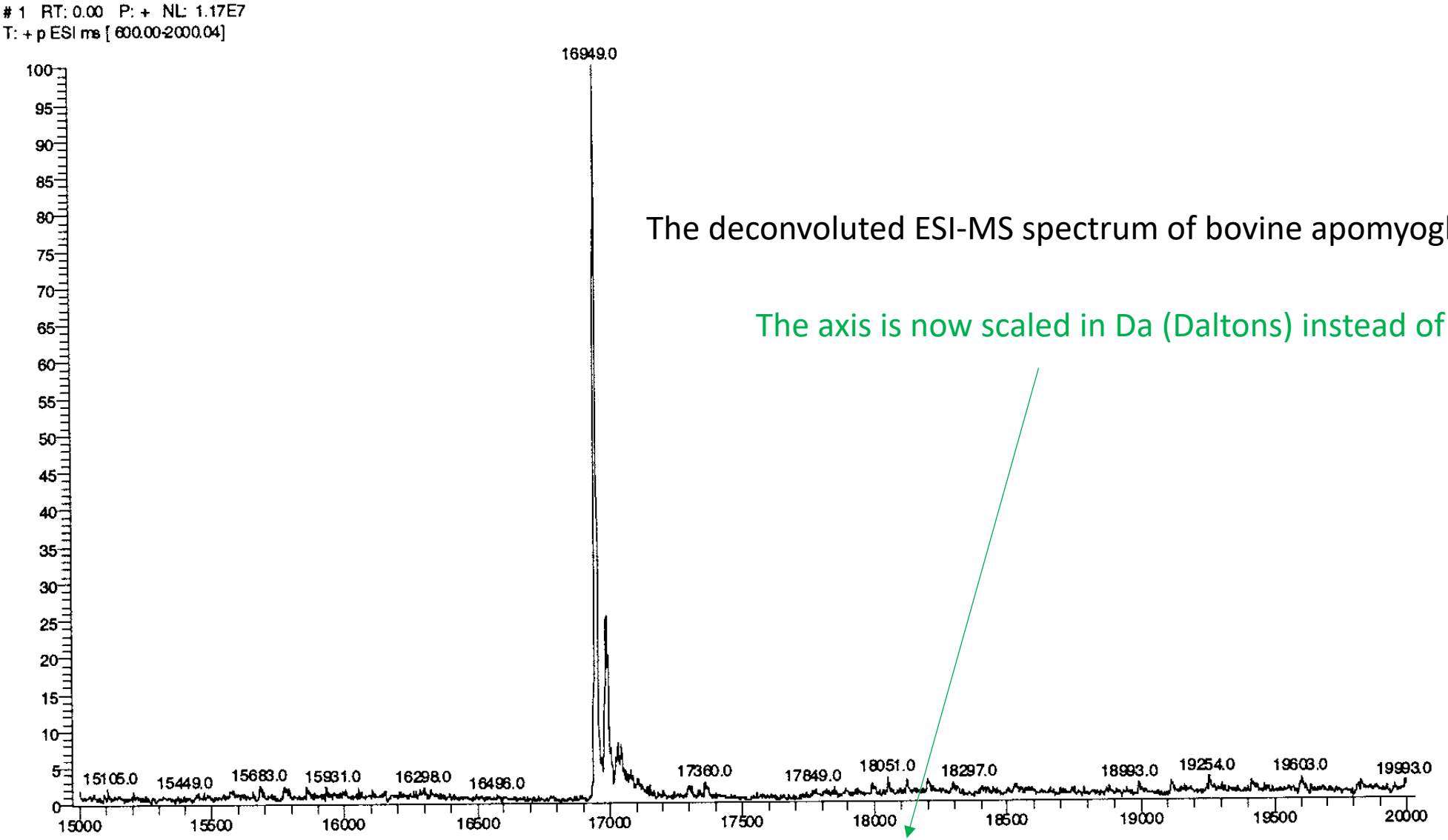
The ESI-MS spectrum of bovine apomyoglobin. What is the molecular weight of this protein?

In the case of peptides, ionization occurs through protonation, thus $m/z = (M_w + nA_{H+})/n \Rightarrow M_w = ((m/z) * n - n)$, where n represents the number of protons and Mw is the molecular weight.

1 extra point for the final exam for making the calculations now. It's just Math.

II. 1. Mass-spectrometry – basic principles

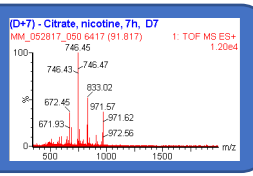




The deconvoluted ESI-MS spectrum of bovine apomyoglobin

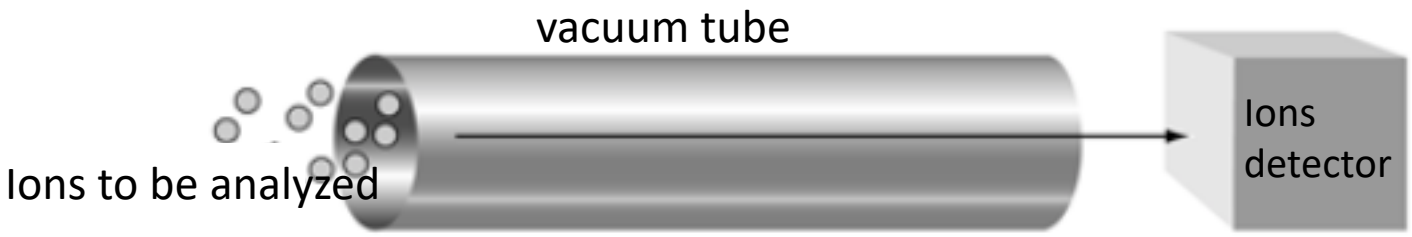
The axis is now scaled in Da (Daltons) instead of m/z.

II. 1. Mass-spectrometry – basic principles



B. Mass-analyzers used in proteomics

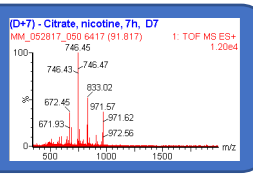
1. The TOF (Time of Flight) mass analyzer operates by **transferring ions generated by the source into a vacuum tube and accelerating them through the tube using a constant electric field. The velocity of the ions is dependent on their charge - the higher the charge, the stronger the acceleration, and thus the higher the velocity.** For ions with equal charges, the velocity depends solely on the mass of the ions - heavier ions will have a slower velocity, while lighter ones will have a faster velocity. **The mass analyzer separates ions based on the time it takes for them to traverse a predetermined flight path length within the vacuum tube of the TOF detector.**

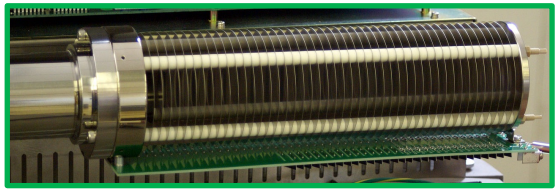


Bendix MA-2 Time-of-Flight Mass Spectrometer, 1960s

An TOF mass analyzer operating based on the principles outlined above is said to function in **linear mode**. Regardless of the ion source, all TOF detectors operating in this mode have low resolution due to the spatial distribution of ions with the same *m/z* (the distribution within the cloud formed by laser pulses in MALDI of ions with the same charge). Since ions with the same *m/z* do not depart simultaneously from the same spatial location, the distance they travel to the detector differs. Consequently, the flight time recorded by the detector for ions with the same m/z will vary and overlap with ions that have very close *m/z* values, leading to **low resolution**.

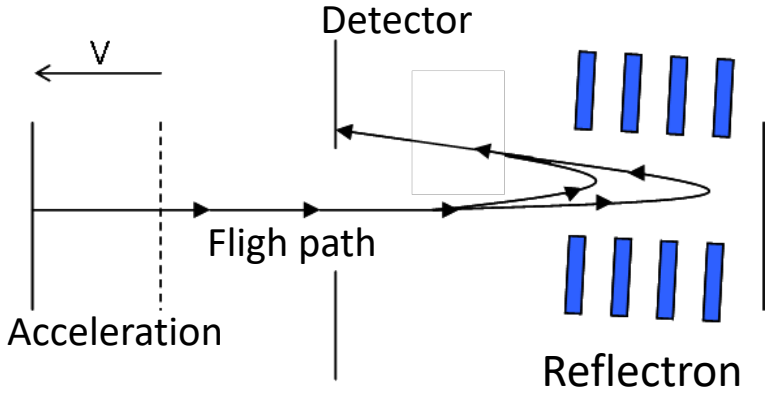
II. 1. Spectrometria de masă – principii generale





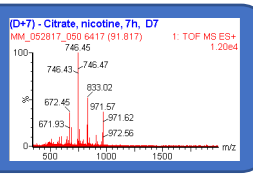
The separation resolution of TOF mass analyzers has been improved by the emergence of two technical innovations:

2. Introduction of a **reflectron** - a device that generates an electrostatic field acting as an ion mirror, reflecting the ion flux back into the the flight tube, but on a slightly different trajectory. High-speed ions will penetrate deeper into the electrostatic field of the reflectron, thus experiencing stronger deceleration and being reflected later. Slower ions will not have enough kinetic energy to penetrate deeply into the reflectron's field and will be reflected towards the detector sooner. In this manner, on one hand, the flight distance is doubled for the same length of the TOF tube, and on the other hand, ions of the same m/z are concentrated inside the reflectron. As a result, the differences in flight time between ions with very close m/z values originating from different spatial points are greatly amplified. They will arrive at the detector at significantly different time intervals, thus improving the instrument's resolution significantly. Presently, TOF mass analyzers have a resolution of 0.001 amu - separating two ions that differ from each other by 0.001 Da.

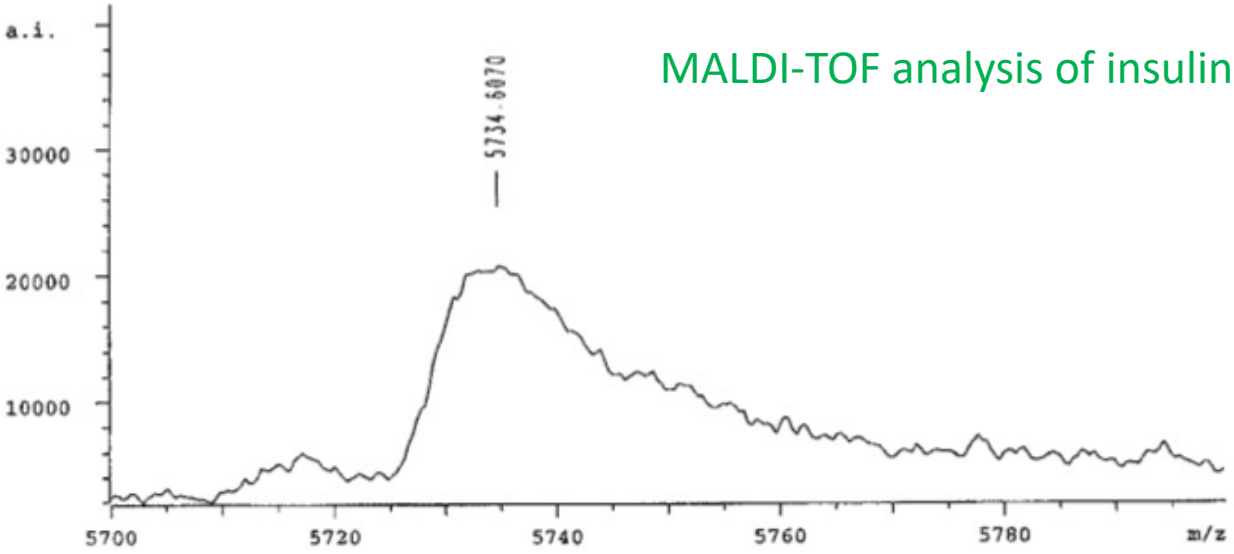


2. Modification of the MALDI ion source by introducing a delay between the laser pulse that generates the matrix-sample explosion and the actual introduction of ions into the mass detector - known as **delayed extraction MALDI**. This delay allows for a uniform distribution of ions at the beginning of the flight tube.

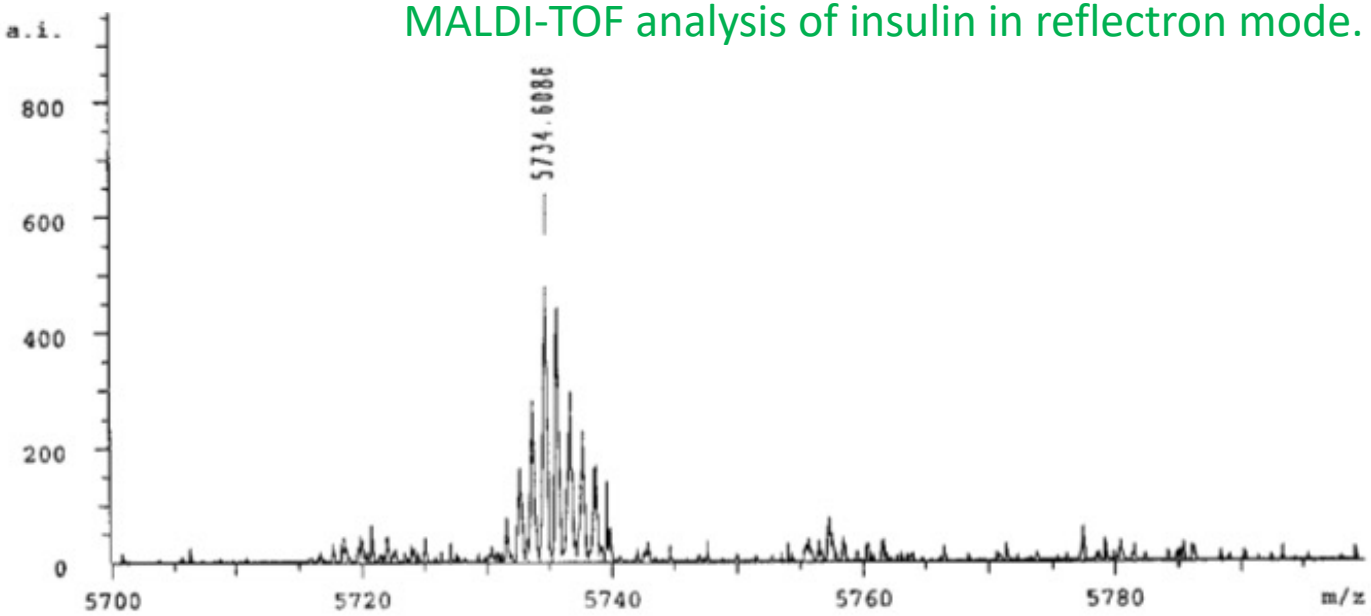
II. 1. Spectrometria de masă – principii generale



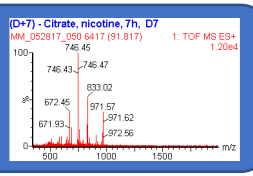
MALDI-TOF analysis of insulin in linear mode.



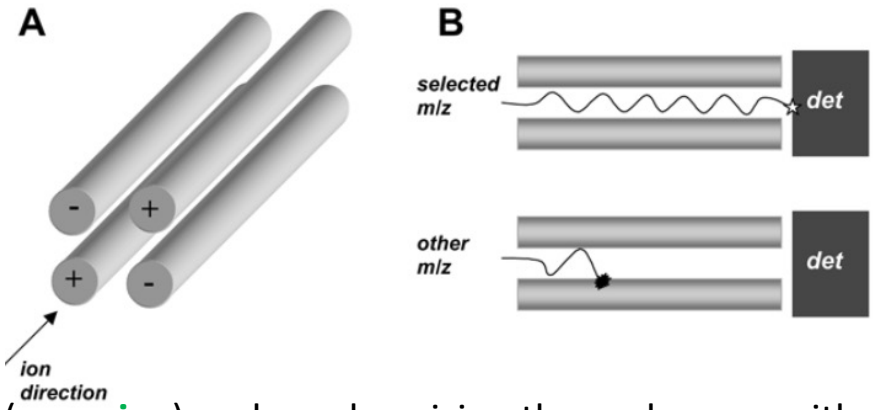
MALDI-TOF analysis of insulin in reflectron mode.



II. 1. Spectrometria de masă – principii generale



2. **Quadrupole mass analyzer** - **consists of 4 parallel metal rods forming 4 electrodes to which controlled, periodically varying voltages are applied at a certain frequency.** This generates a magnetic field that forces the ions to move between the 4 rods in a spiral trajectory towards the opposite end from where they entered the quadrupole. By tightly controlling the applied voltage and the frequencies at which it changes, only ions of a specific m/z will move towards the exit of the quadrupole where the detector is located. By periodically modulating the applied voltage (**scanning**) and synchronizing these changes with the information from the detector, the presence of ions with different m/z values can be identified.

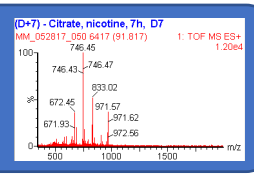


In proteomics, the standalone quadrupole analyzer is less commonly used. Instead, three such analyzers are often grouped and tandem operated to form a mass spectrometer called a **triple quad**. In a triple quad, **the three detectors have different functions**:

- Two of the quadrupole detectors, denoted **Q1** and **Q3**, operate according to the principles described above, separating ions based on *m/z* – **these are working as true mass analyzers**.
- A quadrupole analyzer denoted **q2** functions as a chamber where ions, regardless of *m/z*, are retained for a period of time and can be released simultaneously by changing the applied voltage. Often, this quadrupole is referred to as a **collision cell** because here ions are bombarded with a neutral gas such as N2, He, or Ar. The collision between gas molecules and ion molecules leads to the fragmentation of ions through a process called **collision-induced dissociation** (**CID**).

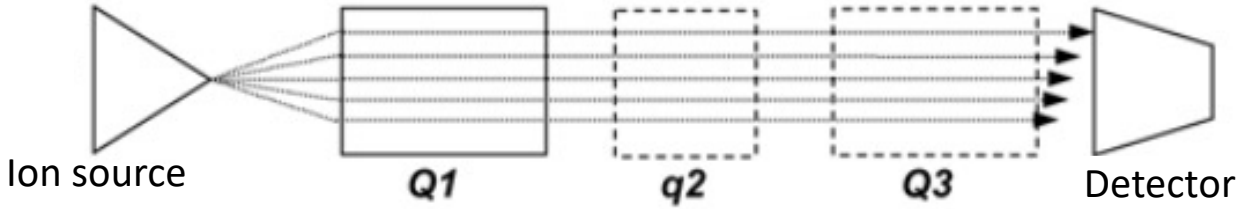
In a triple quad, the three quadrupole detectors are arranged one after the other, with ions passing through them in the order Q1, q2, Q3.

II. 1. Spectrometria de masă – principii generale

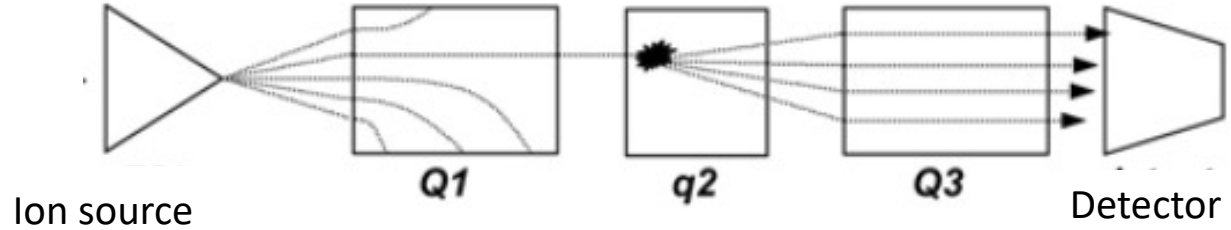


A **triple quad** detector can operate in two modes:

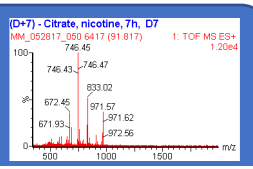
A. Full scan mode (MS^1) - ions originating from the source are separated in Q1, which operates in this case as a true mass analyzer. The separated ions pass without interacting with q2 and Q3 to reach the detector. This mode is generally implemented for 1 second, during which the mass spectrometer identifies all ions entering Q1. **Applied for peptides - all peptides delivered to the mass spectrometer for 1 second by a nanoHPLC system via an ESI interface.**



B. Tandem mode or **MS-MS**, sometimes also denoted as **MS/MS** or **MS^2** - all three quadrupoles operate in synchrony. In this case, Q1 acts as a mass filter, allowing only ions with a specific m/z to pass into q2. **The ions that reach the collision cell are called precursor ions and are fragmented, and the resulting fragments are analyzed based on m/z in Q3.** In this mode, Q3 acts as a mass analyzer, but it does not separate the ions from the sample, but rather the fragments resulting from their dissociation. The *m/z* values of the generated fragments (daughetr ions), together with the *m/z* of the precursor ion, are then used to determine its nature/structure. **This is the operating mode that allows for the fragmentation and sequencing of peptides. In proteomics, a triple quad is alternately used to perform a full scan and identify peptides from the sample, and then in MS/MS mode to determine their sequence from the generated fragments.**

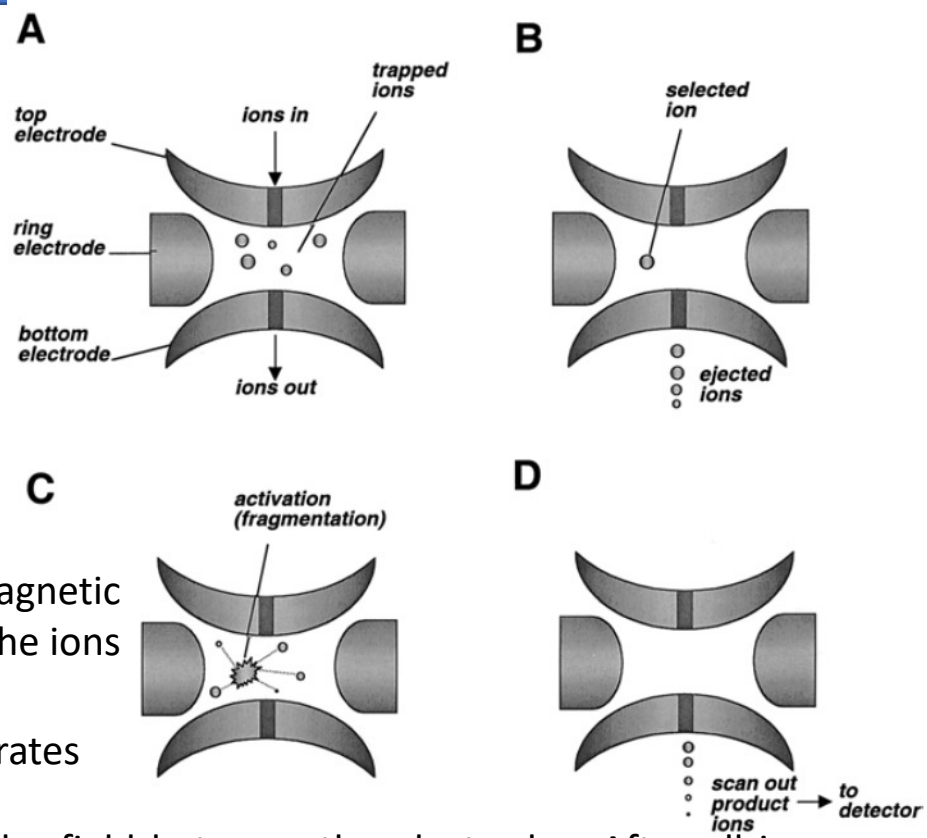


II. 1. Spectrometria de masă – principii generale



3. Ion trap mass analyzer - this type of mass analyzer does not separate ions as they are delivered by the source, as TOF and quadrupole analyzers do. Ions accumulate inside a chamber delimited by 3 electrodes:

- an **top electrode** forming the ceiling of the chamber, equipped with a hole communicating with the source. Through this hole, ions are introduced into the chamber;
- a **bottom electrode** forming the floor of the chamber. This one is also equipped with a hole, but it communicates with the detector;
- a ring electrode corresponding to the walls of the chamber.



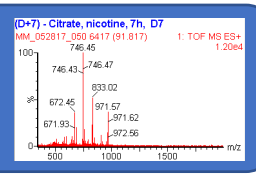
By applying controlled, periodically varying voltage currents, a magnetic field is generated in the space between electrodes, which keeps the ions constantly moving within it - a "trap" for ions.

Similar to a triple quad system, an ion trap detector also operates **alternatively in two modes**:

A. **Full scan mode** (A in the figure) - ions are introduced into the field between the electrodes. After all ions are trapped, by controlled modification of the frequency at which the field-generating currents are applied (scanning), ions will orderly exit the trap based on their m/z.

B. **Tandem mode** (A, B, C, D in the figure) - a new set of ions is introduced into the trap (A). By modifying the magnetic field, ions not of interest for analysis are ejected from the trap, and a single ion for analysis is selected (B). The selected precursor ion is fragmented by CID (C), and through a new modification of the magnetic field, the resulting fragments will orderly exit the trap based on their *m/z* (D).

II. 1. Spectrometria de masă – principii generale

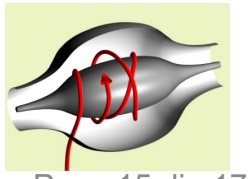


Compared to a triple quad, the ion trap mass analyzer has the advantage that cycling between the two modes as well as fragmentation occurs in the same physical space (inside the trap, ions are not transferred between Q1, q2, and Q3 as in the case of the triple quad). This allows one of the ions resulting from the precursor ion fragmentation to be retained and fragmented again, i.e., performing an **MS/MS/MS** or **MS^3** analysis. In principle, this tandem analysis could be repeated indefinitely - **MS^n** analyses. However, in reality, this approach is rarely used in proteomics because:

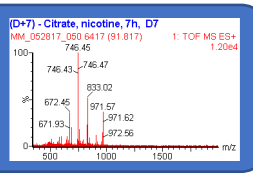
- For peptides, it is currently not possible to anticipate which fragments will result from MS/MS fragmentation, and consequently, it is not possible to select before the actual analysis which fragment will be retained in the trap for further fragmentation and analysis.
- The total number of ions available for analysis decreases with the increase in MS cycles. After an MS/MS of a peptide, the quantity of ions produced is generally not sufficient for a new fragmentation to generate ions detectable by the detector.

The presented mass analyzers can be combined or modified to obtain tandem mass analyzers such as:

- **Q-TOF** mass analyzer - functionally identical to a triple quad, but Q3 is replaced by a TOF analyzer. Full scan (MS1) is thus performed by Q1, fragmentation occurs in q2, but the analysis of the resulting fragments is done in TOF, which has the advantage of significantly better resolution than a quadrupole analyzer.
- **FT-ICR** mass analyzer - Fourier transform ion cyclotron resonance MS - similar in principles to an ion trap, but ions are not evacuated to a detector; instead, their presence is identified based on the modifications they induce in a high-intensity magnetic field. It provides very high resolution but also incurs very high acquisition and operating costs.
- **Orbitrap** analyzer - can be considered a separate type of FT-ICR with considerably lower costs.



II. 1. Spectrometria de masă – principii generale

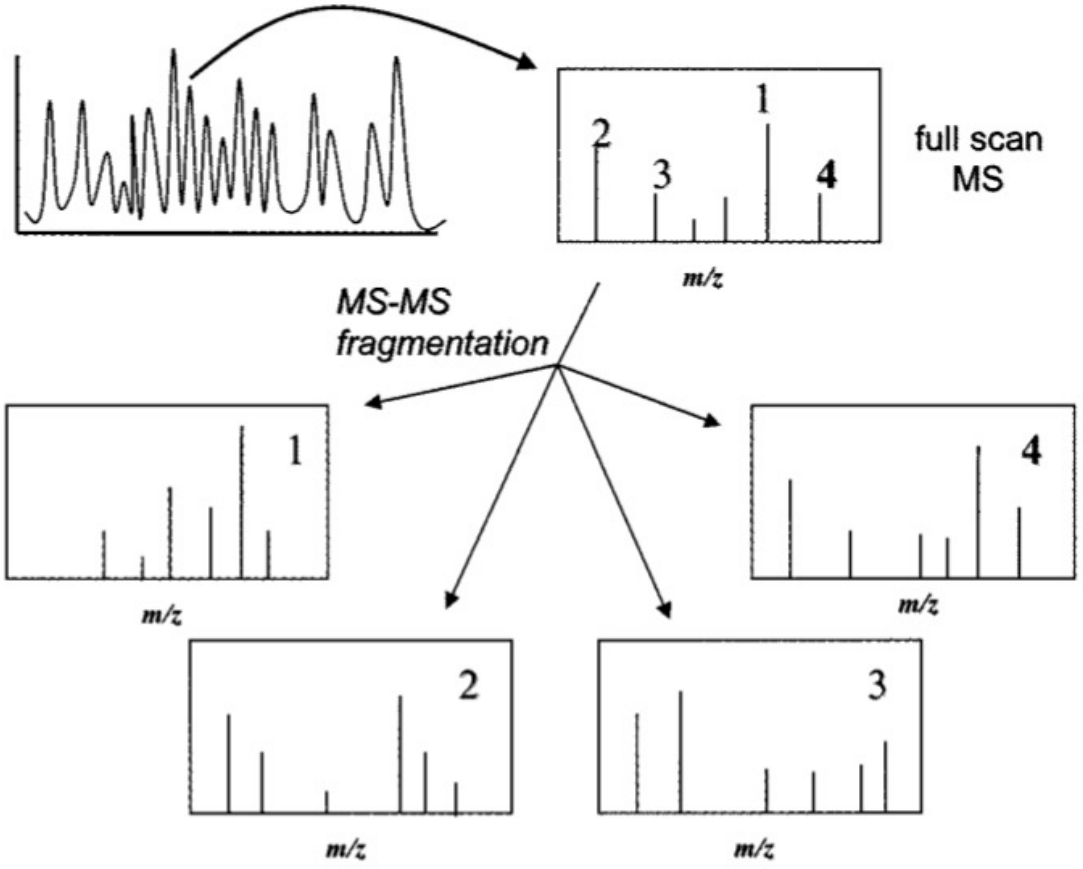


Regardless of their type, mass analyzers capable of performing **tandem MS** analyses can be extremely useful for high-throughput mass analysis of a large number of peptides because they can be programmed to automatically switch between measurement modes as follows:

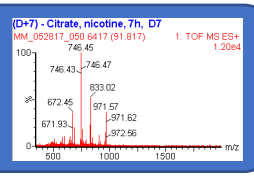
A. The instrument operates in full scan mode and identifies the peptides present in the sample provided by the source.

B. The most abundant peptides are selected one by one and fragmented by CID; MS/MS spectra are recorded for each individual resulted peptide (doughter ion).

C. The instrument returns to full scan mode, and the cycle repeats.



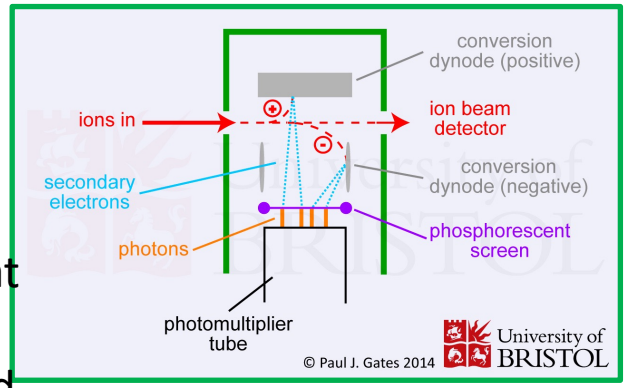
II. 1. Spectrometria de masă – principii generale



C. Ion detectors used in proteomics

In proteomics, various types of ion detectors are used for mass analysis, each with its advantages and limitations. Some of the most common ion detectors used in proteomics include:

- **Electron Multiplier Detectors** (EMD) - These are sensitive and versatile detectors used in a wide range of mass analysis instruments. EMDs work by converting ions into electrons, which are then multiplied in a detector tube, generating a measurable signal proportional to the number of initial ionizations.
- **Photodiode detectors** - These are particularly used in Time-of-Flight Mass Spectrometry (TOF-MS). Photodiode detectors detect light pulses generated by ions entering the detector and generate electrical signals proportional to the intensity of the light.



- **Microchannel Plate** (MCP) detectors - These are sensitive detectors that use a set of microscopic channels to amplify signals from ions. MCP detectors are especially used in tandem mass spectrometry (MS/MS) and offer high sensitivity and good resolution.
- **Channeltron detectors** - These are particularly used in Channeltron Ion Dissociation Mass Spectrometry (CID-MS). CID detectors use a magnetic field to measure the masses of ions and are sensitive to changes generated by ions passing through the detector.

These are just a few examples of ion detectors used in proteomics, and each type of detector has its own characteristics and specific applications in the mass analysis of proteins and peptides.