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ADVANCES IN FOOD ANALYSIS

MASS SPECTROMETRY COUPLED WITH CHROMATOGRAPHIC TECHNIQUES: LC-MS & GC-MS

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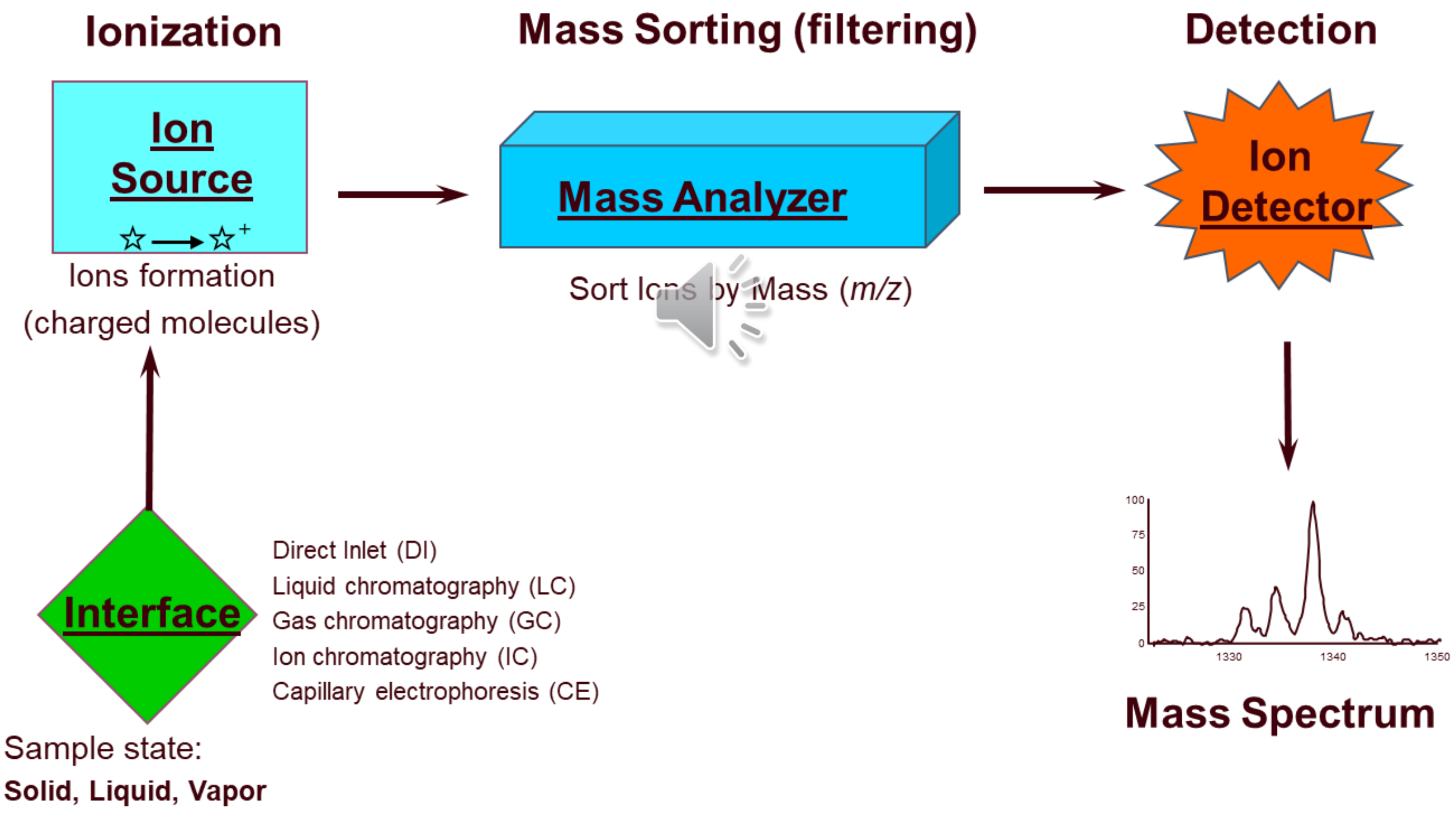
Outline

- **Basic Principles Of Mass Spectrometry Coupled To Chromatography**
- **Mass Spectrometry coupled to LC**
- **Mass Spectrometry coupled to GC**

PART I

MASS SPECTROMETRY COUPLED TO CHROMATOGRAPHY: BASIC PRINCIPLES

Mass Spectrometer Constituents



Ionization

Ion Source

☆ → ☆$^{+}$

Ions formation (charged molecules)

Mass Sorting (filtering)

Mass Analyzer

Sort Ions by Mass (*m/z*)

Detection

Ion Detector

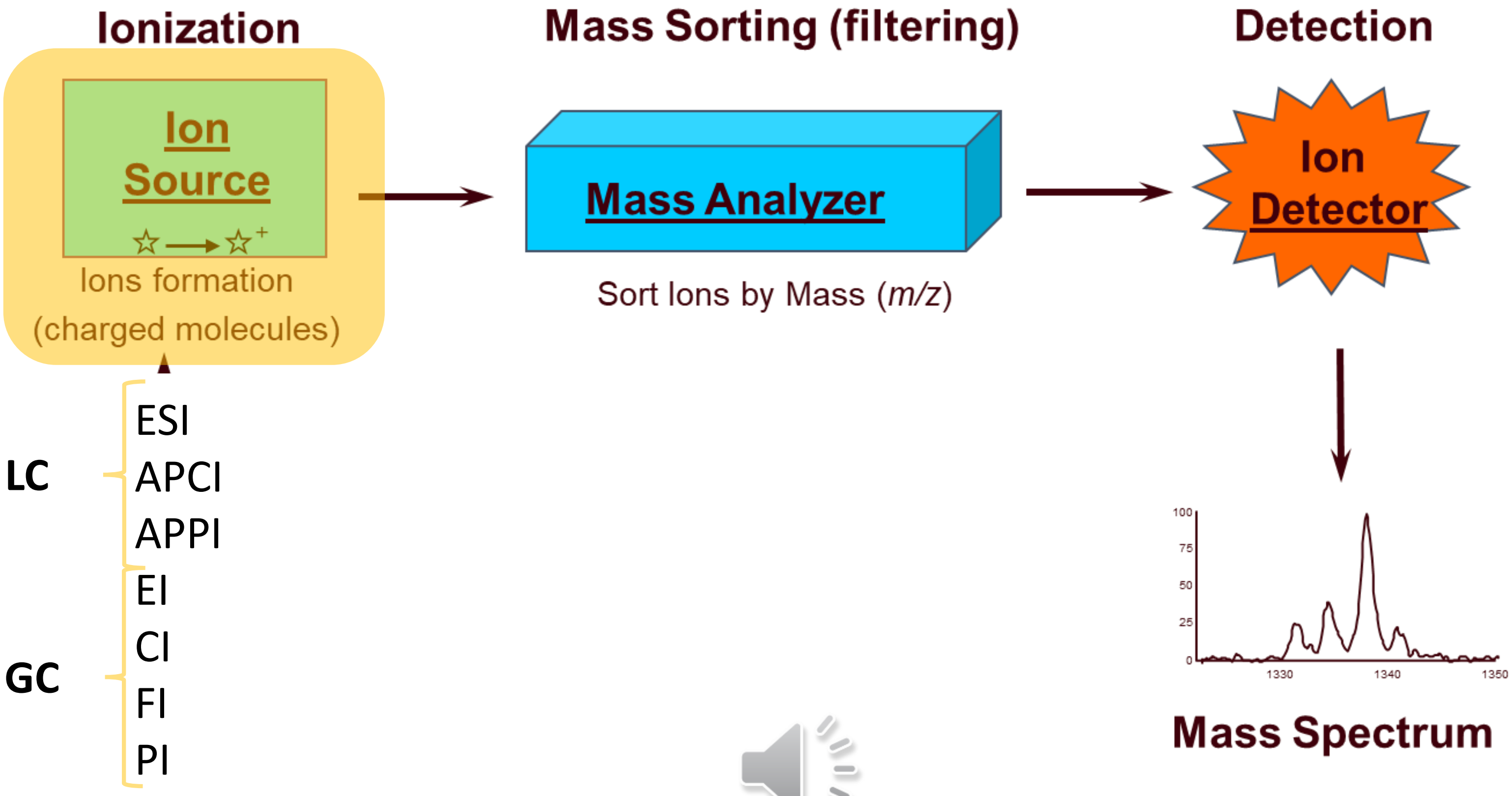
Interface

Direct Inlet (DI)
Liquid chromatography (LC)
Gas chromatography (GC)
Ion chromatography (IC)
Capillary electrophoresis (CE)

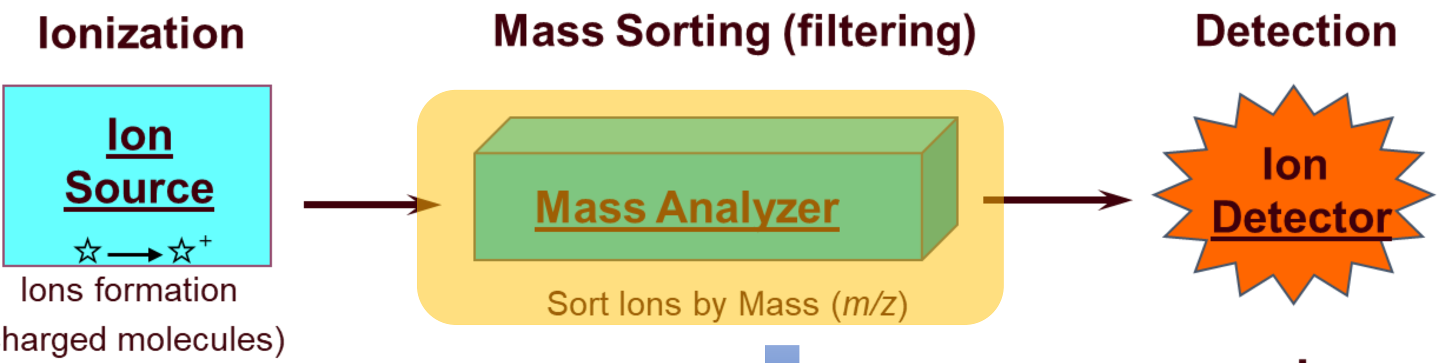
Sample state:
Solid, Liquid, Vapor

Mass Spectrum

Mass Spectrometer Constituents



Mass Spectrometer Constituents



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Ion Source

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(charged molecules)

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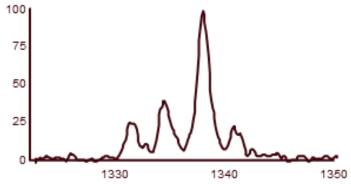
Sort Ions by Mass (*m/z*)

Detection

Ion Detector

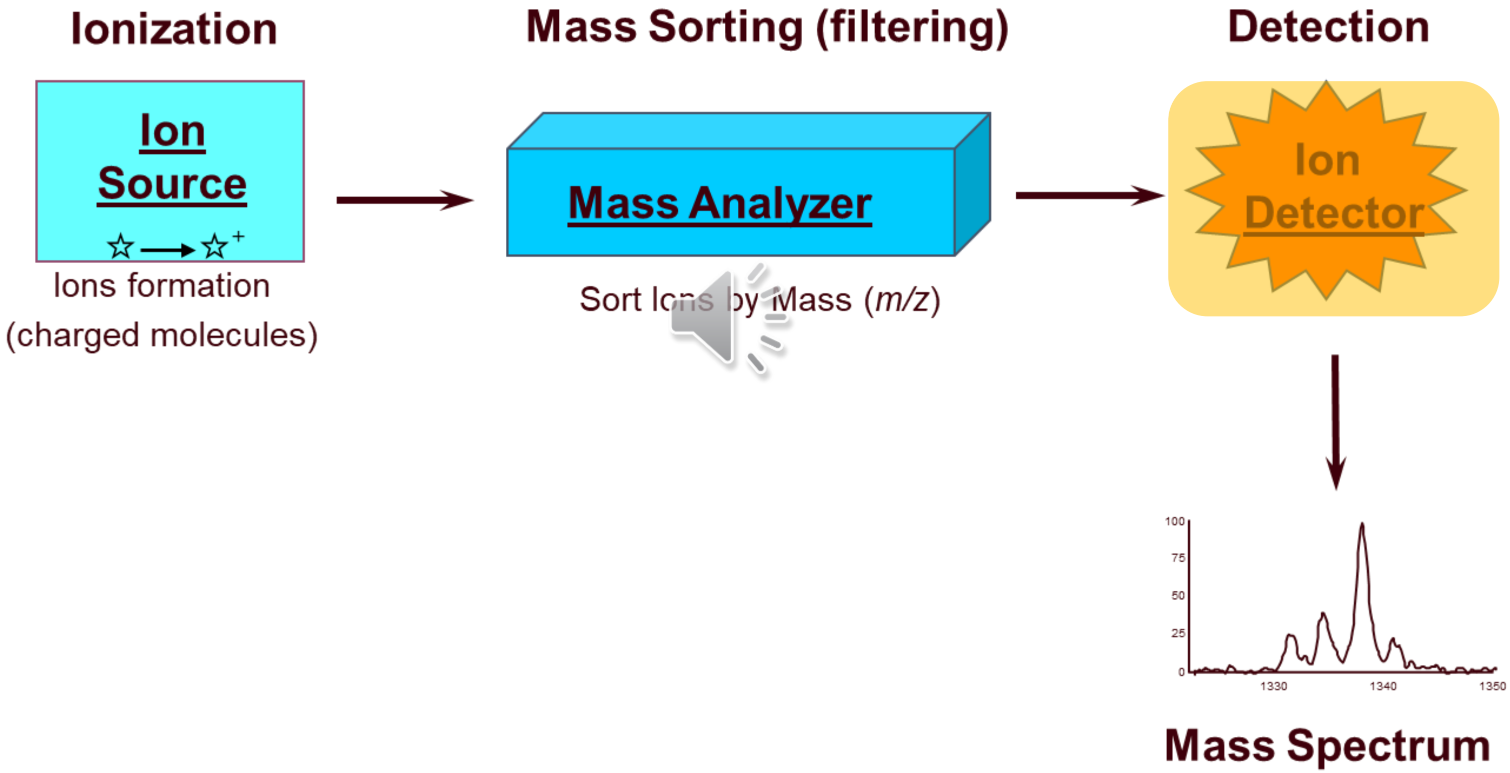
Most common MS technologies used with chromatographic techniques

- **Quadrupole (Q)**
- **Ion Trap (IT)**
- **Orbitrap**
- **Time-of-flight (TOF)**



Mass Spectrum

Mass Spectrometer Constituents



Ionization

Ion Source

☆ → ☆⁺

Ions formation
(charged molecules)

Mass Sorting (filtering)

Mass Analyzer

Sort Ions by Mass (*m/z*)

Detection

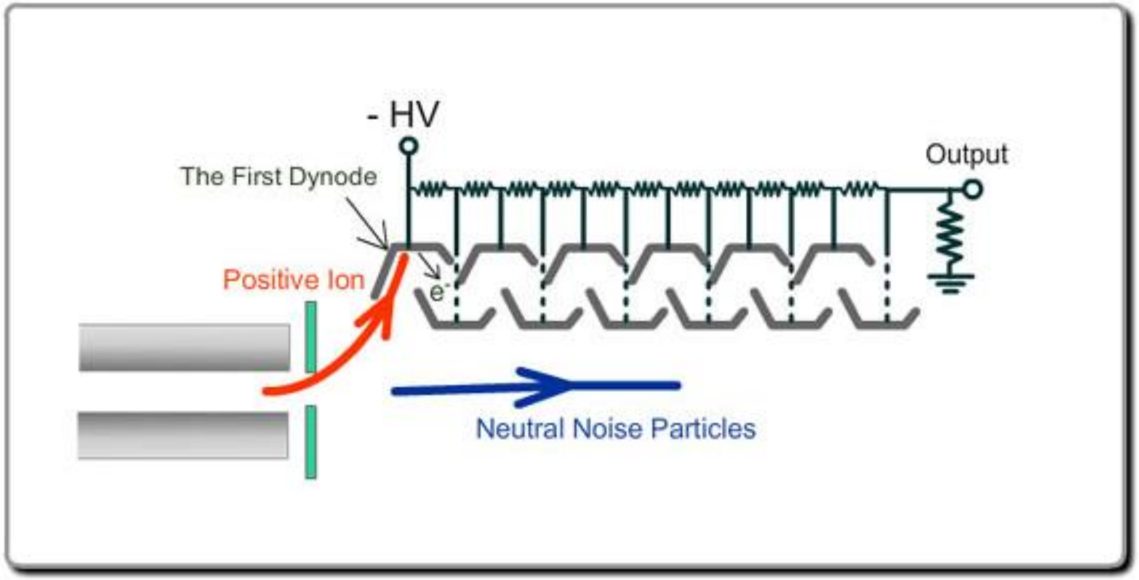
Ion Detector

Mass Spectrum

Electron multiplier

- It converts ions to electrons (electric current)

- Dynodes with voltage difference (usually 90V)



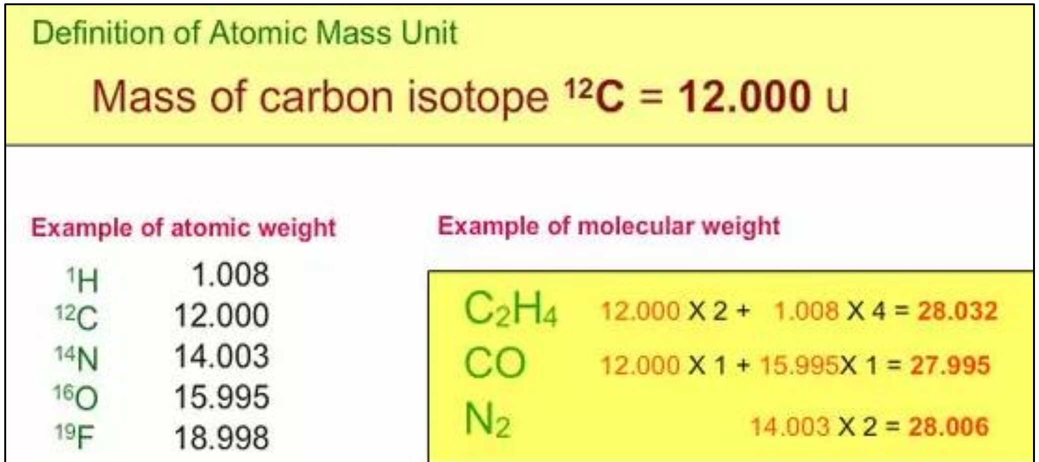
https://www.youtube.com/watch?v=T40QedTy1h8

What is a Mass Spectrometer?

- It is an instrument that measures the mass of microscopic particles such as molecules and atoms.
- It filters/separates ions according to their mass-to-charge ratio (m/z) value.
- In this way, we can identify a molecule and quantify the amount of it in a sample

Molecular Weight

- Atomic mass unit (u) is used for expressing the mass of atoms or molecules. This is the unit in which atomic masses are measured, and it is defined as 1/12th the mass of a ^{12}C atom, based on $^{12}C=12.0000$ u. Using this unit, the mass of ^{1}H is 1.0078u.
- Molecular weight is estimated using the atomic weight of constituent atoms and molecular formula.
- Even if a signal at 28u is observed in a spectrum measured by a low-resolution type of mass spectrometer, you cannot specify this peak. It is either Ethylene C_2H_4, carbon monoxide CO or nitrogen N_2. A magnetic sector-type mass spectrometer, which has high resolution in mass, can resolve C_2H_4, CO and N_2 peaks, and you can successfully determine the compound through a small difference in exact mass.



Definition of Atomic Mass Unit

Mass of carbon isotope $^{12}C = 12.000$ u

Example of atomic weight

^{1}H	1.008
^{12}C	12.000
^{14}N	14.003
^{16}O	15.995
^{19}F	18.998

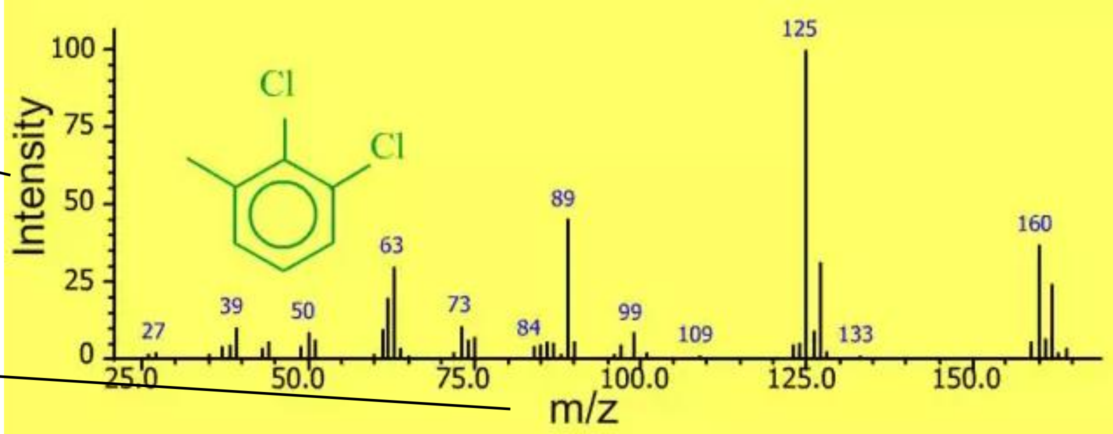
Example of molecular weight

C_2H_4	12.000 X 2 + 1.008 X 4 = **28.032**
CO	12.000 X 1 + 15.995X 1 = **27.995**
N_2	14.003 X 2 = **28.006**

Mass Spectrum

- Ionization produces positively- and negatively-charged atoms, molecules, or fragments called ions.
- A <u>Mass Spectrum is a graphical representation of the mass distribution of the ions.</u>
- The horizontal axis denotes *m/z*, where m is the mass of ion in a unit of u and z is the number of charges of ion.
- From the mass spectrum, we can obtain <u>information about molecular weight and molecular structure</u>, and identify unknown compounds.
- Various types of ions are produced when molecules are ionized in an ionization box.

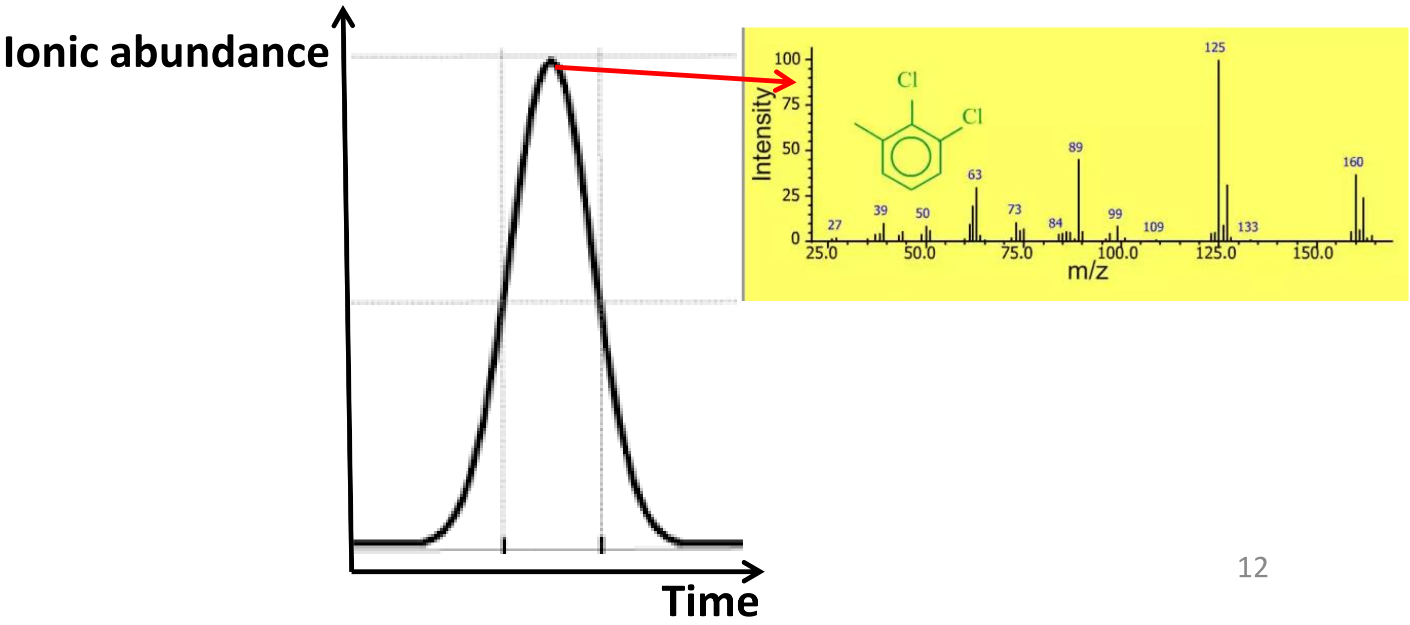
Absolute or relative



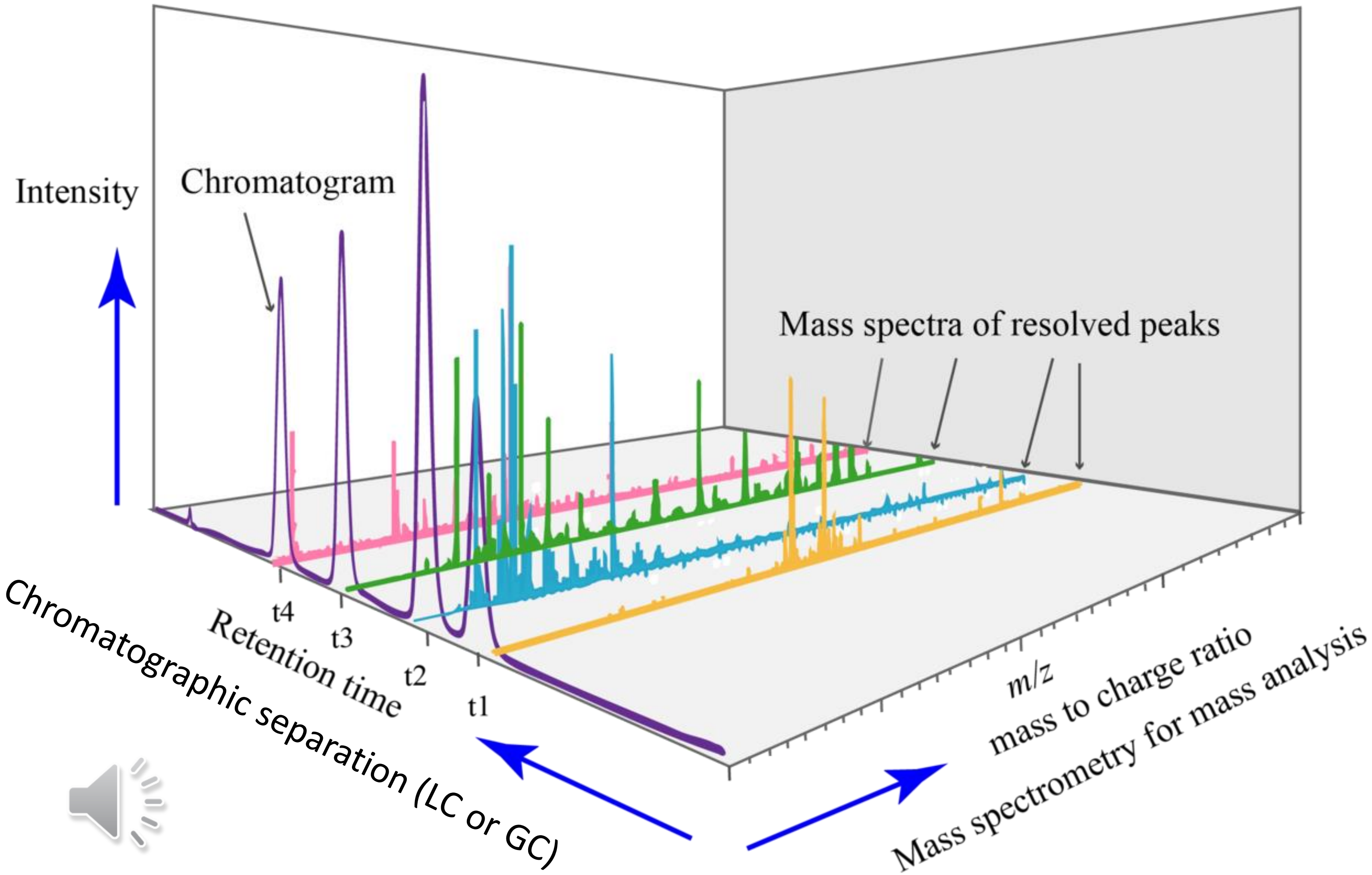
m: mass of ion
z: charge of ion

Ion Chromatogram

- When coupled with chromatography, the components of a mixture are eluted at different elution times from the column.
- The chromatograms obtained are the same as those obtained with other detectors (FID, UV).
- When MS is the detector, the chromatogram is composed of a large set of consecutively acquired mass spectra, each containing spectral information of the eluting compound →Ion Chromatogram

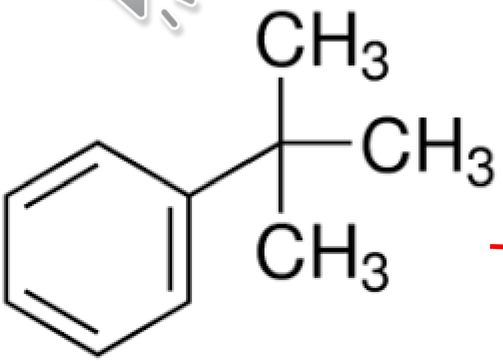


Separation based on both Chromatographic and MS data



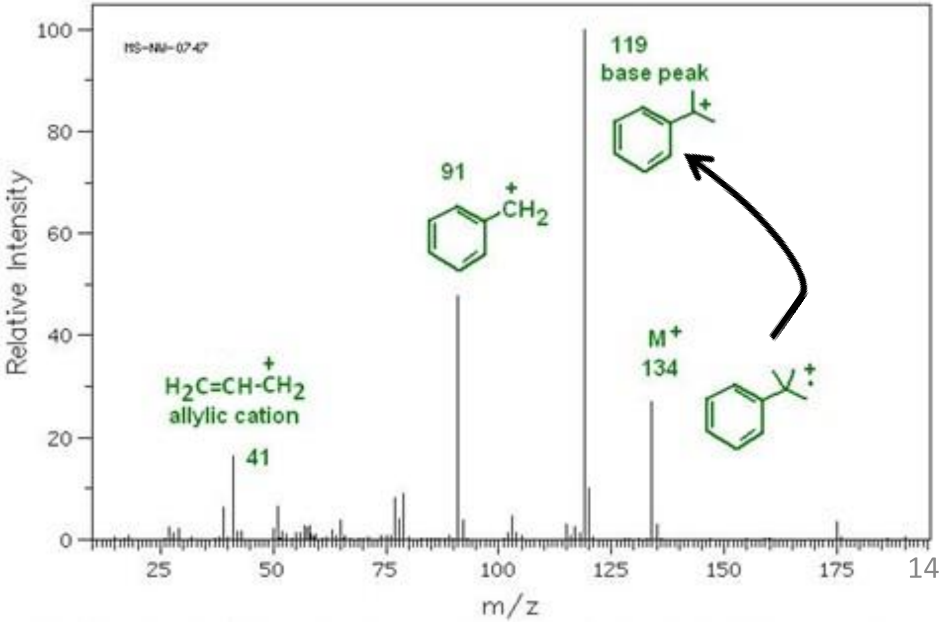
Fragment Ion(s)

- Fragment ions are produced by decomposition of a molecular ion (fragmentation) in the ion source (or in tandem MS experiments).
- There exist many kinds of fragment ions, whose distribution reflects the chemical structure of a compound, according to various ways of fragmentation.
- The fragment ions have smaller masses than the molecular ion.
- Many elements have the natural isotopes. For example, Chlorine with mass number 37 exists in addition to Chlorine 35. The presence of isotopes readily produces the isotope ions in the spectrum accompanied by a main molecular ion peak and fragment peaks. Additionally, we sometimes observe background peaks, arising from chemicals other than samples; for example, water, air, eluting materials from the column and so on.

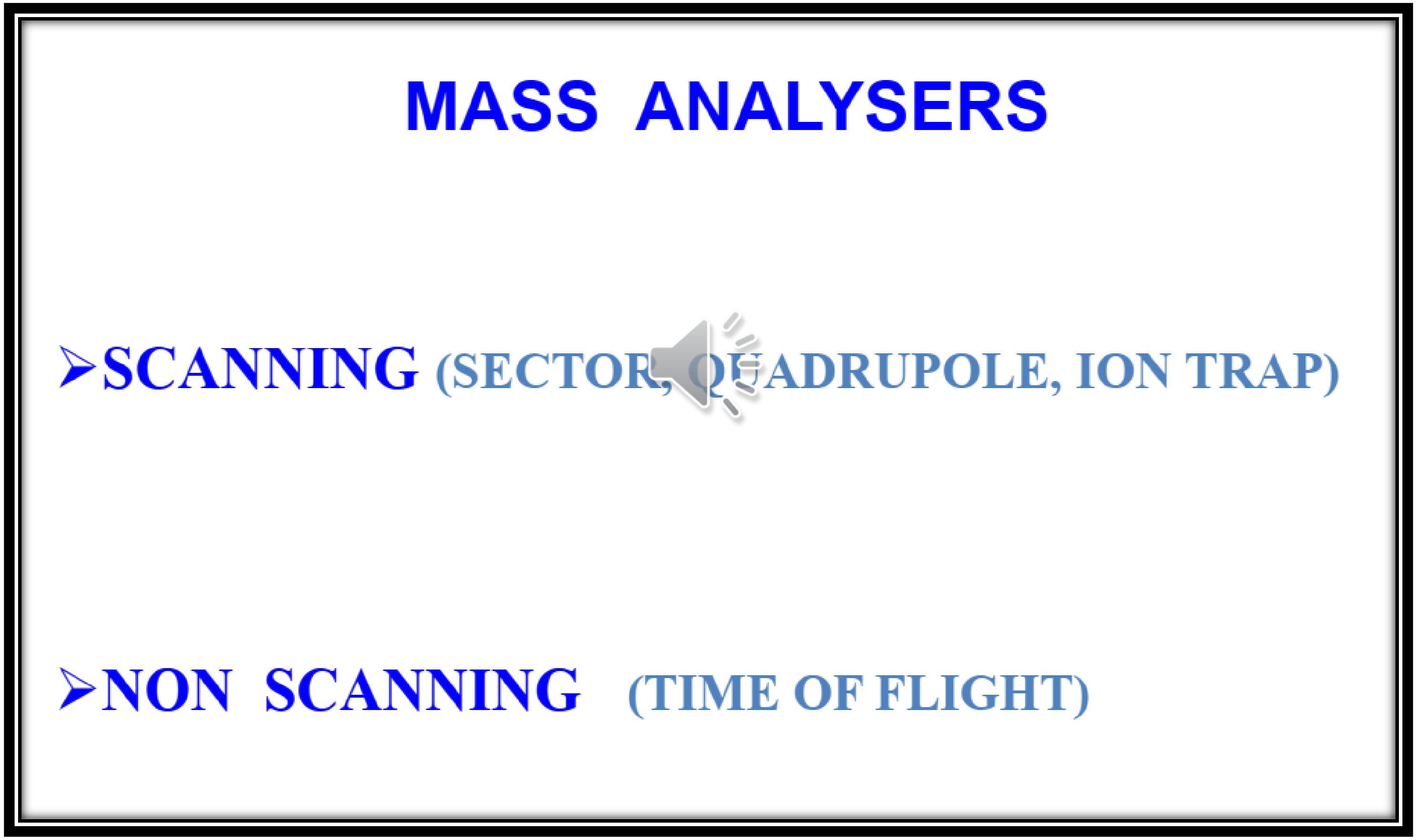


Tert-butyl benzene

$C_{10}H_{14}$ MW (134u)



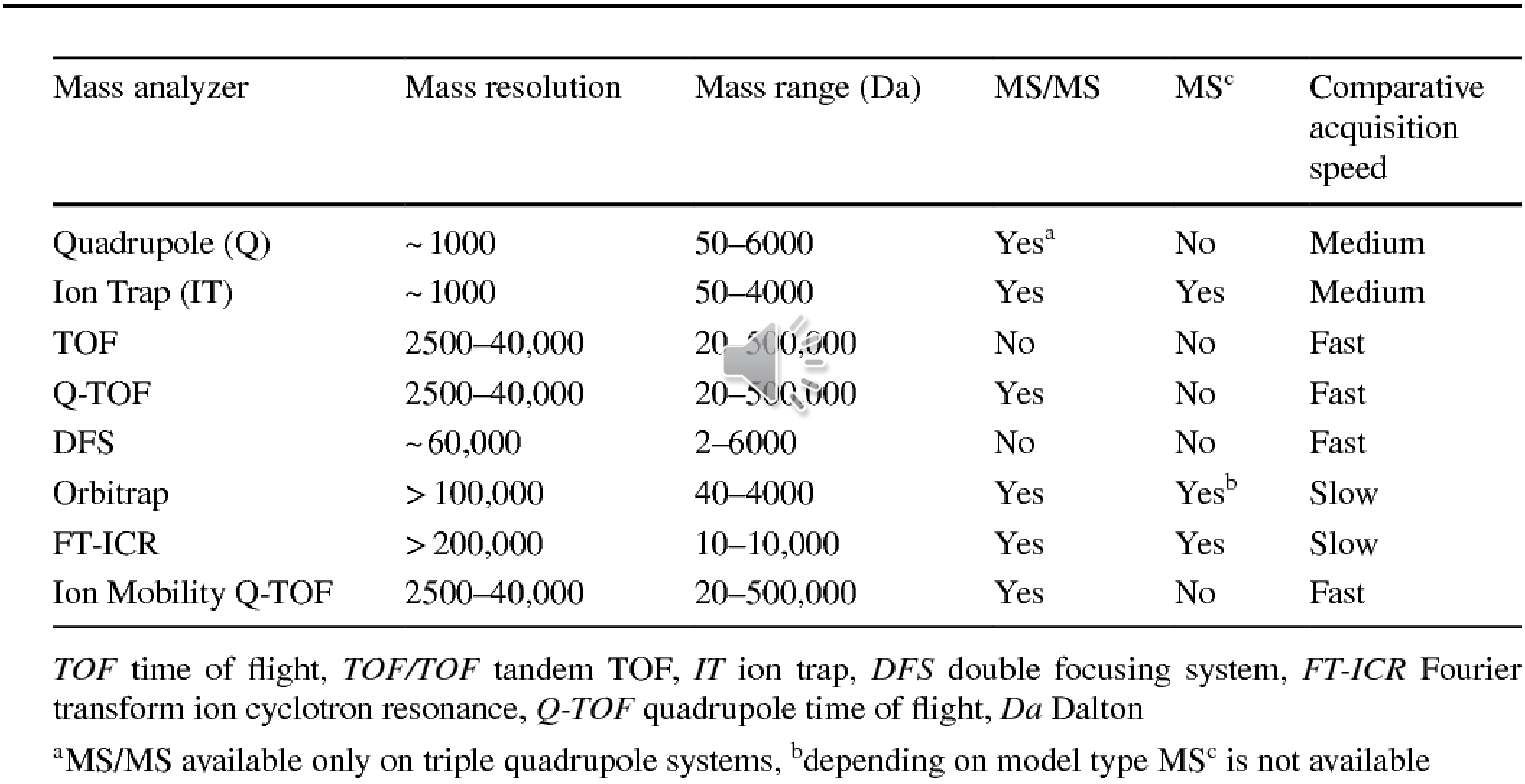
Overview of different MS analyzers



MASS ANALYSERS

- ➤**SCANNING** (SECTOR, QUADRUPOLE, ION TRAP)
- ➤**NON SCANNING** (TIME OF FLIGHT)

Overview of different MS analyzers



Mass analyzer	Mass resolution	Mass range (Da)	MS/MS	MS^c	Comparative acquisition speed
Quadrupole (Q)	~ 1000	50–6000	Yes[a]	No	Medium
Ion Trap (IT)	~ 1000	50–4000	Yes	Yes	Medium
TOF	2500–40,000	20–500,000	No	No	Fast
Q-TOF	2500–40,000	20–500,000	Yes	No	Fast
DFS	~ 60,000	2–6000	No	No	Fast
Orbitrap	> 100,000	40–4000	Yes	Yes[b]	Slow
FT-ICR	> 200,000	10–10,000	Yes	Yes	Slow
Ion Mobility Q-TOF	2500–40,000	20–500,000	Yes	No	Fast

TOF time of flight, *TOF/TOF* tandem TOF, *IT* ion trap, *DFS* double focusing system, *FT-ICR* Fourier transform ion cyclotron resonance, *Q-TOF* quadrupole time of flight, *Da* Dalton

[a] MS/MS available only on triple quadrupole systems, [b] depending on model type MS^c is not available

Basic MS Terminology

- **Mass range -** Range of *m/z* over which a mass spectrometer can detect ions or is operated to record a mass spectrum.
- **Transmission -** The ratio of the number of ions leaving a region of a mass spectrometer to the number entering that region.
- **Mass resolution** - is conventionally defined as the minimum separation between two mass spectral peaks of equal height and width, such that there is a detectable "valley" between them
- **Mass resolving power** - In a mass spectrum, the observed mass divided by the difference between two masses that can be separated: m/Δm. The procedure by which Δm was obtained and the mass at which the measurement was made should be reported.
- **Mass accuracy** - is defined as the difference between *measured accurate mass* and calculated (theoretical) exact mass.

Basic MS Terminology

- **Unified atomic mass unit, *u*** - A non-SI unit of mass defined as one twelfth of the mass of one atom of ^{12}C in its ground state and equal to 1.6605402(10) x 10^{-27} kg.
- **Nominal mass** - Mass of a molecule calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value and equivalent to the sum of the *mass numbers* of all constituent atoms.

  E.g., for CO_2 we calculate the mass by 12 u + 2 × 16 u = 44 u.
- **Exact mass** – Calculated (theoretical) mass of an ion or molecule containing the lighter isotope of each atom.

  It is very close to but not equal to the nominal mass of the isotope (exception for ^{12}C= 12.000000)

  E.g., for CO_2 we calculate the mass by 12 u + 2 × 15.994915 u = 43.989829 u.

Basic MS Terminology

- **Monoisotopic mass** - Exact mass of an ion or molecule calculated using the mass of the most abundant isotope of each element.
- **Relative Atomic Mass:** is calculated as the weighted average of the naturally occurring isotopes of an element.

$$M_r = \frac{\sum_{i=1}^{i} A_i \cdot m_i}{\sum_{i=1}^{i} A_i}$$

A_i being the abundances of the isotopes
m_i their respective isotopic masses

Example: M_r of Cl= 35.4528 u.

$$M_r = \frac{(100 \times 34.968853 \text{ u} + 31.96 \times 36.965903 \text{ u})}{(100 + 31.96)} = 35.4528 \text{ u.}$$

	Isotopic mass	Abundance	Relative abundance
^{35}Cl	34.968853	75.78	100
^{37}Cl	36.965903	24.22	31.96

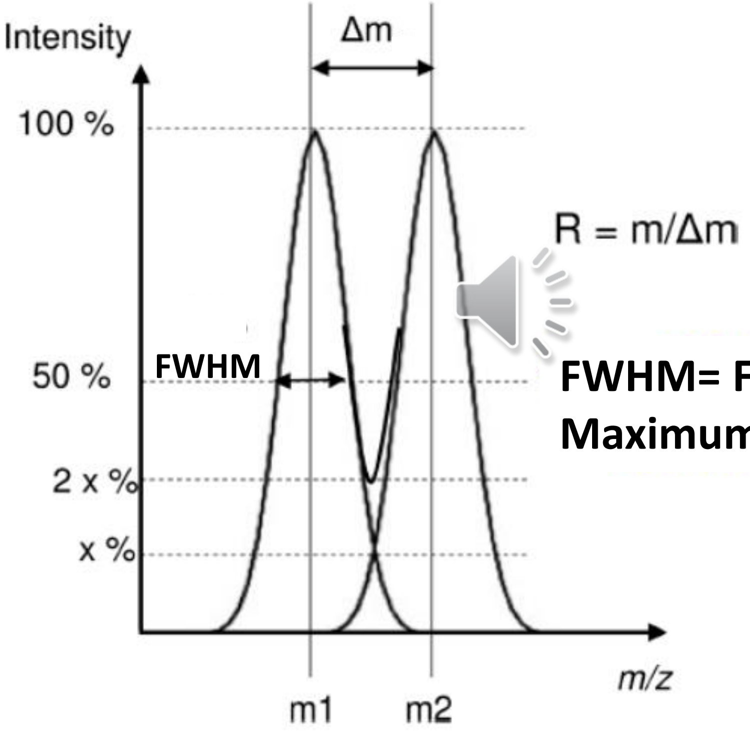
- **Isobaric ions** are ions having the same nominal mass. However, their exact mass is different. Isomers are (almost) perfect isobars.

E.g.

	Nominal masses:	Exact masses:
$N_2^{+\bullet}$	28	28.00559
$CO^{+\bullet}$	28	27.99437
$C_2H_4^{+\bullet}$	28	28.03075

Basic MS Terminology - Mass Resolution and Resolving Power

MASS RESOLVING POWER



FWHM= Full Width at Half Maximum

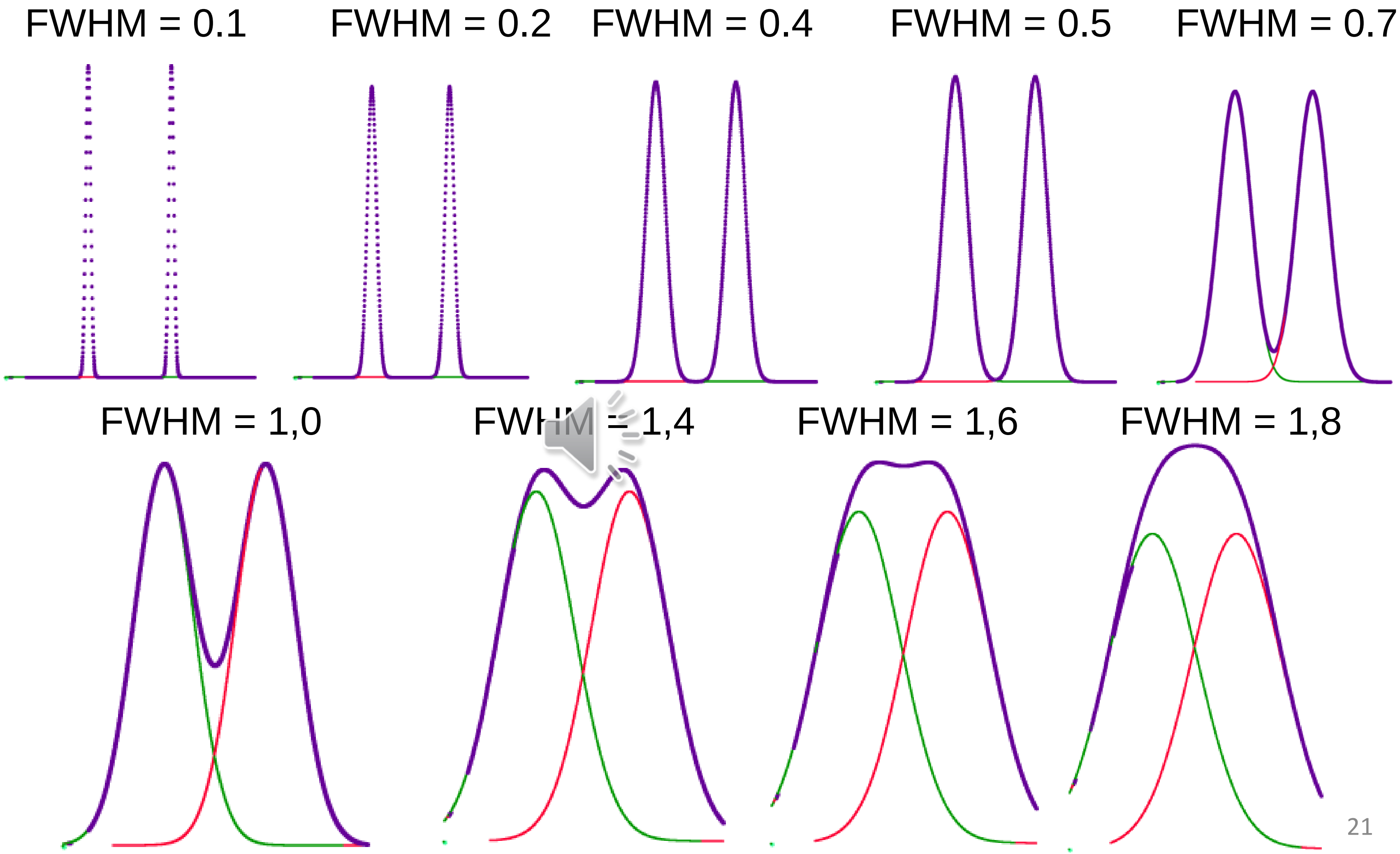
Good mass accuracy can only be obtained from sufficiently sharp and evenly shaped signals that are well separated from each other.
The ability of an instrument to perform such a separation of neighbouring peaks is called *resolving power*. It is obtained from the peak width expressed as a function of mass.

Basic MS Terminology

FWHM= Full Width at Half Maximum

RESOLUTION AND PEAK SHAPE

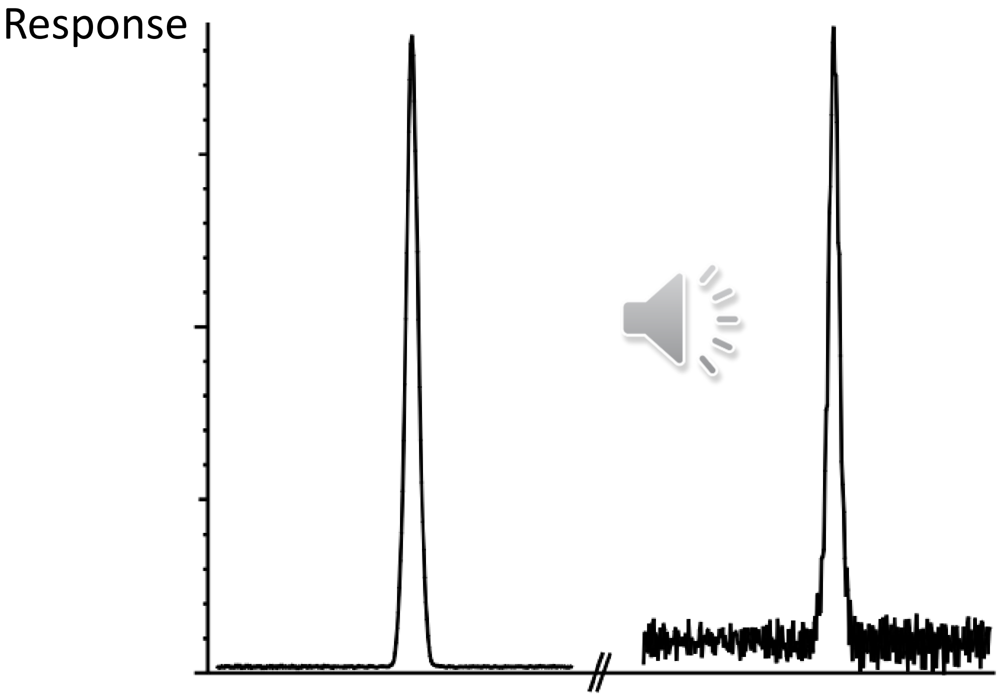
m/z = 1000 and 1001 → 1Da difference between the two peaks



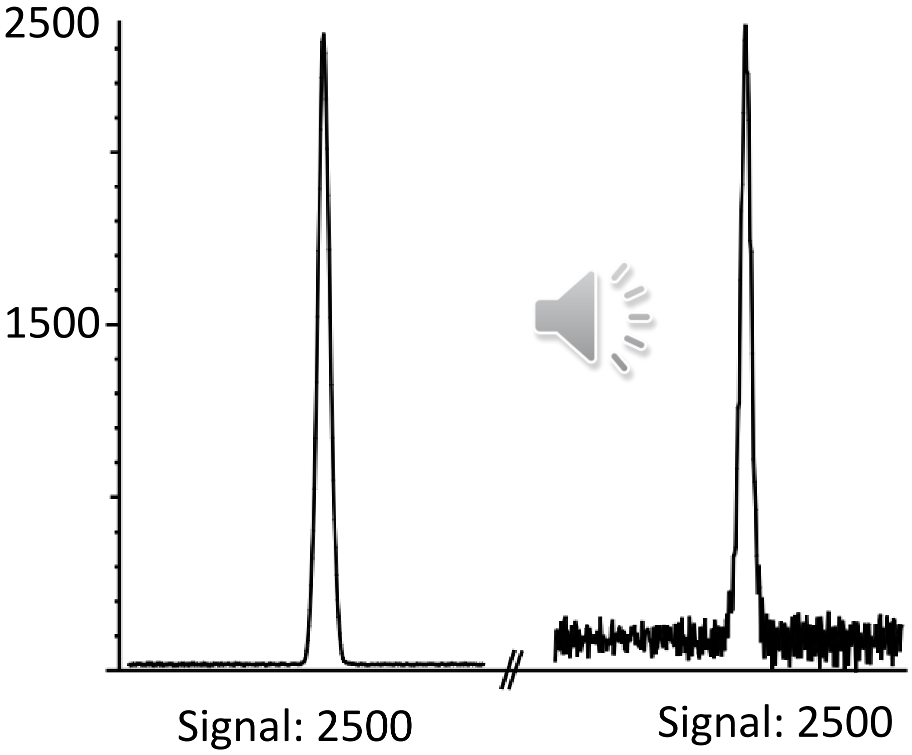
FWHM = 0.1 FWHM = 0.2 FWHM = 0.4 FWHM = 0.5 FWHM = 0.7

FWHM = 1,0 FWHM = 1,4 FWHM = 1,6 FWHM = 1,8

Definition of S/N Ratio



Definition of S/N Ratio



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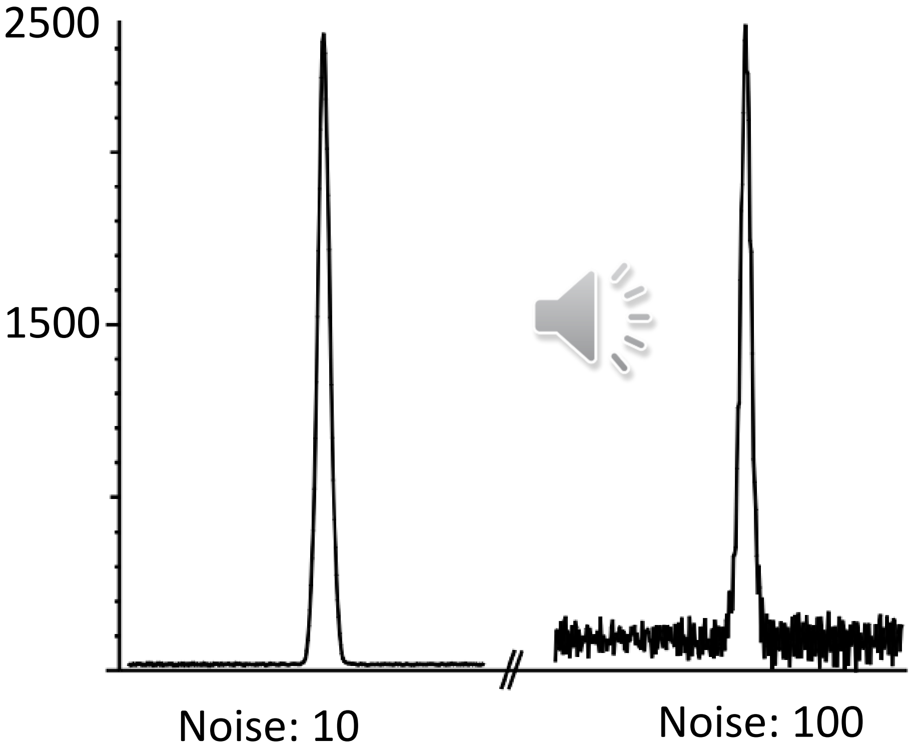
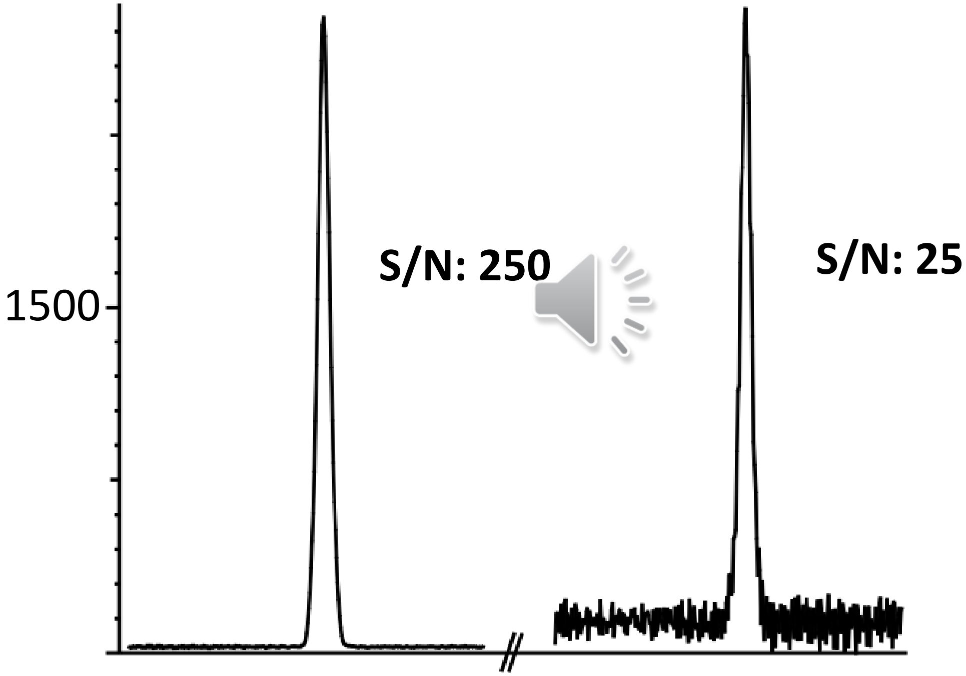


Definition of S/N Ratio



Signal: 2500
Noise: 10

Signal: 2500
Noise: 100

Definition of S/N Ratio

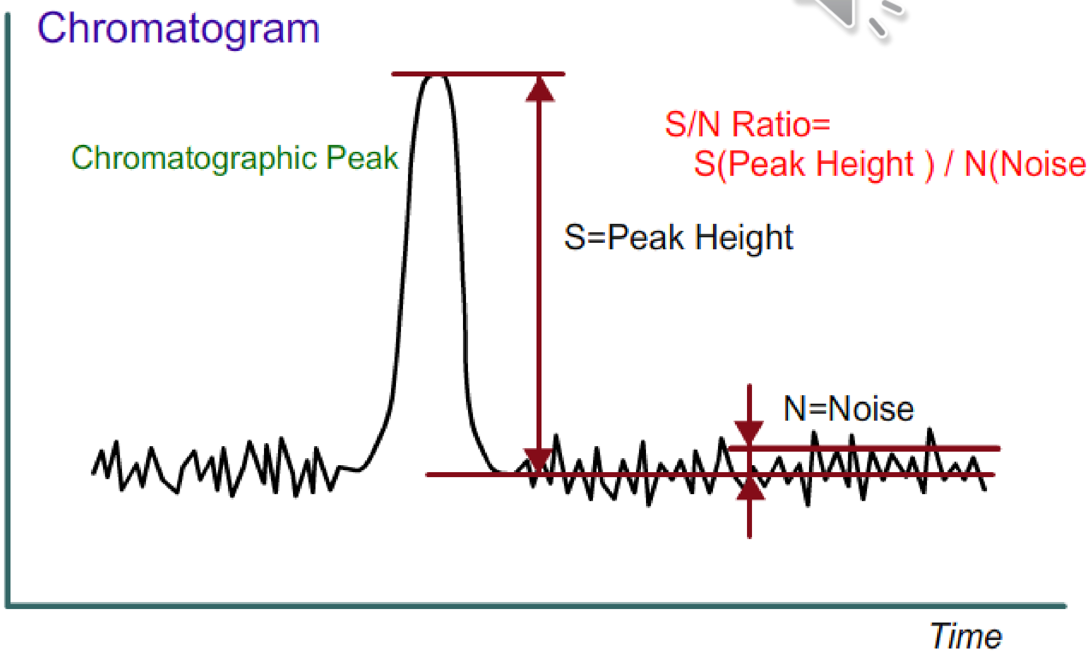
Typical value for a laboratory:

LOD = Limit of Detection

→ **3 σ (Signal is 3x higher than noise)**

LOQ = Limit of quantification

→ **10 σ (Signal is 10x higher than noise)**



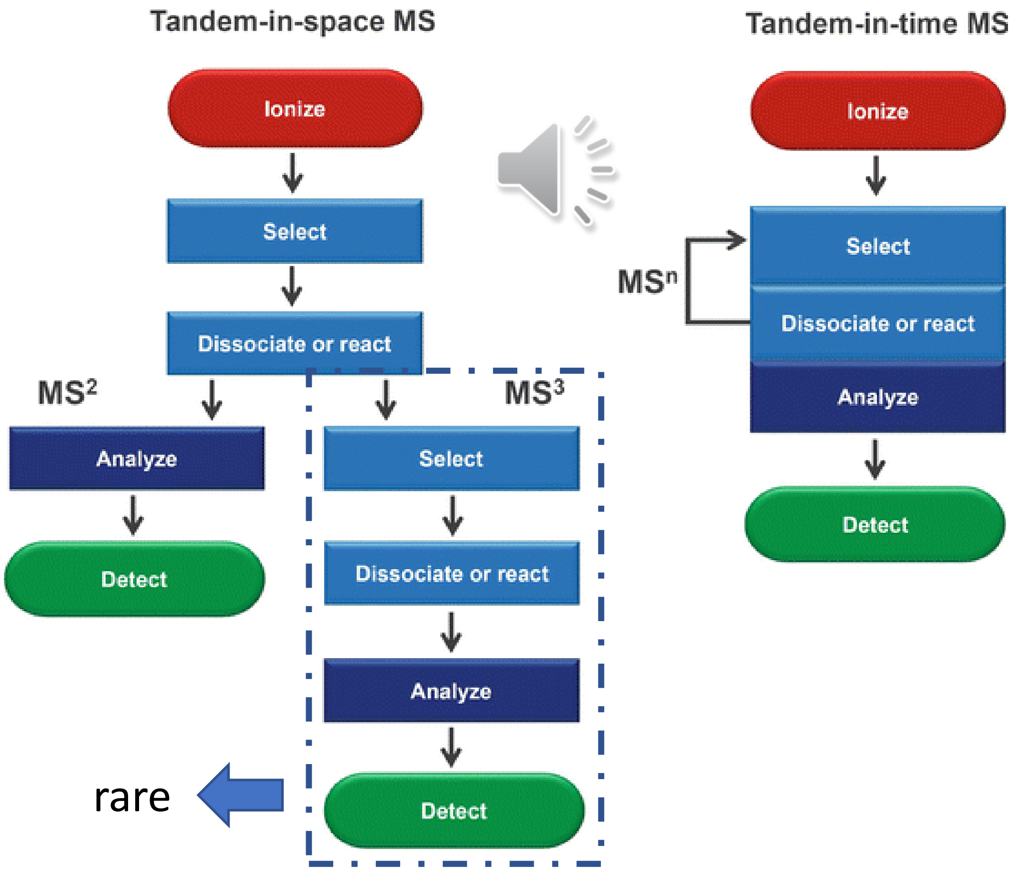
S/N Ratio=
S(Peak Height) / N(Noise in RMS)

Mulditimensional MS
(tandem MS)

Analysis mode in tandem MS

Two stages of mass filtering….two mass analyzer in series:

1. In time: same analyzer with multiple filtering over time → MS^n (ion trap, FTMS..)
2. In space: analyzers physically separated and distinct (quadrupole, sector, tof)



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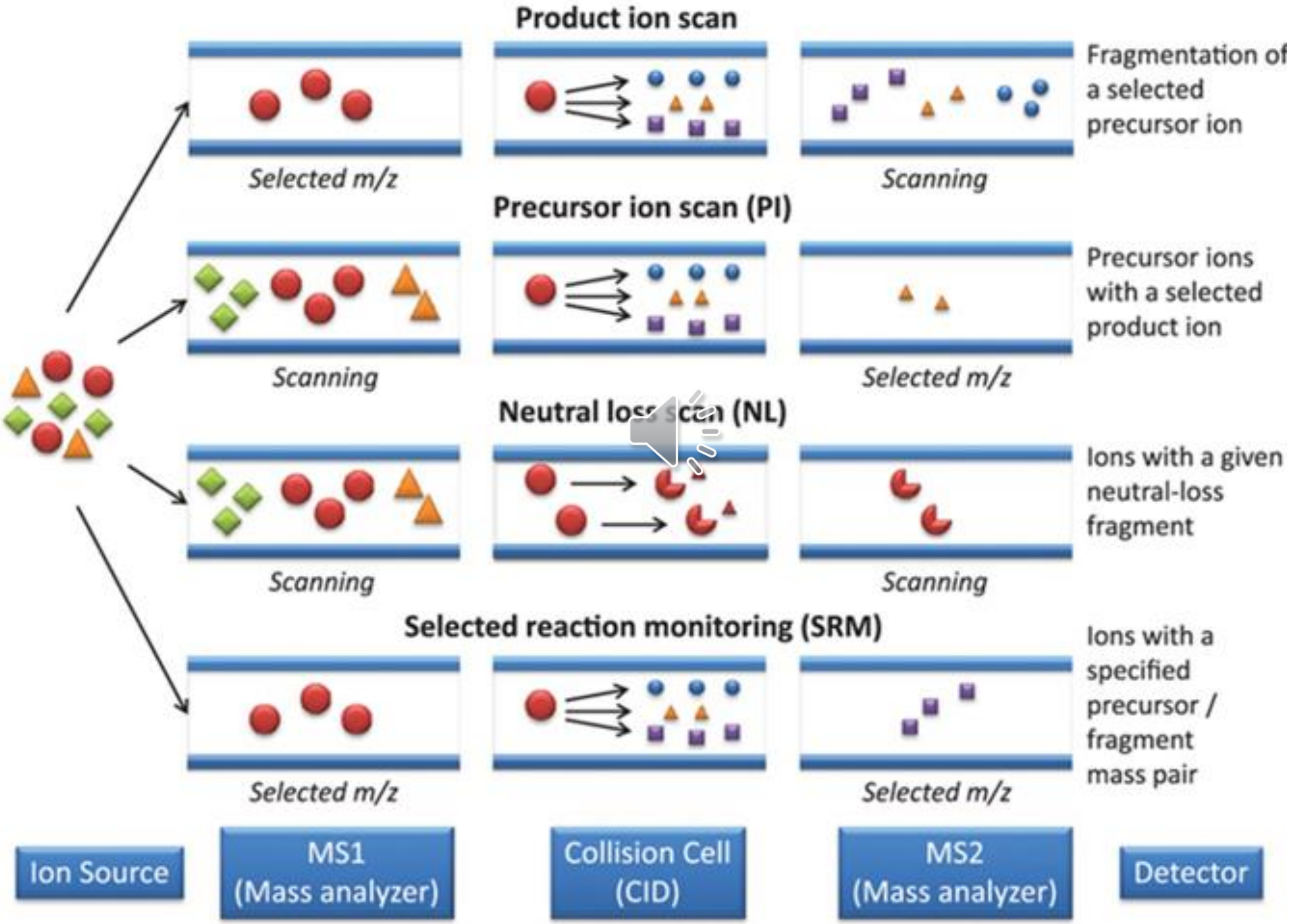
Basic terminological conventions for tandem mass spectrometry

- The term ***tandem mass spectrometry*** or ***mass spectrometry/mass spectrometry*** collectively describes mass spectrometric experiments where mass-analyzed ions are subjected to fragmentations or ion–molecule reactions and the products thereof are collected and mass-analyzed by a second MS stage.
- Instruments are accordingly referred to as ***tandem mass spectrometers***; their stages are denoted MS1, MS2 etc.
- Tandem MS is often abbreviated as MS/MS or MS2. Tandem MS experiments of higher order are referred to as MS3, MS4, … or generally as MSn.
- Ions emerging from MS1 are termed precursor ions, those entering MS2 are called ***product ions***; in higher-order experiments, one may refer to them as nth generation product ions. (The old terms ***parent ion*** and daughter ion are deprecated.)
- Spectra are called tandem mass spectra (never MS/MS spectra).

Analysis mode in tandem MS in space



Product ion scan

Selected m/z — Scanning

Fragmentation of a selected precursor ion

Precursor ion scan (PI)

Scanning — Selected m/z

Precursor ions with a selected product ion

Neutral loss scan (NL)

Scanning — Scanning

Ions with a given neutral-loss fragment

Selected reaction monitoring (SRM)

Selected m/z — Selected m/z

Ions with a specified precursor / fragment mass pair

Ion Source

MS1 (Mass analyzer)

Collision Cell (CID)

MS2 (Mass analyzer)

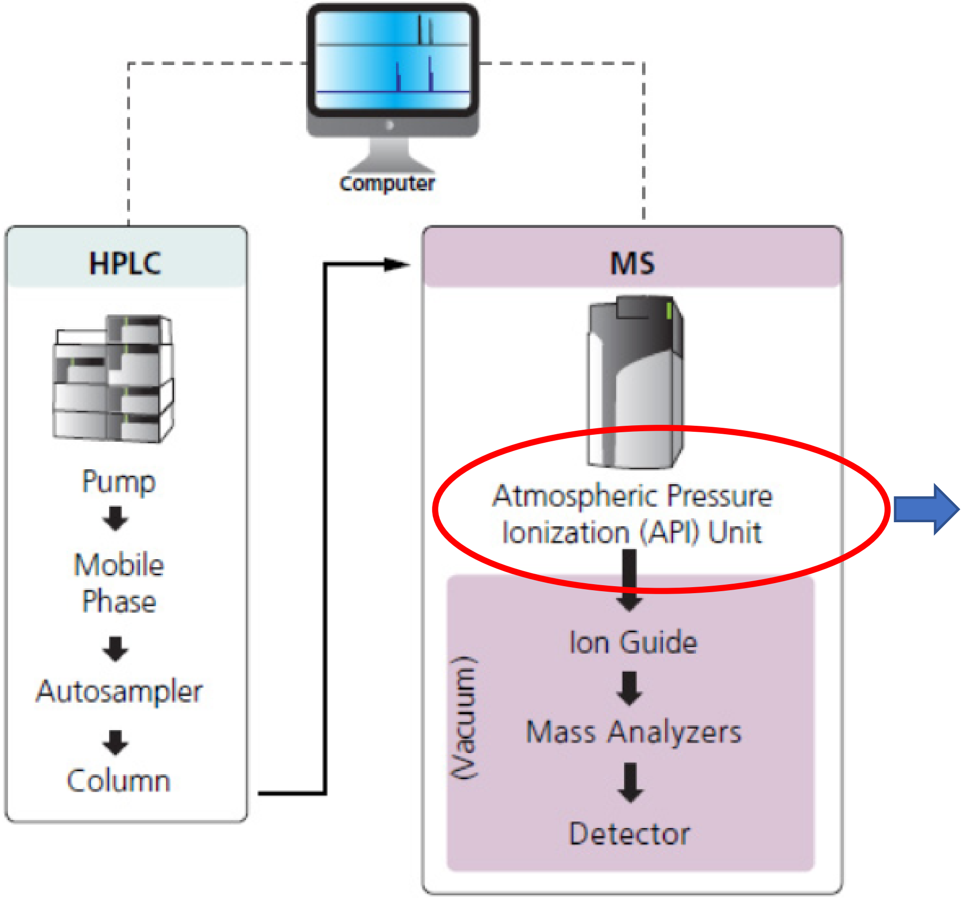
Detector

PART II

MASS SPECTROMETRY COULPED TO LC

Basic instrumentation of LCMS

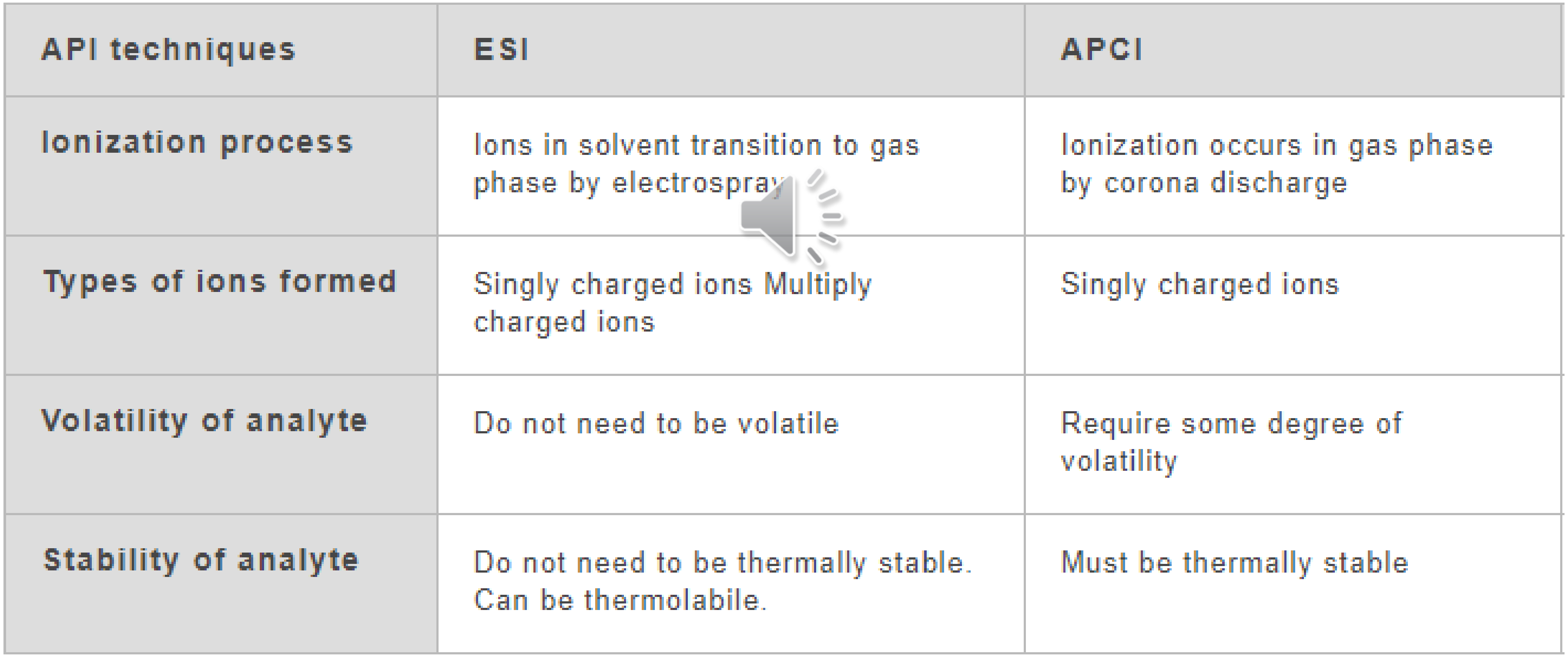
The basic components of an LC-MS system



Device that allows the vaporization, ionization and transfer of the analytes from the LC to the MS

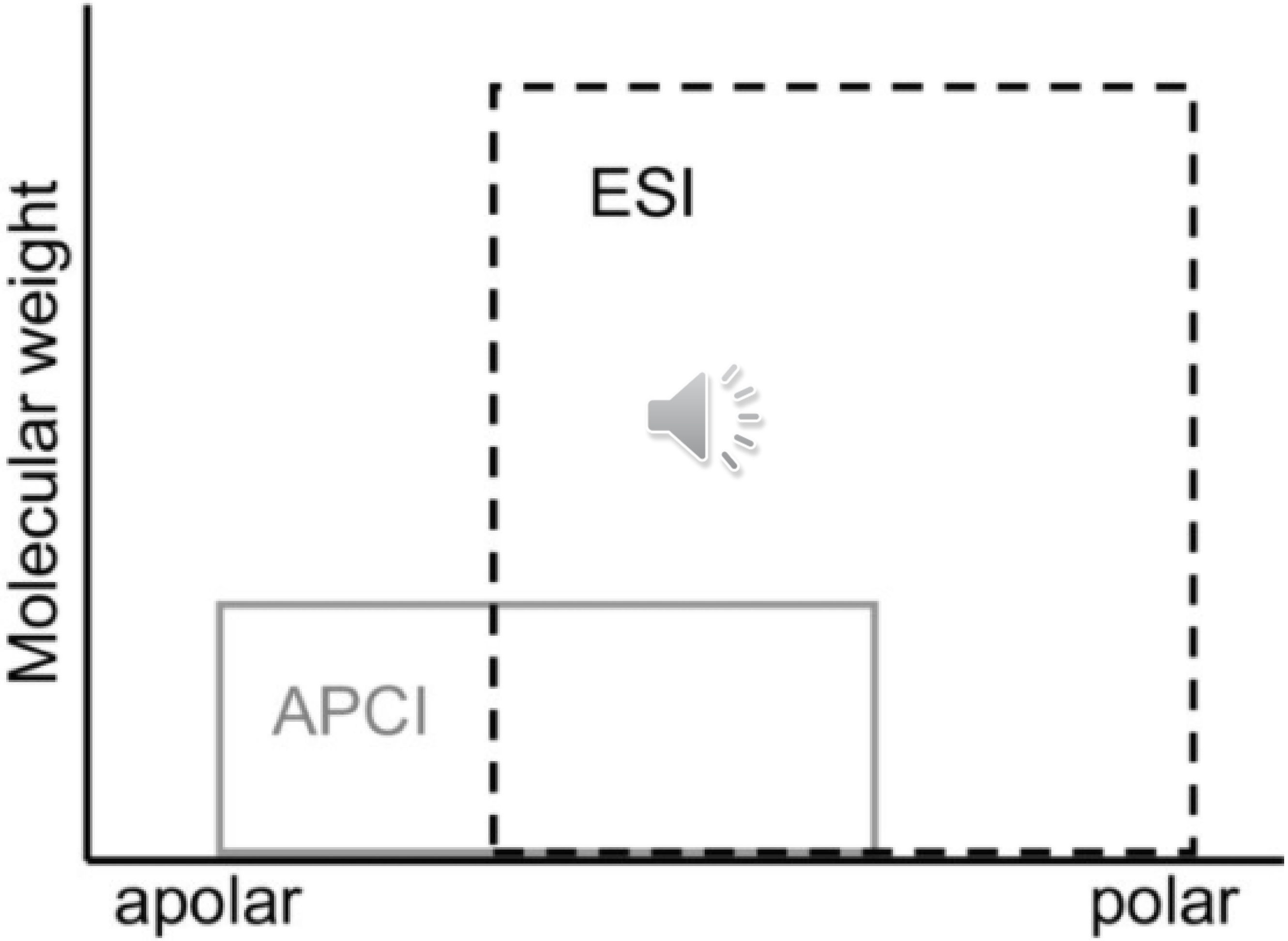
Atmospheric Pressure Ionization (API)

- the selection of API techniques in LCMS is based on the analytes' polarities and properties

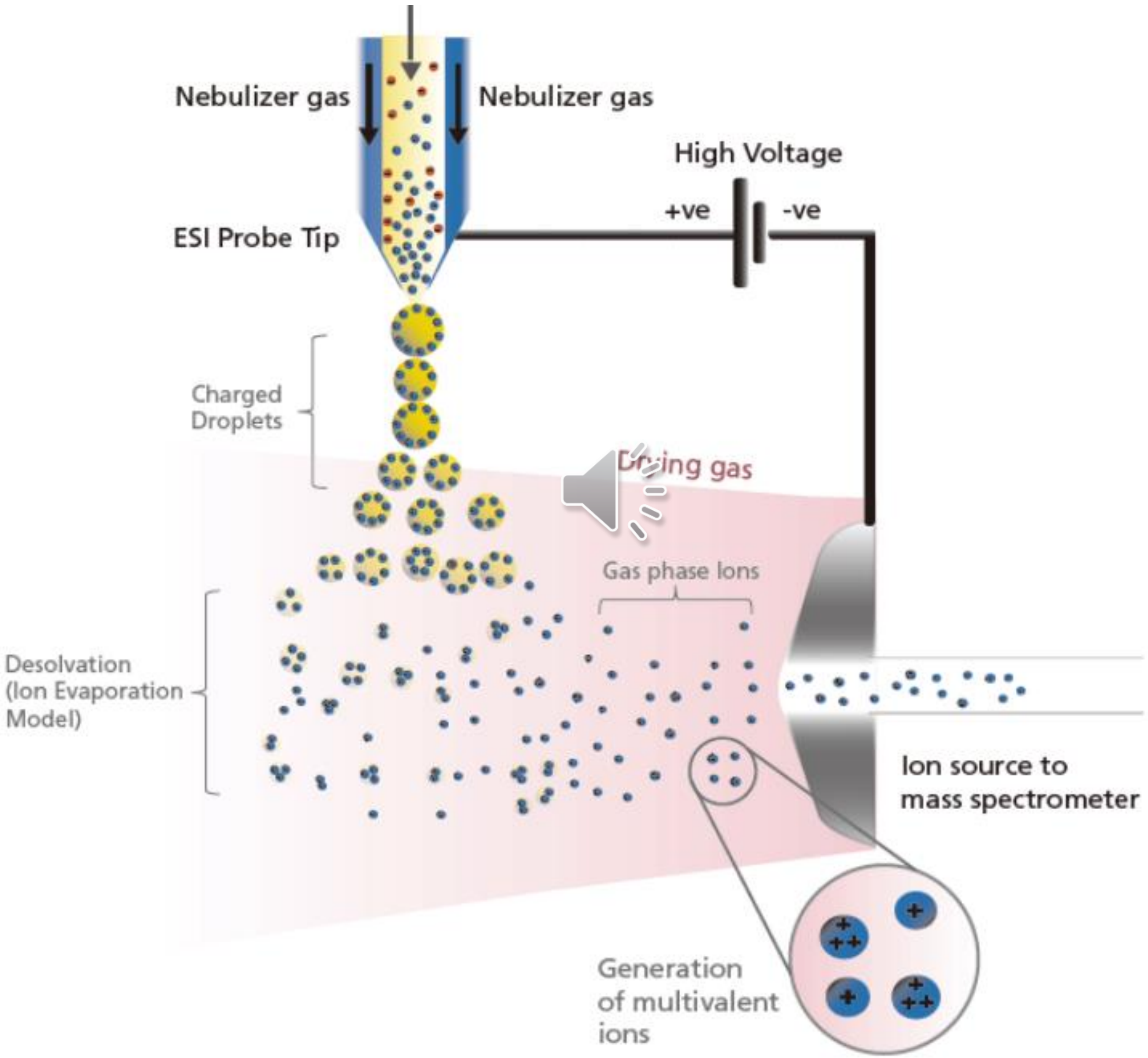


API techniques	ESI	APCI
Ionization process	Ions in solvent transition to gas phase by electrospray	Ionization occurs in gas phase by corona discharge
Types of ions formed	Singly charged ions Multiply charged ions	Singly charged ions
Volatility of analyte	Do not need to be volatile	Require some degree of volatility
Stability of analyte	Do not need to be thermally stable. Can be thermolabile.	Must be thermally stable

Atmospheric Pressure Ionization (API)

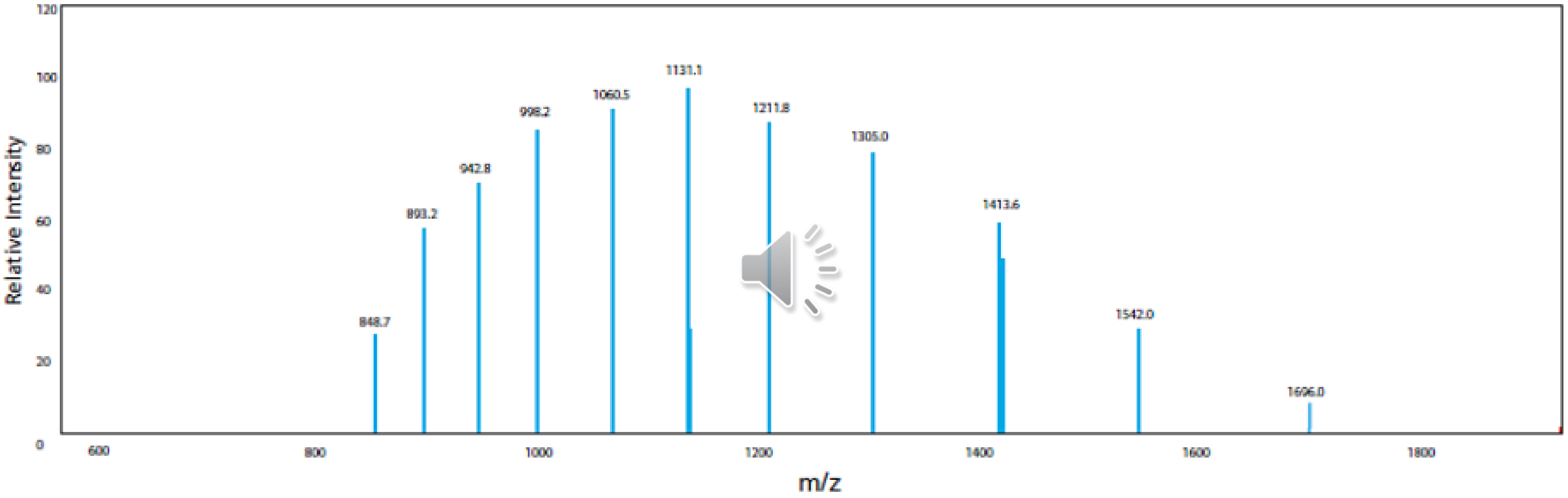


Electrospray Ionization (ESI)



Electrospray Ionization (ESI)

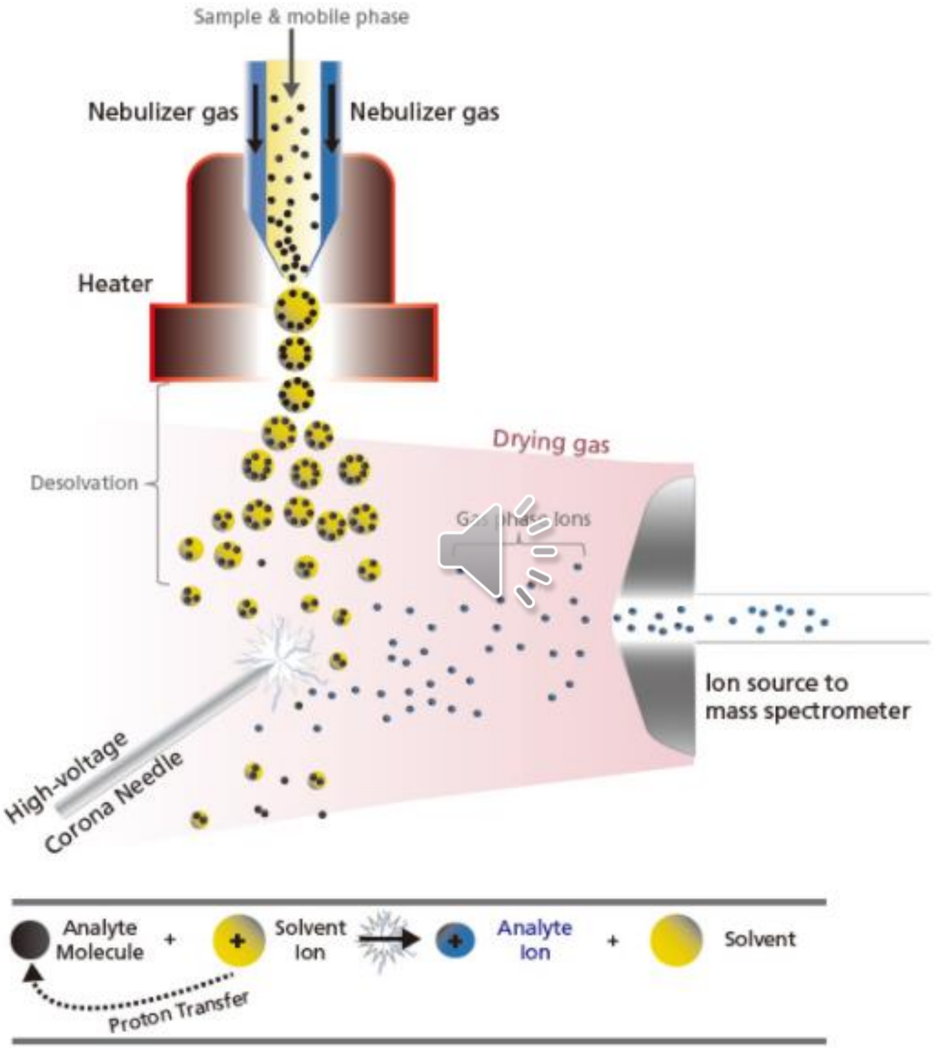
Multiple ions charges



Molecular mass [M + nH]$^{n+}$ *of **myoglobin** calculated using ESI spectrum and deconvolution,*

multivalent ions (n = 10 to 20) of myoglobin is observed in the ESI mass spectrum.

Atmospheric Pressure Chemical Ionization (APCI)



Schematic of the ion-molecular reaction (e.g. proton transfer) in APCI.

Overview of Atmospheric Pressure Ionization (API)

Parameters that can affect the efficiency and sensitivity during the ionization by APIs

- Flow rate of LC or liquid inlet
- Solvents / mobile phases (e.g. types, pH and additives used)
- Properties of analytes (e.g. volatility, thermal stability and ability to form charged species)
- Matrix effects (e.g. suppression and enhancement)
- Output from LC (e.g. peak width)

Overview of Atmospheric Pressure Ionization (API) ESI

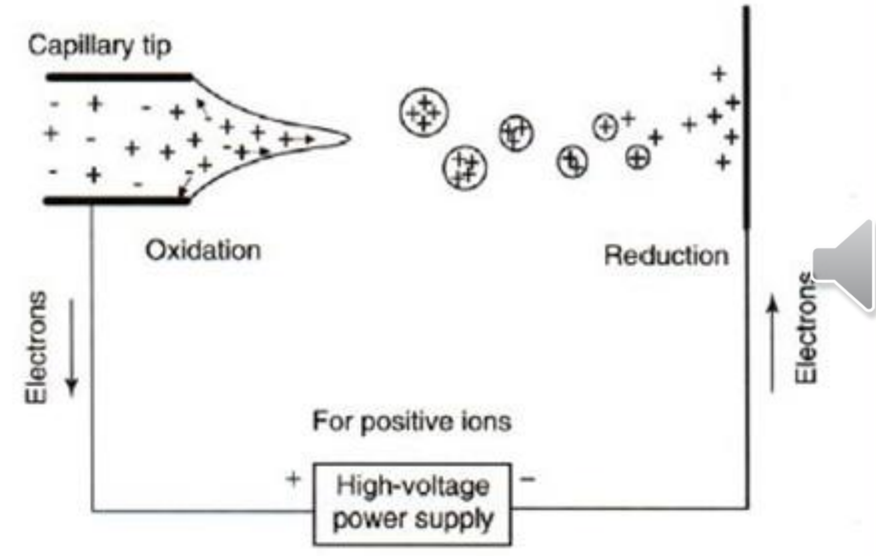
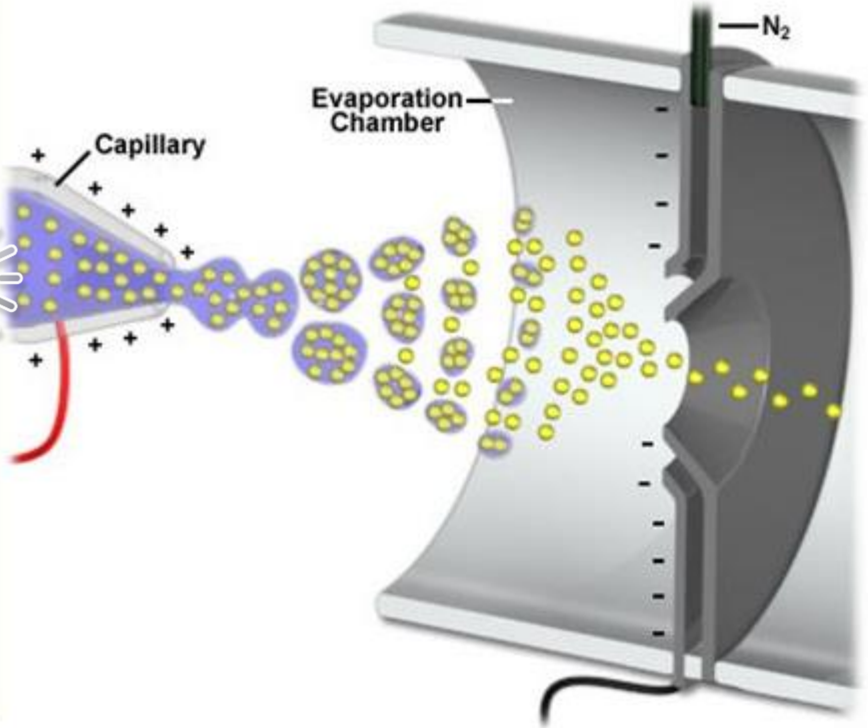
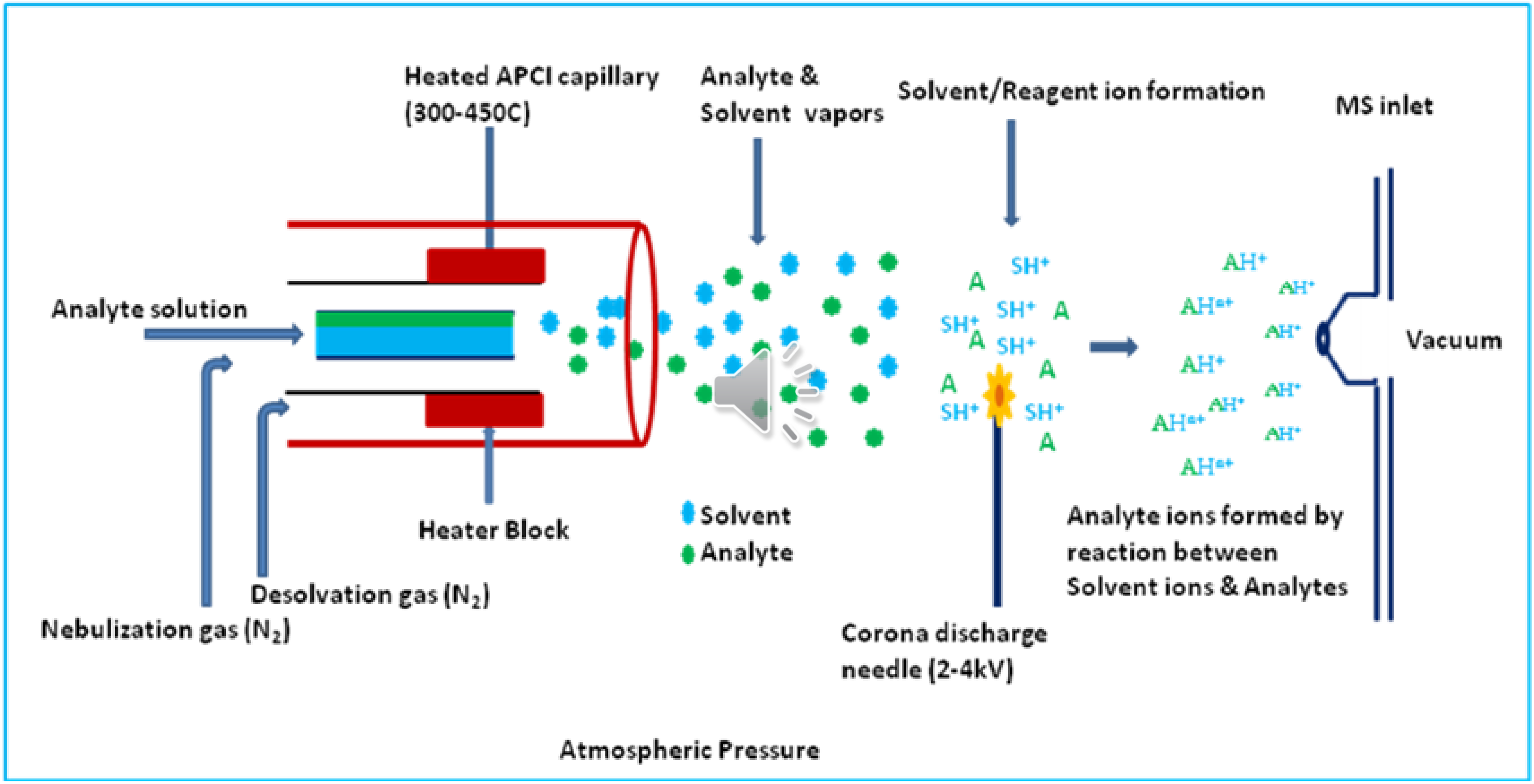


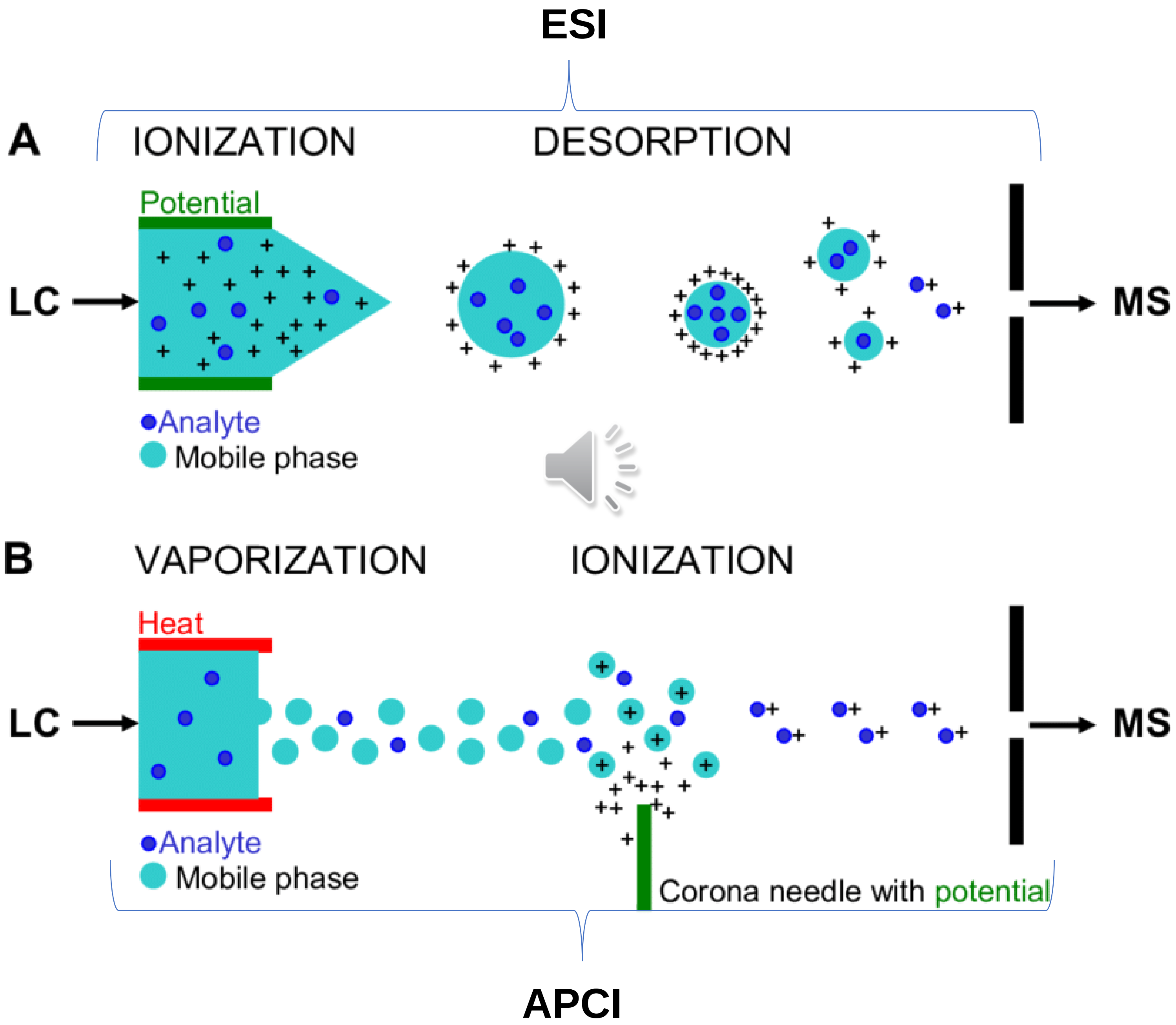
Figure 1.26
Schematic representation of electrochemical process in ESI



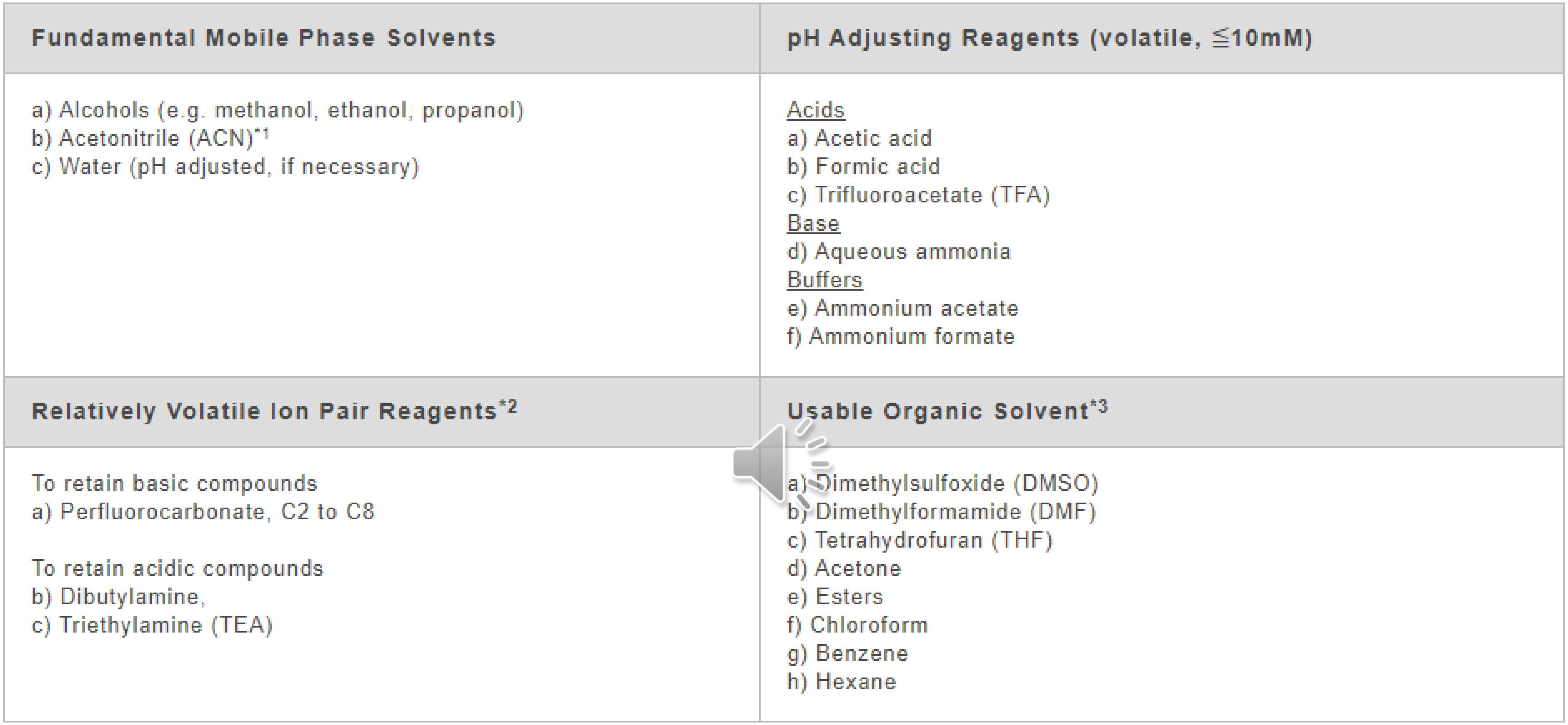
Overview of Atmospheric Pressure Ionization (API)
APCI



Overview of Atmospheric Pressure Ionization (API)



Mobile phases compatible for LCMS



Fundamental Mobile Phase Solvents	pH Adjusting Reagents (volatile, ≦10mM)
a) Alcohols (e.g. methanol, ethanol, propanol) b) Acetonitrile (ACN)[*1] c) Water (pH adjusted, if necessary)	Acids a) Acetic acid b) Formic acid c) Trifluoroacetate (TFA) Base d) Aqueous ammonia Buffers e) Ammonium acetate f) Ammonium formate
Relatively Volatile Ion Pair Reagents[*2]	**Usable Organic Solvent[*3]**
To retain basic compounds a) Perfluorocarbonate, C2 to C8 To retain acidic compounds b) Dibutylamine, c) Triethylamine (TEA)	a) Dimethylsulfoxide (DMSO) b) Dimethylformamide (DMF) c) Tetrahydrofuran (THF) d) Acetone e) Esters f) Chloroform g) Benzene h) Hexane

[*1] *Acetonitrile is not compatible with APCI due to the reduction of nitrile to carbon for negative ionization. In this case, methanol should be used instead.*

[*2] *Use minimally as these substances can remain in the LC and MS system even after changing mobile phase. It is necessary to flush the LC system to remove any traces of these ion-pairing agents.*

[*3] *If a "fundamental mobile phase solvent" is present, it usually does not pose a problem if the mobile phase contains some of these organic solvents. (However, the ionization effect decreases as the concentration of usable organic solvents increases.)*

PART III

MASS SPECTROMETRY COULPED TO GC

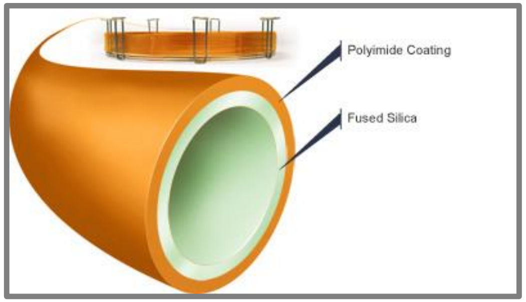
Coupling MS with GC…easy nowadays (Interface)

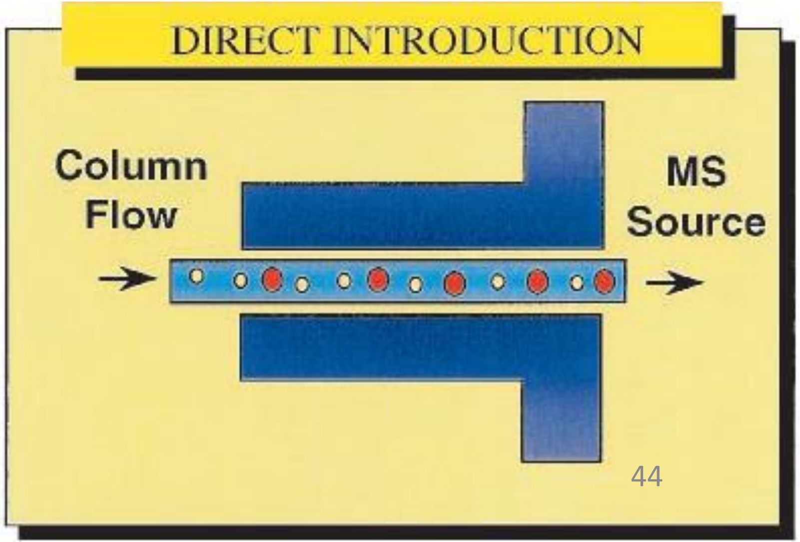
- direct coupling

- the column outlet is introduced directly into the ion source

- no enrichment occurs

- the GC and MS are connected by a heated metallic transfer line





Available Ionization Methods for GC-MS

Hard Ionization:

Good for structural analysis using the generated fragment ions.

- **70eV EI (Electron Ionization)**

Soft Ionization:

Provides molecular formula information.

- Low-energy EI
- CI (Chemical Ionization)
- FI (Field Ionization)
- PI (Photo Ionization)
- APCI (Atmospheric Pressure Chemical Ionization)

The history of ionizations

1921 A. J. Dempster, EI (Electron Ionization)

1950 E. W. Müller, FI (Field Ionization)

1956 F. P. Lossing, PI (Photo Ionization)

1963 R. E. Honig, LD (Laser Desorption)

1966 M. S. B. Munson,
CI (Chemical Ionization)

1969 H. D. Beckey, FD (Field Desorption)

1977 D. F. Hunt,
DCI (Desorption Chemical Ionization)

1981 M. Barber,
FAB(Fast Atom Bombardment)

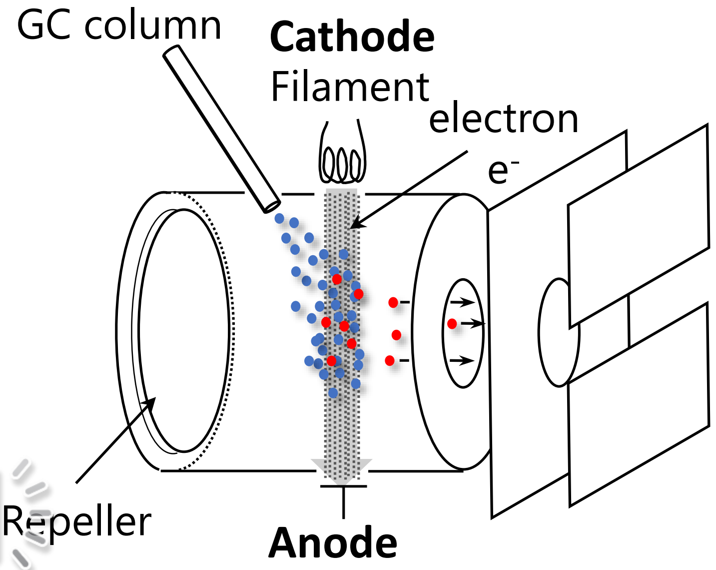
1984 J. B. Fenn, ESI (Electrospray Ionization)

1987 F. Hillenkamp, K. Tanaka,
MALDI (Matrix-assisted Laser Desorption Ionization)

2005 R.B. Cody, J.A. Laramée,
DART (Direct Analysis in Real Time)

Electro Ionization (EI)

- EI dates back to the infancy of MS (early 20th century)
- EI is the most widely used ionization method in GC-MS.
- Since the EI method is the hardest ionization method, many fragment ions are observed.
- The reproducibility of the relative intensity (spectral pattern) of each observed ion is high, and qualitative analysis can be easily performed by comparing the pattern with the EI mass spectrum recorded in databases.
- The number of compounds contained in the NIST database of EI mass spectra exceeds 300,000 and the presence of this database extends the range of applications for GC-MS and GCxGC/MS.
- **Disadvantage**: often, MW information is lacking



● Sample neutral molecule, M
● Ionized molecule or fragments

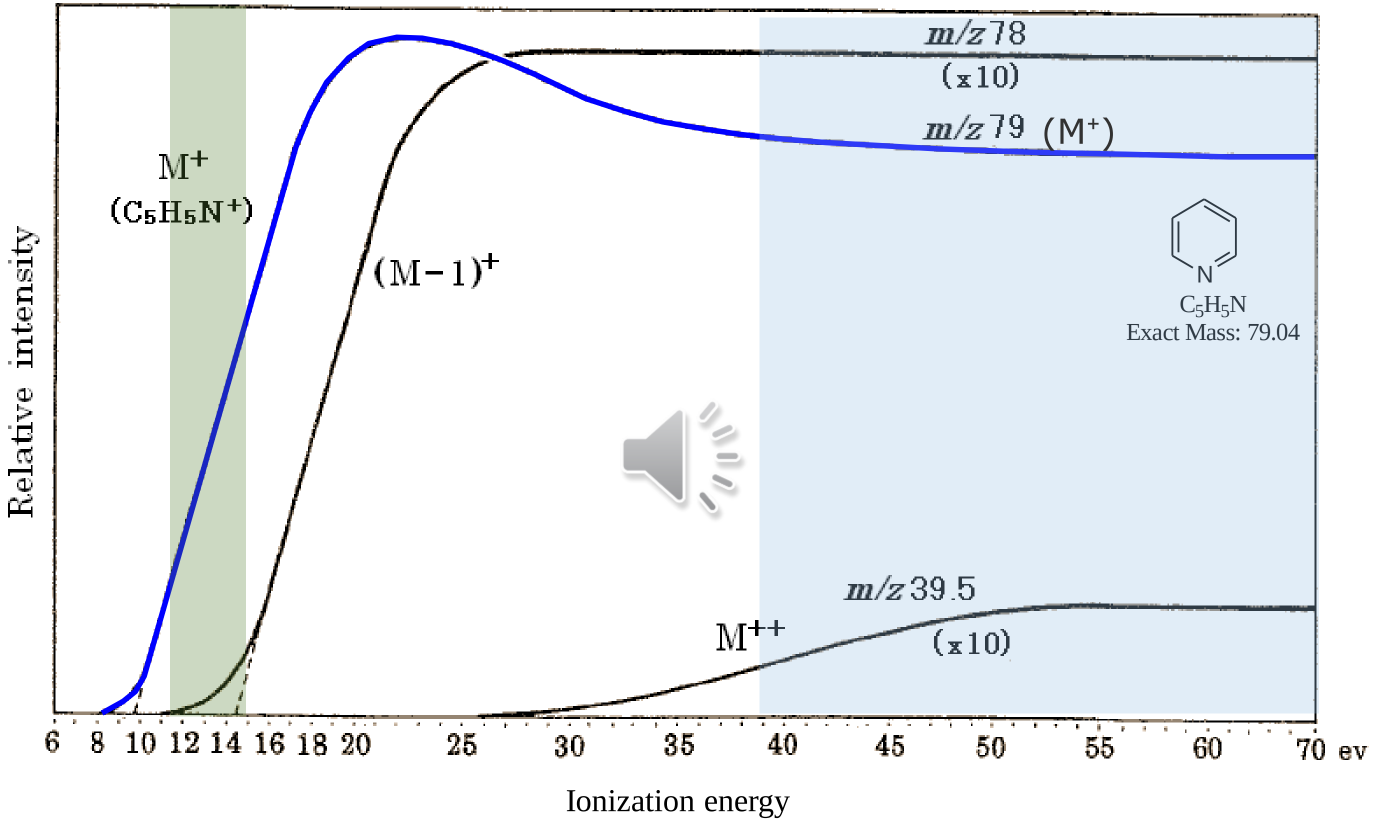
$$M + e^- \longrightarrow M^{+\cdot} + 2e^- \qquad \text{ionization}$$

$$M^{+\cdot} \longrightarrow m_1^+ + n^\cdot \qquad \text{dissociation}$$

https://www.youtube.com/watch?v=22dr-3XDNmQ

Ionization energy and ionization efficiency

Ionization energy and ionization efficiency (pyridine)



- ☐ The total ion intensity is high and stable in the range of 40-100eV.
- ☐ Typically, the total ion intensity is exhibiting a maximum at around 70eV.

GC-EI-MS at 70eV

Advantage	Disadvantage
Sensitivity Reproducibility Library-searchable fingerprint spectra	Extensive fragmentation (loss of molecular ion and/or diagnostic fragments)

unknown analytes, homologous series, or isomers

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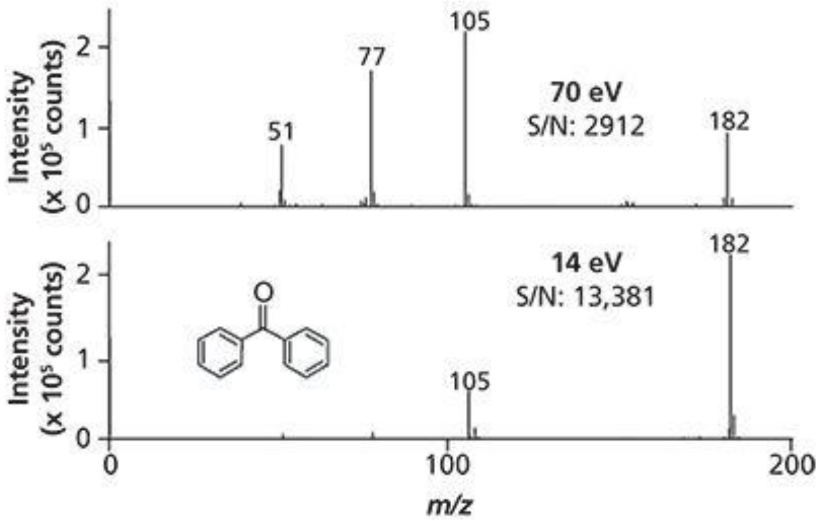
Chromatography

GC-EI-MS < than 70eV

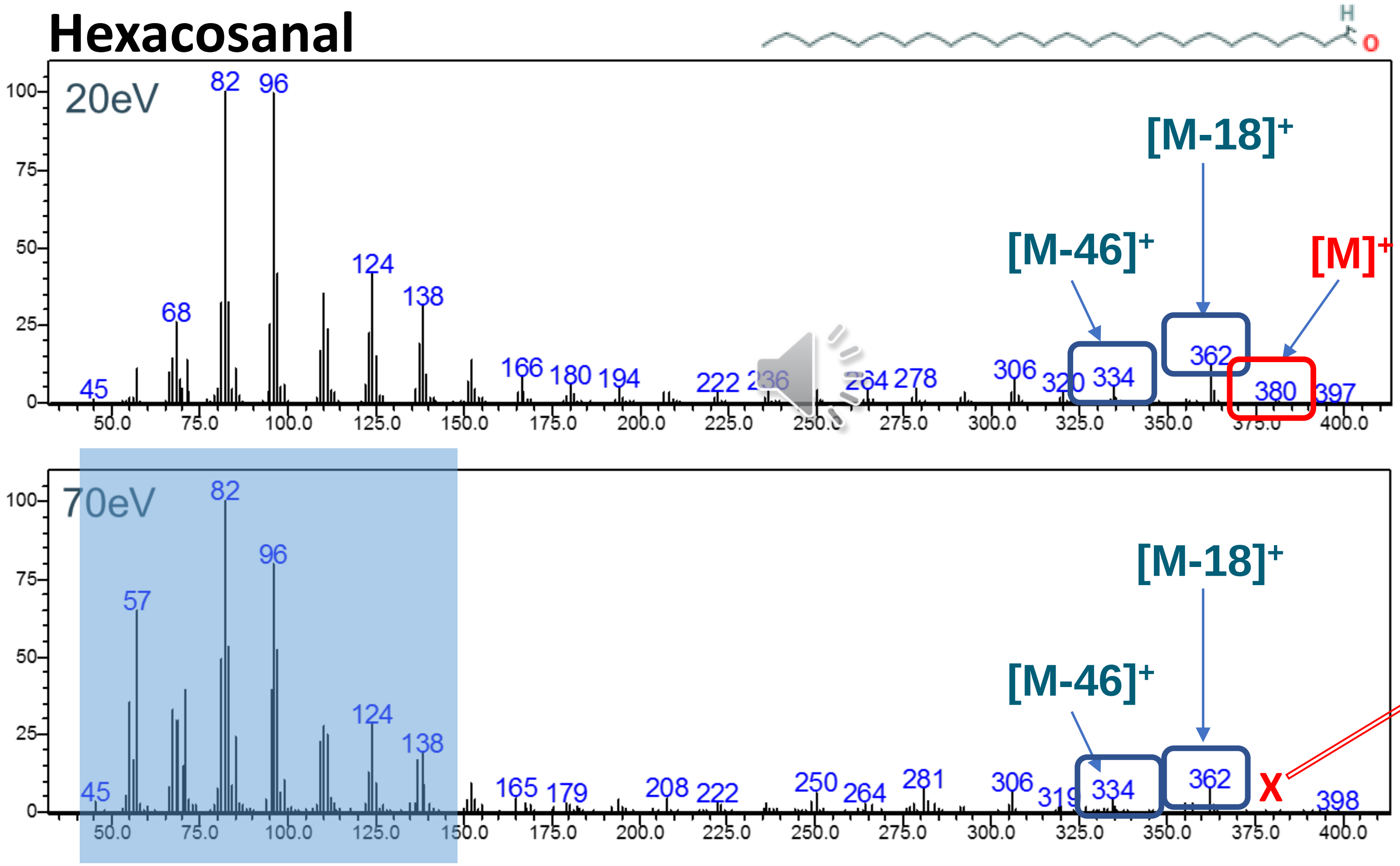
Advantage	Disadvantage
Sensitivity Reproducibility ⬇ **Library-searchable fingerprint spectra**	Extensive fragmentation (increment of **molecular ion** and/or **diagnostic fragments**) ⬆



Just an example!

EI mass spectrum with different ionization energy

No match with MS Library at 70eV



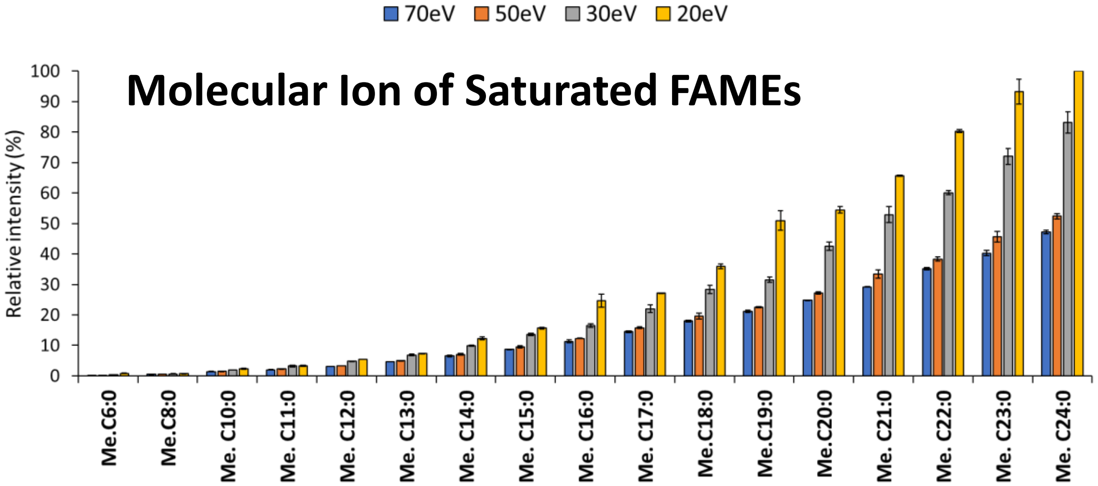
Hexacosanal

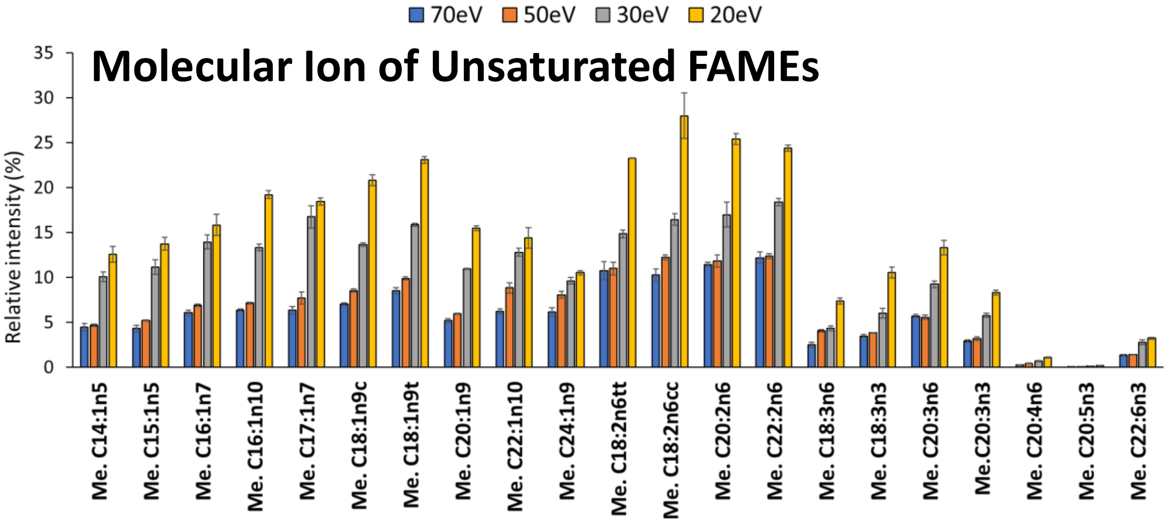
No molecular ion information

Typical fragmentation of saturated aldehydes

Beccaria et al., Anal. Bioanal. Chem., 2018

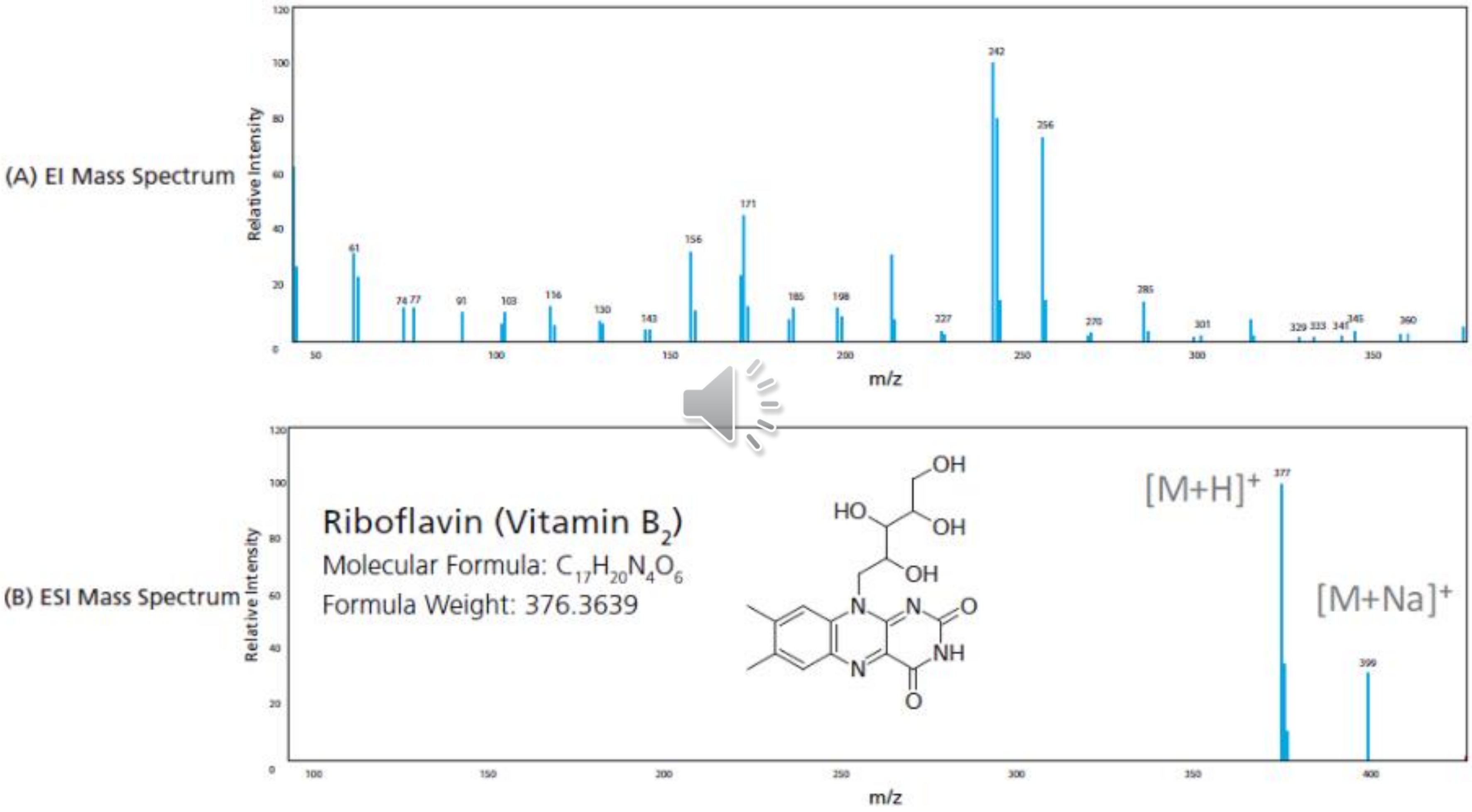
FAME (fatty acid methyl ester) Molecular ion Intensity by GC-EI-MS using different ionization energies





Beccaria et al., Anal. Bioanal. Chem., 2018

Comparison of EI$^+$ (70eV) and ESI$^+$ mass spectra



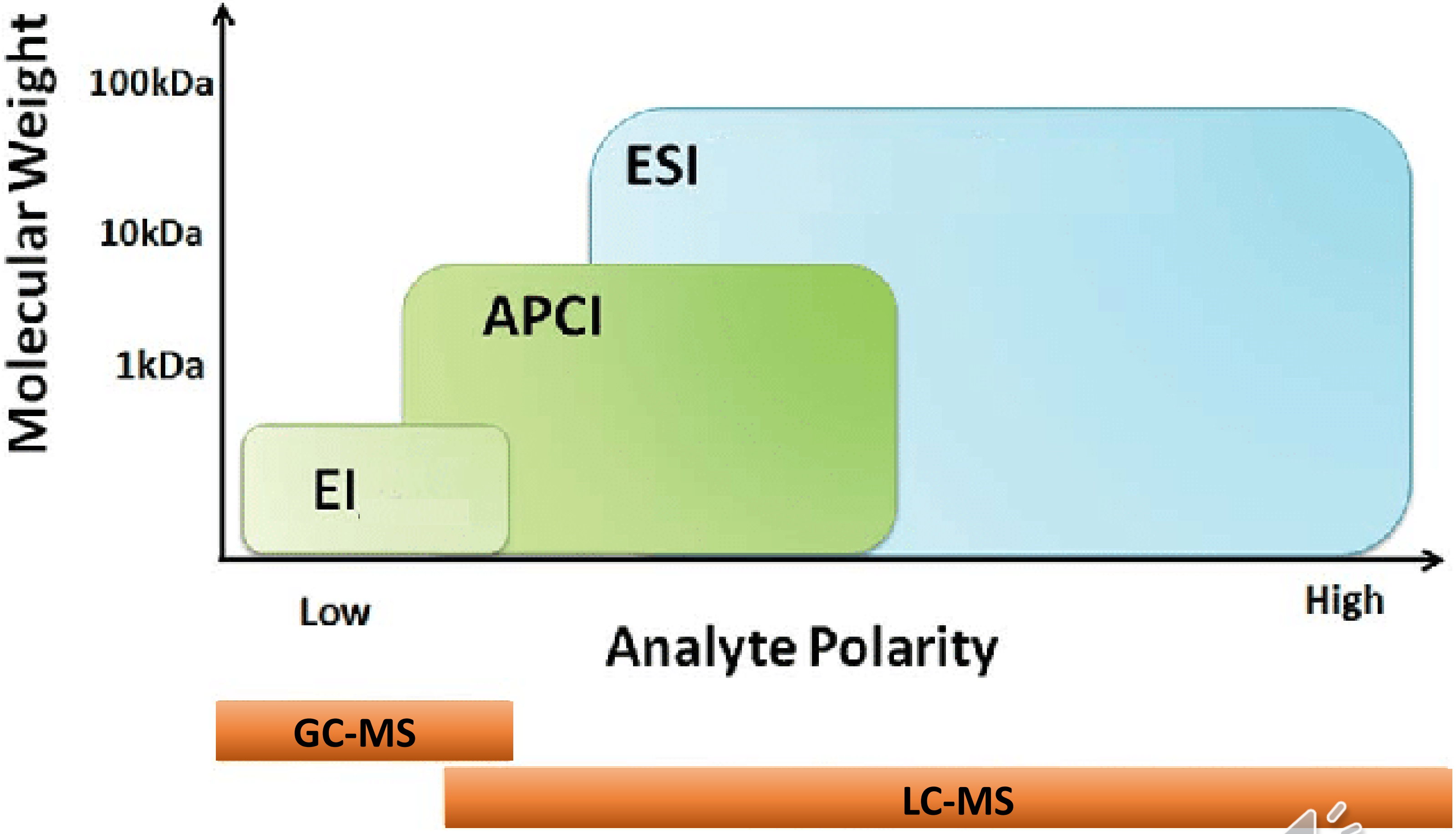
Riboflavin (Vitamin B_2)

Molecular Formula: $C_{17}H_{20}N_4O_6$

Formula Weight: 376.3639

Mass spectra produced from (A) EI (hard ionization) and (B) ESI (soft Ionization)

GC and LC coupled with MS
Interfaces and Range of application



CHROMATOGRAPHY AND MASS SPECTROMETRY

➢ Why Coupling Separation Techniques and Mass Spectrometry?

combining both methods it is possible to eliminate the respective limitations of the individual methods!

✓ Chromatographic techniques coupled with MS decrease the effect of the matrix in the detection of the analytes (matrix effect*) and improve the separation of isomeric compounds

✓ MS alone is a powerful analytical technique, but it often needs pre-sample preparation step(s) before the analysis, it is often subjected to matrix effect* and it the identification of isomeric compounds is not rather easy

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**https://en.wikipedia.org/wiki/Matrix_(chemical_analysis)*