C7895 Mass Spectrometry of Biomolecules

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The course is focused on mass spectrometry of biomolecules, i.e. ionization techniques MALDI and ESI, modern mass analyzers, such as time-of-flight MS or ion traps and bioanalytical applications. However, the course covers much broader area, including inorganic ionization techniques, virtually all types of mass analyzers and hardware in mass spectrometry.

Schedule of lectures for 2010

The lectures will take place in A14-207 every Wednesday 14:00 – 15:50. The changes will be announced in advance.

Consultations

Please contact me in advance to make an appointment.

Content

Preliminary Schedule of the Lectures for 2004

I. **22. 9.** A Brief Historical Perspective: Overview of Techniques and Technology. Basic Concepts of MS (resolution, sensitivity). Isotope patterns of organic molecules. Ionization techniques and sample introduction. Electron impact ionization (EI).

II. **29. 9.** Chemical Ionization (CI). Glow Discharge. Inductively Coupled Plasma (ICP). Field Ionization/Desorption. Fast Atom Bombardment (FAB). Secondary Ion Mass Spectrometry (SIMS). Photoionization (PI). Plasma Desorption (PD)

III. **6. 10.** Laser Desorption (LD). Matrix-Assisted Laser Desorption/ Ionization (MALDI).

IV. **13. 10.** Thermospray (TSI). Ionspray (IS). Electrospray (ESI). Mass Spectrometers: Ion Optics. Wein Filter. Energy Analyzer (E).

V. **20. 10.** Magnetic Sector (B). Quadrupole Filter (Q). Ion Trap (IT).

VI. **27. 10.** Linear Trap (LT). Ion Cyclotron Resonance Fourier Transform Mass Spectrometer (FT-ICR-MS). Orbitrap. Electrostatic Trap. Simulation of Ion Movement (Simion), examples.

Preliminary Schedule of the Lectures for 2004

VII. **3. 11.** Time-of-Flight Mass Spectrometer (TOFMS). Techniques for Enhanced Resolution in TOF MS (Reflector, Delayed Extraction and Orthogonal Extraction).

VIII. **10. 11.** Ion Dissociation (CID, SID, ECD, ETD, IRMPD). Tandem Mass Spectrometry (MS/MS). In-Source Decay (ISD), Post-Source Decay (PSD). New Techniques (TOF-TOF, LIFT). Ion mobility spectrometry (IMS).

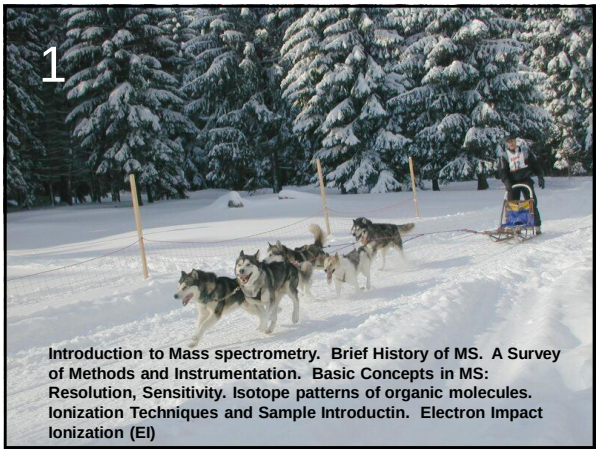
IX. **17. 11.** Vacuum: Principles & Techniques. Detectors. Data Acquisition. Coupling of Separation and MS (on-line, off-line, chips)

X. **24. 11.** Applications: Proteins and Peptides. Protein Identification: Peptide Mapping, Sequence Tag, Accurate Mass Tag.

XI. **3. 12.** Proteins and peptides. Isotope Labelling. ICAT. Sequence Determination. Post-translational Modification.

XII. **10. 12.** Disulfide Bridge Analysis. Proteins. MS databases. DNA, Saccharides, Synthetic Polymers.

XIII. **17.12.** Christmas consultation session



I. Introduction

- **Information sources about mass spectrometry**
- **Brief history of mass spectrometry, a survey of methods and instrumentation**
- **Basic concepts of mass spectrometry**
- **Isotope patterns of organic molecules.**

Study Material

Lecture notes

Advice: please take notes, but do not copy the slides; the English slides will be provided at the end of the semester. The Czech slides can be found on the internet: http:\\bart.chemi.muni.cz

Additional literature

- Robert J. Cotter: *Time-of-Flight Mass Spectrometry - Instrumentation and Applications in Biological Research*, American Chemical Society, 1997.
- Richard B. Cole et al.: *Electrospray Ionization Mass Spectrometry - Fundamentals, Instrumentation & Applications*, John Wiley & Sons, Inc., 1997.

Additional Sources of Information

Internet

Textbook http://www.ms-textbook.com/
Comprehensive information source www.spectroscopynow.com
Laboratories, e.g. www.mpi-muelheim.mpg.de/stoecki/mass_server.html
Protein Prospector prospector.ucsf.edu
Proteometrics - PROWL www.proteometrics.com
Etc etc.

Specialized journals

International Journal of Mass Spectrometry
Journal of Mass Spectrometry
Journal of the American Society for Mass Spectrometry
Mass Spectrometry Reviews
Rapid Communications in Mass Spectrometry

Ionization Techniques and Sample Introduction

Glow discharge (GD)
Electron impact ionization (EI)
Chemical ionization (CI)
Field ionization (FI)
Inductively coupled plasma (ICP)
Fast atom bombardment (FAB)
Secondary ion mass spectrometry (SIMS)
Thermospray (TSI)
Ionspray (IS)
Elektrospray (ESI)
Plasma Desorption (PD)
Laser Desorption (LD)
Matrix-assisted laser desorption/ionization (MALDI)
Coupling of separation and mass spectrometry (on-line, off-line, microdevices)

Mass Spectrometers

Ion optics. Simulation of ion movement, Simion
Energy analyzers
Magnetic sectors
Quadrupole filter
Ion cyclotron with Fourier transformation (ICR-FT-MS)
Ion trap (IT), linear trap (LT)
Time-of-flight mass spectrometer (TOFMS)
New mass spectrometers: Orbitrap, TOF-TOF, LIFT-TOF
Tandem mass spectrometry (MS/MS, MS^n)
Collision induced dissociation (CID)
Surface induced dissociation (SID)
In source and post source fragmentation (ISF and PSD)
Principles of vacuum instrumentation
Detectors a detection electronics
Chromatography - MS (on-line, off-line, in-line, microdevices)

MS Applications

Analysis of biological compounds:

- Proteins, peptide mapping, protein databases, new methods (ICAT)
- Peptide analysis (disulfide bonds, post-translational modifications)
- Nucleic acids
- Saccharides

Analysis of synthetic polymers

and more…

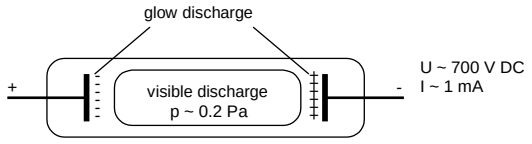
Brief History of Mass Spectrometry

1803 Dalton atomic theory
"mass consists of atoms; all atoms of a kind have the same mass"
… not really: isotopes…

Proof of existence of isotopes:
- optical spectroscopy: a slight shift of spectral lines
  ... requires a very high quality instrument
- MS: easy determination

Glow Discharge Ionization

1880's Crookes: glow discharge

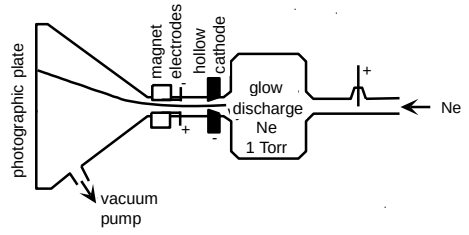


$H_2 + H_2^+ \rightarrow (H_2^+)^* + H_2$

$(H_2)^* \rightarrow H_2 + 4\ h\nu$

The First Mass Spectrometer

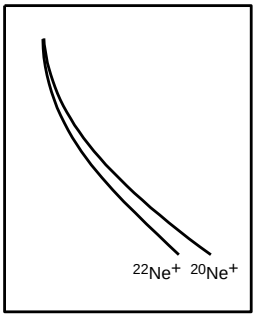
1911 J. J. Thompson: Parabola MS (*Phil Mag*. 1911, 21, 225)
"Rays of positive electricity" 1913



Glow discharge in Ne at 1 Torr, hollow cathode, magnet

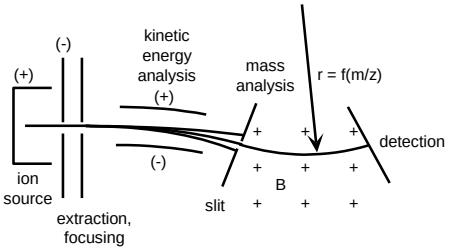
The First Mass Spectrometer

Photographic plate as a detector: ^{20}Ne and ^{22}Ne lines



Magnetic Sector with Energy Analyzer

1919 F. W. Aston: Mass Spectrograph (*Phil. Mag.* 1919, 38, 209)
Magnetic sector with electrostatic energy analyzer



Abundance of most natural isotopes determined by 1930
In Nobel prize ceremony lecture, 1934: "MS is dead, everything's done …"

But the History of MS Goes on ...

1940 C. Berry: Electron impact ionization (EI) for ionization of organic compounds

1950-70 MS applied mostly in structural analysis of organic compounds

1980+ Analysis of heavy molecules due to new ionization techniques: FAB, PD, ESI a MALDI

2006 MS for qualitative,structural and quantitative analysis
Wide scale of commercial mass spectrometers available
MS necessary for analysis of organic and biological molecules
Biospectrometry

Basic Concepts of Mass Spectrometry

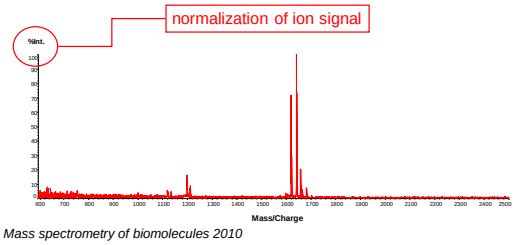
Mass spectrometer
instrument, in which ions are formed from analytes and their mass-to-charge ratio is analyzed

Components of mass spectrometer
1. Ion source chamber (contains device for sample introduction, ion optics)
2. Mass analyzer (ion optics, electrodes, magnets, detector)
3. Vacuum pumps (rough, high and ultrahigh vacuum)
4. Control and data processing unit, software

Mass Spectrum

Ion signal vs. *m/z*

Ion signal
charge, current, often converted to voltage, arbitrary units
signal normalization: intensity of the dominant peak = 100%



Mass Spectrum

Mass, *m*

a.m.u., u, Da (Dalton), molecular weight
number of atom mass units numerically equivalent to molar weight
m/z ... mass-to-charge ratio, Th (Thomson)

Number of charges, *z*:

number of elemental charges of an ion
usually ±1
exceptions, e.g. electrospray-generated ions: $|z| >> 1$

Pozitivní and negative ions, not cations and anions.

Mass spectrometry, not spectroscopy.

Selected Powers in Mass Spectrometry

Mass resolution
A measure of separation of two adjacent peaks.

Two definitions:
1. FWHM (full width at half maximum), $R = m/\Delta m$
2. Max. mass (*m/z*), at which two adjacent peaks with a unit mass difference may be resolved.

Ion energy, *W*
Instead of Joule (J) use electronVolts (eV) and atoms or ions rather than moles
1 eV = 1.6 x 10^{-19} J
simplicity: acceleration voltage = 100 V, charge = 1 ... W = 100 eV
can be easily compared with ionization energy, bond energy, photon energy...

Pressure, *p*
1 atm = 760 Torr = 101 325 Pa = 1.01325 bar = 14.70 PSI

Abbreviations

m	mass of ion, atom, molecule (u, a.m.u., Da)
z	number of charges; (-)
m/z	*mass-to-charge ratio* (Th, Thomson)
e	elemental charge (1.6x10^{-19}C)
U	voltage (V)
E	intensity of electric field (V/m, N/C)
W	energy, labor (eV, J)
v	ion velocity (m/s)
r	*curvature radius* (m)
L	path (m)
λ	mean free path of a molecule
t	time (s)
σ	collision diameter (m)
σ^2	collision cross-section (m^2)
μ	reduced mass (a.m.u., Da, kg)

Abbreviations

n	numerical concentration (m^{-3})
I	current (A), flux (m^{-2}), intensity (-)
T	absolute temperature (K)
p	pressure (Pa, Torr)
R	resolution (-)
f	frequency (Hz)
ω	angular frequency (rad/s, s^{-1})
α	direct proportion
LD	detection limit, also *LOD*, limit of detection (mol, g, M)
S/N	signal-to-noise ratio
RSD	relative standard deviation

Isotope Patterns of Organic Molecules

Carbon isotopes: 99% **^{12}C**, 1% **^{13}C**

Pattern as a function of number of carbon atoms in the molecule:

C:	99% **^{12}C**	1% **^{13}C**	
C_2:	98% **$^{12}C^{12}C$**	2% **$^{12}C^{13}C$**	0.01% **$^{13}C^{13}C$**
C_3:	97% **$^{12}C^{12}C^{12}C$**	3% **$^{12}C^{12}C^{13}C$**	0.04% **$^{12}C^{13}C^{13}C$** 10^{-4} % **$^{13}C^{13}C^{13}C$**

Binomic formula
Relative abundance of the light isotope, *a*
Relative abundance of the heavy isotope, *b*
Number of atoms, *n*
E.g. for $n = 2$: $(a+b)^2 = a^2 + 2ab + b^2$

Monoisotopic molecule contains given atoms in form of a single isotope.
In the case of biomolecules usually carbon atoms in the form of ^{12}C.

Isotope Patterns of Organic Molecules

Relative abundance of molecules (%):

C_{60}:	$^{12}C_{60}$	100
	$^{12}C_{59}{}^{13}C$	66
	$^{12}C_{58}{}^{13}C_2$	21
	$^{12}C_{57}{}^{13}C_3$	4.6
C_{100}:	$^{12}C_{100}$	100
	$^{12}C_{99}{}^{13}C$	110
	$^{12}C_{98}{}^{13}C_2$	60
	$^{12}C_{97}{}^{13}C_3$	22

(normalized with respect to the monoisotopic molecule /only ^{12}C/ = 100 %)

With increasing number of carbon atoms, *n*, the relative intensity of the monoisotopic form decreases, the monoisotopic peak is not dominant any more and intensities of other isotopic forms are comparable (wide envelope) ... see examples below.

Practical Impact of Isotope Abundance

- Decrease of sensitivity
- High resolution *R* necessary at high *m*/*z* for correct determination of *m*/*z*

+ Use of isotopic internal standards. The best internal standards.

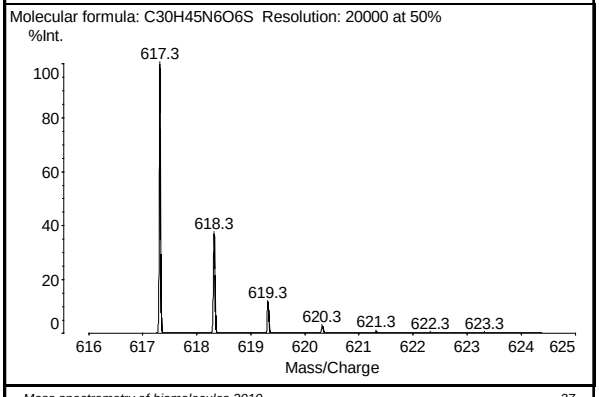
Example:

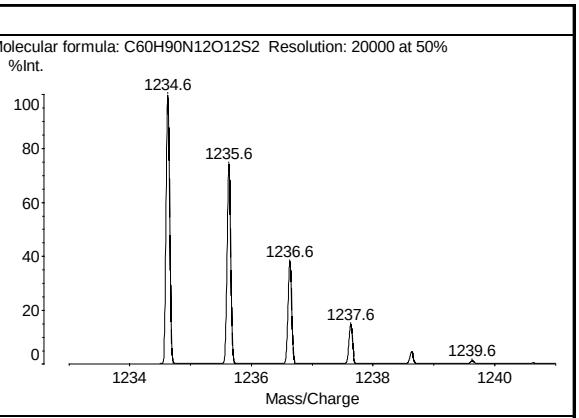
5 peptides/proteins with relative abundance of elements

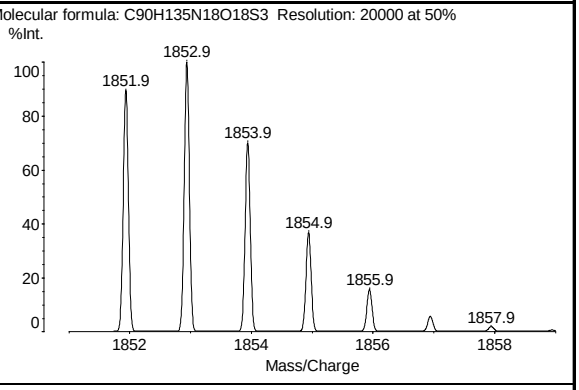
C : H : N : O : S = 30 : 45 : 6 : 6 : 1

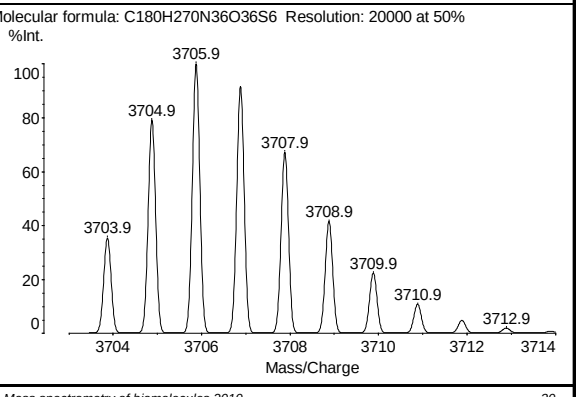
R = 20 000

$C_{30}H_{45}N_6O_6S$	$C_{60}H_{90}N_{12}O_{12}S_2$
$C_{90}H_{135}N_{18}O_{18}S_3$	$C_{180}H_{270}N_{36}O_{36}S_6$ (also shown for more *R*)
$C_{270}H_{405}N_{54}O_{54}S_9$	$C_{360}H_{540}N_{272}\ O_{272}S_{12}$
$C_{900}H_{1350}N_{180}O_{180}S_{30}$	$C_{1800}H_{2700}N_{360}O_{360}S_{60}$

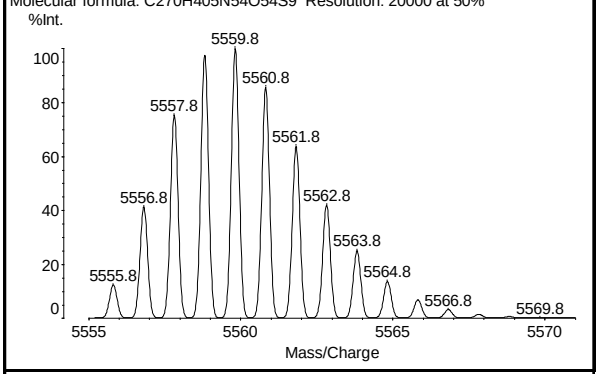




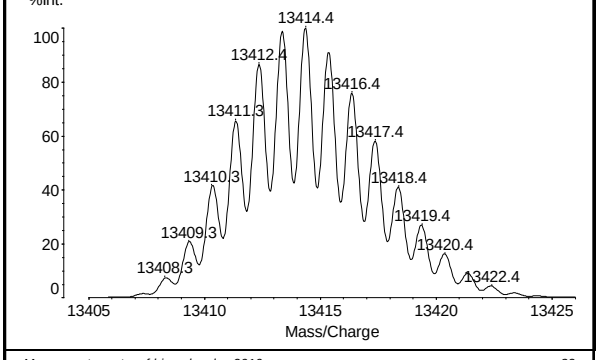




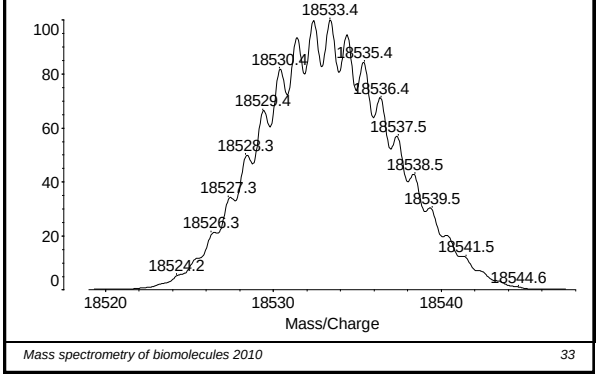
Molecular formula: C270H405N54O54S9 Resolution: 20000 at 50%



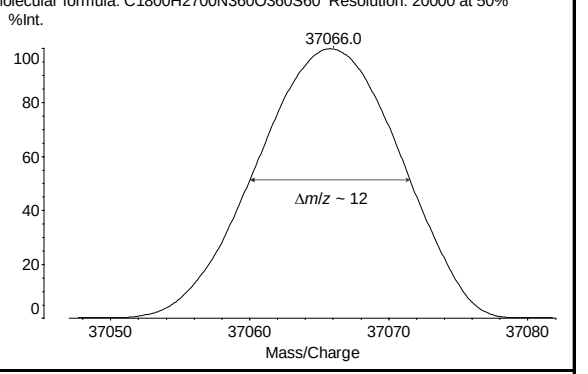
Molecular formula: C360H540N272O272S12 Resolution: 20000 at 50%



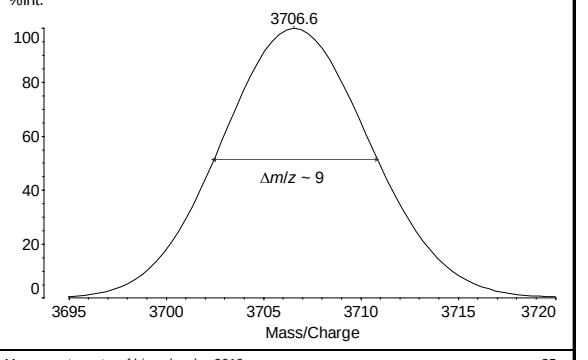
Molecular formula: C900H1350N180O180S30 Resolution: 20000 at 50%



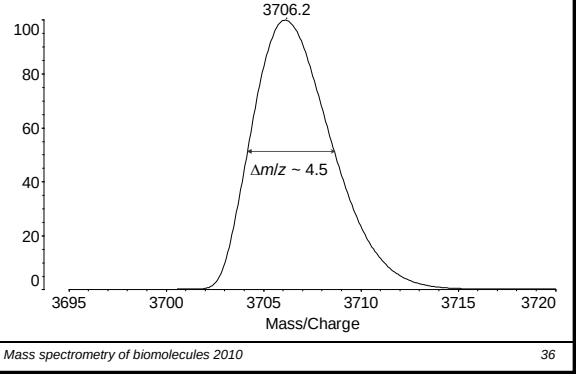
Molecular formula: C1800H2700N360O360S60 Resolution: 20000 at 50%

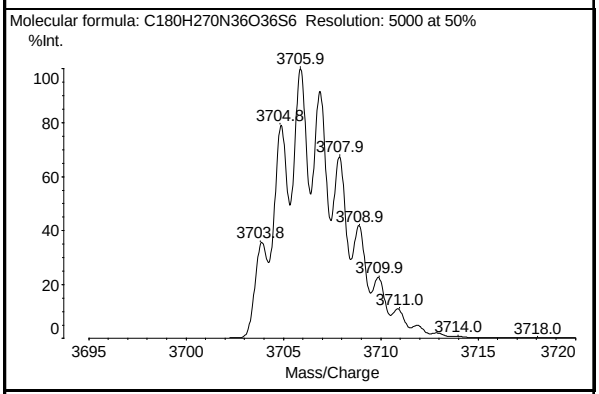


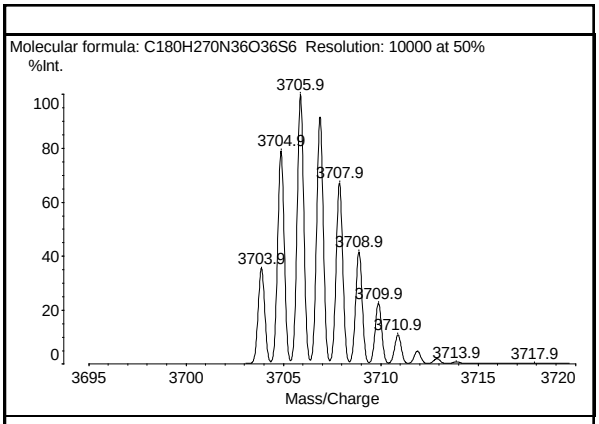
Molecular formula: C180H270N36O36S6 Resolution: 500 at 50%



Molecular formula: C180H270N36O36S6 Resolution: 2500 at 50%

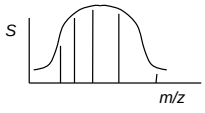






Multiply-charged ions

- Typical example: multiply-charged ions $[M+zH]^{z+}$ by electrospray
  Bell-shaped envelope
- The gaps between peaks are not equidistant (contrary to isotope pattern).



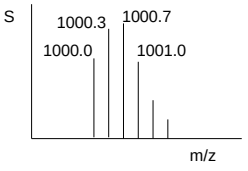
Example:

A small protein with *M.W.* = 10 000 Da

z	4	5	6	7	8	9	10
m/z	2501	2001	1668	1429	1251	1112	1001

What Can Be Said about This Spectrum?

Note: This is a portion of mass spectrum of a single organic compound.
m/z values were determined with accuracy ~ 0.1.



II. Ionization Techniques and Sample Introduction

sample (atm. pressure) → sample (vacuum)

Unwanted phenomena

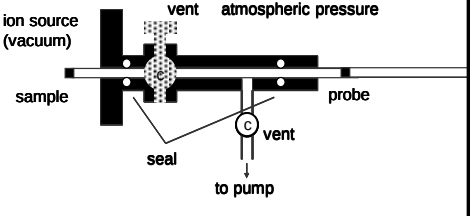
- pressure increase in the ion source
- cooling and freezing of solvent due to solvent evaporation
- adsorption of compound (e.g. water) on the walls of the ion source chamber

Classification of samples acc. to the state

- **Fluid samples**
  gaseous
  liquid (liquid analyte, dissolved analyte)
- **Solid samples**
  volatile (usually light compounds)
  nonvolatile (polar, heavy, polymer compounds)

Sample Introduction

Off-line
On-line



Number of introduced samples

- 1 sample
- more samples
  - in a queue, sample series (discrete samples or continual flow)
  - in paralel

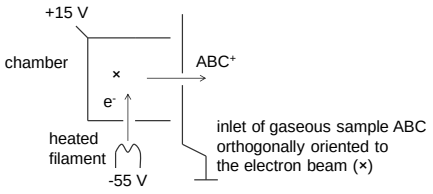
Electron Impact Ionization, EI

Classical ionization technique.

Electrons emitted from a heated filament are accelerated using a medium voltage.

Electron energy, $W(e^-)$ = acceleration potential x charge (1).

Typical energy: 70 eV.



Mechanism of EI

Interaction of electron with analyte molecule ABC:

e^- (fast) + ABC → ABC^+ + 2 e^- (slow)

Total equation,

ABC → ABC^+ + e^-

is characterized by ionization energy ABC, ΔH(ABC).

Ions ABC^+ with energy excess can undergo fragmentation:

$(ABC^+)^*$ → AB^+ + C, A + CB^+ etc

Fragmentation extent depends on electron energy, $E(e^-)$ and on the analyte structure:

a) $W(e^-)$ ~ ionization potential ⇒ production of molecular ions.

Ionization potential of simple organic molecules ~10 – 12 eV.

b) $W(e^-)$ >> ionization potential ⇒ fragmentation.

Type of fragmentation depends on analyte structure; compounds of similar structure have similar fragmentation spectra.

Interpretation of spectra. Spectral libraries (> 100 000 spekter).

Chemical Ionization (CI)

20th of the 20th century A. J. Dempster

The same source as in the case of the EI plus an inlet for reagent gas

Reagent gas (RH)

CH_4, butane, H_2, NH_3 etc.

p(ABC) < 10^{-4} Pa

p(R) ~ 0.1 Pa (λ ~ 0.05 mm, many collisions in the source)

Mechanism of ion formation

1) **production of RH^+**

e^- (fast) + RH → RH^+ + 2 e^- (slow)

2a) **charge transfer**

RH^+ + ABC → RH + ABC^+

Mechanism of EI

Appearance energy (*AE*), at which the fragments AB^+ appears, does not have to be higher than ΔH(ABC)!

ABC → ABC^+ + e^-	Ionization energy ABC, ΔH(ABC)
ABC → AB^+ + C + e^-	Threshold energy AB^+, $AE(AB^+)$
$AE(AB^+) = \Delta H(ABC) + D(ABC^+)$	Dissociation energy ABC^+, $D(ABC^+)$

Absorption of electron during travel through the analyte

Reduction of electron flux, dI during the travel through infinitesimally thin analyte layer:

$dI = -\alpha c I dx$,

after integration:

$I = I_o e^{-\alpha c x}$.

I electron flux (A)

c concentration of ABC, (cm^{-3}) ($c = p/RT$)

x layer thickness (cm)

α cross-section (cm^2) … analogy of ε coefficient in the Lambert-Beerově law

Mechanism of Ion Formation at CI (cont.)

2b) **proton transfer** (more common)

$RH^+ \rightarrow R + H^+$	PA(R) proton affinity
$ABC + H^+ \rightarrow ABCH^+$	$-PA$(ABC)
$RH^+ + ABC \rightarrow R + ABCH^+$	ΔE = PA(R) - PA(ABC)

ΔE < 0: exothermic, preferred reaction

ΔE << 0: energy excess at $ABCH^+$ ⇒ fragmentation of $ABCH^+$ structural analysis

ΔE < 0, $\Delta E \rightarrow 0$ ⇒ ABC^+ a $ABCH^+$ dominate

+ quantitative analysis
+ determination of molecular weight of ABC
+ high ionization efficiency (ABC)
- no structural information

Collisions during CI

Mean free path

Mean path, which a particle travels between 2 collisions

$\lambda = (\sqrt{2}\pi\sigma^2 n)^{-1}$

λ(cm) = 0.66/p(Pa) (only the 1st approximation)

Number of collisions

$z = \pi\sigma^2(8kT/(\pi\mu))^{1/2}$

μ reduced mass, $\mu = (m_1^{-1} + m_2^{-1})^{-1}$

σ collision diameter

σ^2 collision cross-section

CI: $10^{15\text{-}16}$ collisions $\Rightarrow$ high ionization efficiency (ABC)

Comparison of CI *vs.* EI

+ stronger signal
- higher noise
+ overall *S/N* higher (*LOD* of organic compounds ~ pg)

Negative Chemical Ionization

The same ion source as in the case of EI plus an inlet for reagent gas

Mechanism

1. **Production of thermal (slow) electrons**

e^- (fast) + RH $\rightarrow$ $RH^+ + 2\ e^-$ (slow)

W(slow e^-) ~ 3/2 kT

T ~ 400 K $\Rightarrow$ E ~ 0.1 eV

2. **Electron capture**

$ABC + e^- \rightarrow ABC^-$

Preferred by compounds with electronegative groups (PCB, NO_3^- etc.)

LD ~ pg

Sample Introduction for EI/CI

1. Gas/volatile liquid

Residual gas analysis

open ion source in chamber with analyzed gas: p(chamber) = p(gas)

Eluent from a separation column (GC-MS)

Problem: High flow rate of the carrier gas from classical GC columns

a) fraction collection or split flow (splitter may mean reduction of sensitivity)

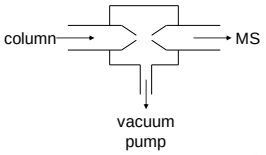
b) continual interface (working without interruption)

i) *particle beam separator*

for analyte (ABC) enrichment in the carrier gas

requires M(ABC) >> M(carrier)

... use He as the carrier gas



Sample Introduction for EI/CI (cont.)

ii) *membrane interface* - sample introduction through a membrane, separation of the carrier gas using a gas-permeable membrane

c) direct introduction from a capillary GC column

lower gas load; lower flow rate of the carrier gas (He)

2. Volatile, thermally stable solid sample

Direct sample introduction on a probe (glass, ceramics, steel). After introduction of the probe, the sample begins to evaporate and undergoes ionization in the gaseous state.

Sample Introduction for EI/CI (cont.)

3. Nonvolatile compounds

- Large molecules
- Molecules with many polar groups

… many interesting compounds (proteins, DNA, saccharides)

a) Generation of volatile derivates and consecutive standard ionization (EI, CI).

Useful for molecules with M < 1000 Da.

Example: esterification, $RCOOH + CH_3OH \rightarrow RCOOCH_3$

b) Application of classical ionization in desorption arrangement. Sample deposited on a probe is inserted into an ion source, in which electrons interact directly with the sample in condensed state.

c) Other ionization techniques

"Soft" ionization: production of molecular ions without their thermal decomposition: FAB, electrospray, laser desorption techniques

Inorganic Ion Sources

Glow discharge

Thermal ionization

Inductively coupled plasma

Other techniques, e.g. laser desorption – also for organics, discussed later

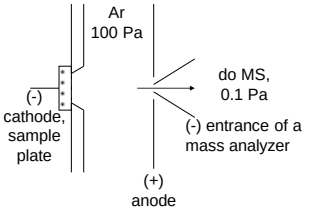
Glow Discharge

The first ion source (J. J. Thompson)

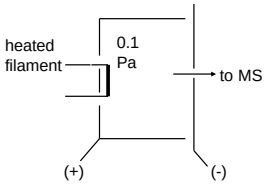
Analysis of solid samples, usually conductive.

Precise and relatively sensitive analysis: *RSD* ~1%, *LOD* ~1 ppb.

Discharge in Ar (*p* ~100 Pa): Ar^+ ions sputter metal atoms (M) from a sample plate and ionize them later ... M^+ ions are formed.



Thermal Ionization (TI)



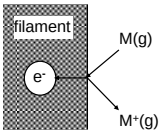
Sample deposited on a filament; the filament is resistively heated.

Evaporation, atomization and ionization:

$MX\ (s) \rightarrow MX\ (g) \rightarrow M\ (g) + X\ (g)$

$M\ (g) \rightarrow M^+\ (g) + e^-$ (filament)

Thermal Ionization



Saha-Langmuir equation

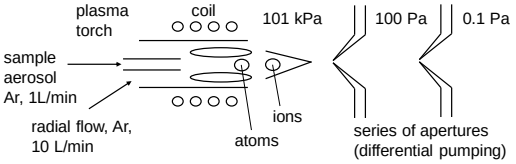
$n(M^+)/n(M) \propto \exp[(W - IE)/(kT)]$

Working function of metal (filament), W ~ 4 eV

Metal		K	Ca	Fe	Zn
IE (eV)	4.6	6.0	7.8	9.4	
(*W* – *IE*) (eV)		-0.5	-1.5	-3.9	-5.4
		efficient			weak

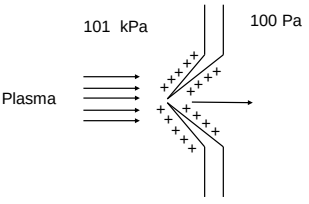
- Three filaments (with different temperature): Substitution of a single filament, which evaporates and ionizes sample too fast.
  - Generation of more stable ion flow (*RSD* only ~ 0.1 % !)
  - Useful for determination of isotopic abundance.

Inductively Coupled Plasma, ICP MS



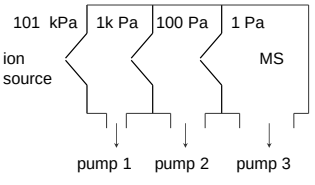
1. Desolvation of MX (aq, aerosol)
2. Evaporation of MX (s)
3. Atomization of MX (g): dissociation to M(g) and X (g)
4. Ionization to M+ (g)

ICP



- Usually 3 vacuum stages (differential pumping).
- Very efficient ionization, $n(M^+)/n(M_{total})$ = 90 – 100%.
- Ions with a single charge prevails.
- Non-equilibrium system.

Differential Pumping



- frequently used concept in mass spectrometry
- used for atmospheric ionization methods

ICP

Supersonic jet

Hot plasma (5000 K) streams via an aperture (slit) into a chamber and expands at supersonic velocity. Random movement of atoms at the atmospheric side is characterized by wide kinetic energy distribution (5000 K) and relatively low translational velocity. The atoms move at supersonic velocity with a very narrow kinetic energy dispersion … supersonic cooling (~300 K). Distribution is later ruined by collisions with molecules of background gas (*barrel shock, Mach disc*).

Efficient ionization

90 - 100 % elements are ionized (very uniform ionization).

Applicable for determination of isotopic abundance (low systematic deviation), elemental composition.

Disadvantages

- not useful for structural characterization of analytes
- interferences

ICP

Detection limits

1 ppt (quadrupole)
10 ppq (magnetic sector)
for comparison: *LD* of ICP-AES and AAS ~ ppm - ppb

ppm	ppb	ppt	ppq
million 10^6	billion 10^9	trillion 10^{12}	quadrillion 10^{15}

Interferences

1. Non-spectral
   Shifts of ionization equlibria as a result of suppression by matrix, acids or easily ionizable elements etc.

2. Spectral
   Isotopic ions
   Izobaric molecular ions

Spectral Interferences in ICP

Formation of molecular ions in ICP

1. Plasma gas and reaction products (Ar^+, Ar^{2+}, ArH^+, ArO^+, ArC^+, ArN^+ etc.)
2. Sample or solvent (hydride ions, OH^+, ClO^+, NO^+, CaO^+, LaO^+ etc.)
3. Chemical ionization of background gas (H_2O^+, H_3O^+, $C_xH_y^+$ etc.)

Elimination of spectral interference

1. Mathematic corrections (e.g. using isotope distribution)
2. Desolvation of aerosol (e.g. by freezing in liquid N_2)
3. Cold plasma (relative shifts of ionization degree)
4. Collision cell (thermalization of ions, shifts of reaction equillibria)
5. Mass spectrometer with high resolution

Spectral Interference in ICP

Examples of isobaric ions and required resolution

Isotope	Interfering ion	Resolution
^{39}K	$^{38}Ar^1H^+$	5690
^{40}Ca	$^{40}Ar^+$	71700
^{41}K	$^{40}Ar^1H^+$	4890
^{44}Ca	$^{14}N^{14}N^{16}O^+$	970
	$^{12}C^{16}O^{16}O^+$	1280
^{52}Cr	$^{40}Ar^{12}C^+$	2380
^{56}Fe	$^{40}Ar^{16}O^+$	2500
^{75}As	$^{40}Ar^{35}Cl^+$	7770
^{80}Se	$^{40}Ar^{40}Ar^+$	9690

Note: higher resolution often means lower sensitivity.

Field Ionization, FI

Very high electric field intensity between sharp spires, $E > 10^9$ V/m
Electron removal due to inner tunnel effect.

Desorption Ionization Techniques

LDI Laser Desorption/Ionization
1963 R. Honig

FD Field Desorption
1969 H. D. Beckey

PD Plasma Desorption
1974 R. D. MacFarlane

FAB Fast Atom Bombradment
1981 M. Barber

SIMS Secondary Ion Mass Spectrometry
1976 A. Benninghoven

MALDI Matrix-Assisted Laser Desorption/Ionization
1988 M. Karas & F. Hillenkamp, K. Tanaka

Field Desorption, FD

H. D. Beckey, *Int. J. Mass Spectrom. Ion Phys*., **1969**, 2, 500-503

Sample is blown (g) or deposited (l, g) on an emitter, a heated metal filament with a specially modified surface

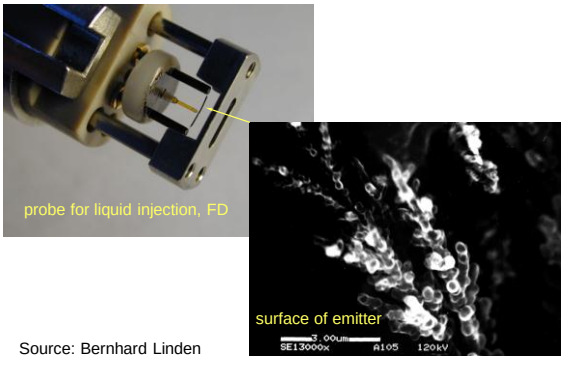
Analytes are formed in very high electric field between sharp spires, $E > 10^9$ V/m; electrons are removed by inner tunnel effect.

Often more ionization mechanisms:

- thermal ionization
- electrospray ... during deposition of liquid samples

Applicable to analysis of organic analytes with *M.W.* < 2 kDa

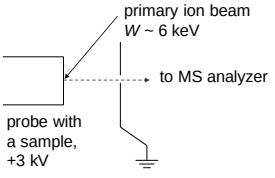
Field Ionization. Field Desorption



Source: Bernhard Linden

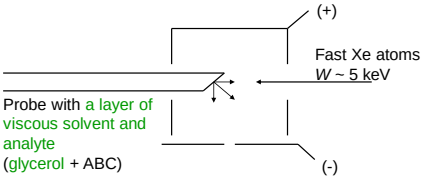
Secondary Ion Mass Spectrometry, SIMS (Fast Ion Bombardment, FIB)

… Ionization by ions



- Primary ion beam may be scanned; the result is MS image of elements/compounds, analyte topography. Imaging resolution is higher than in the case of optical microscopy since the primary ion beam can be focused tighter, ~nm).
- Products … mostly atoms and neutral molecules, also ions. Postionization helpful, e.g. photoionization using a laser.
- Inorganic and organic analysis, also lighter biopolymers at the presence of matrix - "matrix-assisted SIMS".

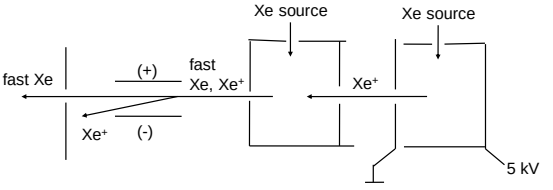
Fast Atom Bombardment, FAB

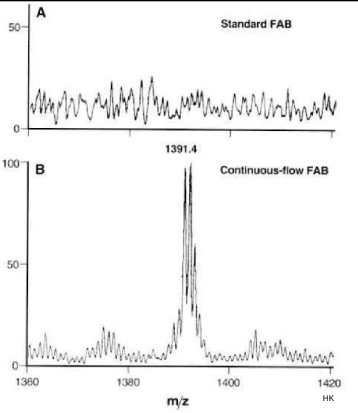


Ionization similar to CI (organic molecules, small peptides …).
Setup: off-line and on-line (flow probe; continuous flow FAB, CF-FAB).
Max. *m/z* ~ 10 000 Da. *LOD* ~ 10 pmol or even ~ 1 pmol (CF-FAB)
Usually $z = \pm 1$.
Formed ions: $ABCH^+$, $[ABC+K]^+$, $[ABC+H]^+$, $[ABC+N_2]^+$, fragments.

Generation of Fast Xe atoms for FAB

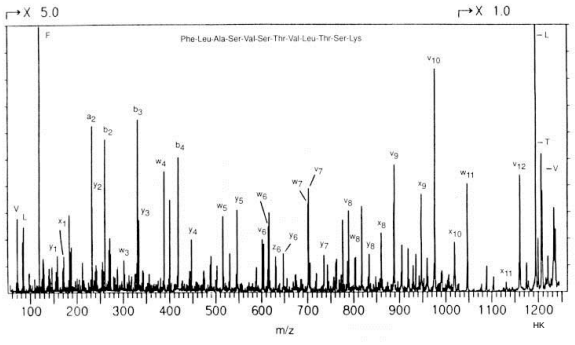
1. Generation of fast Xe^+ ions
2. Conversion of Xe^+ to Xe:
   Xe^+ (fast) + Xe (slow) → Xe (fast) + Xe^+ (slow)
3. Elimination (deflection) of Xe^+





FAB MS of a peptide in normal and CF mode

CF-FAB MS/MS of Human Hemoglobin, chain A



Photoionization, PI

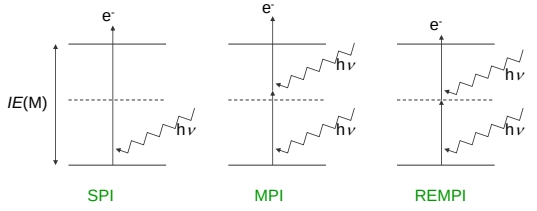
M + *n* hν → M^+ + e-

Necessary condition: *n*hν > *IE*(M)

Photoionization types

1. Single photon ionization, SPI; *n* = 1
2. Multiple photon ionization, MPI; *n* > 1
   - non-selective analysis useful for inorganic analytes
3. Resonance multiphoton ionization (REMPI) *n* > 1
   - if hν = *W* (energy of electron transfer of M)
   - very selective and very sensitive determination
   - aromatic molecules, dyes, drugs

Photoionization Schemes

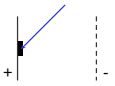


Photoionization

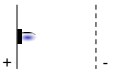
Example of arrangement: LD-PI

PI as a postionization for pro laser desorption

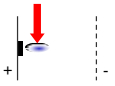
1. Desorption laser pulse



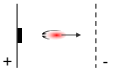
2. Desorption of plume (mostly neutrals)



3. Ionization laser pulse



4. Ion extraction



Plasma Desorption (PD)

1974 R. D. Macfarlane
(R. D. Macfarlane, R. P. Skowronski, D. F. Torgerson, *Biochem. Biophys. Res. Commun.* **1974**, 60, 616.; R. D. Macfarlane, D. F. Torgerson *Science* **1976**, 191, 920.)

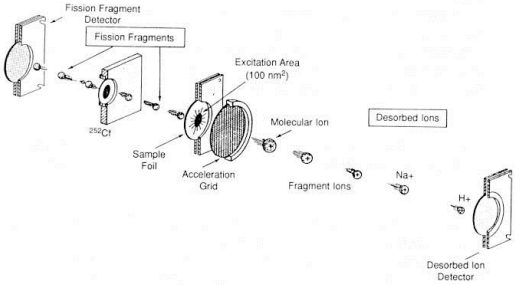
The first technique capable of ionization of heavy compounds (e.g. proteins).

Radioactive californium ^{252}Cf source. Energy of fission fragments ~MeV.

Impact of a fission fragment from the radioactive source on sample deposited on polyester foil may ionize analyte molecule.

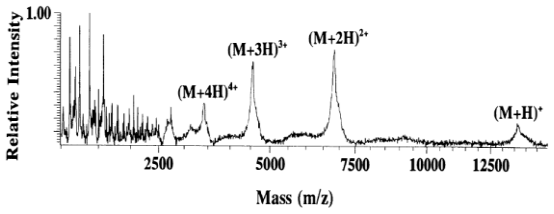
Other fragment from decay of the same ^{252}Cf flies in the opposite direction and triggers signal recording device.

Experimental Setup of Plasma Desorption



(R. D. Macfarlane, R. P. Skowronski, D. F. Torgerson, "New approach to the mass spectrometry of nonvolatile compounds", *Biochem. Biophys. Res. Commun.* **1974**, 60, 616-621.)

Example of Plasma Desorption



Positive PD mass spectrum of ribonuclease A from bovine pancreas

(D. M. Bunk, R. D. Macfarlane *Proc. Natl. Acad. Sci. USA* **1992**, 89, 6215-6219)

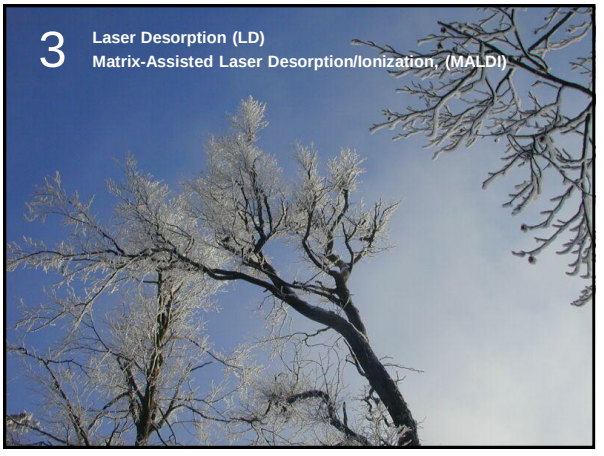
Plasma Desorption Characteristics

Ionization of relatively large organic molecules (~ thousands Da , e.g. insulin, 5 735 Da).

Formation of molecular ions, ion clusters and multiply-charged ions.

Disadvanatges
- radioactive source.
- time-consuming signal accumulation.

MALDI and ESI preferred for ionization of heavy analytes nowadays.



Laser Desorption
Matrix-Assisted Laser Desorption/Ionization

Laser Desorption/Ionization, LDI

After laser discovery in 6th decade of 20th century

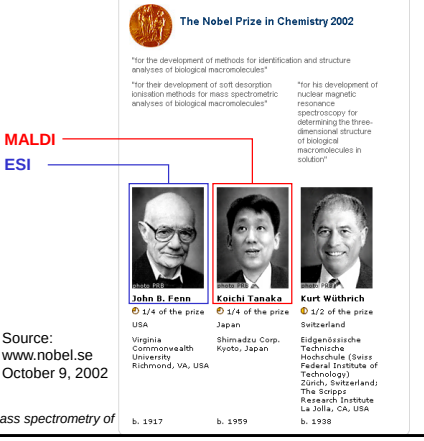
Initially for solid samples: R. Honig, *Appl. Phys. Lett.* **1963**, 2, 138-139.

Later for Organic compounds: M. A. Posthumus, P. G. Kistemaker, H. L. C. Meuzelaar, M. C. ten Neuver de Brauw, *Anal. Chem* **1978**, 50, 985.

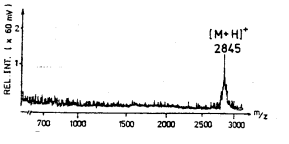
Matrix-Assisted Laser Desorption/Ionization, MALDI

Karas, M; Bachmann, D.; Bahr, U.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Proc.* **1987**, *78*, 53-68.

Tanaka, K.; Waki, H.; Ido, Y; Akita, S.; Yoshida, Y.; Yoshida, T. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151.

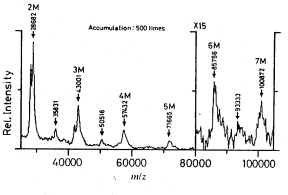


Source:
www.nobel.se
October 9, 2002



1987 Karas & Hillenkamp
melitin
nicotinic acid
m/z = 2845

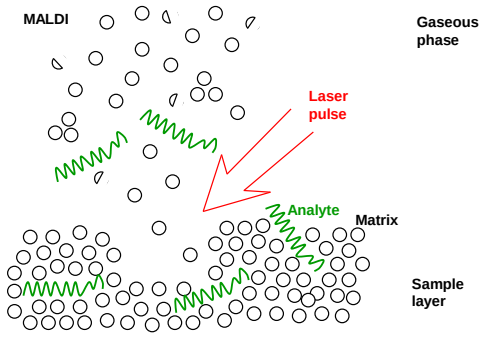
Fig. 7. Matrix-LD spectra of mellitin/NicAc accumulated from 12 single laser shots.



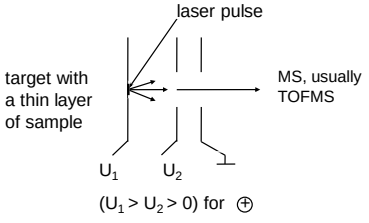
1988 Tanaka
lysozyme
Co/glycerol
m/z = 100 872 (heptamer)

Figure 2. Laser ionization mass spectrum of lysozyme from chicken egg white, mol. wt 14306 Da.

MALDI Schematic (detail)



MALDI Schematic



Principle of MALDI

1. **Very short laser pulse**, typical t ~ ns, max. μs.
   Molecules vaporize before they decompose.
   Collisional cooling: conversion of E_{vib} to E_{trans} .

2. **Energy is absorbed** mostly by **matrix** (M), not by analyte.
   ε(matrix) >> ε(analyte), c(matrix) >> c(analyte)
   Matrix → MH^+, M^+, M*, fragments, fragment ions.
   Analyte, originally "dissolved" in matrix, vaporizes together with matrix.

3. **Matrix actively participates on analyte** (ABC) **ionization**.
   Matrix is excited after absorption of one or more photons.
   Dominant ionization mechanism is **proton transfer**:
   $MH^+ + ABC \rightarrow M + ABCH^+$.

Requirements on Matrix (MALDI)

1. Absorbtion at the wavelength of the used laser (UV, IR).
2. Formation of proper crystals with analyte (empiric rule).
3. Usually acid (efficient proton transfer to ionize na analyte).
4. Stability. Low volatility. Inert (no reaction with analyte).

Types of matrix

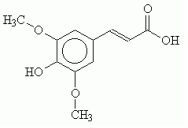
aromatic acids (Karas & Hillenkamp)

glycerol with addition of ultra fine cobalt powder (Tanaka)

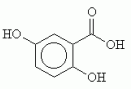
modified surface, e.g. Si - DIOS (Siuzdak), SELDI

Common Matrices for MALDI

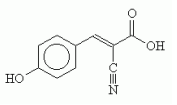
sinapinic acid (SA)
(3,5-dimethoxy-4-hydroxycinnamic acid)



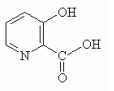
gentisic acid (DHB)
(2,5-dihydroxybenzoic acid)



α-cyano-4-hydroxycinnamic acid **(CHCA)**

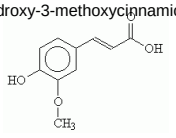


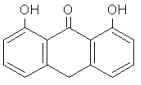
3-hydroxypicolinic acid **(HPA)**



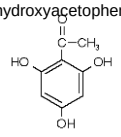
Common Matrices for MALDI

ferulic acid
dithranol (DIT)
(4-hydroxy-3-methoxycinnamic acid)

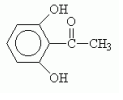




2',4',6'-trihydroxyacetophenone

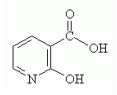


2'-6'-dihydroxyacetophenone **(THAP)**

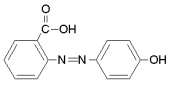


Common Matrices for MALDI

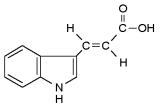
nicotinic acid-N-oxide



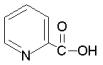
2,-(4-hydroxy-phenlyazo)-benzoic acid **(HABA)**



trans-3-indoleacrylic acid (2-pyridine carboxylic acid)



picolinic acid **(PA)**



Matrices - Applications

peptides < 10 000	CHCA, DHB
peptides, proteins > 10 000	SA, DHB
oligonucleotides < 3 kDa	THAP
nucleic acids > 3 kDa	HPA
synthetic polymers	DHB, DIT, IAA
carbohydrates	DHB, CHCA, THAP

Addition of "comatrices" (e.g. monosaccharides) may lead to improvements in crystallization, sample homogeneity, mass resolution, suppression of fragmentation etc.

Properties of Matrices

CHCA: „hot" matrix
for peptides with M < 10 000 Da
useful for PSD (structure analysis)

DHB: „cold" matrix
universal use

Lasers in MALDI

UV-MALDI

337 nm nitrogen laser (inexpensive and the most common)
355 nm Nd:YAG (3xf) yttrium-aluminum garnet
266 nm Nd:YAG (4xf)
193 nm ArF ... fragmentation!

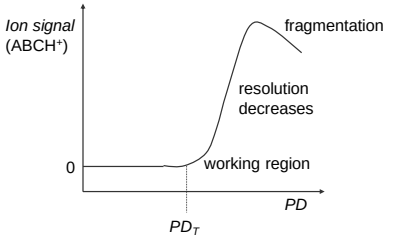
Note: YAG lasers are more expensive, but their life expectancy is much higher. The repetition rate of YAG lasers can reach kHz *vs.* Hz in the case of the nitrogen lasers.

IR MALDI

2.94 μm Er:YAG laser
10.6 μm CO_2 laser

Influence of Laser Energy

Significant dependence of the quality of MALDI mass spectra on laser energy, more exactly on power density, *PD* (power per area).



Influence of Laser Energy

Threshold power density, PD_T … minimum value of laser power per area, at which analyte peaks begin to appear in mass spectrum.

For $PD > PD_T$: signal $(ABCH^+) = k.PD^n$, kde $n = 4 – 6$.
Small variation of power leads to a large variation in ion signal of $ABCH^+$.

In practice, operator usually slowly increases energy during MALDI experiment, simultaneously moves target with sample and observes mass spectra from single laser shots. After the threshold power is established, the power is set approximately 10–30% above it and spectra are accumulated for 10-1000 laser pulses while the target is slowly being moved.

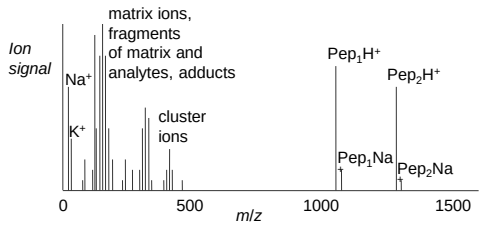
Accumulation of Spectra

Usually average of 10 – 1000 desorptions is recorded to increase signal-to-noise ratio and reproducibility

signal, $S \propto n$
noise, $N \propto \sqrt{n}$
signal/noise, $S/N \propto \sqrt{n}$

MALDI Mass Spectrum

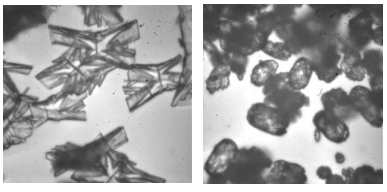
Model spectrum of 2 peptides, Pep_1 a Pep_2 with matrix M



$[ABC+H]^+$, $[ABC+2H]^{2+}$, dimer $[(ABC)_2+H]^+$
adducts with alkaline metals and matrix $[ABC-Na]^+$, $[ABC+K]^+$, $[ABC+MH]^+$
fragment ions of matrix a analyte, cluster ions, e.g. $[M_2+Na]^+$
Matrix suppression – ideally only the peaks of analytes.

MALDI Sample Preparation

MALDI sample = analyte + matrix
c(analyte) = 0.1-10 μM
c(matrix) = 1-100 mM
target: steel, Al, synthetic polymers



detail MALDI vzorku

www.srsmaldi.com

MALDI: Sample Preparation Rules

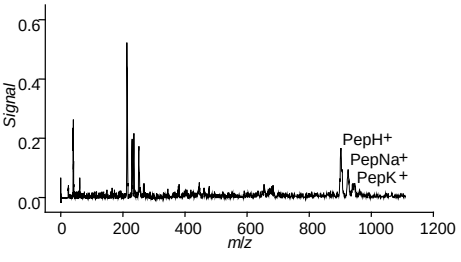
Recrystallization of matrix.
Fresh matrix solution.
Selection of proper solvent (ACN, EtOH, MetOH, acetone, water).
p*H*(matrix) < 4 (adjustment with e.g. 0.1 % trifluoroacetic acid, TFA).
Analyte has to be dissolved.
Purification of analyte prior to MALDI analysis.
Unknown analyte – preparation of a series of solutions with concentration of analyte in wide range.
Deposited samples are usually stable and can be stored (archived)
Thoroughful cleaning of the target

MALDI Sample Preparation Techniques

dry droplet – deposit of mixed solution, dry at room temperature
quick & dirty – mix solutions on target, dry at room temperature
vacuum drying – speed up drying using reduced pressure
fast evaporation – first deposit matrix layer in a volatile solvent
overlayer – matrix layer, then layer of analyte with matrix
sandwich – layers: matrix, analyte, matrix
crushed crystals – matrix crystals crushed and new sample sol'n deposited
acetone redeposition – dissolve dried sample in acetone droplet and dry
spin coating – deposition on rotating target
slow crystallization – slow growth of crystals
electrospray deposition – electrospray aerosol sprayed on target
modified targets – for concentration and more reproducible crystallization often using hydrophilic/hydrophobic interface

Influence of Salt Content

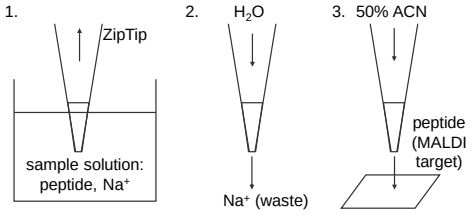
MALDI MS of a peptide sample in the presence of Na and K salts



Sample desalting necessary (not a rule, sometimes ionization based on cationization with Na^+, K^+, Ag^+ ...)
Presence of salts ⇒ adduct formation ↑, sensitivity ↓

Reduction of the influence of salts

- segregation on target ... selection of proper crystal for desorption
- addition of acid (TFA, HCOOH, HCl), NH_4 salts
- target washing (salts are more soluble in water)
- catex on target
- desalting prior to deposition: separation, dialysis, ZipTip (C_{18}) desalting



MALDI Characteristics

+ one of the most ionization techniques applied in mass spectrometry of biopolymers (together with ESI)

+ soft ionization

+ simple spectra, usually $z = +1$ or $z = -1$ (for analytes with electronegative groups)

+ pulse ionization (predestined for coupling with TOF mass analyzers)

+ detection limits ~ amol (for small peptides - best case)

+ fast sample preparation and analysis

MALDI Characteristics

- uneasy quantitative analysis (inner standard needed)

- search for the right spot on the target

- not useful for low-mass analytes due to intensive background in the region of low *m/z* (matrix, fragments and matrix clusters)

- mutual ion signal suppression

MALDI Perspective

MS analysis of large series of biological samples

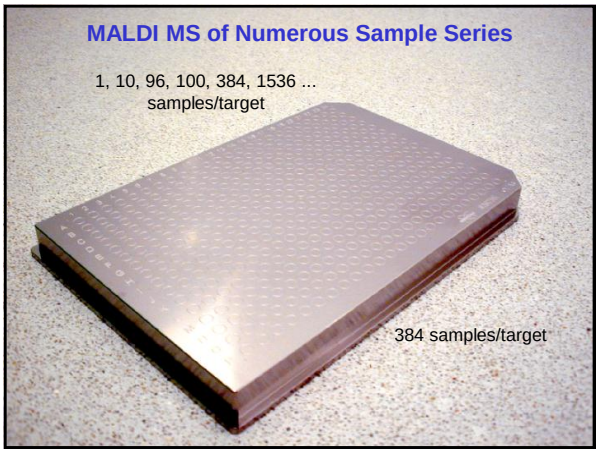
- peptide mapping for identification of proteins
  (MALDI MS of products enzymatic protein digests)
- peptides, proteins, oligonucleotides, saccharides

Micro methods

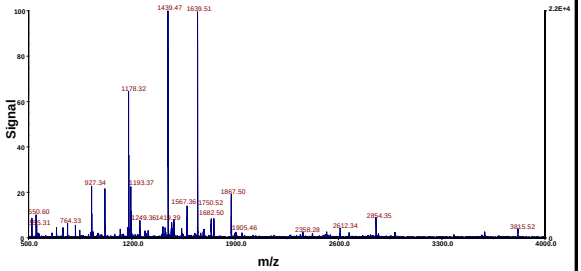
Coupling with separation techniques

- advantage of sample archiving on MALDI target
- recent availability of MS/MS spectrometers for MALDI
- complementar to ESI (various ionization efficiency for various analytes)

MALDI MS of Numerous Sample Series



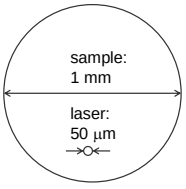
MALDI MS of a Digest: Identification of BSA



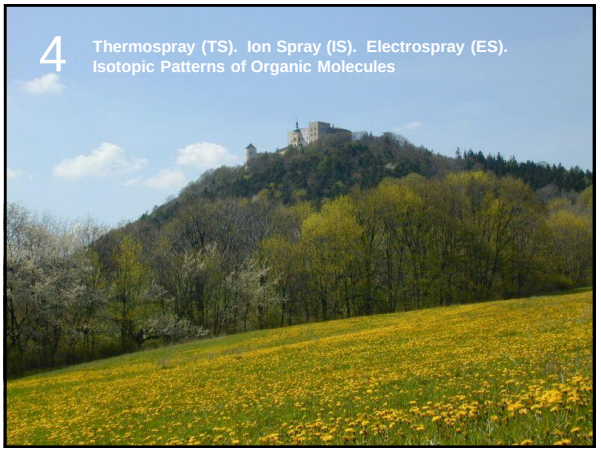
Analyte: 1 pmol of tryptic digest BSA, desalted using ZipTip$_{C18}$
Matrix: 10 mg/ml αCHCA in ACN/0.1% TFA : 70/30
Sample preparation: dried-droplet.
MS: Voyager DE-STR

Micro Methods

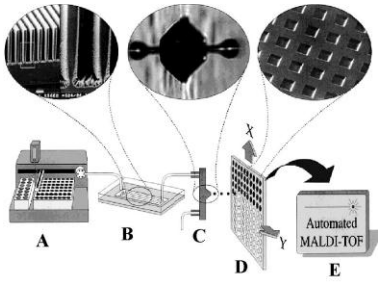
- microtargets, piezoelectric pipetors, deposition from a capillary outlet
- size of laser focus ≈ size of sample ⇒ maximum sensitivity
  high sample density on target



$$\frac{S_{laser}}{S_{sample}} = \frac{50^2}{1000^2} = 0.25\%$$



Micro Methods



(A) sample processing and injection
(B) reactor with immobilized enzyme
(C) micropipetor
(D) nanovials (300 x 300 x 20 μm) on MALDI target
(E) automated MALDI-TOF MS

(Ekstrom, S., Onnerfjord, P., Nilsson, J., Bengtsson, M., Laurell, T., Marko-Varga, G. *Anal. Chem.* **2000,** 72, 286-93)

On-Line Ionization Techniques
Atmospheric Pressure Ionization
(also spray ionization techniques)

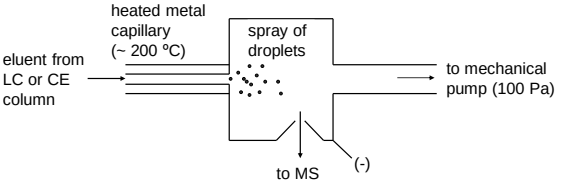
Common attributes

- Analyte: polar, often ionized and dissolved in solution.
- Heated capillaries and other elements of ion source (hundreds °C)
- Additional elements to increase ionization efficiency (electron beam, electric arc)
- Differential pumping
- Often after separation ⇒ 2D separation (MS as the second dimension).
- Ionization process stages:
  1) formation of aerosol droplets
  2) solvent vaporization
  3) ion analysis

Comparison of LDI and MALDI

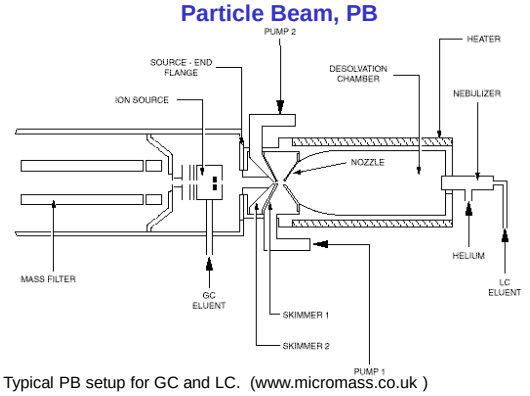
	LDI	MALDI
Ionization	relatively hard	soft
Sample	only analyte	analyte in excess of matrix
Max. *m* (Da)	<10 000	10^6
Typical analyte	small organic molecules, small peptides, synthetic polymers	peptides, proteins, DNA, saccharides, synthetic polymers

Thermospray, TS



- Solution boils in the capillary tip, droplets are generated, then dry aerosol and finally MH^+ ions. Spectra similar as in the case of CI, molecular ions prevail. Electrodes may be inserted into the source to further increase ionization efficiency.
- Electronegative compounds may form negative ions. (An electron source can be inserted into the chamber to promote formation of negative ions M^-.)
- Use of volatile compounds, such as NH_4Ac, to prevent capillary tip clogging.

Particle Beam, PB



Typical PB setup for GC and LC. (www.micromass.co.uk)

Particle Beam

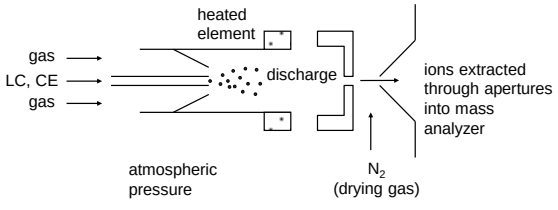
Principle

- Derived from the generator of monodisperse aerosol (R. C. Willoughby, R. F. Browner, *Anal. Chem.*, *56*, 1984, 2626-2631).
- Similar to TSI and heated nebulizer. Additional beam of particles, usually He. Separator of He atoms from ions.
- Additional EI source may be used to increase ion production. Results are EI spectra (with higher noise due to presence of ions and molecules of solvent).

Characteristics

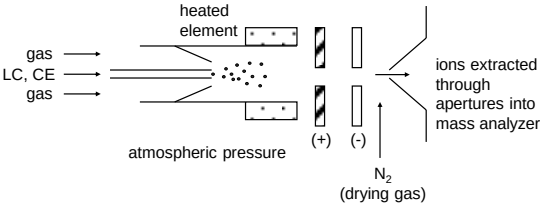
- Spectra similar as in the case of TS. More fragments.
- Less sensitive than TS and ESI.
- Useful for thermally stable, nonionic compounds with medium mass.

Heated Nebulizer



- Spectra similar to CI spectra, usually capture of 1 proton ($[M+H]^+$).
- Low fragmentation (soft ionization).
- Suitable for molecules with medium masses (~2000 Da).
- Structure? ... Additional collision cell needed (MS/MS).

Ion Spray, IS

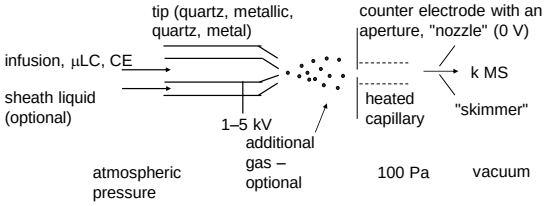


- Only positive ions penetrate through electrodes, negative are removed.
- Formation of multiply charged ions $[M+H]^+$, $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+4H]^{4+}$...
- Packed LC columns with a splitter, micro columns, capillary columns.

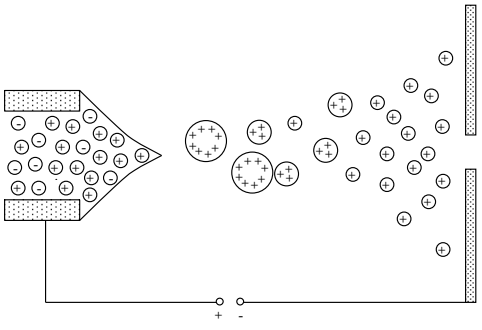
Electrospray (ESI)*, Nanospray+

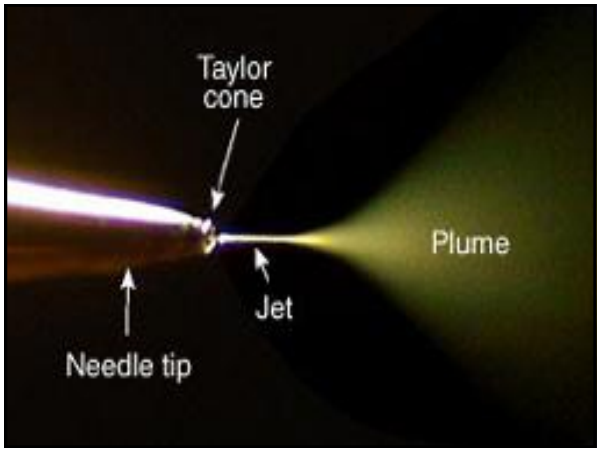
* Yamashita, M; Fenn, J. B. *J. Phys. Chem.* **1984**, *88*, 4451.
* Yamashita, M; Fenn, J. B. *J. Phys. Chem.* **1984**, *88*, 4671.
+ Wilm, M. S.; Mann, M. *Int. J. Mass Spectrom. Ion Processes* **1994**, *136*, 167.

Schematics



ESI Principle





ESI Arrangement

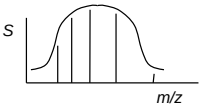
- **Arrangement of needle and aperture (nozzle)**
  ... separation of ions from ballast
  - on-axis
  - off-axis
  - diagonal (tilted) and orthogonal
  - Z-spray
- **Voltage connection**
  - through additional liquid (sheath flow)
  - liquid junction
  - metallic or metallized needle tip (sheathless interface)

ESI Principle

- Formation of Taylor cone in electric field. Concentration of positive charge in the cone, destabilization of the meniscus and emission of droplets with excess of positive charge.
- Volume reduction and increase of surface charge density of the droplets due to solvent evaporation.
- Unsymmetrical fission of charged droplets (Rayleigh stability limit); original droplet loses ~15% of charge, but only 2% of volume.
- Droplet size: μm → nm. Number of charges in a droplet: $10^5 \rightarrow 10$. (Note: Size of a macromolecule ~ nanometers.)
- Formation of secondary ions in gaseous phase, secondary reactions in gaseous phase.
- Ion transfer into the mass spectrometer.
- No discharge; discharge is not desirable.

ESI Characteristics

- Very soft ionization suitable for biomolecules.
- Very high mass limit, *M.W.* ~ 10^6 (*m*/*z* much lower). Ionization of heavy polymers (also virus particle).
- Generation of multiply charged ions
  Bell-shaped envelope. Typical for ion spray and electrospray.
  The gaps between adjacent peaks are not equidistant (in contrast to isotope pattern).



Example:
M.W. = 10 000 Da

z	4	5	6	7	8	9	10
m/*z*	2501	2001	1668	1429	1251	1112	1001

ESI Arrangement

- Additional (curtain) gas – N_2 stream, heated capillary behind the entrance aperture: better desolvation, reduction of cluster formation.
- Optional coaxial stream of liquid through an additional capillary.
- Needle: *i.d.* < 100 μm, *o.d.* 100 μm – 1 mm, *tip* < 100 μm.
- Distance tip – counter electrode: 1 – 3 cm. Flow rate < 10 mL/min.
- Nanospray: smaller dimensions,without additional coax. liquid and forced flow, flow rate < 100 nL/min.

Generation of Multiply Charged Ions

+ Higher *z* reduces *m*/*z* ⇒ mass analyzer with lower *m*/*z* limit can be used for ions with very high *M.W.*
+ Two adjacent $[M+nH]^{n+}$ peaks sufficient for determination of *m* and *z*:
  $(m/z)_n = (m+n)/n$
  $(m/z)_{n+1} = (m+n+1)/(n+1)$
+ Alteration of number of charges, *z* ?
  - pH (solution): pH↓ ⇒ *z*↑
    pH↑ ⇒ *z*↓ or even negative charge promoted
  - β^- emitter or other e^- source (electron capture ⇒ *z* ↓)
- MS of mixture more complex (but can be evaluated). Single compounds are present in several forms and give several peaks in the spectrum ... complex spectra, reduced sensitivity.
- Satellite peaks, e.g adducts $[M+Na]^+$, $[M+K]^+$ etc.

ESI

- Application of electric field (nozzle-skimmer) ⇒ fragmentation in source.
- For structure analysis – additional chamber for collision dissociation after the first mass analyzer.
- ESI signal dependent on analyte concentration, *c*(analyte); at very low concentrations dependent on the amount of analyte (nanospray).
- Signal α *c*(analyte) (10^{-7} – 10^{-3} M), it reaches plateau at higher *c*(analyte).
- ESI closely related to other API:
  e.g. **APCI** (Atmospheric Pressure Chemical Ionization)
  (solvent acts as a reagent gas ... ionization similar to CI)
  - heated needle
  - zero voltage on needle
  - additional elements:
    - electrode for discharge in front of the entrance nozzle
    - piezoelectric element for better nebulization
    - nebulizer (ion spray sometimes called pneumatic ESI)

Factors Influencing ESI

- Type of analyte
- Needle, spray tip (dimensions, arrangement)
- Voltage between needle and counter electrode
- Solution composition (solvents, additives, salts, ion-pair reagents)
- Flow rates of sample, sheath liquid and drying (curtain) gas
- Temperature of the entrance capillary

III. Mass Analyzers

- **Ion optics basics. Simulation of ion movement**
- **Energy analyzer**
- **Mass analyzers**
- **Detection of ions and data acquisition**
- **Vacuum techniques**
- **Coupling of separation to MS. Microfabricated devices**
- **New techniques/instrumentation**

ESI – Some Rules

- Use volatile buffers:
  - CH_3COOH
  - HCOOH
  - TFA (trifluoroacetic acid)
  - NH_4^+ salts of volatile acids
- Keep salt concentration < 20 mM
- Avoid use of sulfates, phosphates etc.
- Orthogonal or Z spray may partially help in the cases the rules mentioned above cannot be applied.
- For positive ionization, pK_a (electrolyte) < pK_a (analyte) – 2
- For negative ionization, pK_b (electrolyte) < pK_b (analyte) – 2
- Proper sample preparation = easier analysis
  desalting, removal of surfactant and other contaminants

Ion Optics

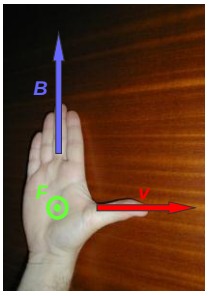
Analogy with light optics

slit	slit
lens	lens
prism, grating	W_{kin}: energy analyzer, deflector m/z: mass analyzer
mirror	ion mirror
optical fiber	ion guide

Differences from light optics

wavelength, λ	kinetic energy, W_{kin} mass/charge, m/z
refraction index (const.)	electric or magnetic fields (tunable, can be altered even during experiment)
Intensity-independent	space charge effects – mutual ion repulsion

Lorentz and Coulomb Forces



Lorentz force at magnetic induction B:

$$\vec{F}_{mag.} = ze\,(\vec{v} \times \vec{B})$$

Coulomb force at el. field intensity E:

$$\vec{F}_{el.} = ze\vec{E}$$

Total:

$$\vec{F} = ze\,(\vec{E} + \vec{v} \times \vec{B})$$

For simplicity only:

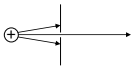
$$F_{el.} = zeE$$

$$F_{magn.} = zevB$$

Elements of ion optics

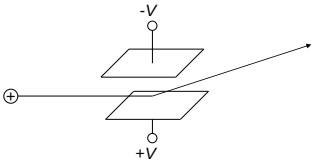
Slit

- restriction of ion beam
- restriction *vs.* transmission

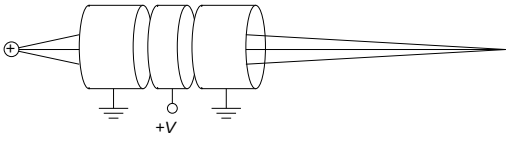


Deflector

- deflection of ions
- 2 deflectors for x, y scans

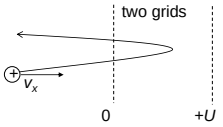


Ion Lens (Electrostatic, Einzel Lens)



- analogy of classical lens
- focal length may be changed by variation of the voltage V
- higher transmission compared to slit
- focal length is not a function of m/z (for ions accelerated in the same ion source). The lens is ideal only for ions with the same kinetic energy and at reasonable numbers (no space-charge effect)
- the schematic above shows only one of the possible arrangements

Ion Mirror (reflector, reflectron)



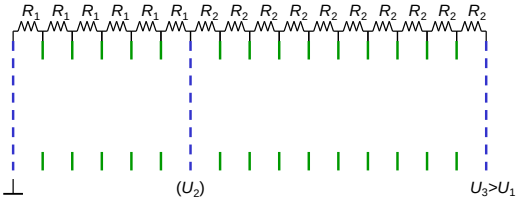
Kinetic energy *vs.* potential energy in electric field:

1) $\frac{mv_x^2}{2} < zeU$ *ion mirror*: ion returns with the same velocity as the velocity, at which the ion entered the mirror

2) $\frac{mv_x^2}{2} > zeU$ *energy filter*: ions with sufficient velocity (energy) penetrate through the second grid … mirror with a partial transmission

Ion mirror: with grids or gridless, with one or two stages, nonlinear fields.
Use: e.g. for correction of initial energy dispersion in TOF MS.

Construction of Dual Stage Ion Mirror



grids: definition of equipotential planes
rings: shielding of the ground potential (vacuum apparatus walls)
potential at the rings defined using a series of resistors and U_3
U_1 ... acceleration voltage

Ion Characteristics

Characteristics of a single ion

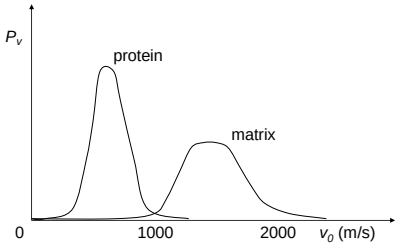
m/z	mass/charge
v	velocity (kinetic energy, $W = mv^2/2$)
t	time
x, y, z	coordinates
α	angle (... direction)
t_0	time of ion formation (index$_0$ … ion formation)

Characteristics of ion groups

(usually for the ions of the same type, i.e. the same m/z)
… described by dispersions of v (W), x, y, z, t_0, α

Ion Properties

Example: Initial velocity dispersion at MALDI
(P_v … fraction of ions moving at velocity interval <v, $v+\Delta v$>)



Simulation of Ion Movement

- Exact calculation of ion movement complex even for relatively simple electric and magnetic fields.
- Simulation of ion movement: program **Simion**

Development of ion optics using the program Simion:

1. Input of geometry (schematics of electrodes).
2. Input electrode potential, define magnetic field.
3. Definition of ions (number n, v_0 , x_0, y_0, z_0, α_0, ϕ_0).
4. Movement simulation $\Rightarrow$ result (graphical representation, text).

Simion

Example: Simulation of electrostatic lens using program Simion.

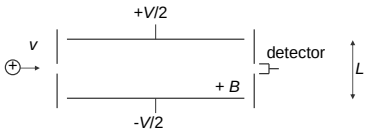
Lens: 3 segments
diameter, d = 36 mm
length of segments, y_1 = 28 mm, y_2 = 26 mm, y_3 = 32 mm
gaps between segments, l = 2 mm
ion origin in xyz [0, 0, 0] mm
potentials, U_1 = 0; U_2 = tunable; U_3 = 0

Ions: number, n = 5
mass, m = 100
kinetic energy, W_0 = 100 eV
coordinates xyz [0, -30, 0] mm
$\alpha_0(i)$ = (-4 + 2i)° , where i = 0 … n – 1

Aim: Verify function of the lens for voltage of the middle ring, U_2 = 0, 85, 100, 120 a 133 V.

Solution: Presented in the lecture

Wein Velocity Filter



- Transverse electric field between two flat electrodes
- Transverse magnetic field
- Electric intensity field vector E is perpendicular to the vector of magnetic induction B
- Ions are drawn by electric and magnetic forces with opposite direction

Wein Velocity Filter

For ion flying on axis of the filter:

$$zeE = zevB \qquad \left(E = \frac{V}{L}\right)$$

$$\boxed{v = \frac{E}{B}}$$

... only ions with specific speed pass through the filter.

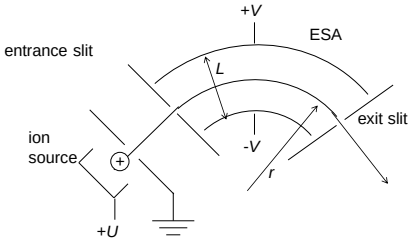
In case all ions were accelerated by voltage U:

$$\frac{mv^2}{2} = zeU$$

$$\boxed{\frac{m}{z} = \frac{eB^2}{E^2}2U}$$

… Wein filter can be used for mass analysis (m/z).

Energy Analyzer; Electrostatic Analyzer (ESA, E)



Energy Analyzer

Acceleration: $\frac{mv^2}{r} = zeE$, where $E = \frac{2V}{L}$.

Energy: $\frac{mv^2}{2} = zeU$

Radius of curvature:

$$r = \frac{2U}{E}$$

- Radius of curvature is directly proportional to ion kinetic energy.
- Not a function of *m/z*.
- Even thick ion beam (parallel ion trajectories) are focused.
- Using electrodes curved also axially, ions can be focused in two dimensions.
- Sector – shape of a slice, a portion of a circle

Mass Analyzers

Magnetic sector (MAG, B)

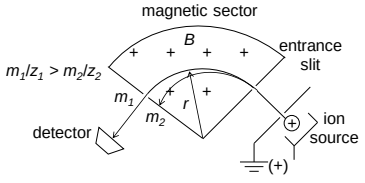
Quadrupole analyzer (Q, q)

Ion cyclotron (ICR-FT-MS)

Ion trap (IT), Linear trap (LT)

Time-of-flight (TOF)

Magnetic Sector (MAG, B)



Vector of magnetic induction *B* is perpendicular to the velocity vector of ions streaming from the ion source into the sector.

Principle of Magnetic Sector

1.) Lorentz force = centrifugal force:

$$Bzev = \frac{mv^2}{r}$$

$$\frac{m}{z} = \frac{eBr}{v}$$

2.) Kinetic energy = acceleration energy:

$$\frac{mv^2}{2} = zeU$$

$$\boxed{\frac{m}{z} = \frac{eB^2r^2}{2U}}$$

Principle of Magnetic Sector

$$\boxed{\frac{m}{z} = \frac{eB^2r^2}{2U}}$$

Scan types:

- Scan ***U***, const. *B*. Problems with low extraction efficiency at low values of *U*.
- Scan ***B***, const. *U*. Difficult originally, prevailing nowadays.
- Move the exit slit, ***r***. Const. *U*, *B*. Usually not used.

Peak switching

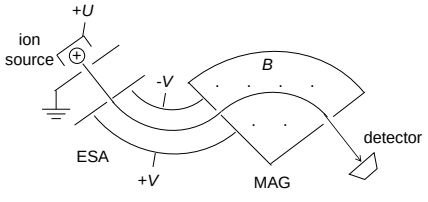
Stepwise change of one of the variables (*B*, *U*). Suitable for monitoring of limited number of ion types.

Tandem Electrostatic Analyzer – Magnetic Sector

Double focusing mass spectrometer

Forward-geometry, ESA-MAG, EB

1. energy analyzer: selections of ions with a certain kinetic energy
2. magnetic sector: mass analysis



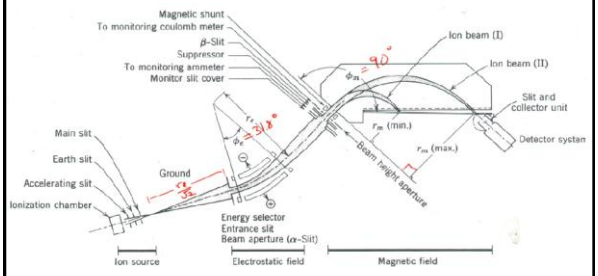
Tandem EB (ESA-MAG)

- Dispersion of ion characteristics (v, x, α) and instability of fields (B, U) reduce quality of spectra.
- Placing ESA prior to MAG solves problem with velocity (kinetic energy, W_{kin}) dispersion of ions entering into mass sector.

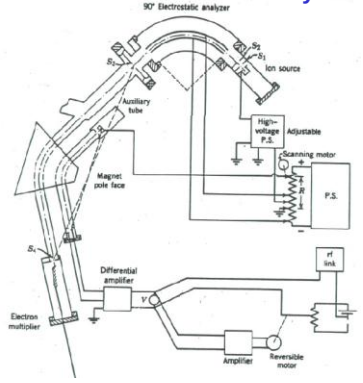
Practical EB geometries:

1. **Nier-Johnson**
   90º ESA + 60º MAG
   exit focused for given radius, r
   suitable for scanning spectrometers
2. **Mattauch-Herzog**
   31.8º ESA + 90º MAG
   single focal plane for ions with different m/z
   suitable for planar detectors (photographic plate, array)
3. **Matsuda**
   suitable for compact instruments

Mattauch-Herzog Geometry



Nier-Johnson Geometry



Other Combinations of E and B

Reverse-geometry, BE

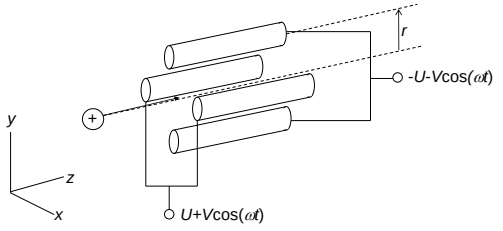
1. magnetic sector: mass filter
2. energy analyzer: sorting of ions according to kinetic energy

MIKES (Mass-Analyzed Ion Kinetic Energy Spectrometry)
the first MS/MS technique, tandem mass spectrometry (1973)

1. Magnetic sector allows passing of only ions with certain m/z.
2. Metastable ions may undergo decay in the region between magnetic and energy analyzer.
3. Daughter ions from the decay are sorted according to kinetic energy in the energy analyzer.

A variety of **hybrid instruments** based on E and B: EBE, BEB, EBEB, BEBE ...

Quadrupole Filter (Q, q)



4 rods with hyperbolic (also round, square or other) cross-section
U ... DC voltage component
V ... AC (RF) voltage component
ω … angular frequency, $\omega = 2\pi f$, f = 1 - 3 MHz, phase shift 180º

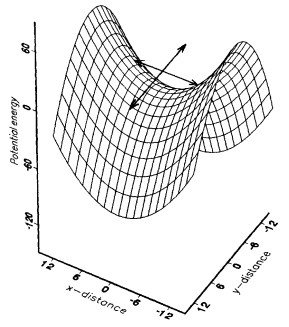
Quadrupole Filter

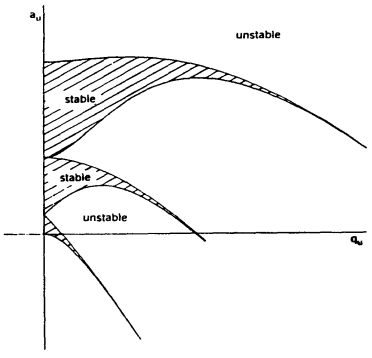
xz plane: heavy ions pass
yz plane: light ions pass

... only ions with a specific m/z will pass through the quadrupole filter, all other ions are deflected.

Note: important exception is m/z-unselective RF quadrupole

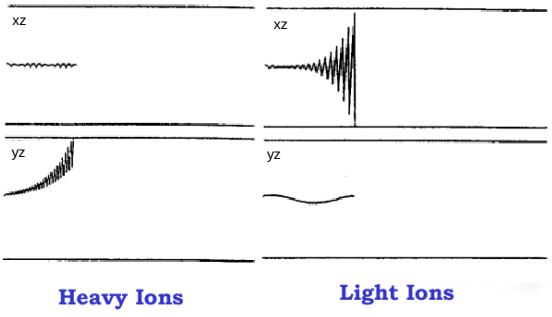
Quadrupole field

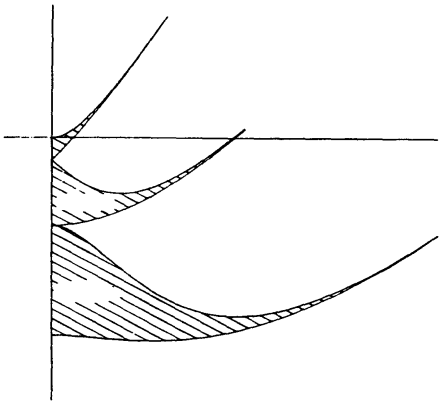




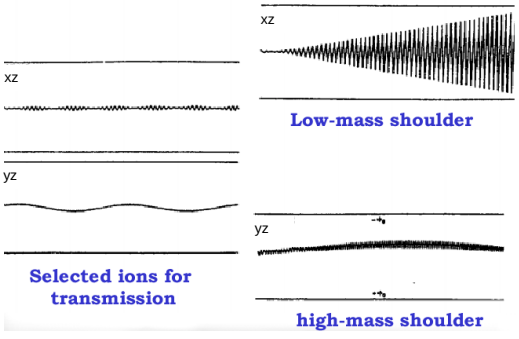
Graphical representation of stable solutions of the Mathieu equation plotted in (a,q) space.

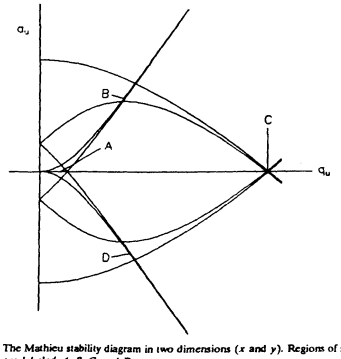
Ion Trajectories in Quadrupole Filter





Ion Trajectories in Quadrupole Filter

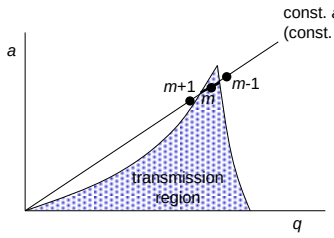




The Mathieu stability diagram in two dimensions (x and y). Regions of simultaneous overlap are labeled A, B, C, and D.

Stability Diagram

... graphic representation of the solution of the Mathieu equations, which describe trajectories of ions in the quadrupole filters.

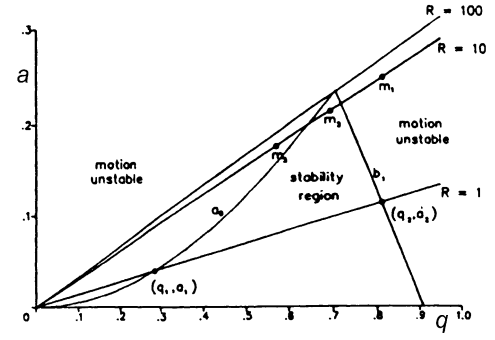


$$a = \frac{8eU}{\left(m/z\right)\omega^2 r^2}$$

$$q = \frac{4eV}{\left(m/z\right)\omega^2 r^2}$$

Resolution

… is determined by the value (a/q) of the slope of the scan line



Properties of Quadrupole Filter

MS scan

r a f usually const.; U a V scanned simultaneously *at* const. U/V.

Upper *m/z* limit: 2 000 - 4 000.

$$\max\left(m/z\right) \sim \frac{0.316V}{r^2 f^2} \quad [\text{V, cm, MHz}]$$

To increase the upper m/z limit: $V\uparrow$, $r\downarrow$, $f\downarrow$.

Properties of Quadrupole Filter

To increase resolution: (Resolution, $R < 10\,000$, usually $R < 2\,000$.)

- For acceleration voltage, U_{acc} (towards the quadrupole):

$$\frac{m}{z} = \frac{2eU_{acc}}{v^2} \qquad v = \frac{l}{t}$$

$$t = \sqrt{\frac{l^2 \frac{m}{z}}{2eU_{acc}}}$$

For given quadrupole length, l, the voltage U_{acc} must be low enough to enable sufficient number of ion oscillations, tens to hundreds at the given frequency, ω, during the time of flight, t, through the filter.

- Better manufacturing, very low mechanical tolerances.

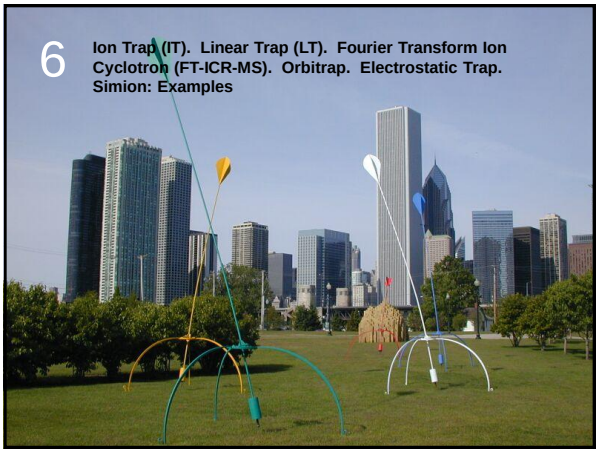
Quadrupole Filter - Notes

External source

- fringe field, especially at the entrance
  - responsible for unstable trajectories; significant fraction of ions does not get inside
  - mass discrimination of heavy ions, which spend longer period in the fringe field, are more influenced
- solution
  - entrance electrostatic lens
  - entrance RF quadrupole (q, RF only), hexapole or octopole
    ... only RF component: $a = 0$, $U = 0 \Rightarrow$ unselective filter, through which ions of all *m/z can pass*

Triple quadrupole (QqQ)

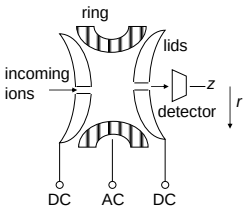
- popular tandem mass spectrometer for collisional dissociation
- middle quadrupole (q, RF only) serves as a collision chamber

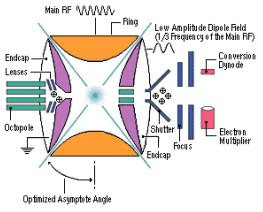


Quadrupole Ion Trap (Ion trap, IT)

1953 Wolfgang Paul (Paul trap) – almost unnoticed
1983 Finnigan – commercial instrument (one of best selling MS's)

Schematics of ion trap

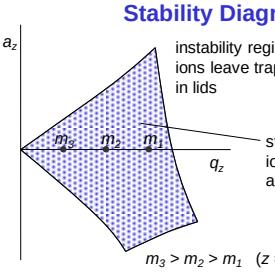




DC: constant voltage or ground
AC: constant + alternate (RF) component, $U + V\cos(\omega t)$

Bruker, Model ESQUIRE-LC

Stability Diagram (Ion Trap)



instability region:
ions leave trap through apertures in lids

stability region:
ions oscillates inside trap, they are trapped (storage mode)

$m_3 > m_2 > m_1$ ($z = 1$)

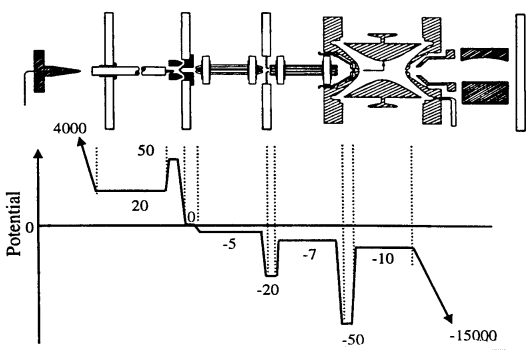
$$a_z = \frac{16eU}{(m/z)\omega^2 r^2}$$

$q_z \propto V/(m/z)$ for movement along z axis ($a = 0$, $U = 0$)

$$q_z = \frac{8eV}{(m/z)\omega^2 r^2}$$

Scan: by increasing V. First lighter, then heavier ions are expelled from the trap. (Also resonant ejection helps to improve resolution.)

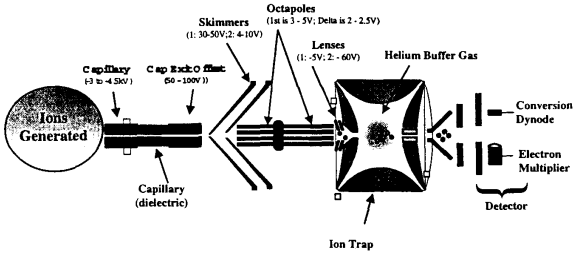
Potential Energy Diagram for LCQ Deca



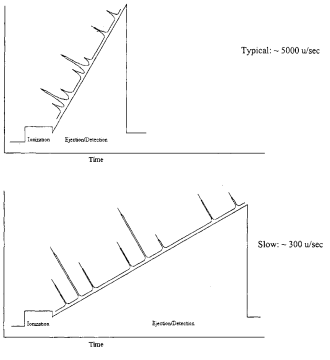
Ion Trap

- **Experiment stages:** ionization, accumulation, scan a detection.
- **Accumulation**: ions are being trapped even during ionization pulse – the result is very low detection limits. The total time of experiment depends on following requirements:
  Sensitivity ... Longer accumulation means higher sensitivity.
  Scan time ... Wider *m/z* range, higher *m* means longer scan (see next page).
  Resolution ... Longer scan means slower (and longer) scan.
- **Resonance ejection**
  of ions of specific *m/z* by application of RF voltage on trap lids. A hole in the stability diagram is created. It is used to increase resolution even in regular scan mode.
  (1983, G. Stafford: "mass selective instability mode")
- **Buffer gas** (low mass gas: He, 1 mTorr)
  Much better results than with analyte itself.
  Collisions of analyte with buffer gas molecules lead to better distribution of energy among analyte ions and keeps ions in the center of the trap. Advantageous in GC-MS.

LC MSD Ion Trap: Ion Path



Increased Resolution by Decreasing the Scan Rate



Resolution Reference:
J. C. Schwartz, J.E. P. Syka, I. Jardine, J. Am. Soc. Mass Spectrom., 2 (1991) 198-204.

Ion Trap

- **MS/MS** – trap can replace tandem of two mass spectrometers

  CID, CAD: collision-induced (activated) dissociation

- **High *R*** … < 10 000, usually ~ 5 000

- **Upper mass limit**, m/z_{max} ~ 70 000 (resonance ejection)

- **Miniaturization**: ion micro trap, trap on a chip (also quadrupole filter)
  + total size only 1 cm
  + useful in space exploration
  - much lower mechanical tolerances, demanding manufacturing
  - low parameters (R, m/z_{max})

Ion Trap

- **Ion transmission**
  only half ions may be detected

- **Physical restrictions**
  dimensions, voltage and frequency limited.

- **Maximum dynamic range** (10^6 for single ion type) limited:
  - minimum by sensitivity (usually 10; even 1 ion may be detected)
  - maximum by space charge effect: for number of trapped ions higher than 10^6 the trap does not function properly – due to mutual ion repulsion. Note that the charge limit involves sum of charges of all ion types!

Linear Trap

Linear trap or "2D trap" is based on quadrupole ion filter/ion guide. The previously described ion trap is sometimes called "3D trap".

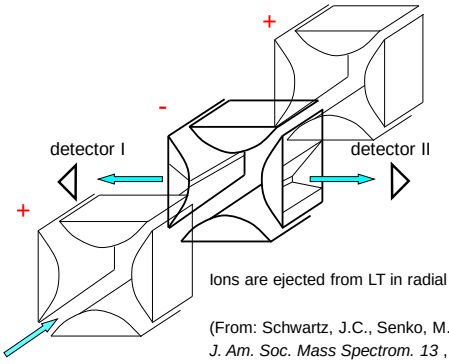
Ions are injected into the quadrupole and trapped by elevating potential on lids. (Ions oscillate in a potential well of RF-only quadrupole.) Later the ions can be gradually ejected and detected through the rods or lids.

Two approaches of ion ejection from LT:

A. Schwartz, J.C., Senko, M.W., Syka, J.E.P., *J. Am. Soc. Mass Spectrom. 13* , **2002**, 659.

B. Hager, J.W., *Rapid Comm. Mass Spec. 16*, **2000**, 512.

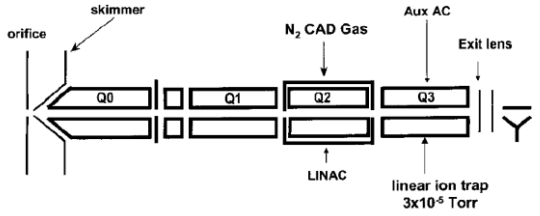
Linear Trap with Radial Ejection



Ions are ejected from LT in radial direction.

(From: Schwartz, J.C., Senko, M.W., Syka, J.E.P., *J. Am. Soc. Mass Spectrom. 13* , **2002**, 659.)

Linear Trap with Axial Ejection



Ions are ejected from the LT (Q3) along quadrupole axis.

(From: J. C. Y. Le Blanc *et al. Proteomics 3,* **2004**, 859-869.)

Advantages of 2D Ion Traps

Capture Efficiency: 50-75% (3-D: 5 % - 20%, and depends on mass)

Extraction Efficiency: 25-50% (3-D : 20%)

Sensitivity increased by a factor of 5-10

Ion Capacity: 20 - 30 times larger than 3-D

Linear range increased more than 2 orders, up to 10^6

Resolution: ~10 000 at scan rate 300 a.m.u./sec

Wider variety of ion handling (accumulation, scans...)

Differences b/w Quadrupole Filter and Traps

Quadrupole filter

- no ion storage
- only ion with certain *m/z* can pass through a filter at a time

... scanning instrument, loss of ions and hence lower sensitivity
(except in single ion monitoring)
... no space charge effects, hence higher dynamic range

Comparison of Quadrupole Analyzers

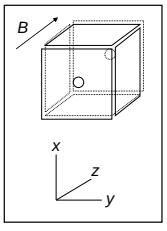
	QIT	QQQ	LQIT
Sensitivity	++	-	++
Dynamic range	-	++	+
Mass range	-	+	+
MS^3	++	-	++
Neutral loss scan/Precursor scan	-	+	+
m/z accuracy	+	-	+
Resolution	+	-	+

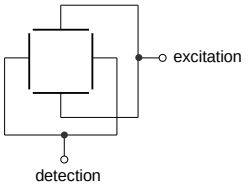
Ion Cyclotron (FT-ICR-MS)

(Fourier Transform - Ion Cyclotron Resonance - Mass Spectrometer)

…another type of ion trap

Cubic trap consisting of two pairs of electrodes and two lids

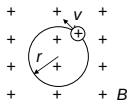




FT-ICR-MS

Ion movement in magnetic field:

$$Bzev = \frac{mv^2}{r}$$



Angular (cyclotron) frequency:

$$\omega = \frac{v}{r} = \frac{Be}{\left(m/z\right)}$$

FT-ICR-MS Characteristics

- (superconducting) magnet with very high magnetic induction $B \sim 10$ Tesla
- very low pressure, $p < 10^{-7}$ Pa (UHV, ultra high vacuum)
  ... high cost ~ $700 000
- limited dynamic range ~ 10^3, 100 – 10^5 ions

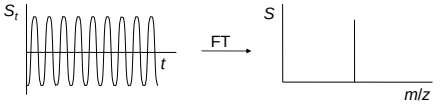
+ very high precision and accuracy, *m/z*, ~ ppm
+ very high resolution, $R \sim 10^6$
+ multiplex (Fellgett) advantage of FT … ion signal is acquired for all *m/z* during entire acquisition period ⇒ improvement of *S/N* 10^3x, reduction of acquisition period ~10^6x (FT-ICR *vs.* ICR)
+ suitable for MS^n (CID), commonly $n = 2 – 3$, even $n = 10$ demonstrated

Other geometries of cell: cylindric, hyperbolic (Penning)

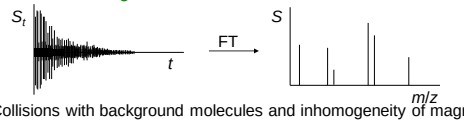
Fourier Transform

... transformation of signal from time domain to frequency (*m/z*) domain

Ideal signal of ions with single *m/z*



Real FT-ICR-MS signal



- Collisions with background molecules and inhomogeneity of magnetic field result in signal decrease. Higher p ⇒ more collisions ⇒ lower R.
- Lorentz peak shape.

FT-ICR-MS Principle

Ionization for FT MS

1. internal: analyzer = source (e.g. EI, LDI)
2. external: ionization in an external source and introduction to the cell
   - ion guides, quadrupoles, electrostatic lens
   - differentially pumped chambers (ions are formed at the first cell at higher pressure and then introduced into analyzer cell through an aperture along *z*-axis)

Excitation

1. Pulse ... high amplitude, extremely short duration
2. Chirp ... fast scan through frequencies in required interval
3. SWIFT ... Stored Waveform Inverse Fourier Transform
   profile of the excitation waveform generated by iFT of required *m*/*z* profile
   (iFT = inverse FT, transformation from frequency (*m*/*z*) to time domain

FT-ICR-MS Principle

Detection

1. nondestructive (inductive) – ions attract electrons in the detection plates during passage nearby: non-destructive detection (FT-ICR)
2. destructive – ions strike the detection plates (ICR)

Data acquisition

- Higher sampling frequency ⇒ higher upper *m*/*z* limit.
  Nyqist criterion: the highest achievable frequency = sampling frequency/2
- Longer acquisition period, *t* ⇒ higher resolution and lower *m*/*z* limit.
- Result ... necessity of storage of many data points
  - heterodyne frequency mixer (shift of signal frequency down allows to use lower sampling frequency)

FT-ICR-MS Principle

Z-trapping

- DC voltage ~1-5 V on the lids to keep ions inside (and not to leave in *z*-axis direction.
- Presence of an additional electric field causes magnetron oscillations (~10 Hz) of ions in addition to cyclotron oscillation (~ MHz). The result of combined movement are more complex calibration, peak shift and higher loss of heavy ions.

FT-ICR-MS

Resolution

$$\frac{m}{\Delta m} \alpha \frac{Bt}{\frac{m}{z}}$$

Upper *m/z* limit. From the mean quadratic speed of thermal movement,

$$v = \sqrt{\frac{2kT}{m}} \quad ,$$

which is responsible for pre-excitation of ions, it can be expressed:

$$r = \frac{mv}{zeB} = \frac{\sqrt{2mkT}}{zeB} \quad .$$

In a trap characterized by *B* and *r*, *m* of ions that can be stored is lower than

$$m = \frac{(zeBr)^2}{2kT}$$

.

New Mass Spectrometers

Mass spectrometer of future?

... not a single solution

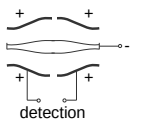
Trends:

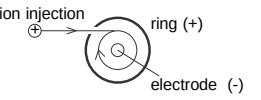
- **Withdrawal of magnetic sector instruments.**
- **Hybrid spectrometers**
  Modern spectrometers and their hybrids gain popularity (ion traps, orthogonal TOF analyzers).
- **New spectrometers?**
  Linear quadrupole traps (discussed earlier)
  Electrostatic trap "Orbitrap"
  Linear electrostatic trap

Electrostatic Trap "Orbitrap"

A. Makarov, HD Technologies Ltd., USA, *ASMS Conference* **1999**

rings (+)
electrode (-)





Principle

- Ions are injected into axially symmetric electric field and circulate around the middle electrode.
- Ion oscillations along the electrode axis are inductively detected, mass spectra are obtained after Fourier transform, *f* = f(*m*/*z*).

Orbitrap Properties

+ Very high resolution – close to FT-ICR-MS (R = 50 000).

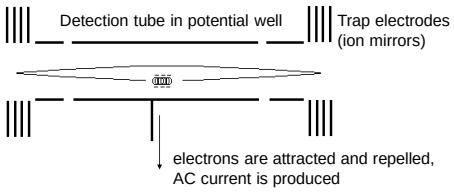
+ Very high precision and accuracy of m/z… close to FT-ICR-MS.

+ No magnet necessary.

+ No RF generators necessary.

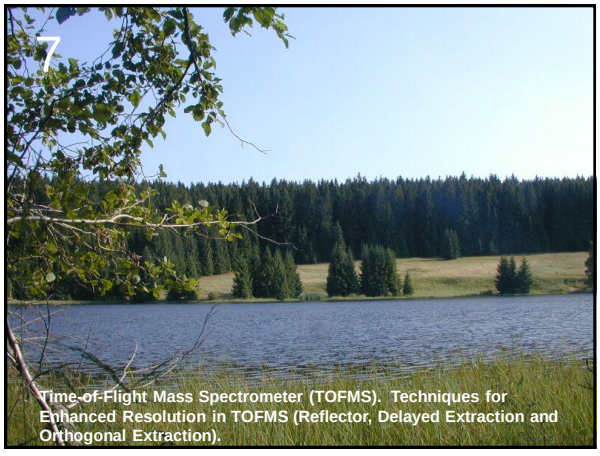
- Ultrahigh vacuum needed as in the case of FT-ICR-MS.

Linear Electrostatic Trap



Detected frequency = f(m/z)

(Benner, *Anal. Chem*. **1997**, *69*, 4162-4168)



TOFMS History

TOF = Time-of-flight
m/z calculated from the time of ion travel

1946 TOF Principle
(W. E. Stephens, *Phys. Rev.*, **1946**, *69*, 691)

1948 First instrument, „Ion Velocitron“
(A. E. Cameron, D. F. Eggers, *Rev. Sci. Instrum.* **1948**, *19*, 605)

1955 Ion source with 2 stages, time-lag focusing
(Wiley, W. C.; McLaren, I. H.; *Rev. Sci. Instrum.*, **1955**, *26*, 1150)

TOFMS History

1973 Ion mirror
(B. A. Mamyrin, V. I. Karataev, D. V. Schmikk, and V. A. Zagulin, *Sov. Phys. JETP* **1973**, *37*, 45)

1995 Delayed (pulse) extraction
(Whittal, R. M.; Li, L., *Anal. Chem.* **1995**, *67*, 1950-1954;
Brown, R. S.; Lennon, J. J., *Anal Chem.* **1995**, *67*, 1998-2003;
Vestal, M. L.; Juhasz, P.; Martin, S. A, *Rapid Commun. Mass Spectrom.* **1995**, *9*,1044-1050)

TOFMS Principle

1. Pulse formation of ions

2. Acceleration of ions
The same acceleration energy, *W(el.)* for ions with the same z.
Transformation *W(el.) = W(kin.)*

3. Drift (flight) of ions:
Separation of ions according to m/z; $W(kin.) = mv^2/2$

4. Impact of ions on detector
Acquisition of signal in time, $I(t)$

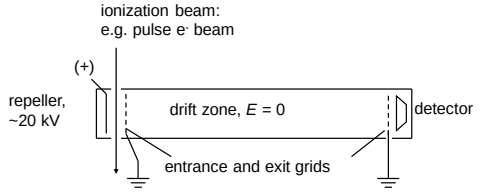
5. Determination of m/z from the time of flight
Transformation $I(t) \rightarrow I(m/z)$

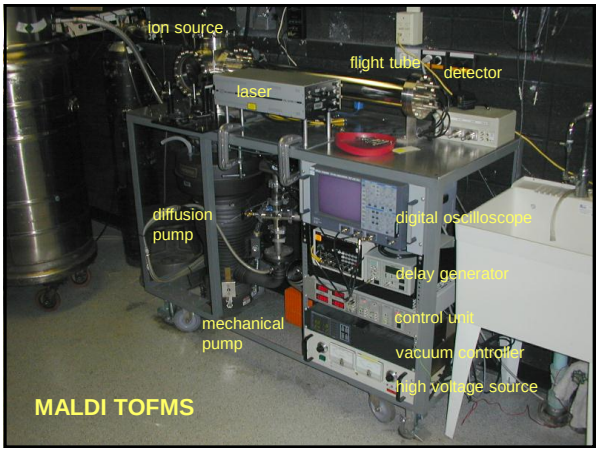
TOFMS Geometry

Linear geometry

... the simplest arrangement

... ions are not focused optimally on the detector



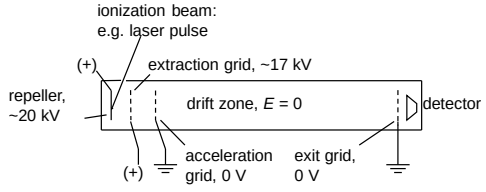


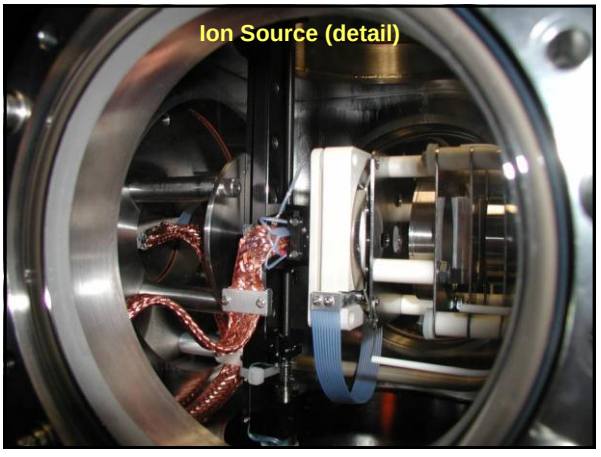
TOFMS Geometry

Wiley-McLaren linear geometry (1955)

... adjustment of the extraction electric field does not depend on the value of the total acceleration voltage

... allows focusing of ions on the detector at the end of the flight tube



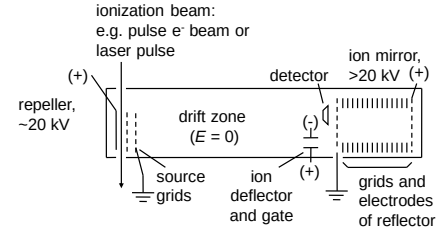


TOFMS Geometry

TOF MS with ion mirror (reflector)

... for higher resolution

... for structural analysis (PSD)

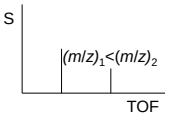


TOFMS Principle

1. Pulse ionization (pulse width ~ ns): fast generation of ion plume (LDI, MALDI, PD, EI …)
2. Extraction and acceleration of ions in electric field
3. Separation of ions in drift zone at $E = 0$ (field-free region, flight tube)
4. Detection of ions, signal acquisition and transformation to m/z domain

acceleration energy: $zeU = \frac{mv^2}{2}$ kinetic energy

$v = \frac{L}{t}$ — length of drift zone; flight time



$$\frac{m}{z} = 2eU\frac{t^2}{L^2}$$

TOFMS Principle

Exact relation includes also the time spent in in the ion source and the detector regions:

voltage: U_s 0 0 U_d

distances: s L d

$t_{TOF} = t_s + t_L + t_d$

$$t_s = \sqrt{\frac{2ms^2}{zeU_s}} \qquad t_L = \sqrt{\frac{mL^2}{2zeU_s}} \qquad t_d = \frac{d}{U_d}\sqrt{\frac{m}{2ze}}\left(\sqrt{U_s} + \sqrt{U_s + 4U_d}\right)$$

Note: *1)* t_s, t_L, t_d and hence t_{TOF} are proportional to $(m/z)^{1/2}$

2) for rough estimate t_{TOF}: $t_{TOF} \sim t_L$ ($L >> s, d$)

TOFMS Calibration

$m/z=(2eU/L^2)t^2$

⇓

$m/z=k_1(t-t_0)^2$

⇓

$t=c_0+c_1(m/z)^{1/2}$

$t=c_0+c_1(m/z)^{1/2}+c_2(m/z)$

$t=c_0+ c_{-1}(m/z)^{-1/2} +c_1(m/z)^{1/2}+c_2(m/z)$

correction of data acquisition trigger

correction of the original ion velocity before extraction pulse

correction of non-ideal extraction pulse shape

Principal Advantages of TOFMS

No theoretical upper *m/z* limit

Ideal for pulse ionization

Fellgett advantage: for each of pulses, entire spectrum recorded, no need for scanning

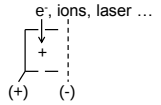
Very short duration of acquisition of a spectrum (~10^{-4} s)

High ion transmission ... a prerequisite of high sensitivity

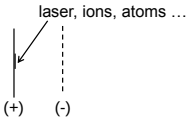
Simplicity

Ion Sources for TOFMS

1. Ion source for gaseous samples



2. Ion source for condensed samples (desorption)



Ideal Ion Source for TOFMS

All ions are created:

- in time t_0 (with period of formation, $\Delta t = 0$)
- at distance x_0 (x … flight axis of TOFMS)
  (preferably in a single point [x_0, y_0, z_0])
- with the same velocity v_{x0} (, which does not have to be zero,) along x-axis.
  (preferably $v_{y0} = 0$ a $v_{z0} = 0$)

Real Ion Source for TOFMS

Ions are characterized by initial dispersions of
time (time of creation t_0),
space (position of creation x_0, y_0, z_0),
velocity v_0 (energy W_{kin0}) and
angle α (deviation of the flight x-axis).

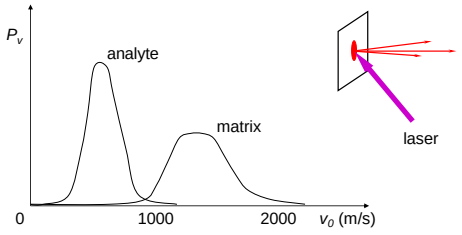
Result: drop of resolution, R

Wiley-McLaren ion source for gaseous samples:

Adjustment of voltage on the first grid allows to find a compromise between contribution of v_0 a x_0 dispersions to reach optimal resolution (ion focusing into detector plane)

Ion Properties

Example: Dispersion of initial ion velocity in MALDI
(P_v … probability of occurrence of molecules with velocity in $<v_0, v_0+\Delta v>$)



Pulse Generation of Ions

1. **Extraction using constant electric field (DC)**
   pulse ionization beam + constant extraction field

2a. **Pulsed extraction, PE (delayed extraction, DE)**
   pulse ionization beam + pulse extraction field

2b. **Orthogonal extraction**
   continuous generation (introduction) of ions + pulse extraction field

Enhancement of Resolution in TOFMS

1. **High acceleration voltage**
2. **Ion mirror.**
3. **Pulse extraction**

... important decision when buying TOFMS

1. High Acceleration Voltage

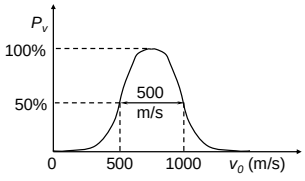
$$\frac{mv^2}{2} = \frac{mv_0^2}{2} + zeU \quad \Rightarrow \quad v = \sqrt{v_0^2 + \frac{2zeU}{m}}$$

contribution of desorption — contribution of electric field

Increase of acceleration voltage will minimize contribution of initial velocity dispersion of analyte.

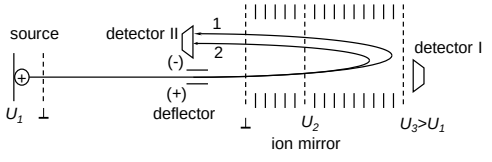
Influence of Acceleration Voltage

Př.: MALDI TOFMS of a peptide, m = 2000 Da, z = 1, v_0 = 750 m/s, Δv_0(FWHM) = 500 m/s, L = 1 m. (2 ions: v_{01} = 500 m/s, v_{02} = 1000 m/s.)



***U* = 1 kV**: t_1 = 101.673 μs, t_2 = 101.284 μs — ***R* ~ 130**
***U* = 10 kV**: t_1 = 32.190 μs, t_2 = 32.177 μs — ***R* ~ 1300**

2. Ion Mirror (reflector, reflectron)



- Ions 1 and 2 with same m/z and velocities v_1 a v_2: faster ion penetrates deeper into the ion mirror and its trajectory is longer.
- If adjusted properly, two ions will hit the detector II simultaneously.
  (B. A. Mamyrin, V. I. Karataev, D. V. Schmikk, and V. A. Zagulin, *Sov. Phys. JETP* **1973**, *37*, 45;
  Mamyrin, B. A.; Shmikk, D. V.; *Sov. Phys.* **1979**, *49*, 762)

Construction of Ion Mirror

Linear ion mirror ... *E* is constant along the ion mirror axis

Single stage ... intensity of electric field, *E* is constant in the entire ion mirror (Alikhnov)

Dual stage ... ion mirror is divided in two regions with different *E*

(B. A. Mamyrin, V. I. Karataev, D. V. Schmikk, V. A. Zagulin, *Sov. Phys. JETP* **1973**, *37*, 45)

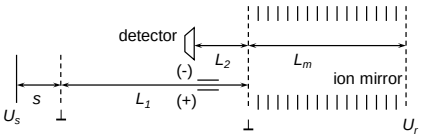
Nonlinear ion mirror ... *E* varies along the ion mirror axis

Quadratic field (D. R. Jardine, J. Morgan, D. S. Alderdice, P. J. Derrick, *Org. Mass Spectrom.* **1992**, *27*, 1077)

Curved field (T. J. Cornish, R.J.Cotter, *Rapid Commun. Mass Spectrom.* **1993**, *7*, 1037-1040)

+ additional patents (Japan, Soviet Union)

Single Stage Ion Mirror



$t_{TOF} = t_s + t_L + t_r$ ... sum of periods ion spends in source, tube and mirror

$$t_{TOF} = t_0 - \frac{v_0}{a} + \frac{1}{2}\sqrt{\frac{2s}{a}}\left(1 - \frac{L}{2s} + \frac{2a}{a_r}\right)\frac{v_0^2}{2as} - \frac{1}{8}\sqrt{\frac{2s}{a}}\left(1 - \frac{3L}{2s} + \frac{2a}{a_r}\right)\left(\frac{v_0^2}{2as}\right)^2 + \ldots$$

$$t_0 = \sqrt{\frac{2s}{a}}\left(1 + \frac{L}{2s} + \frac{2a}{a_r}\right)$$

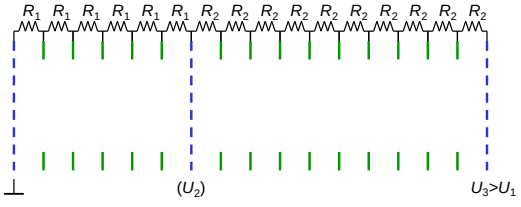
$L = L_1 + L_2$ ... drift zone, E=0

a ... acceleration in source, $a=qE_s/m$

a_r ... acceleration in mirror, $a=qE_r/m$

(Moskovets, E. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 150-155)

Construction of Ion Mirror



grids: definition of equipotential plane

rings: electric shielding of vacuum apparatus walls

voltage on the rings is defined by series of resistors

Single Stage Ion Mirror

$$t_{TOF} = t_0 - \frac{v_0}{a} + \frac{1}{2}\sqrt{\frac{2s}{a}}\left(1 - \frac{L}{2s} + \frac{2a}{a_r}\right)\frac{v_0^2}{2as} - \frac{1}{8}\sqrt{\frac{2s}{a}}\left(1 - \frac{3L}{2s} + \frac{2a}{a_r}\right)\left(\frac{v_0^2}{2as}\right)^2 + \ldots$$

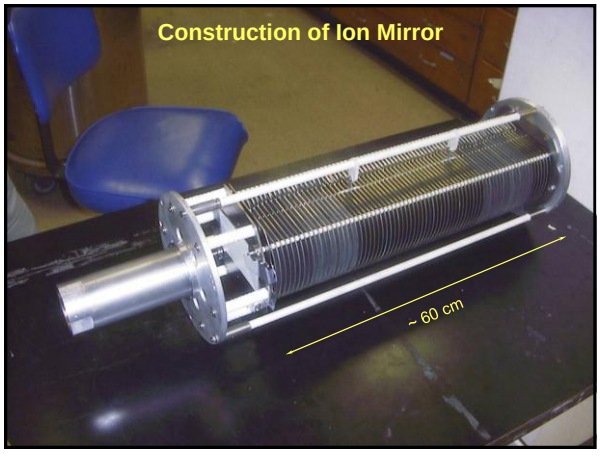
contribution of initial velocity, v_0

t_0 ... t_{TOF} ion with zero initial velocity, v_0= 0

$$t_0 = \sqrt{\frac{2s}{a}}\left(1 + \frac{L}{2s} + \frac{2a}{a_r}\right)$$

source — drift zone — mirror

Increase of *R by minimization of the 3rd term*: $1 - \frac{L}{2s} + \frac{2a}{a_r} = 0$



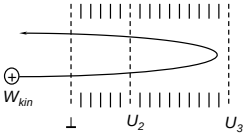
Dual Stage Ion Mirror

Two-stage ion mirror ... ion mirror is divided in 2 stages with different *E*

For positive ions:

- positive voltage on the middle grid, $0 > U_2 > U_3$
  - a variety of arrangements, various ratio of stage lengths, e.g. ~1:2, ~2:3
  - short 1st stage with higher *E* allows shortening of the entire mirror length
- negative voltage on the middle grid:
  - space focusing in the 1st stage
  - energy focusing in the 2nd stage

Dual Stage Ion Mirror



$t_{TOF} = f(W_{kin}, U_2, U_3)$
energy dispersion ... the main reason of peak broadening and low *R*

Mamyrin:
Solution $\frac{\partial}{\partial W_{kin}} f(W_{kin}, U_2, U_3) = 0$ and $\frac{\partial^2}{\partial W_{kin}^2} f(W_{kin}, U_2, U_3) = 0$

Dual Stage Ion Mirror

+ Applicable to energy dispersion below ~20%.
E.g. for energy dispersion < 5% theoretical resolution ~100 000

- Only ions that penetrate deep into the ion mirror (85-100%) are focused.

- Mass spectra of ions with wider energy dispersion have to be recorded at several values of the potential on the mirror. The resulting spectrum is stitched from these several segments.

Nonlinear Ion Mirror

Aim: t_{TOF} independent on their W_{kin} for ions of all *m/z*

In **quadratic** field $U(z) = \frac{k}{2}(z-a)^2 + C$

$$\frac{d^2z}{dt^2} = -\frac{q}{m}k(z-a)$$

ion reflection ≠ $f(W_{kin0})$

z ... axis of potential change
a ... parabola minimum
k a *C* ... constants
f ... frequency of oscillations

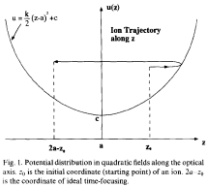


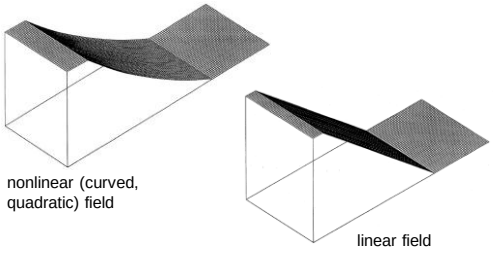
Fig. 1. Potential distribution in quadratic fields along the optical axis. z_0 is the initial coordinate (starting point) of an ion. $2a - z_0$ is the coordinate of ideal time-focusing.

(A. A. Makarov, E. N. Raptakis, and P. J. Derrick, *Int. J. Mass Spectrom. Ion Processes* **1995**, *146/147*, 165.)

Nonlinear Ion Mirror

Other practical fields:
axially symmetric hyperbolic potential
planar hyperbolic potential
axially symmetric hyperlogarithmic potential

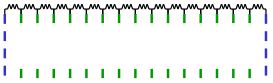
Nonlinear Ion Mirror



(T. J. Cornish, R. J. Cotter "Non-linear field reflector", US Patent 5 464 985)

Nonlinear Ion Mirror

Usually quadratic field or its approximation



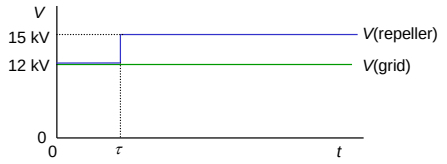
Creation of nonlinear field
using unequal resistors in series
using unequal spacers between rings and/or grids

+ simultaneous focusing of ions with wide dispersion of W_{kin} , e.g. products of post-source fragmentation, for entire *m/z* range
- more complex calibration
- ideal only for ions moving on the axis of the reflector
... only limited *R* can be achieved in practice

3. Pulsed Extraction
(Delayed Extraction Time-Lag Focusing)

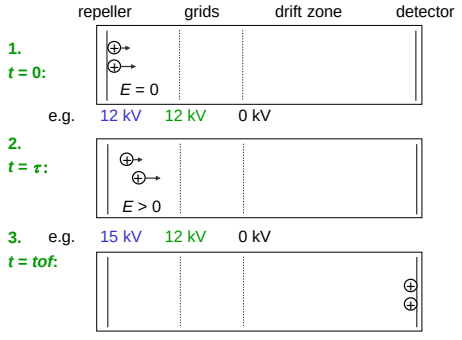
Extraction field is turned on after a delay:

1. Laser pulse at $t = 0$
2. Expansion of ions at zero extraction field for a period τ (delay)
3. Extraction field is turned on at $t = \tau$, with fast rise time

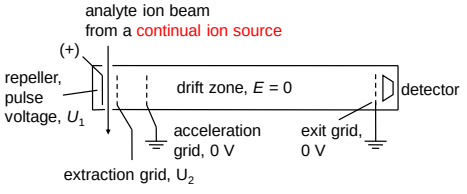


(Brown, R. S.; Lennon, J. J.; *Anal. Chem.* **1995**, *67*, 1998)

Pulsed Extraction



Orthogonal Extraction



Pulse extraction for continual ion sources

Analyte stream is extracted by pulse extraction field orthogonal to the entering ion beam.

Usually with ion mirror for higher resolution.

Other Factors Influencing Resolution in TOFMS

- non-ideal ion source
- ion optics
- tube length dilatation
- properties of detector
- sampling frequency of A/D converter
- sample preparation
- acceleration voltage fluctuations

E.g. acceleration voltage fluctuations:

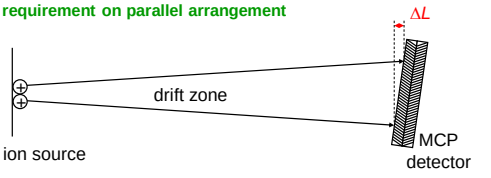
m = const. $Ut^2 \Rightarrow R^{-1} = \frac{dm}{m} = const.\left(\frac{dU}{U} + 2\frac{dt}{t}\right)$

dt given by max. frequency of AD converter, detector speed and time dispersion of ion generation

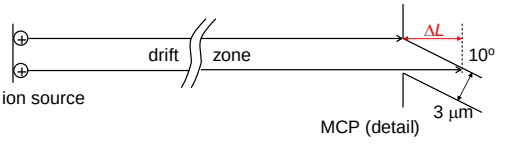
dU given by high voltage power supply (drifts and noise)

Importance of Alignment in TOFMS

Strict requirement on parallel arrangement



MCP with narrow channels



Importance of Alignment in TOFMS

Resolution:

$$R = \frac{m}{\Delta m}$$

$$R = \frac{t}{2\Delta t}$$

$$\boxed{R = \frac{L}{2\Delta L}}$$

$$\frac{m}{z} = 2eU\frac{t^2}{L^2}$$

$$t = L/v$$

E.g. for R = 15 000 and L = 3 m:

$$\boxed{\Delta L \leq 100\ \mu m}$$

Note: MCP with channel angle 10° and diameter 10 μm:
ΔL = 10 μm/tan(10°) = 57 μm

Influence of Grids

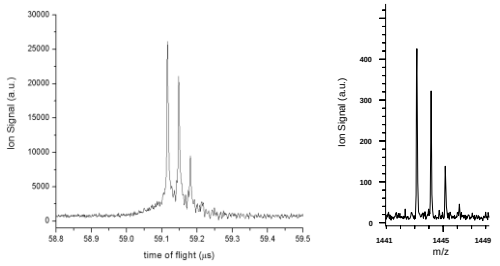
Grids ... electrodes transparent for ions
... in ion source, ion mirror and in front of detectors

MS with grids
+ precise definition of potential
- ion losses (impact, deflection, reaction)
- secondary ion formation on grids, sputtering of grid material
- field penetration

Gridless MS
- higher resolution without losses on grids
- more complex design (extra curvature of ion trajectories, lens effect)

For details, see: T. Bergmann, T. P. Martin, H. Schaber *Rev. Sci. Instrum.* **1989**, *60*, 347.

Influence of Grids



TOF with grids **Gridless TOF**

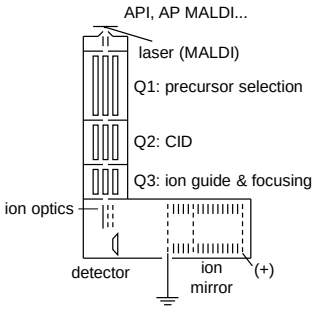
Note: grids do not have to always mean drop of resolution

Comparison of TOFMS Systems

system	geometry	extraction	*R*
TOFMS	linear	DC	500
DE-TOFMS	linear	pulse	5 000
rTOFMS	reflector	DC	10 000
DE-rTOFMS	reflector	pulse	20 000
oTOFMS	reflector	pulse	10 000

Tandem Mass Spectrometer QTOF

Ion beam is wide after exit from Q3 (space dispersion - x_0), but ions have negligible energy dispersion - v_0 in the direction of flight



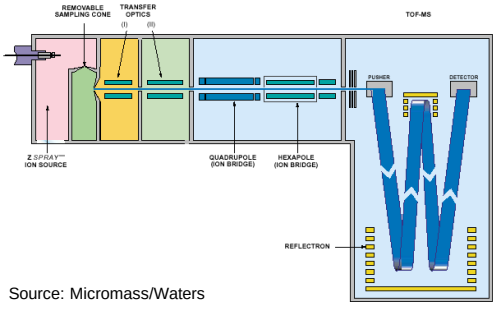
Hybrid Ion Source for QTOF

Exchangeable ion source

... continual and pulse ion techniques

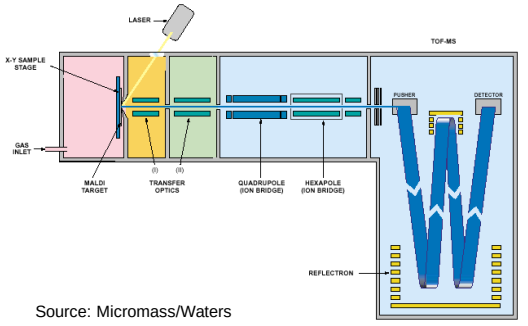
Example: QTOFMS: API
AP MALDI

Hybrid QTOF MS for API and MALDI



Source: Micromass/Waters

Hybrid QTOF MS for API and MALDI



Source: Micromass/Waters

Technical Notes

Target (MALDI): 384 spots on target sized as microtitration well plate, sufficient area for collection of eluent from a separation column

Ion optics: with or without grids (gridless)

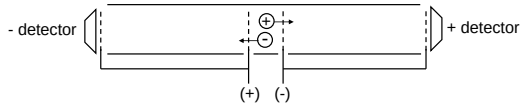
Collision chamber in ion source: increase of fragmentation degree

length of the drift zone (flight tube): typically 1-2 m, but even 10 cm or 5 m

Ion guide – it may be placed in the tube to increase ion transmission (wire in the flight axis with ~ -50 V for positive ions)

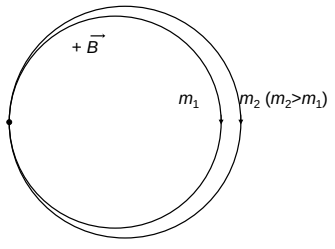
TOF: Other Geometries

TOF MS for simultaneous detection of positive and negative ions



MALDI Target

TOF: Other Geometries



360º magnetic sector - no separation in space
- separation in time

Other Technical Notes

Detectors: microchannel plate, MCP
usually double MCP
electron multiplier
hybrid detector (scintilating layer + photomultiplier)

Detection electronics: AD converter (8 bits, 0.5 - 4 GS/s)
TD converter + segmented detector

Commercial TOFMS

Applera	Mariner	API	oTOF
	Voyager	MALDI	TOF
	4700	MALDI	TOF/TOF
	QSTAR	API, MALDI	QoTOF
Bruker	Biflex, Proflex, Reflex	MALDI	TOF
	Autoflex, Omniflex, Ultraflex	MALDI	TOF (LIFT)
JEOL	AccuTOF	API	oTOF
LECO	Renaissance	API (ICP)	TOF
	Jaguar	API	oTOF
Micromass/Waters	M@LDI	MALDI	TOF
	QTOF	API	oTOF
	QTOF Ultima	API, MALDI	oTOF
Thermo/Finnigan	Tempus	EI, CI	oTOF
Kratos/Shimadzu	Kompact, Axima	MALDI	TOF

etc. etc.

Comparison of Common Mass Spectrometers

MS	max. *m/z*	*R*
quadrupole filter	4 000	2 000
magnetic sector	20 000	50 000
ion traps	10 000	5 000
FT-ICR-MS	100 000	100 000
TOFMS	1 000 000	10 000

Note:

The values in the table are approximate, as they describe average instruments, the parameters of research-grade systems might be significantly higher (differences in order of magnitudes).

TOFMS Summary

- Unlimited theoretical upper *m/z* limit. Practical limit: ionization and detection efficiency, metastable ion decay.
- Entire spectrum recorded at once.
- High speed of data acquisition (1 spectrum in ~10^{-4} s).

  E.g. insulin (*m/z* = 5735) accelerated by 15 kV overcomes 1-m drift zone in ~ 50 μs.
- High ion transmission, especially in linear mode (> 50 %).
- High resolution (*R* > 10 000).
- Simplicity and relatively low cost.
- Fragmentation techniques available (ISD, PSD, TOF/TOF ... next lecture)

TOFMS Perspective

Advantages of TOFMS

upper *m/z* limit, speed and high sample throughput, high sensitivity and resolution, relatively lost cost

Huge spread of TOFMS in the last decade

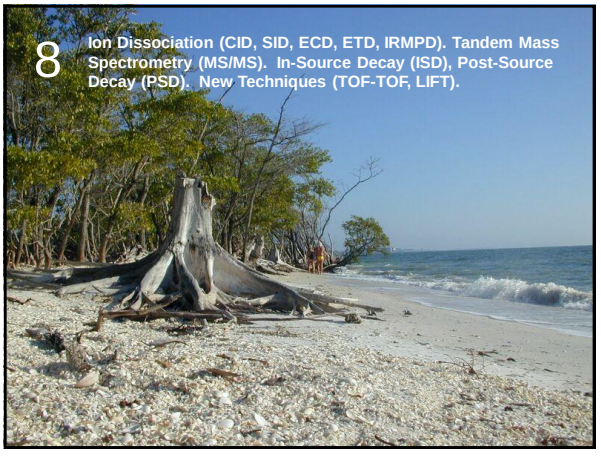
MALDI + PE TOF, rTOF MS

API, AP MALDI + oTOF MS

New techniques for MS/MS (TOF-TOF, LIFT, QTOF)

Competition

LT, FT-ICR and hybrid MS



Tandem Mass Spectrometry

(Tandem MS, MS/MS, MS^n)

ionization → MS_1 → precursor ion → fragmentation → MS_2 → daughter ions

1. ionization ⇒ mixture of ions
2. first MS ⇒ selection of parent ion, precursor ion
3. fragmentation of ions (e.g. in collision cell)
4. second MS ⇒ analysis of fragmentation products, daughter ions

Arrangement

1. in space – two or more discrete *m/z* analyzers in a series
2. in time – consecutive ionization, precursor selection, fragmentation and scan in a single *m/z* analyzer (ion trap)

Fragmentation Classification

Dissociation ... monomolecular reaction, *t*(induction) << *t*(dissociation)

Fragmentation can be induced by:

1. **Collision** w/ atom or molecule
   collision-induced dissociation, CID
2. **Collision** with surface
   surface-induced dissociation, SID
3. **Photon** (photodissociation, PD)
   e.g. infrared multiphoton dissociation, IRMPD using CO_2 laser
4. **Electron** (electron capture dissociation, ECD)

Note: It is difficult sometimes to determine the exact cause of ionization, e.g. the decay in MALDI (in- and post-source decay, ISD and PSD) may be induced by both collisions and photons.

Fragmentation Induced by Collisions

1. collisions with molecules of background (collision) gas in collision cell
   at elevated pressure, *p* ~100 Pa
   CID, collision-induced dissociation
   CAD, collisionally activated dissociation

2. excessive excitation during ionization, e.g. high laser power at MALDI
   ISD, in-source decay ... in TOFMS
   PSD, post-source decay … in TOFMS

3. collisions with surface: **SID**, surface-induced dissociation
   - *surface*: layer of an organic compound (polymer or monolayer of small organic molecules, e.g. alkanthiols) on a suitable substrate (Au)
   - *collisions as a result of acceleration by electric field,* e.g. voltage nozzle-skimmer

Ion Stability

1. **Stable**: lifetime, $\tau > 10^{-6}$ s
   Ion flights through entire MS without decomposition.

2. **Metastable**: lifetime, $\tau \sim 10^{-7}$ - 10^{-6} s
   Ion is decays during flight in the *m/z* analyzer.

3. **Unstable**: lifetime, $\tau < 10^{-7}$ s
   Ion fragments in the ion source.

Note: This is historical classification according to the time ions spend in magnetic sector.

Reactions: unimolecular, bimolecular

Ion Collisions

Fragmentation
- intended fragmentation aimed at elucidation of analyte structure
- usually with atoms of rare gases

Elastic collisions
Kinetic energy is conserved.

Inelastic collisions
Part of kinetic energy is converted into inner ion energy:
$E_{in} <= E(M_t/(M_t + M_i))$
t = target, i = ion
Use heavy target to transfer more energy on ion.

1 eV/ion ~ 100 kJ/mol (100 kJ/5 g tatranky)

Low-Energy Collisions

Collision Energy: 1 – 100 eV
vibrational excitation, *t* (ion-target interaction) ~ 10^{-14} s

Collision efficiency
usually sufficient due to many collisions in collision cell

Instrumentation
triple quadrupole filter, ion traps, hybrid MS

The most widespread technique nowadays.

High-Energy Collisions

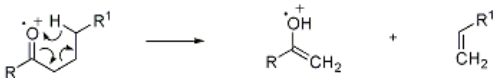
Collision energy: keV
electron nature of excitation, *t* (ion- target interaction) ~ 10^{-15} s
converted energy ~ 1 – 3 eV

Collision efficiency
He – reduces angular scatter of products
(scatter has negative impact on *m/z* analysis)
Ar, Xe – enables more efficient energy conversion

Instrumentation
hybrid sector instruments, TOF/TOF

Example: McLafferty Rearrangement

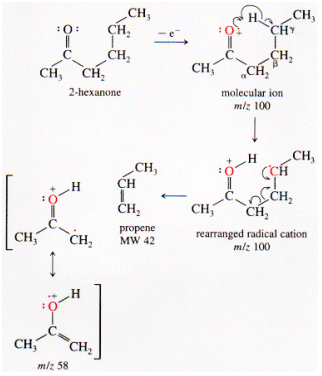
Electron impact-induced breakage of carbonyl compounds with hydrogen in γ position and formation of enolic fragment and olefin:



F. W. McLafferty, *Anal. Chem.* **31,** 82 (1959).

D. G. I. Kingston *et al., Chem. Rev.* **74,** 215 (1974); K. Biemann, *Mass Spectrometry* (New York, 1962) p 119;
Djerassi *et al., J. Am. Chem. Soc.* **87,** 817 (1965); **91,** 2069 (1969); **94,** 473 (1972)
M. J. Lacey *et al., Org. Mass Spectrom.* **5,** 1391 (1971); G. Eadon, *J. Am. Chem. Soc.* **94,** 8938 (1972); F. Turecek, V. Hanus, *Org. Mass Spectrom.* **15,** 8 (1980).

Mechanism of McLafferty Rearrangement



Why MS/MS?

1. Structure elucidation of organic compounds

2. Analysis of mixture of analytes as a substitute of separation – MS combination.
- All ions are ionized simultaneously at MS/MS. CID and daughter-ion scan are done for selected precursors.
- Attention! Mutual ion suppression in complex mixtures during ionization.

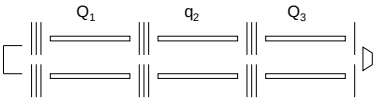
3. Improvement of S/N
- Higher selectivity ⇒ reduced noise
- Single ion monitoring

Mass spectrometers for MS/MS

1. Triple quadrupole filter (Triple quad, QQQ, TQ, Q3)
2. Ion traps (IT, LIT, FT-ICR)
3. BE (magnetic sector – electrostatic analyzer) with reverse geometry
4. TOF/TOF MS
5. Hybrid spectrometers, such as QTOF, IT-TOF, EBQ etc.

Classification of tandem mass spectrometry
1. in space – 1, 3, 4, 5
2. in time – 2, 5 (IT-TOF)

Triple quadrupole, QQQ, QqQ, Q3



Classical instrument for low-energy CID (MS/MS, MS^2)

Q_1: MS_1
q_2: RF-only, collision chamber
Q_3: MS_2

Scan Types in MS/MS

1. Single Ion Monitoring
Set Q1: only ABC^+ pass
Set Q3: only one of ABC fragments pass – e.g. AB^+, A^+, BC^+…
… very selective and sensitive proof of ABC

2. Daughter Ion Scan
Set Q1: only ABC^+ pass
Scan Q3: spectrum of ABC^+ fragments
… structure elucidation

3. Parent Ion Scan, Precursor Ion Scan
Scan Q1: spectrum of original ions ABC^+, ABF^+, DEF^+ etc.
Set Q3: detect only specific fragment, e.g. AB^+
… for identification of a group of similar compounds (with the same functional group or structure motif)

4. Neutral Loss Scan
Scan Q1 and Q3 simultaneously while keeping constant difference between the *m/z* of transmitted ions
… loss of the same neutral (compounds with the same functional group.)

Other Mass Spectrometers for MS/MS

Tandem magnetic and electrostatic sector with reverse geometry (BE)

1. Isolation of precursor ABC^+ in B.
2. Fragmentation in collision chamber.
3. Kinetic energy analysis in E.
   Daughter ions are characterized with W; $W = f(m/z)$.
   More sophisticated combinations: EBE, EBEB.

EBqQ

EB: precise precursor selection
q_1: RF-only (collision cell)
Q_2: selection/analysis of products

Tandem quadrupole filter - oTOFMS (QTOFMS)

TOFTOF

Other Mass Spectrometers for MS/MS

Tandem IT - TOFMS (IT-TOFMS)

IT: accumulation and MS^n option in the 1st stage.
TOF: sensitive detector with high mass resolution&accuracy as the 2nd stage.

Ion traps: IT, FT-ICR-MS

- MS^n option
- the same spectrometer as for MS, only software upgrade needed
- the same price
- Procedure:
  1. isolation of precursor (after accumulation of all ions)
  2. excitation of precursor (amplitude boost) for a longer period
  3. product scan (or back to the 1st step for MS^n, n >2)

Tandem LT – FT-ICR-MS

plenty of scan types and operational modes

TOF in Tandem MS

... for structural analysis and/or identification

In-source decay (ISD)

Post-source decay (PSD)

Collision-induced dissociation (CID, TOF/TOF)

In-Source Decay (ISD)

- Also called In-Source Fragmentation (ISF)
- Technique for study of molecular structure
- Elevated laser power at MALDI leads to excessive "heating" (vibrations) of molecules/ions of analyte and fragmentation of analyte in the source
- Intensity of fragments << intensity of parent ion ($[M+H]^+$)
- Pulse extraction necessary to:
  - reach sufficient resolution and sensitivity
  - prolong ion stay in the ion source (more collisions)

ISD Characteristics

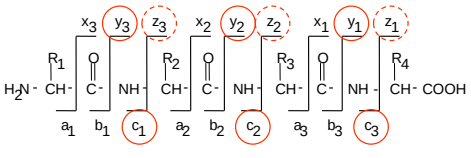
\+ Ion mirror not necessary.
\- Pulse extraction needed.
\- Preparation of clean analyte required (no option of precursor selection).
\- May require special sample preparation (e.g. higher salt concentration)

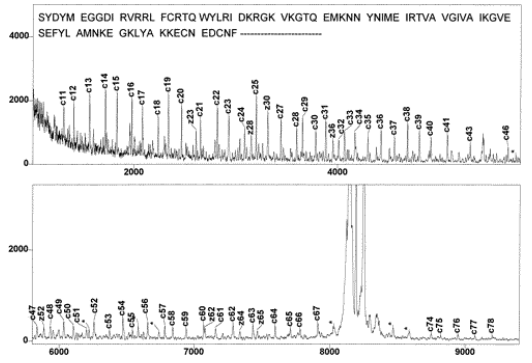
Use: MALDI TOFMS of peptides, saccharides

Examples of ISD

Fragmentation rules … according to relative strength of bonds.
Typical fragmentation products of peptides:

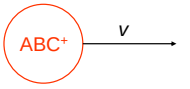
c, y, z





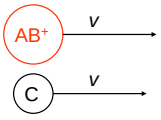
ISD. Analyte: 20 pmol KGF analogue. Matrix: sinapinic acid. Laser: N_2.
(V. Katta, D. T. Chow, M. F. Rohde *Anal. Chem.*, **70**, 4410-4416, 1998.)

Precursor ABC^+

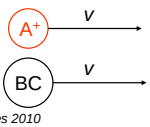


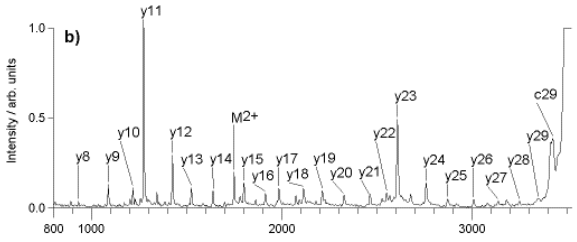
$$\frac{m_{ABC^+}v^2}{2} > \frac{m_{AB^+}v^2}{2} > \frac{m_{A^+}v^2}{2}$$

Fragmentation 1



Fragmentation 2



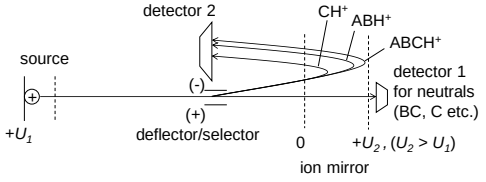


ISD

Analyte:	oxidized bovine insulin, chain B
Matrix:	thiourine
Laser:	Er:YAG (λ = 2936 nm)

(http://medweb.uni-muenster.de/institute/impb/research/hillenkamp/publicat/abs-cm99.html)

Ion Mirror as an Energy Analyzer in PSD TOFMS



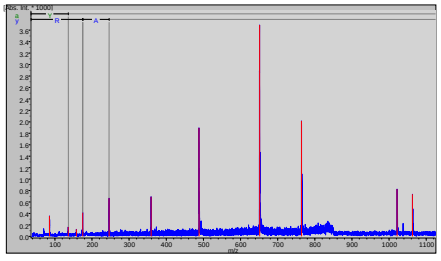
Clasical ion mirror allows focusing of only product ions with < 20 % energy dispersion and hence in < 20 % *m/z* interval. Total PSD spectrum has to be put together from few PSD spectrum increments obtained at different reflector potential.

Quadratic ion mirror allows focusing of products ions in wide *m/z* interval.

Post-Source Decay (PSD)

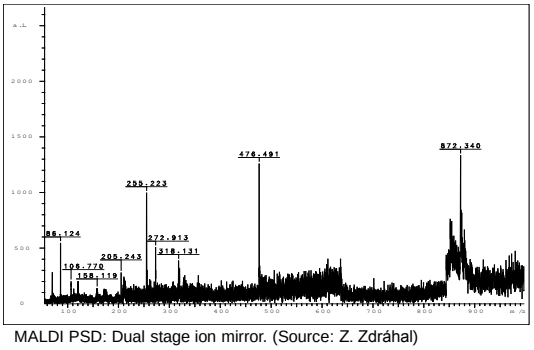
- Ion selector (ion gate): selection of precursor (*m/z* interval 1 - 20 Da).
- Analysis of product ions in ion mirror:
  - Fragment ions of analyte are formed during flight through the drift zone (as a result of excessive excitation), e.g.:
  - $ABC^+ \rightarrow AB^+ + C$
  - $ABC^+ \rightarrow A^+ + BC$ etc.
  - Kinetic energy conservation for the first equation:
  - $m_{ABC}v^2/2 = m_{AB}v^2/2 + m_Cv^2/2$.
- The heavier the fragment ion is, the higher kinetic energy it has and the deeper it penetrates into the ion mirror $\Rightarrow$ longer flight time.

MALDI PSD of a Derivatized Peptide YLYELAR



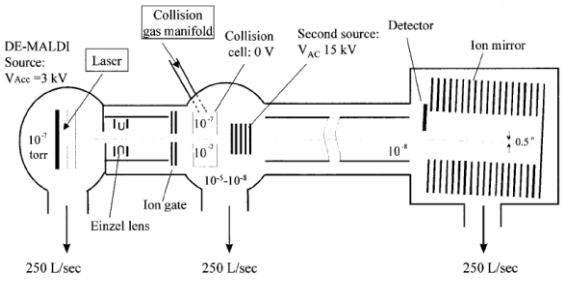
Spectrum composed from spectral increments recorded at different ion mirror potential. Only ions that penetrate deep into the mirror were focused. Dual stage ion mirror used. (Source: Z. Zdráhal)

MALDI PSD of a Peptide



MALDI PSD: Dual stage ion mirror. (Source: Z. Zdráhal)

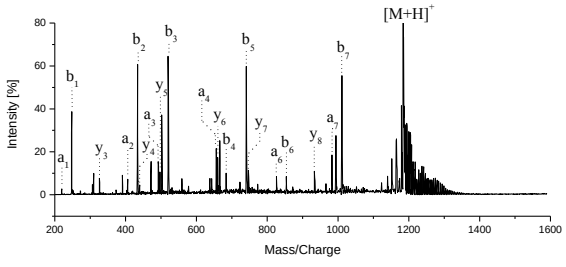
TOF/TOF



TOF-based MS/MS

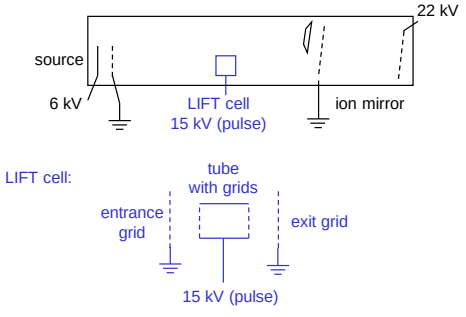
(Medzihradszky, K. F.; Campbell, J. M.; Baldwin, M. A.; Falick, A. M.; Juhasz, P.; Vestal, M. L.; Burlingame, A. L. Anal. Chem. 2000, 72, 552).

MALDI PSD of a Peptide



pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
MALDI PSD, a **quadratic-field reflector** used.
(O. Šedo et al. *Anal. Chim. Acta* **2004**, *515*, 261-269)

LIFT - TOFMS



Collision-Induced Dissociation (CID) in TOFMS

Fragmentation results of collision of precursor ion with gas molecule

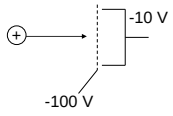
Collision chamber

- additional collision chamber in TOF ion source (negligible effect)
- inserted between two TOF analyzers (TOF/TOF)
- in hybrid instrument (e.g. QqTOF)

Ion Detection

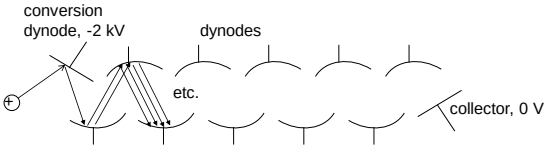
Faraday cup
Electron multiplier
Channeltron
Microchannel plate, MCP
"Daly" detector
Array detectors
Photographic plate
Hybrid detectors, e.g. MCP-diode array

Faraday Cup



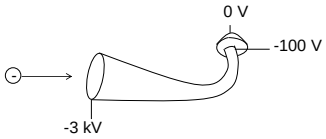
- Very small currents
  E.g.: 100 ions/s ⇒ current = 1.6 x 10^{-17} A

Electron Multiplier



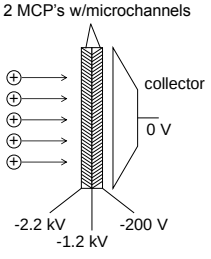
- Analogy of photomultiplier; conversion dynode (instead of photocathode) followed by a series of dynodes with decreasing potential and a collector. Electron multiplier is not encapsulated in a glass bulb.
- Conversion of ions to electrons on the first dynode (e.g. Cu-Be alloy). Multiplications of electrons on dynodes. Electron capture on collector, generation of current.
- Gain ~ 10^6

Channeltron



- Glass tube with a layer of semiconducting PbO inside and applied voltage on the ends. Current flowing through the PbO layer forms potential gradient along the tube (rather than dynodes kept at discrete potential values). Electron multiplication similar to electron multiplier.
- Conversion dynode necessary for detection of positive ions.
- Gain ~ 10^6

Microchannel Plate (MCP)

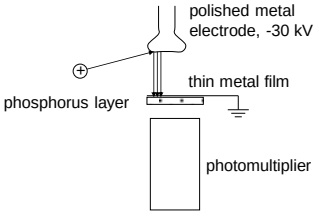


Dimensions of MCP
- thickness ~1 mm
- diameter 1 - 10 cm.

Microchannels
- slightly tilted,
- diameter ~ 3 - 20 μm.
- covered with a PbO layer,
  ... formation of gradient along microchannels (electron multiplication on continuous dynodes)
- Chevron structure in sets of more MCP's.

Array detector with large sensitive area useful in TOFMS
Gain of single MCP ~10^3, dual MCP ~10^6 or higher.

"Daly" Detector



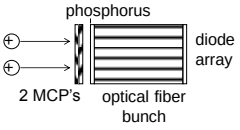
Ion → electron(s) → 4 photons (phosphorus)
+ Very low noise.
+ Very high gain (photomultiplier usually works in photon counting mode).
- Light interferes.
- Very low pressure (p < 10^{-5} Pa) necessary.

Array Multichannel Detectors

… e.g. for magnetic sector

Photographic plate
- historical detector, time-consuming processing

Combination of MCP and diode array



Incorporation of optical fiber bunch for higher positional resolution.

Other detectors ... combination of the detectors discussed before
E.g. MCP and phosphorus scintillator … isolation of high voltage

Data Acquisition in MS

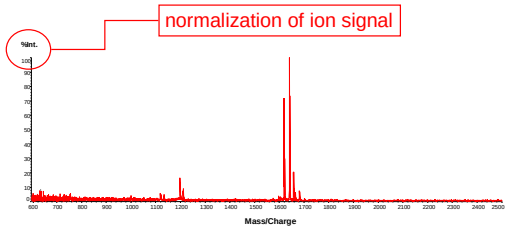
ions
↓ charge, *q*
electrons
↓ *I* = *q*/*t* (*t* ... time)
current
↓ *U* = *IR* (*R* ... impedance)
voltage
↓
recording device

Different schematic for photographic plate and scintillating detectors

Mass Spectrum

y-axis: current, voltage, charge, ions, counts???

a.u. (arbitrary units) or **relative intensity**



Mass Spectrum Acquisition

I(m/z) = f(X) = f(t)

Scanning instruments		**Scanned *X***	**Scan period**
MAG	magnetic sector	*B*, *U*	~ 1 s
Q	quadrupole filter	*U*, *V*, *f*	~ 0.1 s
IT, LT	ion traps	*U*, *V*, *f*	~ 0.1 s

Direct signal acquisition in time:

TOF		~ 100 μs
FT-ICR	ion cyclotron	~ 1 s

Other non-scanning instruments may use array detectors.

Recording Devices

digital converters
- **A/D converter (analog to digital)**
- **T/D converter (time to digital)**

photographic plate
chart recorder
analog oscilloscope with a camera

A/D Converter (ADC)

Measurement (digitization) of voltage

Basic parameters

number of bits (resolution)
sampling frequency, number of samples pre second [Sample/s]

max. frequency (cut-off frequency)
polarity: unipolar (negative), bipolar
input voltage range
max. input voltage
number of data point (memory length, buffer size)
stability
etc.

A/D Converter

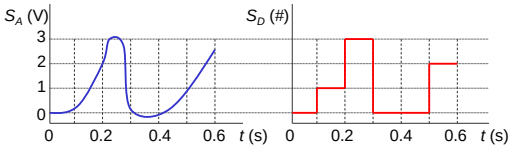
Example:

2-bit converter with input voltage range 0-3V and sampling frequency 10 S/s

number of levels = 2^2:

period, $T = 1/10 = 0.1$ s

level	high-bit	low-bit
0	0	0
1	0	1
2	1	0
3	1	1



Signal Precision and Accuracy

Precision and (accuracy) of measurement given by number of bits of ADC

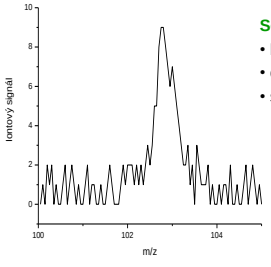
Example: for 8-bit ADC, $2^8 = 256$ levels:

deviation might be up to 1/2 of the difference between two adjacent levels

min. rel. deviation > $(2x(\text{number of levels} - 1))^{-1} = (1/2x255)^{-1} \sim 0.2$ %

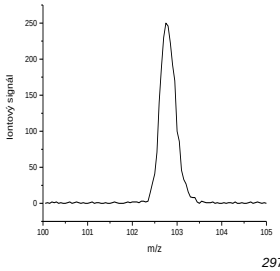
number of bits	1	8	12	16	24
number of levels	2	256	4 096	65 536	16 777 216
min. rel. deviation (%)	50	0.2	0.01	$8x10^{-4}$	$3x10^{-6}$
dynamic range (rel. deviation < 10%)	-	50	800	13 000	3 000 000

Selection of ADC



Solution:

- higher detection range
- converter with higher number of bits
- signal accumulation (averaging)



Possible reasons:

low signal level

converter with low number of bits

Acquisition Speed

... given by sampling frequency of ADC

Factors: scan period
scan range
required resolution
required quality of peaks (number of data points/peak)

Example 1:

Requirements on a Q: scan period = 0.1 s, unit resolution in *m/z* range = 200 – 2 000, >10 pts/peak. What is min. sampling frequency?

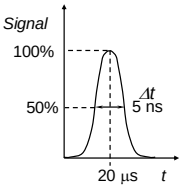
min. sampling frequency = (2 000-200)x10/0.1 = 180 ksample/s

memory length = (2 000-200)x10 = 18 000 samples

… for 16-bit converter: 36 kByte (1 data point = 19 bits or 2 bytes)

ADC Speed

Example 2: How fast ADC is needed for TOFMS in order to record ion flying 20 μs with resolution 2000, provided the peak should be characterized by 10 points?



t = 20 μs, R = 2 000

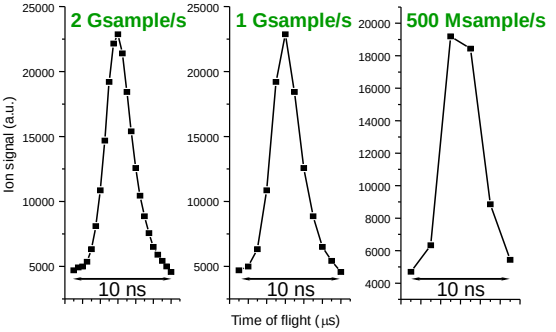
$R = m/\Delta m = t/(2\Delta t)$

$\Delta t = t/2R$ = 20 μs/4000 = 5 ns (FWHM)

entire (~10 points) … 10 ns $\Rightarrow t_{sample}$ = 1 ns (1 ns/sample)

sampling frequency, $f = 1/t_{sample}$ = 1 Gsample/s

Influence of Sampling Frequency of ADC (in TOFMS)



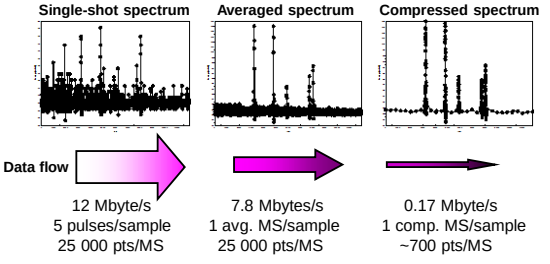
ADC: Current State of Art

High number of bits: 24 bits
- but only at low sampling frequency (1 kHz)

High sampling frequency: 20 Gsamples/s
- but only with low-bit ADCs (8 bits)

High performance
- data accumulation (averaging) directly in PC card
- on-board compression (algorithms without and with data loss)
- fast data transfer from card to PC
  ... PCI card in computer, direct PC memory access

Use of Accumulation and Compression in TOFMS



Application: CE–MS, simultaneous 8 CE eluents, 12 comp. MS/s/eluent
⇒ 31.3 Mbyte/3-minute CE
Hardware averaging and compression using multiple digital signal processors (FastFlight, EG&G).

Available ADCs

Digital oscilloscopes
up to 20 Gsamples/s
max. deviation of *t*: 1 ps
bandwidth up to 8 GHz
Tektronix, LeCroy, Agilent etc.

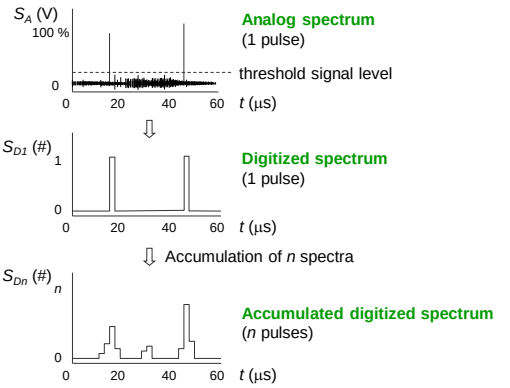
PC cards (PCI)
up to 5 Gsample/s, 8bit, with accumulation
Aqiris, Signatec, GaGe etc.

T/D converter (TDC)

Pulse counters
Impact of ion on detector → pulse → amplifier → discriminator → counter

TDC (time to digital converter)
1-bit A/D converter
2 levels: 0 a 1

Parameters:
time resolution
dead time
number of channels
etc.



TDC

Only one of a group of ions is used during one acquisition period (single channel TDC).

Suitable for acquisition of relatively low signals with high repetition rate.

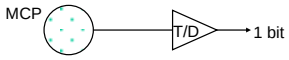
Relatively low cost (compared to ADC).

Applications:
TOFMS with high frequency of extraction pulses (>kHz)
ESI - oTOFMS
AP-MALDI – oTOFMS
TOFMS with low numbers of ions
PD – TOFMS

TDC

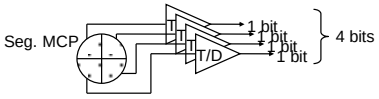
TDC with multiple channels
... to increase dynamic range
... using a multiple channel TDC in combination with a segmented detector

Classical TOF detector



TDC

Segmented TOF detector



Ions hit randomly four segments with similar probabilities
...total ion fluence might be higher

TDC

Current state of the art
Time resolution ~10 ps at millisecond range
Montáž do stojanu nebo do PC (PCI sběrnice)

Manufacturers: LeCroy, Kore Technologies etc.

Informatics & Bioinformatics

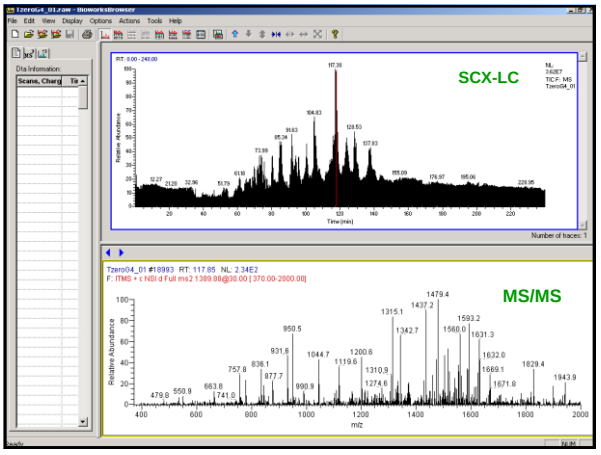
Hardware: IBM PC platform most popular

Software: Windows XP

Exponential growth of performance: Moore law

Example:
Evaluation of MS/MS spectra using SEQUEST program

SCX - LC - MS/MS of Y2H yeast digest (the next slide)



PC Cluster for evaluation of SCX-LC-MS/MS

1x master PC (2 processors P4, 3 GHz)
8 x slave PCs (2 processors P4, 3 GHz)
interconnected with 1-Gbit network

PC Cluster for evaluation of SCX-LC-MS/MS

Vacuum Instrumentation Background

Evangelista Torricelli (1608-1647)
Pressure unit: 1 Torr = 1 mm Hg

Pressure, *p*
p = force/area [Pa, N/m^2]
1 atm = 760 Torr = 101 325 Pa = 101.325 bar = 14.70 psi
1 Torr = 133 Pa

Vacuum
gaseous state with p < 101325 Pa

Mean free path of a molecule
Mean path of a molecule (atom, ion, particle) travels between 2 collisions
$\lambda = (\sqrt{2}\pi\sigma^2 n)^{-1}$
λ(cm) = 0.66/p(Pa) ... only rough estimation for air at 25°C

Gas Flow Classification

1. **Turbulent flow** (p > 10 kPa)
   very short λ
   many collisions between molecules

2. **Viscous, continuous flow** (p = 1000 - 0.1 Pa, „rough" vacuum)
   λ = μm - cm:
   still much shorter compared to dimension with vacuum apparatus
   more collisions molecule – molecule than molecule – wall

3. **Molecular flow** (p < 0.01 Pa, "high" or "ultra high" vacuum, UHV)
   λ > m:
   collisions of molecules with walls of vacuum apparatus prevail
   usual situation in mass spectrometer
   (exceptions: collision cells, CI, ion mobility spectrometry)

Gas Flow Regimes

Gas Flow Rate, *Q*
[Q] = Pa m^3/s, Pa L/s

***C* … conductance** of a tube with diameter D and length L
$C = f(D, L, p, \text{gas}, T)$, [$C$] = L/s

At molecular flow regime, conductance is not a function of pressure p:
$C \propto D^3/L$ (approximation)

At viscous flow, conductance C depends on pressure p:
$C \propto pD^4/L$ (approximation)

Design of a vacuum apparatus
- Short thick connectors $\Rightarrow C\uparrow \Rightarrow$ faster pumping.
- In serial connections, the thinnest tube (bottleneck) limits pumping of the entire system: $1/C_{tot} = \Sigma 1/C_i$

Pumping Speed

Pumping speed, *S* $S = \frac{Q}{p}$ [S] = L/s

$1/S_{chamber} = 1/S_{pump} + 1/C_{tot}$

Appropriate design: $C_{tot} > S_{pumpa}$
i.e., using expensive efficient pump together with long narrow tubes or hoses does not make sense

Notes:
- In molecular flow regime pump does not suck gas. Pump acts as a trap; molecules that come to the pump will not return to chamber.
- Pumping speed varies with gas type (drops with gas *M.W.*).
- Pressure is not constant throughout the system, positioning of pressure sensor matters.
- Outgassing. Time needed for reaching sufficient vacuum is prolonged by presence of volatile compounds adsorbed (water, sample, finger prints) and absorbed in the system (gases and water in gaskets, plastics).

Vacuum Pumps

Mechanical pumps (rotary oil, membrane, roots)

Diffusion pump

Turbomolecular pump

Cryotrap, sorption traps, chemisorptions traps

Rotary Oil Pump

- One, two or even more stages.
- Rotor immersed in oil
  - min. pressure limited by oil vaporization
  - trap for oil vapors needed (to keep apparatus clean - oil contamination!)
  - trap for oil mist on outlet needed (to keep operator's lungs clean)
  - periodic oil changes required
- Pressure > 0.1 Pa … "rough" vacuum, pumping speed, *S*: 1 – 500 L/s (typical)
- The most common rough pump in commercial systems.
- Other mechanical pumps: membrane pump (no contamination, but higher limit *p* and lower pumping speed)

Turbomolecular Pump

- Series of turbines rotating at extremely high speed (up to 50 000 rpm). Gas molecules are reflected by turbine blades. Perimeter speed of turbine blades > speed of gas molecules.
- Dependence of inlet and outlet pressure on gas molecular weight:
  $\ln (p_{out}/p_{in}) \propto \sqrt{m}$
- UHV pump, limit pressure ~10^{-8} Pa
- Pumping speeds up to ~ 3000 L/s
- Commonly used UHV pump in commercial systems.

Diffusion Pump

- Special non-volatile oil heated with a heater at the base of the pump, heated oil vapors flow upwards through a central tube, stream as circular jets downwards on cooled walls, condense and flow down to the base. Molecules of pumped gas are drawn by oil jets.
- Often used in combination with a trap cooled with water or liquid nitrogen; this may prevent oil backstreaming or increase pumping speed (solvent vapors).
- UHV (ultra-high vacuum) pump, limit pressure ~10^{-6} Pa, pumping speed up to ~ 10 000 L/s.
- Reliable, low maintenance.
- Slow start, backstreaming.

Cryotrap

- Sorbent (e.g. charcoal) cooled down to 4 K (liquid He).
- Regeneration necessary.

UHV System Heating

Molecules of background gas (especially water) adsorb on inner walls of mass spectrometer. Slow desorption of gas molecules continues to elevate pressure and prolongs pumping time. Pumping can be fastened by heating of the entire system (e.g. by wrapping the system using a resistively-heated tape), which shift equilibrium from adsorption to desorption.

Pressure Sensors (Pressure Gauges)

Hydrostatic pressure gauge

- Pressure determined from difference of liquid levels.
- Range down to 1 kPa, or 0.1 Pa for special constructions.

Mechanical gauge

- Determination of pressure from deviation of elastic membrane.
- Reading of the deviation - mechanical
  - change of capacitance (range 10^{5} – 10^{-2} Pa)

Thermocouple gauge (Pirani)

- Bimetallic thermocouple and resistively heated filament.
- Heat transfer from filament to thermocouple dependent on pressure and type of gas.
- Range *p*: 100 Pa - 0.1 Pa or 100 kPa - 0.1 Pa (convectron).

Pressure Gauges

Ion gauge

Bayard & Alpert: ion tube with heated cathode.

- Three electrodes in a glass bulb: spiral (+), collecting wire (-) in the center of spiral and heated filament (-) outside the spiral (heated cathode).
- The same setup as in the case of open ion source: electron from heated filament are extracted towards the spiral, ionize gas, ions and electrons are captured on the wire and current is measured. $p = f$(current). Note: current depends on gas composition!
- Pressure range: 10^{-2} - 10^{-10} Pa (high vacuum, UHV)

Penning: ion tube with cold cathode.

- ions are formed in electric discharge in magnetic field.
- Pressure range: 1 - 10^{-4} Pa, some constructions down to 10^{-10} Pa.

Note: commercial spectrometers employ thermocouple and ion gauges.

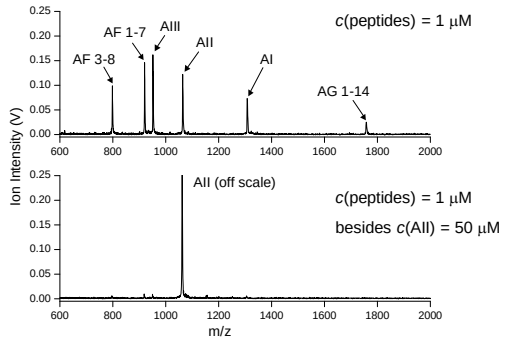
Coupling Separation to Mass Spectrometry

Why separation?

... analysis of very complex samples, mixtures of many analytes, e.g. common sample in proteomics contains >10^2 peptides

Simple MS or even MS/MS is not powerful enough for several reasons:
- high probability of occurrence of 2 or more analytes with the same *m/z*
- overlap of isotopic envelopes of analytes with close *m/z*
- mutual ion suppression
- limited dynamic range of mass spectrometer
- resolution of the 1st stage at MS/MS often unsatisfactory (R_1 ~ 500)
- removal of contaminants
- source of additional information about analytes

Ion Suppression in Peptide Mixtures



Classification of Interfaces

Gas → vacuum
- jet separator for analyte enrichment in carrier gas (particle beam)
- membrane interface
- capillary column or classical column + splitter

Liquid → vacuum
- API ionization techniques (ionization directly from liquid at atmospheric pressure)
- Flow probes (FAB)
- Sample deposition on a target
  - moving belt (deposition at atmospheric pressure – transport, differential pumping - ionization)
  - Fraction collection

Separation - ESI MS

Most common techniques:

2D GE - MS
- 2-dimensional gel electrophoresis on polyacrylamid gel
- band excision and protein processing
- common planar technique for protein separation

RPHPLC – ESI MS
- liquid chromatography on reverse phase – ESI MS
- common column technique for peptide analysis

Data dependent scan ... one MS scan followed by few MS/MS scans (discussed again later)

Separation of biomolecules: MALDI or ESI ?

ESI MS

+ Separation compatible
+ Commercially available, routinely used
+ ESI-MS/MS well established
+ High sensitivity
+ Easy automation
- No sample archiving only on-line
- Minor components not analyzed in MS-MS mode
- Quantification *vs.* MS-MS
- LC gradient often slow

MALDI MS

+ Simpler spectra
+ Separation decoupled from mass spectrometry
+ Capability of sample archiving

MALDI Interface

Common strategy:
1. Deposition of liquid sample on target
2. Sample drying
3. Target insertion into mass spectrometer and analysis

Regime
- On-line
- Off-line
- In-line

Addition of MALDI matrix
- Mixing with analyte solution (sheath flow, T, liquid junction)
- Deposition of analyte solution on target precoated with matrix layer

Collection of eluent
- Discrete fractions
- Continuous streak

MALDI Interface

Off-line

Targets with hydrophilic spots (anchorchip).
Micromethods using piezoelectric pipetors and microtargets.
Deposition using electrospray

On-line

Flow probe with or without frit
Nebulizer for aerosol generation

In-line

ROBIN Interface
Deposition on target at subatmospheric pressure, moving belt interface

Off-line vs. on-line

+ Separation decoupled from mass analysis
+ Sample archiving

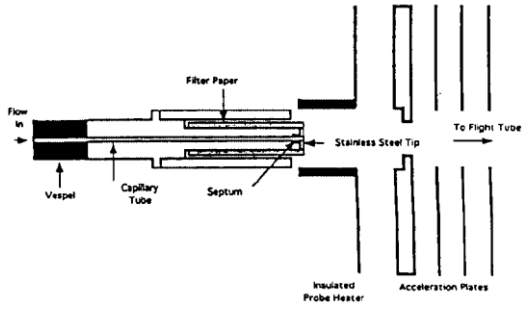
Sample Archiving (MALDI)

Strategy for separation - MALDI MS and MS/MS

1. Deposition of eluent on target
2. MALDI MS analysis (<10% sample consumed)
3. Analysis of MALDI MS data
4. MALDI MS-MS selected peaks (detail analysis)

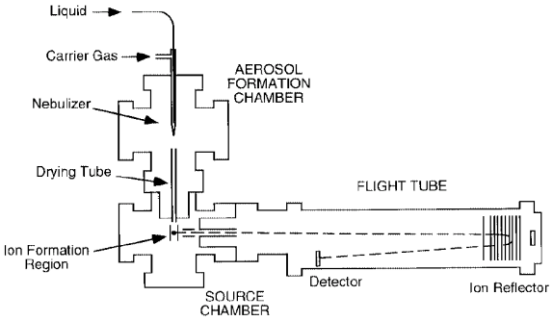
(The overall procedure may be interrupted anytime after the first step.)

Flow Probe (on-line)



(Whittal, R. M., Russon, L. M., Li, L. J. *Chromatogr. A* **1998,** 794, 367-375.)

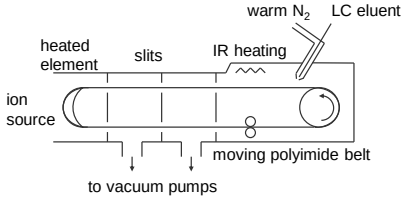
Nebulizer for Aerosol Generation (on-line)



(Fei, X., Wei, G., Murray, K. K. *Anal. Chem.* **1996**, 68, 1143-1147.)

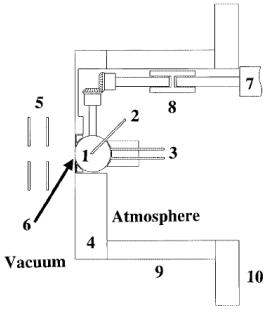
Moving Belt Interface (in-line)

Interface for continuous introduction of nonvolatile sample in volatile solvent into mass spectrometer



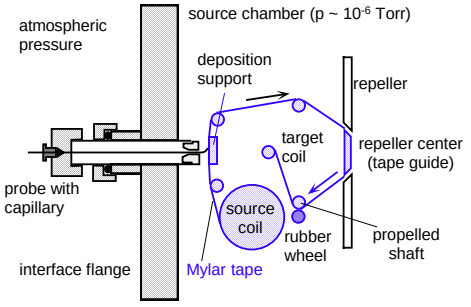
(McFadden W. H., Schwartz H. L. and Evans S. *J. Chromatogr.* 122, 389, 1976.)

ROBIN (Rotating Ball Inlet), in-line

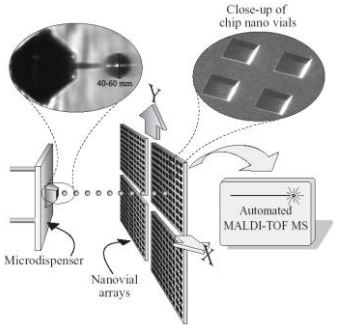


Orsnes, H., Zenobi, R. *Chem. Soc. Rev*. **2001,** 30, 104-112.

Vacuum or Subatmospheric Deposition
(in-line, off-line)

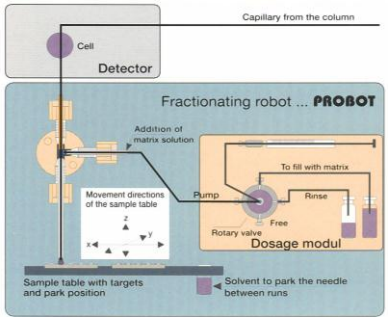


Micromethods



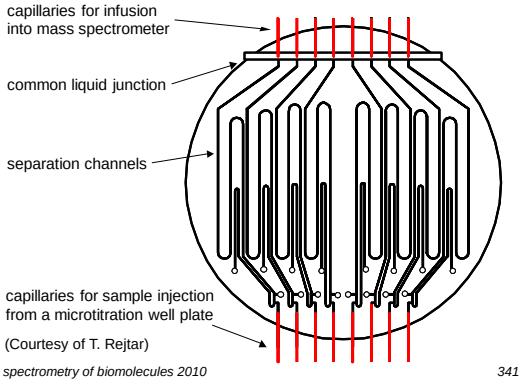
(Laurell, T.; Nilsson, J.; Marko-Varga, G. *Trends Anal. Chem.* **2001**, 20, 225-231.)

Automated Fraction Collection (off-line)



E.g. commercially available Probot (www.lcpackings.com)

Example of μdevice for Paralell Analysis



Microfluidic Devices and Mass Spectrometry

Why connect micro and macro? ... possible advantages of μdevices:

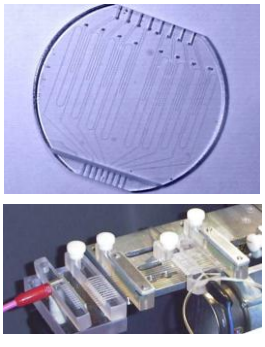
- integration of more steps, "lab on a chip" (sample preparation, purification, injection, separation...)
- paralell analysis (repeated motif on a single device)
- low cost – single use μdevice (serial production)
- all chemistry on the μdevice, MS as a detector

Basic types:

1. 2D array
   - affinity array: first selective preconcentration and/or capture of specific compounds, then MALDI MS directly from the μdevice
   - array of μvials with tips for ESI: higher reproducibility

2. Fluid μdevices (μdevices with channels)
   - systems for paralell analysis – for ESI, MALDI ...
   - systems integrating several steps

μdevice *vs.* Conventional Device



Biological Applications of MS

Genome, proteome, metabolome.
Small organic molecules, biomolecules, drugs, petrochemical products.
Biopolymers (DNA, proteins, carbohydrates).
Synthetic polymers.

Proteomics

Characterization of peptides and proteins – protein complement of genome.
Nowadays the main and the most perspective application of mass spectrometry.

Genome ↔ proteome. Level of protein expression (gen → protein) varies.

Genome is static, proteome dynamic: expression depends on type and function of protein, location in cell, state and health of cell. Function of organism is directly related to proteome.

Proteomics

Proteome analysis much more complex than genome analysis:
- more building blocks (amino acids)
- higher variability – modification of amino acids
- no existing amplification method for proteins analogical to PCR
- low levels of many proteins (e.g. regulatory proteins)

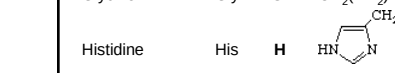
Differential proteomics

Determination of relative protein expression (presence or absence) in v influenced and healthy organism (organ, tissue, cell).

Functional proteomics

Determination of all interactions (protein-protein, protein-DNA, etc.) in given organism (organ, tissue, cell).

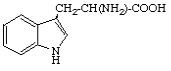
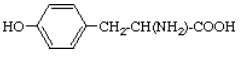
IUPAC: Amino Acids

Trivial name	Symbols		Formula
Alanine	Ala	**A**	CH_3-CH(NH_2)-COOH
Arginine	Arg	**R**	H_2N-C(=NH)-NH-$[CH_2]_3$-CH(NH_2)-COOH
Asparagine	Asn	**N**	H_2N-CO-CH_2-CH(NH_2)-COOH
Aspartic acid	Asp	**D**	HOOC-CH_2-CH(NH_2)-COOH
Cysteine	Cys	**C**	HS-CH_2-CH(NH_2)-COOH
Glutamine	Gln	**Q**	H_2N-CO-$[CH_2]_2$-CH(NH_2)-COOH
Glutamic acid	Glu	**E**	HOOC-$[CH_2]_2$-CH(NH_2)-COOH
Glycine	Gly	**G**	CH_2(NH_2)-COOH
Histidine	His	**H**	

IUPAC: Amino Acids

Trivial name	Symbols		Formula
Isoleucine	Ile	**I**	C_2H_5-CH(CH_3)-CH(NH_2)-COOH
Leucine	Leu	**L**	$(CH_3)_2$CH-CH_2-CH(NH_2)-COOH
Lysine	Lys	**K**	H_2N-$[CH_2]_4$-CH(NH_2)-COOH
Methionine	Met	**M**	CH_3-S-$[CH_2]_2$-CH(NH_2)-COOH
Phenylalanine	Phe	**F**	C_6H_5-CH_2-CH(NH_2)-COOH
Proline	Pro	**P**	
Serine	Ser	**S**	HO-CH_2-CH(NH_2)-COOH

IUPAC: Amino Acids

Trivial name	Symbols		Formula
Threonine	Thr	**T**	CH_3-CH(OH)-CH(NH_2)-COOH
Tryptophan	Trp	**W**	
Tyrosine	Tyr	**Y**	
Valine	Val	**V**	$(CH_3)_2$CH-CH(NH_2)-COOH

Development of Techniques for Ionization of Peptides and Proteins

1963	**LDI**	Laser Desorption/Ionization (R. Honig)
1969	**FD**	Field desorption (H. D. Beckey)
1974	**PD**	Plasma desorption (R. D. McFarlane)
1976	**SIMS**	Secondary ion mass spectrometry (A. Benninghoven)
1981	**FAB**	Fast atom bombardment (M. Barber)
1984	**ESI**	Electrospray (J. B. Fenn)
1988	**MALDI**	Matrix-Assisted Laser Desorption/Ionization (M. Karas & F. Hillenkamp, K. Tanaka)
1994	**nano-ESI**	Nanoelectrospray (M. S. Wilm, M. Mann)

Current Ionization Techniques in Proteomics

FAB
max. *m* ~ 10 000 Da
LD ~ 20 pmol (classical FAB), < 1 pmol (CF-FAB)

ESI
max. *m* ~ 100 000 Da (*z* >> 1)
LD < 10 fmol (routine)
LD ~ amol or 10^{-9} M (selected applications)

MALDI
max. *m* ~ 10^6 (practically unlimited – TOF analyzer)
relatively least vulnerable to contaminants
LD < 10 fmol (routine)
LD ~ amol or 10^{-9} M (selected applications)

Mass Spectrometers in Proteomics

MALDI MS	high sample throughput TOF
ESI MS-MS	structure elucidation QqTOF, IT, FT ICR
New instrumentation	MALDI TOF-TOF, MALDI LIFT TOF MALDI QqTOF

- fast identification in MS mode
- option of later detail analysis in MS/MS mode
  (result-dependent analysis)

Separation Methods in Proteomics

GE
gel electrophoresis on polyacrylamide gel

HPLC
high performance liquid chromatography on reverse phase

IEC
ion-exchange chromatography

AC
affinity chromatography

CE
capillary electrophoresis

Centrifugation
common centrifugation, gradient centrifugation

Gel Electrophoresis (GE)

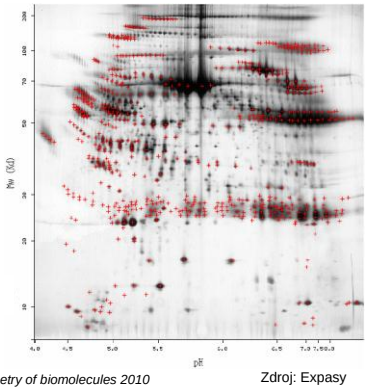
2D SDS PAGE

2D: 2-dimensional electrophoresis
1. dimension: according to p*I* (isoelectric focusation)
2. dimension: according to size of SDS after denaturation (charge/size = const.)

PA: polyacrylamide gel as separation medium

- Useful for separation of tens of thousands proteins, for very simple mixtures 1D GE sufficient
- Fast identification and protein characterization on 2D gel is the most common analysis of current proteomics
- Approximately 5% proteins and 30% peptides exhibit abnormal migration
- PTM's influence apparent protein *m* (errors up to 50%)
- Problematic protein transport from gel matrix (electroblot on a membrane)

Example: 2D GE of Human Plasma

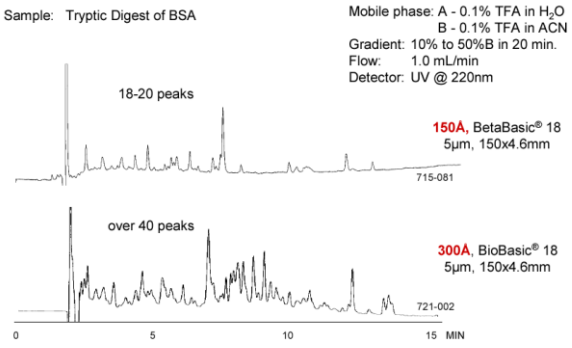


Zdroj: Expasy

RP HPLC

- Column: classical, capillary (ϕ ~300 μm) or nano LC (ϕ ~75 μm)
- Packing: C_{18}, particle size: 3 – 10 μm (RP ... reverse phase)
- Gradient elution, e.g.:
  - organic phase: ACN (acetonitrile) + 0.1% TFA (trifluoroacetic acid)
  - aqueous phase: 0.1% TFA
  - start: 10% organic phase + 90% aqueous phase
  - end: 80% organic phase + 20% aqueous phase
- Organic solvent promotes peptide solubility, enables detection in UV (230 – 240 nm) and evaporates fast, which is convenient for fraction collection, e.g. in MALDI MS.
- Standard technique of column separation for peptides and proteins

Example: RP HPLC of BSA Digest



(Source: ThermoFinnigan, 2002)

Enzymatic Protein Digestion

Most commonly used enzyme is **trypsin** (modified, e.g. TPCK to suppress chymotrypsin activity, methylation etc.).

Other enzymes and reagents: lysine, chymotrypsin, CNBr etc.

Products of enzymatic cleavage

- specific fragments of analyzed protein

  E.g. trypsin cleaves on C-terminus of amino acids K or R, if P is not a neighbor. (Actual rules more complex):

  N terminus-X-X-X-X-X-K-Y-Y-Y-Y-Y-Y-C terminus (Y≠P)
- non-specific fragments of analyzed protein
- artifacts (modification of amino acids, e.g. oxidative, due to PA gel etc.)
- fragments of enzyme (autolysis)
- keratin fragments ()

Enzymatic Protein Digestion in 2D Gel

1. Wash the gel slices for at least 1 hr in 500 microliters of 100 mM ammonium bicarbonate. Discard the wash.
2. Add 150 microliters of 100 mM ammonium bicarbonate and 10 microliters of 45 mM DTT. Incubate at 60 degrees centigrade for 30 min.
3. Cool to room temp and add 10 microliters of 100 mM iodoacetamide and incubate for 30 min in the dark at room temperature.
4. Discard the solvent and wash the gel slice in 500 microliters of 50% acetonitrile/100 mM ammonium bicarbonate with shaking for 1 hr. Discardthe wash. Cut the gel into 2-3 pieces and transfer to a 200 microliter eppendorf style PCR tube.
5. Add 50 microliters of acetonitrile to shrink the gel pieces. After 10-15 min remove the solvent and dry the gel slices in a rotatory evaporator.
6. Re-swell the gel pieces with 10 microliters of 25 mM ammonium bicarbonate containing Promega modified trypsin (sequencing grade) at a concentration such that a substrate to enzyme ratio of 10:1 has been achieved. (If the amount of protein is not known, add 0.1-0.2 microgramsof modified trypsin in 10 microliters of 25 mM ammonium bicarbonate).After 10-15 minutes add 10-20 microliters of additional buffer to cover the gel pieces. Gel pieces need to stay wet during the digest. Incubate 4 hrs to overnight at 37 degrees Centigrade.Proceed to step 8 if further extraction of the gel is desired (recommended)-otherwise continue with step 7.
7. Approximately 0.5 microliters of the supernatant may be removed for MALDI analysis and/or the supernatant acidified by adding 10% TFA to a final concentration of 1% TFA for injection onto a narrow-or microbore reverse phase column. (If necessary the sample's volume may be reduced~1/3 on a rotatory evaporator.)
8. Extraction (Optional)- Save supernatant from step 7 in tube X, and extract peptides from gel twice with 50 microliters of 60%acetonitrile/0.1% TFA for 20 min. Combine all extracts in tube X (using the same pipet tip to minimize losses), and speed vac to near dryness.Reconstitute in 20 microliters of appropriate solvent. Proceed with chromatography or MALDI analysis.

(http://www.abrf.org/ResearchGroups/ProteinIdentification/EPosters/pirgprotocol.html)

Enzymatic Protein Digestion

protein $\xrightarrow{\text{enzyme}}$ peptide mixture

Reasons for moving from high *m/z* region to low *m/z* region:

1. Better parameters of mass spectrometers (higher resolution, precision and accuracy of *m/z* determination, sensitivity)
2. Narrower isotopic envelope (simpler spectra, higher sensitivity)

Note.:

digestion = enzymatic cleavage

digest = mixture of protein fragments, products of digestion

modifications before digestion:

- reduction of -S-S- bridges using, e.g., dithiothreitol (DTT)
- alkylation –SH using, e.g., iodoacetamide (IAA) $\Rightarrow$ *m*: + 57 Da
- purpose: „unfolding of protein" ... better approach for enzyme

Strategy of Analysis in Proteomics

Basic Analysis Types

1. **Identification** (confirmation of presence of a known protein in sample)
2. **Relative quantification** in differential proteomics
3. **Sequencing of unknown** protein/peptide (de novo sequencing)

Sample is usually mixture of many proteins/peptides

⇓⇓⇓⇓⇓⇓

Analysis is based on combination of separation with MS and MS/MS

Identification

Amino acid sequence of many proteins has already been described and it is not necessary to analyze it again.

Strategy of Analysis in Proteomics

Top-down
- protein isolation
- protein processing, enzymatic cleavage
- analysis of peptides (MS, MS/MS)

Bottom-up
- enzymatic cleavage
- separation of peptides
- MS and MS/MS analysis of peptides

Some Useful Terms

genotype	genetic "equipment" of an organism, disposition
phenotype	actual state of organism, result of interaction with environment
in vivo	in living organism
in vitro	outside living organism, in artificial environment

http://www.meta-library.net/gengloss

Protein Identification

Information known prior analysis:
- Sample source (organism)
- Isoelectric point (p*I*) from 2D PAGE
- Molecular weight of protein from 2D PAGE, or possibly from MALDI MS
- N- and/or C- terminal portion of sequence (Edman degradation)

... The information is not sufficient for protein identification

Methods for protein identification

A. 2D GE + X + MS (peptide mass fingerprinting, peptide mapping)
B. 2D GE + X + RPHPLC + MS and MS/MS (sequence tag, AMT)
C. X + 2D LC (e.g. IEC and RHPLC) + MS and MS/MS
D. X + AC (afinity chromatography) + MS and MS/MS (IMAC, ICAT)
E. Strategy using X and isotopic reagents/standards
X ... selective enzymatic cleavage

Protein Identification

- MS is used to obtain information specific for protein
- Comparison of specific characteristics of analyzed protein with a protein library (containing specific characteristics) of known proteins.

Specificity is based on:

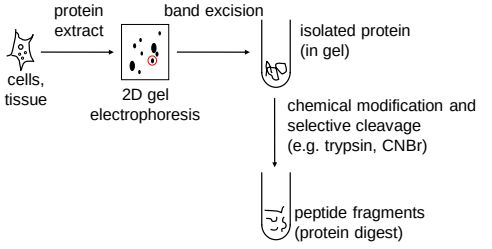
1. Analysis of more peptides related to the protein
   peptide mass fingerprinting (peptide mapping), MS analysis of products of enzymatic cleavage of the protein
2. Detailed analysis of a single peptide of the protein
   a) **sequence tag, MS-tag** – MS/MS analysis of a portion of the protein (fragmentation of a peptide in gaseous phase)
   b) **accurate mass tag, AMT** – determination of amino acid composition of a portion of the protein (accurate *m* of product of enzymatic cleavage)

- Requires accurate MS analysis and a quality database
- MS-based identification is much faster and more sensitive than chemical methods, such as Edman degradation)
- Negative result of database search may mean new protein discovery!!!

Peptide Mass Fingerprinting, PMF
(Peptide Mapping)

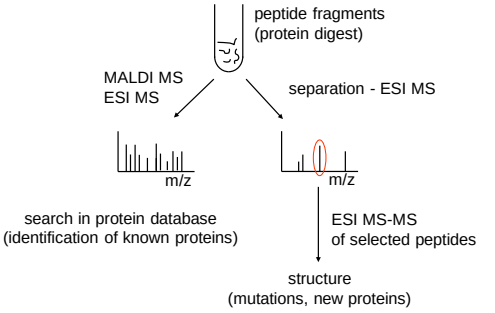
Confirmation of protein identity using MS of products of enzymatic cleavage

1. Separation (and possibly quantification) of proteins: 2D GE
2. Band excision, modifications, enzymatic cleavage



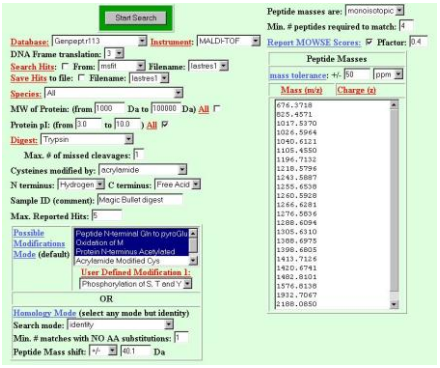
Peptide Mass Fingerprinting, PMF
(Peptide Mapping)

3. MS analysis and evaluation of results



Example of Database Search: MS Fit

Input:



Example of Database Search: MS Fit

Output:

MS Fit – Protein Identification from *m/z* values of peptide fragments using MS Fit program on http://prospector.ucsf.edu

Drawbacks of PMF

- Sample with a single protein needed, if possible, max. 2-3 proteins before cleavage
- *m/z* accuracy < 100 ppm or even better (<10 ppm) needed
- Better *m/z* accuracy ⇒ more confident answer from database search
- Absence of peptides in digest usually lower problem than presence of unexpected peptides
- Usually 4-5 peptides (in conjunction with high mass accuracy) sufficient for confident protein identification

PMF: Theory and Practice

Extra (unexpected) peaks

- non-selective cleavage (e.g. due to chymotryptic activity of trypsin)
- impurities (e.g. keratins)
- unsatisfactory protein isolation (additional proteins in band)
- enzyme autolysis
- post-translational modifications (PTM's), artifacts

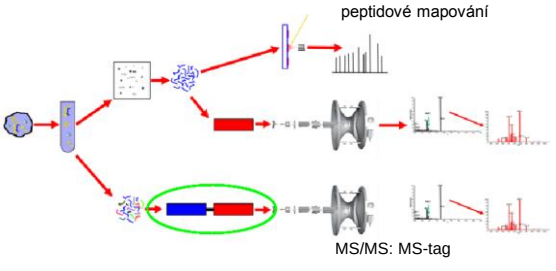
Missing peaks

- low-soluble peptides
- adsorption of peptides
- mutual suppression of peptides
- non-selective cleavage
- insufficient digestion (sterical restrictions)
- PTM's, artifacts

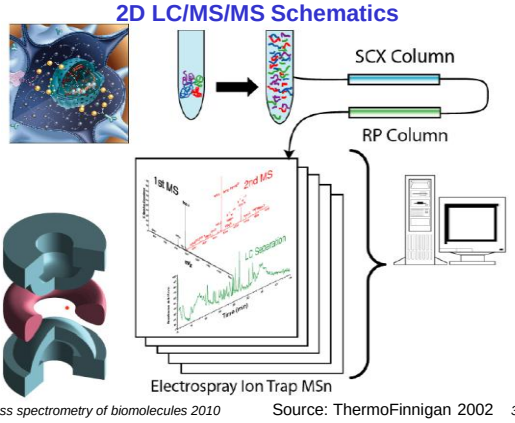
Sequence-Tag Method (MS-Tag)

- Protein identification based on knowledge of a portion of short portion of protein sequence (sequence-tag, MS-tag), usually sequence of a single peptide
- Precise determination of amino acid sequence of a protein fragment from digestion – usually using ESI-MS/MS or MALDI-PSD-MS mass spectrum or peak list
- Required length of sequence 3 or more amino acids
- The longer sequence is known, the more unambiguous identification
- The known portion of sequence is being searched for in protein databases
- Other information of protein: organism, *M.W.*, p*I* (both from 2D GE), etc.

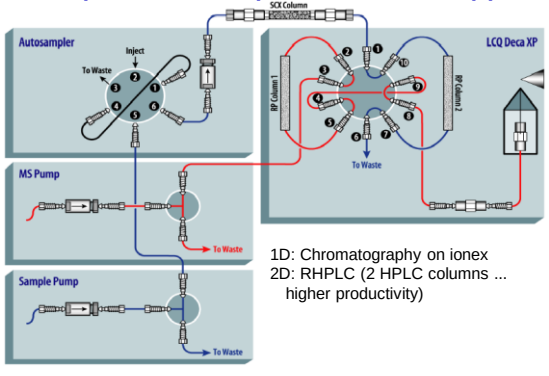
Basic Strategy of MALDI MS and LC - ESI MS



2D LC/MS/MS Schematics

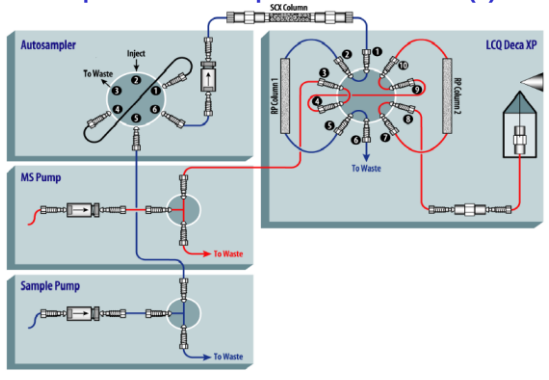


Experimental Setup for 2D LC/MS/MS (1)

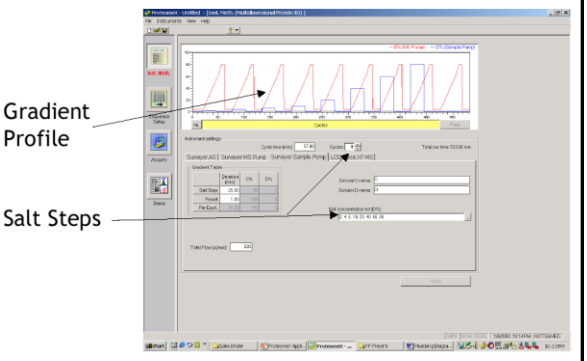


1D: Chromatography on ionex
2D: RHPLC (2 HPLC columns ... higher productivity)

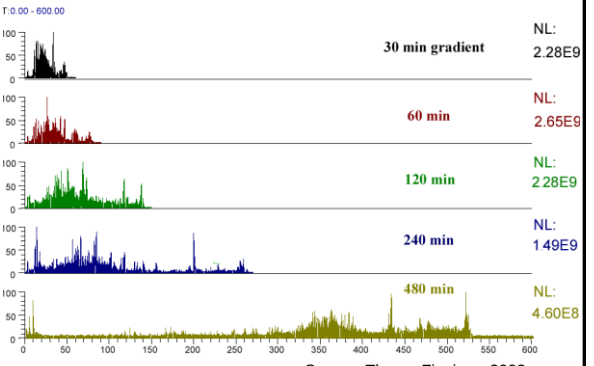
Experimental Setup for 2D LC/MS/MS (2)



Control of 2D LC/MS/MS Experiment



Results of 2D LC/MS/MS Experiment



LC-ESI MS and MS/MS:

Dynamic analysis LC-ESI MS/MS (data-dependent analysis):

- One MS sken followed by few MS/MS scans of main precursors
- In the case of more complex mixtures even MS^3 scans possible (e.g. for detection of phosphorylation)

Accurate Mass Tag (AMT)

- Identification of protein on basis of knowledge of accurate mass of a single peptide fragment (after enzymatic digestion of the protein)
- Accurate determination of protein mass of peptide using FT ICR MS

atom	H	C	N	O	S
m [Da]	1.0078246	12.000000	14.00307	15.994915	31.972072

- Typical number of amino acid residues in peptide chain ~ 10
- With precision of determination m/z < 1 ppm, there is high probability that the measured mass correspond only to a single amino acid composition as shown on the next slide

AMT

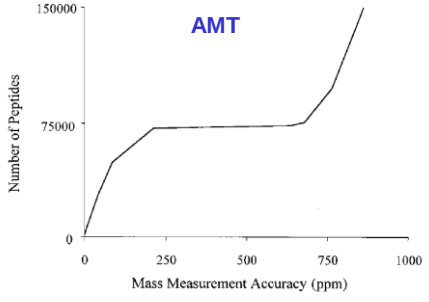


Figure 2. Number of 10-mer polypeptides at mass 1178.4 Da that can be distinguished as a function of mass measurement accuracy. As mass measurement accuracy improves beyond 100 ppm (~0.1 Da) the number of distinguishable polypeptides increases significantly.

(T. P. Conrads, G. A. Anderson, T. D. Veenstra, L. Paša-Tolić and R. D. Smith *Anal Chem.* **2000**, *72*, 3349-3354.)

Other Strategies of Proteome Analysis

- Not aimed at complex proteome analysis
- Based on column separation techniques (rather than on 2D GE)
- Simplification of the original mixture – selection of specific proteins/peptides, e.g. containing cystein (ICAT) or phosphorylated amino acid (IMAC) etc.
- Possible losses due to incomplete analysis are not dramatic and are compensated for by simpler and shorter analysis
- Additional tricks, such as use of isotope labels provide quantitative information about analyte

Isotope Labels MS of biomolecules

Unstable isotope labeling

- in radiochemistry

Stable isotope labeling

- common in MS
- basic approaches
  - use of inner standards labeled with isotope
  - incorporation of compound labeled with isotope into organism (useful for differential proteomics of lower organisms)
  - derivatization of analyte using reagent labeled with isotope

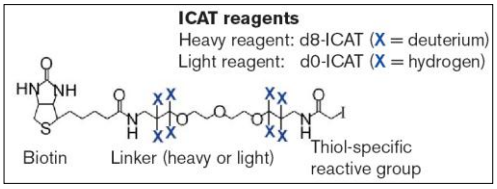
Some Methods and Abbreviations

GIST	Global Internal Standard Technology (^{2}H, ^{13}C, ^{15}N)
ICAT	Isotope-Coded Affinity Tags (cystein-containing peptides capture on affinity columns)
PhIAT	Phosphoprotein Istope-coded Afinity Tag (fosforylované peptidy)
IMAC	Ion Metal Afinity Chromatography (phosphopeptide capture on affinity columns)
AQUA	Absolute QUAntification (synthesized isotopically labeled peptides as internal standards)
SILAC	Stable Isotope Labeling with Amino acids in Cell culture (culture growth in normal and enriched media)
MUDPIT	Multidimensional Protein Identification Technology (SCX – RHPLC – MS/MS)

etc. etc.

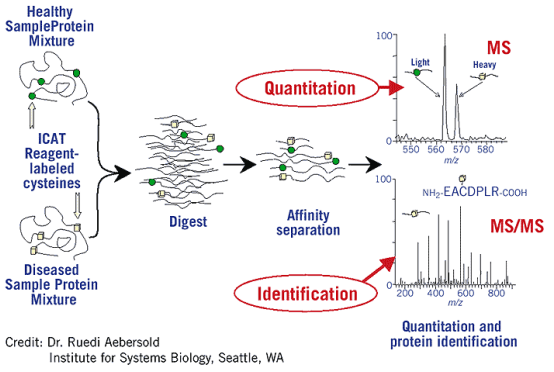
ICAT (Isotope-Coded Affinity Tags)

ICAT ... isotope-coded affinity tags (R. Aerbersold)
Determination of differences in protein expression from relative signal of peptides



1. Protein fractionation
2. Enzymatic digestion of entire sample
3. Isotope labeling (ICAT)
4. Mixing and MS analysis

ICAT



Credit: Dr. Ruedi Aebersold
Institute for Systems Biology, Seattle, WA

ICAT

Note

- Proteins can be labeled before digestion (step # 2 and 3 exchanged)

Drawbacks of 1st generation ICAT

- Mass difference of the isotope labels was equal to 8, which might lead to interferences in MS/MS spectra
- Slightly different retention of analytes labeled with light and heavy reagent. Result: the ratio b/w light and heavy form cannot be found from the ratio of ion intensities during HPLC MS/MS; integration across entire LC peak was necessary

2nd generation ICAT

- Use 9 ^{13}C atoms in the link (instead of 8 ^{2}H atoms)
- The same elution profile of light and heavy forms and less interferences

ICAT Characteristics

Advantages

+ Suitable for a range of protein samples (body fluids, cells, tissues, cultures)
+ Significant simplification of mixture
+ Compatibility with other methods suitable for analysis of minor proteins

Disadvantages

- Relatively large label (~500 Da)
- Not suitable for proteins without cystein (e.g. 8% in the case of yeast)

Determinations of Protein/Peptide Sequence

Classical analysis

- Edman degradation
- Determination of terminals (N, C)

Disadvantages: analysis time, relatively high cost and sample consumption

Current strategy: combination of more methods

- preparative separation
- enzymatic digestion
- isotope labeling
- MS
- MS/MS, ISF, PSD
- MS^n

Determination of Peptide Sequence using MS

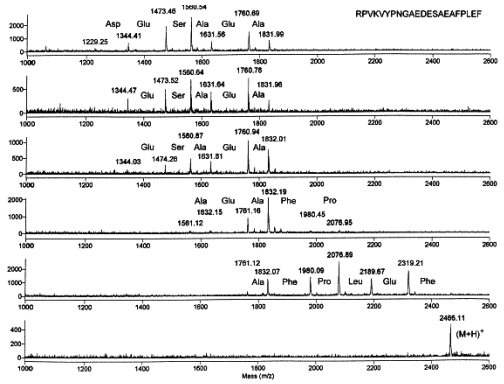
Combination of chemical cleavage and MS

1. Generation of mixture of peptide fragments differing by one amino acid:

a) phenyl isothiocyanate + $A_1A_2A_3..$ → phenylisothihydantoin$A_2A_3..$ + A_1
phenyl isothiocyanate + $A_2A_3..$ → phenylisothihydantoin$A_3..$ + A_2
etc.
phenyl isothiocyanate in low amounts as terminating reagent forms small fraction of phenylcarbamate of each peptide

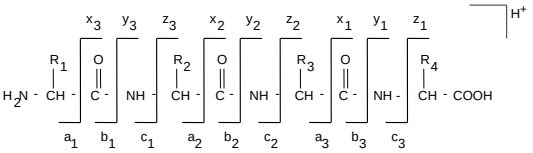
b) alternative strategy uses application of carboxypeptidase for different time periods or in different amounts ⇒ formation of different digests

2. MALDI MS of peptide fragment mixtures.
amino acid is determined from distance of adjacent peaks of the same type, sequence from the peak order



Patterson, D. H., Tarr, G. E., Regnier, F. E. and Martin, S. A., *Anal. Chem.*1995, *67*, 3971.

Schematics of Peptide Fragmentation



immonium ions of amino acids: 

Roepstorff & Fohlman, Biomed. MS 1984, 11, 601.

Determination of Peptide Sequence using MS/MS

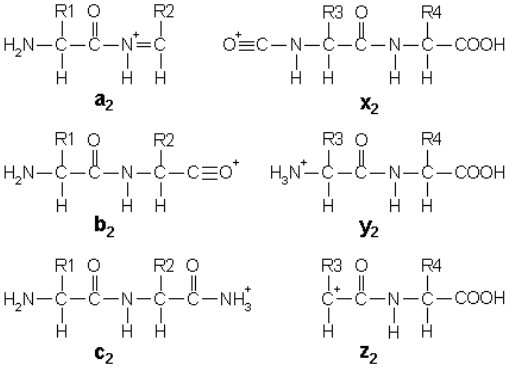
CID
- low-energy CID currently most popular fragmentation method of peptides
- IT, TQ, QTOF

ISD
- requires isolation of pure analyte
- MALDI TOF

PSD
- lower quality of spectra compared to CID, sometimes spectra stitching needed
- MALDI TOF

Note
- high-energy CID, SID, photodissociation, dissociation after electron capture are not used routinely yet
- Leu/Ile … isomers, Gln/Lys ... isobars

Fragments of Peptides



Peptide Fragmentation

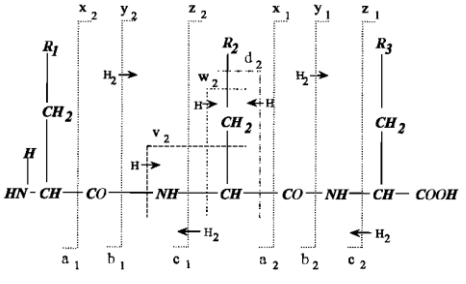
Fragmentation of 1 or 2 bonds in peptide chain
- fragments containing N terminus (a, b, c)
- fragments containing C terminus (x, y, z)
- these ions may be formed even after breakage of 2 bonds of the chain
- loss of NH_3, H_2O, CO_2

Fragmentation of side chain (amino acid residue)
- usually loss of a portion of side chain of amino acid
- fragments type d, w, v

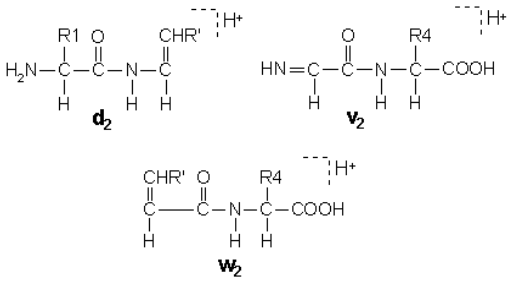
Inner fragments
- do not contain either N or C terminus ... lower analytical significance

Immonium ions
- useful information about amino acid presence; *m(IM)= m(AKres)* - 27

Schematics of Peptide Fragmentation



Other Types of Peptide Fragments



amino acid	*m*(mono)	*m*(immonium)	*m*(accompanying ions)
A	71.03712	44	
R	156.10112	*129*	59,70,73,87,100,112
N	114.04293	*87*	*70*
D	115.02695	*88*	*70*
C	103.00919	*76*	
E	129.04260	*102*	
Q	128.05858	101	56,84,*129*
G	57.02147	30	
H	137.05891	**110**	*82*,*121*,*123*,*138*,166
I	113.08407	**86**	44,*72*
L	113.08407	**86**	44,*72*
K	128.09497	*101*	*70*,84,112,129
M	131.04049	*104*	61
F	147.06842	**120**	*91*
P	97.05277	**70**	
S	87.03203	60	
T	101.04768	74	
W	186.07932	**159**	77,117,**130**,132,**170**,**171**
Y	163.06333	**136**	*91*,*107*
V	99.06842	72	41,55,69

m ... amino acid residuum

Calculation of Molecular Weight of Peptide/Protein

Sum of amino acid residua masses

+

Mass of terminals:

usually 1 Da (H) for N terminus (-NH_2)

and 17 Da (OH) for C terminus (-COOH)

+

Mass of proton, 1 Da – ion is usually in $[M+H]^+$ form

(but e.g. 23 Da for adduct $[M+Na]^+$ etc.)

CID Fragmentation

Types of ions in CID

1. b and y ion series
2. accompanying peaks
   - –17, NH_3 loss from Gln, Lys, Arg
   - –18, H_2O loss from Ser, Thr, Asp, Glu
   - a (tj. b – 28), satellite fragment series of b type (CO loss)
3. immonium ions of amino acids.
4. internal fragments – especially from Pro in the direction of C terminus

Precursor selection in CID MS/MS:

z = 2: $[M+2H]^{2+}$

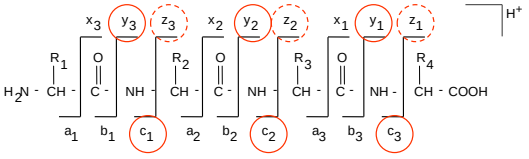
Ion with two charges provides higher quality CID spectra compared to ions with *z* = 1 a 3

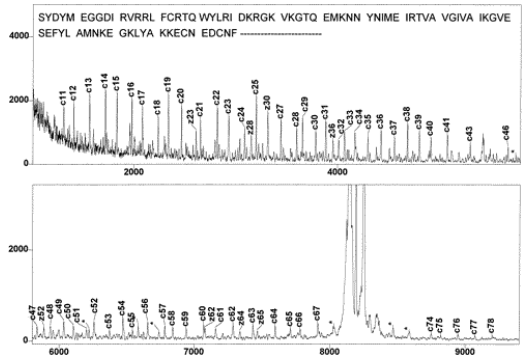
ISD fragments

Fragmentation rules … according to relative bond strength.

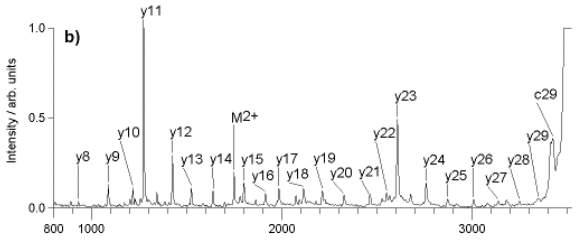
Typical products of peptide fragmentation:

c, y, z





ISD. Analyte: 20 pmol KGF analog. Matrix: sinapinic acid. Laser: N_2.
(V. Katta, D. T. Chow, M. F. Rohde *Anal. Chem.*, **70**, 4410 -4416, 1998.)



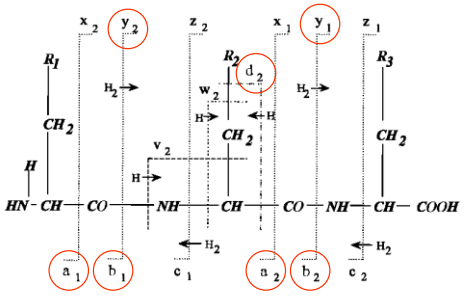
ISD
Analyte: oxidized B chain of bovine insulin
Matrix: thiourea
Laser: Er:YAG (l = 2936 nm)

(http://medweb.uni-muenster.de/institute/impb/research/hillenkamp/publicat/abs-cm99.html)

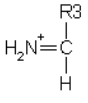
PSD Fragments

Typical products of peptide fragmentation: a, b, y, z, d.
Accompanying peaks –17 (loss of NH_3) a –18 (loss of H_2O).
Immonium ions of amino acids.
Internal ions – especially from Pro to the C terminus

(B. Spengler *J. Mass Spectrom. 32*, 1019-1036, **1997**)



immonium ions of amino acids:



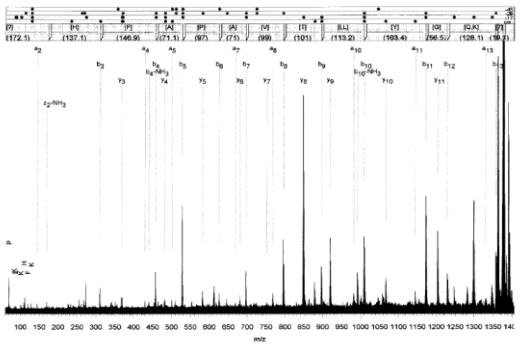
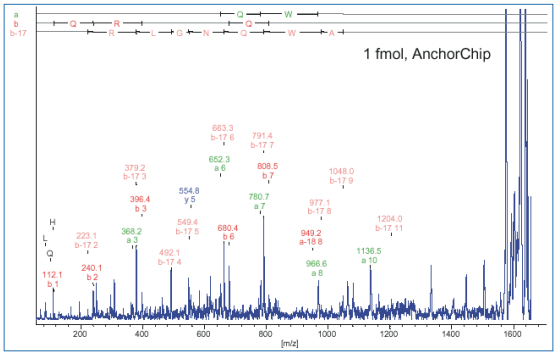


Figure 18. MALDI-PSD mass spectrum of the peptide at $[M+H]^+ = 1375.8$ u acquired on ALADIM I, Institute of Laser Medicine, Düsseldorf. Labeled are the ion signals interpreted as *N*-terminal and *C*-terminal ions. The bar above the labels describes the amino acid sequence determined. Dots in the top part of the figure correspond to neighborhood analysis of peaks (presence of a-type neighbors to b-type ions, satellites, etc.).

PSD, source: B. Spengler *J. Mass Spectrom. 32*, 1019-1036, **1997**



PSD of bombesin, source: Bruker Daltonics, GmbH 2000

Evaluation of MS/MS spectra of peptides

Manual and a semiautomatic deduction of sequence

- localization of ion series of *b* and *y* type (accompanying series *a*)
- identification of immonium ions
- identification of inner fragments
- interpretation complicated by occurrence of other fragments
- not all peptides are product of specific digestion, e.g. not all tryptic fragments are terminated with with R or K on C-terminus

... unambiguous determination of sequence of unknown peptide from CID is often impossible

Automated determination of sequence

- MS/MS spectrum of unknown peptide is compared with a database of computer-generated spectra of all peptides, which have *m/z* of precursor
- e.g. algorithm Sequest (J. R. Yates III et. al., 1994)

... much more successful than the deductive approach

Determination of Protein Sequence

Procedure

1. **Enzymatic digestion**
   production of more types of digests to generate overlapping fragments
   - various length of digestion
   - various enzymes (trypsin, V8 ...)
   - random cleavage, "shotgun sequencing"

2. **Determination of at least partial sequence of peptides**
   RPHPLC ESI MS/MS

3. **Deduction of sequence using PC**
   total sequence determined from combined portions of peptide sequences

Helpful Techniques for Sequence Determination

Protein hydrolysis in $H_2{}^{18}O$
identification of terminus: C-terminus will not be labeled

Hydrolysis of protein in mixture of $H_2{}^{18}O$ a $H_2{}^{16}O$ (1:1)
1. MS: dublets of tryptic peptides (except fragment of protein C-terminal)
2. MS/MS: both dublet peaks of a peptide selected as precursor for MS/MS; only fragments containing C terminus of a peptide will generate doublet fragments ... easier to survey spectra

Esterification (methylation) of carboxyl groups of a peptide
Δm = +14 / carboxyl group (-COOH $\rightarrow$ -$COOCH_3$)
comparison of original and resulting spectra
similar technique based on derivatization of amino group of a peptide

Note 1: exchange reaction may run even on C terminus
Note 2: more complex isotope patterns with masses M ($2x^{16}O$), M+2 ($1x^{16}O$,$1x\ 2x^{18}O$) a M+4 ($2x^{18}O$)

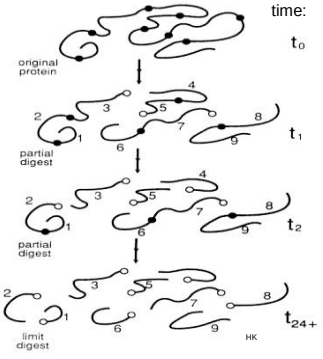
Determination of Protein Sequence

Example:

Various extent of fragmentation due to various length of digestion and use of isotope labels

Determination of terminus: hydrolysis in $H_2{}^{18}O$ (C-terminus will not be labeled)

Note: exchange reaction may run even on C terminus



Mutation Detection

Detection of exchange/absence of an amino acid(s) in protein chain

Procedure:
1. Enzymatic digestion
2. Analysis of mass spectra
   - missing peptides
   - superfluous peptides
3. MS/MS analysis of unknown (superfluous) peptides and their comparison with normal proteins/peptides, analysis of peak shifts

Post-Translational Modifications (PTM)

- modifications after translation RNA $\rightarrow$ protein on ribosome
- cannot be explained based on genome
- related significantly with protein function
- more than 200 PTM types described (database Delta Mass)
- often only small fraction of protein modified $\Rightarrow$ sensitivity
- requires selective isolation of peptides with modified amino acid
- bond between peptide and modifying group often weak

Post-Translational Modifications (PTM)

Common PTMs:
- phosphorylation
- glycosylation
- acylation (fat acid esters, acetyl)
- attachment of glycosylphosphatidyl inositol
- proteolytic products
- carboxylation (of glutamic acid)
- deamidation of asparagine and glutamine

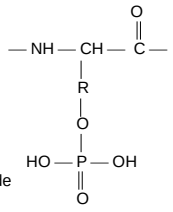
Some modifications might be quite complex, e.g. oligosaccharide modifications of glycoproteins (many types of sugars and many sites on protein that can accommodate sugars – O, N). For structural elucidation, combined methods using MS^n and enzymatic cleavage might be used (N-glycosidase, O-glycanase etc.).

Phosphorylation

- reversible PTM related to cellular regulatory mechanisms

- monoesteric bond of phosphoric acid group to side chain hydroxyl of

1. serine (167 Da)
2. threonine (181 Da)
3. tyrosine (243 Da)



MS/MS identification of phosphorylation

1. **Loss of H_3PO_4** (neutral loss scan: 98 Da) usually provides also ion $[M+H-98]^+$
2. **Detection of ion PO_3^-** (79 Da) in negative mode
   Parent ion scan for *m/z* (product) = 79
   Other negative ions: $H_2PO_4^-$ (97 Da), PO_2^- (63 Da)
3. **Fragment peak shifts** in MS/MS spectra

Phosphorylation

Identification procedure:

1. Preparation of mixture of proteolytic peptides (containing phosphopeptides)

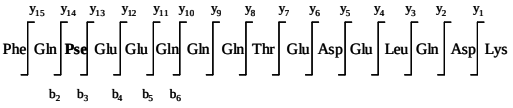
2. Separation of phosphopetides using e.g. HPLC, CE, affinity chromatography, such as Immobilized Metal Affinity Chromatography (IMAC), see Porath, J. *Protein Expression Purif.* **1992**, *3*, 263.

3. MS/MS analysis of phosphopeptides

Relatively stable monoester bond
⇒ **peak shifts in MS/MS spectra (Δm = + 80 Da)**

amino acid	M (residue)	M (monoester H_3PO_4)
serine	87	167
threonine	107	187
tyrosine	163	243

Example: ESI MS/MS of Oligopeptide with Monophosphorylated Serine

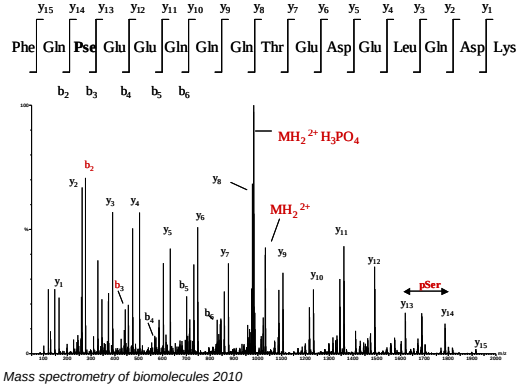


Information sources:

1. Molecular peaks MH_2^{2+} a $(M - H_3PO_4)H_2^{2+}$, mass difference Δm = 98 ($\Delta m/z$ = 49)
2. b-ions: $\Delta m/z$ (b_3-b_2)= 167
3. y-ions: $\Delta m/z$ (y_{14}-y_{13})= 167

(credit: K. R. Jennings)

Example: ESI MS/MS of Oligopeptide with Monophosphorylated Serine



Disulfide Bridge Analysis

cysteine (-SH) → cystin (-S-S-), intra- and inter- molecular bridges

Number of cysteines – using alkylation of cysteine

- e.g. alkylation of a single cysteine with vinylpyridine increase mass of protein/peptide by 105 Da ⇒ number of cysteines = Δm/105

Localization of cysteines in protein chain

Enzymatic cleavage and splitting of the digest into 2 aliquots:

1. aliquot ... MS analysis
2. aliquot ... reduction -S-S- (e.g. with dithioerythritol), then MS analysis

Comparison of the 2nd spectrum with the 1st spectrum:

1. Δm = 2 Da ⇒ peptide with an **intramolecular** -S-S- bond
2. the first peak disappear, two other peaks with lower *m/z* show up ⇒ **intermolecular** bridge between 2 peptides

Knowledge of sequence of amino acid (MS/MS) enables exact localization of -S-S- bridge in protein chain.

Influence of S-S Bridges in ESI-MS

Structures of lysozyme (β–sheet and α–helix) stabilized by 4 S-S bridges

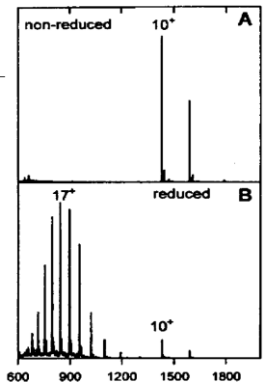
Reduction with 1,4-dithiothreitol (DTT) removes S-S bridges

⇓

„Unfolded" protein with more exposed basic amino acid residues

⇓

Higher number of charges

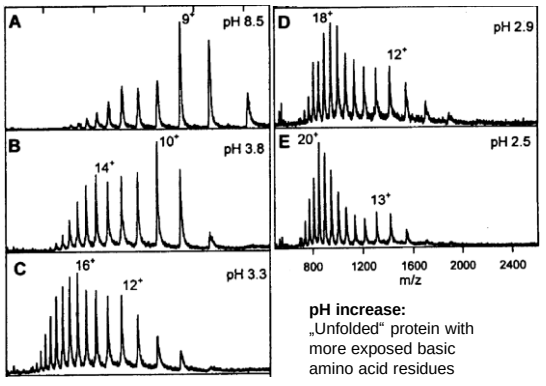


Noncovalent Interactions

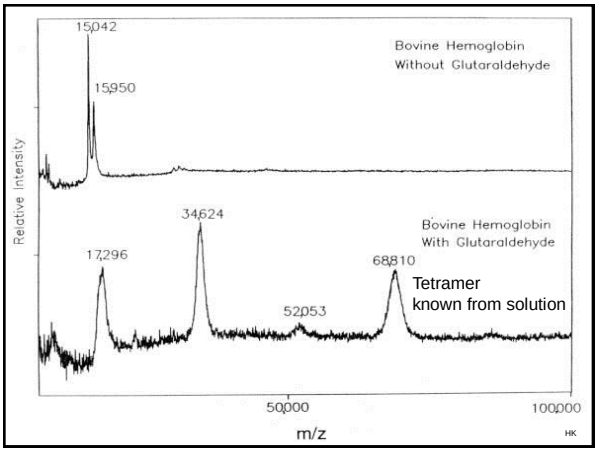
- Interactions with low mass ligands (+metal ions), other proteins, oligomers etc.
- The relation between occurrence of the complex in (g) and (l) phase is not always clear
- Complexes dissociate during ionization and MS analysis

Example:

1. Complexes of bovine hemoglobine are stabilized in form of tetramer by cross-linking with glutaraldehyde (bridges between lysine residues). Then MALDI MS (see next slide)
2. ESI for complexes of proteins with ligands (e.g. metaloproteins with drugs) ... soft ionization, which barely removes water molecules. Study of interactions between 2 proteins is more difficult – use of soft extraction conditions, favorable for formation of complexes in solution.



pH increase:
„Unfolded" protein with more exposed basic amino acid residues



Examples: Nanospray MS

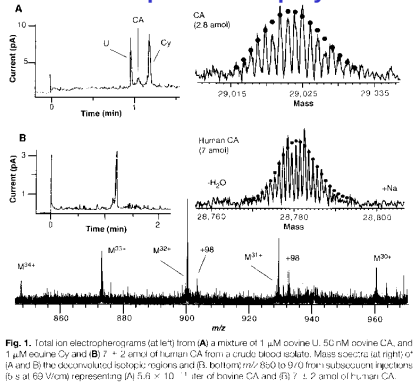


Fig. 1. Total ion electropherograms (at left) from (**A**) a mixture of 1 μM bovine U, 50 nM bovine CA, and 1 μM equine Cy and (**B**) 7 + 2 amol of human CA from a crude blood isolate. Mass spectra (at right) of (A and B) the deconvoluted isotopic regions and (B, bottom) *m/z* 850 to 970 from subsequent injections (*b* s at 69 V/cm) representing (A) 5.6 × 10 ⁻¹ ter of bovine CA and (B) 7 ± 2 amol of human CA.

CA = carbonic anhydrase, Cy = cytochrome
Valaskovic, G. A.; Kelleher, N. L.; McLafferty, F. W. *Science* **1996**, 273, 1199.

Scientific Databases on Internet

The databases below are just a few examples of many:

NCBI (National Center for Biotechnology Information)
Medline: www.ncbi.nlm.nih.gov
Scirus: www.scirus.com
ScienceDirect: www.sciencedirect.com

Databases for organic chemistry
NIST Chemistry WebBook (NIST)
Spectra Online (ThermoGalactic)
Spectral Data Base System, SDBS (NI AIST)

Scientific Databases on Internet

Internet sources for protein identification using MS

- Eidgenossische Technische Hochschule (MassSearch) www.cbrg.inf.ethz.ch
- European Molecular Biology Laboratory (PeptideSearch) http://www.mann.embl-heidelberg.de/GroupPages/PageLink/peptidesearchpage.html
- Swiss Institute of Bioinformatics (ExPASy) www.expasy.ch/tools
- Matrix Science (Mascot) www.matrixscience.com
- Rockefeller University (PepFrag, ProFound) prowl.rockefeller.edu
- Human Genome Research Center (MOWSE) www.seqnet.dl.ac.uk
- University of California (MS-Tag, MS-Fit, MS-Seq) prospector.ucsf.edu
- Institute for Systems Biology (COMET) www.systemsbiology.org
- University of Washington (SEQUEST) http://fields.scripps.edu/sequest/index.html

Scientific Databases on Internet

Other links:

UMIST (pepMAPPER) http://wolf.bms.umist.ac.uk/mapper/

European Molecular Biology Laboratory, Heidelberg (PeptideSearch) www.narrador.embl-heidelberg.de

Global Proteome Machine http://h112.thegpm.org/tandem/thegpm_tandem.html

Protein (peptide) databases

Genpept – NCBI GenBank

NBRF - National Biomedical Research Foundation

Swissprot - Swiss Institute of Bioinformatics

Owl - Leeds Molecular Biology Database Group

Delta Mass – protein posttranslational modification database

Useful Tools on Internet

Tools

MS-Comp – suggestion of possible combinations of amino acids, mass table of dipeptides

MS BLAST 2 – short sequences (6 amino acids)

BLAST a FASTA – protein homology

GlycoSuite DB, GlycoSciences – saccharides analysis

Mass calculators (GPMaw, SHERPA, PAWS, MW Calculator)

etc.

MS of Nucleic Acids and Oligonucleotides

- Ionization: usually more efficient for negative ions. Analysis typically in negative mode.
- Ionizaton technique: **MALDI** (usually IR MALDI)
- Less successful than in the case of proteomics
- Higher extent of fragmentation and more salt adducts
- Proper desalting essential … prevention of formation of a series of adducts with Na and K
- MALDI of heavy DNA (>100 kDa): linear TOF MS, IR laser, pulse extraction

MALDI MS of Nucleic Acids and Oligonucleotides

Typical matrices

3-hydroxypicolinic acid

2',4',6'-trihydroxyacetophenon

picolinic acid

Typical applications

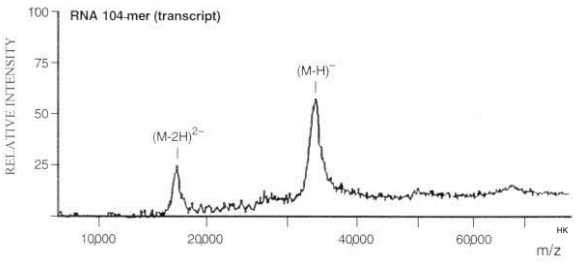
- characterization of synthetic and biologic oligonucleotides (determination of *M.W.*)
- analysis of PCR products, mutation analysis (absence or exchange of nucleotides)
- DNA sequencing
  - Sanger sequencing (classical method)
  - Sequencing using exonuclease
  - Fragmentation (in gaseous phase)

MALDI MS of Nucleic Acids and Oligonucleotides

- MALDI MS spectra of very heavy NA (>2 thousands nucleotides)
- *m/z* accuracy: < 1% ... the best of current methods
- excellent sensitivity: < 1 fmol

Berkenkamp, S; Kirpekar, F; Hillenkamp, F *Science* **1998**, *281*, 260-262.

IR MALDI MS of RNA



Sequencing NA using Exonuclease and MALDI MS

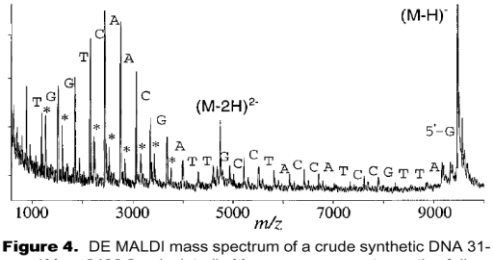
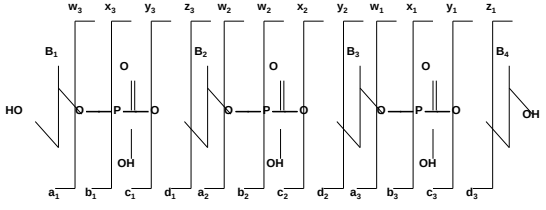


Figure 4. DE MALDI mass spectrum of a crude synthetic DNA 31-mer (M_r = 9486.2 calculated). Mass measurements on the failure products define the sequence up the 3′ trinucleotide (see Table 1). Asterisks indicate +80-Da satellite peaks: instrument, 1.3 m linear; matrix, 3-HPA.

(Juhasz, P.; Roskey, M. T.; Smirnov, I. P.; Haff, L. A.; Vestal, M. L.; Martin, S. A.; *Anal. Chem. ;* **1996**; *68*(6); 941-946)

Oligonucleotides Fragmentation in Gaseous Phase

Schematics of fragmentation of oligonucleotides in gaseous phase and marking of fragments



Saccharides Analysis

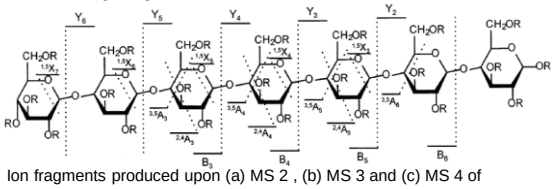
- Soft ionization methods: MALDI, ESI
- MS^n for determination of sequence and structure
- Enzymes used for partial cleavage of saccharides prior MS analysis
- Common monosacharides: glucose, manose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuramine acid (sialic acid).
- Determination of complete structure of oligosaccharides is more difficult than in the case of proteins and nucleic acids
  - result of isomeric nature of sugars and possible branching
  - knowledge of building blocks and sequence is not sufficient for structural elucidation. Type, location and optical configuration of each glycosidic bond needed

Nomenclature for MS/MS Oligosaccharides

- Nomenclature of MS fragments with charge on non-reducing side: A, B and C; fragments with charge on reducing side: X, Y and Z depending whether a circle or a glycosidic bond is cleaved.
- Lower index of B, C, Y and Z fragment ions determines number of cleaved glycosidic bonds, upper index of A and X fragment ions determines bonds that were cleaved. Lower indexes a, b etc. give cleaved branch of nonlinear saccharides.
- Fragments B, C, Y and Z together with mass differences can be used for determination of sequence and branching.
- MS is suitable for determination of optical isomery of glycosidic bond. Method is based on selective oxidation of β-anomer of derivatized hexoses using CrO_3; ketoester is formed.

MS^n of Oligosaccharides

a) MS^2 of 1497.8, $[M+Na]^+$ >



Ion fragments produced upon (a) MS 2 , (b) MS 3 and (c) MS 4 of permethylated maltoheptaose.

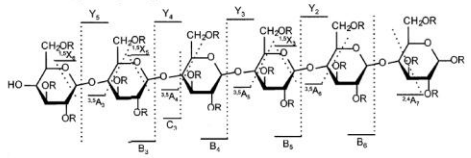
Glcβ1—4Glcβ1—4Glcβ1—4Glcβ1—4Glcβ1—4Glcβ1—4Glc

Maltoheptaose

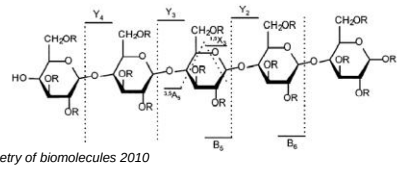
Source: Weiskopf, A. S.; Vouros, P. and Harvey, D. J. *Rapid. Commun. Mass Spectrom.* ***1997***, *11*, 1493–1504.

MS^n of Oligosaccharides

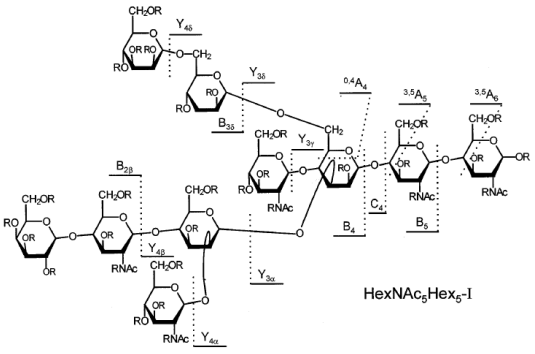
b) MS^3 of 1279.6, [M+Na]$^+$ > Y_6 >



c) MS^4 of 1075.5, [M+Na]$^+$ > Y_6 > Y_5 >



MS^n of Oligosaccharides



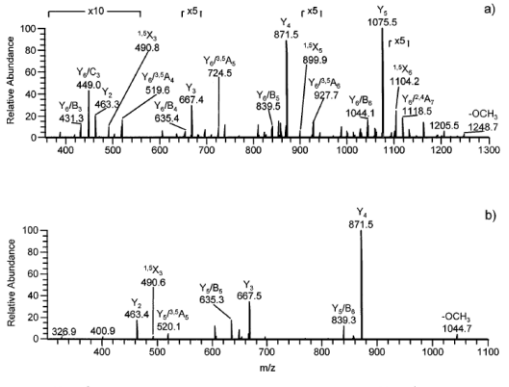
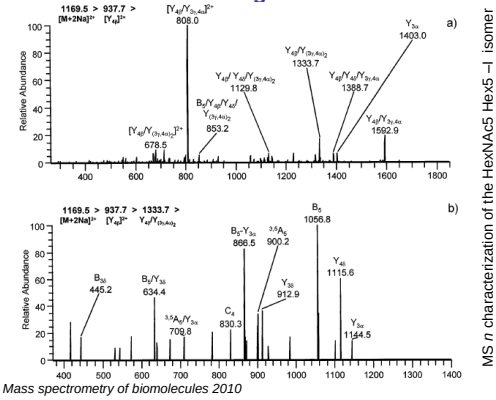


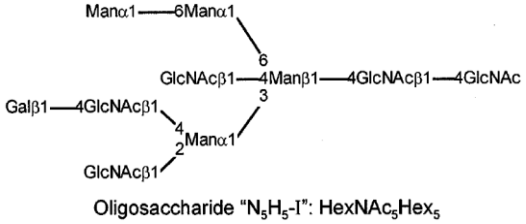
Figure 3. (a) MS^3 of permethylated maltoheptaose, *m/z* 1497.8 → 1279.6 →. (b) MS^4 of permethylated maltoheptaose, *m/z* 1497.8 → 1279.6 → 1075.5 →.

MS^n of Oligosaccharides



MS *n* characterization of the HexNAc5 Hex5 –I isomer from chicken ovalbumin. (a) MS 3 – *m/z* 1169.5 → 937.7 →, (b) MS 4 — *m/z* 1169.5 → 937.7 → 1333.7 →

MS^n of Oligosaccharides



Oligosaccharide "N_5H_5-I": $HexNAc_5Hex_5$

Lipids

Fat acids, acylglycerols, derivates of cholesterol, phospholipids and glycolipids

FAB, DCI (desorption chemical ionization), MALDI

Negative mode ([M-H]$^-$ ions detected)

Fragmentation

phospholipid negative ion

fragments of sacharides

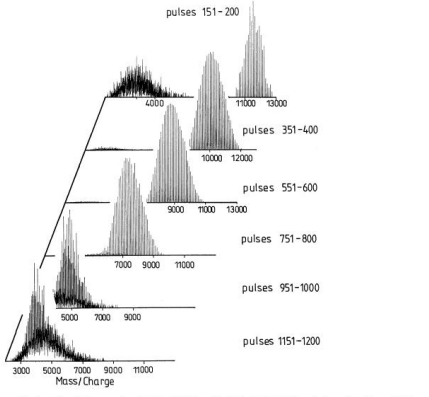
Analysis of Synthetic Polymers

MALDI TOFMS
high M_{max}, simple spectra, number of charges $z = 1$
alternative of GPC

Analysis
Building unit (monomer)
Terminal groups
Absolute molar weight
Polydispersion (mean m, dispersion width)

Challenges
Adduct formation with Na^+, K^+
Mass discrimination … ionization and detection efficiencies = f(m)
Correct transformation from time to mass domain

Example: Carman Jr, H.; Kilgore, D.; Eastman Chemical Company, USA, *ASMS 1998*



Source: Esser, E. *et al. Polymer* **2000**, *41*, 4039–4046

MALDI-TOF spectra of fractions from SEC separation of PMMA 10 900, on-line SEC–MALDI-TOF analysis, matrix: dithranol, LiCl.

Other Topics

... all topics were not covered, for example:

Fragmentation – chemistry of reactions in gaseous state

Ion mobitlity mass spectrometry

Isotope dilution/enrichment, quantification in MS

Preparative MS, history: preparation of ^{235}U

etc. etc.

Questions and Consultation

Training for examination

A consultation meeting can be scheduled in beginning of January

V. Questions and Consultation

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Dept. Analytical Chemistry 43, tel.: 541 129 271, preisler@chemi.muni.cz

Please download updated study material in pdf format:
http://147.251.29.118/MSBio/MSBio.htm

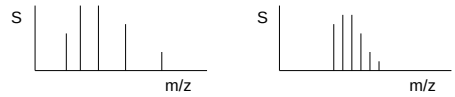
Please report any discrepancies/errors in the pdf document to me. Thank you.

Other Consultations in my office.

Exam dates
December? January? February?

Exemplary Questions

1. Compare MALDI and ESI (advantages vs. disadvantages).
2. What can be said about 2 different spectra of the same (single) peptide? (*m/z*-axis origin is not in zero, only a portion of the spectra is displayed.)



3. For 2 adjacent peaks of the same compound in ESI-MS, following values were determined: $(m/z)_1$~ 1001 and $(m/z)_2$ ~ 1501. Determine *m*.
4. Time of flight of ion $C_2H_8O_2N^+$ in TOFMS is 14.8 μs. Calculate the mass of a singly-charged ion, which hits the detector at 8.94 μs? What organic ion can it be?
5. What variables do have impact on resolution in TOFMS? Positive or negative influence?
6. Compare numbers of ions that can be detected during acquisition of a single spectrum using FT-ICR-MS and quadrupole filter?

Exemplary Questions

7. Why instruments that can monitor both isotopes at the same time are preferred for isotope ratio determination?
8. Can a quadrupole filter be used for analysis of a protein with *M.W.* 30 kDa?
9. What ionization method and what mass spectrometer would you suggest for:
   a) explosive detection on airport
   b) sequencing of short peptides
   c) semiquantitative determination of ~70 samples in geologic sample
   d) identification of elemenal impurities in thin surface layer of sample
10. What is the origin of Lorentzian peak profile in FT-ICR-MS?
11. What is mutual orientation of equipotential level of *U* and electric field intensity vector *E*?
12. What will be the difference between velocities of two ions with *m* = 100 a.m.u., *z* = 2, initial velocity v_{01} = 100 m/s and v_{02} = 200 m/s after acceleration by 1 kV and 10 kV?

Exemplary Questions

13. What determines the practical upper *m/z* limit in TOF MS?
14. Compare challenges in determination of peptides and DNA oligomers using MS.
15. You are about about to analyze peptides/proteins in a soup. Adding of what compound will you try to avoid prior to MS analysis? How would you modify the soup and what ionization technique will you use?
16. What detector fits ion trap better: MCP, channeltron, electron multiplier, photographic plate or Faraday cup?
17. What is the influence of time dispersion (of ion formation) on resolution of ion trap?
18. What is *m* of amino acid A, its residuum (in peptide chain) and its immonium ion?
19. Explain the plateau on the graph of number of peptides vs. *m/z* accuracy (AMT method) between 200 and 700 ppm accuracy.
20. Compare advantages and disadvantages of 2DGE and column separation techniques in proetomics.

Exemplary Questions

21. What parameters of mass spectrum may improve if signal is recorded using a) FT ICR MS, b) TOF MS, c) IT for longer period?
22. Why does mass spectrometer need to be evacuated prior to any measurement?
23. What is the most significant reason of limited resolution in MALDI TOF MS? Explain principles of techniques leading to resolution improvement in MALDI TOF MS.
24. What is the difference between units u, Da a Th?
25. What is the difference between energy and velocity dispersions of ions?
26. In what region of TOF mass spectrometer are analytically important fragments generated during a) MALDI ISD, b) MALDI PSD?