Laboratory Techniques and Medical Device Approvals

Mass spectrometry of biomolecules I

Theory and intact biomolecule analysis

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Mass spectrometry: Introduction

Use

compare measured mass with theoretical mass

- Identity of biomolecules: exact mass
- Structure elucidation (molar mass, fragmentation, isotopic pattern)
- Quantitation (10 $^{-12}$ g ca 10^{-15} mol bei 1000 Da)

Advantages

- very high selectivity
- fast & sensitive UV detection: you chose one wavelength!

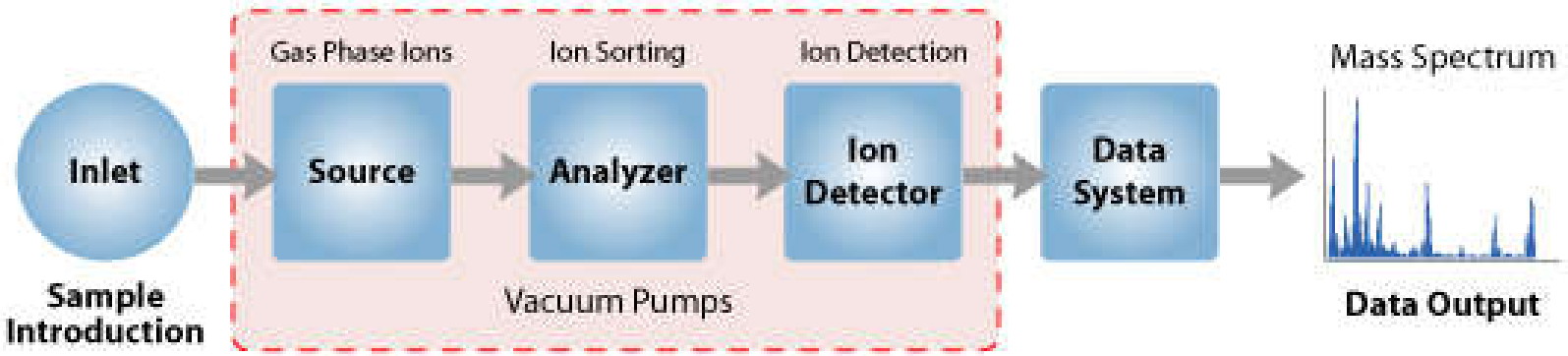
Disadvantages

- expensive & requires extensive knowledge and experience
- only under vaccum possible molecules need to be in gas phase
- salts are not tolerated (e.g. biological/physiological buffers)

  blood, urin, … would contaminate mass spectrometry



Components of a mass spectrometer



Ion generation (ion source):

Electrospray ionisation (ESI)

Matrix-assisted Laser Desorption/Ionization (MALDI)

ionization of biomolecules

Ion separation (analyzer):

Quadrupol

Time-of-flight (TOF)

Electrical ion trap

Electromagnetic ion trap (ion cyclotron)

calculate m/c

Ion detector:

Multichannel plate

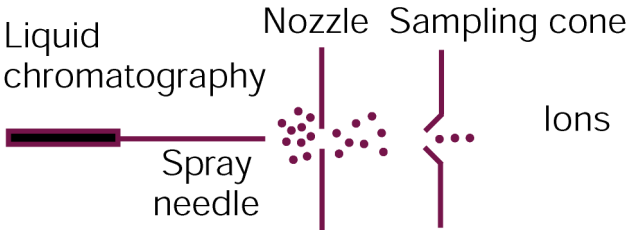
Electron multiplyer

Axial oscillation image current induced on the outer electrode (orbitap)

creates amplitude out of m/c, records ions that pass the electrode

MS – Ion source for macromolecular biomolecules

Electrospray ionization (ESI)

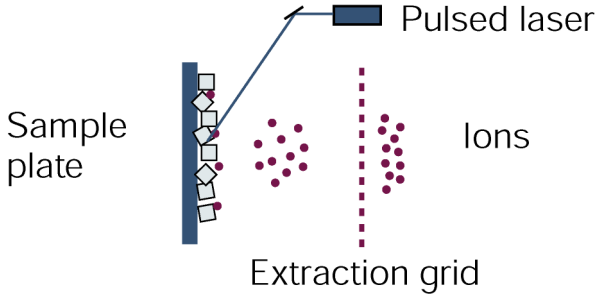






John. B. Fenn
Nobel price for chemistry 2002

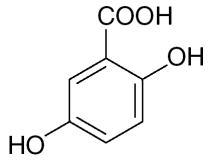
Matrix-Assisted Laser Desorption Ionization (MALDI)







Koichi Tanaka
Nobel price for chemistry 2002



2,5-Dihydroxy-benzoesäure

let plate with liquid of sample + benzoesäure dry, from solid phase directly to gas phase

Electrospray, an old, known phenomenon

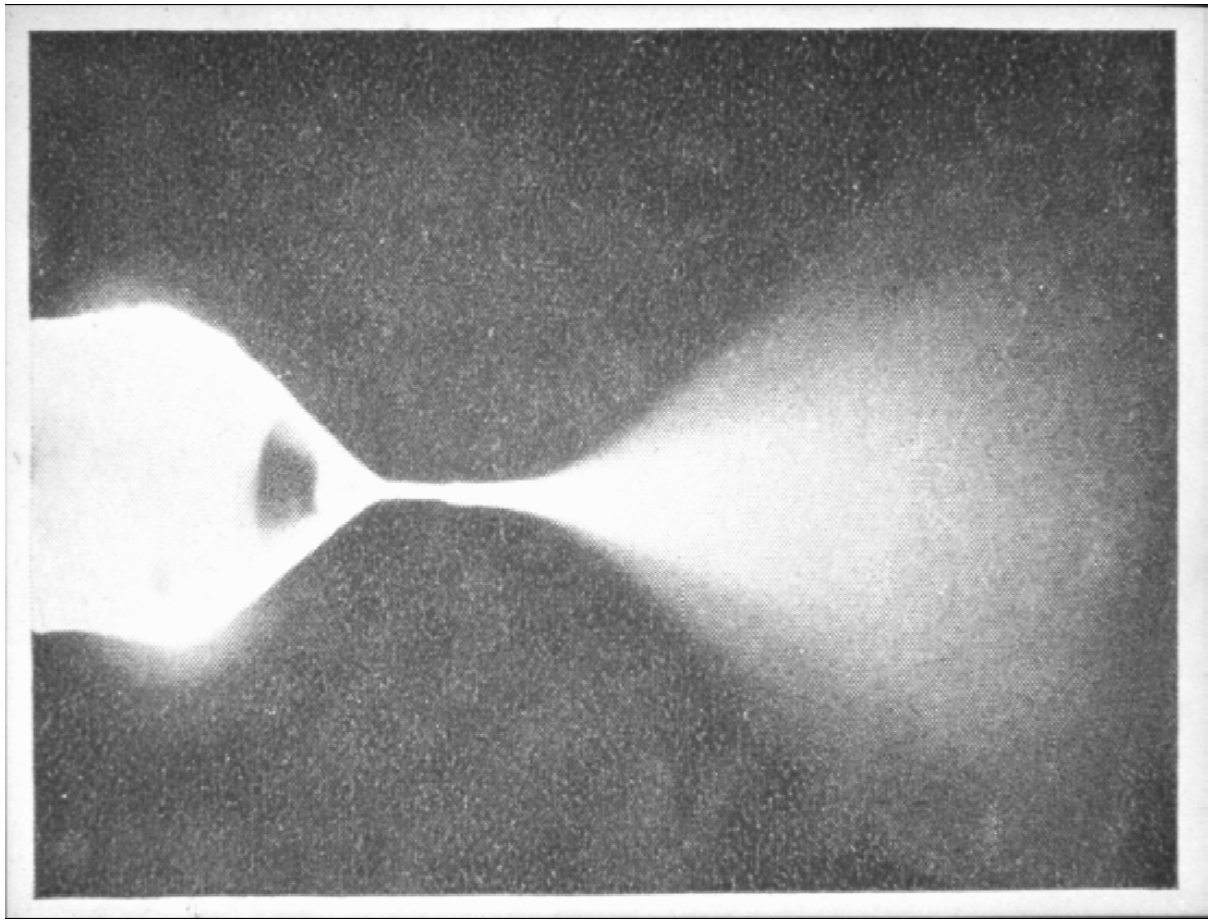


18. century: Abbé Nollets experiments

> ...As soon as the person had contact to the high voltage generator she bleeded no longer normally when injured but the blood sprayed from the wound

F. Lemière, LC•GC Europe "LC-MS Supplement", Dec 2001, p29-35

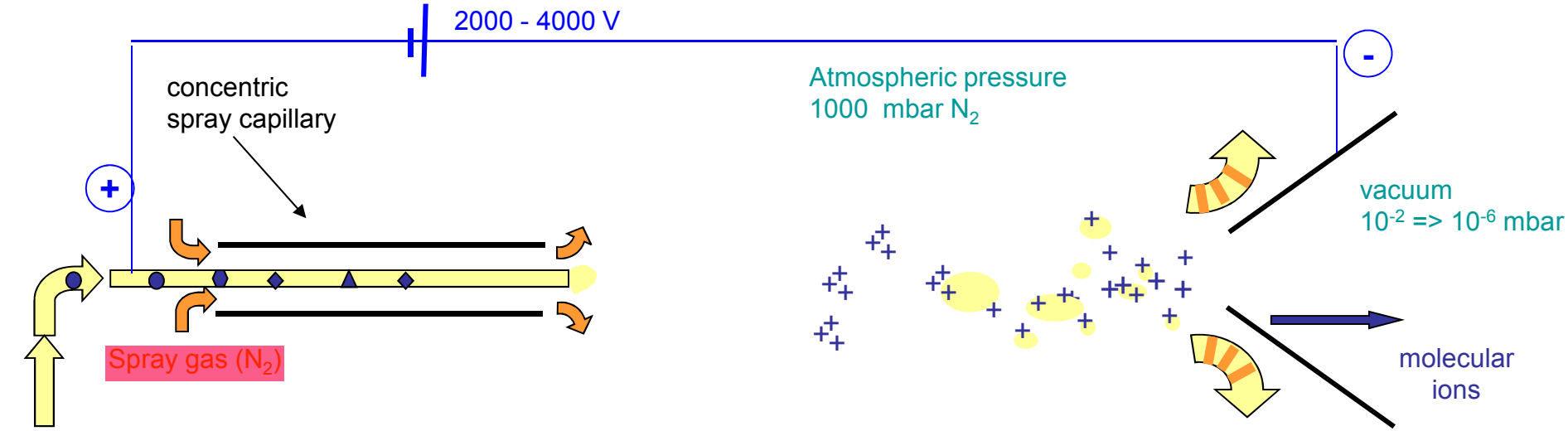
An old phenomenon



J. Zelene, Phys.Rev.,10, 1-6, (1917)

at the beginning large droplets + many protons in droplets, organic solvent will get away, droplets are smaller, droplets will explode! (see next slide)

Electrospray ionization (ESI) - principle



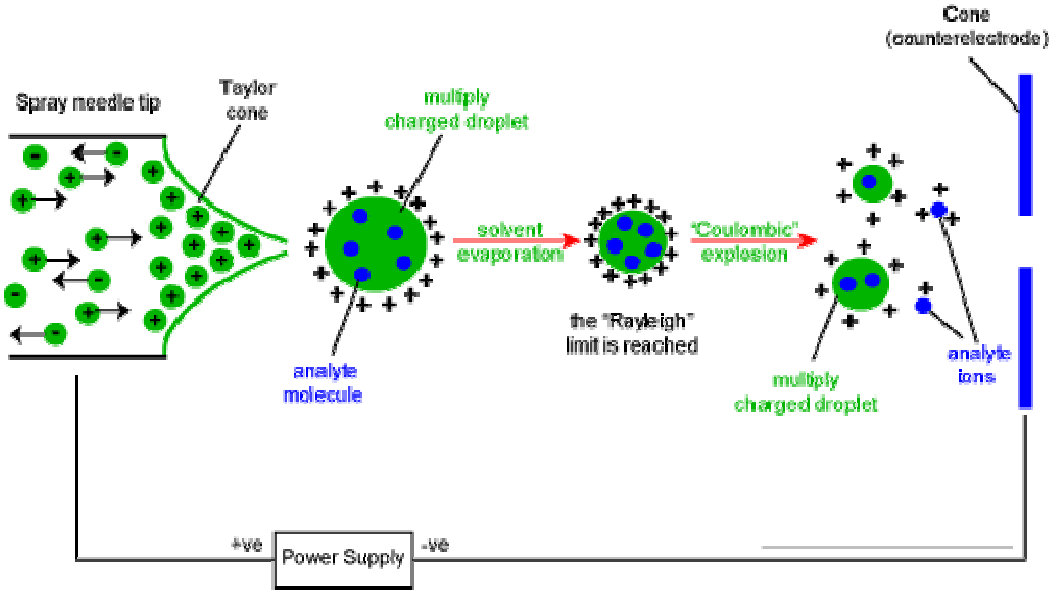
- Soft ionization unter atmospheric pressure by protons
  => acidic solvents (ACN / H2O / 0.05% formic acid)
- coupling with HPLC & capillary electrophoresis possible
  => online detection

+

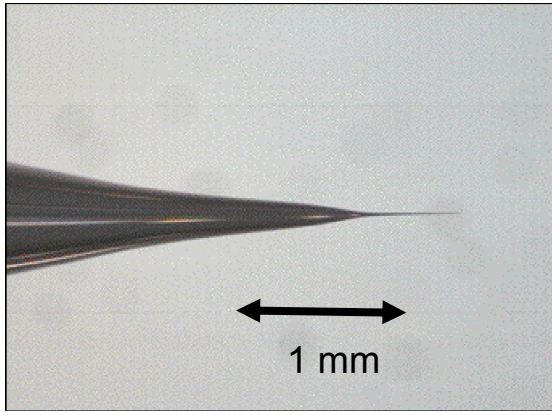
- multiple charges per analyte molecule possible
  => complex spectra
- only volatile buffer components are compatible
  => limited separation buffer, no ion pairing reagents

-

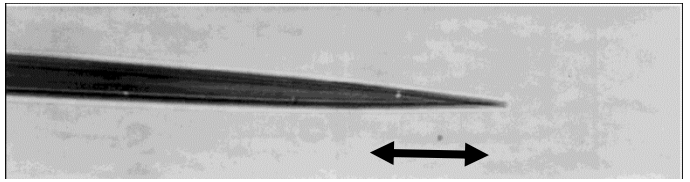
Mechanisms of ion formation in ESI



Nano-Spray-Needle



Drawn out glass capillary

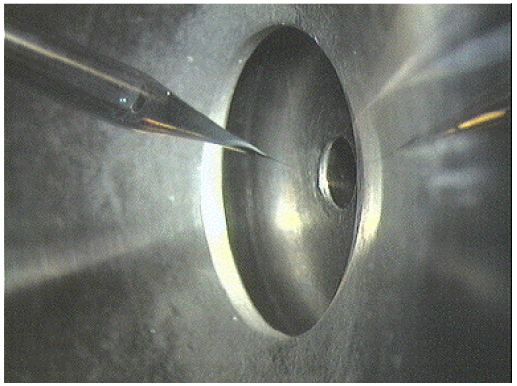


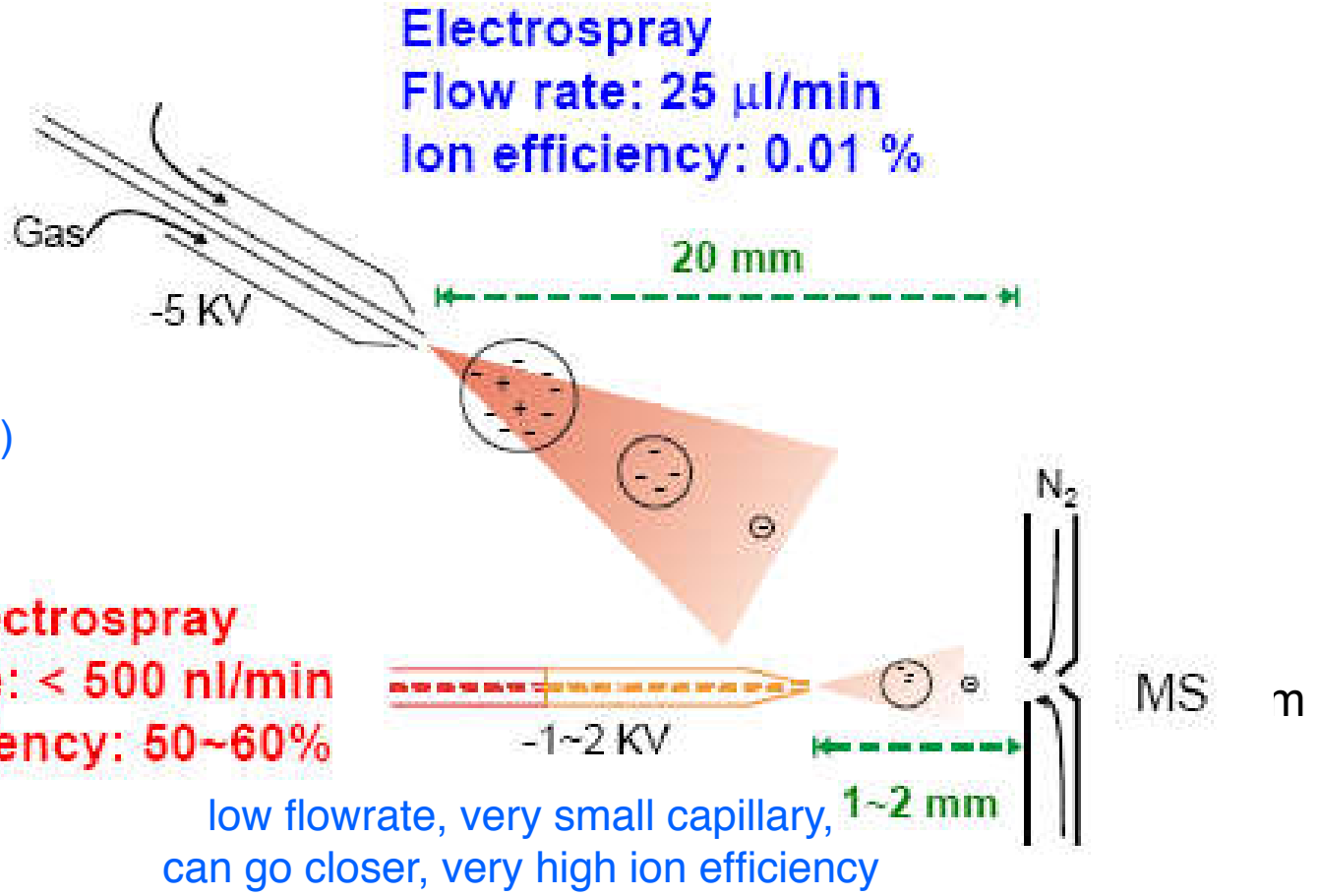
„open“ capillary



Quelle: M. Wilm, DPFG Summerschool 2008, Brixen

Comparison of the ionisation efficiency for electrospray and nano-electrospray

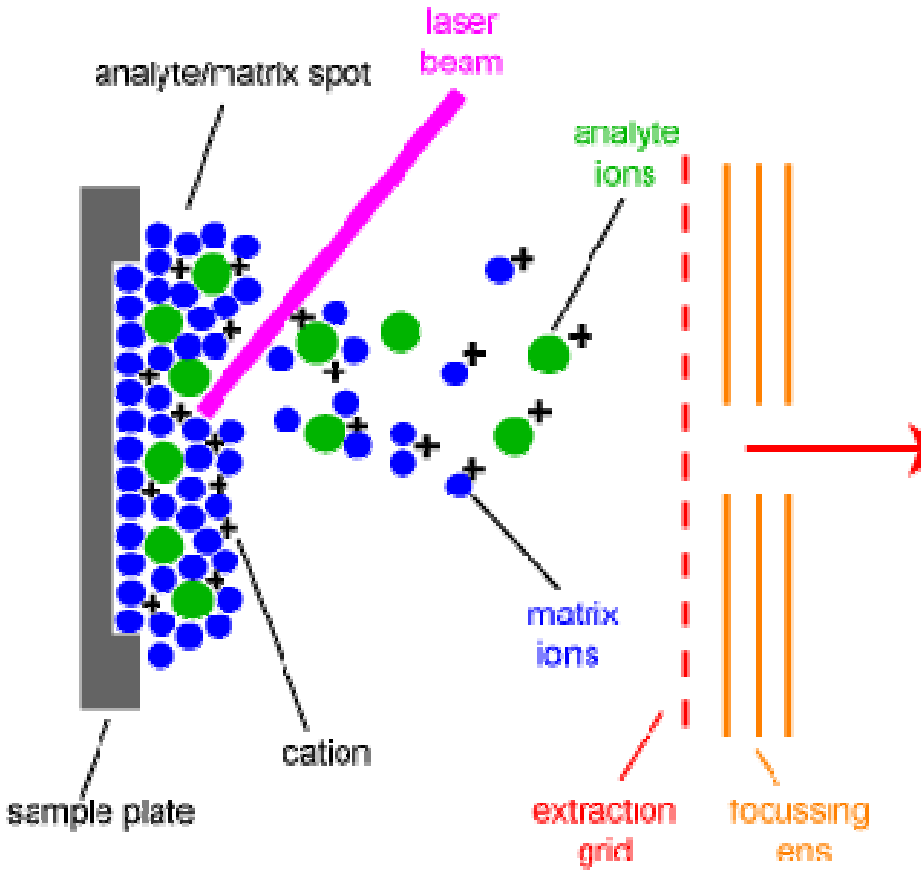




mass sensivity (same signal!)

low flowrate, very small capillary,
can go closer, very high ion efficiency

Mechanism of Matrix-assisted Laser Desorption/Ionization (MALDI)



i) Formation of a „solid" solution:
Matrix is in excess; analyte molecules are isolated from each other

ii) *Matrix Excitation*: laser beam excites the chromophor of the matrix
➔ Vibrational excitation,
➔ Desintegration of solid solution spot
➔ Matrix evaporates away and leaves free analyte in gas phase

iii) *Analyte ionisation*:
By proton transfer from matrix to analyte
Cation attachment to the analyte (Na+)

Mechanisms of Matrix-assisted Laser Desorption/Ionization (MALDI)

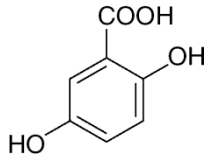
each droplet different sample, much higher throughput than ESI, you can reanalyze your sample

Matrix for the MALDI process:

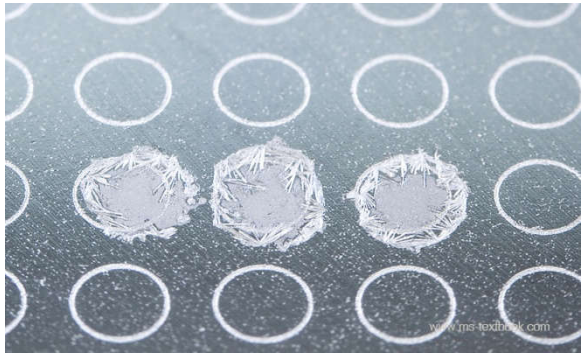
- Strong absorption of the laser wavelength
- Highly sublimable
- Proton donator
- ➔ small organic acid



MALDI target (stainless steel)



2,5-Dihydroxy-
Benzoic acid (DHB)



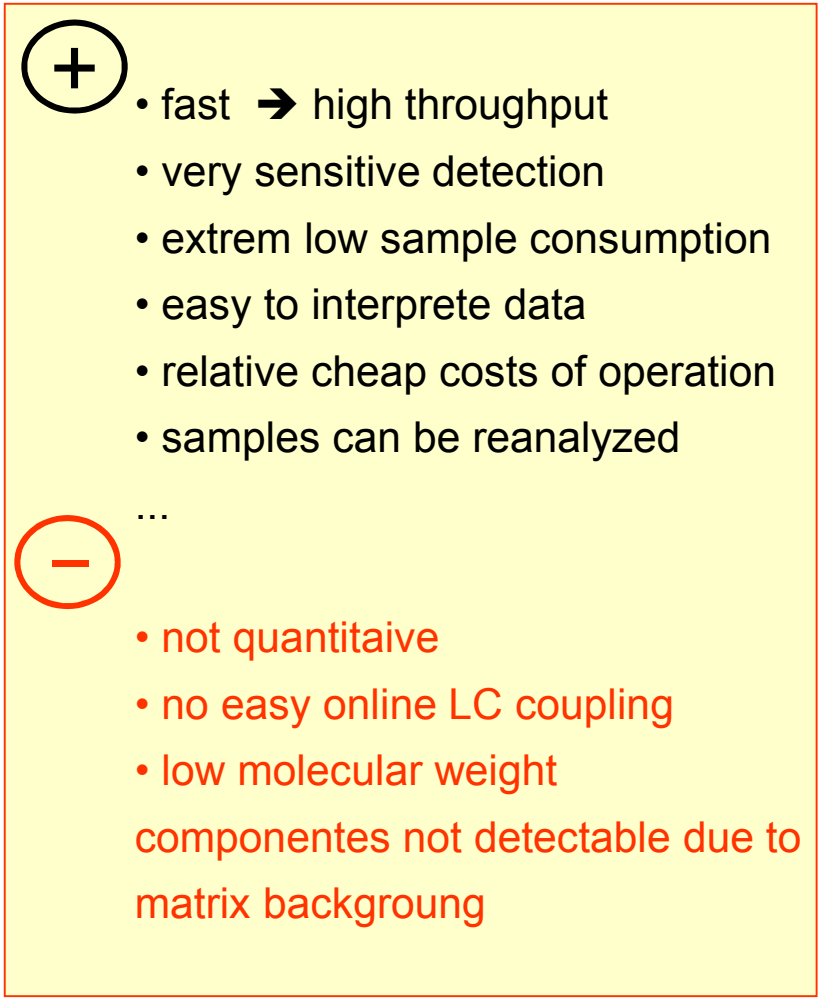
crystallized 2,5-DHB matrix



MALDI spots

crystals are not homogenous, if you shoot middle or crystal you might get different signals -> not quantitative

Advantages/Disadvantages Matrix-assisted Laser Desorption/Ionization (MALDI)

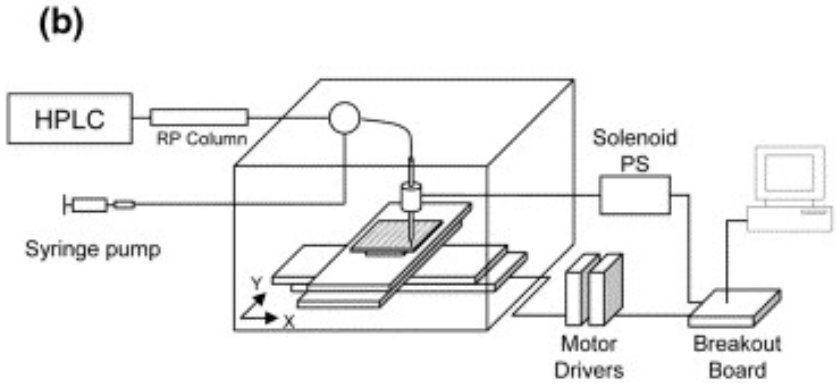


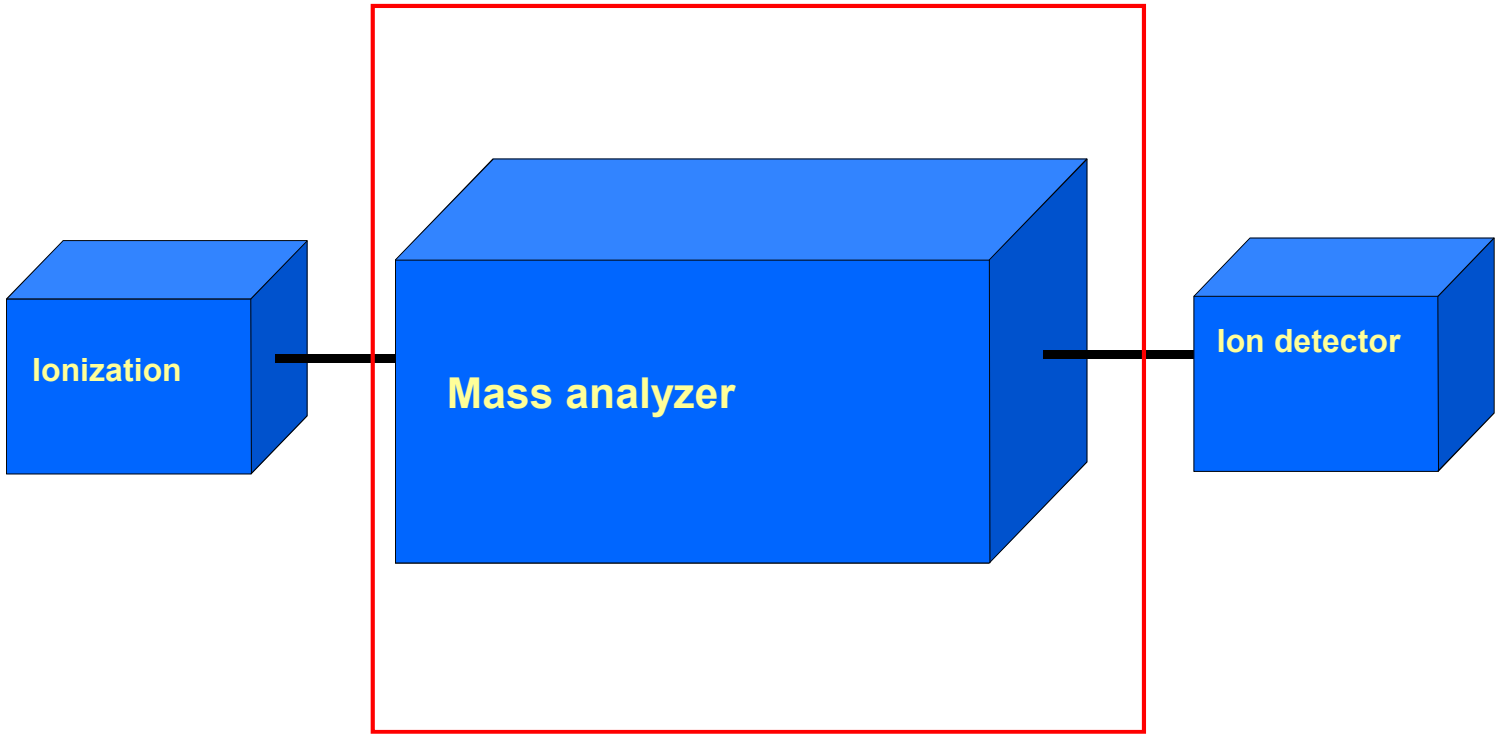
Coupling liquid chromatography and MALDI

Fraction collection directly on a MALDI target



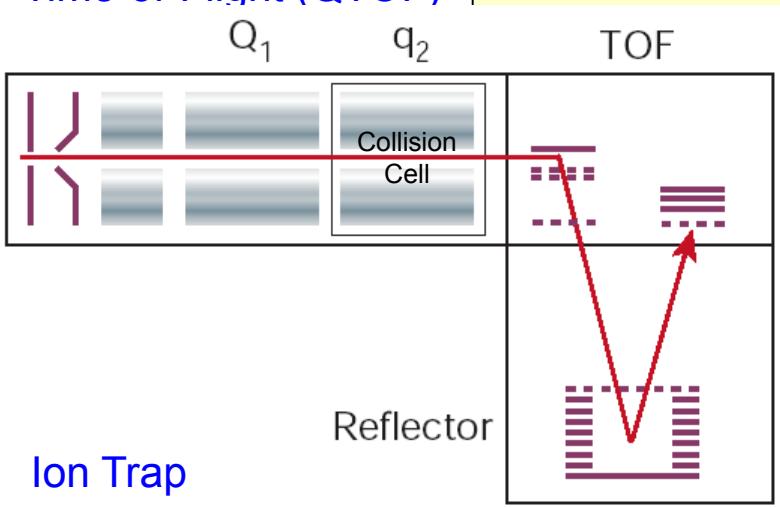
Continuous deposition of the LC eluent on a MALDI target




Ionization
Mass analyzer
Ion detector

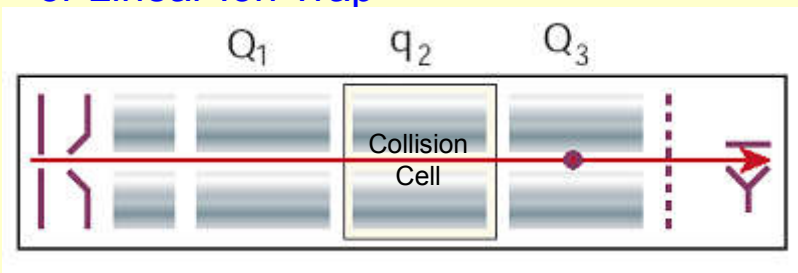
Mass analyzers - overview

Quadrupole
Time-of-Flight (QTOF)

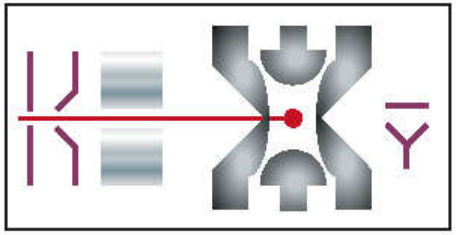


Triple Quadrupole
or Linear Ion Trap

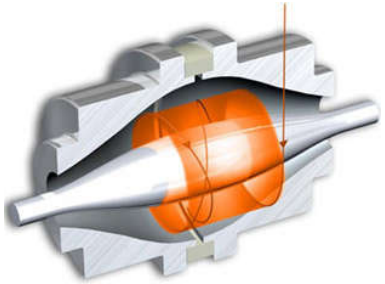
detect quantity of fractions



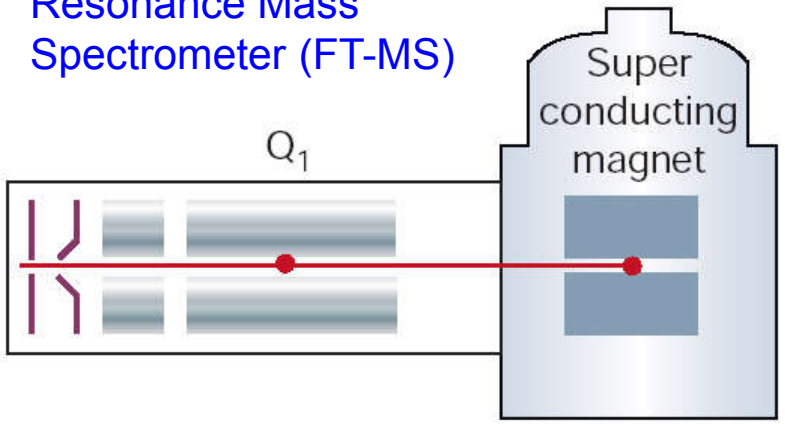
Ion Trap



OrbiTrap



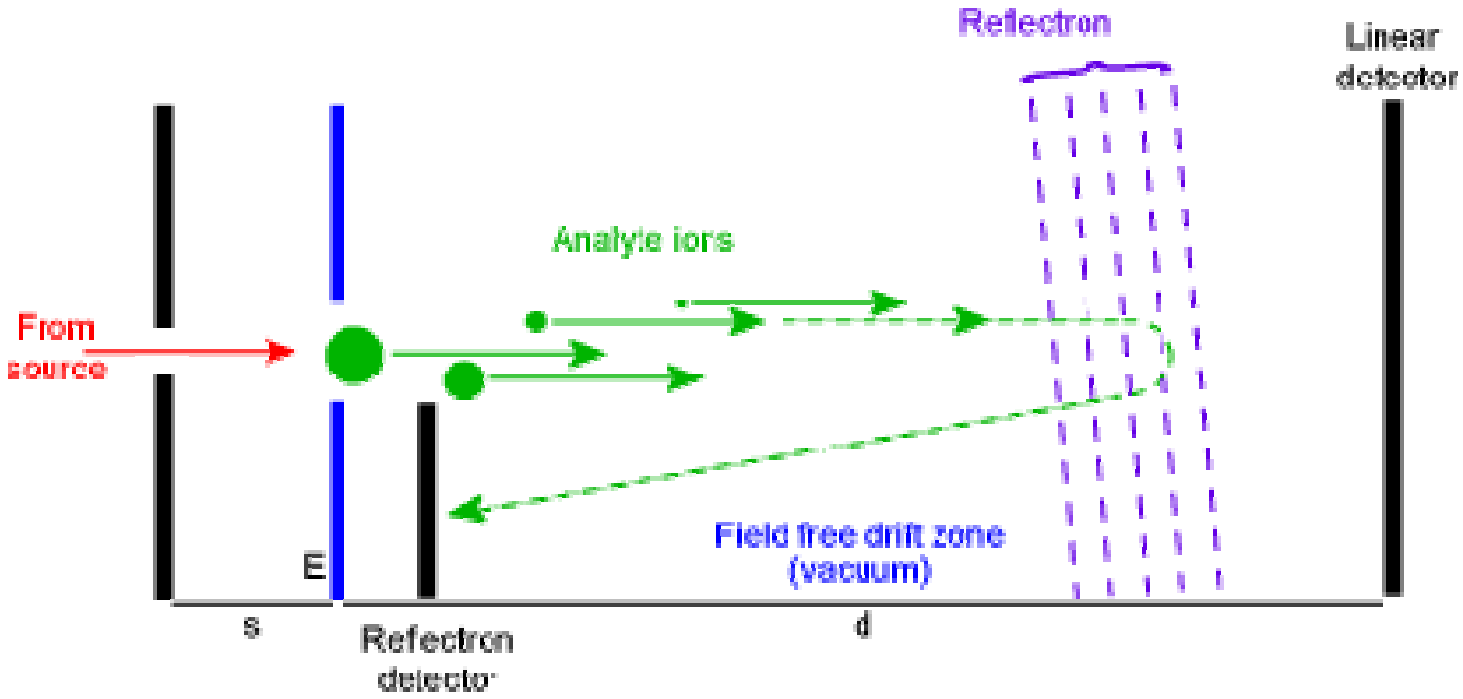
Fourier Transform
Ion Cyclotron
Resonance Mass
Spectrometer (FT-MS)



1/2*m*V^2=E*z*e
1/2*m*(l/t)^2=E*z*e
determine m/z, next slide

Time of flight mass analyzer – how does it work ?

smaller ions will reach detector first!, bigger ions later



at one point of time, orthogonal, get the same initial energy

1. Ions are introduced from source (MALDI) or previous analyzer (quadrupole) and clock is started
2. All the ions receive the same initial kinetic energy using an electric pulse (E_{el} = E*z*e)
3. **Ions drift along the field free drift zone and are separated by their ~ z/m**
4. Ions reach the detector after different flight times
5. Calculation of m/z from their flight time

Time of flight mass analyzer – TOF equation

good for unknown or more than one ions

$$\frac{m}{z} = 2e\mathbf{E}s\left(\frac{t}{d}\right)^2$$

m/z	mass-to-charge ratio of the ion
E	extraction pulse potential
s	length of flight tube over which E is applied
d	length of field free drift zone
t	measured time-of-flight of the ion

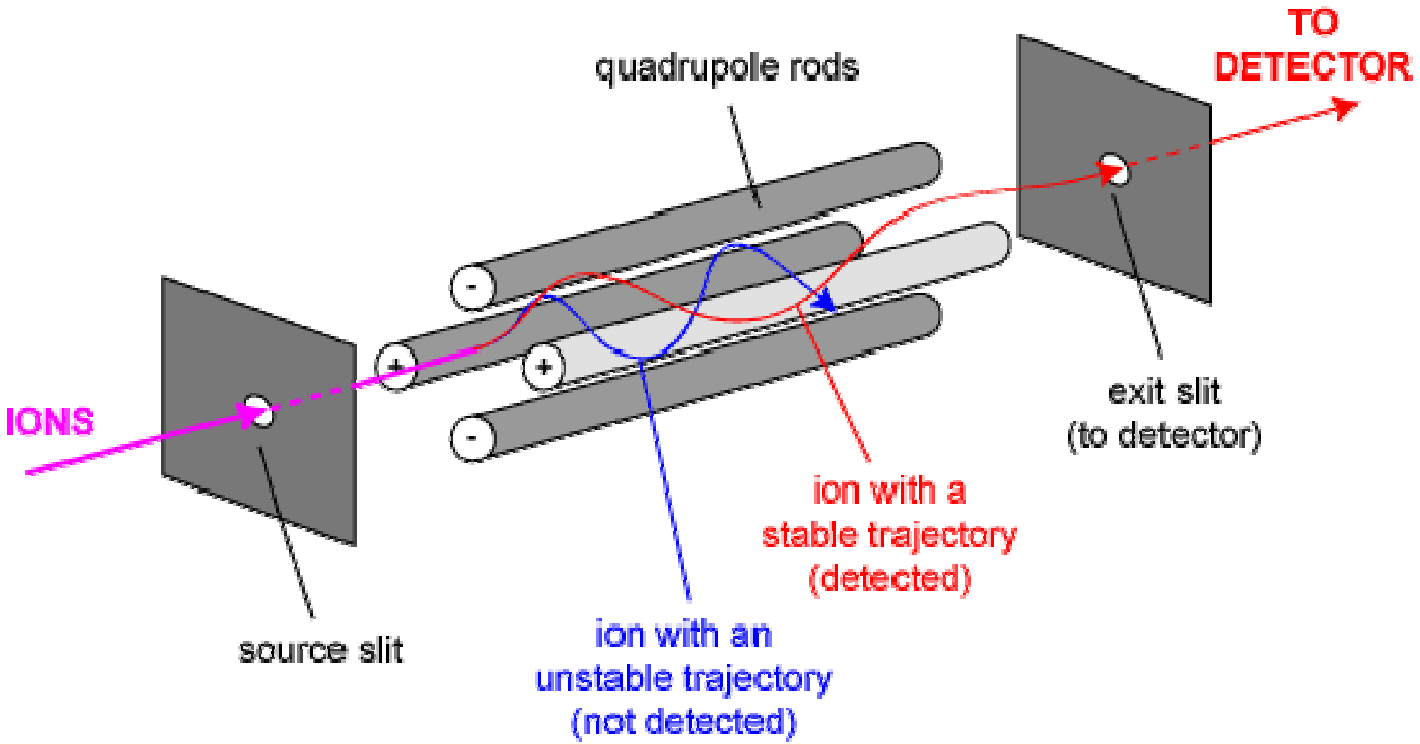
⊕

- extreme sensitive (all ions are detected)
- unlimited mass range
- speed of analysis (full spectrum recording in seconds)
- intact mass of proteins possible

long tube needed for good resolution

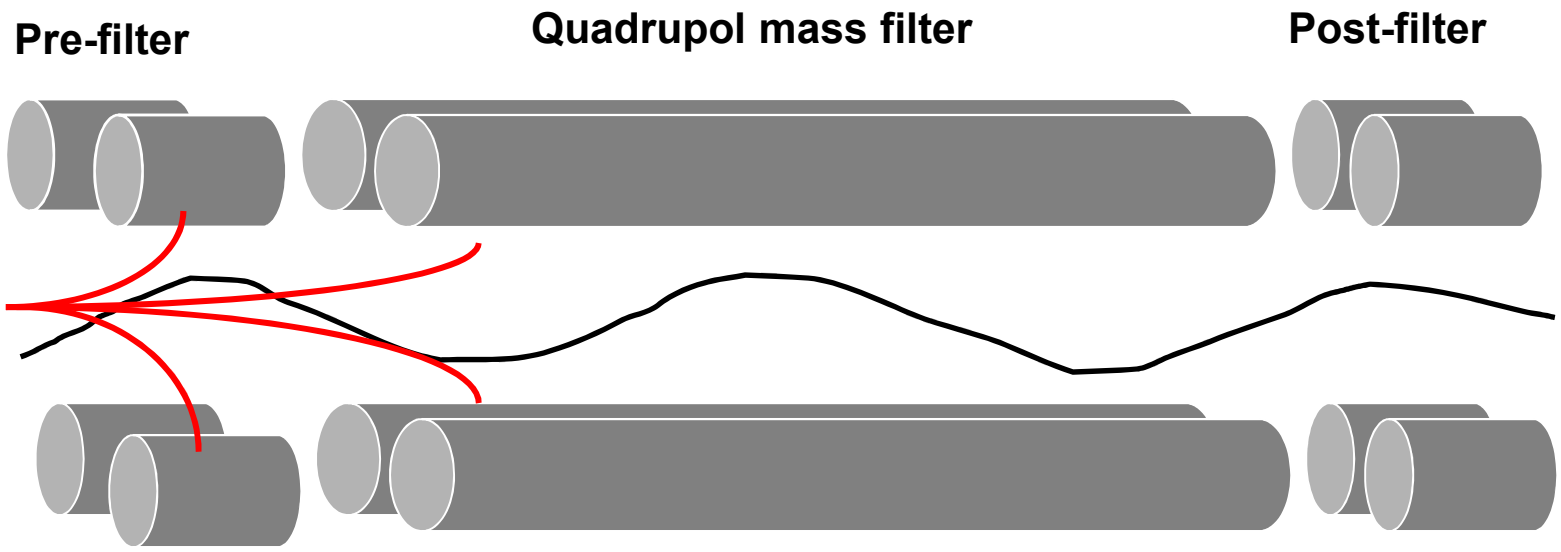
for preknown ions!

Quadrupole mass analyzer



- A quadrupole mass analyser consists of four parallel rods that have fixed DC and alternating RF potentials applied to them.
- Ions produced in the source of the instrument are focussed and passed along the middle of the quadrupoles.
- Their motion will depend on the electric fields so that only ions of a particular *m/z* will have a stable trajectory and thus pass through to the detector.
- The RF is varied to bring ions of different *m/z* into focus on the detector and thus build up a mass spectrum.

Quadrupol mass analyzer - Scheme



Repelled Ions

stable (resonant) ions

Quadropoles are often used to focus the ion beam
Quadrupole mass analyzer is relatively slow, needs scanning

Commercial instruments



maXis QTOF-MS
(Bruker)

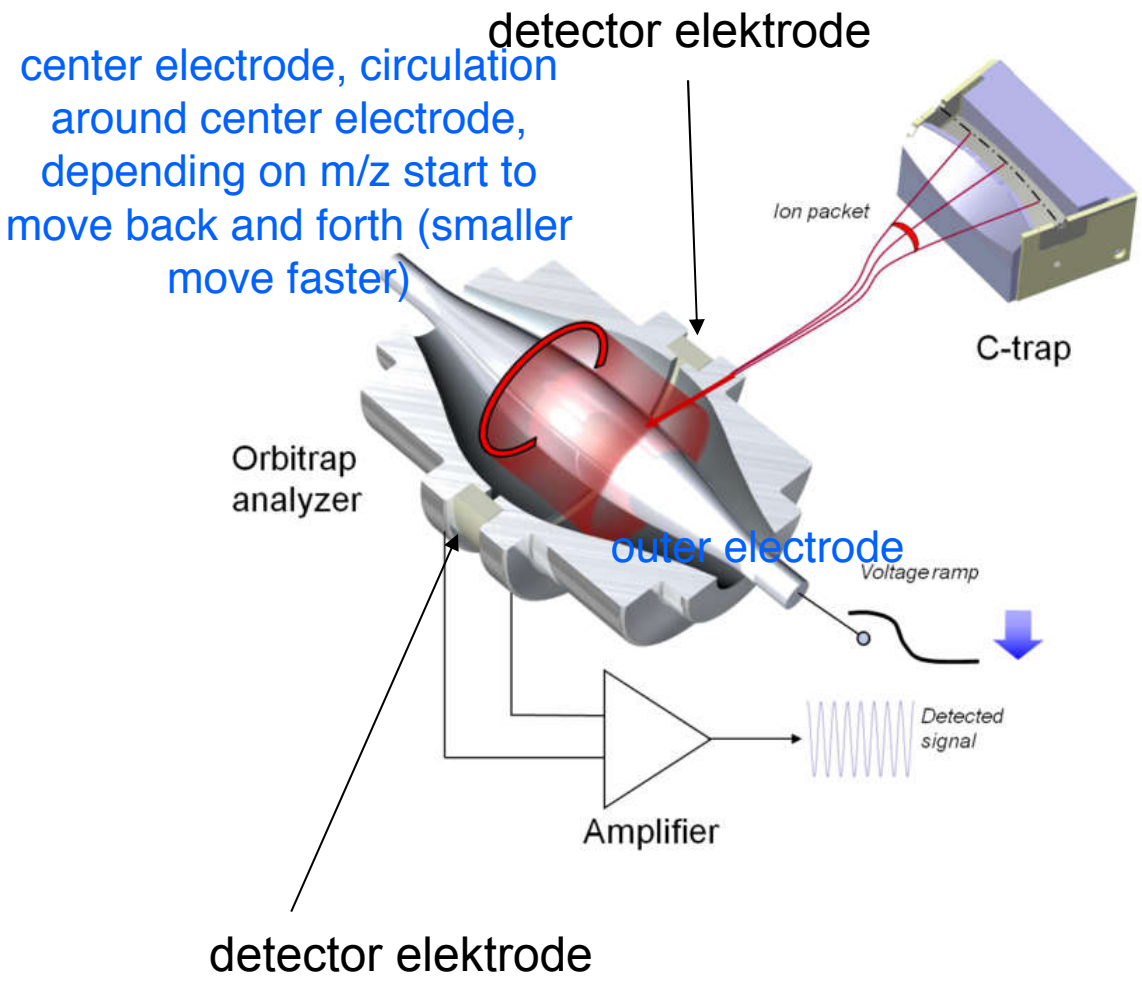


6530 QTOF-MS
(Agilent)



Xevo™ QTOF-MS (Waters)

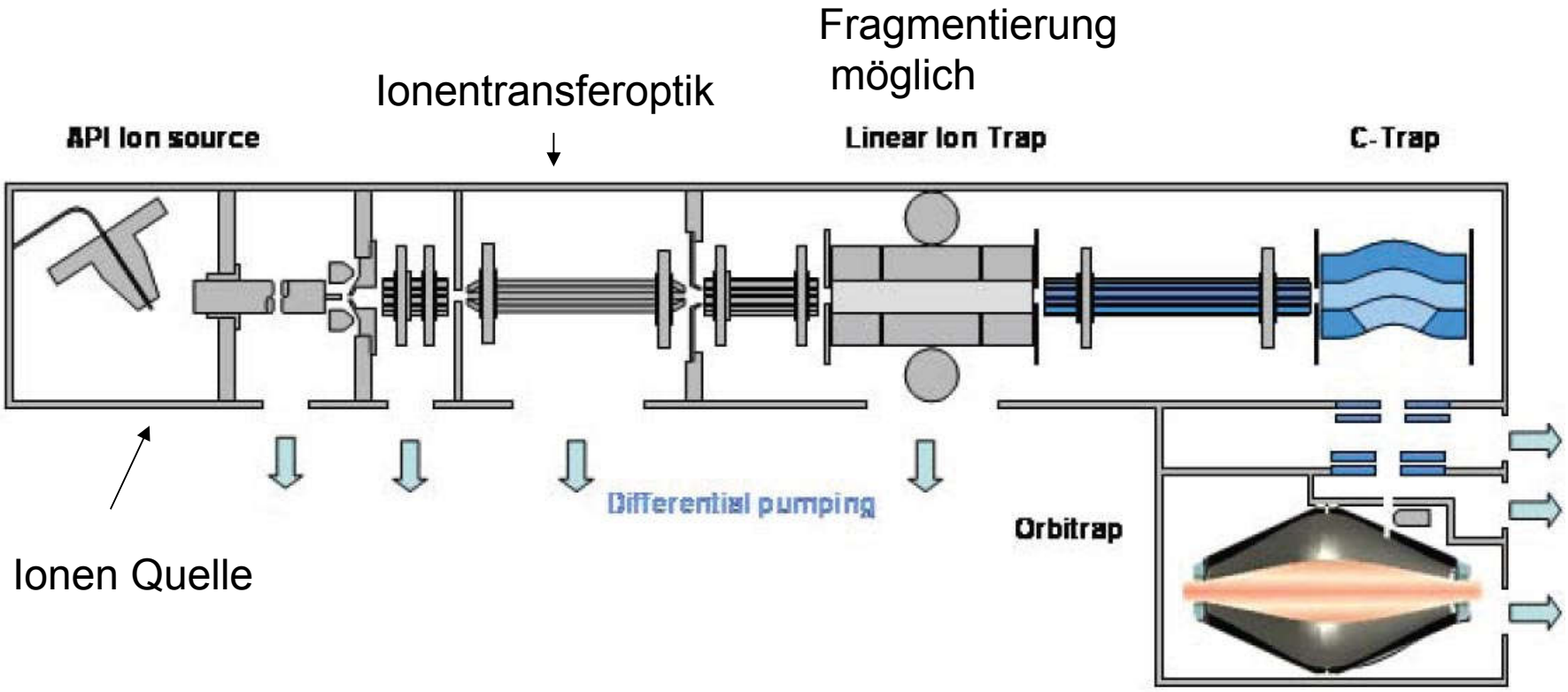
Orbitrap mass analyzer - principle



Introduced by Alexander Makarov by the end of 1990

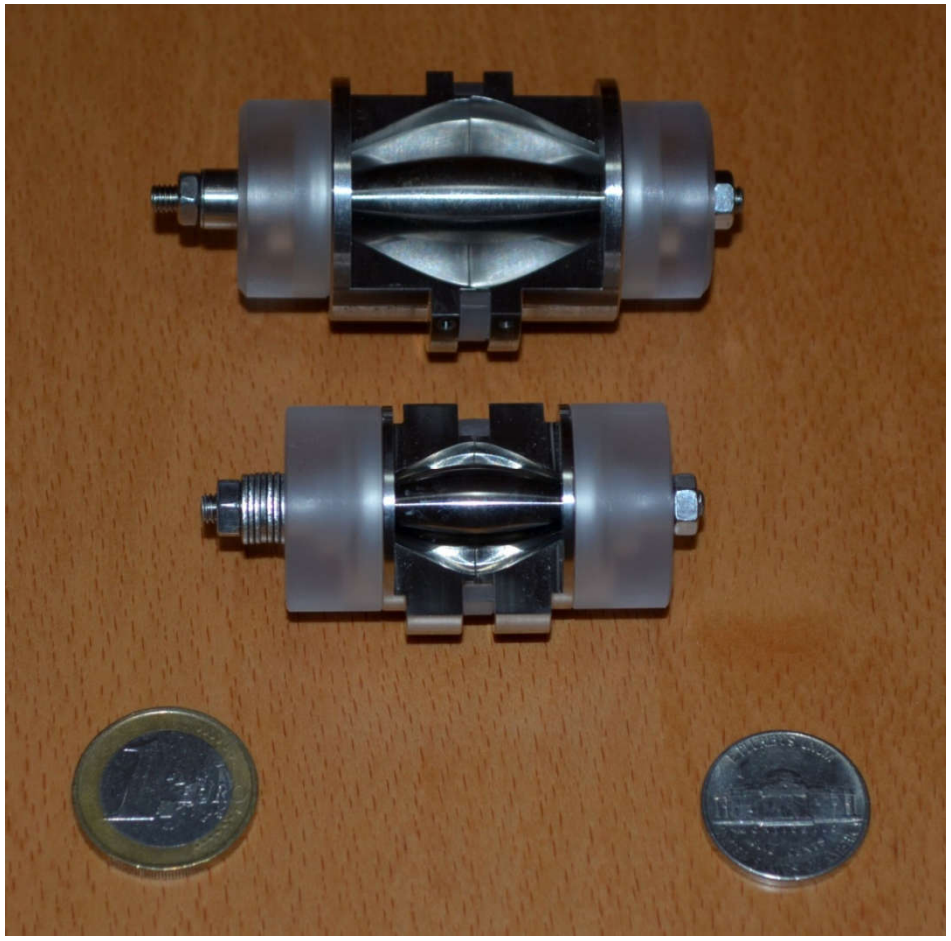
- The ions transferred from the C-Trap are captured by rapidly increasing the voltage on the center electrode of the Orbitrap.
- The trapped ions assume circular trajectories around the center electrode.
- Their axial oscillations, along the center electrode are detected.

Orbitrap mass analyzer – hybrid instrument



- very high mass resolution (oscillations can be recorded extremly exact)
- high sensitivity of the linear ion trap (can be filled with many ions)

Orbitrap mass analyzer – commercial instrument



Messzelle der Orbitrap



LTQ Orbitrap

Advantages and disadvantages of different mass analyzers

NMI schafft Ergebnisse

	Ion trap	Quadrupole	Time-of-flight	Orbitrap
Sensitivity	+	+	+	(+)
Speed	+	-	+	(+)
Resolution	-	-	+	+
Mass accuracy	-	-	(+)	+
Mass range	+	-	+	(+)
Fragmentation	+	+	-	-

By combining different analyzer one can obtaine the desired properties for different analysis types.

Mass spectrometry of intact biomolecules

- ESI spectra
- MALDI spectra
- Isotopic distribution for proteins and peptides
- Charge distribution for proteins
- Mass resolution (definition)

Mass spectrum of peptides

Example:

positive charged amino acids (basic amino acids)
negative charged amino acids

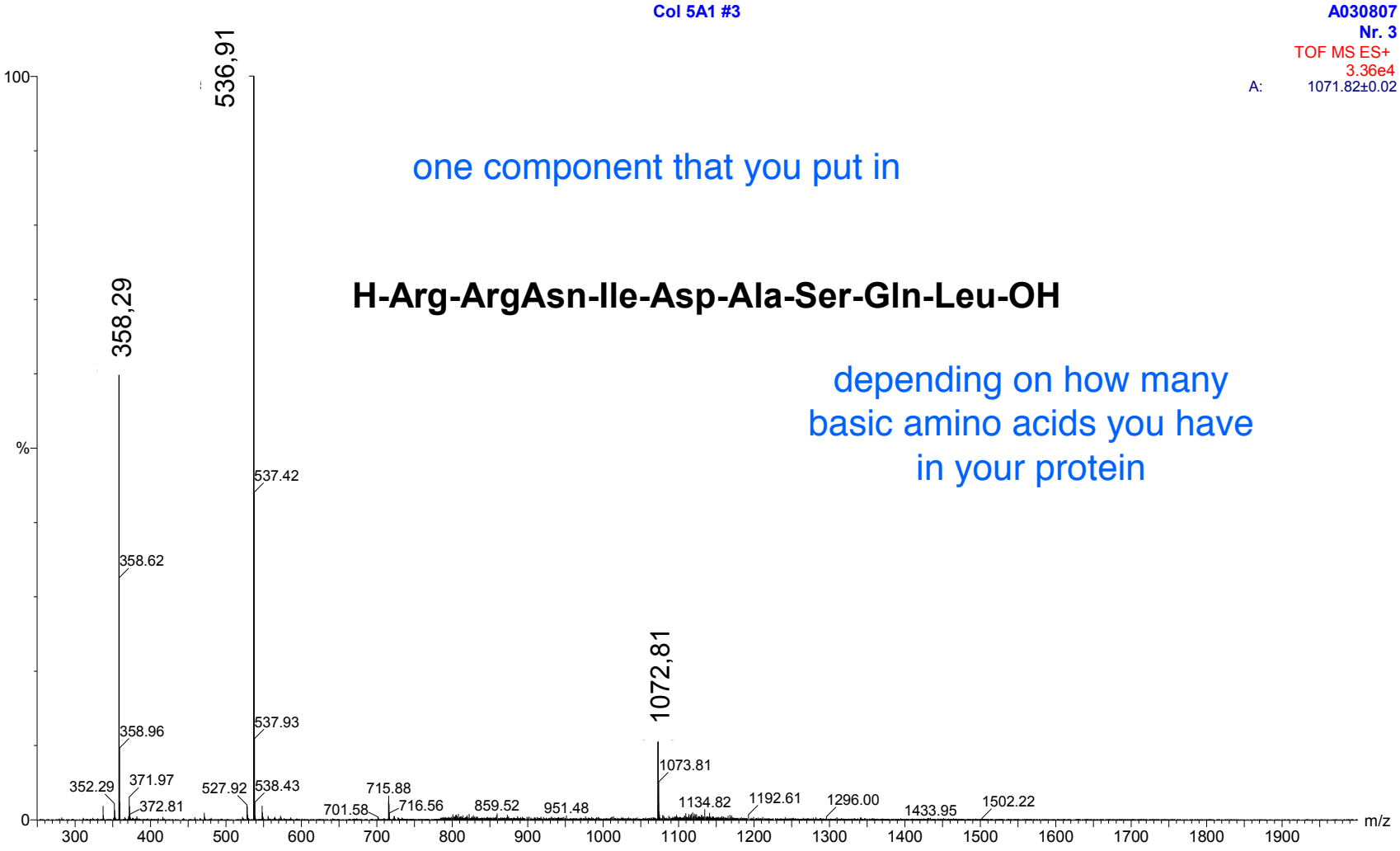
H- Arg – Arg – Asn – Ile – Asp – Ala –Ser – Gln – Leu – OH

Monoisotopic mass: 1071,58 Da

can’t measure neutral molecules ;)

How does the ESI and MALDI spectrum will look like ?

Electrospray mass spectrum of a peptide



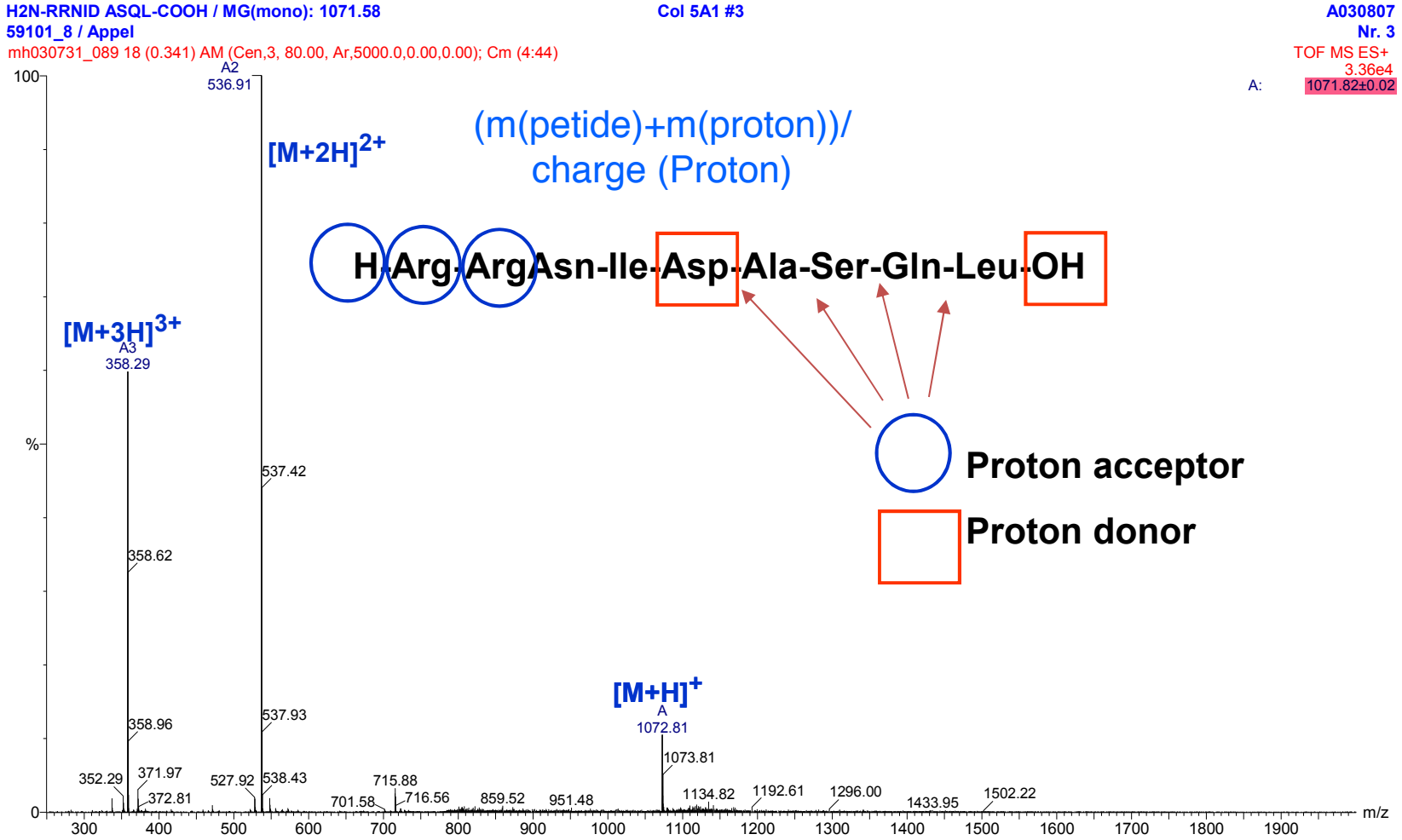
one component that you put in

H-Arg-ArgAsn-Ile-Asp-Ala-Ser-Gln-Leu-OH

depending on how many
basic amino acids you have
in your protein

by adding one proton you add one dalton

Electrospray mass spectrum of peptides

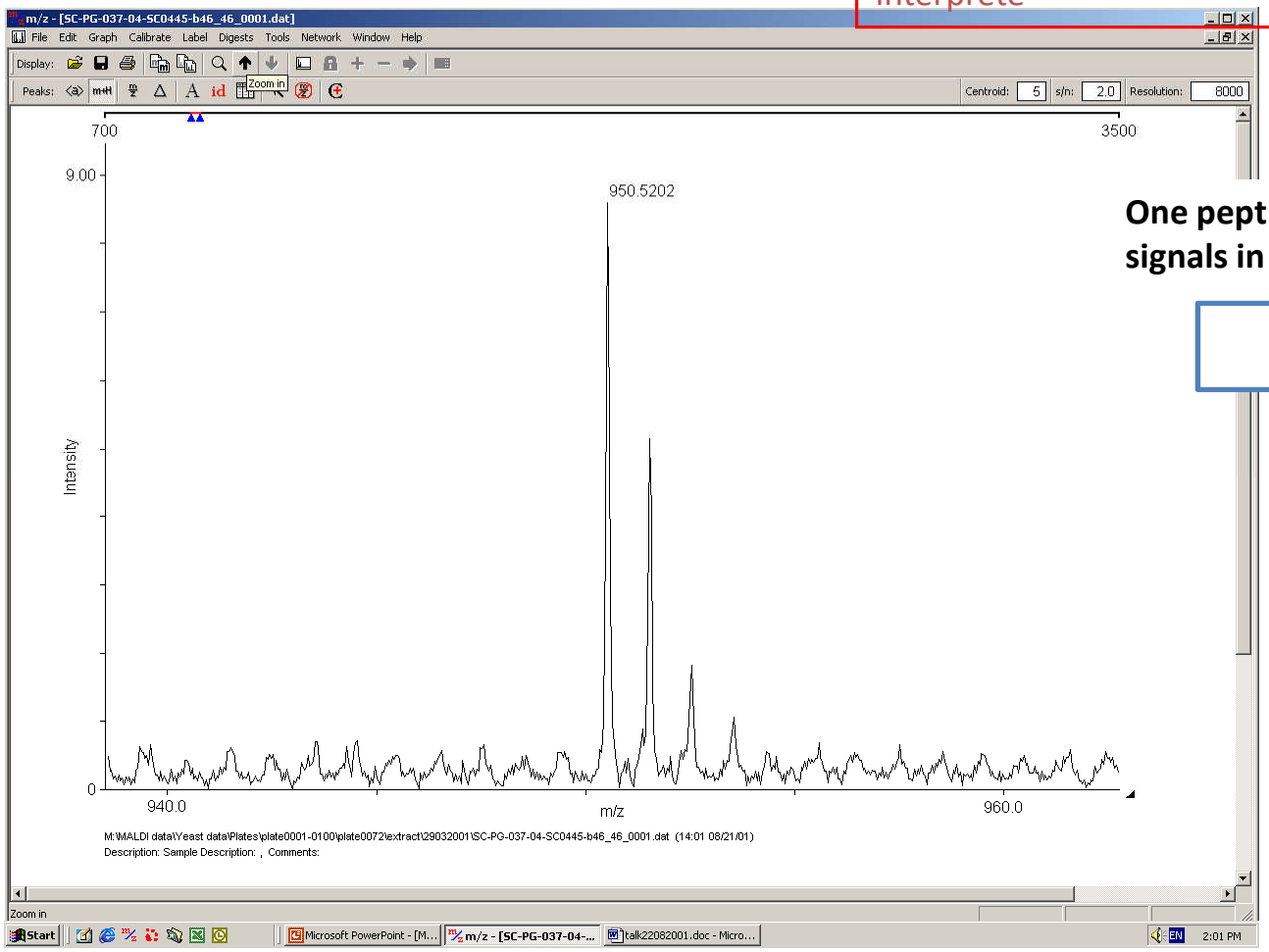


Statistical distribution of charges due to statistical attachment of protons.

MALDI mass spectrum of a peptides

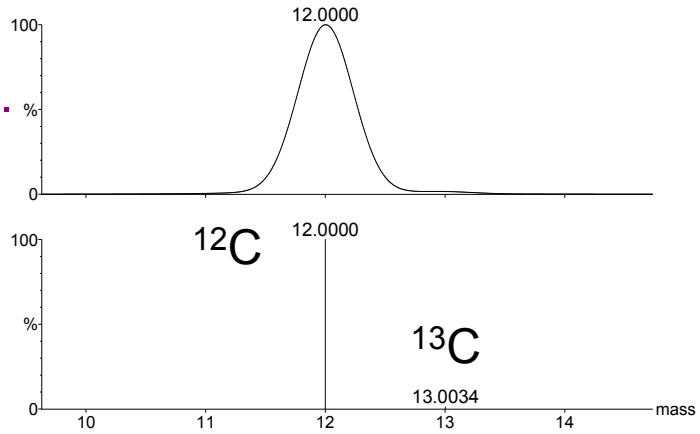
MALDI: introduces usually one protein
Electrospray: more than one possible

MALDI process results in singly charge molecular ions ➔ spectra are easy to interprete



One peptide = multiple signals in the mass spec

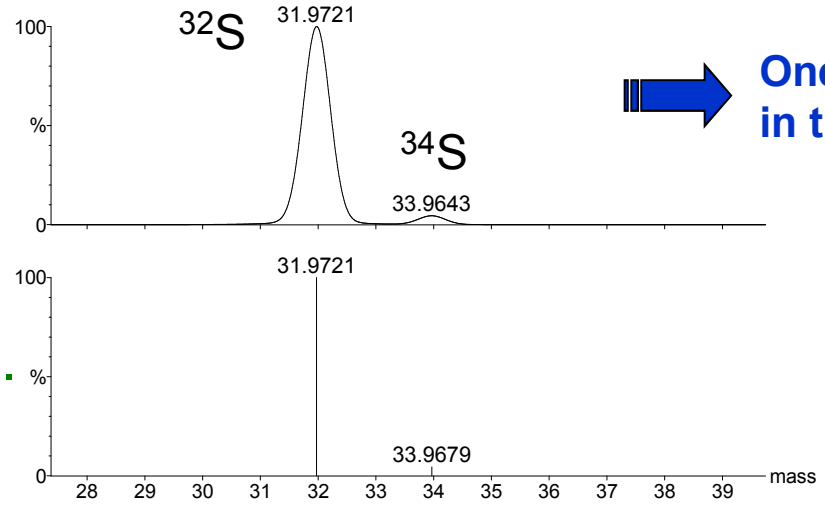
Elemental isotopes



Most elements have different stable isotope that are separated by the mass spectrometer

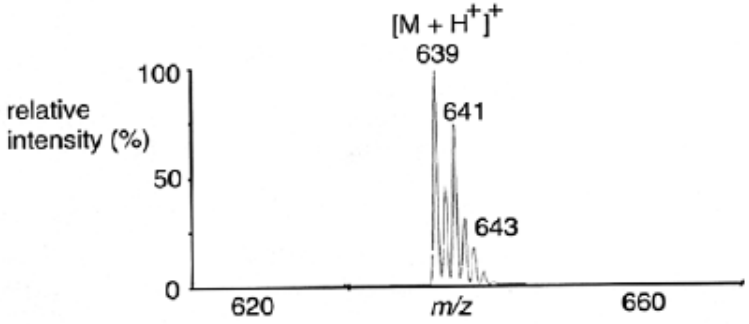
E.g.:

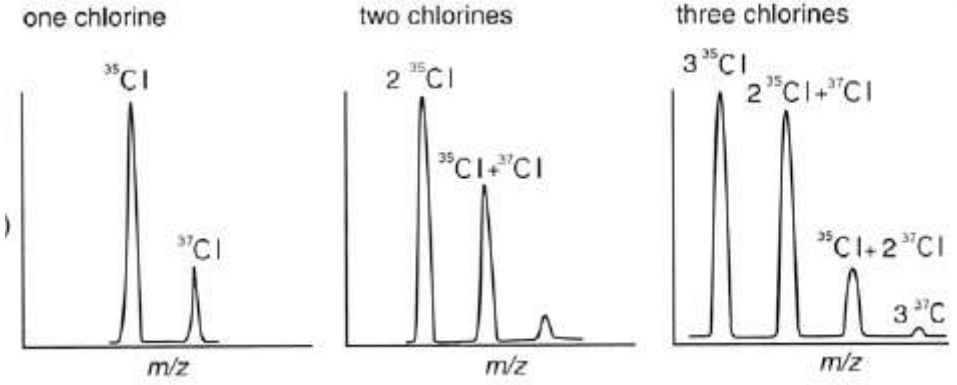
$^{12}C : {}^{13}C$	= 98,9% :1,1%
$^{1}H : {}^{2}H$	= 99,985% : 0,015%
$^{14}N : {}^{15}N$	= 99,634% : 0,366%
$^{15}O:{}^{16}O:{}^{17}O$	= 99,762% : 0,038% : 0,2%
$^{32}S:{}^{33}S:{}^{34}S:{}^{36}S$	= 95,02% : 0,75% : 4,21% : 0,02%



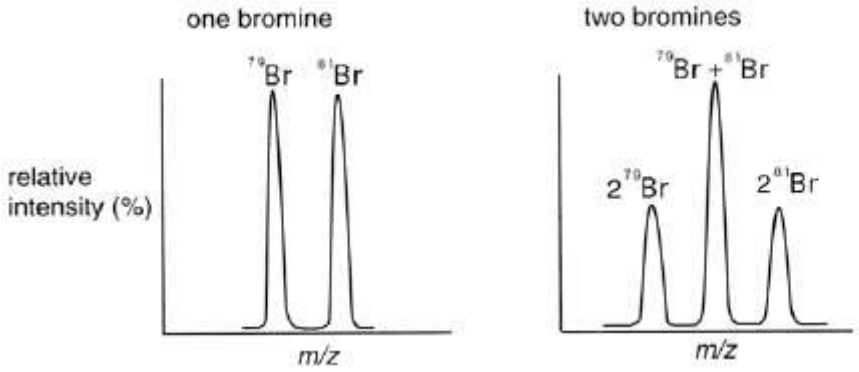
One element shows multiple signals in the mass spectrum

Elemental isotopes





Isotopic pattern reveals
That the molecule contains
2 chlorine atoms



How does a protein mass spectrum look like?

Isotopic distribution for peptides

The calculation of the molecular mass using the different isotopic masses results in multiple masses per sum formula (isotopic distribution)

The calculation of the molar mass using the average elemental mass results defined mass for each summ formula.

1. Molecular mass (M)↔ Molar mass (MG) :

AGDFR

$C_{24}H_{36}N_8O_8$ M = 564,2656 amu (Da) MG: 564,5990 g/mol Δ : 0,3334 amu

AGDFR TRESE IILAD

$C_{72}H_{117}N_{21}O_{26}$ M = 1691,8479 amu MG: 1692,8469 Δ : 0,999 amu

AGDFR TRESE IILAD ERTDF STDAE QWERT

$C_{150}H_{230}N_{44}O_{56}$ M = 3543,6502 amu MG: 3545,7392 Δ : 2,089 amu

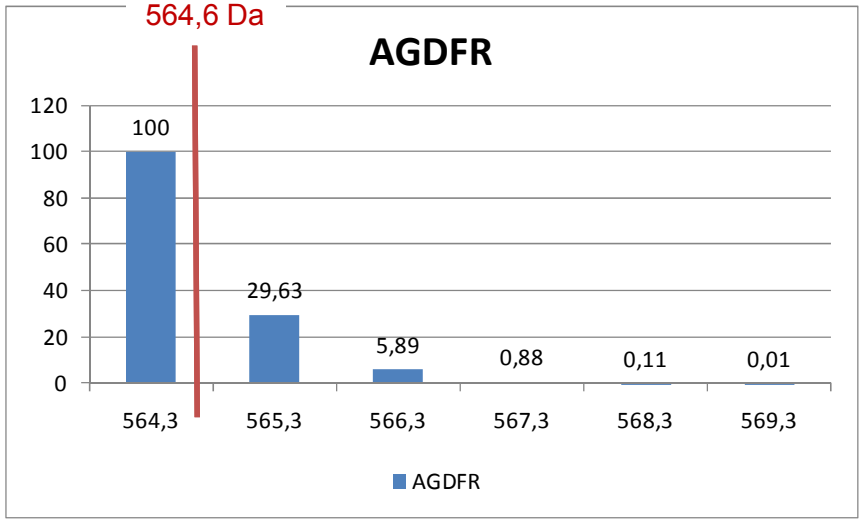
AGDFR TRESE IILAD ERTDF STDAE QWERT WERSD FYYGD RTVDG FGTRF YPSDF RTESR FGTRE

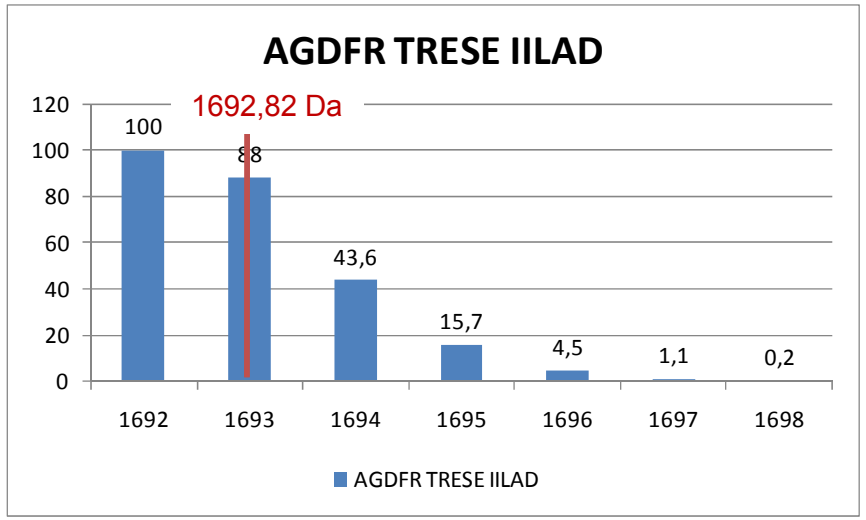
$C_{343}H_{496}N_{98}O_{115}$ M = 7827,5977 amu MG: 7832,3028 Δ : 4,7051 amu

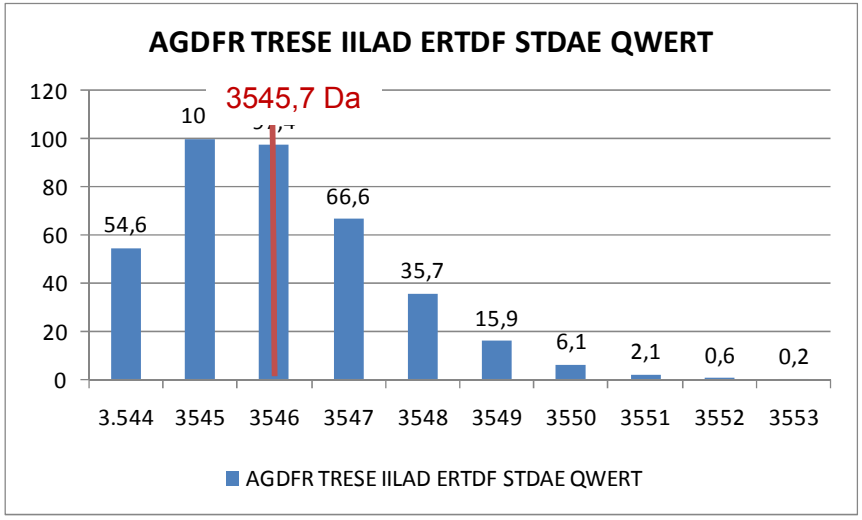
Calculated isotopic distribution

characteristic for peptides!

statistical distribution







Average mass:

Weighted by frequency of the isotope masses:

Quelle: http://www.geocities.com/junhuayan/pattern.htm

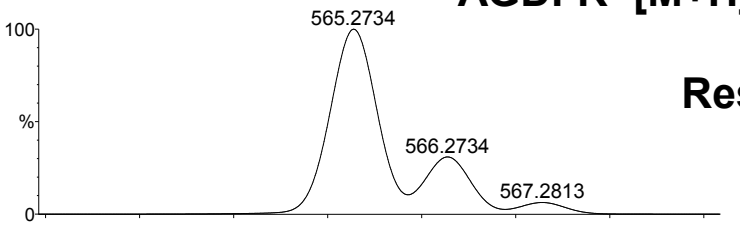
Isotopic distribution for peptides

Multiply charged ions

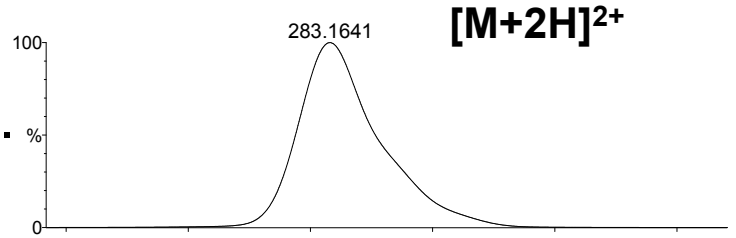
mass + charge unknown
can find charge out (see box on right)

The isotope distribution for peptides can be used for the identification of the charge state
Singly charged = isotope distance 1 amu
Doubly charged = isotope distance ½ amu
Triply charged = isootope distance 1/3 amu
....

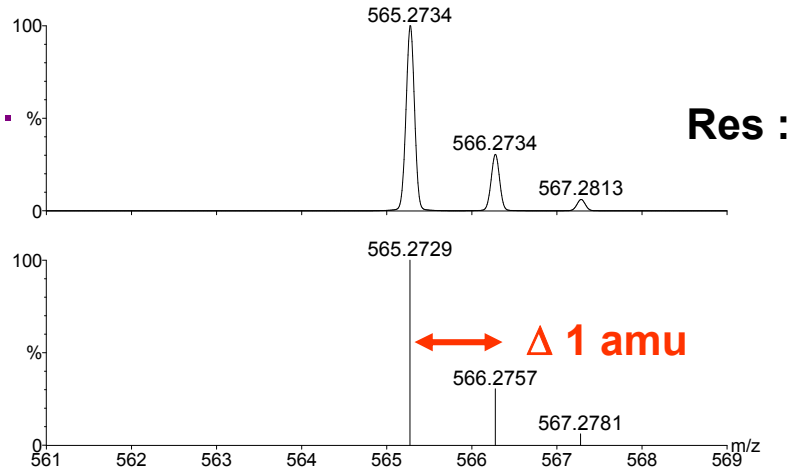
AGDFR [M+H]⁺

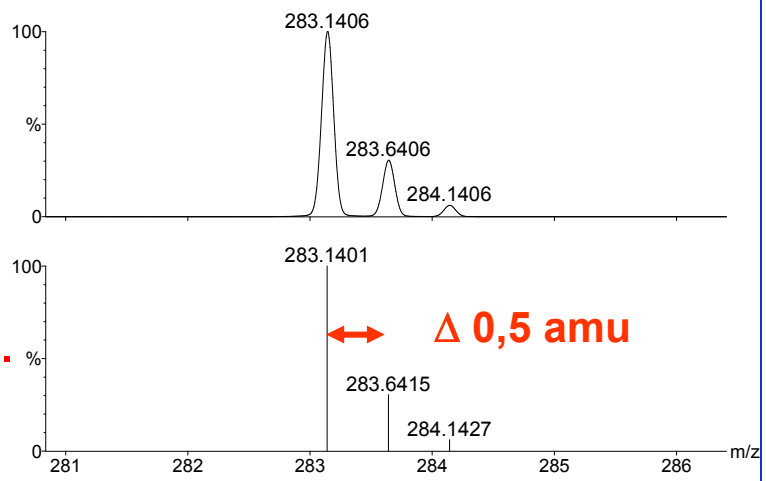


Res : 0,5 amu .



Res : 0,1 amu



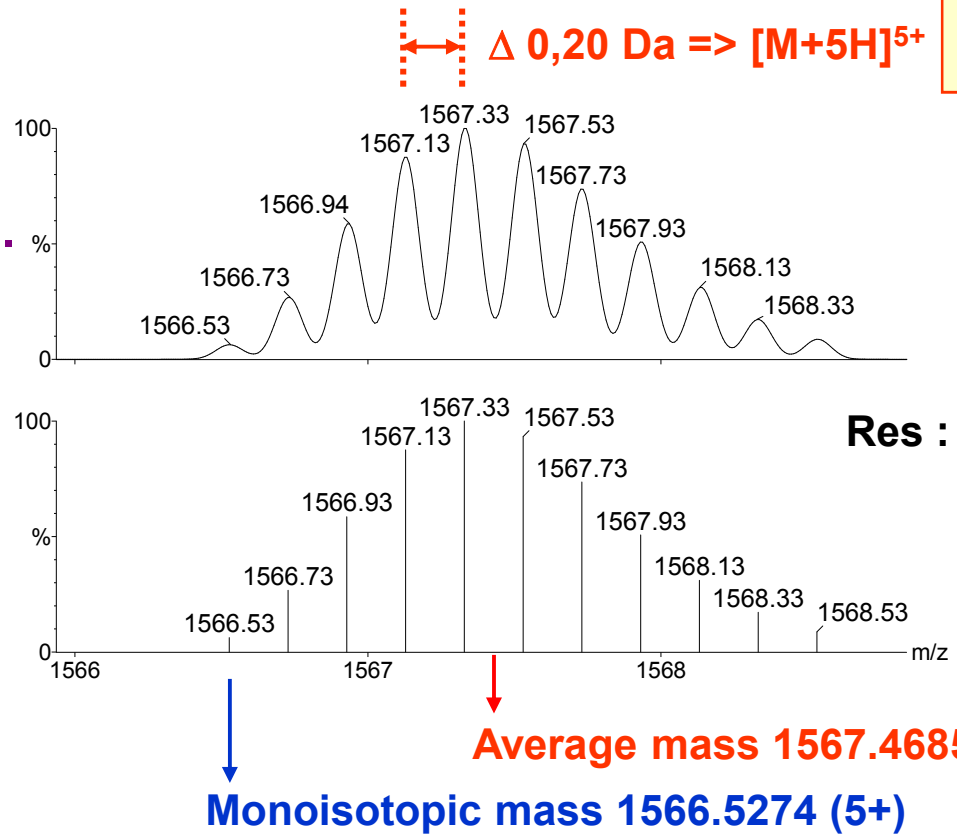


Isotope distribution for peptides

For high molecular weight proteins

Only the average mass can be determined.

The monoisotopic mass is not detectable any more.



Res : 0.1 amu

Average mass 1567.4685 (5+)

Monoisotopic mass 1566.5274 (5+)

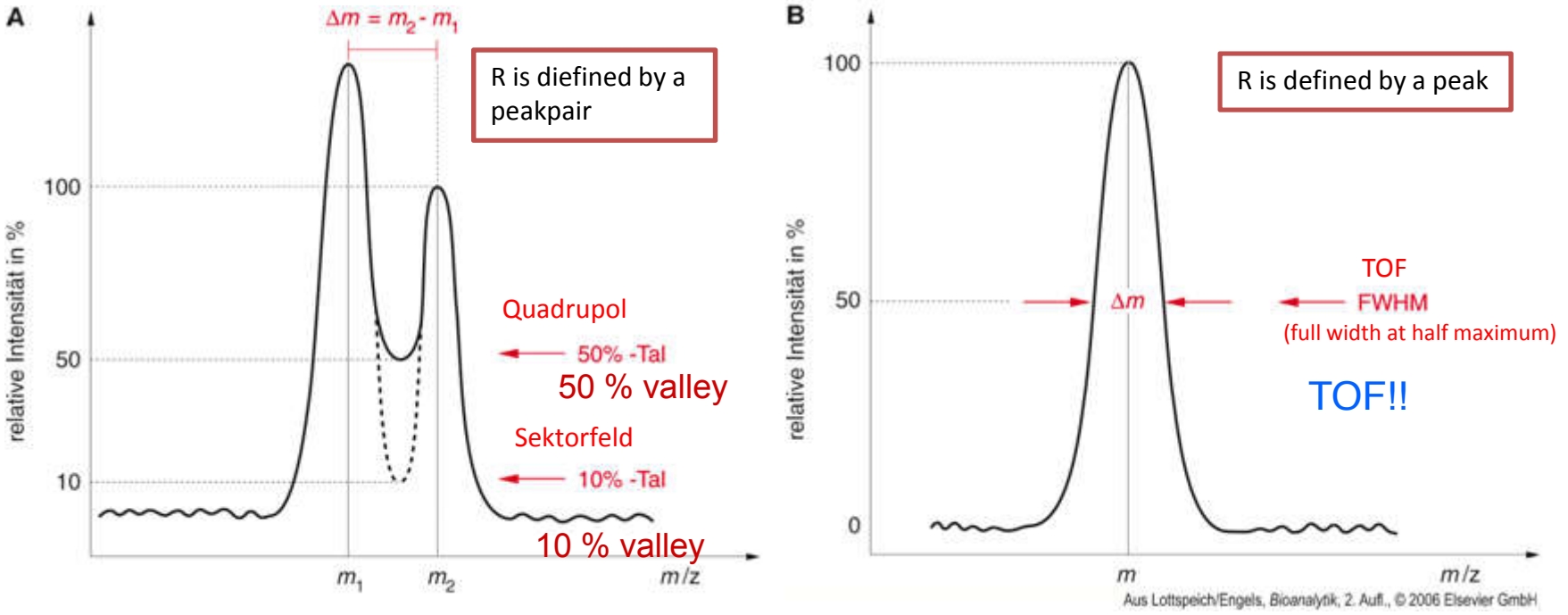
AGDFR TRESE IILAD ERTDF STDAE QWERT WERSD FYYGD RTVDG FGTRF YPSDF RTESR FGTRE

Mass resolution

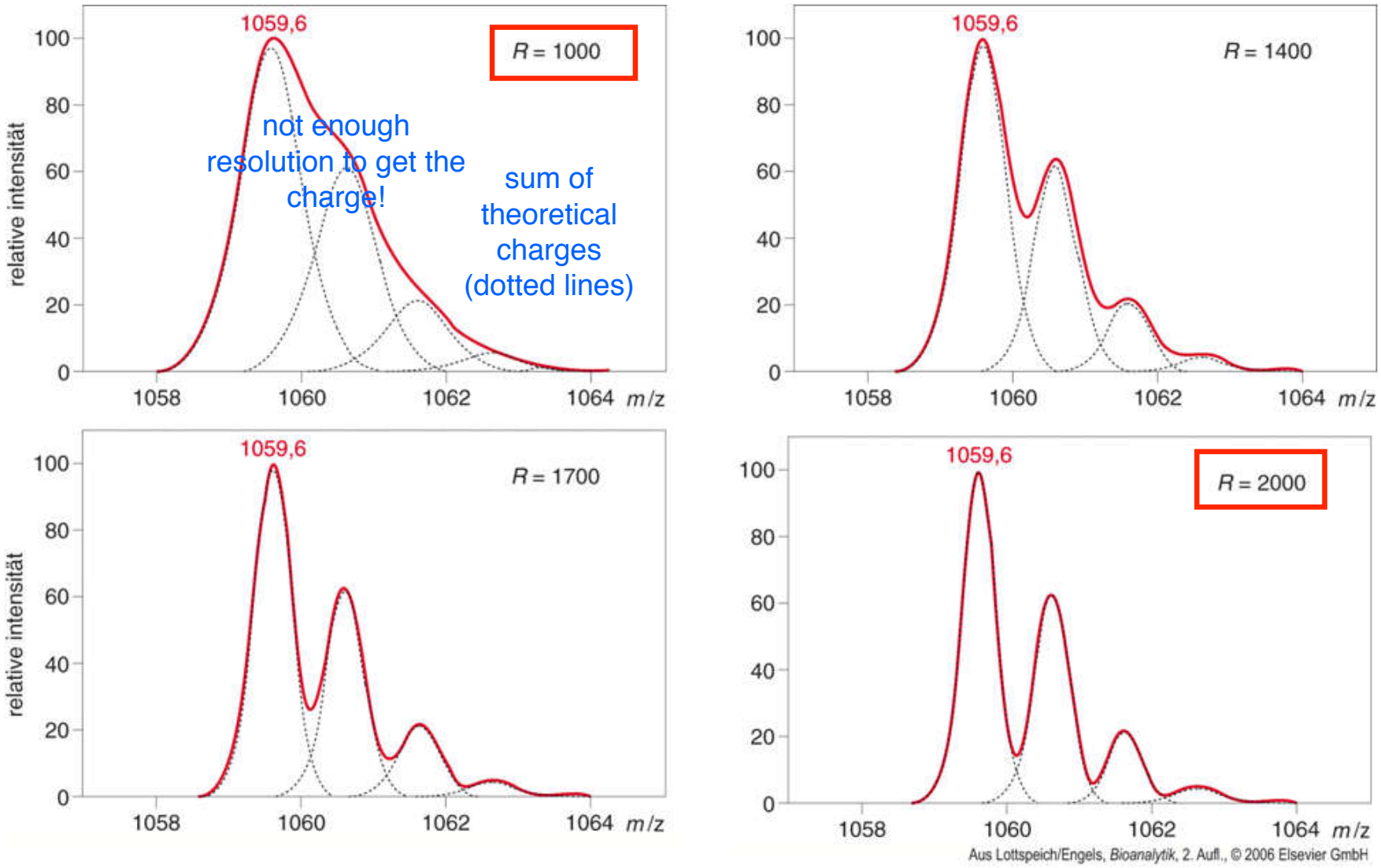
Is the capability of a mass spcetrometer to resolve two ions with minimal mass difference.

$$R = \frac{m}{\Delta m} = \frac{m_1}{(m_2 - m_1)}$$

Different definitions for different mass spectrometers



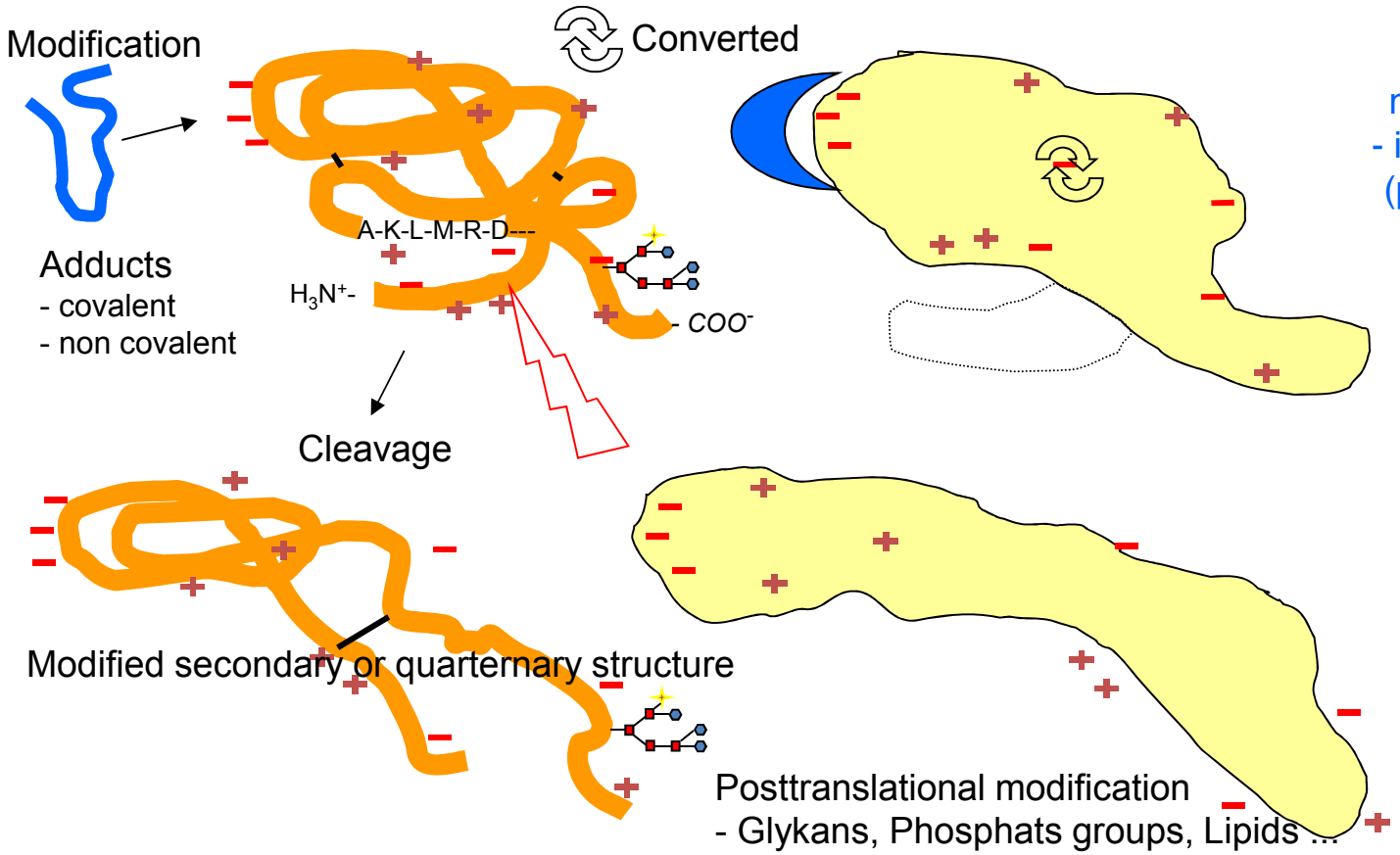
Mass resolution



Aus Lottspeich/Engels, *Bioanalytik*, 2. Aufl., © 2006 Elsevier GmbH

Mass spectrometry of proteins

not straight but 3D structures (coiled), hard to detect how many protons you can add



mass calculation:
- isotopic resolution (pattern gives you charge)
- charge states

study different confirmations of proteins