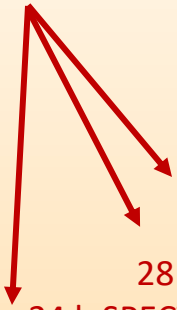
# ADVANCED BIOLOGICAL METHODOLOGIES
# 80 hours



28 h PROTEOMICS (Prof. B. Manconi)

28 h STRUCTURAL BIOLOGY (Prof. D. Piano)

24 h SPECTROSCOPY (Prof. F. Cesare Marincola)

AIMS

At the end of the module, the students will have: an indispensable knowledge to understand and apply the basic notions of mass spectrometry and proteomic techniques in biochemistry and biomedical research.

## PREREQUISITES:

The knowledge of organic chemistry, physics, and biochemistry is ESSENTIAL!

For this module, knowledge of applied biochemistry, specifically chromatography and electrophoresis, is also important.

Information on the ABM course is available on this link

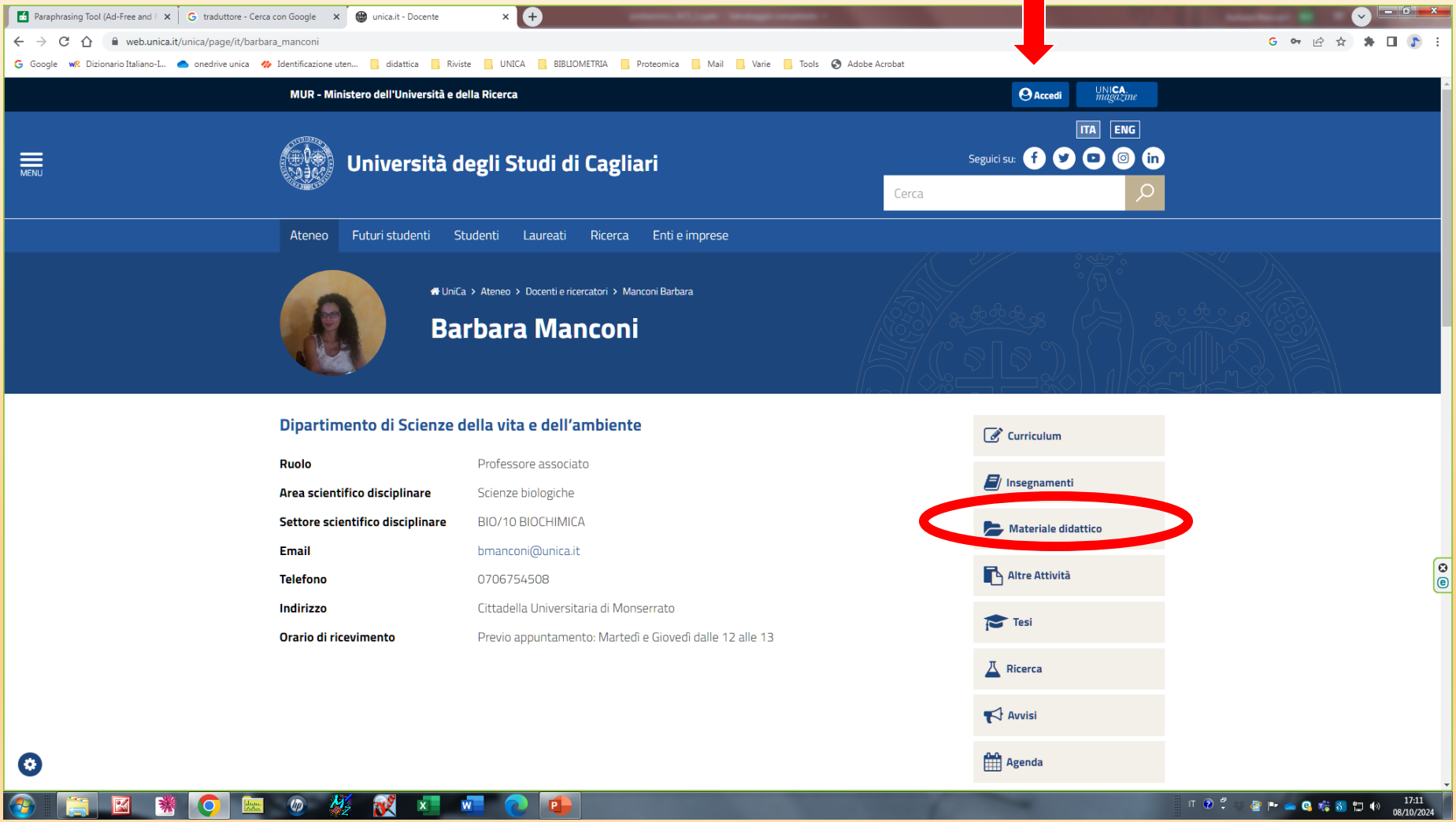
**Texts**: (free choice among the following texts):

- Peter Wyatt Proteomics: Principles, Techniques and Analysis. Syrawood Publishing House
- Edmond De Hoffmann, Vincent Stroobant Mass Spectrometry: Principles and Applications-Wiley
- Throck Watson O. David Sparkman. Introduction to Mass Spectrometry: Instrumentation, Applications and Strategies for Data Interpretation, Wiley-Backwell
- Josip Lovric': Introduciong Proteomics. Wiley-Backwell

- The slides of the lectures will be available for ACS students (**Barbara Manconi web page**)

https://web.unica.it/unica/page/it/barbara_manconi

ESSE3 credentials



• Teacher are available every day by appointment (via e-mail) for further information or clarification on the topics discussed in the lectures.

Prof. Barbara Manconi: bmanconi@unica.it

Tutor: Dr. ssa Cristina Contini (cristina.contini93@unica.it)

**CONTENTS of Proteomics module**.

16 h of classroom lessons (from October 14 to November 6)

Introducing Proteomics: aims, structural, expression and functional proteomics, application fields.

Mass spectrometry (MS) applied to protein study. Basic concepts of MS. Ionization sources (MALDI, ESI, and Ambient Ionization). Low and high resolution mass analyzers. Coupling with gel-free and gel-based separation techniques.

Gel-free and gel-based proteomic approaches. Principles of chromatography: reverse phase chromatography (RP-HPLC). Electrophoretic separations: SDS-PAGE, Two-dimensional electrophoresis (2D PAGE). Spot picking and processing.

Bio-informatics tools for analysis of mass spectrometry data.

MS Methods for protein identification. Peptide Mass Fingerprinting. Tandem mass spectrometry, principles and application to the protein sequencing, *de novo* sequencing.

Bottom-up, shut-gun, middle-down and top-down proteomics. Principles, protocols and applications.

Quantitative strategies, label-free and label-based, relative and absolute, SILAC, AQUA-peptide, XIC, SIM, SRM, MRM and PRM.

12 h of Laboratory experiences: take note!

November 24 (h14.30-18.30)

December 1 and 15 (h14.30-18.30)

you must visit the occupational doctor for health surveillance

## Examination and verification of learning

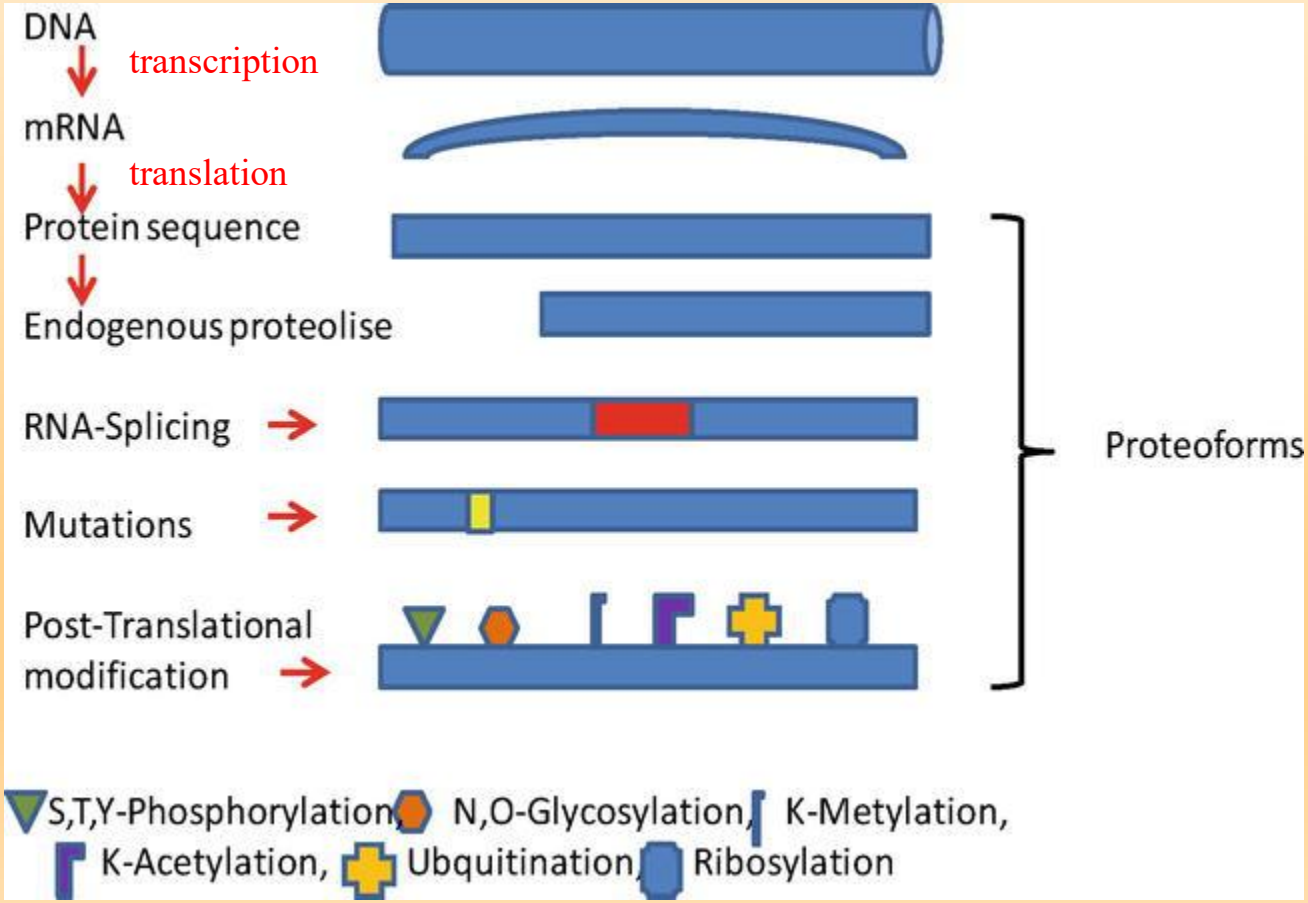
"For the final exam, I will provide you with a list of scientific articles on proteomics. You will choose one article of your interest, study it, and prepare a short PowerPoint presentation of about 10 minutes. During the exam, there will be questions both on the content of the scientific article and on the topics covered in class."

The score of the proteomics module will average with the scores of the other two modules of the course.

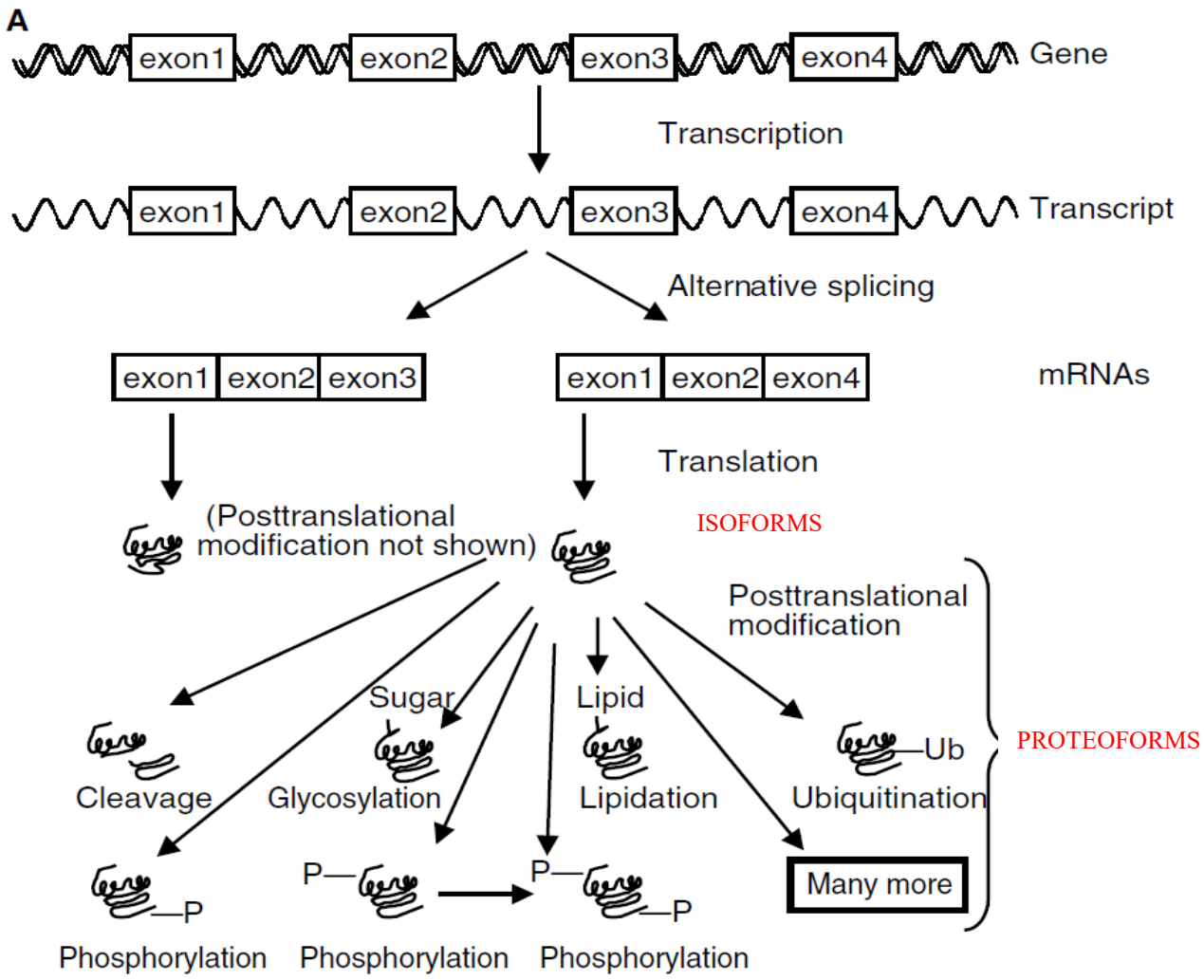
# PROTEOME

Proteome is a blend of the words "protein" and "genome". It was coined in 1994 by a Ph.D student Marc Wilkins at Macquarie University,which founded the first dedicated proteomics laboratory in 1995

The term “PROTEOME” indicates the set of all the proteins expressed by a given genome at a given time in the life of a cell, tissue or organism, including all the isoforms and post-translational modifications (PTMs).



The information obtained by genome study is not sufficient to fully understand the cellular phenotype.




*J. Mass Spectrom. 36, 1083-1091, 2001*

***35000 Genes → 1 Milion of Proteins***

**$3x10^9$ base pairs**

**35000 genes (human)**

⇩

**x Polymorphisms x Alternative splicing (P-A)**

⇩

**(35000 xPxA) Mature mRNA**

⇩

**x Conformation x PTM (CxM)**

⇩

**(35000 xPxAxCxM) Several Proteoforms of a same protein**

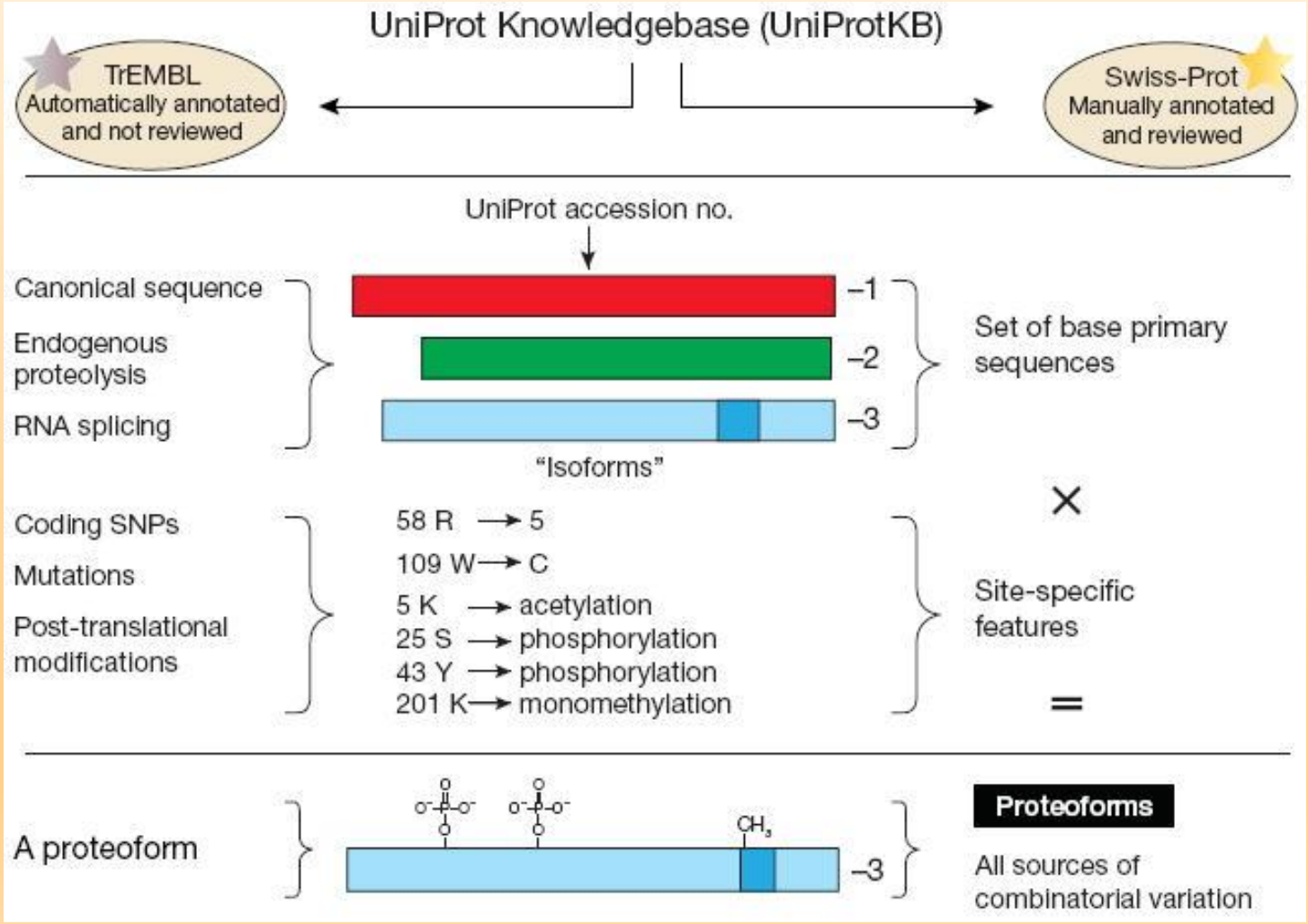
⇩

**x Interactions (I)**

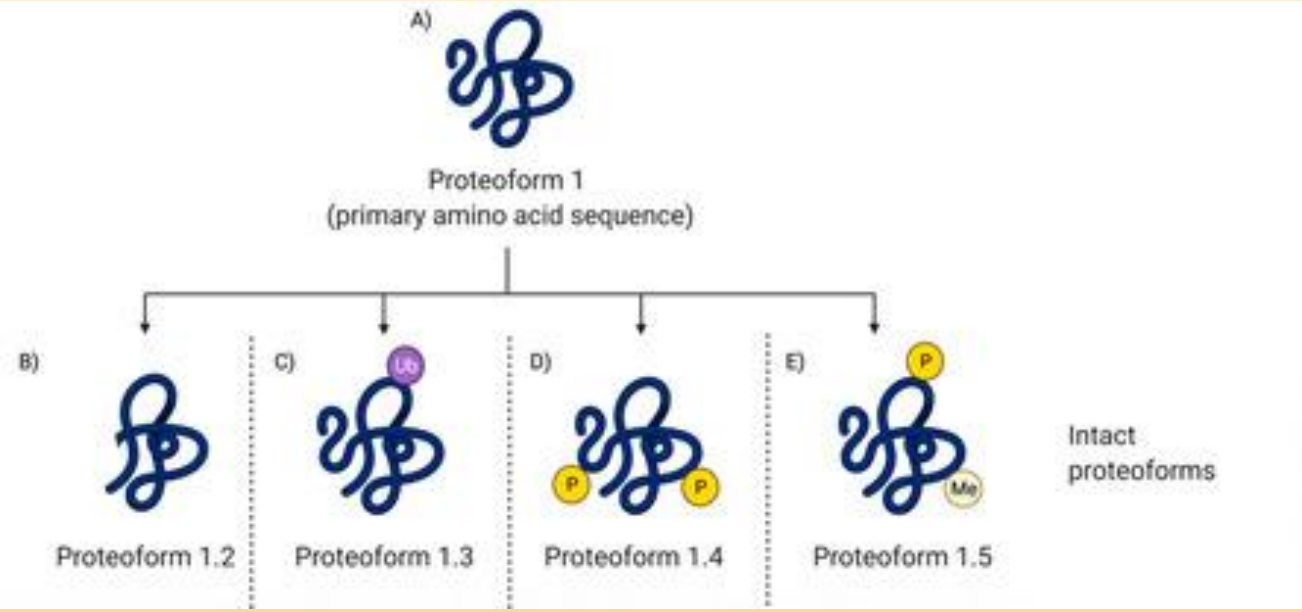
⇩

**(35000 xPxAxCxMxI) Interactions**

The term ‘proteoform’ designates all the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and post-translational modifications




Nat Methods. 2013 Mar; 10(3): 186–187.
Proteoform: a single term describing protein complexity


A)
Proteoform 1
(primary amino acid sequence)
B)
C)
Ub
D)
P
P
E)
P
Me
Proteoform 1.2
Proteoform 1.3
Proteoform 1.4
Proteoform 1.5
Intact
proteoforms

# PROTEOMICS

Proteomics is the large-scale study of proteins.

**Aims of the PROTEOMICS**:

A) To provide a complete view of the proteins present within the biological system under study

B) To provide a profile of the qualitative and quantitative changes that proteins can undergo as a result of different stimuli (physiological, pathological, pharmacological, toxicological, environmental ...)

C) To obtain information on the function and interactions of proteins within the various cellular pathways.

# WHAT IS PROTEOMICS?

The analysis of complete complements of proteins: identification and quantification; modifications; interactions; and activities. FUNCTION.

AND HOW DO THESE CHANGE DURING A BIOLOGICAL RESPONSE?


Science 291 (2001) 1221.

The genome is defined "static" because it remains constant for a given cell and identical for all cells in an organism, and does not change much within the species.

The proteome is "dynamic" over time, it changes in response to external factors, or to physiological conditions (age, biological cycles…) and it differs largely between the different cell types.

For these reasons, there is a need to develop methodologies, and analytical strategies that allow to study the proteome in the most accurate way.

## CLASSICAL (or EXPRESSION) PROTEOMICS

It studies the pattern of expression of proteins in cells, tissues or in biological fluids

## FUNCTIONAL PROTEOMICS

It studies the physical and functional interactions among proteins and the pathways in which they are involved

## STRUCTURAL PROTEOMICS

It studies the three-dimensional structure of the proteins

Simultaneous analysis of hundreds and in some cases thousands of different proteins

Analysis of complex protein mixtures extracted from TISSUES, CELL CULTURES, BIOLOGICAL FLUIDS (SERUM, PLASMA, URINE, SALIVA, CSF, CELL CULTURE MEDIUM, SYNOVIAL FLUID...)

**EXPRESSION PROTEOMICS**

IDENTIFICATION

CHARACTERIZATION OF POST-TRANSLATIONAL MODIFICATIONS AND ISOFORMS

QUALITATIVE AND QUANTITATIVE COMPARISON, that is the definition of the differential expression of the same proteome under different physiological, environmental or pathological conditions.

**Of great interest in clinical diagnostics and preventive medicine to identify multiple biomarkers of disease.**

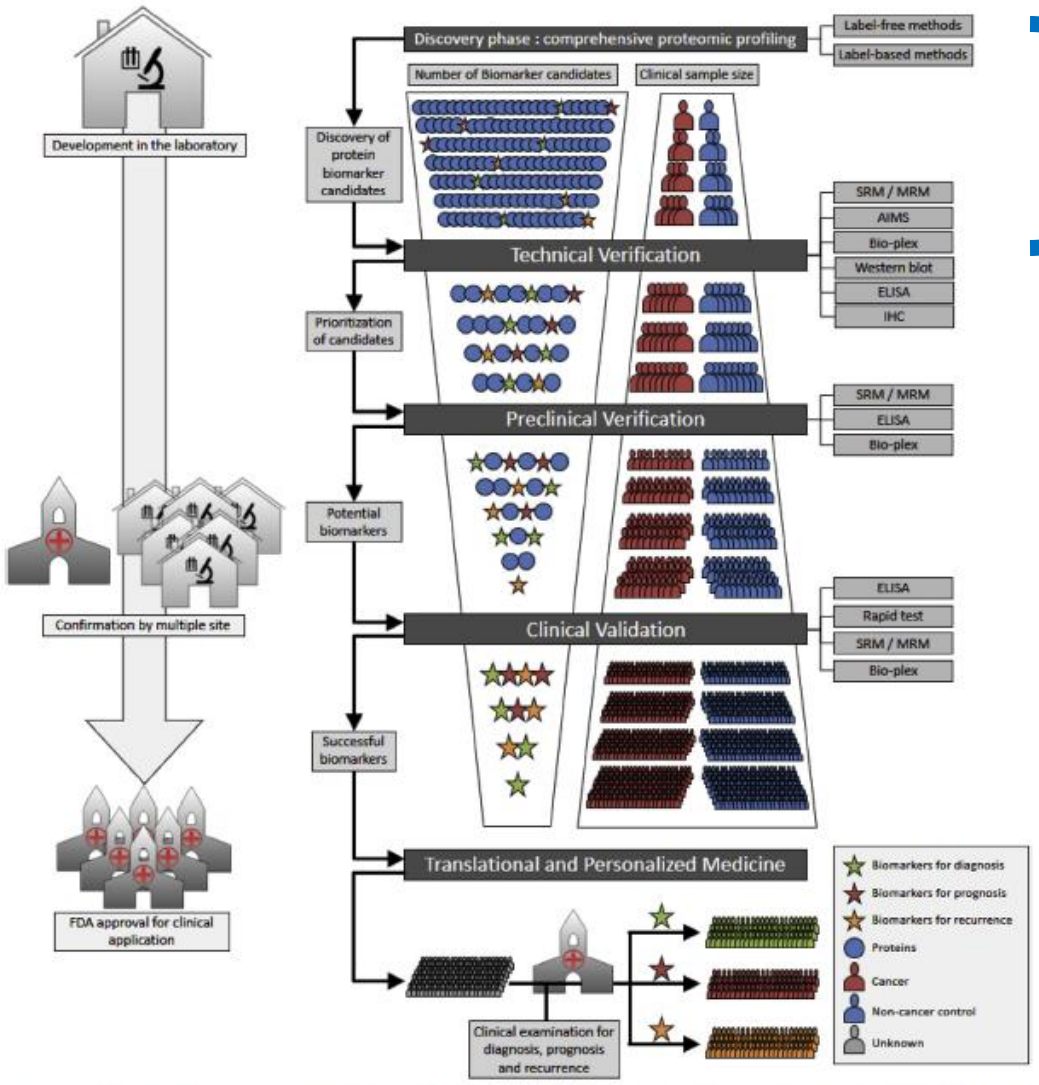



**Fig. 2 – Biomarker discovery pipeline for clinical application. The pipeline starts from a clearly defined, unmet clinical need for a biomarker or biomarker panel capable of distinguishing diseased patients from non-diseased individuals among a specific population. In the discovery phase, samples prepared from a small number of individual samples, or pooled samples, are used to profile proteomic changes and generate a list of biomarker candidates. Individual variations may cause uncertainty and add cost to subsequent biomarker verification and validation steps. For selection or prioritization of biomarker candidates, a targeted quantification assay is then used to bridge the gap between the discovery phase in the laboratory and validation in clinics. Progressing through technical and pre-clinical verification, multiplexed targeted or advanced methods are used on an increasing number of samples, ultimately leading to the selection of a single or several promising biomarkers for use in the final development of a high-throughput assay for translational and personalized medicine using a large number of samples from multiple sites or hospitals. Hospital-based clinical studies will be essential for acceptance and use of results from marker discovery studies in a clinical setting.**

PROTEOMICS: 1° STEP to obtain a comprehnsive proteomic profiling of a set o clinical samples

Work-flow of multiple disease biomarker discovery (approved by FDA)

JOURNAL OF FOOD AND DRUG ANALYSIS 27 (2019) 387–403

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfda-online.com

Review Article

**Development of biomarkers of genitourinary cancer using mass spectrometry-based clinical proteomics**

Yi-Ting Chen [a,b,c,d,*], Cheng-Han Tsai [a], Chien-Lun Chen [e,f], Jau-Song Yu [a,b,g], Ying-Hsu Chang [h,i,**]

To perform a proteomic investigation is essential to apply MASS SPECTROMETRY analysis

# MASS SPECTROMETRY

How to define the mass spectrometry?

- It is a technique allowing the characterization and identification of molecules on the base of the **molecular mass value**, and of the structural characterization (i.e. aminoacid sequence in the case of proteins).
- A mass spectrometer (MS) separates and detects only charged species (ions), and measures the mass to charge ratio (m/z).

The mass spectrometer measures the m/z ratio
Dalton/unit of electric charge (Thomson)

Why Ionize ? Is Difficult to manipulate neutral particles on molecular scale. If they are charged, then we can use electric fields to move them around.

# Applications of mass spectrometry

Applications of mass spectrometry in proteomics - Characterization of proteins and protein complexes, sequencing of peptides, and identification of postranslational modifications (PTMs), Biomarker discovery and profiling.

Applications of mass spectrometry in metabolomics - Cancer screening and diagnosis, global metabolic fingerprinting analysis, biomarker discovery and profiling, biofuels generation and use, lipidomics studies, and metabolic disorder profiling.

Applications of mass spectrometry in food analysis - Pesticides and residues, bioactive compounds (Polyphenols, vitamins etc.)

Applications of mass spectrometry in environmental analysis - Drinking water testing, pesticide screening and quantitation, soil contamination assessment, carbon dioxide and pollution monitoring, and trace elemental analysis of heavy metals leaching.

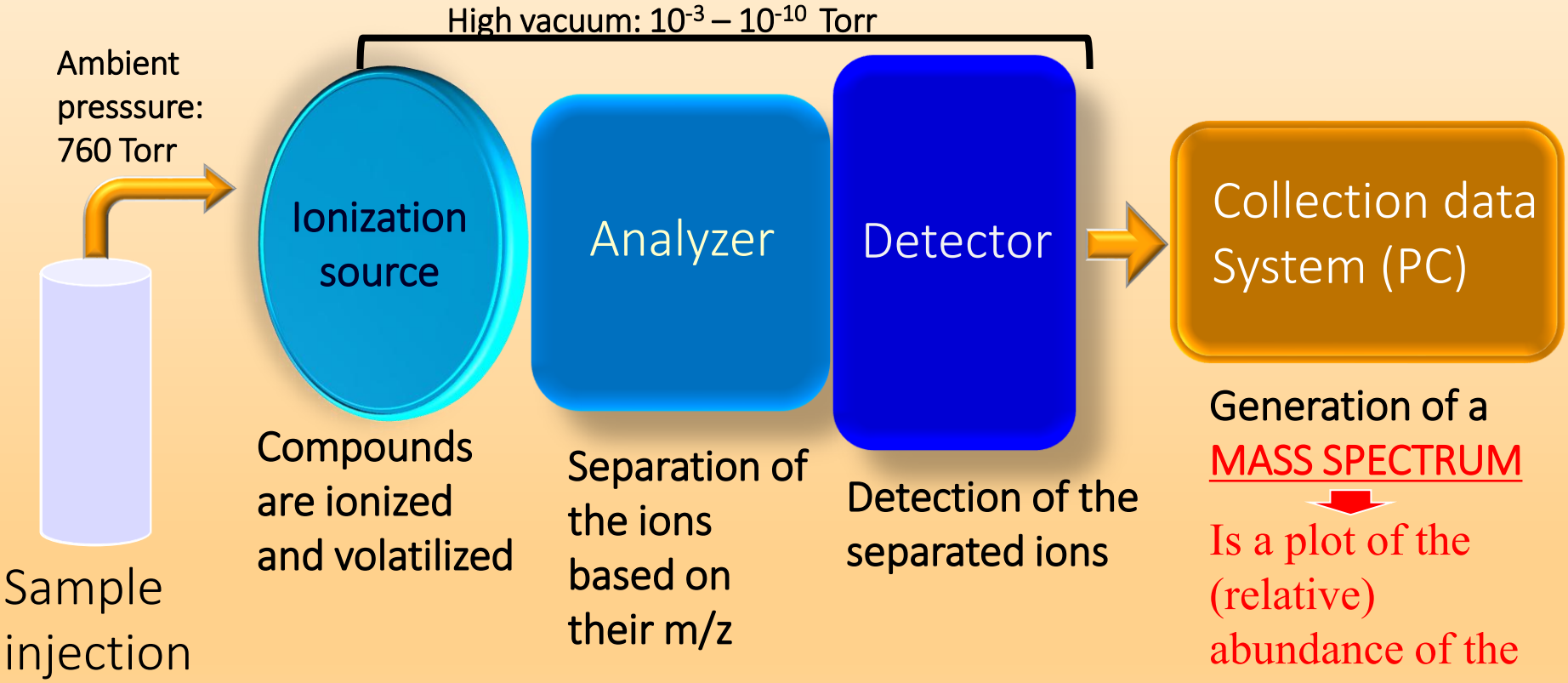
Applications of mass spectrometry in pharmaceutical analysis - Drug discovery and absorption, distribution, metabolism, and elimination (ADME) studies, pharmacokinetic and pharmacodynamic analyses, metabolite screening, and preclinical development.

Applications of mass spectrometry in forensic analysis - Analysis of trace evidence (e.g., fibers in carpet, polymers in paint), arson investigation (e.g., fire accelerant), confirmation of drug abuse, antidoping, and identification of explosive residues (bombing investigation).

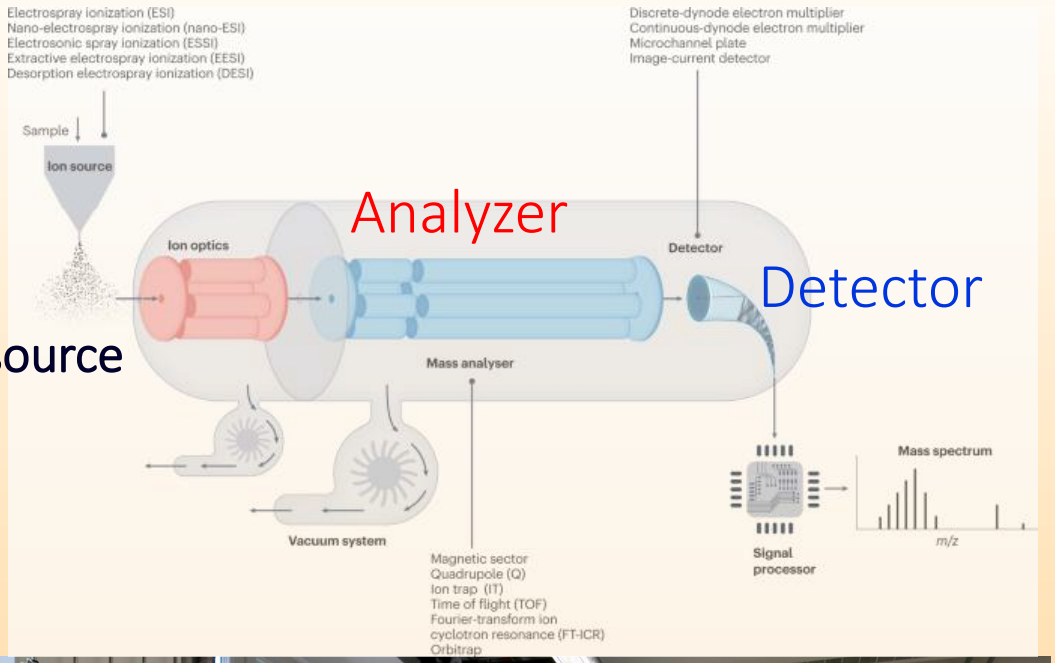
Clinical applications of mass spectrometry - Clinical drug development, Phase 0 studies, clinical tests, disease screening, drug therapy monitoring, analysis of peptides used for diagnostic testing, and identification of infectious agents for targeted therapies, functional compounds (vitamins, antioxidants), metabolomics, and proteomics

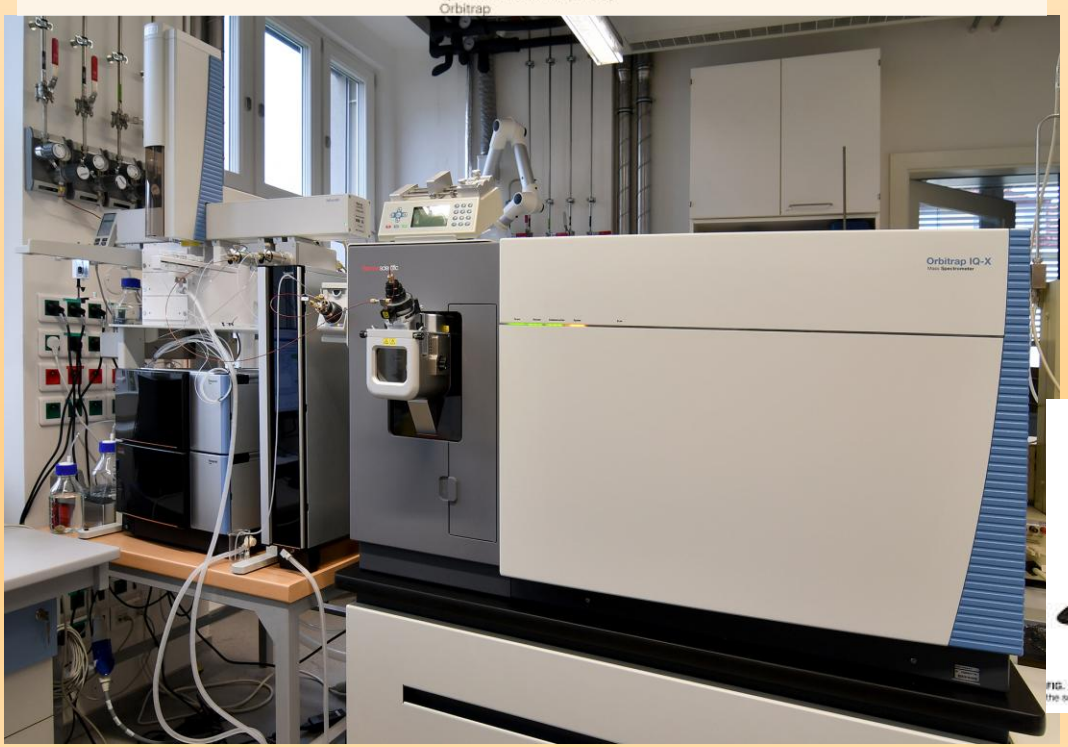
"The basis in MS (mass spectrometry) is the production of ions, that are subsequently separated or filtered according to their mass-to-charge (*m/z*) ratio and detected.

Niessen, W. M. A.; Van der Greef, J., *Liquid Chromatography–Mass Spectrometry: Principles and Applications*, 1992, Marcel Dekker, Inc., New York, p. 29.



Ionization source




Collection data System (PC)


1) Allows the volatilization of the compounds present in the sample and, therefore, their transfer into the gaseous phase>> ions get vaporized in a vacum

2) Promotes the ionization of the compounds

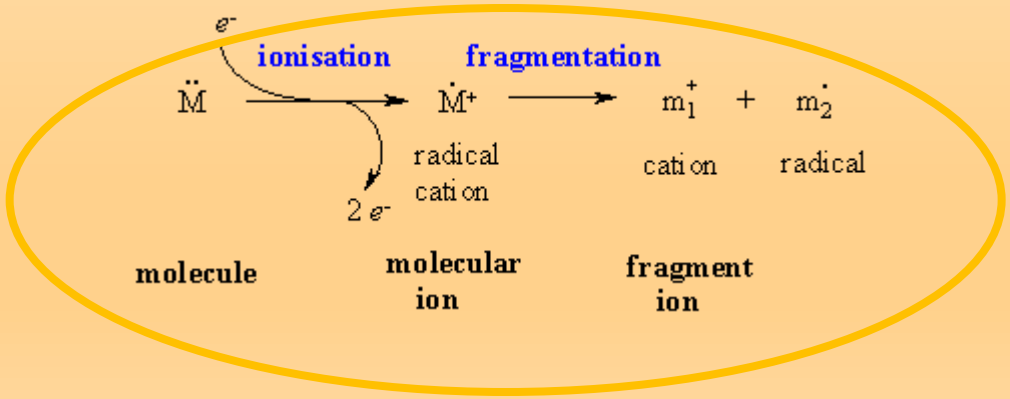
# How does the ionization of molecules take place?

## IONIZZAZIONE:

**Molecule** ⟶ **molecular ion**

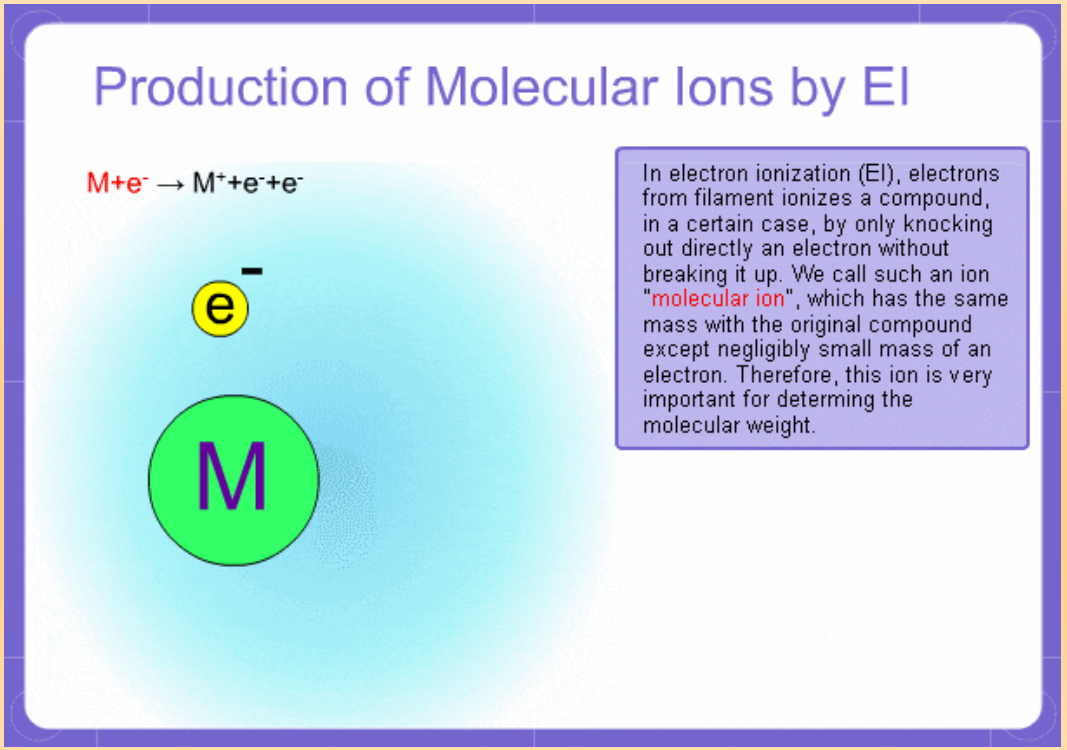
$$M + e^- \longrightarrow M^{+\bullet} + 2e^-$$

**positive radical molecular ion**




Different kinds of ions are generated from different types of ionization souces

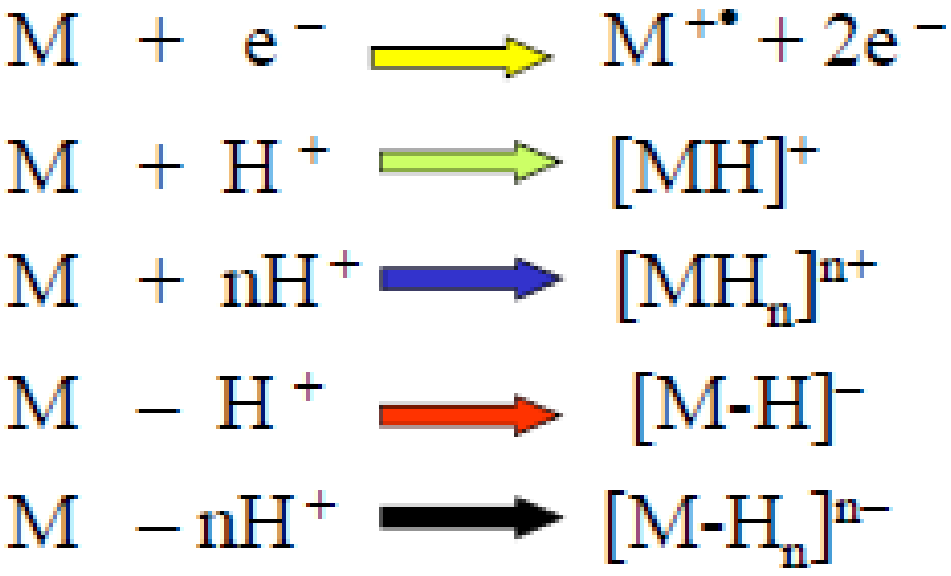
- **Molecular Ion**
  - Molecular ion (M): A radical cation formed by removal of a single electron from a parent molecule in a mass spectrometer = MW.
  - For our purposes, it does not matter which electron is lost; radical cation character is delocalized throughout the molecule; therefore, we write the molecular formula of the parent molecule in brackets with:
    - A plus sign to show that it is a cation.
    - A dot to show that it has an odd number of electrons.



# How does the ionization of molecules take place?

## IONIZZAZIONE:

**Molecule → molecular ion**




Different kinds of ions are generated from different types of ionization souces

**positive radical molecular ion**

**positive mono-charged molecular ion**

**positive multiple-charged molecular ion**

**negative mono-charged molecular ion**

**negative multiple-charged molecular ion**

Several types of ionization techniques can be used by different ionization sources

- ATMOSPHERIC PRESSURE IONIZATION (API)
  - ELECTROSPRAY (ES)
  - ATMOSPHERIC PRESSURE CHEMICAL IONIZATION (APCI)
- ELECTRONIC IMPACT (EI)
- CHEMICAL IONIZZATION (CI)
- FAST ATOM BOMBARDMENT (FAB)
- MATRIX ASSISTED LASER DESORPTION IONIZATION (MALDI)
- AMBIENT IONIZATION (AI)

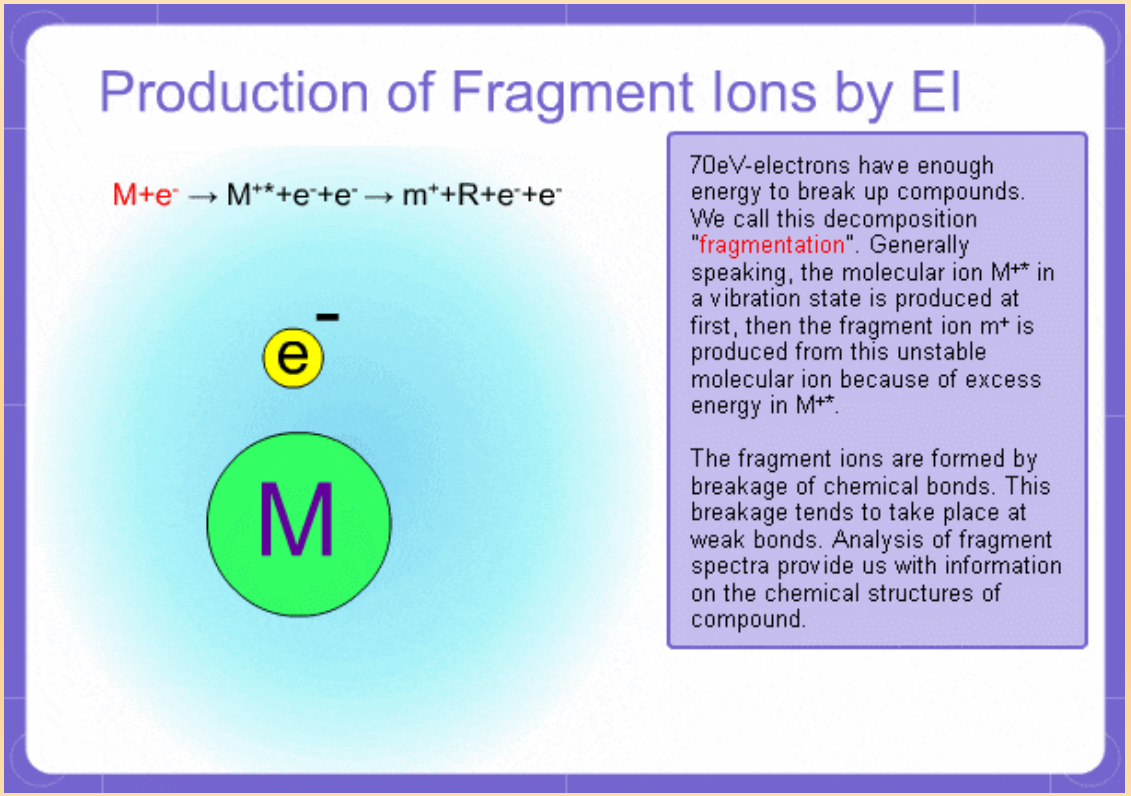
# Ionization techniques are grouped into HARD and SOFT Techniques

## HARD IONIZZATION

Electronic impact (EI)

- Large excess of energy on the molecular ion
  - Extended fragmentation of the molecule

The detection of the fragments by MS provides many structural information, principally on the functional groups (IN SOURCE),




$$M + e^- \longrightarrow M^{+\bullet} + 2e^-$$

**positive radical molecular ion**

# Ionization techniques are grouped into HARD and SOFT Techniques

## SOFT IONIZZATION

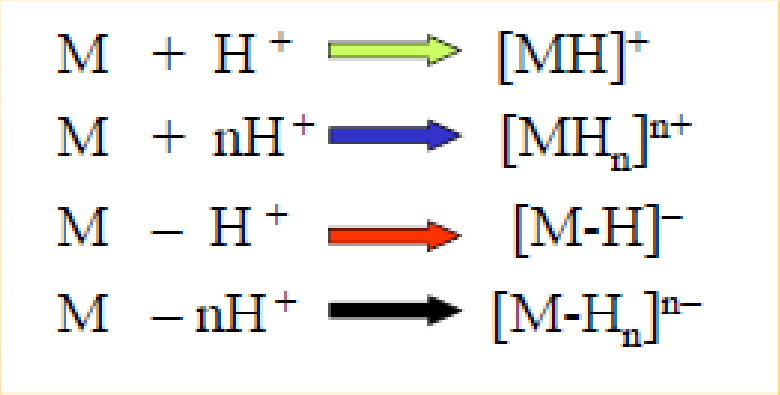
Electrospray, MALDI, chemical ionization, APCI

It produces protonated or deprotonated ions by applying little energy.

Little or no fragmentation

Determination of the mass value of the molecule.

To obtain structural information additional energy has to be applied on the molecule to induce its fragmentation (NOT IN SOURCE, BUT IN the ANALYZER)

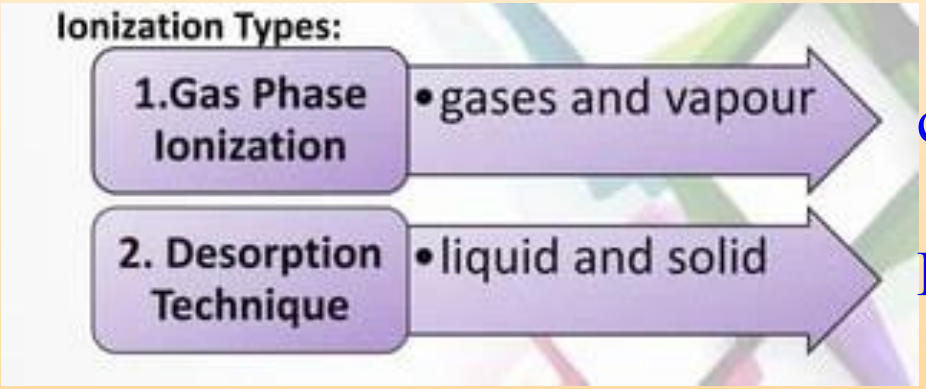



positive mono-charged molecular ion
positive multiple-charged molecular ion
negative mono-charged molecular ion
negative multiple-charged molecular ion


State of the analytes

Type of ion source

SOFT IONIZZATION




chemical ionization

Electrospray, MALDI,

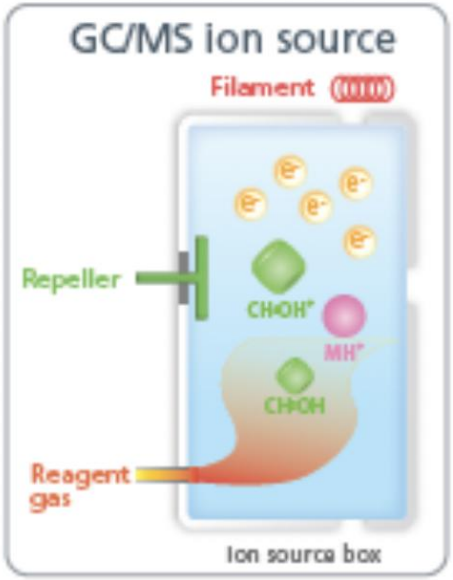
# CI source (CHEMICAL IONIZATION)

**This is a soft technique requires molecules in vapor (gas) phase before their introduction in the source: it ONLY APPLIES TO VOLATILE MOLECULES**

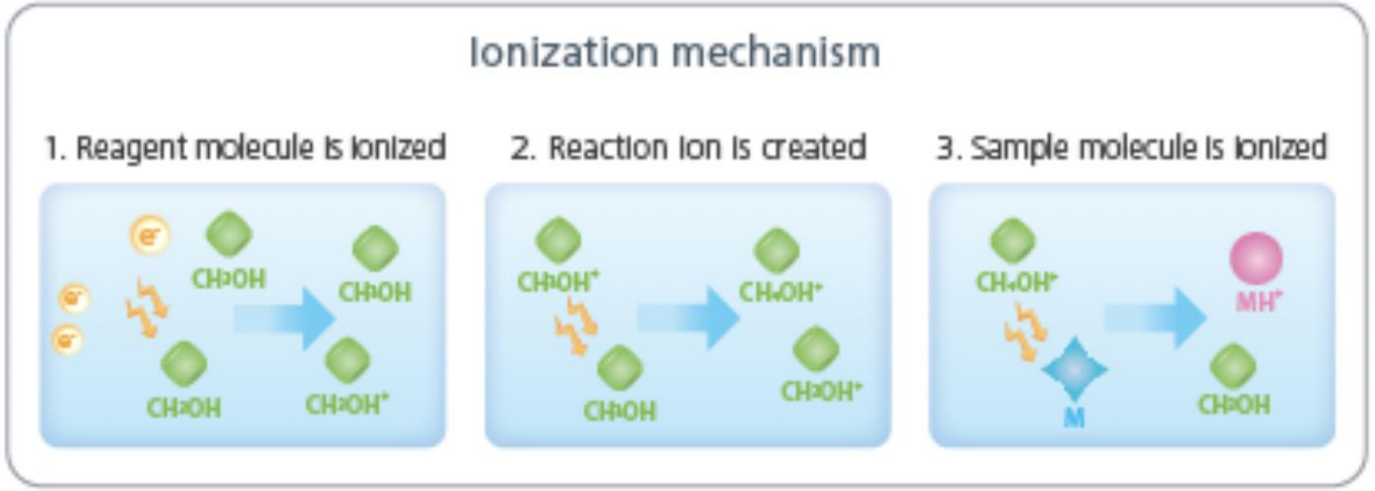
## Common reagents:

- $CH_4$ (PA = 5.7 eV)
- $NH_3$ (PA = 9.7 eV)
- Isobutano (PA = 8.7 eV)

Chemical ionization **uses a reagent gas to ionize sample molecules through ion–molecule reactions in the gas phase**. The reagent gas (e.g., methane) first undergoes electron ionization, generating a molecular ion which then reacts with gas molecules generating ions.




https://www.youtube.com/watch?v=DAOHFI5WhNU




Principles of SMCI

**M = molecules in vapor phase**

The molecular ion of the reagent gas has to:

a. Transfer of a proton ($H^+$)

$$M + [BH]^+ \rightarrow [MH]^+ + B$$

$$PA_M > PA_B$$

PA= affinità protonica in fase gassosa

A great difference of proton affinity (PA) between reagent and molecules

# For not volatile molecules: Ionization for DESORPTION (named also ionization in condensed phase)

MOLECULES IN CONDENSED PHASE
(LIQUID OR SOLID)

(ionization and volatilization together)

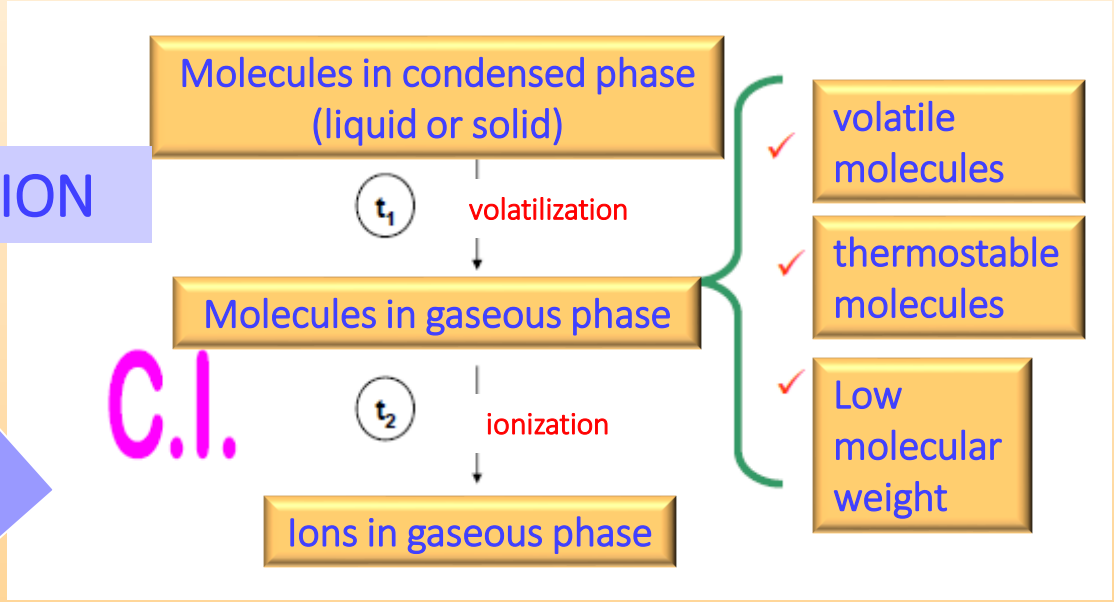
In only one step: desorption

IONS IN GASEOUS PHASE

# Molecules suitable for chemical ionization:

## non volatile molecules

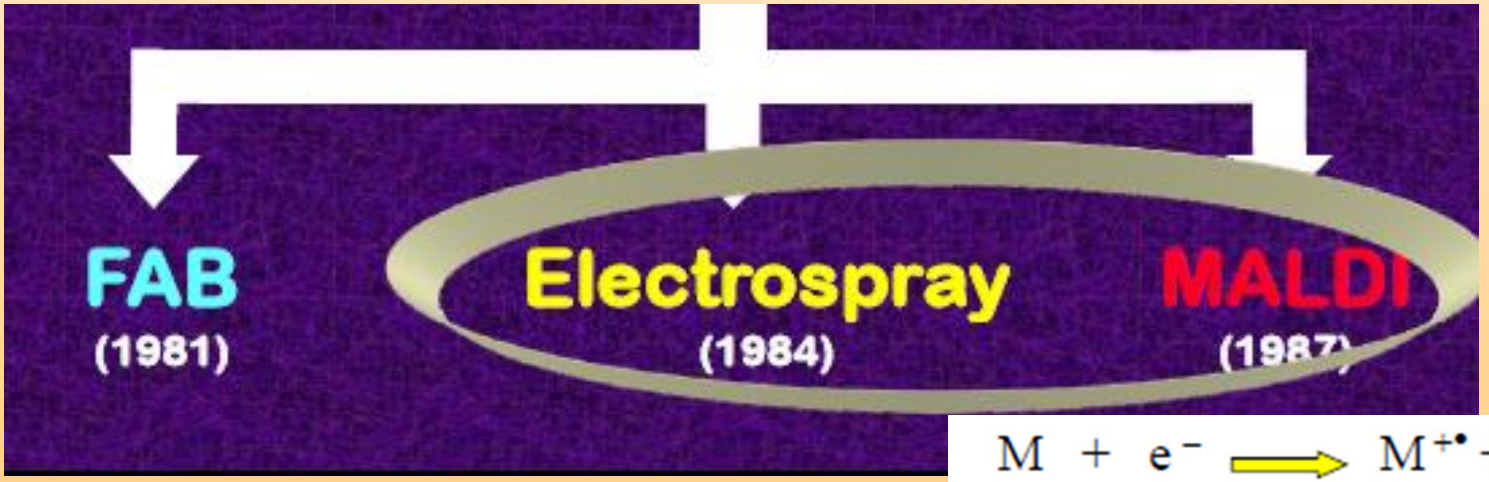
DESORPTION




CONDENSED PHASE ionization techniques have allowed applying mass spectrometry to problems of biomedical interest.
They allow the ionization of biological macromolecules because they work with condensed phases (liquid or solid)

# DESORPTION IONIZATION

They involve a rapid addition of energy to molecules in the condensed phase (liquid or solid) with the consequent production of stable ionic species in the gas phase, generally without loss of electrons, and with minimal fragmentation.




$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

$$M + H^+ \rightarrow [MH]^+$$

$$M + nH^+ \rightarrow [MH_n]^{n+}$$

$$M - H^+ \rightarrow [M\text{-}H]^-$$

$$M - nH^+ \rightarrow [M\text{-}H_n]^{n-}$$

CONDENSED PHASE ionization techniques (based on DESORPTION phenomenon)

- **FAB source** (fast atom bombardment), now little used
- **ESI source (**Electro-Spray Ionization) allows to analyze a large spectrum of molecules and it is applied on different fields.
- **MALDI** source (Matrix Assisted Laser Desorption and Ionization) allows analyzing macromolecules without limits to their mass.
- **AI** source (AMBIENT IONIZATION), for samples not processed or minimally processed in their original environment.

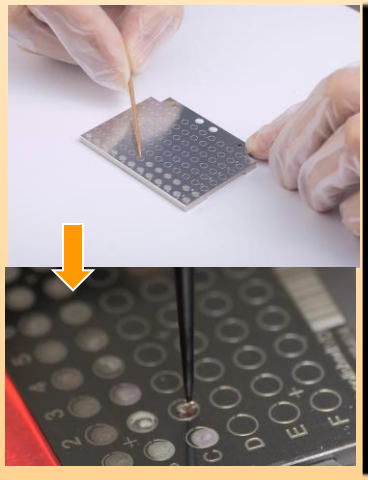
# FAB Fast Atom Bombardment (1975-1980)

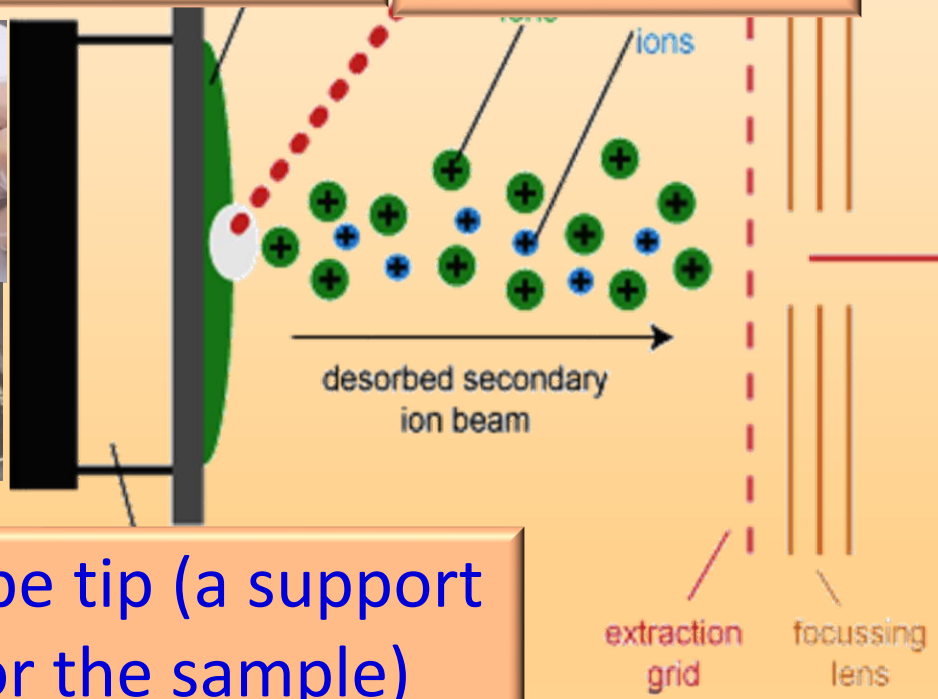
Analyte mixed with a non-volatile chemical protection environment, called matrix (ie glycerol), deposited on a plate

Analyte bombarded by fast-moving neutral atoms BEAM

The atoms are typically inert heavy gas such as argon or xenon

University of BRISTOL



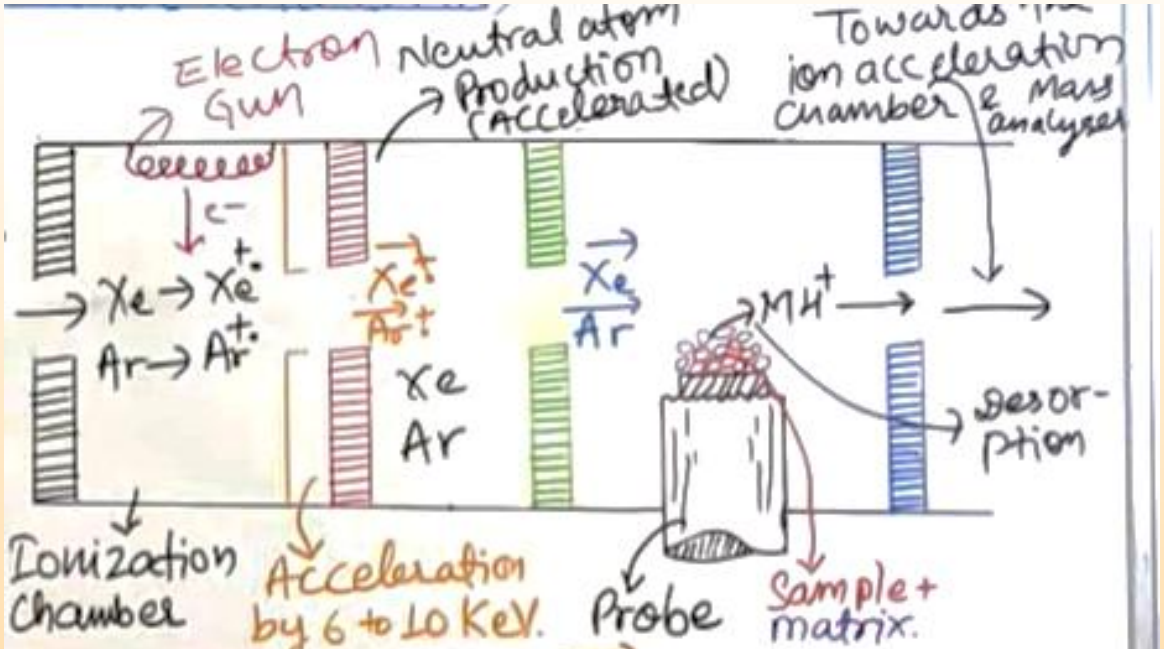



ions are pushed by the electric field towards the analyzer

Ions to analyzer

Probe tip (a support for the sample)

FAB is very effective for the analysis of non-volatile, thermally unstable polar compounds. The ionization products $[M+H]^+$ and $[M-H]^-$ are produced in pairs in FAB, facilitating the analysis of positive ion mass spectrometry and negative ion mass spectrometry. The FAB has high sensitivity and consumes less sample. However, FAB is not suitable for separating samples.




$Xe + \bar{e} \rightarrow Xe^{+\cdot} + 2e^-$ → $\overrightarrow{Xe^{+\cdot}}$

$Ar + \bar{e} \rightarrow Ar^{+\cdot} + 2e^-$ → $\overrightarrow{Ar^{+\cdot}}$

$\overrightarrow{Xe} + \overrightarrow{Xe^{+\cdot}} \rightarrow \overrightarrow{Xe} + Xe^{+\cdot}$

$\overrightarrow{Ar} + \overrightarrow{Ar^{+\cdot}} \rightarrow \overrightarrow{Ar} + Ar^{+\cdot}$

} Removed by the electric field.

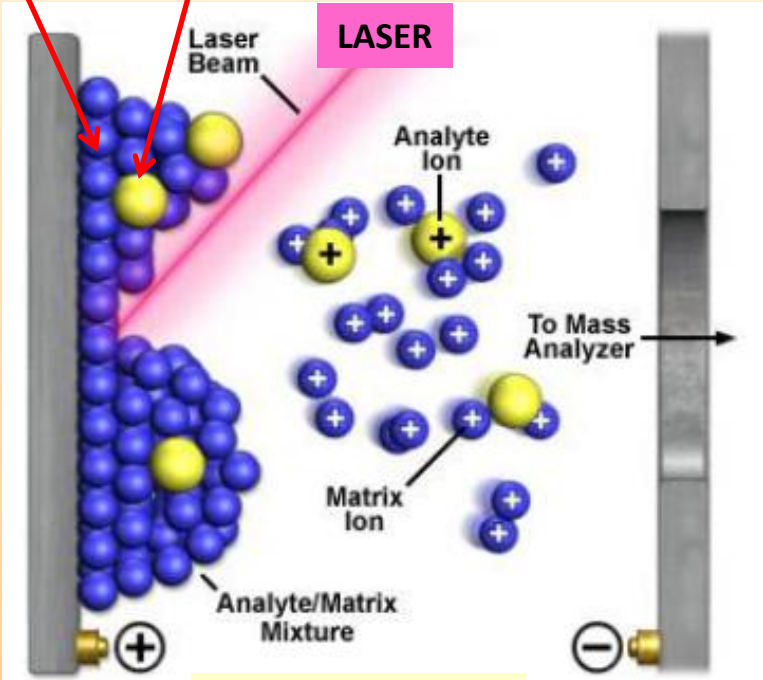
$\overrightarrow{Xe}$ or $\overrightarrow{Ar}$ (Accelerated neutral atoms) will be bombarded to the sample-matrix mixture & ionize the sample due to translational energy.

Glycerol-$H^+ + M \rightarrow [MH]^+$ Quasi molecular ion.

# MALDI: MATRIX ASSISTED LASER DESORPTION IONIZZAZION

Crystals of matrix

Crystals of analyte mixed with the matrix




The energy supplied by the laser beam leads to the sublimation of the analyte ions.

Monocharged or bi-charged ions are formed in the gaseous phase, and they are pushed by the electric field towards the analyzer

Method suitable for compounds with molecular weights (MW) up to 300 kD.

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

$$M + H^+ \rightarrow [MH]^+$$

$$M + nH^+ \rightarrow [MH_n]^{n+}$$

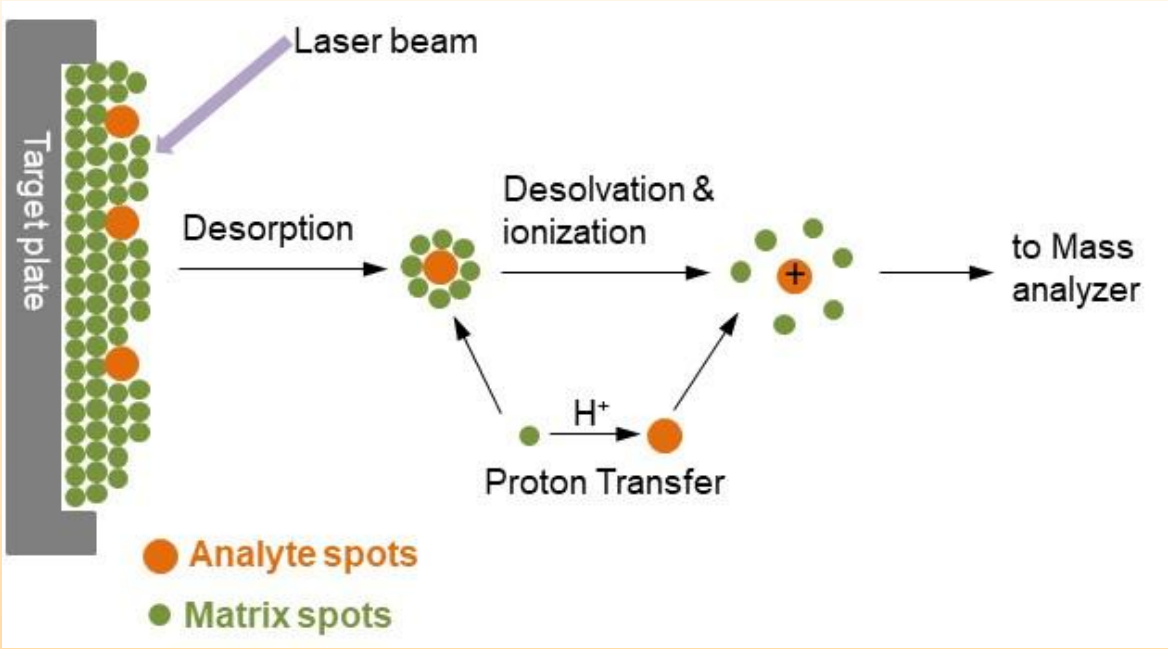
$$M - H^+ \rightarrow [M\text{-}H]^-$$

$$M - nH^+ \rightarrow [M\text{-}H_n]^{n-}$$

# What happens with MALDI?

**1)** The analyte is mixed with a **large quantity of matrix.** As the mixture dries, crystals are formed on the sample material, that are critical for efficient ionization.

The mixture is seeded on a steel plate (TARGET) on which crystallization will take place




**2)** The Target is introduced into the ionization chamber (under vacuum) and the crystals shot with a pulsed laser beam.

Traditionally UV laser (Nitrogen lasers at 337 nm) have been used as a source for MALDI-

The energy transferred from the laser beam to the matrix causes its sublimation.

**3)** The matrix carries the analyte molecules and transfers protons to the analyte thus allowing the formation of positive ions in the gaseous phase.

Ions are frequently monocharged

**MATRIX**

- Soluble in the same solvent as the analyte. They must mix homogeneously.
- The MATRIX absorbs at the wavelength of the laser, so that the laser energy is deposited on the matrix and not on the analyte. Nitrogen lasers at 337 nm
- Ability to absorb laser energy and transfer it as excitation energy to solid-state analyte molecules= desorbtion
- Ability to ionize analyte molecules, through the transfer of $H^+$ ions.
- The MATRIX does not covalently modify the analyte

# Choosing the matrix

| Matrix | Application |
|---|---|
| ***Sinapinic Acid*** (3,5-dimethoxy-4-hydroxy cinnamic acid) | Peptides and proteins greater than 10 kDa in mass |
| ***CHCA*** (a-cyano-4-hydroxycinnamic acid) | Peptides and proteins less than 10 kDa in mass |
| ***THAP*** (2,4,6-Trihydroxyacetophenone) | Small oligonucleotides less than 3.5 kDa in mass |
| ***HPA*** (3-hydroxypicolinic acid) in diammonium citrate | Large nucleotides greater than 3.5 kDa in mass |

Matrices are chosen to be weak acids, that is, good proton donors

## a. Transfer of a proton ($H^+$)

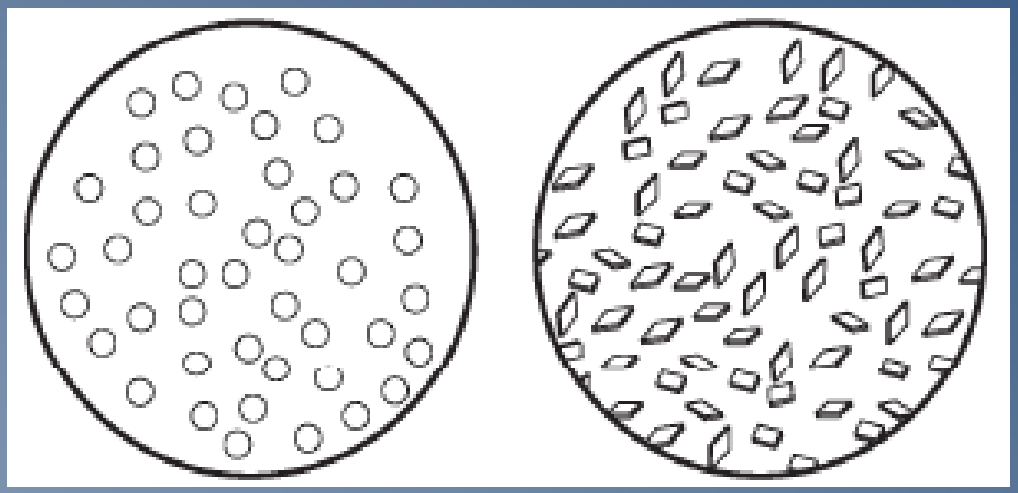
$M + [BH]^+ \rightarrow [MH]^+ + B$

$PA_M > PA_B$ PA= affinità protonica in fase gassosa

**positive mono-charged molecular ions**

**positive multiply-charged molecular ions**

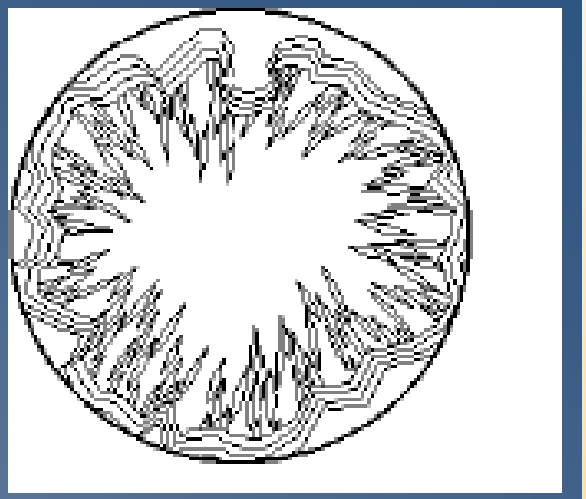
# Different matrices, different crystals



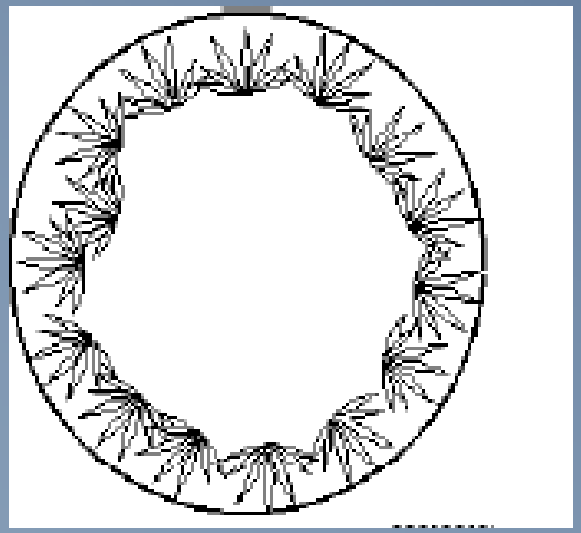
HCCA



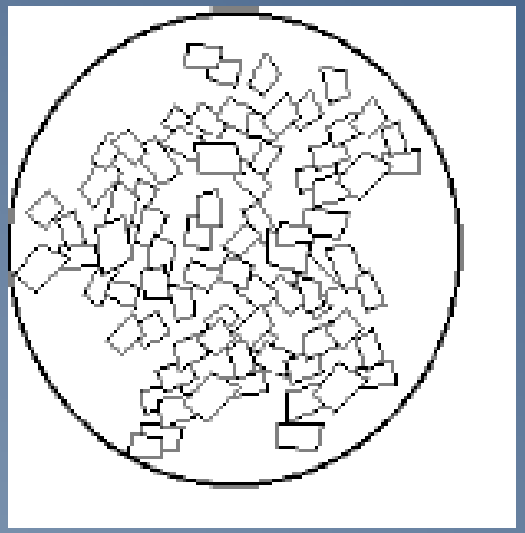
SIN



DHB



3HPA

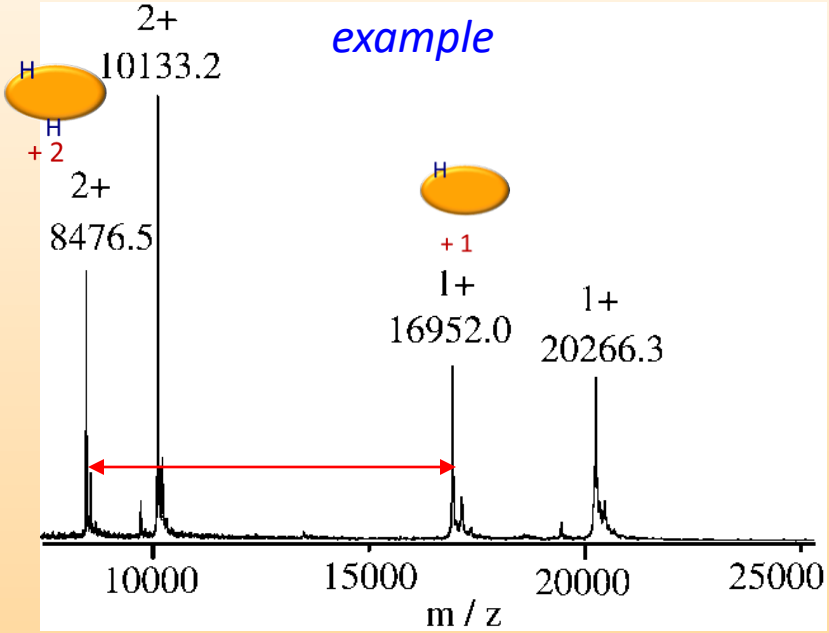


THAP

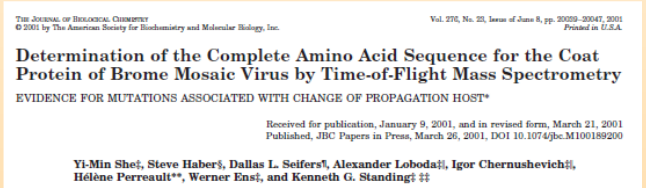
https://www.youtube.com/watch?v=tyLdUkXT9wA

# The MALDI source usually generates mono-charged and bi-charged ions.

REMEMBER!! The mass spectrum is a plot of the (relative) abundance of the produced ions as a function of the m/z ratio.




MALDI/TOF m/z spectrum of the BMV-P coat protein. The measurement was performed on the MALDI/TOF mass spectrometer using myoglobin (16,951 Da) as an internal standard and sinapinic acid as matrix.


Determination of the Complete Amino Acid Sequence for the Coat Protein of Brome Mosaic Virus by Time-of-Flight Mass Spectrometry

EVIDENCE FOR MUTATIONS ASSOCIATED WITH CHANGE OF PROPAGATION HOST*

Received for publication, January 9, 2001, and in revised form, March 21, 2001
Published, JBC Papers in Press, March 26, 2001, DOI 10.1074/jbc.M100189200

Yi-Min She‡, Steve Haber§, Dallas L. Seifers¶, Alexander Loboda‡‖, Igor Chernushevich‡‖, Hélène Perreault**, Werner Ens‡, and Kenneth G. Standing‡ ‡‡

**Important!!**

For the myoglobin with mass 16951.0 Da, MALDI source can produce a mono-charged ion (THE protein ACQUIRES **1 $H^+$**), **at m/z =** (16951.0 +1)/1= 16952.0 $[M+H^+]^{+1}$

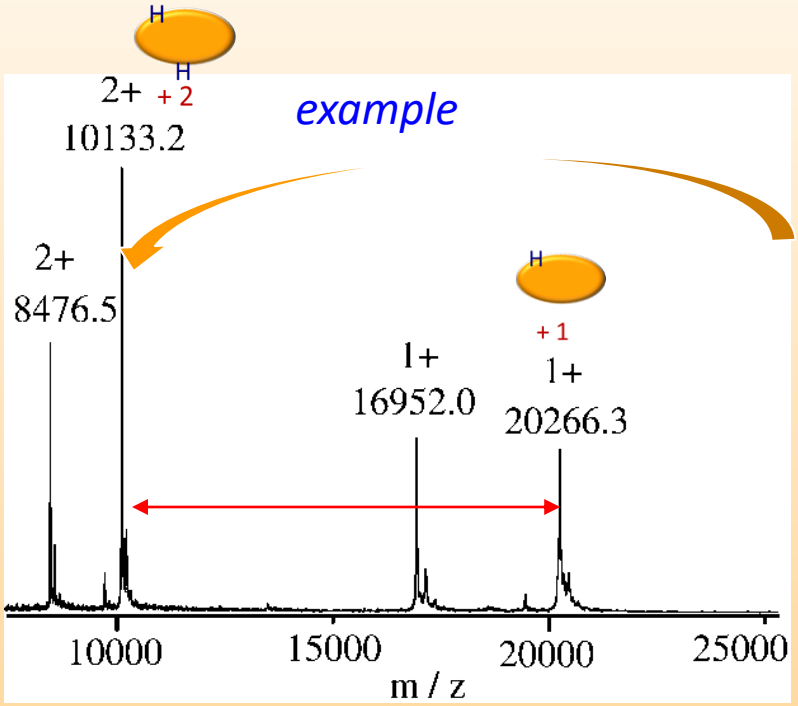
And the bi-charged ion (THE Protein ACQUIRES **2 $H^+$**), **at m/z =** (16951.0 +2)/2= 8476.5 $[M+2H^+]^{+2}$

**If the charge of the m/z ion is known, we can determine the mass of the peptide/protein**.

If the MS spectrum reveals a bi-charged ion at 8476.5 m/z the mass value of the parent protein is: **[M] = (m/z * z)-z = (8476.5 x 2) - 2 = 16951.0 Da**

**If the charge of the m/z ion is known, we can determine the mass of the peptide/protein.**

**[M] = (m/z * z)-z =**

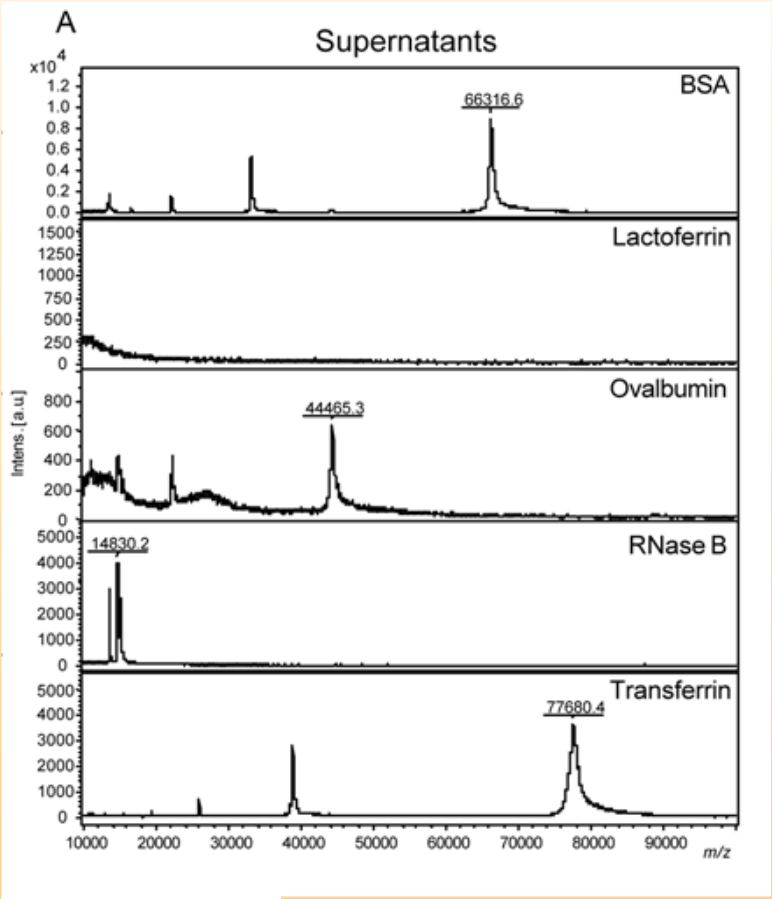



let's try to determine the molecular mass value of the virus parent protein

If the MS spectrum reveals a bi-charged ion at 10133.2 m/z the mass value of the parent protein is: **[M] = (m/z * z)-z = (10133.2 x 2) - 2 = 20264 ±1Da**

If the MS spectrum reveals a mono-charged ion at 20266.3 m/z the mass value of the parent protein is: **[M] = (m/z * z)-z = (20266.3 x 1) - 1 = 20265 ± 1Da**

# *Qualitative and quantitative applications of MALDI-TOF*




Journal of Biomolecular Techniques
16:405–411 © 2005 ABRF

**Selective Isolation of Glycoproteins and Glycopeptides for MALDI-TOF MS Detection Supported by Magnetic Particles**

*Katrin Sparbier, Sonja Koch, Irina Kessler, Thomas Wenzel, and Markus Kostrzewa*

J Am Soc Mass Spectrom 2002, 13, 1015–1027



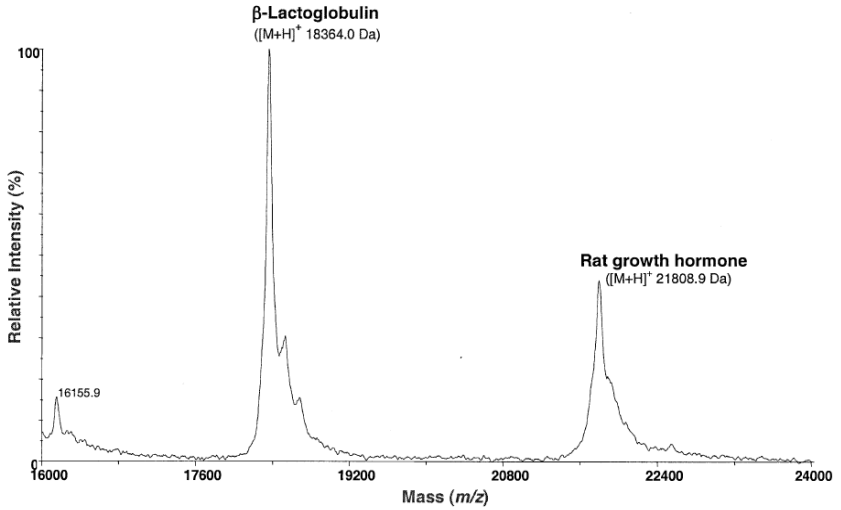



**Figure 1.** A representative MALDI mass spectrum for growth hormone ([M + H]$^+$ 21,808 Da) in a rat pituitary extract. $\beta$-Lactoglobulin (15 nmol, [M + H]$^+$ 18,364 Da) was added as an internal standard and caffeic acid was used as the MALDI matrix.

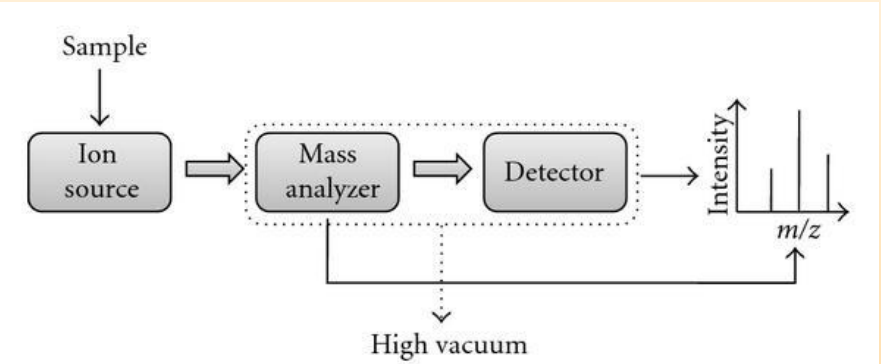
**Practical Quantitative Biomedical Applications of MALDI-TOF Mass Spectrometry**

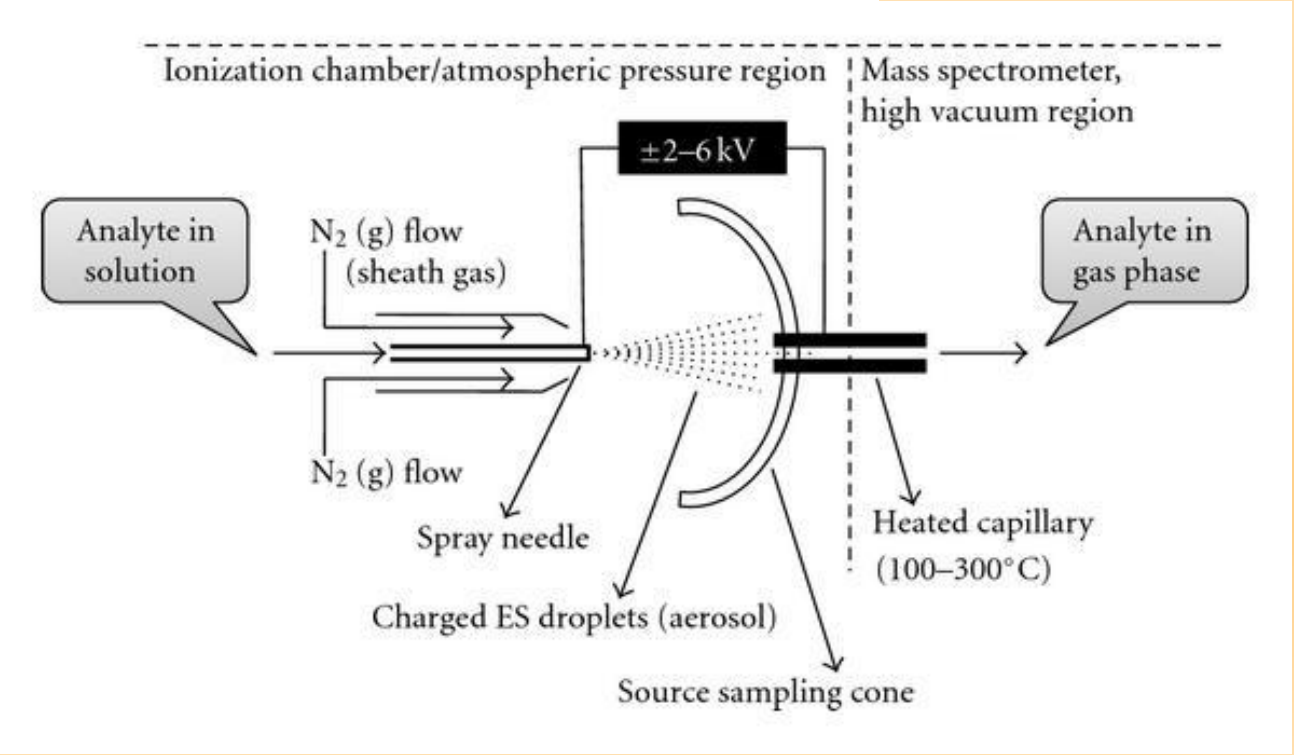
Martin Bucknall and Kim Y. C. Fung
Ray Williams Biomedical Mass Spectrometry Facility, University of New South Wales, Sydney, Australia

Mark W. Duncan
Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado, USA

# ESI-ElectroSpray Ionisation

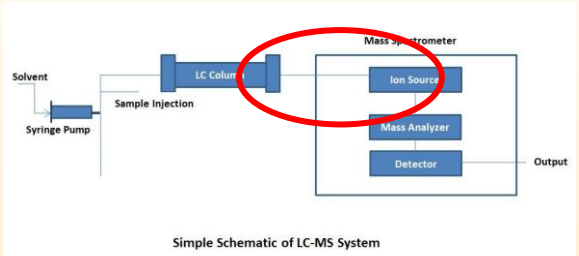
The molecules are introduced into the ion source in the liquid phase (directly injected or separated in HPLC or CE (capillary electrophoresis) and are converted to ions in gas phase



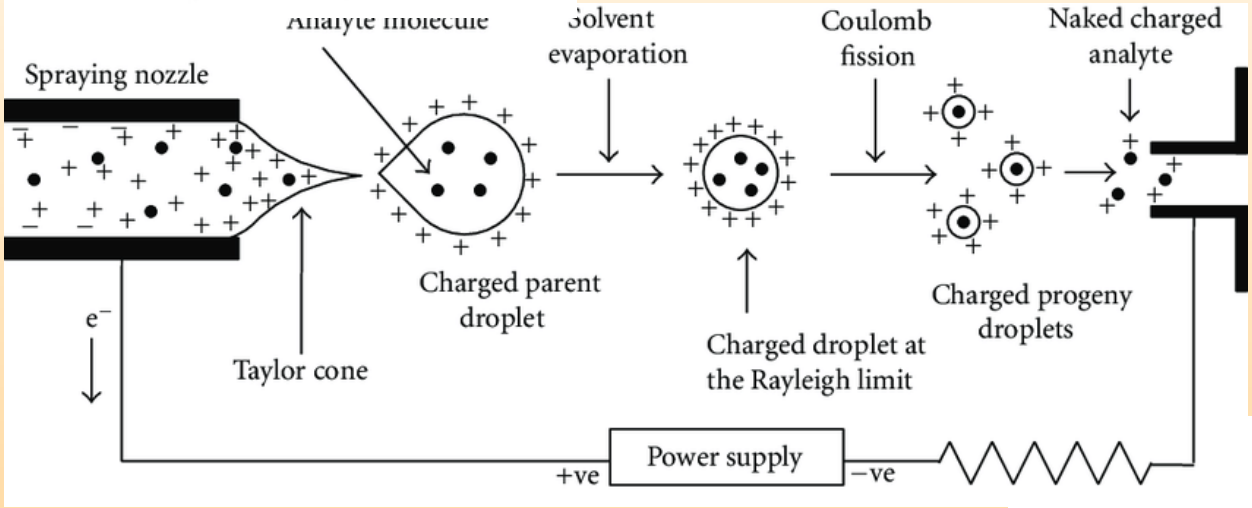




Generally a dilute analyte solution is injected by a mechanical syringe pump through a hypodermic needle or stainless steel capillary (~0.2 mm o.d and ~0.1 mm i.d) at low flow rate (typically 1–20 $\mu$L/min). A very high voltage (2–6 kV) is applied to the tip of the metal capillary (typically located at 1–3 cm from the spray needle tip). This strong electric field causes the dispersion of the sample solution into an aerosol of highly charged electrospray (ES) droplets. A coaxial sheath gas (dry $N_2$) flow around the capillary results in better nebulization. This gas flow also helps to direct the spray emerging from the capillary tip towards the mass spectrometer. The charged droplets diminish in size by solvent evaporation, assisted by the flow of nitrogen (drying gas). The heated capillary (typically 100–300°C) causes the complete desolvation of the ions passing through it.

# ESI-ElectroSpray Ionisation



Simple Schematic of LC-MS System



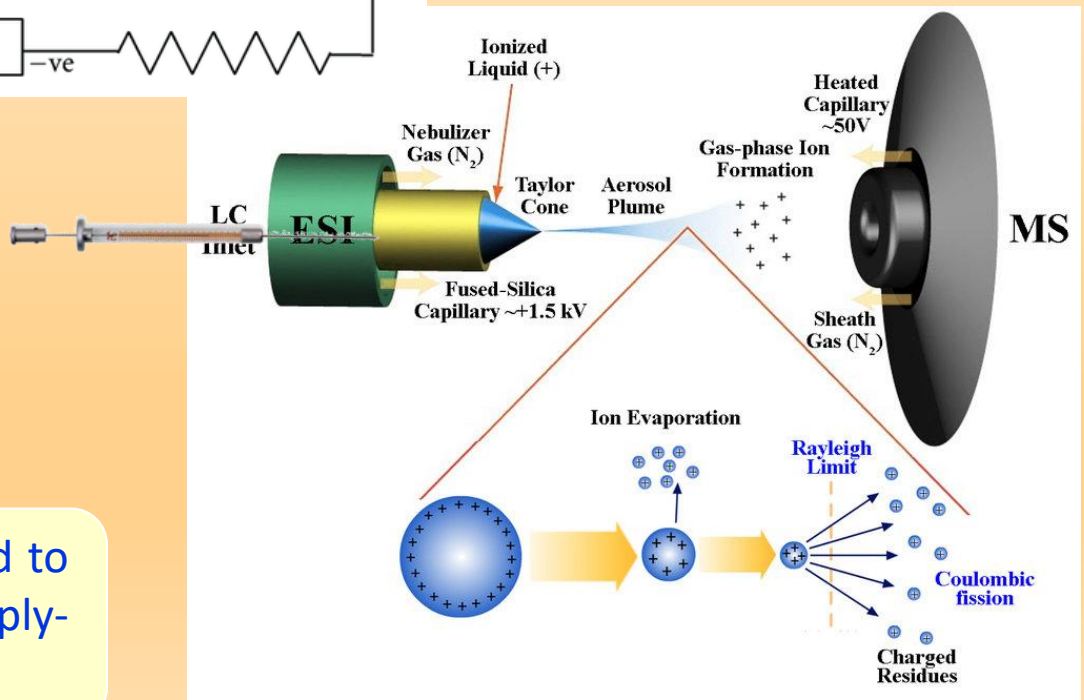

as the size of the droplets decreases, the repulsion between charges of the same sign increases cousing droplet to explode

Solvent droplets containing the charged molecules come out from the capillary:

negative positive

Depends on the solvent

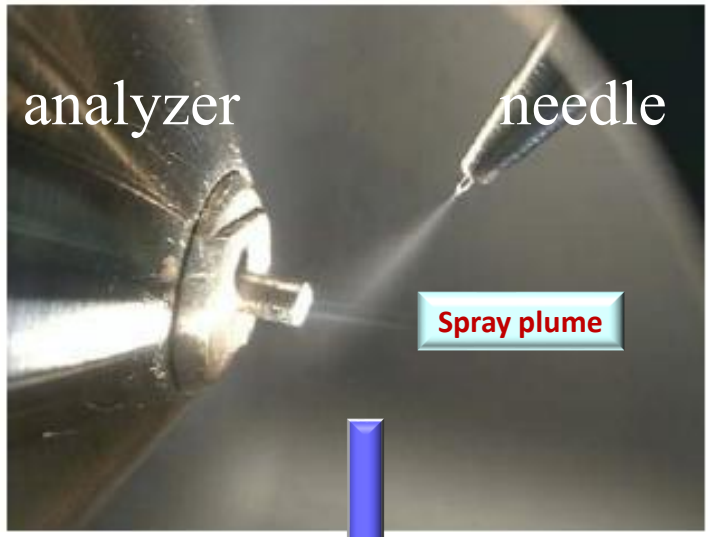
The solvent has to be evaporated to obtain Mono-charged and Multiply-charged molecular ions

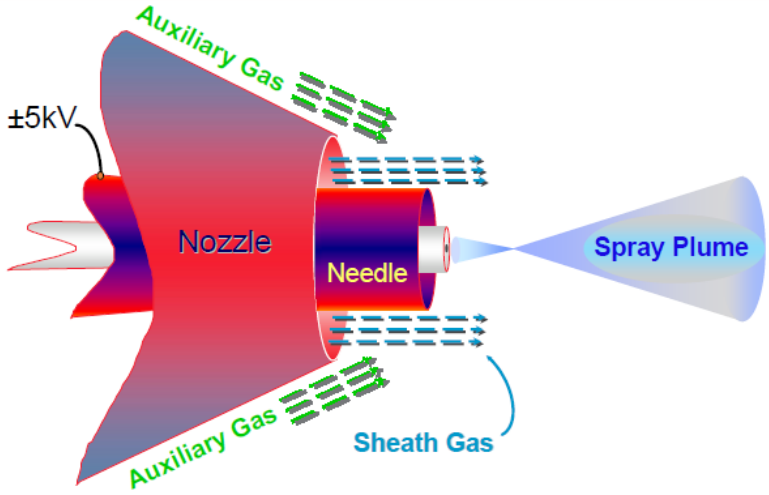


# Evaporation occurs because:

The **high potential** applied to the end of the electrospray needle allows to spray the solution into a myriad of charged droplets, containing the sample.

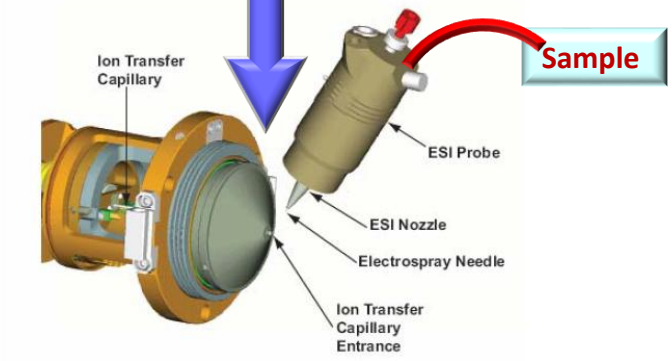
The **nitrogen gas** is applied to the spray plume to help its nebulization and to improve the desolvation of the droplets




Thermo 17 ThermoFisher SCIENTIFIC

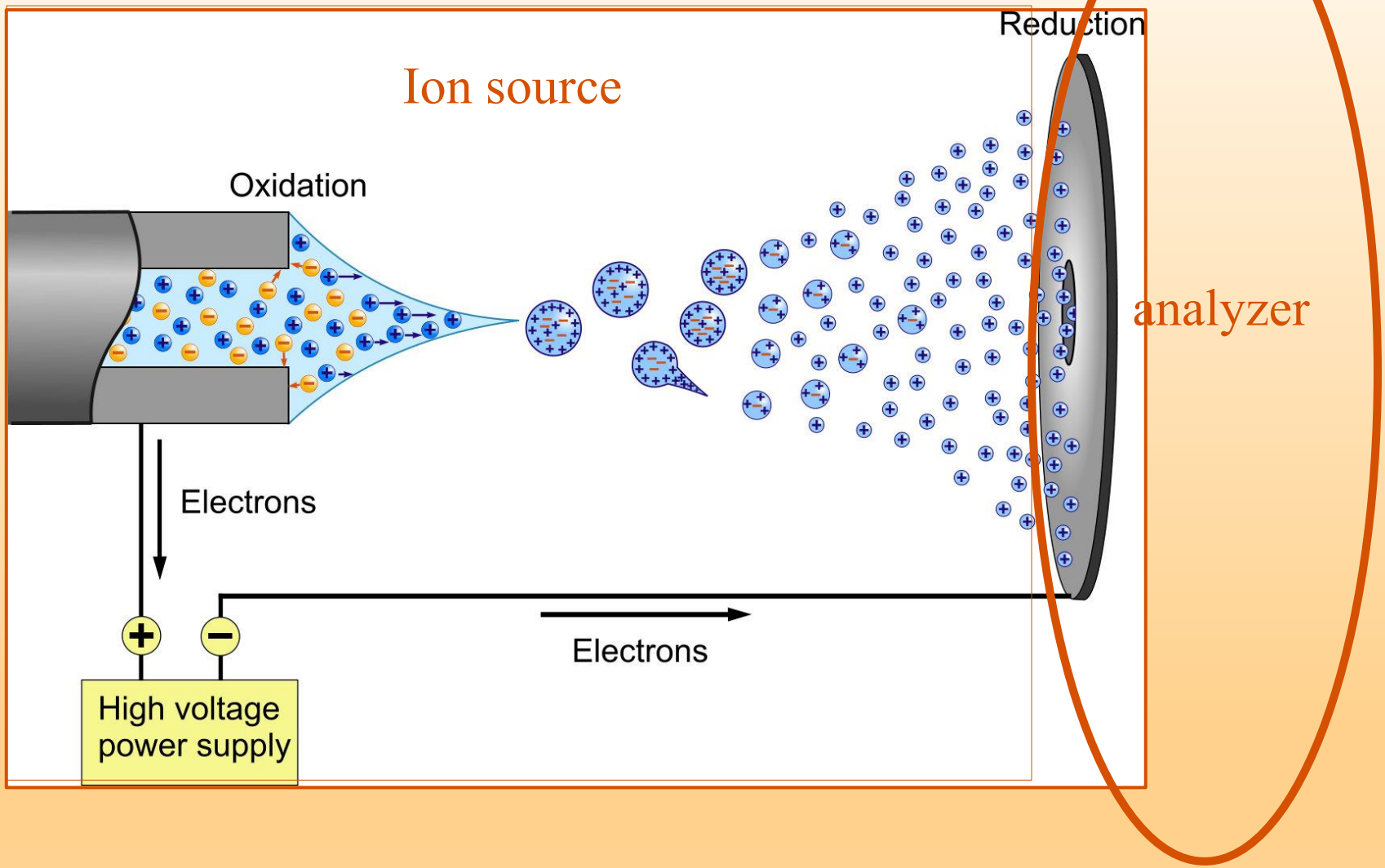
When sheath gas is used, nitrogen is applied as an inner coaxial gas (when used in tandem with auxiliary gas), helping to nebulize the sample solution into a fine mist as the sample solution exits the ESI or APCI nozzle. When auxiliary gas is being used, nitrogen flows through the ion source nozzle, the vapor plume is affected; the spray is focused and desolvation is improved. The auxiliary gas also helps lower the humidity in the ion source. Typical auxiliary gas flow rates for ESI and APCI are 10 to 20 units. Auxiliary gas is usually not needed for sample flow rates below 50 µL/min.




Thermo 25 ThermoFisher SCIENTIFIC

The ESI probe includes the ESI sample tube, needle, nozzle, and manifold. Sample and solvent enter the ESI probe through the sample tube. The sample tube is a short section of 0.1 mm ID fused-silica or metal capillary tubing that extends from a fitting secured to the ESI source housing, through the ESI probe and into the ESI needle, to within 1 mm from the end of the ESI needle. The ESI needle, to which a large negative or positive voltage is applied (typically ±3 to ±5 kV), sprays the sample solution into a fine mist of charged droplets. The ESI nozzle directs the flow of sheath gas and auxiliary gas at the droplets. The ESI manifold houses the ESI nozzle and needle and includes the sheath gas and auxiliary gas plumbing. The sheath gas plumbing and auxiliary gas plumbing deliver dry nitrogen gas to the nozzle.

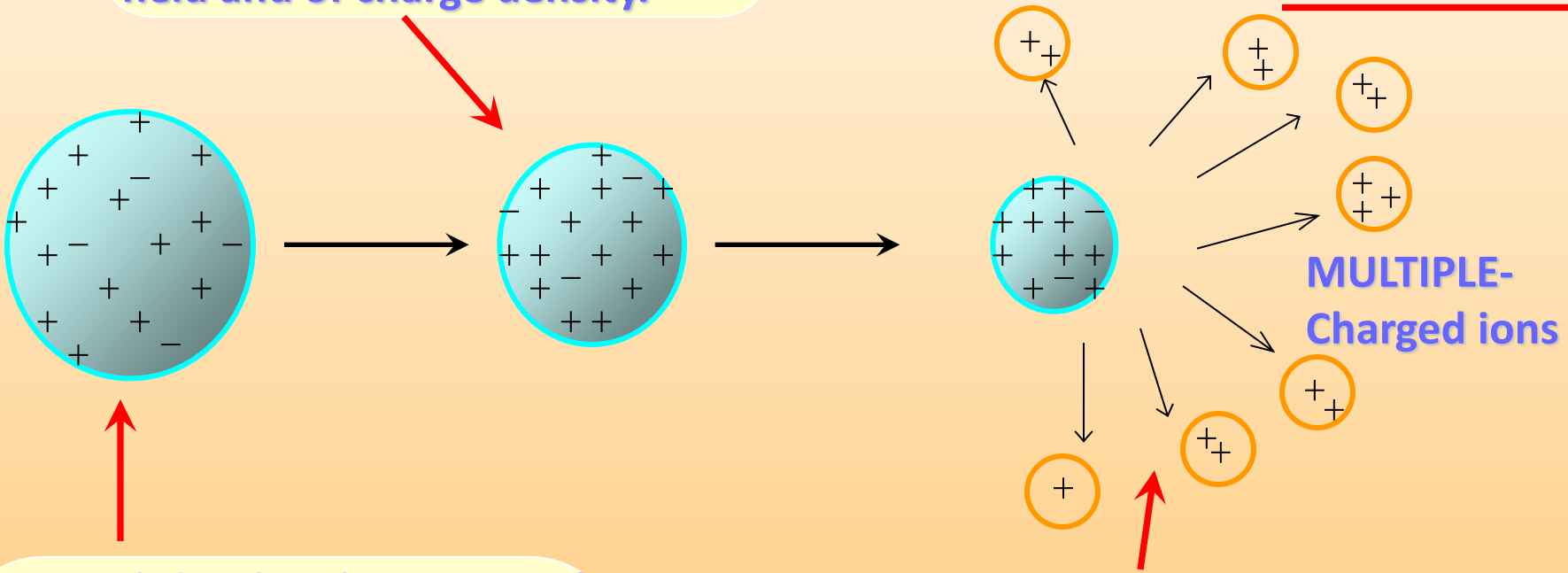
# Where do ions go?



# Formation of multiply-charged ions in the ESI source

Solvent vaporization promotes the increase of drop electric field and of charge density.

MULTIPLY-Charged ions are released in the gas phase and are pushed towards MS analyzer by electric field



MULTIPLE-Charged ions

Aerosol droplets have ions of opposite charge. However, one is dominant. In acidic solution usually the positive one.

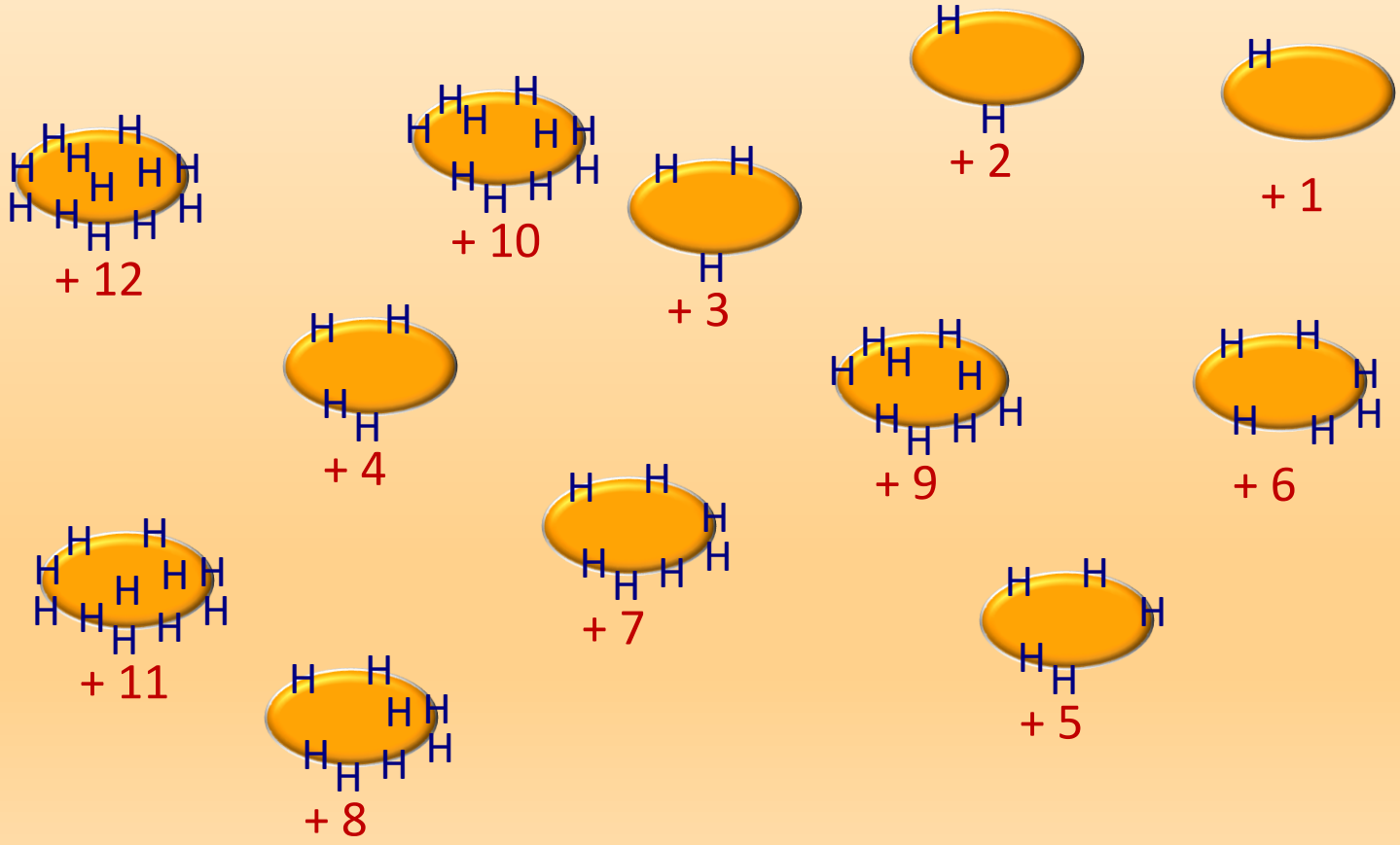
When the desolvation reaches a critical point, the drop explodes (coulombic explosion)

https://www.youtube.com/watch?v=i0PofjRIoMg

Protein with mass = 10000 Da

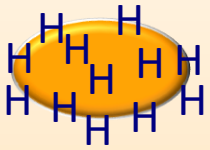
In an acidic solution (pH < 3) it tends to acquire protons. For a protein with 12 positively charged groups (which?), it could generate 12 differently protonated multiply-charged ions.

ESI source facilitates protonation and generation of multiply-charged ions from solvent droplets >>> transition to the gas phase



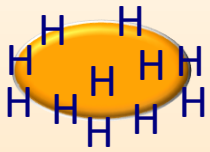
The multiple-charged ions of the same protein have different m/z ratios (MASS/CHARGE) (Thomson)

Protein with mass = 10000 Da



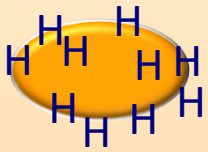
+ 12

m/z = (10000 +12)/12 = 834.3 $[M+H^+]^{+12}$



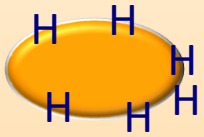
+ 11

m/z = (10000 +11)/11 = 910.1 $[M+H^+]^{+11}$



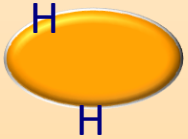
+ 10

m/z = (10000 +10)/10 = 1001 $[M+H^+]^{+10}$



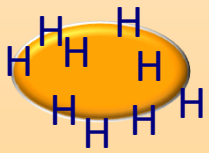
+ 6

m/z = (10000 +6)/6 = 1667.7 $[M+H^+]^{+6}$



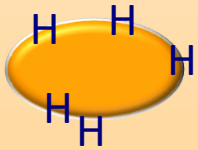
+ 2

m/z = (10000 +2)/2 = 5001 $[M+H^+]^{+2}$



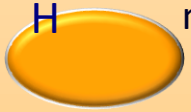
+ 9

m/z = (10000 +9)/9 = 1112.1 $[M+H^+]^{+9}$



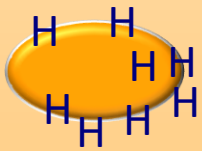
+ 5

m/z = (10000 +5)/5 = 2001 $[M+H^+]^{+5}$



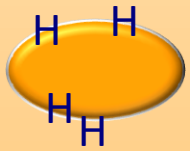
+ 1

m/z = (10000 +1)/1 = 10001 $[M+H^+]^{+1}$



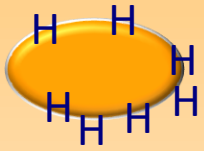
+ 8

m/z = (10000 +8)/8 = 1251.0 $[M+H^+]^{+8}$



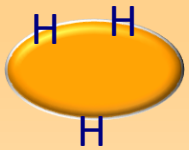
+ 4

m/z = (10000 +4)/4 = 2501 $[M+H^+]^{+4}$



+ 7

m/z = (10000 +7)/7 = 1429.6 $[M+H^+]^{+7}$



+ 3

m/z = (10000 +3)/3 = 3334.3 $[M+H^+]^{+3}$

**ESI MS SPECTRUM: is a plot where the multiple-charged ions with different m/z values are related to their relative abundance.**

In the ESI MASS SPECTRUM a Gaussian of multiple-charged ions generated by the same protein is detected.

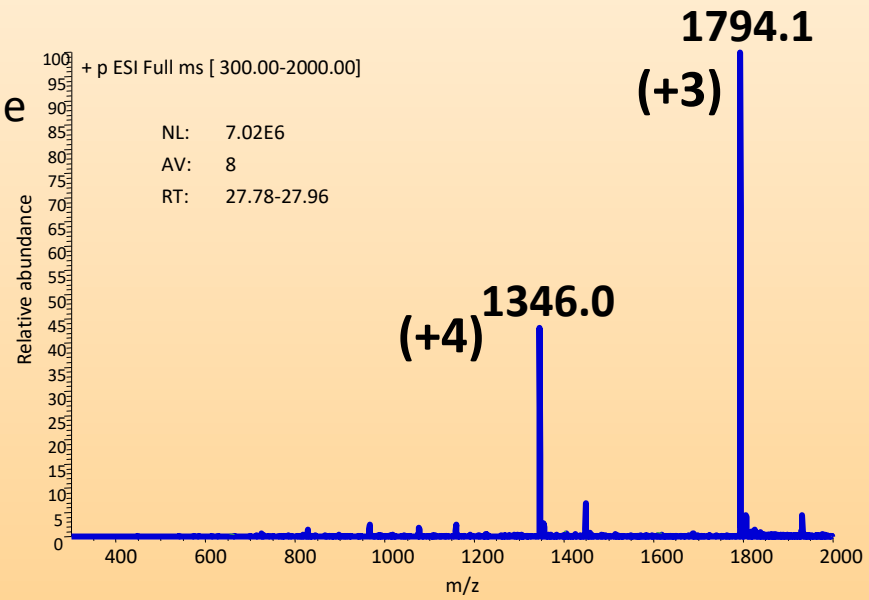
If more than 1 protein is present, the gaussians of m/z ions produced by every protein are superimposed.

**At the same setting of ESI parameters, at the same experimental and instrument conditions: EACH PEPTIDE/PROTEIN GENERATES A CHARACTERISTIC ESI SPECTRUM regardless of the sample.**

# ESI MS spectrum of a peptide = is simple, with few m/z ions

Example: ESI MS spectrum of Statherin, salivary phosphopeptide with 43 amino acid residues,

- capacity of proton acquisition = 5 $H^+$ = until 5 positive charges (ammino-terminal end, 1 Lys, 3 Arg),
- Nr of Theoretical m/z ions = 5
- Experimentally, we can observe the +3 and the +4 ions in the spectrum,
  they are enough to recognize the peptide




m/z = (5979 +4)/4 =
1346 $[M+H^+]^{+4}$

m/z = (5979 +3)/3 =
1794 $[M+H^+]^{+4}$

You have selected STAT_HUMAN (P02808) from UniProtKB/Swiss-Prot:

Statherin precursor
Signal in positions 1-19 has been removed.

*Information within the data-bank*

- Chain Statherin at positions 20 - 62 [Theoretical pI: 6.25 / Mw (average mass): 5219.76]

| mass | position | #MC | modifications | conflicts | variants | alternative isoforms | peptide sequence |
|---|---|---|---|---|---|---|---|
| 5219.7605 | 20-62 | 0 | PHOS: 21, 22 | 5379.7205 | 62:MISS 25-34 | | DSSEEKFLRRIGRFGYGYGP YQPVPEQPLYPQPYQPQYQQ YTF |

**Can we calculate the m/z values expected for a protein with a known mass value?**

**We calculate the m/z on the base of the theoretical mass of the protein, the detection of accurate m/z values in the experimental ESI spectrum and their correspondence with the theoretical ones is an important factor for protein identification**

*Example: Statherin*

You have selected STAT_HUMAN (P02808) from UniProtKB/Swiss-Prot:

Statherin precursor
Signal in positions 1-19 has been removed.

- Chain Statherin at positions 20 - 62 [Theoretical pI: 6.25 / Mw (average mass): 5219.76]

| mass | position | #MC | modifications | conflicts | variants | alternative isoforms | peptide sequence |
|---|---|---|---|---|---|---|---|
| 5219.7605 | 20-62 | 0 | PHOS: 21, 22 | 5379.7205 | 62:MISS | 25-34 | DSSEEKFLRRIGRFGYGYGP YQPVPEQPLYPQPYQPQYQQ YTF |

*Theoretical average mass (Mav) of the mature peptide (with 2 phosphorylations)*

| | | | | | |
|---|---|---|---|---|---|
| Moleclular mass | | 5379.7 | | | |
| | | | 0 | Eventual Mod | |
| | | | 0 | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | m/z | | | | |
| | | | | | |
| 1 | 5380.70 | (5379.7+1)/1 = (M+n)/n | | | |
| 2 | 2690.85 | (5379.7+2)/2 | | | |
| 3 | 1794.23 | (5379.7+3)/3 | | | |
| 4 | 1345.93 | (5379.7+4)/4 | | | |
| 5 | 1076.94 | (5379.7+5)/5 | | | |

Can I see these ions in the mass spectrum?

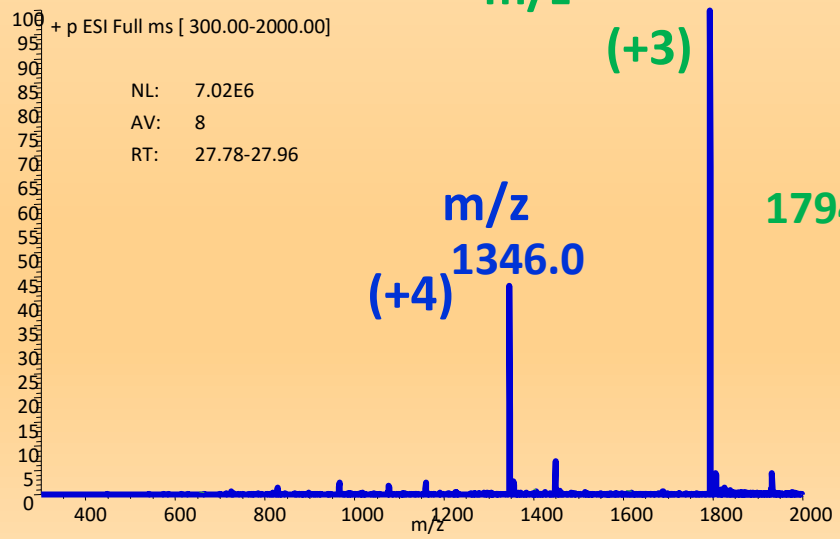
In the ESI MS spectrum:

- It is not necessary to observe all the possible ions (the m/z range in the plot is usually 200- 2000 m/z)
- It is not necessary to observe the monocharged ion

**IF we DON'T know the M of the protein…**
**how do we derive protein mass from the ESI spectrum?**

**DECONVOLUTION**

**In the ESI spectrum of statherin we observe 2 consecutive ions (+3) and (+4)**
**We know the charge…**




**Ion with charge «+3»** → **[M] = (m/z * z)-z =** → **1794.1*3 – 3 = 5379.3 Da**

**Ion with charge «+4»** → **1346.0*4 – 4 = 5380.0 Da**

**It continues in the next…..**

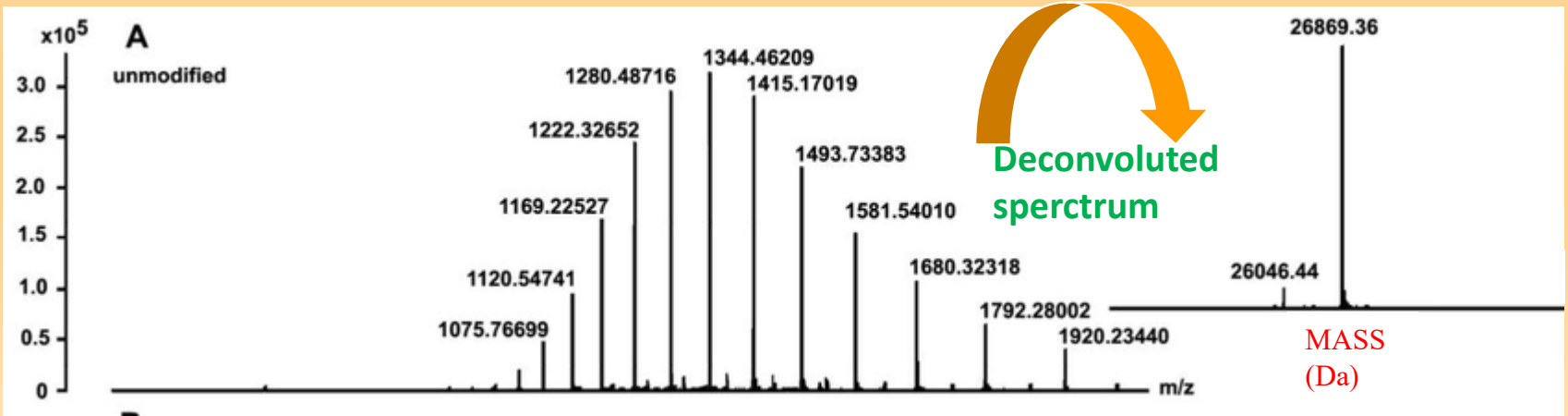
**We can average the two values = (5379.3+5380)/2 = 5379.7 Da**

**(Experimental (deconvoluted) average mass value)**

**The experimental average mass (Mav) corresponds to the expected Mav of the peptide statherin (Th. Mav = 5379.7 Da)**

This is an important information to identify a peptide/protein.

More accurate and precise is the experimental Mav more accurate and precise is the identifcation



The procedure that allows determining the experimental MW starting from the m/z is known as:

**DECONVOLUTION**

It is a procedure that can be performed manually for a simple ESI spectrum where the charge of the multply-charged ions is well determined

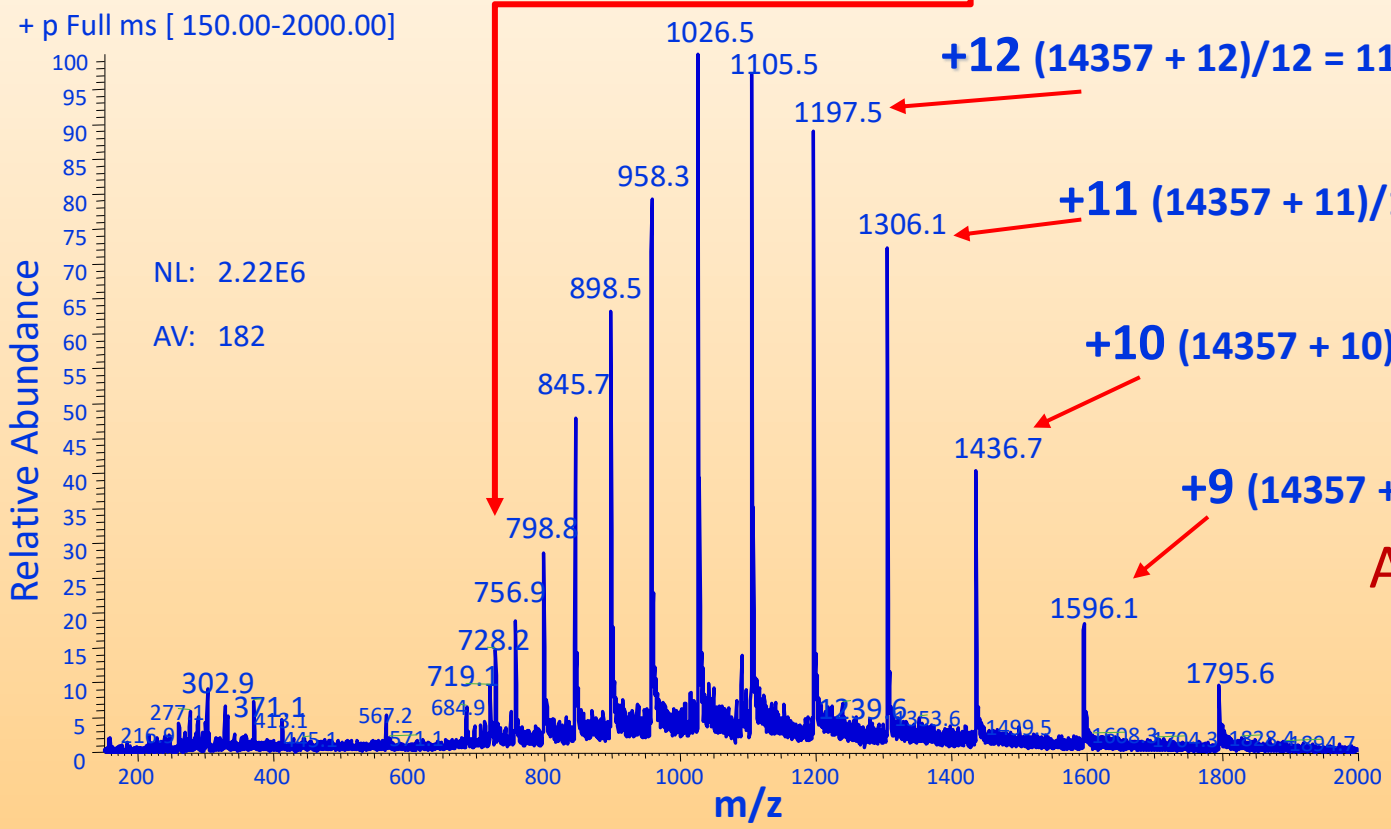
It is quickly and easily performed automatically by an appropriate software, able to individuate the charge of the m/z ions and calculate the mass values.

**ATTENTION!**

**We talk about peptides when the molecular mass is under 10 kDa,**

**about proteins when the molecular mass is major than 10 kDa,**

ESI MS spectrum of a protein = can be complex, with many m/z ions. Longer sequence = bigger probability to have protonable residues and thus to generate many multiply-charged ions at low m/z value




**+12** (14357 + 12)/12 = 1197.5 12 $H^+$

**+11** (14357 + 11)/11 = 1306.1 11 $H^+$

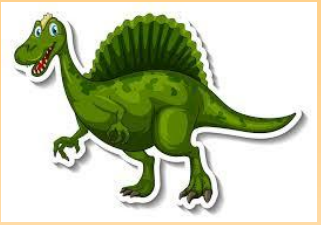
**+10** (14357 + 10)/10 = 1436.7 10 $H^+$

**+9** (14357 + 9)/9 = 1596.1

Acquires 9 $H^+$

as the charge increases , the m/z value decreases

**We observe a gaussian of different ions at different m/z values originating from the same protein with theoretical Mav = 14357 Da**



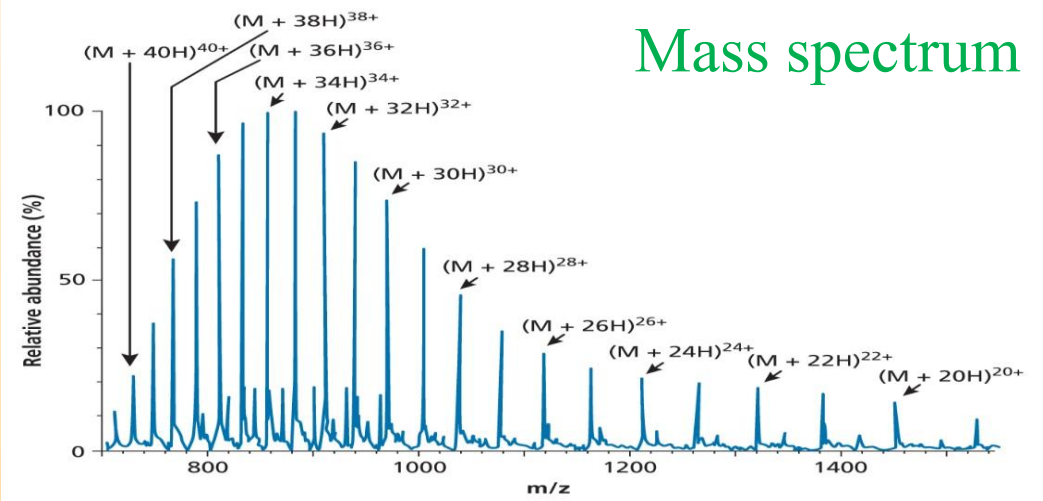
Big proteins can be detected with a ESI source, untill 70000 Da (working in the best conditions), since big proteins may generates, in the source, many highly protonated ions (with low m/z values and thus detectable in the range of the ESI spectrum)

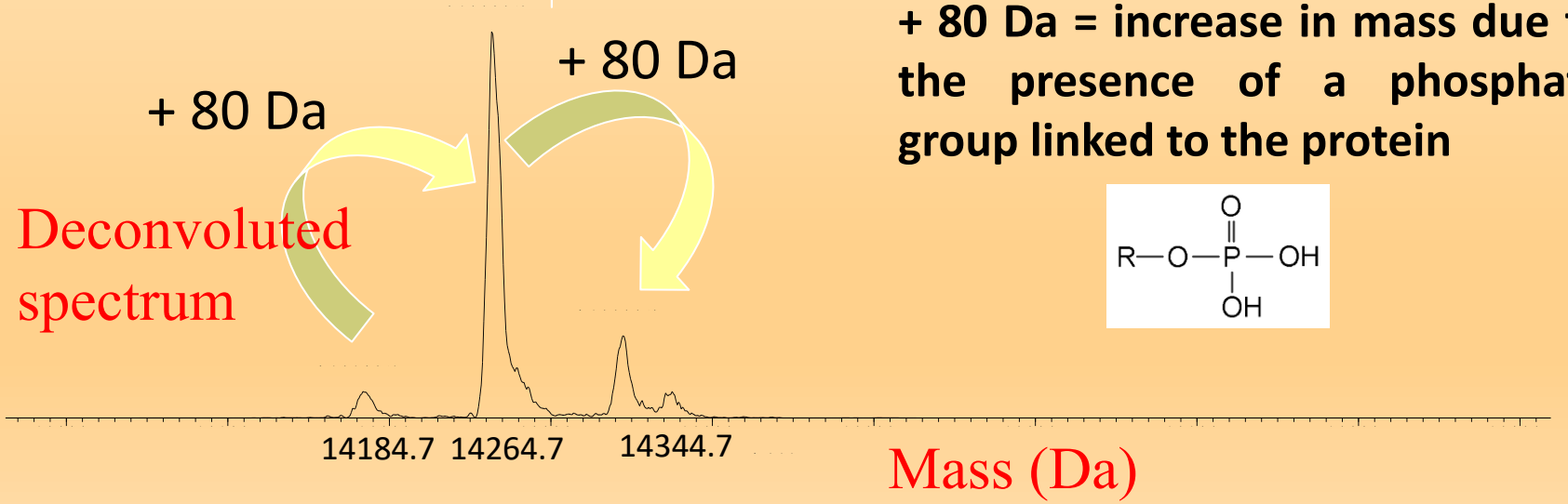
# Detection of PTMs

ESI-MS analysis of human salivary proteins

**Three almost overlapping gaussians of multiply-charged ions: series A, B and C**

**They can belong to proteins correlated each other**

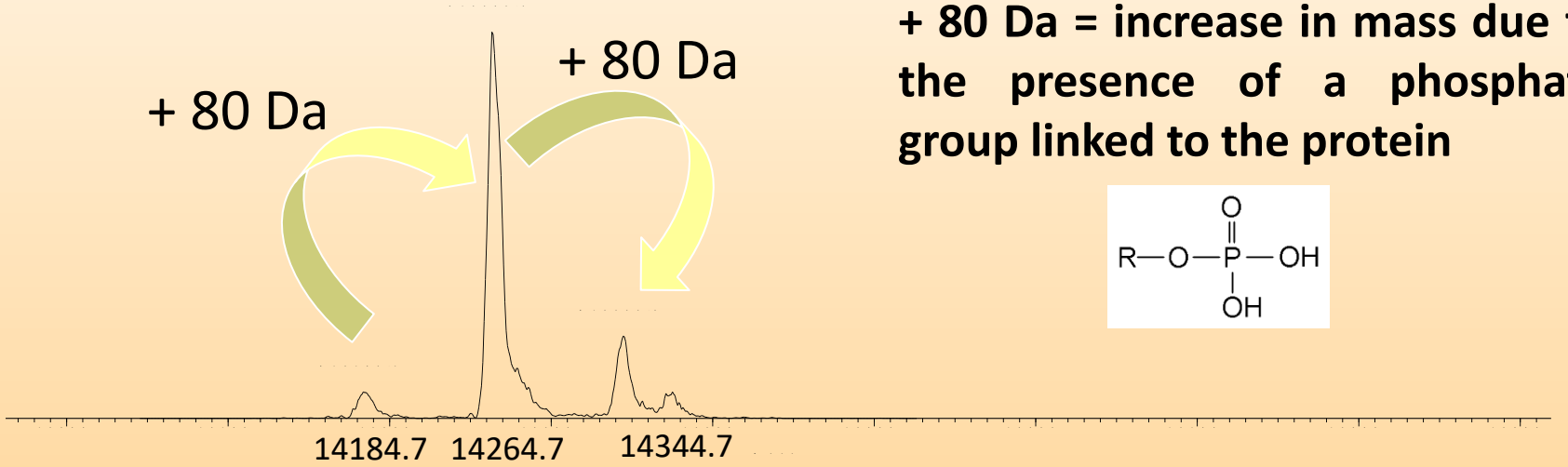



**+ 80 Da = increase in mass due to the presence of a phosphate group linked to the protein**

# Indeed…

**The deconvolution of the previous mass spectrum revealed three main mass values differing of 80 Da:**




**+ 80 Da = increase in mass due to the presence of a phosphate group linked to the protein**



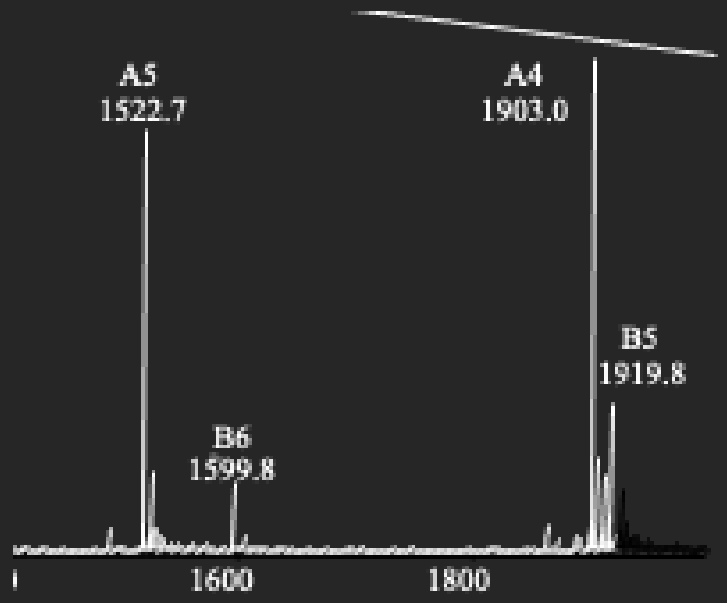

**These experimental average mass values correspond to those of three proteoforms of the same protein present in human saliva:**

| | |
|---|---|
| **Cystatin S (theoretical Mav: 14184.7 Da)** | |
| **Cystatin S1 (theoretical Mav: 14264.7 Da)** | = Cyst S mono-phosphorylated |
| **Cystatin S2(theoretical Mav: 14344.7 Da)** | = Cyst S di-phosphorylated |

# ADVANTAGES OF ESI IONIZATION

- The system is easily adaptable to automation by interfacing with liquid chromatography.
- There is not interferences from the mobile phase.
- Very sensitivity for high mass values (> 400 m/z)

Based on the EI mass spectrum of compound A, determine its Average molecular mass value (Mav)




Ion with charge «5»

Ion with charge «4»

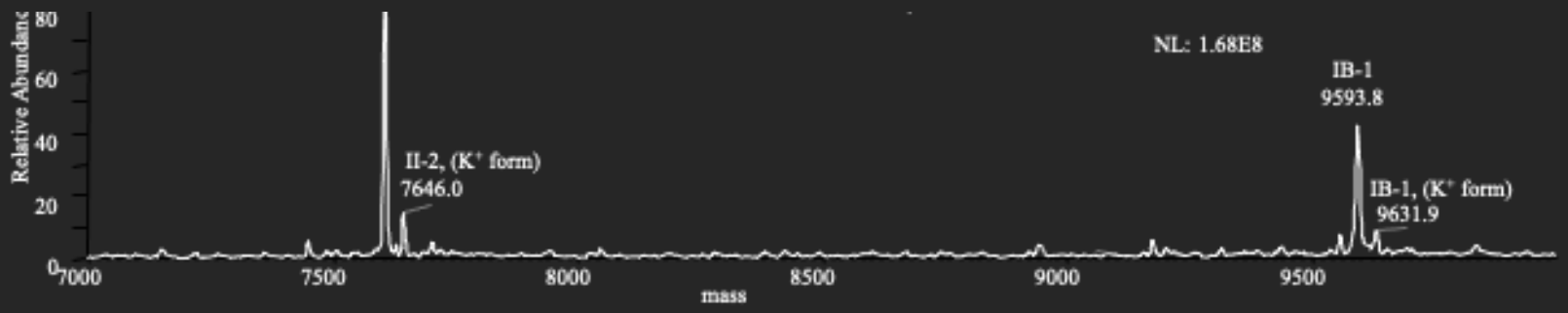
[M] = (m/z * z)-z =

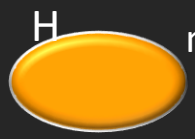
1522.7*5 – 5 = 7608.5 Da

1903.0*4 – 4 = 7608.9 Da

Mav= 7608.5+7608.0/2= 7608.2

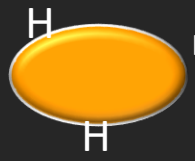
# Based on the deconvoluted ESI mass spectrum of compound IB-1, determine the +1, +2 and +3 m/z values



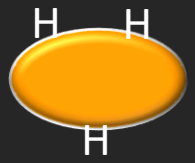

+ 1

m/z = (9593.8+1)/1 = 9594.1 $[M+H^+]^{+1}$




+ 2

m/z = (9593.8 +2)/2 = 4798.9 $[M+H^+]^{+2}$




+ 3

m/z = (9593.8 +3)/3 = 3198.0 $[M+H^+]^{+3}$

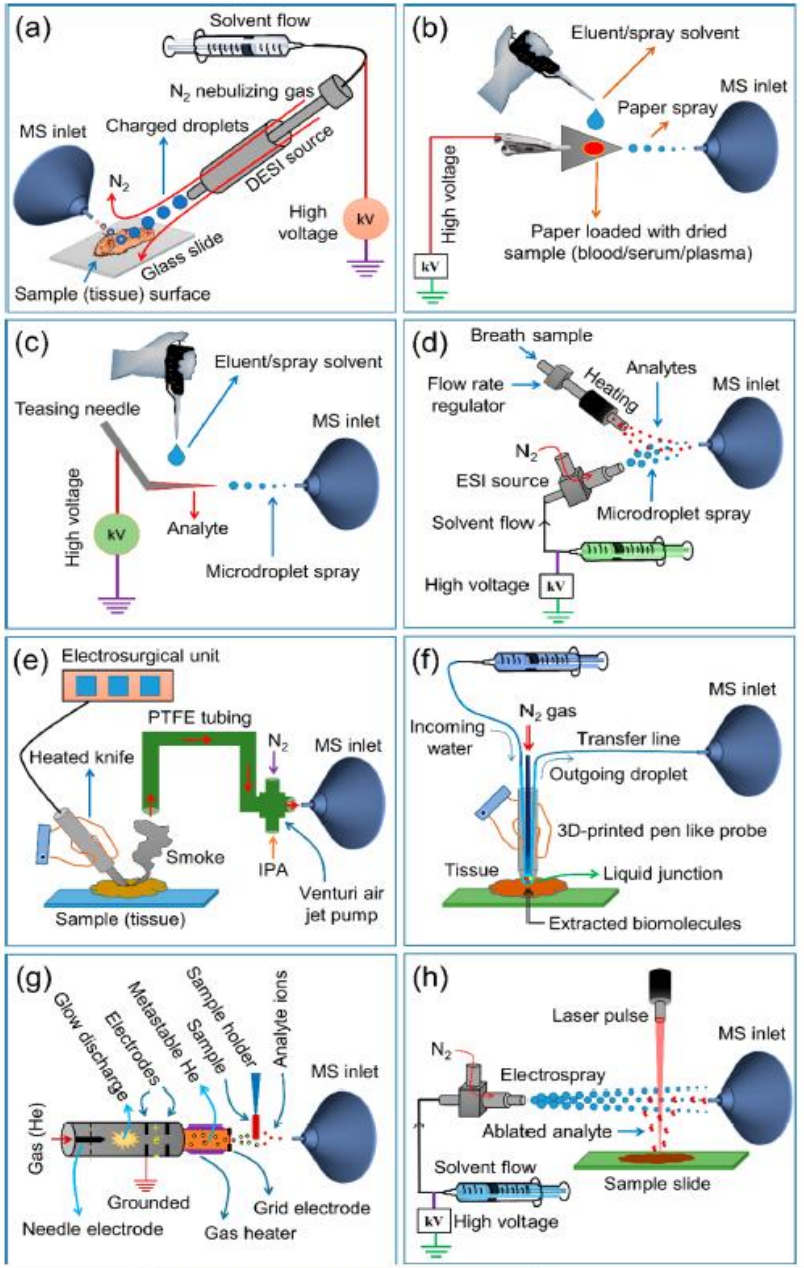
https://www.youtube.com/watch?v=9AWBAI-Owzk&t=149s

# AI: AMBIENT IONIZZATION

Instantaneous capture of molecular fingerprints from **native samples**.

AI refers to a variety of **combinations of sample introduction systems**, and desorption and ionization methods that allow **direct analysis of sample surfaces in open-air conditions**, with little or no sample pretreatment and, in most cases through noninvasive procedures

http://pubs.acs.org/journal/acsodf




Empowering Clinical Diagnostics with Mass Spectrometry
Shibdas Banerjee*
Cite This: ACS Omega 2020, 5, 2041−2048
Read Online

**Figure 3.** Schematic diagrams of (a) desorption electrospray ionization mass spectrometry (DESI-MS), (b) paper spray ionization mass spectrometry (PSI-MS), (c) touch spray ionization mass spectrometry (TSI-MS), (d) extractive electrospray ionization mass spectrometry (EESI-MS), (e) rapid evaporative ionization mass spectrometry (REIMS) or iKnife, (f) MassSpec Pen, (g) direct analysis in real-time mass spectrometry (DART-MS), and (h) matrix-assisted laser desorption electrospray ionization mass spectrometry (MALDESI-MS).

If you consider the molecular weight of the analytes in relation to the polarity, it is evident that **the several ionization techniques are apropriate for different kinds of molecules** (different for dimension and polarity)

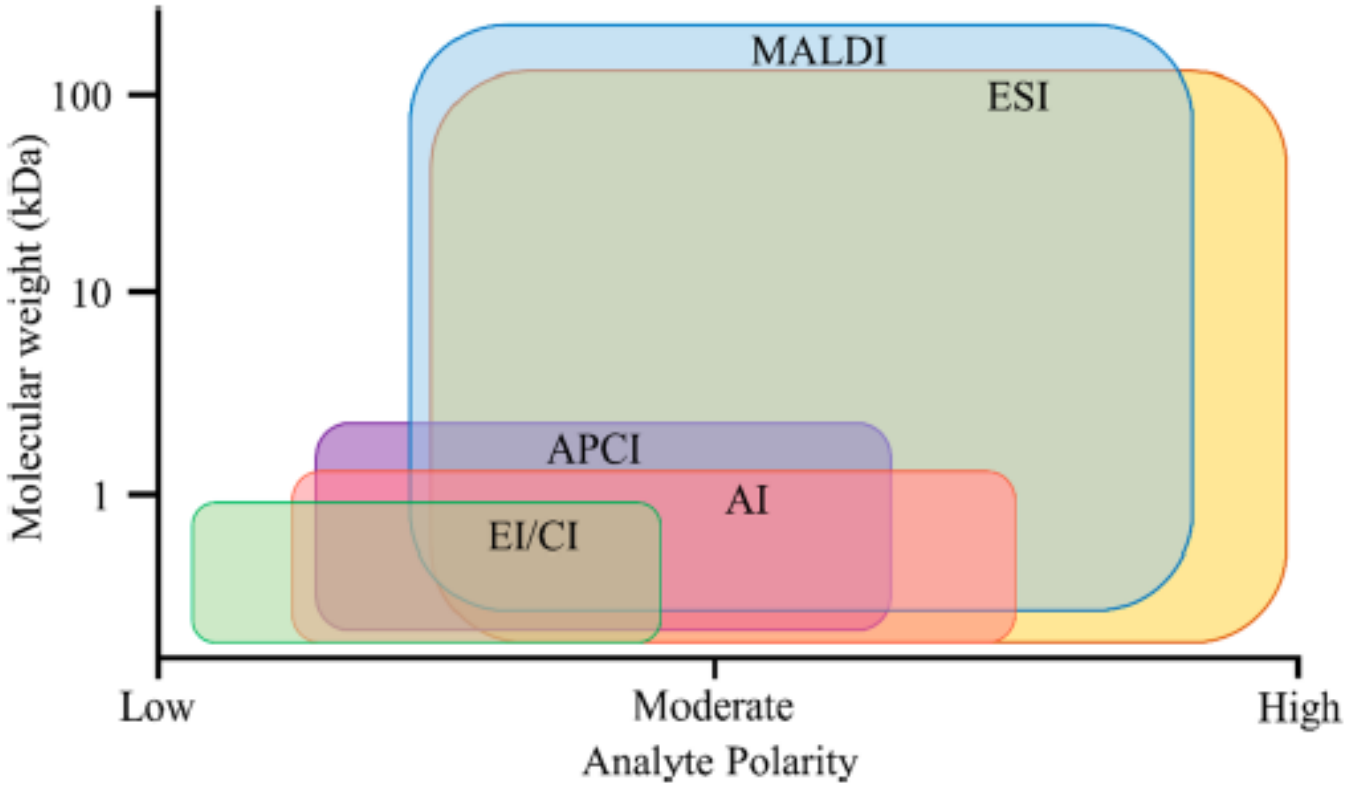



Figure 1. Portrayal ranges of different ionization techniques in the discovery of biomarkers of various molecular weights and polarity.

Empowering Clinical Diagnostics with Mass Spectrometry
Shibdas Banerjee*
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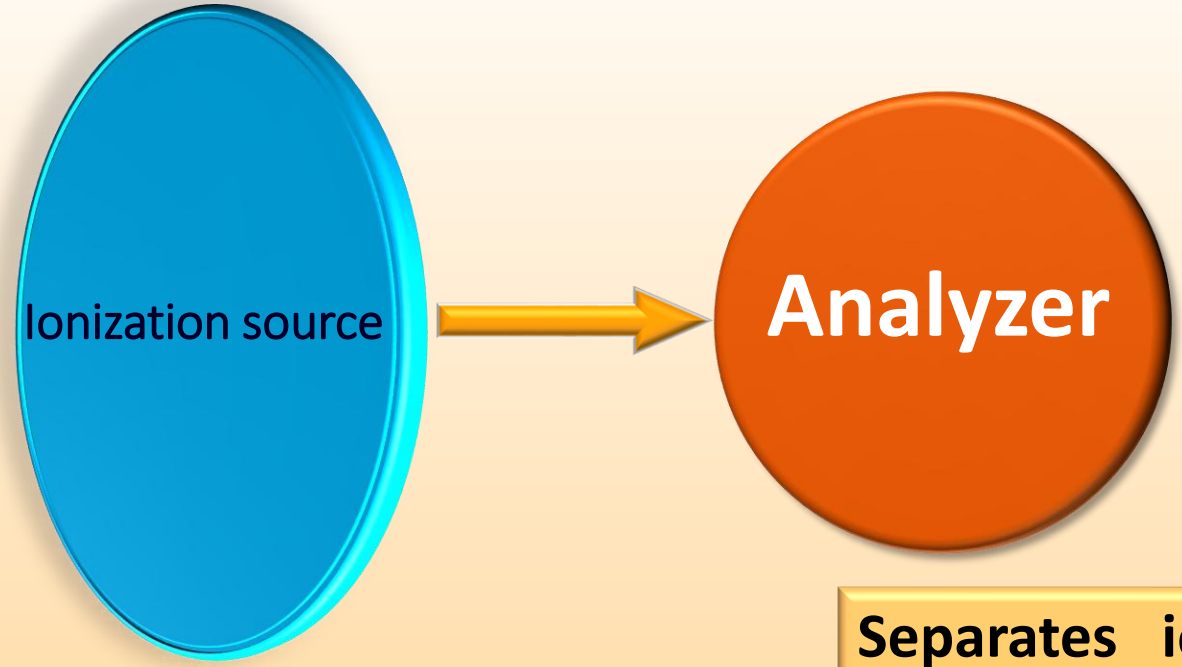
Read Online

# TYPES OF IONIZATION AND INFORMATION OBTAINED

| | MW | Struttura |
|---|---|---|
| EI | No | Yes |
| CI | Yes - low MW | Limited |
| | | |
| ES | Yes - low MW | Yes* |
| APCI | Yes - medium MW | Yes* |
| MALDI | Yes - high MW | Very limited |

* Through MS/MS (Tandem MS) experiments

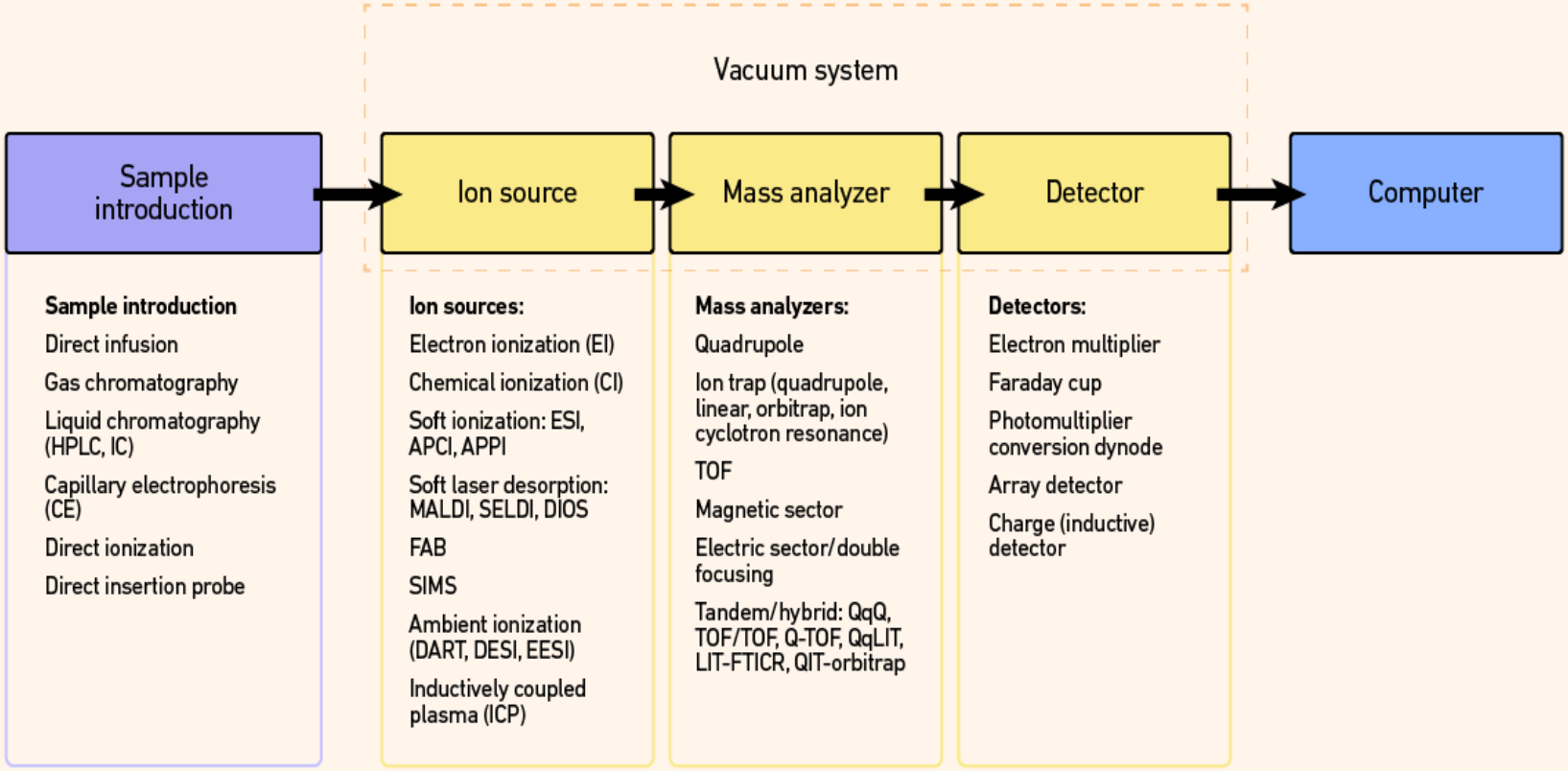


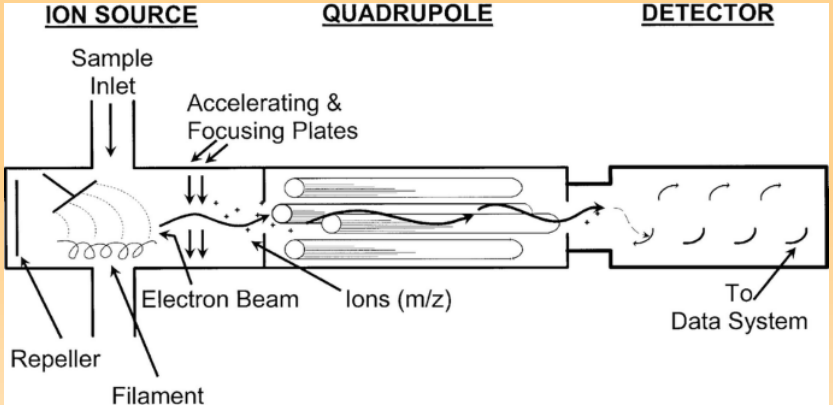

**It must generate ions in the gas phase. The ions are routed to the analyzer that must separate them**

**Separates ions with different m/z value**

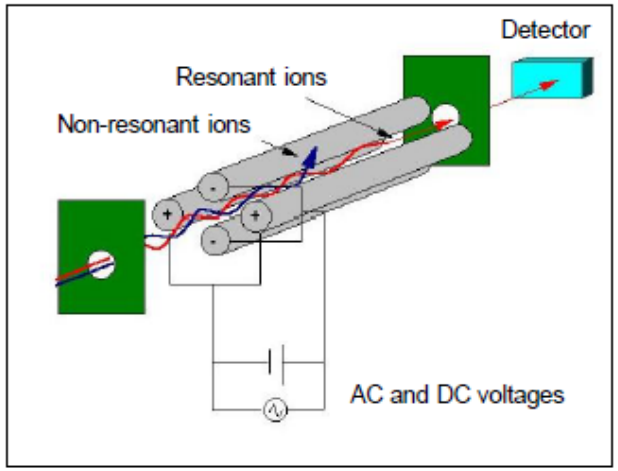
- Magnetic analyzer
- Quadrupole analyzer
- Ion-trap analyzer
- TOF (time of flight)
- Etc….

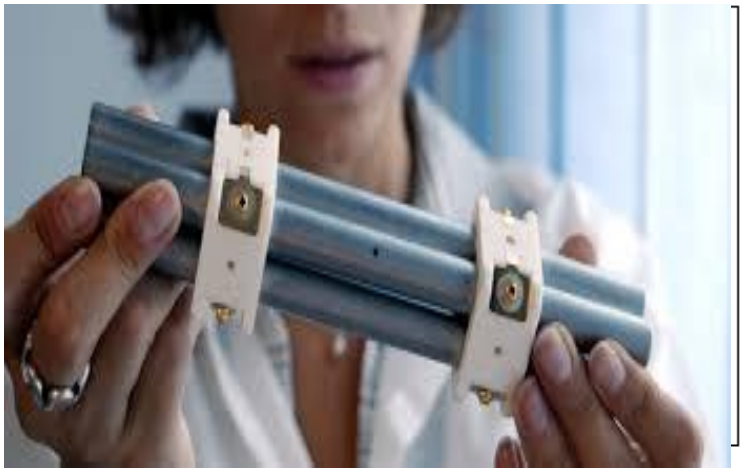
# Mass Spectrometer Varieties



# Quadrupole Mass Analyzer (THE SIMPLIEST)

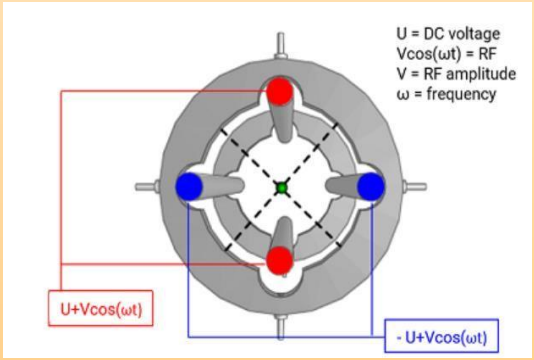


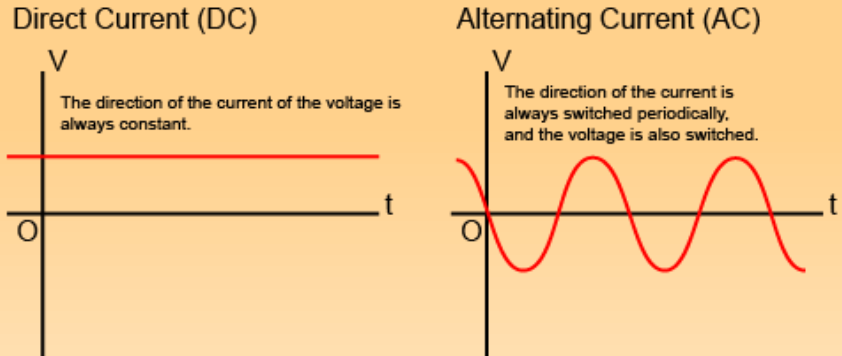
**It is a small and** cheapest **analyzer. Usually, quadrupoles have a low resolution and can only detect m/z up to 4000 Th.**

9 www.chemistry.adelaide.edu.au Angeletti, Einstein CoM 2007 W.M. Keck MS Lab

- It consists of four cylindrical rods, set parallel to each other located into a straight tube under vacuum.
- The opposite rods carry like charges
- Each opposite rod pair is connected electrically with direct current (DC) , and an alternating current (AC) voltage in order to affect the movement of ions.

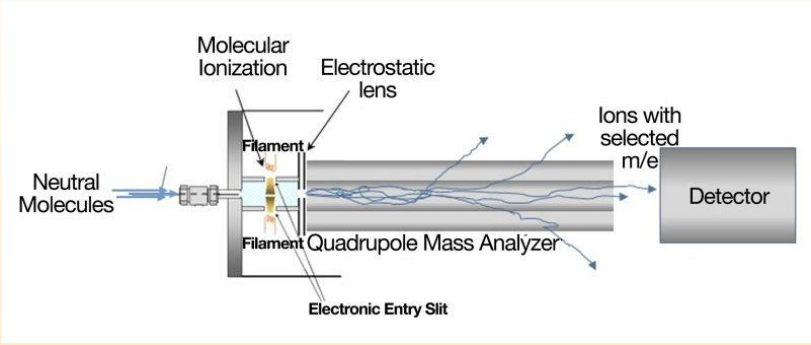



**Positive DC voltage repels positive ions**
**Negative DC voltage attracts positive ions**
**The chance of ion hitting the rod depends on the mass and charge of the ion and also on the strength of the field and frequency of the oscillation**



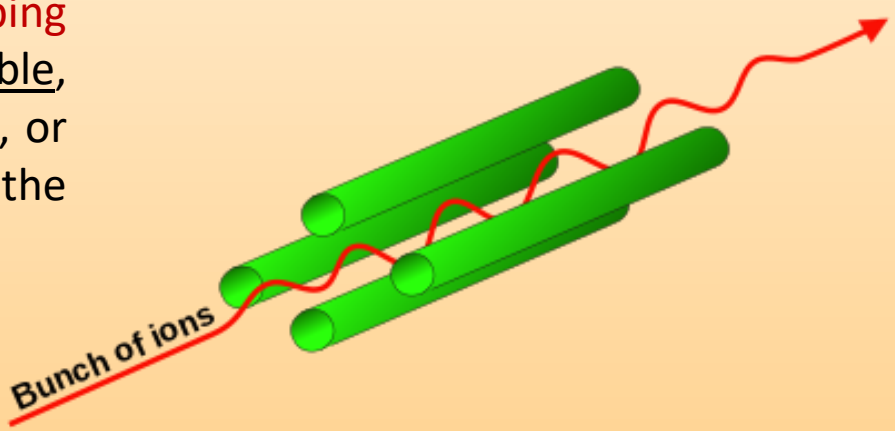

**Radio frequency** (**RF**) is the oscillation rate of an alternating electric current

# Quadrupole Mass Analyzer




- When a DC voltage is superimposed onto the AC voltage, ions of a specific *m/z* can be tuned to travel the quadrupole, while ions of higher or lower *m/z* values will be lost by collisions with the quadrupoles through unstable trajectories.

- Due to the electromagnetic field the Ions travel down the quadrupole between the rods describing oscillating trajectories, which can be stable, allowing the ion to escape from the quadrupole, or unstable leading to the collision of the ion with the bars of the quadrupole.




- The selection of ions with a specific *m/z* ratio is achieved by changing the polarity and the voltages that are applied to the rods.

- **Scanning a spectrum is achieved by simultaneously varying continuous and alternating voltage, keeping their ratio constant.**

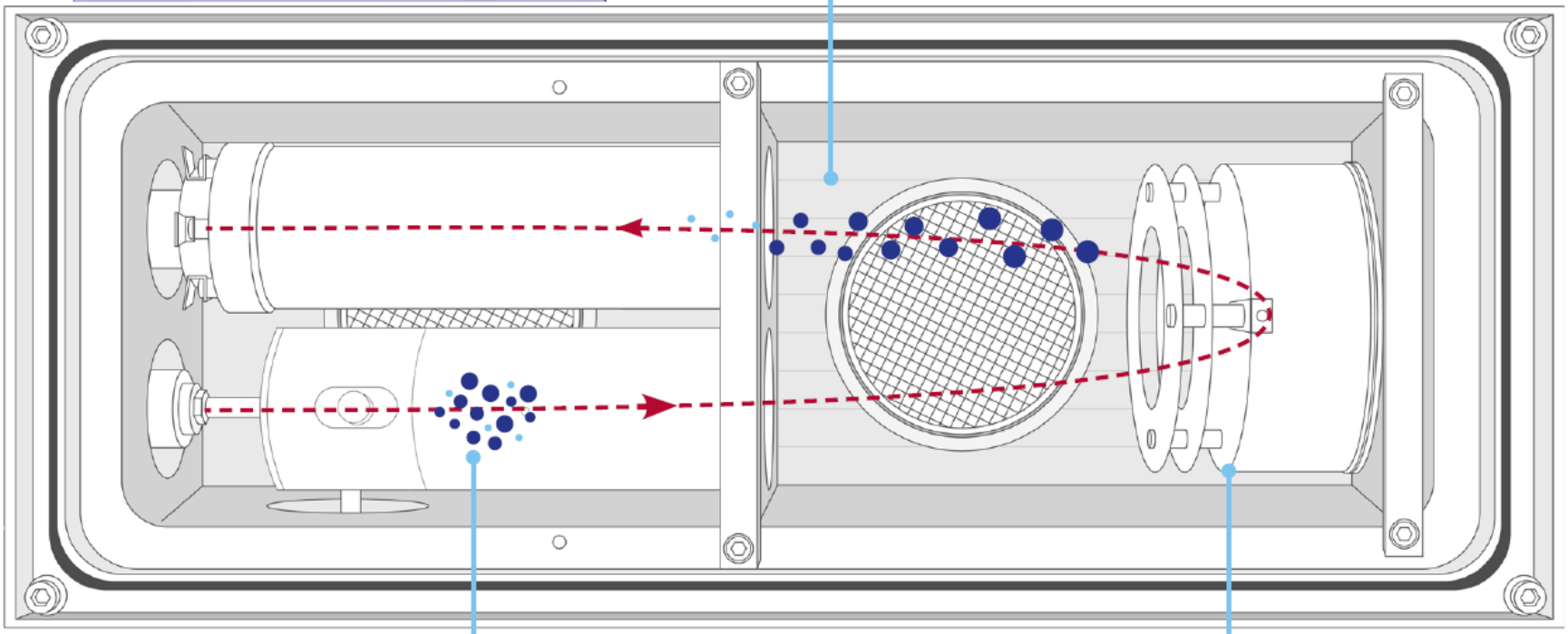
https://www.youtube.com/watch?v=eg-Vj_9xvP8

https://www.youtube.com/watch?v=qxPb9vFWdqo

# Time-of-flight analyzer (TOF)

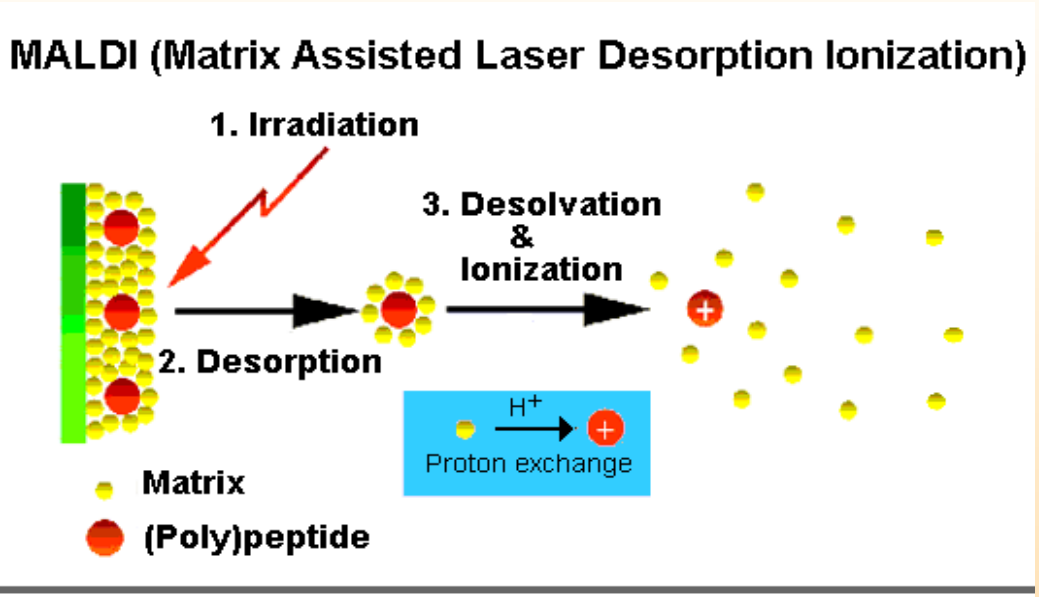
4 Molecular mass can be deduced from time of flight using appropriate calibration mixtures (peptides and proteins of known mass).

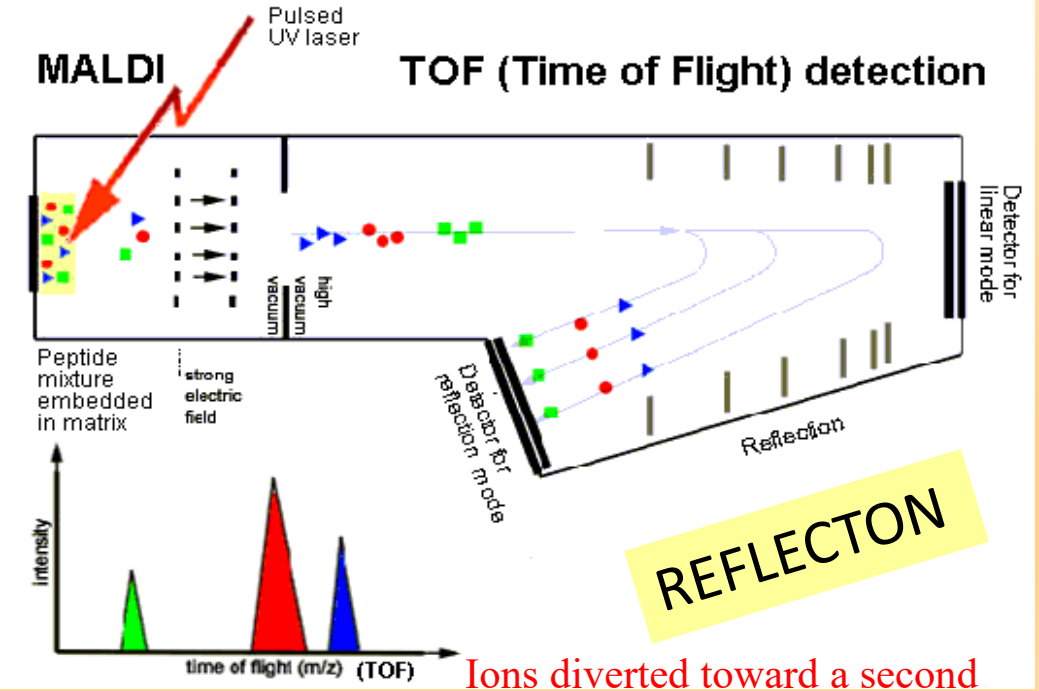
3 Small ions travel faster than large ones, thereby separating the ions (by m/z) before they reach the detector



1 Ions are accelerated into the flight tube from the ion source with the same initial kinetic energy

2 The multi-stage reflectron improves resolution by extending the flight path




REFLECTON

Ions diverted toward a second detector to extend the time of flight

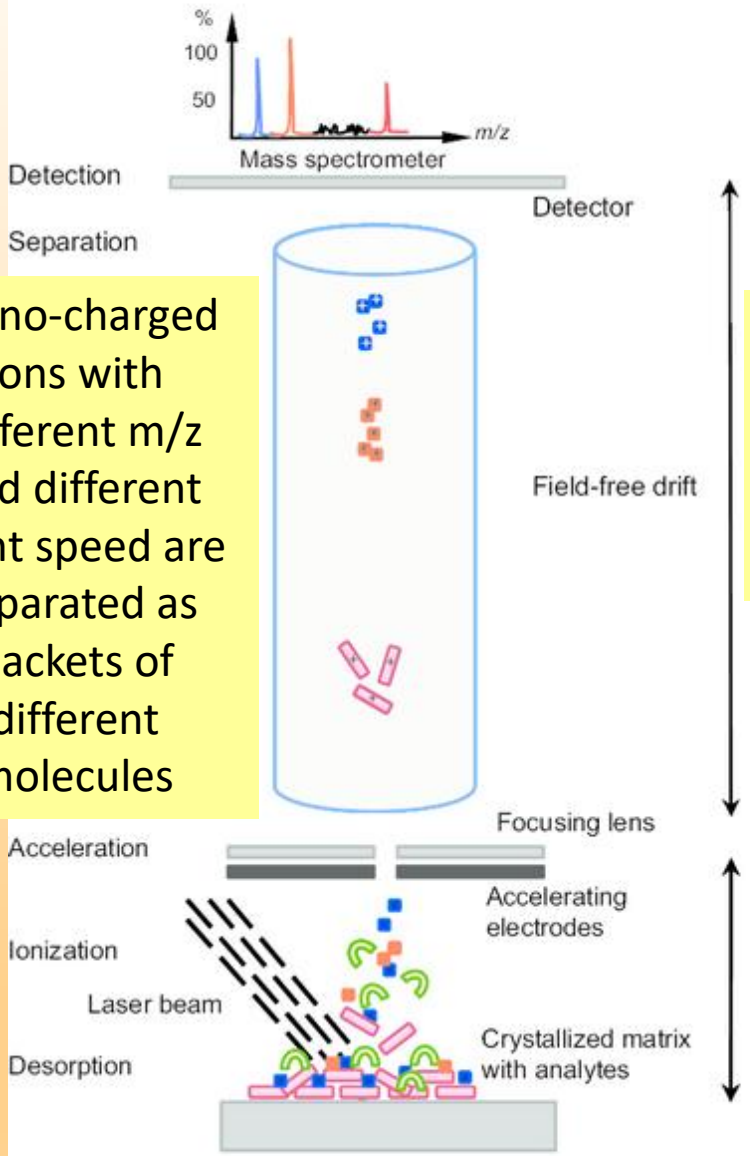
High sensitivity and virtually unlimited mass measuring range.

very big molecules ("Dumbo" proteins) can also flight in the TOF.....

MALDI-TOF film

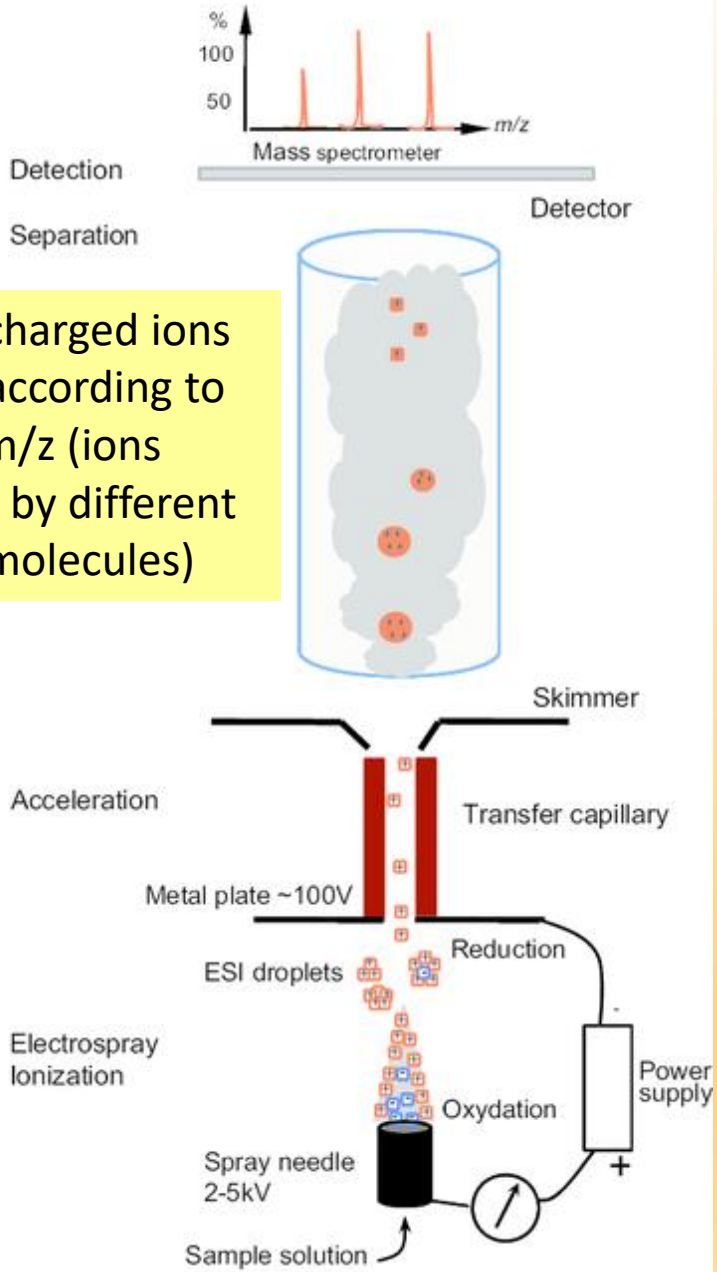
https://www.youtube.com/watch?v=feBxX2zLLjs

## MALDI-TOF MS



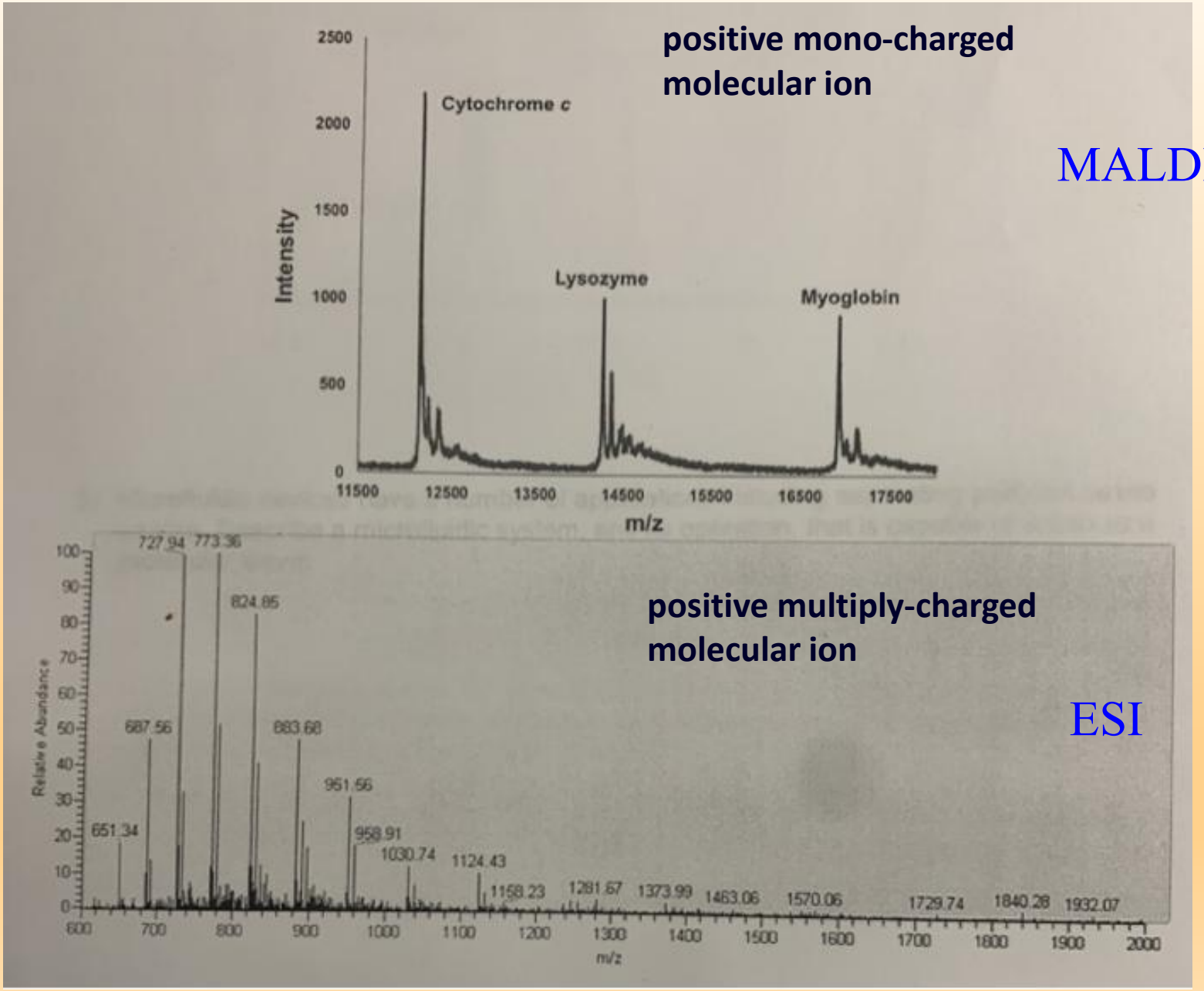

Mono-charged ions with different m/z and different flight speed are separated as packets of different molecules

## ESI-TOF MS




Multiply-charged ions scanned according to their m/z (ions generated by different parent molecules)

Lavigne JP, Espinal P, Dunyach C, et al. Mass spectrometry: A revolution in clinical microbiology? (2012) Clinical Chemistry and Laboratory Medicine DOI: 10.1515/cclm-2012-0291


positive mono-charged
molecular ion
Cytochrome c
Lysozyme
Myoglobin
Intensity
m/z
positive multiply-charged
molecular ion
Relative Abundance
m/z

MALDI

*vs*

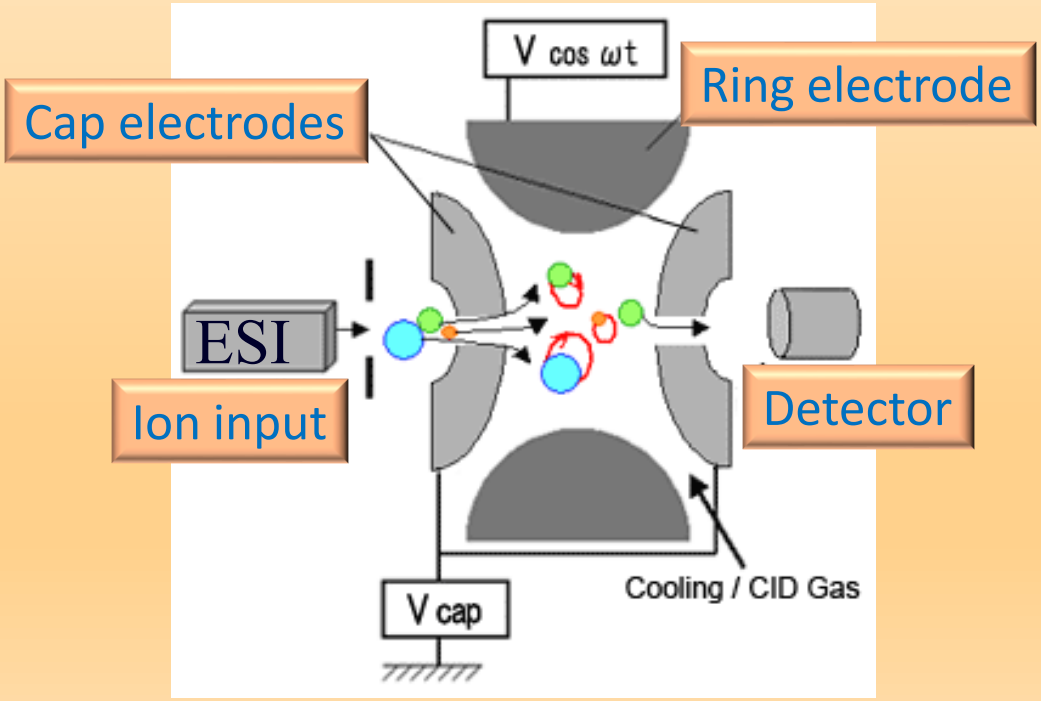
ESI

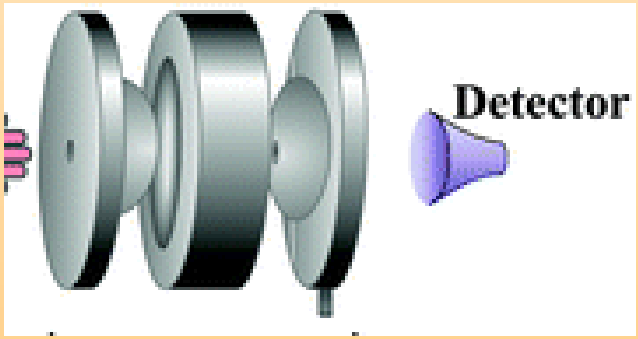
# ION TRAP (IT) ANALYZER

**It is the evolution of the quadrupole, also called Quadrupolar Ion Trap or 3D Ion Trap.**

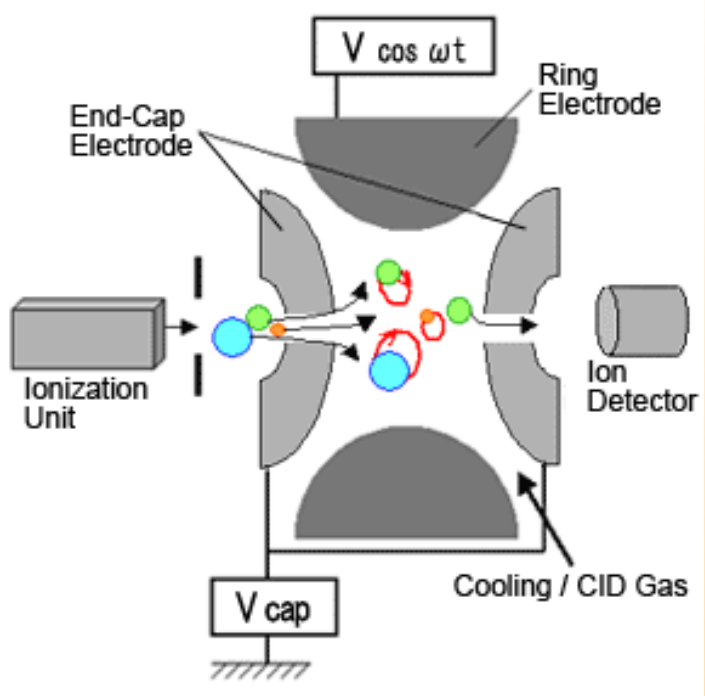
The ion trap is equipped with a **RING ELECTRODE** (to which continuous voltage is applied) and two **LOWER and UPPER CAP ELECTRODES**, to which alternating voltage is applied. They have the shape of two convex surfaces.

The ions are trapped in the space between these three electrodes by AC (oscillating) and DC (static) electric fields.




instead of ions passing through the quadrupoles, ions are trapped in a circular electrode in an RF field.




https://www.shimadzu.com/an/lcms/support/intro/lib/lctalk/61/61intro.html

When the **ions** are introduced, they remain **temporarily trapped and move with stable oscillations, depending on their m/z ratio.**

The high frequency voltage is **gradually increased** and at the same time the oscillations of the ions are destabilized:

**As the main RF voltage changes, ions of different mass-to-charge ratios eject through the slots in the linear trap. Ions are focused toward the ion detection system where they are detected.**

Typically, ion traps are coupled to ESI ionization sources>> ESI-IT.

# LINEAR ION TRAP (LIT) or 2D Ion Trap

It is an evolution of the Ion Trap.

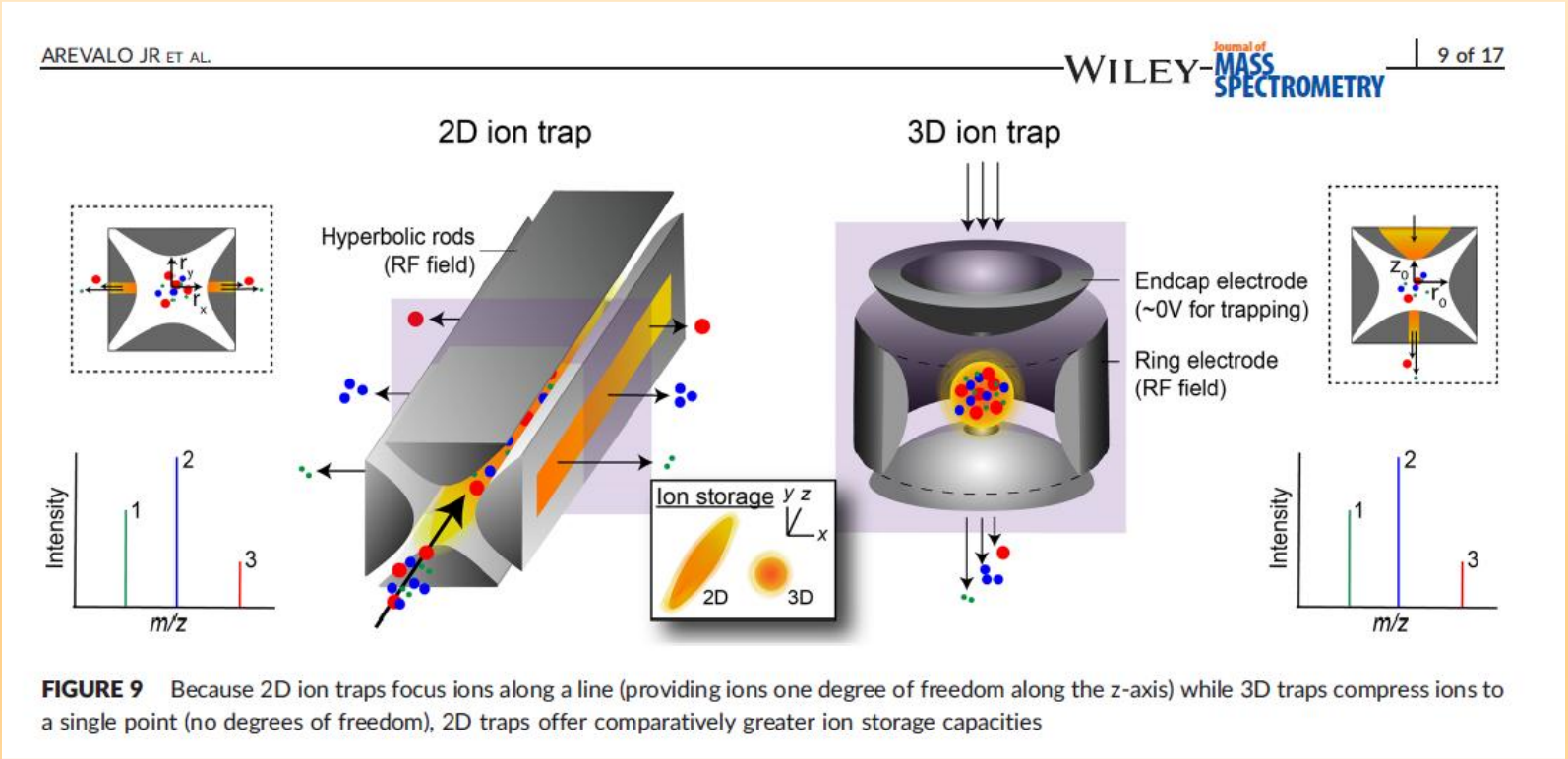



**FIGURE 9** Because 2D ion traps focus ions along a line (providing ions one degree of freedom along the z-axis) while 3D traps compress ions to a single point (no degrees of freedom), 2D traps offer comparatively greater ion storage capacities

Mass spectrometry and planetary exploration: A brief review and future projection. Ricardo Arevalo Jr, Ziqin Ni,| Ryan M. Danell. J Mass Spectrom. 2020;55:e4454.

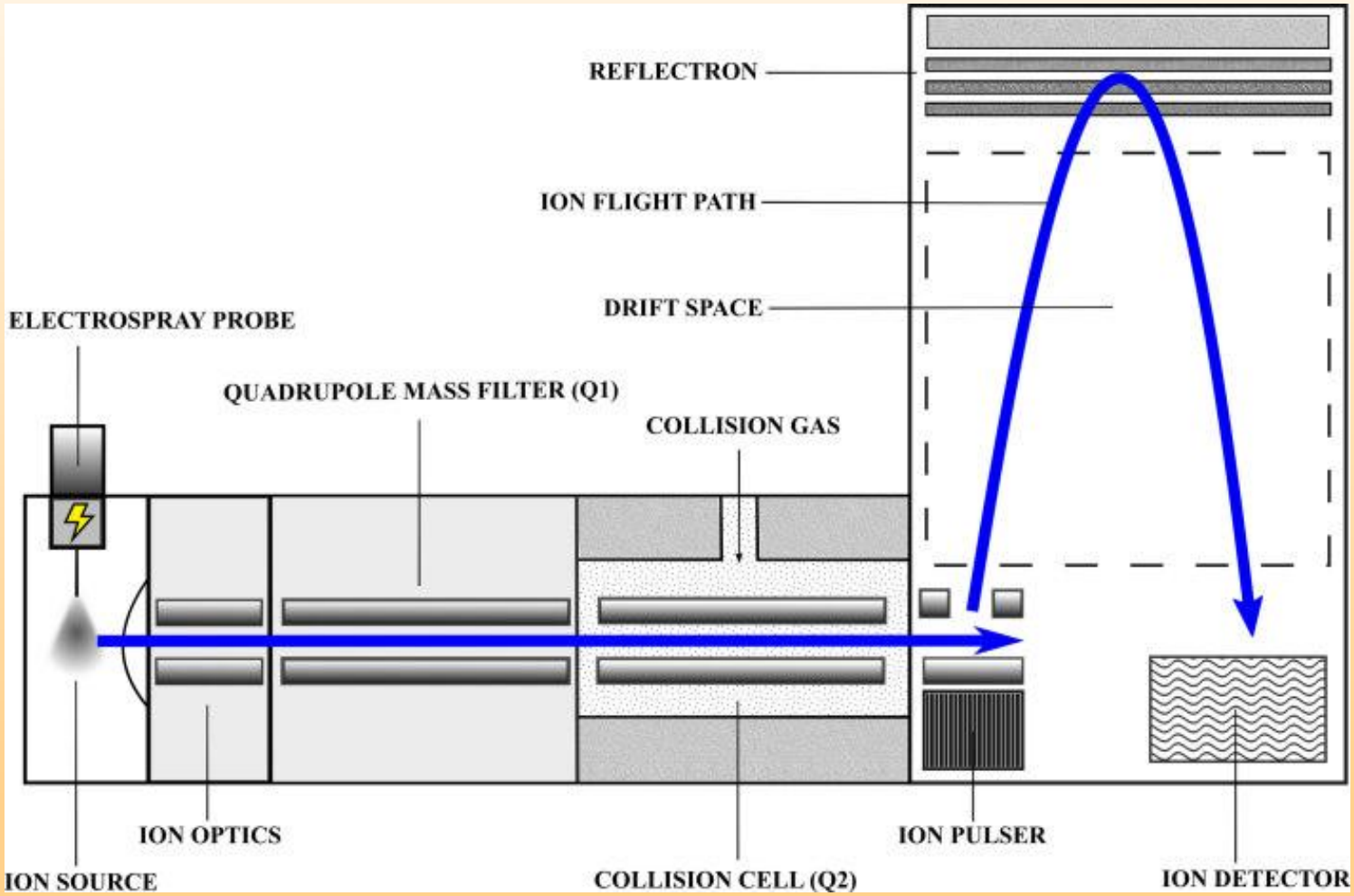
# Sectorial or hybrid mass spectrometers

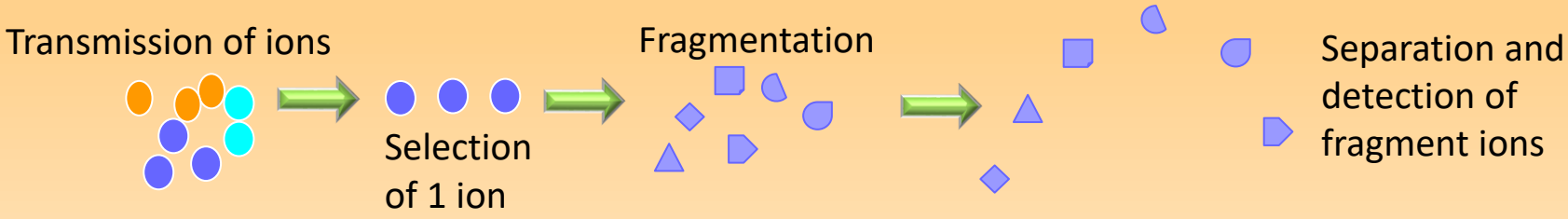
- HIGH SENSITIVITY
- HIGH MASS RESOLUTION (ACCURACY AND PRECISION IN MASS MEASUREMENTS)
- POSSIBILITY TO FRAGMENT THE PARENT MOLECULES (SMALL OR BIG MOLECULES) BY TANDEM-MS EXPERIMENTS, in case of proteins, this characteristique allows to sequence the protein

Triple quadrupole (QQQ)
TOF-TOF
Q-TOF
ORBITRAP

# Q-TOF

https://www.youtube.com/watch?v=W-DRL-V2Rkg



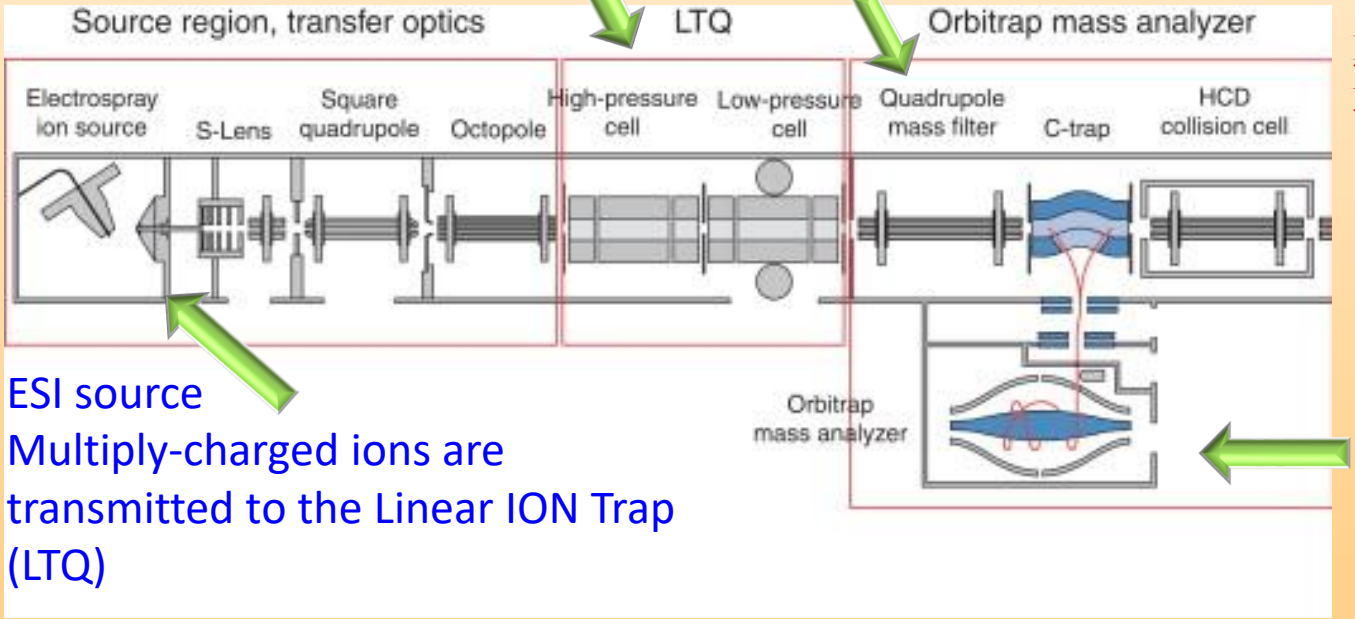



# ORBITRAP ELITE

LTQ:

1) separation and detection of all the ions
2) Trapping and fragmentation of all the ions

Transmission of ions and fragments to C-Trap




ESI source
Multiply-charged ions are transmitted to the Linear ION Trap (LTQ)

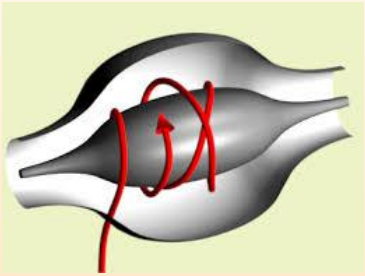
https://www.youtube.com/watch?v=KjUQYuy3msA

https://www.youtube.com/watch?v=zGeH5CmuVFQ

C-trap: stabilization of the ions and transmission
Orbitrap: detection of ions and their fragments in high resolution

Thousand of multiply-charged ions can be analyzed together, at the same time, thus thousand of proteins present in a sample can be detected in the same analysis.

Orbitrap mass spectrometry is a type of mass analyzer. It works based on 2 main principles. Similar to the moon orbiting around the Earth, it causes ions to orbit around the metal rod in the middle.



A voltage is applied to the electrode, causing ions to orbit the central electrode while they also oscillate back and forward along the central axis.

By measuring the frequency of this back and forward motion along the central axis it is possible to determine the mass-to-charge ratio of the ions and turn this signal into a mass spectra.

The result is a mass spectrogram that is extremely high in its resolution as well as very accurate. In addition, the orbitrap mass analyzer is extremely robust due to its simple structure.

## Key features and applications

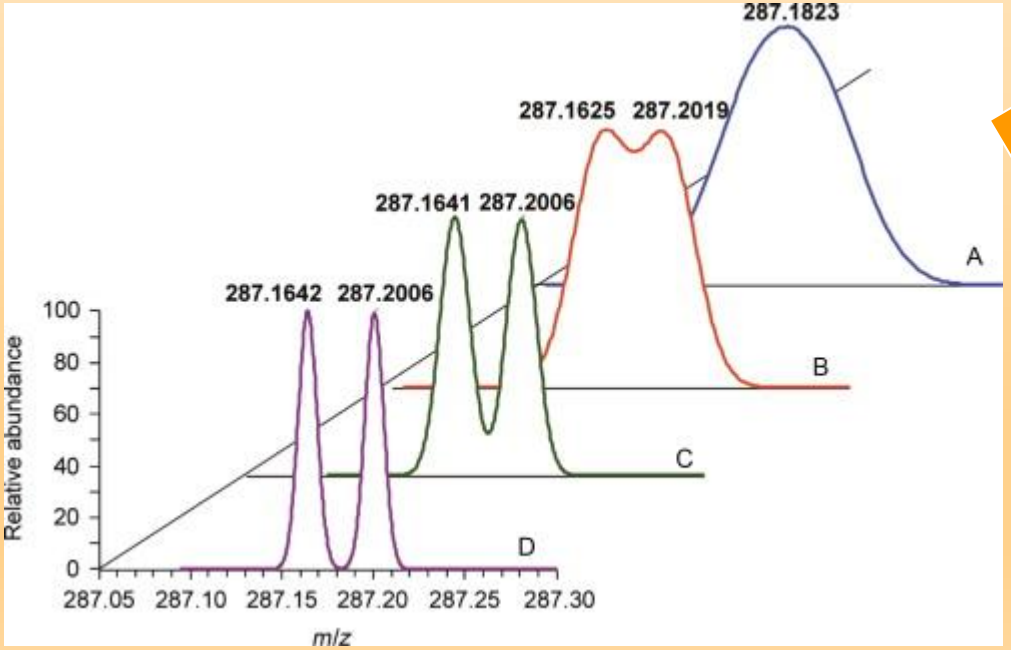
High resolution and accuracy: Orbitrap analyzers are known for providing high-resolution and accurate-mass measurements.

Simple structure: Its design is relatively simple and robust, making it suitable for a variety of analytical applications.

Analytical power: It is often used in non-targeted analysis, such as in proteomics, metabolomics, and biomarker discovery.

Flexibility: Some Orbitrap-based mass spectrometers combine an Orbitrap with other mass analyzers, such as a quadrupole and linear ion trap, to provide greater flexibility and analytical power.

high-resolution mass spectrometry (HRMS) provides highly accurate/exact mass measurements to several decimal places, allowing it to distinguish between molecules with very similar masses, low-resolution mass spectrometry (LRMS) determines Average mass values



# SOME DEFINITIONS

- **Atomic mass unit (amu) or Dalton:** unit of measurement used to measure mass defined as 1/12 of the mass of a Carbon-12 ($_{12}C$).
- **Molecular mass:** mass of a specific molecule expressed in amu
- **Average molecular mass (Mav):** is calculated by taking for each element (C, O, N, H, P…) the average atomic mass, which is the mean of the exact masses of the naturally occurring isotopes of that particular element. It is expressed in amu.
- **Isotopes:** atoms having the same atomic number (Z) but different number of neutrons.

- **Monoisotopic mass:** exact mass of a molecule calculated by considering for each element the exact mass of the most abundant isotope present in nature
- **Accurate mass:** molecular mass measured experimentally. It is an approximation of the exact monoisotopic mass

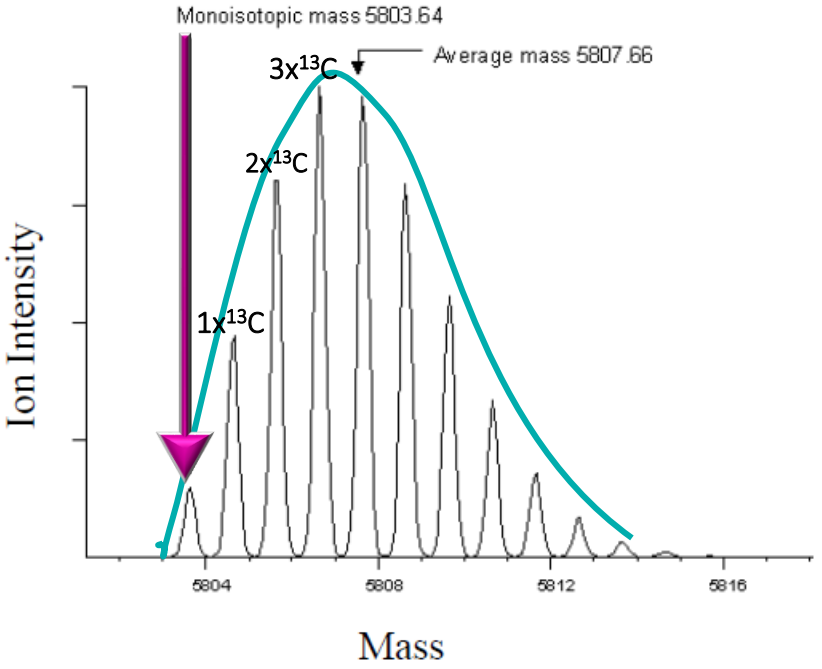
What do Monoisotopic Mass and Average Mass represent?
There are isotopes for the elements, their mass is different, and the mass spectrometer is able to distinguish them!

| Isotope | Natural Abundance % | Atomic mass [$^{12}C$ = 12(.000000..) ] | |
|---|---|---|---|
| $^{1}H$ | 99.985 | 1.007825 | |
| $^{2}H$ | 0.015 | 2.014102 | |
| $^{12}C$ | 98.900 | 12.000000 | |
| $^{13}C$ | 1.100 | 13.003354 | ← Not negligible! |
| $^{14}N$ | 99.640 | 14.003074 | |
| $^{15}N$ | 0.360 | 15.000108 | |
| $^{16}O$ | 99.760 | 15.994915 | |
| $^{17}O$ | 0.040 | 16.999133 | |
| $^{18}O$ | 0.200 | 17.999160 | |
| $^{31}P$ | 100.00 | 30.973763 | |
| $^{32}S$ | 95.040 | 31.972074 | |
| $^{33}S$ | 0.760 | 32.971461 | |
| $^{34}S$ | 4.200 | 33.967865 | ← Not negligible! |
| $^{35}Cl$ | 75.800 | 34.968855 | |
| $^{37}Cl$ | 24.200 | 36.965896 | ← Not negligible! |

- Natural Abundance:
  - $^{12}C$: 98.93%
  - $^{13}C$: 1.07%
- Use Monoisotopic Mass (all $^{12}C$ molecule) For High Mass Accuracy

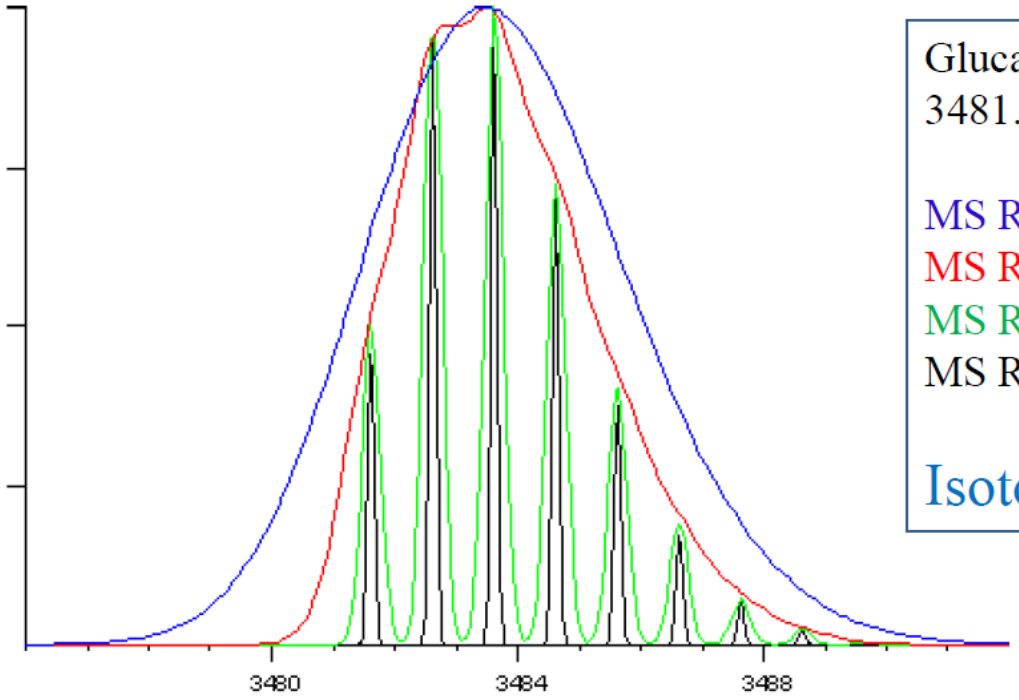
The monoisotopic mass corresponds to the molecule in which only $^{12}C$s are present. Isotopic masses are also detectable with
1 x $^{13}C$, 2x $^{13}C$, 3x$^{13}C$.....

The average mass: is the mean value of all isotopic masses revealed. It takes into account all possible isotopes.



The ability of a mass spectrometer to measure values of average or monoisotopic masses depends on its resolving power

The ability of a MS to distinguish one ion from another of very similar m/z's.




Glucagon at Different Resolutions
3481.62 $MH^+$

MS Resolution @ 1,000
MS Resolution @ 3,000
MS Resolution @ 10,000
MS Resolution @ 30,000

Isotopes Resolved!

Matrix Science



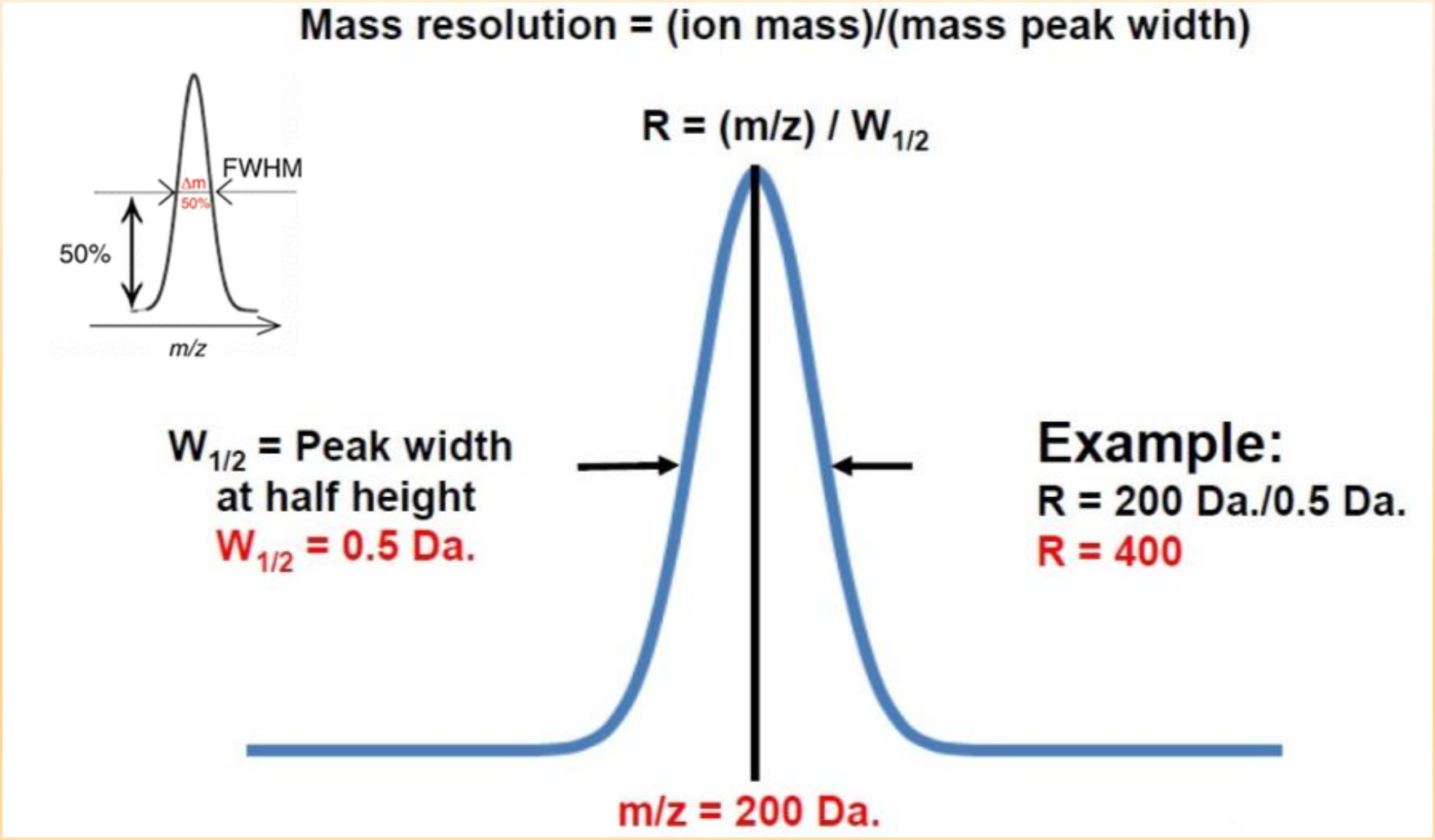
https://www.youtube.com/watch?v=qUhi98o1qUU&t=17s

# RESOLUTION (in the case of one peack)

Given one mass M1 the mass resolution is:

$$R=(m/z)/W_{1/2}$$

FWHM: Full Width at Half Maximum

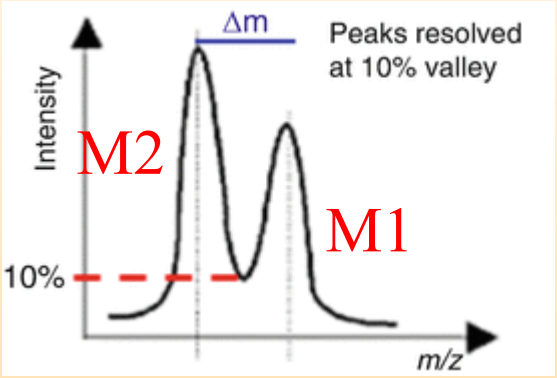


## One more definition: The resolving power (in the case of two adjacent pecks)

Given two masses M1 and M2 (M1 smaller than M2), I can reveal them as distinctive masses if the resolving power of the analyzer is at least equal to:

***Resolving Power = M1/(M2 - M1)***

Example:
To separate the ions 156.093896 and 156.081320 (very similar values) the required resolution is=
156.081320/(156.093896 - 156.081320) = **12400**




Mass Spectrometry and High Resolution Mass Spectrometry: An Overview.
Gianluca Giorgi. 2017, Molecular Technologies for Detection of Chemical and Biological Agents pp 89-101

Currently there are instruments with resolutions up to about 450000.
The level of information that can be obtained from a mass spectrometer depends on its resolving power. HIGH RESOLUTION instruments provide the mass of the ions with HIGH PRECISION and HIGH ACCURACY, information that exclusively defines the elementary composition of the corresponding ions, and thus, is necessary for identification of the analyte.

**Example: the low-resolution mass spectrometer provides a unique signal at mass value: 28 Da;**
**in a High-resolution mass spectrometer they generate three separate signals, they can be resolved.**

| | |
|---|---|
| CO | 27.994914 |
| $N_2$ | 28.006146 |
| $C_2H_4$ | 28.031298 |

# Mass Spectrometry

## Definitions and concepts

- Resolution (resolving power)

  RP(FWHM) = measured mass / peak width at 50% peak intensity

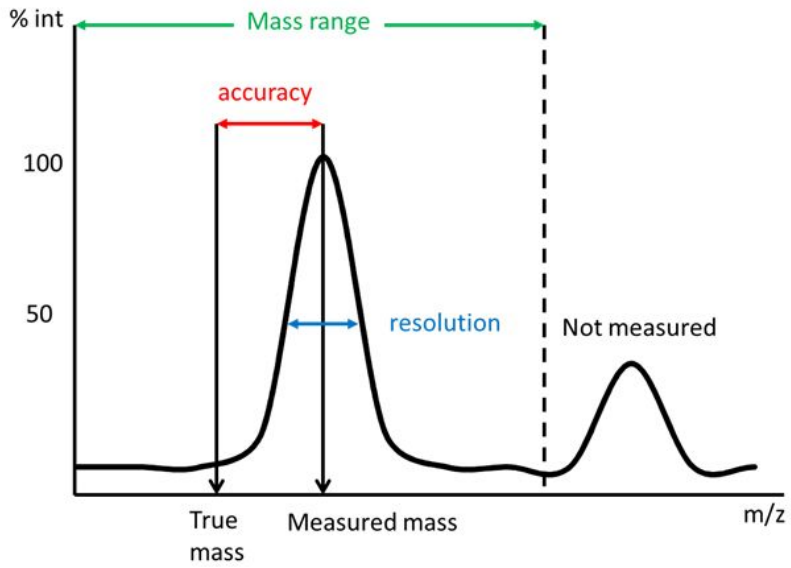
- Accuracy

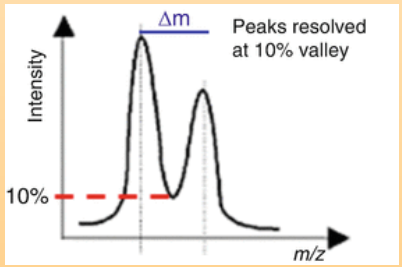
  Difference in true mass and measured mass

- Mass range

  Range of ions that can be detected (typically 50-1000 m/z)

Full Width at Half Maximum



**ACCURACY =** the proximity of the experimental measurement to the true value (exact mass). When a measurement is close to the true value, we say it is accurate, and when it is not, we say it is inaccurate.

**Normally, mass measurement error would be used to describe the accuracy.**

**Theoretical mass = 156.093896 Da**
**Experimental mass = 156.093900 Da it is an accurate measure**
**= 156.15 Da it is not an accurate measure**

---

**PRECISION =** the repeatability of the measurement reflecting random errors. Random errors cause measurements to fall on both sides of the average experimental measurement and affect the precision of the set of measurements. When a set of mass measurements of one ion species lie close together we say the measurements are precise, and when not we say the measurements are imprecise.

**High precision, low error by the instrument**

1° measure (analysis) = 156.093900 Da
2° measure (analysis) = 156.093901 Da
3° measure (analysis) = 156.093900 Da
4° measure (analysis) = 156.093899 Da
Etc….

**Low precision, high error by the instrument**

1° measure (analysis) = 156.093900 Da
2° measure (analysis) = 156.125000Da
3° measure (analysis) = 156.073000 Da
4° measure (analysis) = 156.060000 Da
Etc….

Two other items of terminology which must be clarified are:

**Repeatability**—this is the short-term precision of multiple replicate experimental measurements made under similar conditions, i.e., the same instrument, operator and over a limited time, normally the same day.

**Reproducibility**—refers to differences among experimental measurements made under different circumstances i.e., a measurement of the same quantity made by different operators, even different instruments and often with a significant time difference between groups of measurements.

# Mass Spectrometry

## Definitions and concepts

- Resolution (resolving power)

  RP(FWHM) = measured mass / peak width at 50% peak intensity

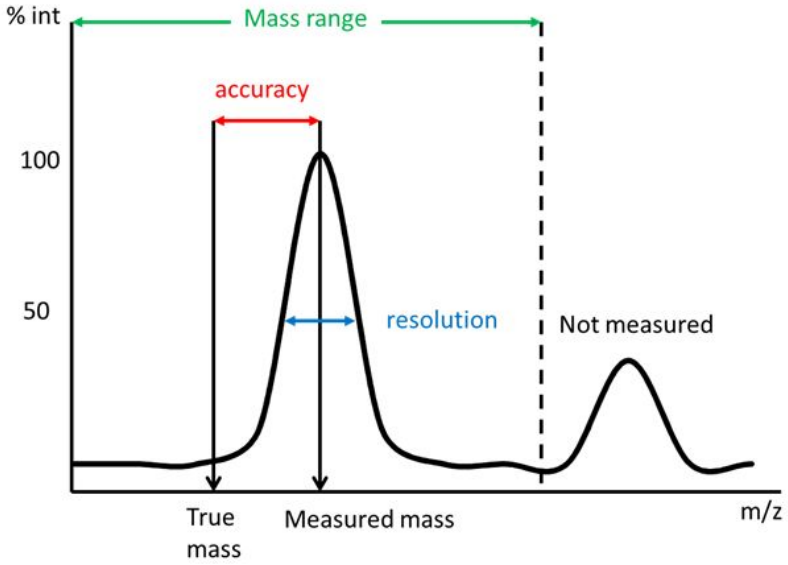
- Accuracy

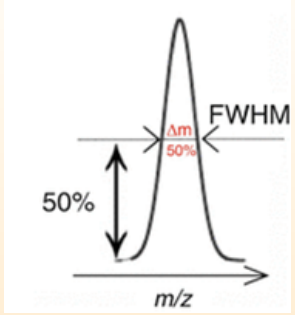
  Difference in true mass and measured mass

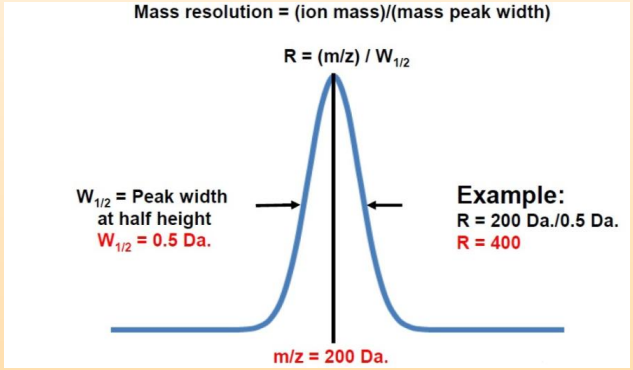
- Mass range

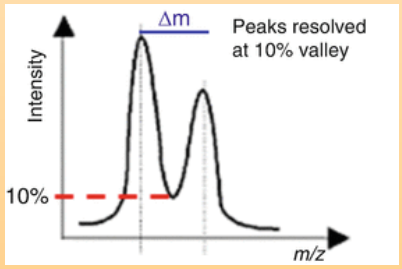
  Range of ions that can be detected (typically 50-1000 m/z)

Full Width at Half Maximum



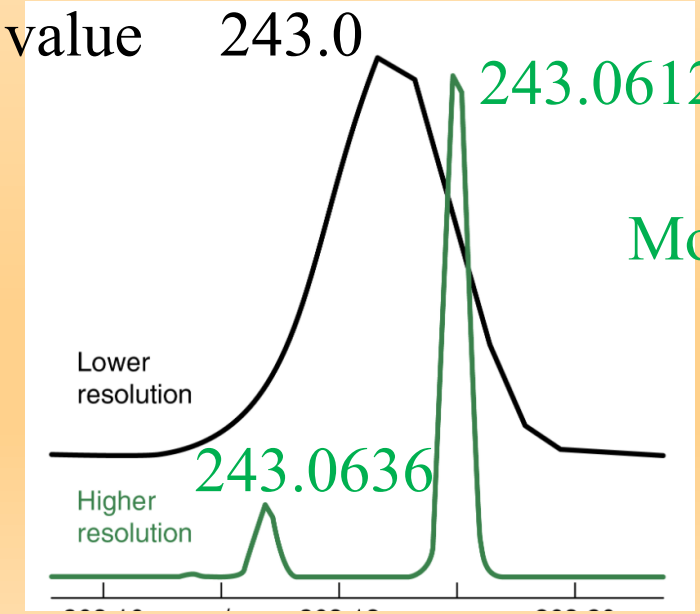



What mass resolving power of the analyzer is required in a mass spectrometry esperiment to distinguish between ion A (243.0612) and ion B (243.0636)?

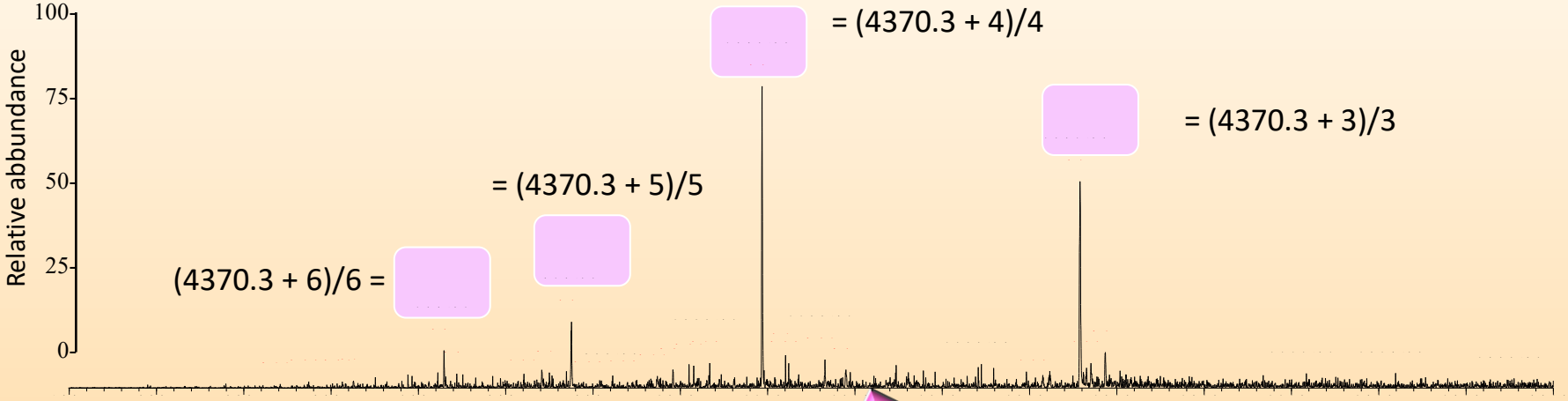
To separate the ions 243.0636 and 243.0612
(very similar values) the required resolution is=
243.0636/(243.0636 - 243.0612) = 101.266 FWHM
(100.000)

Average mass value 243.0



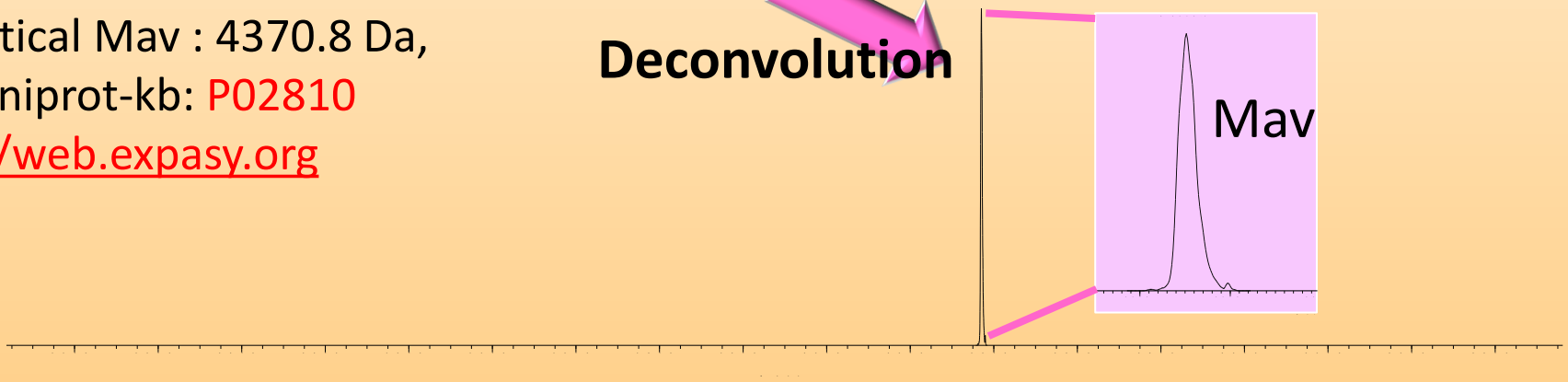

Monoisotopic mass values

# ESI mass spectrum obtained for P-C peptide by a low-resolution mass spectrometer (LR-MS resolution = 6000)




Theoretical Mav : 4370.8 Da,
code Uniprot-kb: P02810
https://web.expasy.org

**Deconvolution**




Display the list of masses in raw text format to be exported into an external application

- Chain Peptide P-C at positions 123 - 166 [Theoretical pI: 12.01 / Mw (average mass): 4370.78]

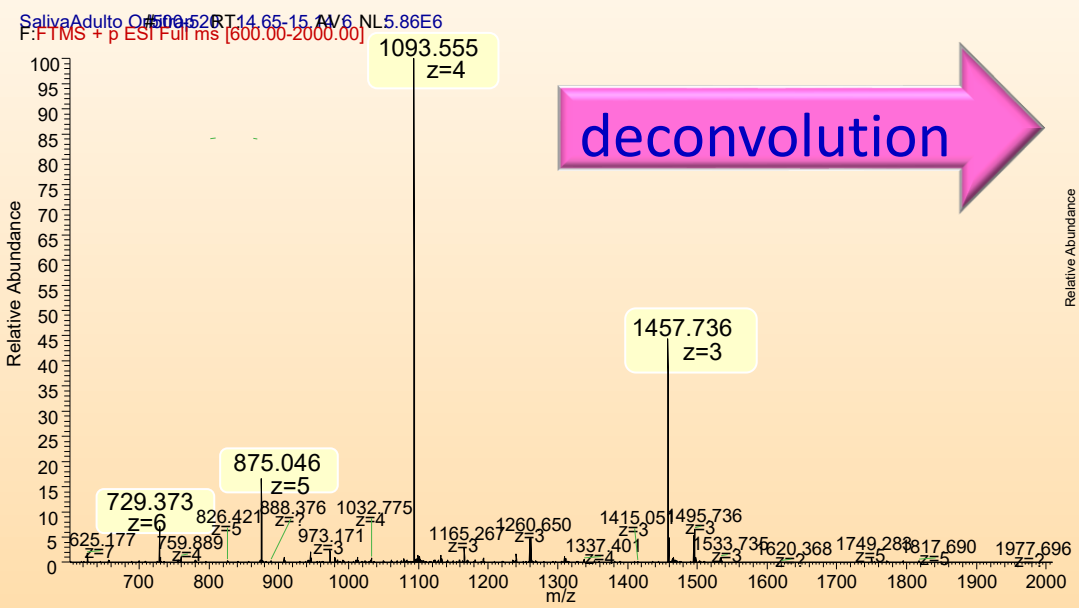
| mass | position | #MC | modifications | conflicts | variants | alternative isoforms | peptide sequence |
|---|---|---|---|---|---|---|---|
| 4370.7762 | 123-166 | 0 | | | 163:Q->K | | GRPQGPPQQGGHQQGPPPPP PGKPQGPPPQGGRPQGPPQG QSPQ |

100.0% of sequence covered (you may modify the input parameters to display also peptides < 500 Da or > 100000000000 Da):

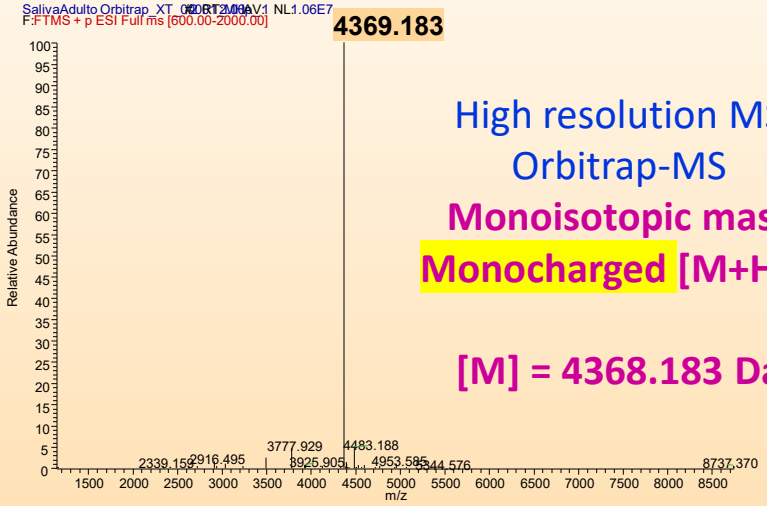
1) The HRMS can separate the isotopic ions of all the multiply-charged ions belonging to a certain protein
2) The HRMS can recognize a difference in mass (Δm) among the values of the isotopic ions of each multiply-charged ion
3) On the base of this Δm, the HRMS gives an exact charge to each multiply-charged ion
4) The HRMS, on the base of the charges, is able to determine the exact monotisotopic mass value of the protein

**DECONVOLUTION PROCESS IN HIGH RESOLUTION ANALYSIS**

ESI mass spectrum obtained for P-C peptide via a high-resolution mass spectrometer (60000) (HRMS) ORBITRAP ELITE




deconvolution




High resolution MS
Orbitrap-MS
**Monoisotopic mass**
**Monocharged [M+H]$^{+1}$**

**[M] = 4368.183 Da**

Monoisotopic mass
[M+H]$^{+1}$ measured with very high accuracy and precision

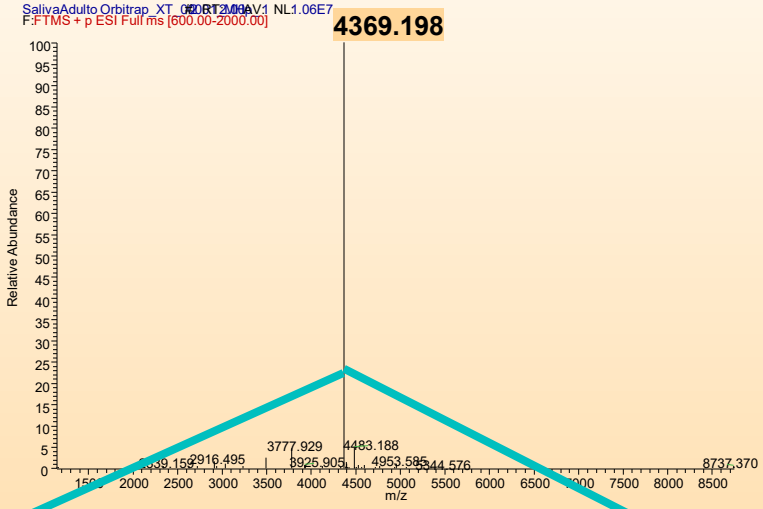
Chain Peptide P-C at positions 123 - 166 [Theoretical pI: 12.01 / Mw (average mass): 4370.78 / Mw (monoisotopic mass): 4368.18]

| mass | position | #MC | modifications | conflicts | variants | alternative isoforms | peptide sequence |
|---|---|---|---|---|---|---|---|
| 4369.1833 | 123-166 | 0 | | | 163:Q->K | | GRPQGPPQQGGHQQGPPPPP PGKPQGPPPQGGRPQGPPQG QSPQ |

Theoretical Monoisotopic monocharged mass
[M+H]$^{+1}$
code Uniprot-kb: P02810
https://web.expasy.org

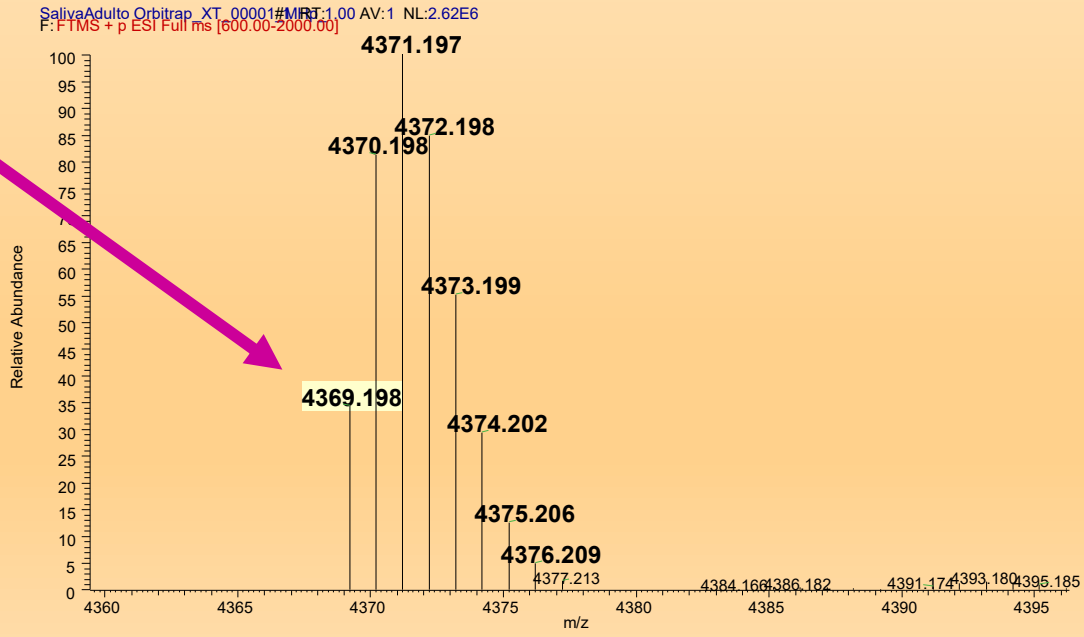
ESI mass spectrum obtained for P-C peptide via a high-resolution mass spectrometer (60000) (HRMS)

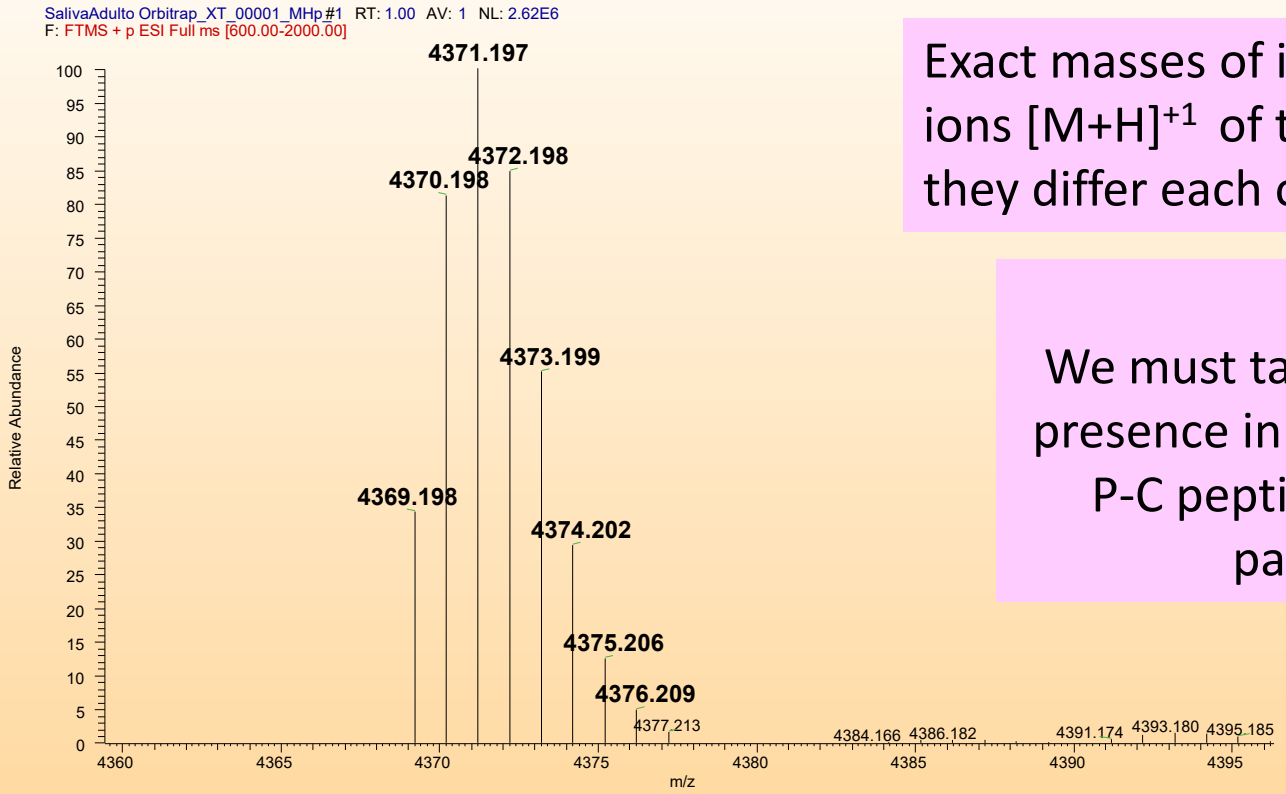
MS high resolution
Orbitrap-MS
Monoisotopic mass
$[M+H]^{+1}$




The HRMS spectrometer measures the isotopic masses of the peptide from the monoisotopic one, with an accuracy and very high precision

Distribution Isotopic masses $[M+H]^{+1}$ measured for P-C peptide: differ by 1,






Exact masses of isotopic monocharged ions $[M+H]^{+1}$ of the P-C peptide: they differ each other by 1 Da

Why?
We must take into account the presence in the structure of the P-C peptide of isotopes, in particular $^{13}C$

M monoisotopic monocharged [M] = **4368.198** Da

$[M+H]^{+1}$ = (4368.198 + 1)/1 = **4369.198 m/z**

$[M+H]^{+1}$ + 1 $^{13}C$= (4368.198 + 1+1)/1 = **4370.198 m/z**

$[M+H]^{+1}$ + 2 $^{13}C$= (4368.198 + 1+2)/1 = **4371.198 m/z**

$[M+H]^{+1}$ + 3 $^{13}C$= (4368.198 + 1+3)/1 = **4372.198 m/z**

**Etc.....**

The **distance (mass difference)** between the isotopic peaks of a molecule, measured in MS, is related to the charge of the multiply-charged ions.
This mass difference (Δm), detectable especially in analyses made with high-resolution MS, is considered by programs that operate the deconvolution of mass spectra and provide monoisotopic mass values with great accuracy.

**Isotopic masses**
**Mono-charged ion: isotopic m/z ions distance themselves 1**
**Bi-charged ion: Δm is 0.5**
**Tri-charged ion: Δm is 0.33**
**Tetra-charged ion: Δm is 0.25**
**Penta-charged ion: Δm is 0.20**
**……**

## Example:

HRMS spectrum of an ion at m/z = 1015.016

$\Delta m = 0.5$

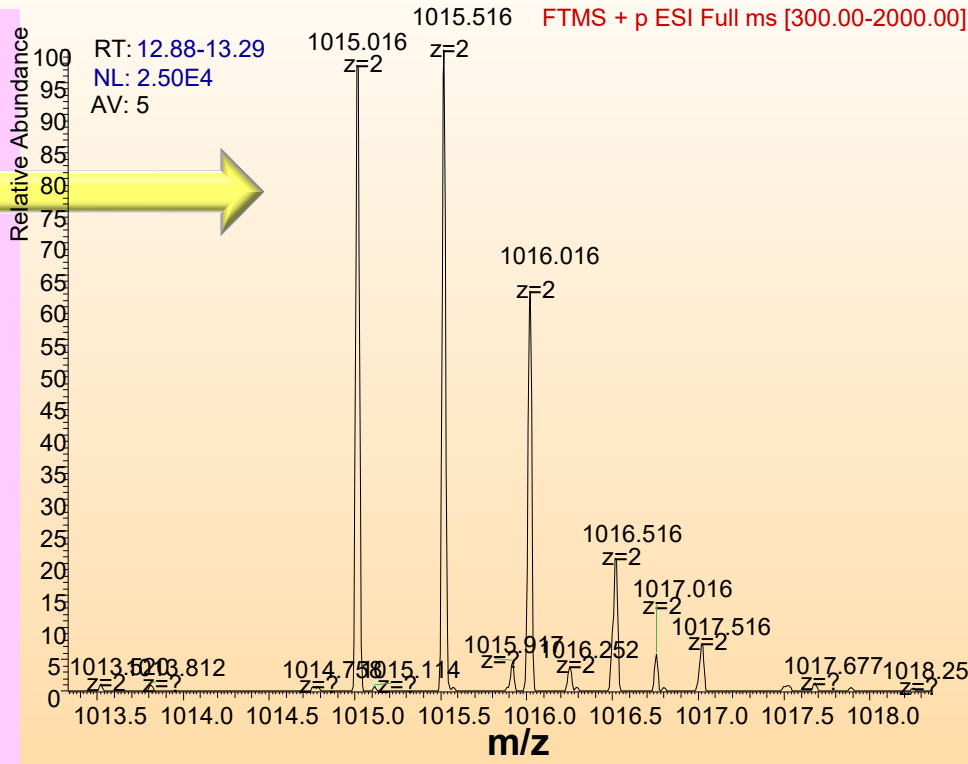
Computed charge +2,

It is a BI-CHARGED ion $[M+2H]^{+2}$

→ Thus, the mass of the peptide to which it belongs to is =

(1015.016 x 2) -2 = 2028.032 Da

(Monoisotopic Mass)




The HRMS gives charge +2 to the ion 1015.016 m/z, because it can separate its isotopic ions and recognize a difference in mass between them (Δm) of 0.5

Isotopic bi-charged ions:

[M] = **2028.032** Da

$[M+2H]^{+2}$ = (2028.032 + 2)/2 = **1015.016 m/z**

$[M+2H]^{+2}$ + 1 $^{13}C$= (2028.032 + 2 + 1)/2 = **1015.516 m/z**

$[M+2H]^{+2}$ + 2 $^{13}C$= (2028.032 + 2 + 2)/2 = **1016.016 m/z**

$[M+2H]^{+2}$ + 3 $^{13}C$= (2028.032 + 2 + 3)/2 = **1016.516 m/z**

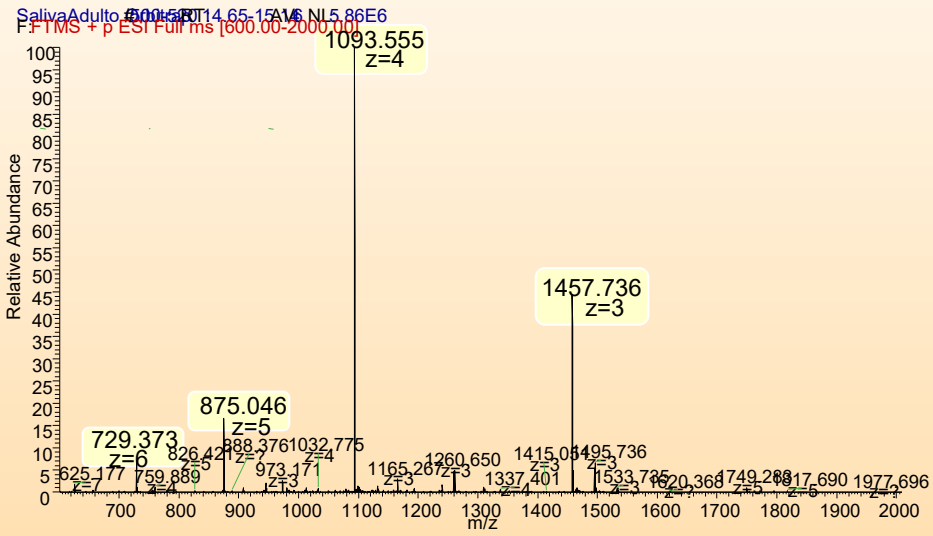
**Etc…..**

Δm = 0.5 Da

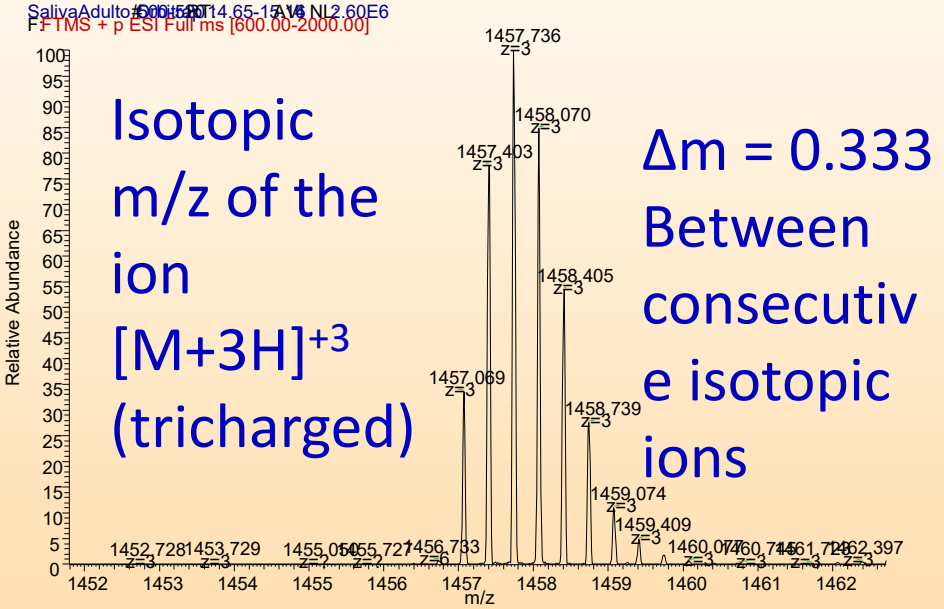
Δm = 0.5 Da

Δm = 0.5 Da

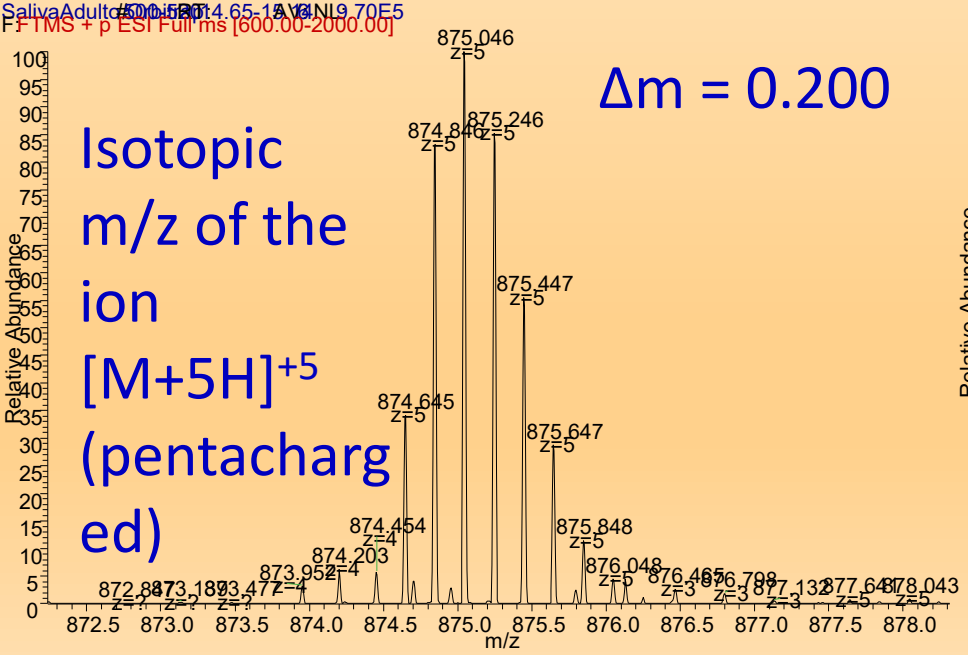
A very accurate mass value is obtained because the resolution on each multiply - charged isotopic ion is very high




Isotopic m/z of the ion $[M+3H]^{+3}$ (tricharged)

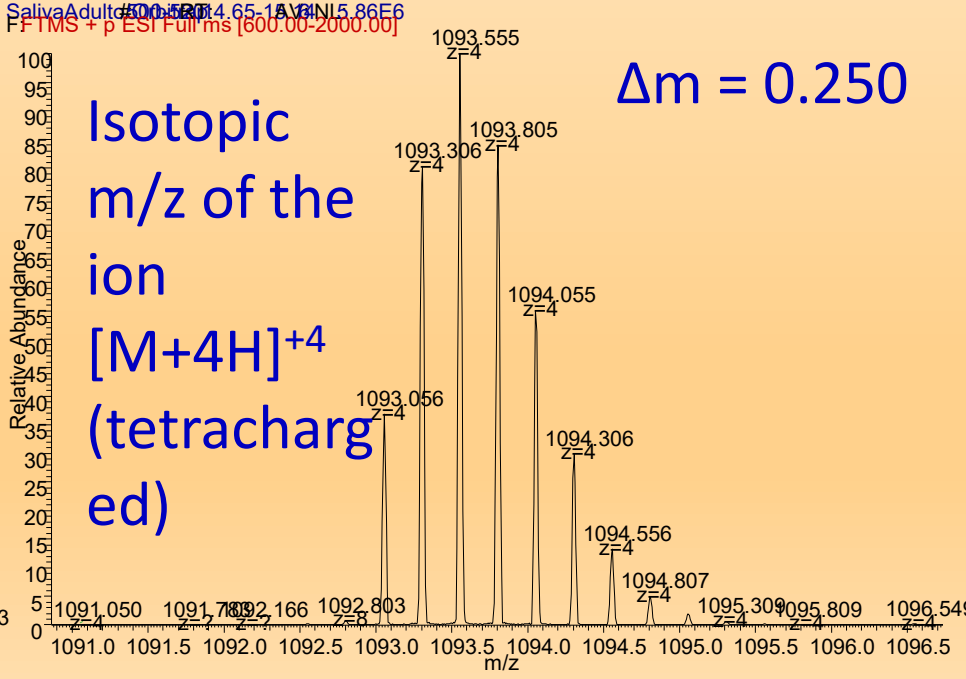



$\Delta m = 0.333$ Between consecutive isotopic ions




Isotopic m/z of the ion $[M+5H]^{+5}$ (pentacharged)

$\Delta m = 0.200$




Isotopic m/z of the ion $[M+4H]^{+4}$ (tetracharged)

$\Delta m = 0.250$