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Prematerial concepts for UHPLC-QTOF users

JYU-Mslab 21.11.2023

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1. Concepts: Mass spectrum

Mass spectrum is a two dimensional presentation of the MS analysis:

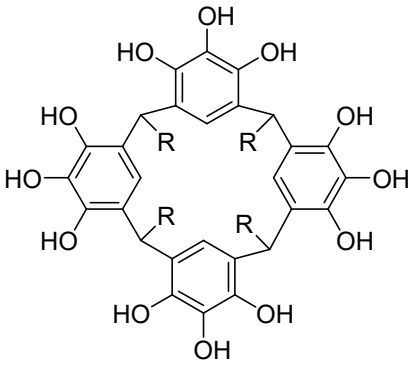
- x-axis: *m/z* ratios of the ions
- y-axis: relative or absolute abundance of the ions

m/z ratio =
monoisotopic molecular mass of the ion / its charge

(NOTE: not molar mass!)

mass peak =
signal from an ion at certain *m/z* value

mass range =
m/z range on which spectrum is measured or shown



$R=CH_2CH_3$
MW 664
P1

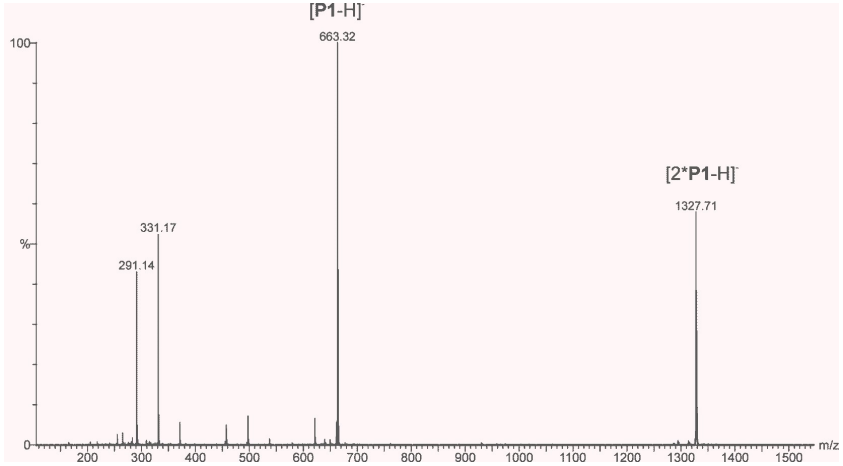


Fig. 1. Pyrogallarene (P1) ESI-MS mass spectrum on negative polarization

- The height of a mass peak represents the relative or absolute abundance
- This can be shown as absolute intensity (counts, a.I.) or as relative intensity (r.I.)
- In relative intensity presentation there are two possibilities:

a) the highest peak gets a value of 1.0 or 100 % and the other are shown relative to that
b) the total ion current gets a value of 1.0 or 100 % and the peaks are shown as fractions of ion current

Total ion chromatogram (or current) = TIC
=sum of the peak intensities at certain time = sum of all ions arriving on detector

Extracted ion chromatogram = EIC
= sum of peak intensities for certain ion(s)

Base peak
= the highest peak in the spectrum

NOTE 1: mass spectrum shows ions only from chosen polarization (+ or -), not on opposite polarization ions or neutral molecules

NOTE 2: peak has intensity and ion has abundance

mass list =
numeric representation of mass spectrometric result.

In mass list *m/z* values and corresponding peak intensities are shown as a list.

2. Ions

- Mass spectrometry can only observe ions (molecule with non-zero electrical charge). Neutral compounds must be ionized prior the analysis.

- Ionization can take place in various ways depending on sample and ionization technique used → different ion types are produced.

- Ions are marked in square brackets and charge is marked as upper index.

e.g. $[M]^{+}$, $[M+X]^{-}$, $[M+Na]^{+}$, $[M+32H]^{32+}$

NOTE: Molecules under analysis are often marked with letter M, other notations are also possible, but use of letters H and K should be however avoided.

2.1 Radical ions:

- *Radical ions* are formed when compound donates or accepts an electron.
- *Molecular ions* are radical ions and they are formed when neutral intact compound (M) donates or accepts an electron.
- Molecular ion is radical cation ($M^{+\bullet}$) or radical anion ($M^{-\bullet}$) and it has uneven number of electrons (*odd-electron ion,* $OE^{+\bullet}$).
- Generally only singly charged ions are formed (the highest possible charge state is +2).
- Radical anion is formed through capture of an electron, which is often unfavourable and rare incident. Radical anion can form if molecule contains strongly electronegative groups.
- Molecular ions are often formed during electron ionization (EI) and if the molecular ion has enough energy, it commonly dissociates further.

NOTE: sometimes notification $M^{\bullet+}$ is seen, but in MS charge is marked prior the unpaired electron.

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

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

2.2 Protonated / deprotonated ions:

- Ionization takes place if molecule donates or accepts a proton H^+.

$$M + H^+ \longrightarrow [M+H]^+$$

$$M - H^+ \longrightarrow [M-H]^-$$

- Protonated / deprotonated ions have even number of electrons (*even-electron ions* EE^+/EE^-)
- Common ionization event in electrospray ionization (ESI)
- Protonation is common *e.g.* with amines, which have relatively high proton affinity.
- Depending on analyte, also multiply charged ions can be formed:

$$M + nH^+ \longrightarrow [M+nH]^{n+}$$

$$M - nH^+ \longrightarrow [M-nH]^{n-}$$

  - If there are multiple sites, which can accept or donate a proton, electron or another charged group and multiply charged ion is large enough
  - tendency to form multiply charged ions increases when the size of a molecule increases (better stabilization of multiple charges)
  - for example proteins and peptides commonly exist as multiple charged ions due to large number of polar groups. Large proteins can stabilize even 100+ charge.

2.3 Adduct ions:

- When neutral molecule adopts an ion (other than proton) positive or negative *adduct ion* is formed.
- Common ionization event in electrospray ionization (ESI).
- Generally ions at low charge states are formed.
- Alkali metal and ammonium adducts are common cationic adducts (common with carbonyl compounds which does not have acidic hydrogen).
- Halide and carboxylate adducts are common anionic adducts.

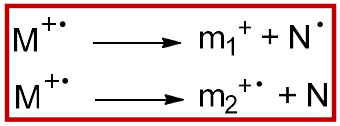
$$M + Na^+ \longrightarrow [M+Na]^+$$
$$M + NH_4^+ \longrightarrow [M+NH_4]^+$$
$$M + Cl^- \longrightarrow [M+Cl]^-$$
$$M + CH_3COO^- \longrightarrow [M+CH_3COO]^-$$

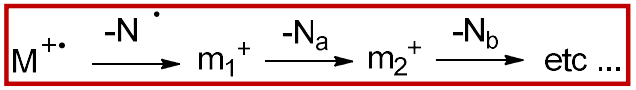
NOTE: Na^+, K^+ and Cl^- are present in small quantities almost everywhere and observation of them as adduct formers is common even if they are not added in sample.

Cluster ion = $A_xB_y^{n+}$ (complex ion consisting of neutral molecules and anions or cations).

2.4 Fragment ions:

- Ionic dissociation products resulting from ion fragmentation (dissociation) are called *fragment ions* *(earlier daughter ion*).
- *Precursor ion* is the ion where fragment ions originate from (earlier *mother ion*).
- Fragment ions are formed as a result of covalent bond dissociation
- Dissociation can take place during ionization or during tandem MS experiment.
- Fragment ions give structural information on analytes.
- Fragment ions can fragment further, forming a *dissociation pathway*.
- $OE^{+\bullet}$ ions can dissociate to EE^{+} fragment ion and radical or to $OE^{+\bullet}$ fragment ion and neutral molecule.
- EE^{+} ions can only dissociate to EE^{+} fragment ions and neutral molecules.





NOTE: if precursor ion is singly charged, exist fragment ions on smaller *m/z* value than that of precursor ion.

If precursor ion is multiply charged, it can produce fragments which have higher *m/z* values than that of precursor ion.

3. Units

- Mass spectrometry measures the ratio between mass and charge *m/z*, which is unitless symbol.
- Mass (molecular mass M_r or molecular weight MW) is expressed in atomic mass units (u).

  1u = 1.660540 x 10^{-27} kg = 1 Da (NOT: g/mol!)

  1u = 1/12 ^{12}C atomic mass

- Especially in the case of biomolecules Dalton (Da) is often used.
- Charge of an ion is expressed as elemental charge (e = 1.602177x10^{-19} C).

NOTE 1: before 1961 amu was used, which is 1/16 of ^{16}O atomic mass

NOTE 2: in some books (not common) unit Th is used, Th= thomson = *m/z*

NOTE 3: *m/z* is writen with italics and located at the front of number

4. Mass values

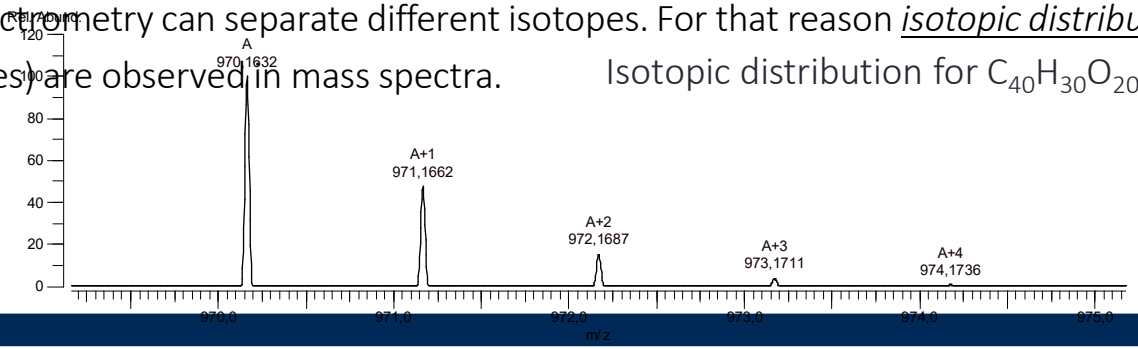
- Mass of the material consisting of certain element is a sum of its isotopes and their natural abundances.

- Isotopes are variants of same element, which have different mass (same number of protons, but different number of neutrons in nucleus)

- For example carbon isotope ^{12}C nucleus consists of 6 protons and 6 neutrons (12.000000 u). Carbon isotope ^{13}C has 7 neutrons in nucleus (13.00335 u). These have natural relative abundances of 98.93 % and 1.07 %.

- Mass spectrometry can separate different isotopes. For that reason *isotopic distributions* (envelopes) are observed in mass spectra.

Isotopic distribution for $C_{40}H_{30}O_{20}N_{10}^{+}$

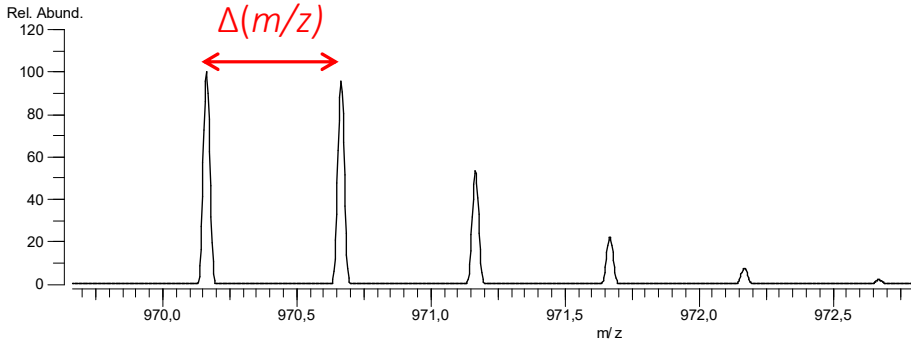


- Molecular mass difference between common isotopes ~ 1 u.
- Ion charge state can be determined using isotopic patterns.
- Difference between peaks originating from different isotopes is 1/z.

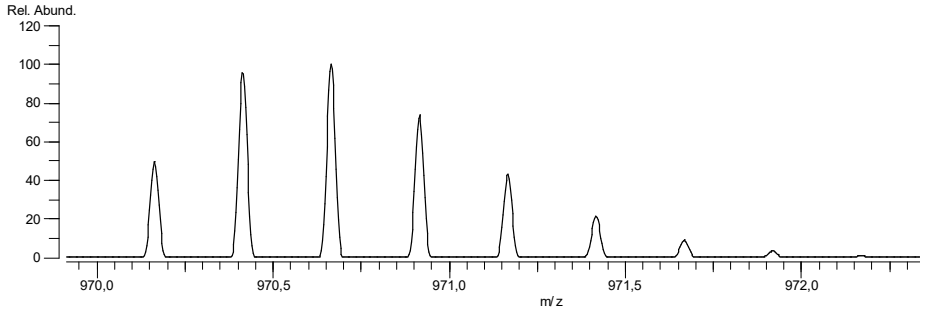
$\Delta(m/z) = 1/z \rightarrow z = 1 / \Delta(m/z)$

- Singly charged ion shows isotopic distribution where separation of isotopic peaks is ~ 1, for doubly charged ion separation is ~ 0.5 (1/2) and for ion at charge state 100^{+} ~ 0.01 (1/100).

Isotopic distribution for $C_{80}H_{60}O_{40}N_{20}^{2+}$



Isotopic distribution for $C_{160}H_{120}O_{80}N_{40}^{4+}$



Molecular mass can be presented in different ways:

1. *nominal mass* is obtained if most abundant atomic weights rounded to integer are used (12 u, 1u, 16 u...).

Used with low resolution instruments and MS/MS experiments

Example. $CH_3OH = {}^{12}C^{1}H_4{}^{16}O = 12 + 4x1 + 16\ u = 32\ u$

2. *monoisotopic mass (exact mass*) is a sum of atomic mass values for the lightest isotopes*

Used for accurate mass determinations and identification of ions.

Example. $CH_3OH = {}^{12}C^{1}H_4{}^{16}O = 12.0000 + 4x1.0078 + 15.9949\ u = 32.0262\ u$

3. *average mass* (molar mass) is average (averaged over all natural isotopes) mass of each element

Used to prepare samples with known concentration

Example. $CH_3OH = 12.0112 + 4x1.0079 + 15.9994\ u = 32.0423\ u$

4. *isotopic mass* is like monoisotopic mass, but any other isotope than the lightest is used

Example. ${}^{13}CH_3OD = {}^{13}C^{1}H_3{}^{2}H^{16}O = 13.0034 + 3x1.0078 + 2.014 + 15.9949\ u = 34.0357\ u$

*NOTE: some books refer to most abundant, not lightest isotope

Molecular mass vs. *m/z* :

- Mass spectrometer measures *m/z* values for ions, not molecular mass values for neutral molecules

- For a positive ion without adduct formation:

$m_{ion} = z(m/z)_{ion}$ molecular mass for ion

$m_{neutral} = m_{ion} + zm_e$ ($m_e = 0.000549$ u) molecular mass for neutral molecule M (z=+)

- For negative ions:

$m_{neutral} = m_{ion} - zm_e$

- For multiply charged protonated or adduct ion $[m+zA]^{z+}$:

$m_{neutral} = z[(m/z)_{ion} - (m_A - m_e)]$

NOTE: To calculate exact m/z values you need to take charge state into account!

Tools to calculate m/z values:

- *ChemDraw, ChemSketch:* molecular formula and mass values from structure drawing
- Agilent Isotope distribution calculator
- *MS softwares, Exact Mass Calculator, ChemCalc:* monoisotopic mass, *m/z* value and isotopic distributions from molecular formula

Warning!!! All softwares don´t take mass of an electron into account in the case of ions. This should be checked for new software before use.

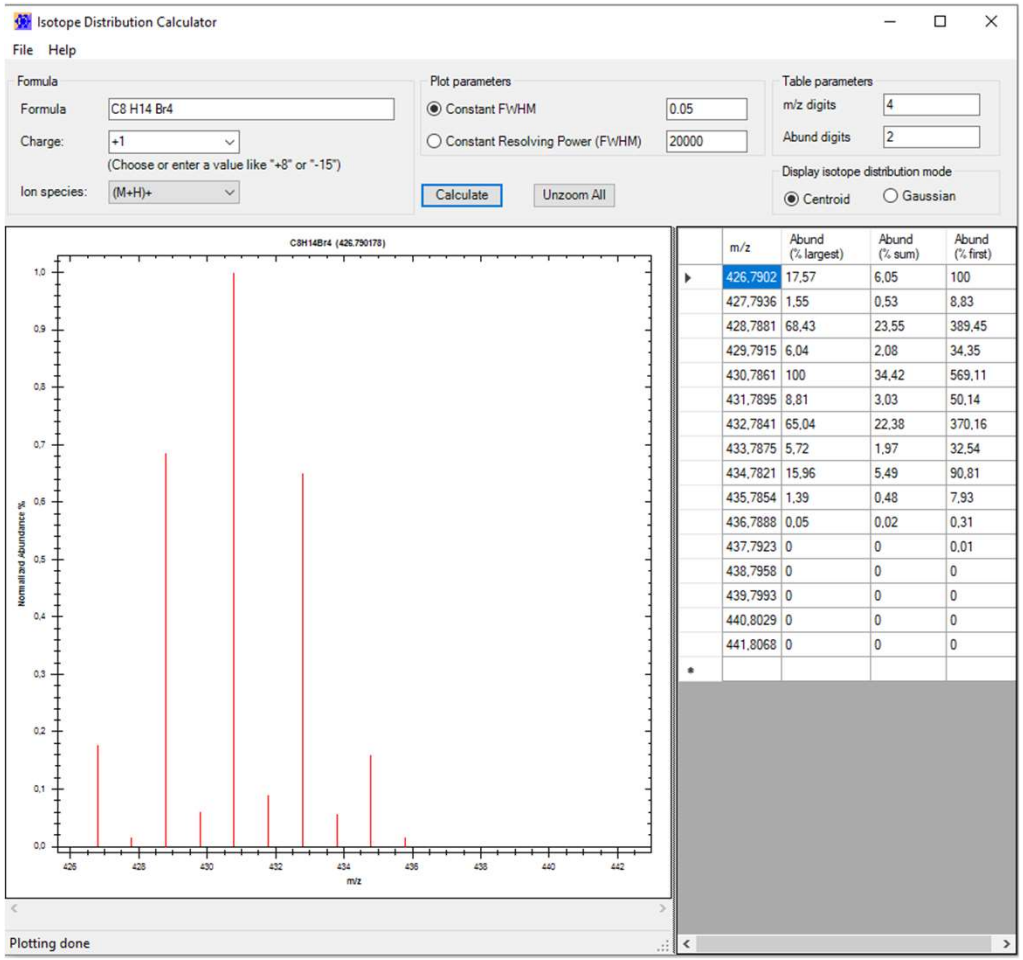
ChemCalc available at www.chemcalc.org:

1. Insert molecular formula and charge of an ion at formula field, *i.e. C500H604++++*
2. Adjust resolution
3. Press Submit

Molecular weight = average mass

Monoisotopic mass = monoisotopic mass for neutral

Monoisotopic experimental mass = theoretical *m/z* value (exact mass)



Accurate mass

- Nominal mass is not sufficient for determination of elemental composition
- *accurate mass* = **experimentally** measured monoisotopic mass or *m/z* value for an ion, which can be used to determine molecular formula.
- exact mass = **theoretical** monoisotopic mass or *m/z* value for an ion according to molecular formula
- *mass accuracy* = difference between accurate mass (experimental) and exact mass (theoretical)
- In calculations usually most abundant or lightest isotopic mass is used
- *mass defect* = difference between nominal mass and exact mass.

$$m_{\text{mass defect}} = m_{\text{nominal mass}} - m_{\text{exact mass}}$$

For example: for ^{1}H mass defect is -0.00782 u and for ^{16}O 0.005085 u. (^{12}C does not have mass defect).

- Mass accuracy can be described in two ways:

Absolute mass accuracy $\Delta m/z = m/z_{theor} - m/z_{exp}$ *(mu or mDa)*

Relative mass accuracy $\delta m/m = (\Delta m/z \,/\, m/z_{theor}) \times 10^6$ (in ppm)

- High resolution (HR) accurate mass result is valid if mass accuracy is < 3 mDa (absolute) or < 5 ppm:n (relative)
- Otherwise low resolution (LR) nominal mass result is used.
- As the mass of compound increases (or number of different elements), the number of possible compositions increases.

→ the larger the compound, the higher mass accuracy (absolute) is needed for unambiguous determination of elemental composition.

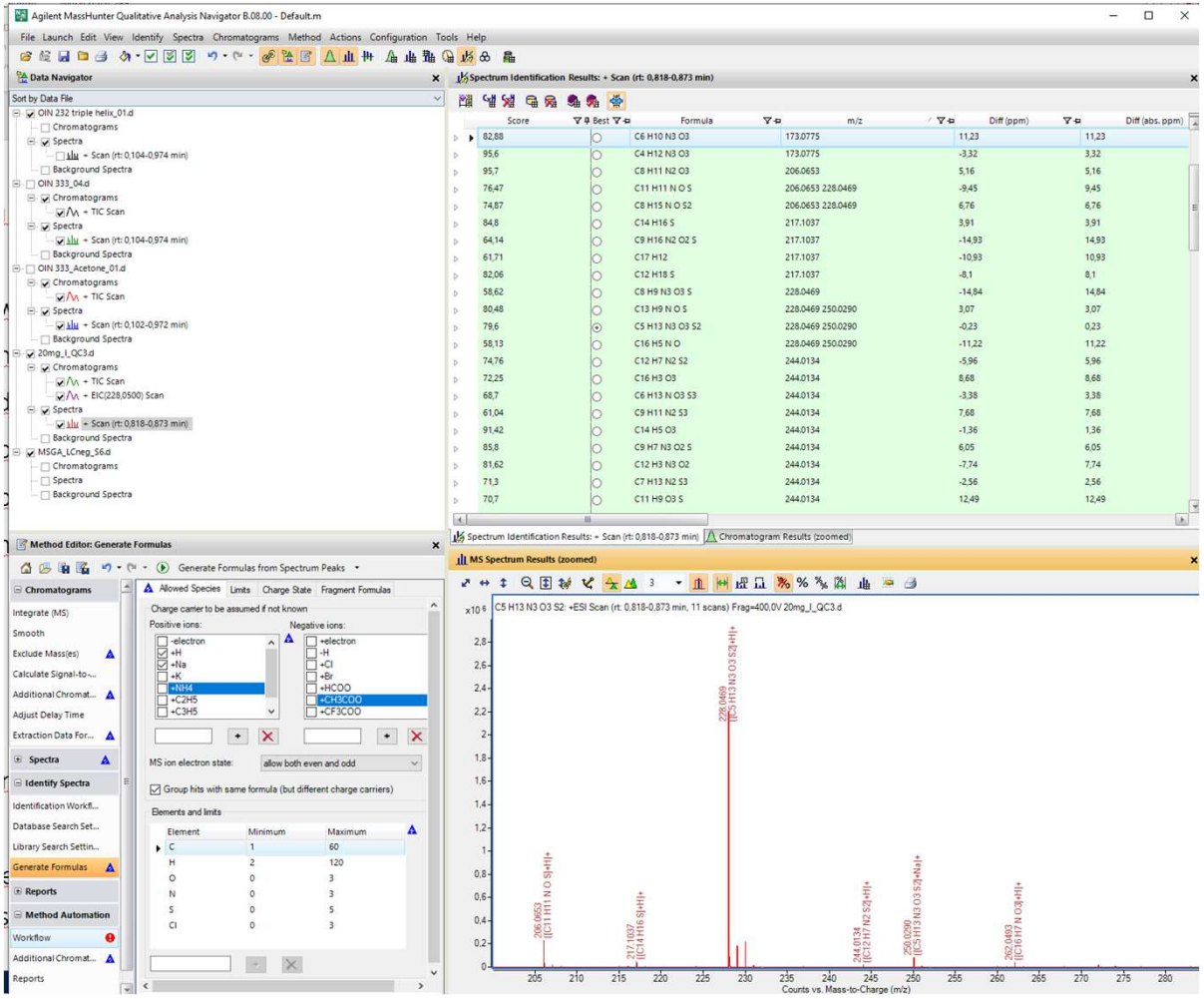
The number of resulting molecular formulae can be limited using other available information.

- what elements can be included?
- how many?
- double bond equivalent?
- parity of electrons?
- polarity?
- mass accuracy?

examination of isotopic patterns can further increase the accuracy of determination.

- Several softwares (esim. iFit, Sigma-Fit™, TargetAnalysis™) also apply combination of accurate mass and isotopic distributions to increase reliablity of accurate mass determination.

- Agilent instruments use identify spectra to generate potential formulae for MS spectrum



Accurate mass is used to verify molecular formula of new synthesised compounds along other characterization information

for example: (+)ESI-TOF observed ion, experimental and theoretical m/z

Compound **21h**: Prepared according to the general procedure using 3-phenylpropanal (46 mg, 45.3 µL); Reaction time: 7 h (plus Wittig reaction, 2 h); Eluent: 10-15 % EtOAc in hexane; Yield: 51 mg as colorless oil (81%); R_f = 0.28 (5 % EtOAc in hexane, 2 times run); $[\alpha]_D$ = + 3.52 (*c* = 1.05, CH_2Cl_2); FT-IR (film, cm^{-1}): 3027, 2948, 2360, 2341, 1720, 1655, 1602, 1551, 1495, 1454, 1435, 1199, 1030, 699; ^1H NMR (250 MHz, $CDCl_3$): δ 7.33-7.08 (m, 10H), 6.76 (dd, J_1 = 8.6, J_2 = 15.6, 1H), 5.72 (d, J = 15.6, 1H), 4.51(dd, J_1 = 5.4, J_2 = 12.4, 1H), 4.30 (dd, J_1 = 8.0, J_2 = 12.4, 1H), 3.72 (s, 3H), 2.90-2.56 (m, 5H), 2.51-2.39 (m, 1H), 1.91-1.66 (m, 2H); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 166.0, 146.9, 140.5, 138.2, 128.9, 128.60, 128.57, 128.2, 126.6, 126.3, 123.9, 76.9, 51.6, 45.0, 39.9, 37.3, 32.8, 31.5; HRMS (*m/z*): $[M+Na]^+$ calcd for $C_{22}H_{25}NO_4Na^+$ 390.1681, found 390.1688; Enantiomeric excess (99.85%) was determined by HPLC (Chiralpak IB column, hexane/*i*-PrOH = 95:05, flow rate 0.80 mL/min, λ = 230 nm, rt): t_R (major) = 27.13 min, t_R (minor) = 31.66 min.

- Accurate mass verifies interpretations and needs to be calculated for that reason! Especially important when isobaric ions need to be separated.

- Accurate mass limit (below 3 mDa / 5ppm) is often pursued and mass accuracies should be in line within one spectrum.

- In high throughput analysis accurate mass is used as one recognition point.

samples	Ion	Formula	m/z_{exp}	m/z_{theor}	Δ (mDa)
1	$[Ag(L1)]^+$	$C_{18}H_{16}N_6Ag$	423.0475	423.0482	0.7
2	$[Ag(L2)]^+$	$C_{18}H_{16}N_6Ag$	423.049	423.0482	-0.8
3	$[Zn(L1)Cl]^+$	$C_{18}H_{16}N_6ZnCl$	415.0473	415.0411	-6.2
4	$[Zn(L2)Cl]^+$	$C_{18}H_{16}N_6ZnCl$	415.0392	415.0411	1.9
5	$[Cu(L1)Cl]^+$	$C_{18}H_{16}N_6CuCl$	414.0399	414.0416	1.7
	$[Cu(L1)Cl+DMSO]^+$	$C_{20}H_{22}ON_6SClCu$	492.0544	492.0555	1.1
	$[Cu(L1)_2]^+$	$C_{36}H_{32}N_{12}Cu$	695.2189	695.2163	-2.6
	$[Cu(L1)_2Cl]^+$	$C_{36}H_{32}N_{12}CuCl$	730.1845	730.1852	0.7
6	$[Cu(L2)Cl]^+$	$C_{18}H_{16}N_6CuCl$	414.0412	414.0416	0.4
	$[Cu(L2)Cl+DMSO]^+$	$C_{20}H_{22}ON_6SClCu$	492.0559	492.0555	-0.4
	$[Cu(L2)_2Cl]^+$	$C_{36}H_{32}N_{12}CuCl$	730.1852	730.1852	0.0

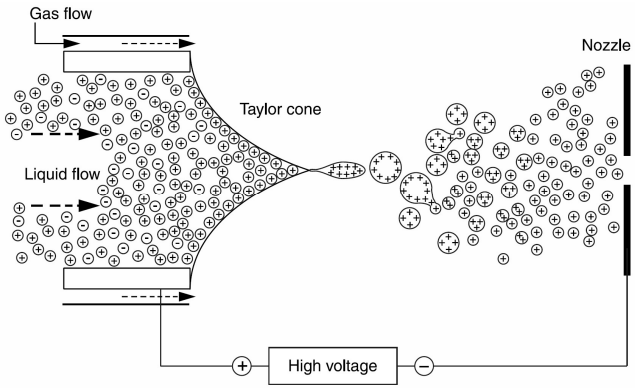
2. Ionization: Electrospray ionization (ESI)

Operational principle:

- Dillute sample solution (~ 2-20 µM) is run through thin metal capillary (0.1-0.2 mm), which is in high electric field (ca 10^6 V m^{-1}).

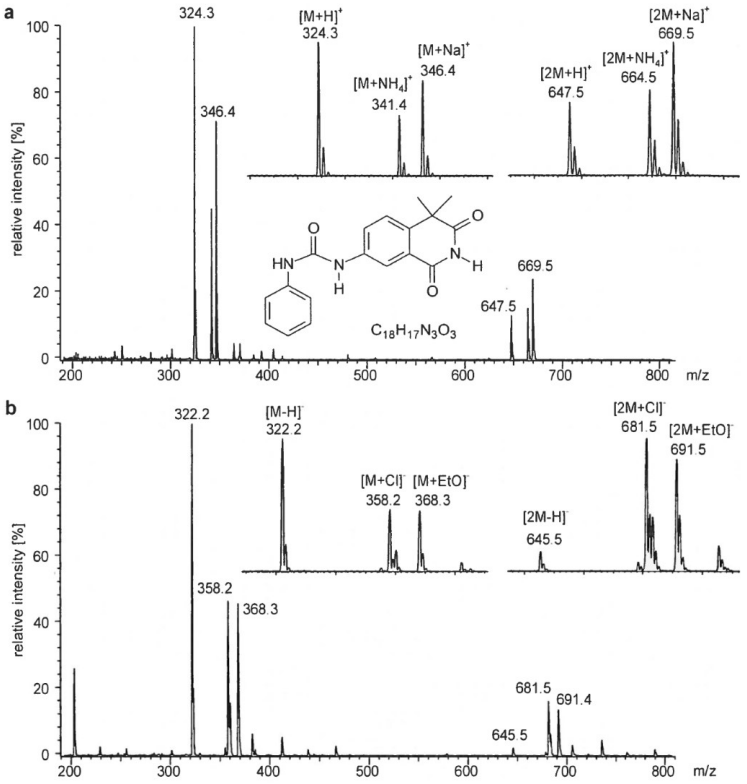
→ Charged droplets are formed.

→ Heated dry gas evaporate solvent from droplets until gas phase ions are formed



- ”soft” ionization technique → usually only intact analyte ions are observed

- even relatively labile or fragile analytes do not fragment:
  - proteins, peptides and other biomolecules
  - organometallic compounds
  - weak noncovalent complexes

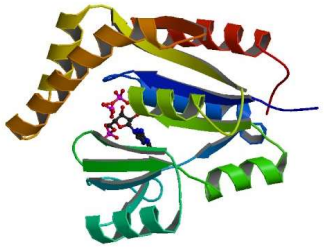


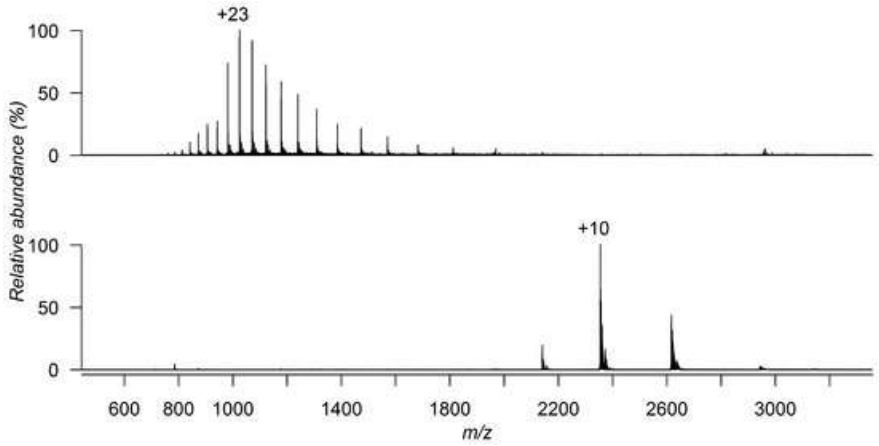
Pos. and neg. polarization ESI mass spectra for a anti-HIV medicin.

Ions formed in ESI

- Protonated/ deprotonated ions: $[M + nH]^{n+}$, $[M - nH]^{n-}$
- Adduct ions: $[M+Na]^{+}$, $[M+K]^{+}$, $[M+NH_4]^{+}$, $[M+Cl]^{-}$, $[M+CH_3COO]^{-}$
- Multimer formation ($[2M + nH]^{n+}$, $[4M - nH]^{n-}$) is often observed especially if concentration is increased and compound contains functional groups receptive for dimerization.
- Only small amount of energy is transferred during ionization.

- Also multiply charged ions are often observed ($[M + zH]^{z+}$ tai $[M - zH]^{z-}$)
- Benefits:
  - enables analysis of large ions with instruments with limited *m/z* range
  - increases mass accuracy
  - increases sensitivity of detection
  - enables polypeptide conformation analyses (= native MS)





ESI-MS spectrum of Plasmodium vivax guanylate kinase (PvGK) under denaturing conditions (50:50 (v/v) acetonitrile-water, 1% formic acid) (top) and native conditions (10 mM ammonium acetate solution) (bottom). (Read, J.A. et al., *Proteins* **2001**, *43*, 175-185; Alison E. Ashcroft *Nat. Prod. Rep.*, **2005**,*22*, 452-464).

- charge states depend on:
  - structure, size and shape of a molecule
  - solvent
  - pH

NOTE: ion charge in ESI ≠ ion charge in solution!

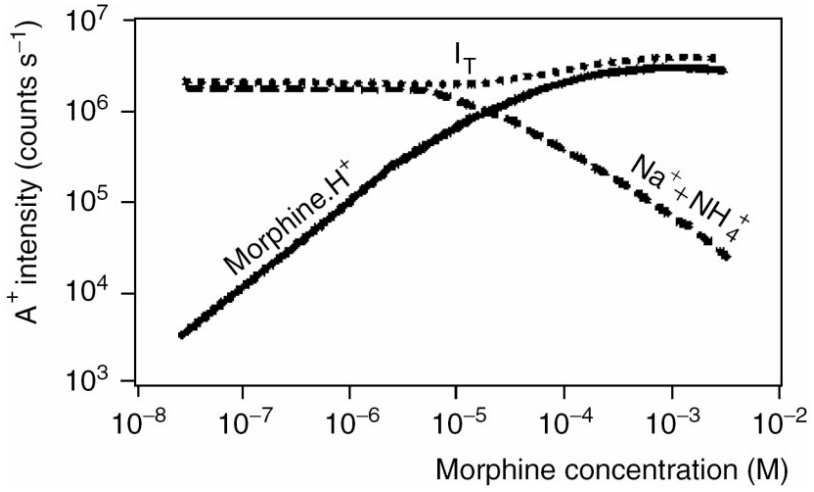
Typical ions formed during the ESI.

compound type	ionization reaction	observed ions	example
Ionic	–	M^+ (M^-)	metals, cations
Basic	protonation	$[M + H]^+$	amines, nitrogen compounds
Many basic groups	multiple protonation	$[M + zH]^{z+}$	peptides, proteins
Acidic	deprotonation	$[M – H]^-$	carboxylic acids
Many acidic groups	multiple deprotonation	$[M – zH]^{z-}$	oligonucleotides
neutral, polar	adduct formation	$[M +Na]^+$, $[M + NH_4]^+$, $[M + Cl]^-$ ...	saccharides, carbonyl compounds
(neutral, unpolar	electrochemical electron transfer	$M^{+\bullet}$, $M^{-\bullet}$	fullerens, ferrocene)

Concentration dependency of ionization efficiency when using ESI:

- *Ion current* = produced ions / time, is not linearly dependent on concentration.
- Ion current depends on voltage, but is not dependent on flow rate or analyte concentration.
- Flow rate (concentration) has to be sufficient for production of stable signal, but ion current is constant depending on voltage.

ion current and concentration of different ions as morphine is added to solution which contains Na^+ and NH_4^+ ions.



- ESI response to concentration is linear on range <10 – 100 µM, which makes quantitative use difficult
- For concentrated samples ionization can be suffocated.
- Different ions present in solution influence their individual intensities → often liquid chromatography inlet have to be used for quantitative use
- ESI is not compatible with strong salt concentrations
- *Suppression* = contaminant or other analytes decreasing effect on analyte intensity.

Ion transfer efficiency, ITE :

is influenced by several variables:

- analyte (ionization, pKa, surface activity, chemical and physical factors)
- solvent (volatility, conductivity, surface tension, polarity)
- additives, matrices (buffers, contaminants)
- ESI source parameters (voltages, dry gas temperature and flow rate, source geometry etc)

- Best sensitivity is obtained on ionic compounds
- ionization efficiency is generally good, if compound is hydrophopic and contains acidic or basic group (alkyl amines, carbocylic acids)
- if compound is hydrophilic (large number of polar groups in a small molecule) ionization efficiency can be decreased.

NOTE: ESI-MS only reflects the relative abundances of analytes in solution.

All variables have to be taken into account in quantitation and selection of internal standard!

7. ESI-MS spectra interpretation

Mass spectra interpretation is the systematic analysis of mass spectra for molecule identification.

ESI-MS spectrum interpretation involves determination of ions formed in relation to measured sample: what ions the observed peaks represents?

Possible strategy:

1. What is measured? (= preliminary information)
2. What are the *m/z* values and the charge states for the most intense peaks (isotopic patterns of most intense peaks)
3. Is the peak for intact molecule found (the suspected ion according to proposed product)?
4. Mass differences between most intense peaks? Are there characteristic mass differences?

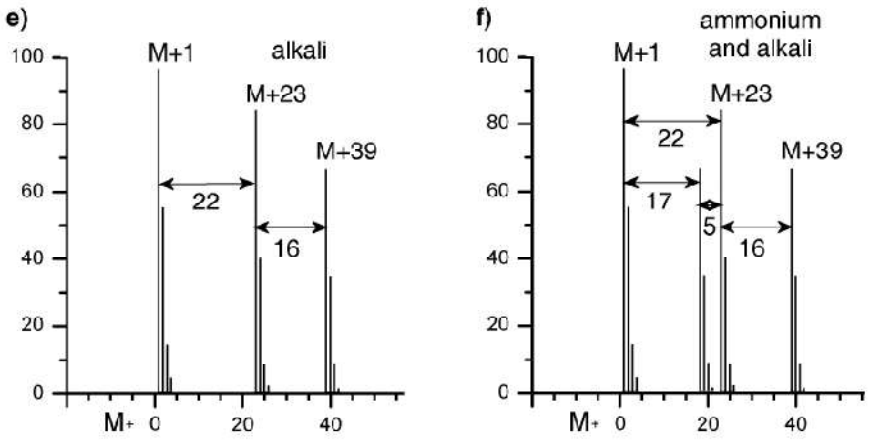
Preliminary information aids the spectrum interpretation:

- What is measured?
  - What sample can contain: mixture, purified compound, possible side products, modifications...
  - Sample history: possible impurities, how synthesized, how treated, purified etc...
  - Expected polarization for ions, expected charge state?
  - Sample properties? (labile, is it easily fragmented, does it form adducts, does it form easily multimers)
- How is sample prepared?
  - Solvent(s)
  - Additives (metal cations, acids, bases, buffers)
- What was earlier measured?
  - Carryover from previous samples
  - Buffers, acid, cation, calibrant traces in the instrument

Typical ions for adduct formation in ESI:

ion	m/z	neutrals	u
NH_4^+	18	MeOH	32
Na^+	23	ACN	41
K^+	39	H_2O	18
Cs^+	133	CH_3COOH	60
H^+	1		
Cl^-	35		
CH_3COO^-	59		
OH^-	17		

- Ion identification is facilitated if mass differences between near by ions are calculated

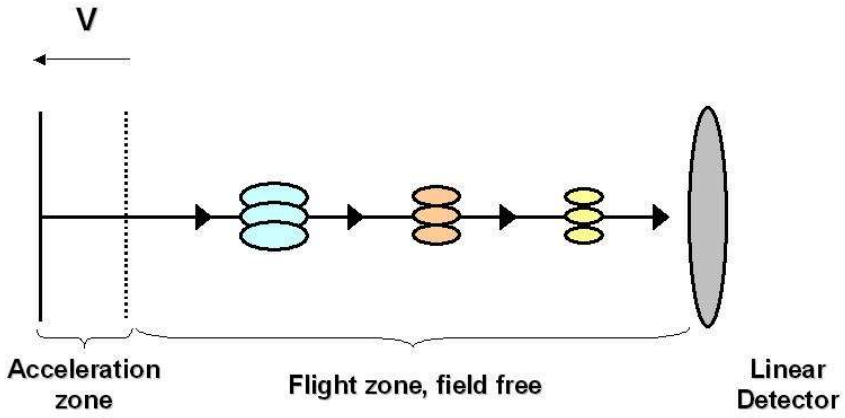


pair of ions	Δm
$[M+H]^+$ vs. $[M+NH_4]^+$	17.0265
$[M+H]^+$ vs. $[M+Na]^+$	21.9819
$[M+H]^+$ vs. $[M+K]^+$	37.9559
$[M+Na]^+$ vs. $[M+K]^+$	15.9739
$[M-H]^-$ vs. $[M-2H+Na]^-$	21.9819
$[M-H]^-$ vs. $[M+Cl]^-$	35.9767

8. QTOF mass analyzers

- Operation of TOF (time-of-flight) is based on different flight times of ions with different mass and charge in field free region.
- Ions are expelled from ion source as packages and accelerated by potential V_s.
- Ions arrive to detector at different times depending on their *m/z* value (small ions arrive first).
- Flight time can be expressed following way and *m/z* solved because the term in parantheses is constant

$$t^2 = m/z(d^2/2V_s e)$$



- Ion reflector TOF instruments have better resolution than linear ones

- Ion reflector consists of several ring electrodes with increasing potential, which form deaccelerating electric field

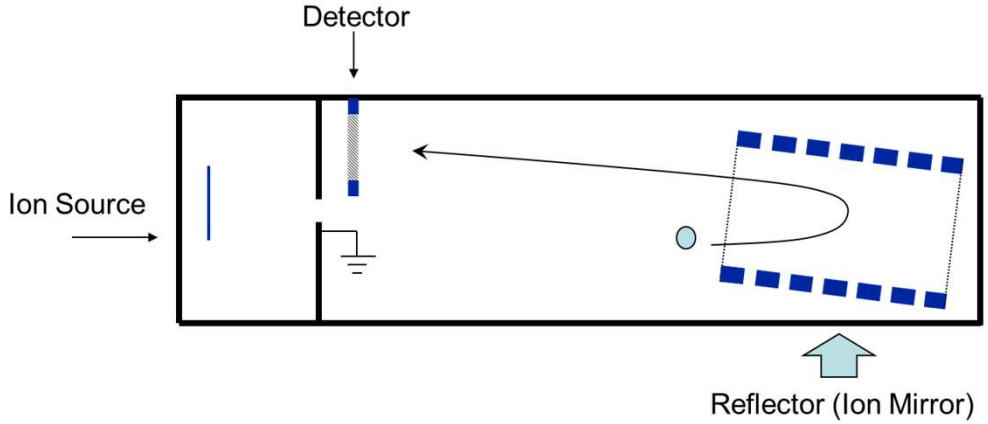
- Ion speed increases until ions turn back to their original direction.

→ Ions with larger kinetic energy fly further in reflector than ions with lower kinetic energy

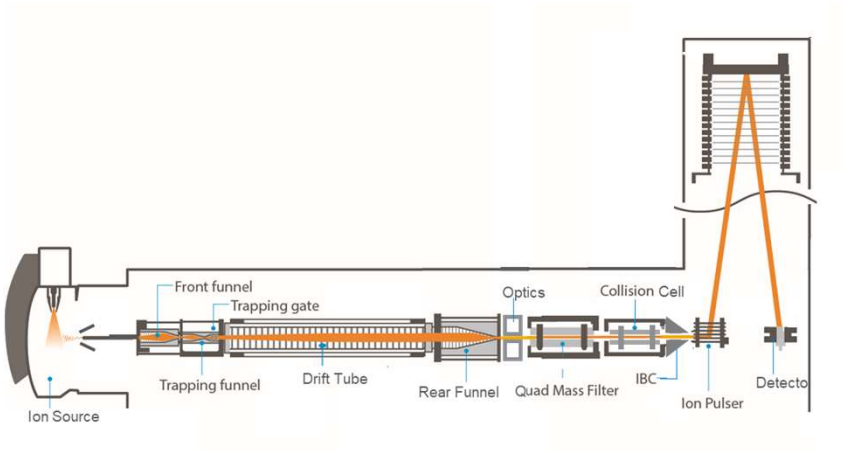
→ Small kinetic energy ions fly shorter path than ions posessing larger energy

→ Kinetic energy distribution narrows

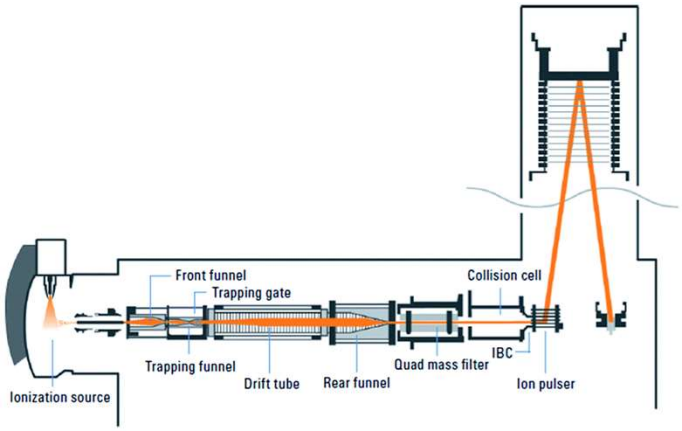
→ Ions of same *m/z* arrive to detector at the same time, but distance is different



Agilent 6530 and 6560 configurations

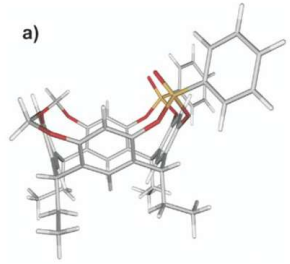


Agilent 6560 ESI-IM-QTOF



Agilent 6530 ESI-QTOF

MS/MS experiments in QTOF instruments (CID)



In CID (*collision induced dissociation, MS/MS experiment*)

ion of interest is isolated:

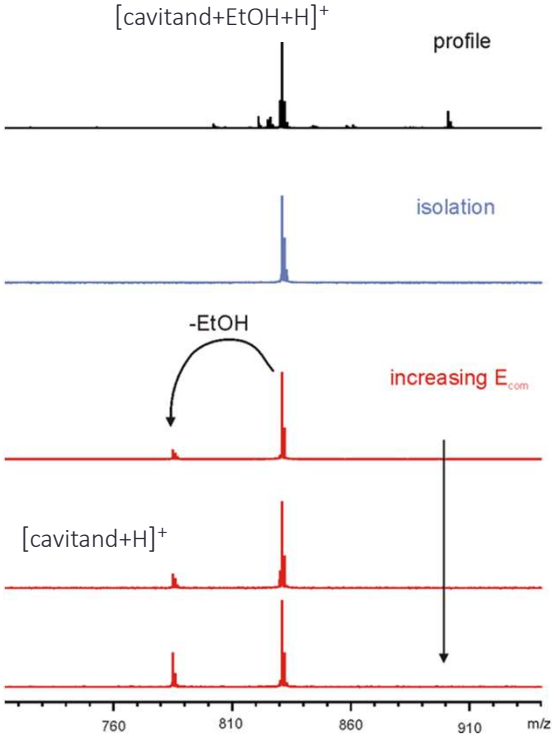
→ Ion is thermally activated by electric field

→ ion collides with inert gas molecule (N_2)

→ fraction of ion kinetic energy is transformed to rotational/vibrational energy

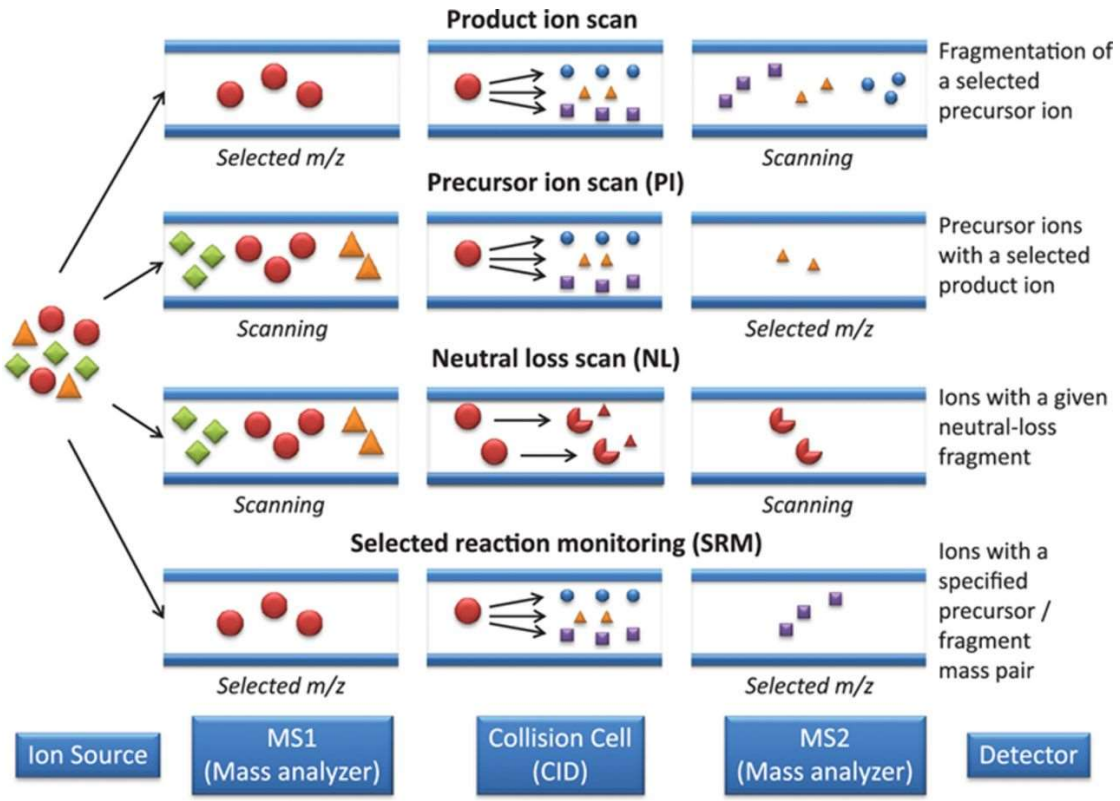
→ ion fragments / dissociates

- Energy redistribution takes place and low-energy dissociation prevails (weakest bond dissociates, ergodic dissociation method)
- With QTOF instruments MS/MS experiments are tandem in space experiments and fragmentation takes place in quadrupole and mass analysis in TOF



Scan modes on QQQ and QTOF instruments

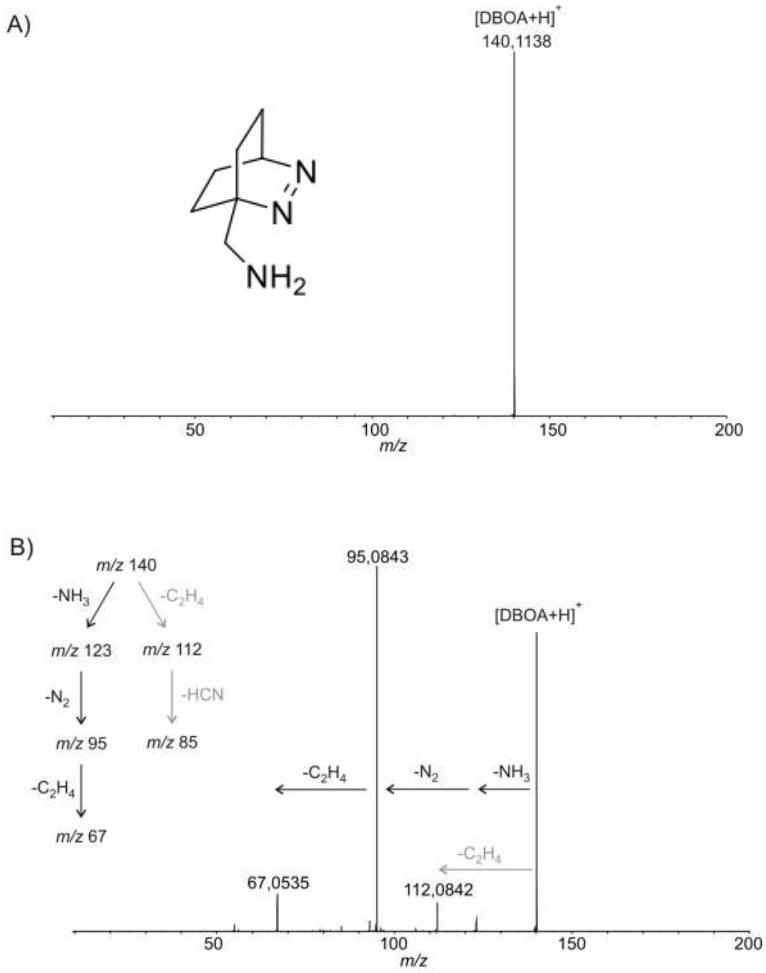
Several different scan options are possible depending on information needed:



→ MRM = multiple reaction

CID experiments are used for:

- To get structural information
- To get identification feature
- To study gas-phase properties of ions
- To study interaction strength (in supramolecules)
- To sequence peptides (b and y ions are formed) or other polymers.



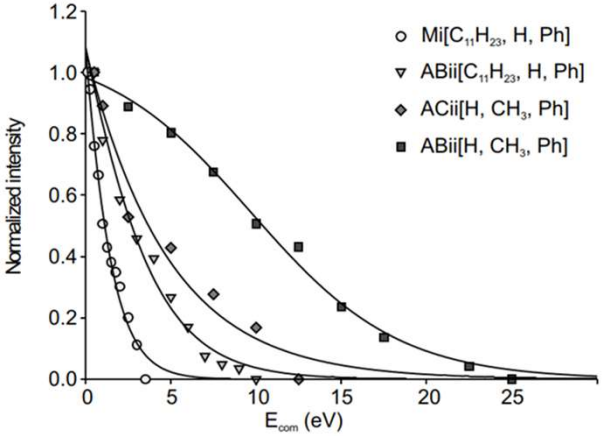
ER-CID

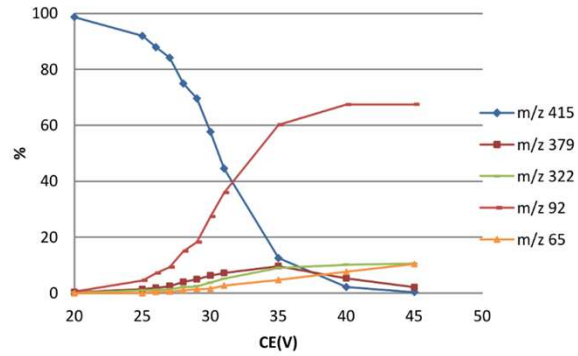
- Energy-resolved CID
- Fragmentation / dissociation of complexes is followed as a function of collisional energy
- Center-of-mass energies (E_{com} /E_{CM}) or thresholds can only be defined accurately in FTICR
- With other instruments only relative comparison is possible.
- Typically $E_{com}^{50\%}$ or $E_{CM}^{0.5}$ values are determined
- Lab frame energy values can be corrected with ion mass using equation:

$$E_{CM} = E_{LAB}\frac{m_N}{m_N+m_{AB}}$$

m_n = mass of neutral

m_{AB} = mass of ion





Ion mobility mass spectrometry (IM-MS)

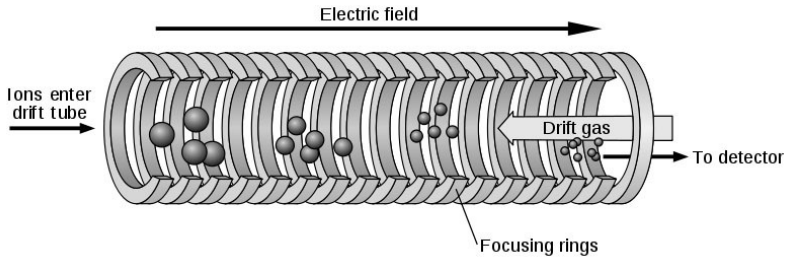
Ion mobility drift tube separates the ions according to their mobility in electric field and in presence of drift gas (He, N_2)

→ drift time t_d, ion mobility K_0

Ion mobility K_0 depends on ion charge, drift gas and **CCS (collision cross section (Ω, CCS)**, which depends on size, shape and charge state of the ion

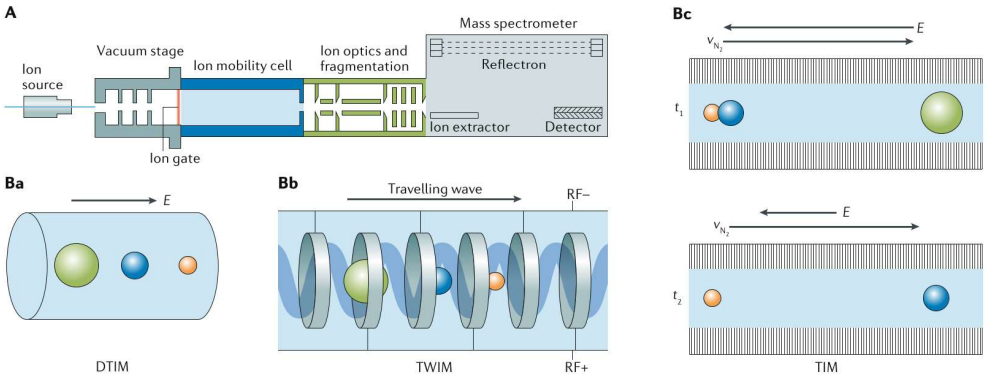
→ 2D separation: mobility (K_0 and CCS) (shape) & *m/z* value

→ Ions with different structure but same *m/z* can be separated



IMS Methods

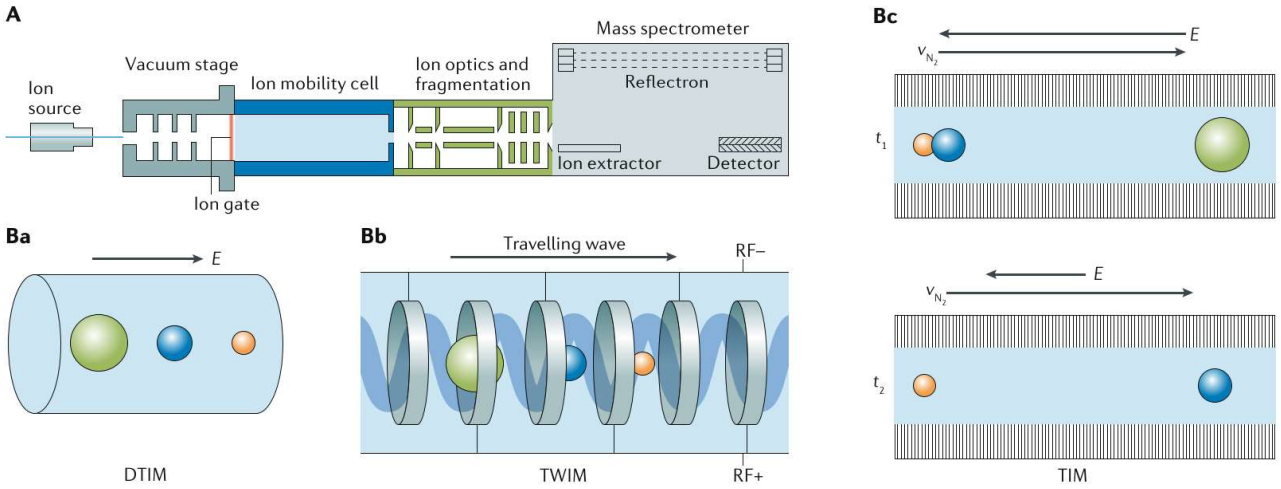
1. Drift-time IMS (DTIMS)
   - Highest resolving power, decreased sensitivity (ions are lost)
   - Direct measurement of collision cross-sections (CCS)
2. Aspiration IMS (AIMS)
   - low resolution, both polarities can be measured at the same time, can operate in continuous manner → ions are not lost
3. Differential-mobility / Field-assymmetric waveform IMS (DMS / FAIMS)
   - continuous ion monitoring , high-separation selectivity



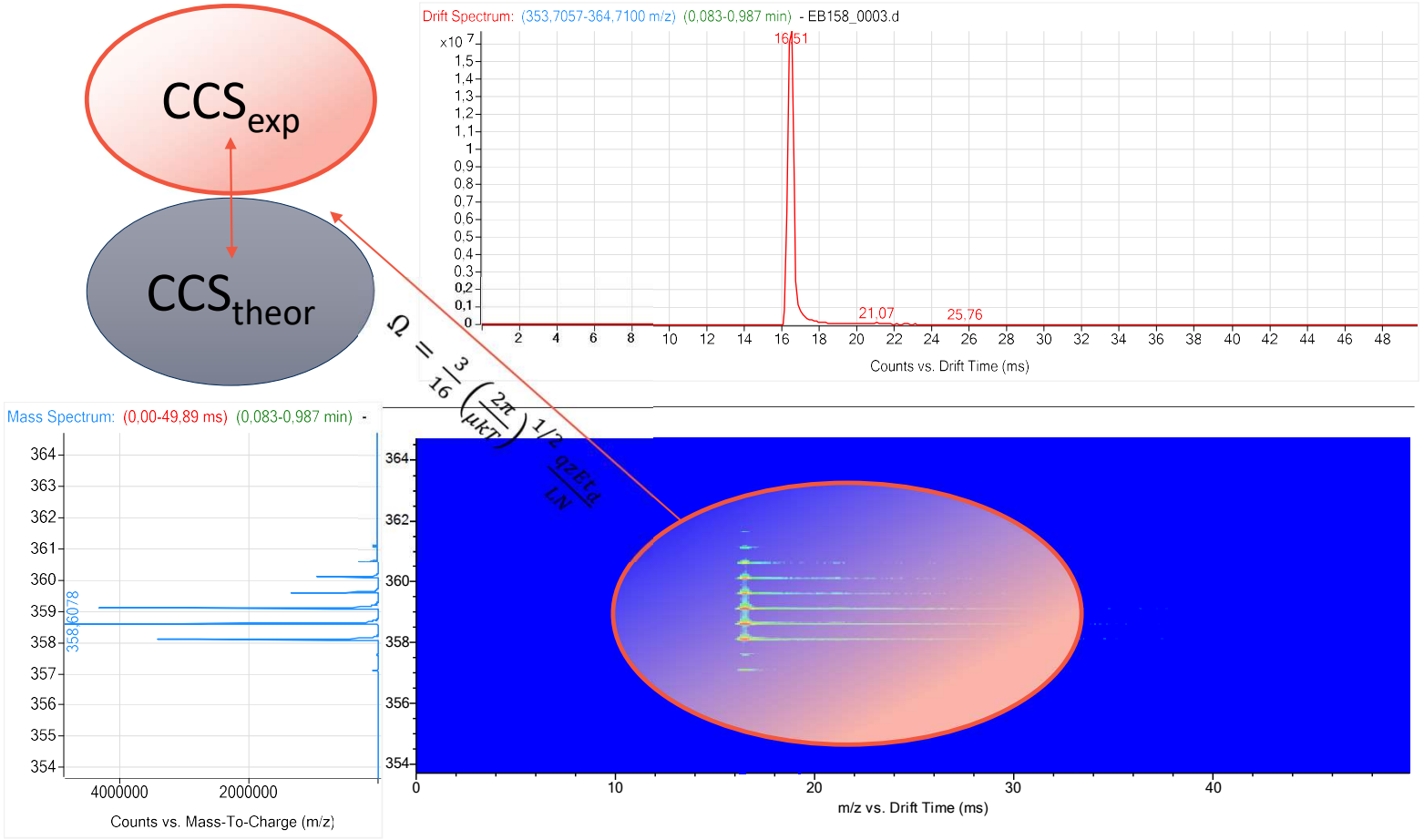
IMS Methods

4. Travelling-wave IMS (TWIMS)
   - low resolution, good sensitivity, need for calibrants if CCS are required

5. Trapped IMS (TIMS)
   - High resolution, good sensitivity, need for calibrants if CCS are required

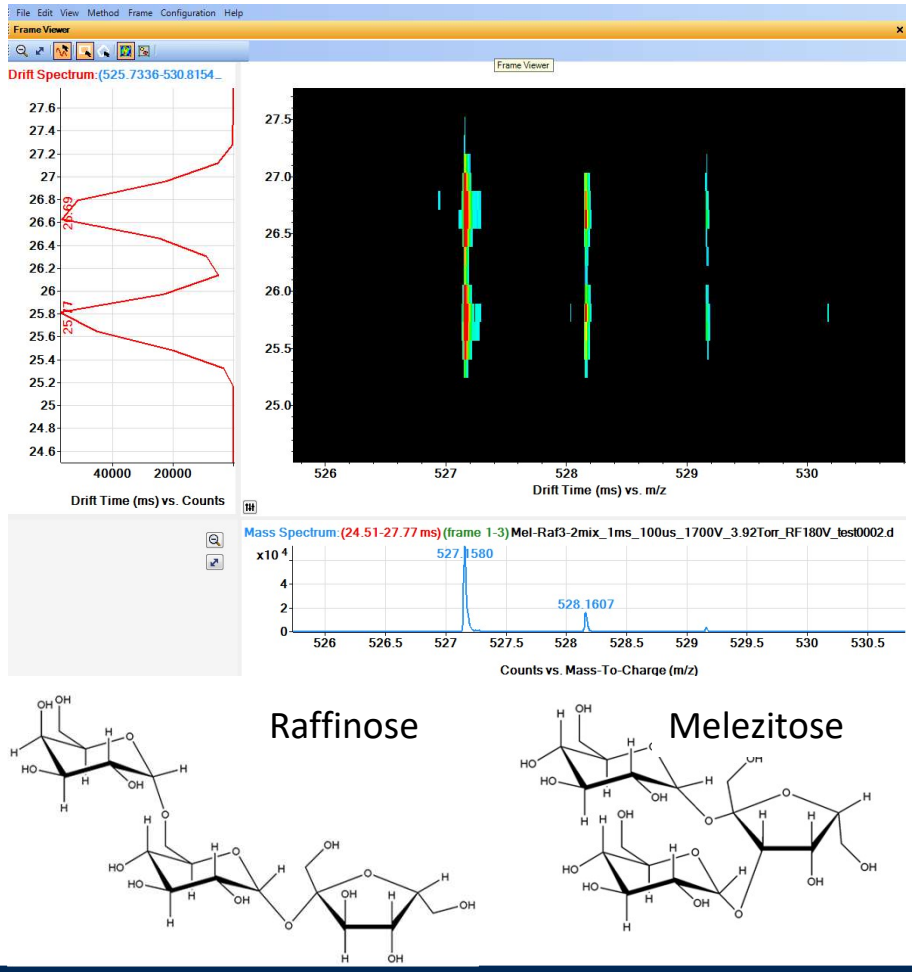


Ion-mobility and mass spectra



What is it good for?

- Separation of isomers, isobars, conformers, (enantiomers)
- Structural chemistry in gas phase (polymers, endo/exo complexes...)
- Measurement of collision cross sections (CCS)
- Conformational changes and structural dynamics (substrate binding, folding/unfolding)
- Separation of compound families according to mass-mobility correlation
- Gives additional identification feature (K /CCS)
- Reduction of noise



What is CCS?

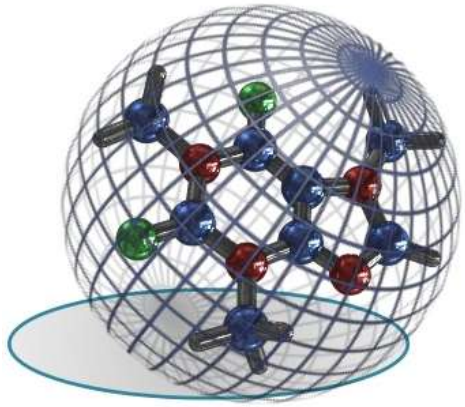
CCS is an observational property of ion which averages all geometric orientations and ion-gas interaction types during the experiment (CCS, Ω given in $Å^2$)

The CCS is characteristic for each ion in a given drift gas at a defined temperature and electric field.

Experimental CCS values can be compared with calculated CCS values

Is obtained by calibration (TWIMS, TIMS) or directly from drift time in DT-IMS, where it is directly related to drift time and is obtained from Mason-Schamp equation

$$\Omega = \frac{3}{16}\left(\frac{2\pi}{\mu kT}\right)^{1/2}\frac{qzEt_d}{LN}$$



Ω = the integrated CCS
μ = the reduced mass of the ion and the drift gas
k = Boltzmann's constant
q = elementary charge
z = charge number
E = the electric field
t_d = the drift time
L = length of the drift tube
N = neutral gas number density.

4. Work practises in ESI-MS lab

Safety aspects

Normal safety protection is generally used

- eye protection (safety goggles)
- skin protection (gloves, lab coat)
- (ear protection?)

Gas cyliders should be stored appropriately and replacement bottles ordered in time

- Typical gases: N_2, He, Ar, CO_2
- Not toxic, but replaces air
- Tied at appropriate places
- Take care that pressure control valve is sealed and works
- Uncontrolled release of compressed gas is dangerous!

The instrument should be run down, if service is done

- No voltages (ion source has normally ~ 4.5 kV voltage)
- No hot gas flows (heated gas flows might have ~ 200 °C)

Remove your gloves when working with PC!

Severe risks:

- electric shock
- spatters from solvent
- burns by acids
- Exposure on toxic substances

General practises

ESI-MS is a sensitive instrument and work practises should be chosen accordingly!
MS lab is also not personal space and other users should be taken into account!

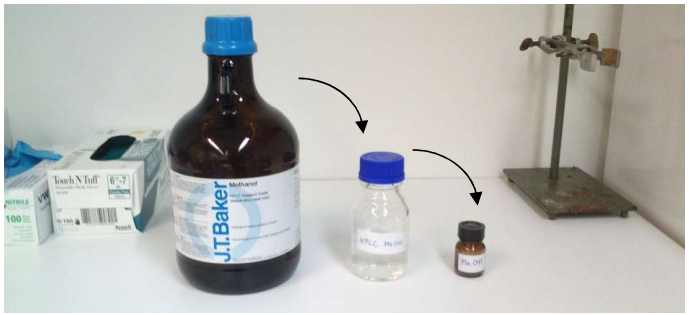
Organisation

- Consumables should be kept in order
- Samples and solvents should be marked correctly (dates, codes, concentrations…)
- Samples and solvents should be stored appropriately (rt, fridge, freezer…)
- Weighing of samples should be done in correct order and carefully
- Stock solutions and sample solutions should never get mixed
- IF YOU ARE UNSURE ABOUT YOUR SAMPLE, DO NOT MEASURE THAT

NOTE: If you don´t follow the instructions, JYU Mslab cancel your rights use instrumentation!

Cleanliness

- Solvents always (at least) HPLC grade
- Solvent systems to avoid contaminations
- Personal equipment, personal solvents
- Disposable consumables
- Purity in weighing and sample preparation
- Sample should not contain solid particles!!!



Shared labs

- Care for other users and instrument!
- Cleaning the infusion line
- Notice shortages in consumables
- Cleaning the balance station and tables besides
- Taking care of instrument performance (gas cylinders, pump oils etc)

Sample preparation for ESI-MS

General:

- ESI response on concentration is linear on range 1 – 100 µM
- ESI is not compatible with high salt and detergent concentrations (unvolatile salts, high buffer concentrations, organic detergents)
  - (NaCl), phosphate buffers, PEGs…
  - Adduct formation, ion suppression, contamination…
  - Strong acids are not suitable
- *Ion suppression* = intensity decreasing effect of an impurity or other analyte
- Sample should be soluable in chosen stock solution solvent (and not contain ANY solid particles!)
- Sample need is often low (concentration, volume)

- Almost all polar solvents can be used in sample preparation (methanol (MeOH), ethanol (EtOH), isopropanol (i-PrOH), acetonitrile (ACN / MeCN), water (H_2O), ammonium acetate/bicarbonate buffers

- Unpolar solvents and solvents which form stable ions are difficult to use (hydrocarbons, DMSO, DCM, $CHCl_3$)

- Ionization can be enhanced by adding additives (metal acetates, carboxylic acids, acetate and formate buffers (1-20mM)

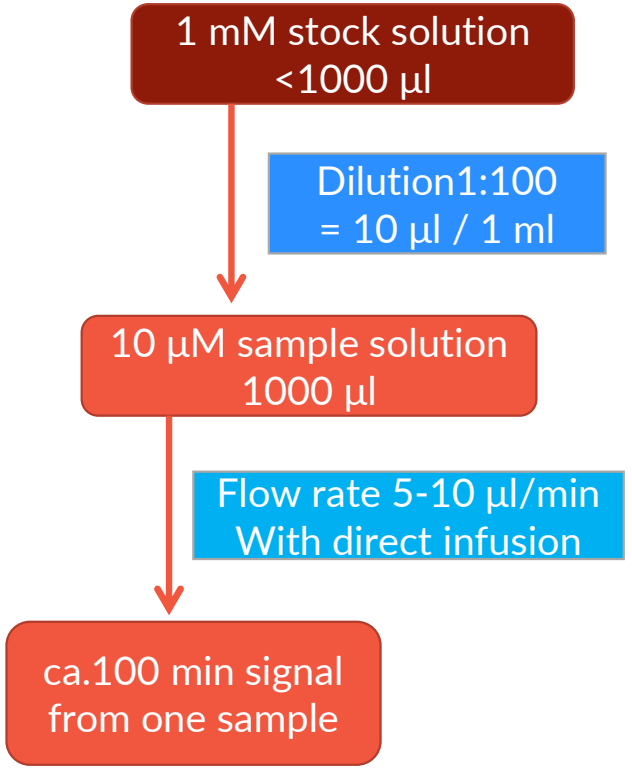
Sample preparation in practise:

Usually it is not possible to prepare the sample solution directly.

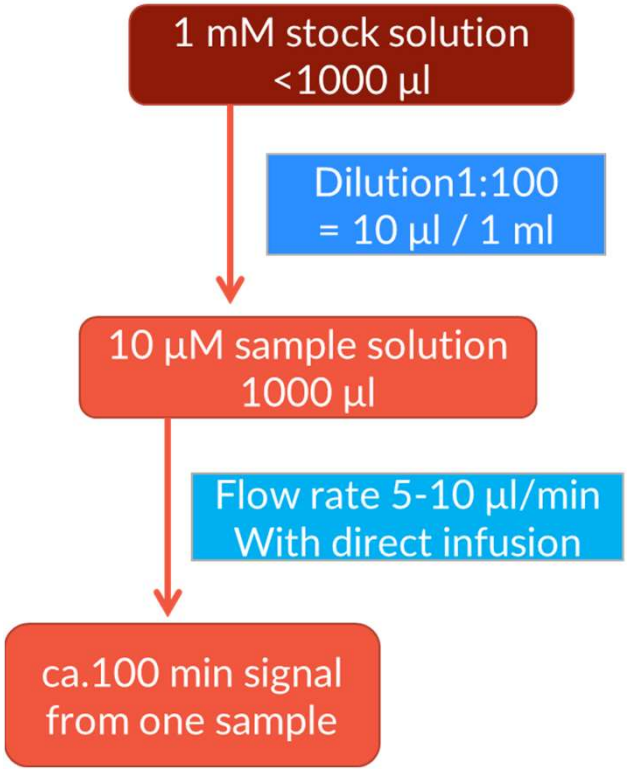
→ First **stock solution** is prepared (in 1.5 ml glass vial)

For stock solution solvent is chosen according to solubility of the sample (= any solvent can be used!)

Convenient stock solution concentration is 1-10 mM (has to be known) and volume 100-1000 µl.



- **sample solution** is then prepared from stock solution by dilution
- Solvent for sample solution should be ”ESI compatible” (MeOH, H_2O, ACN and their mixtures)
- If you use direct infusion, sample compound should be stable and ionize well in that solvent, but it is not necessary that sample is directly dissolved in it.
- Suitable sample solution concentration is usually 1-20 µM and volume 250-1000 µl.



Calibration and accurate mass determination

Accurate mass = experimentally determined monoisotopic mass (or m/z) which can be used to obtain molecular formula

Absolute mass accuracy: $\Delta m/z = m/z_{theor} - m/z_{(exp)}$

Relative mass accuracy: $\delta m/m = (\Delta m/z \,/\, m/z_{theor}) \times 10^6$ (ppm)

Principally used for:

- to screen known substances (Doping / drug analysis, food, environmental, toxicological analysis)
- To characterize unknown substances (border guards, customs laboratory, forensics, organic synthesis)

- Compared to other methods, MS requires only small amount of sample and is relatively reliable

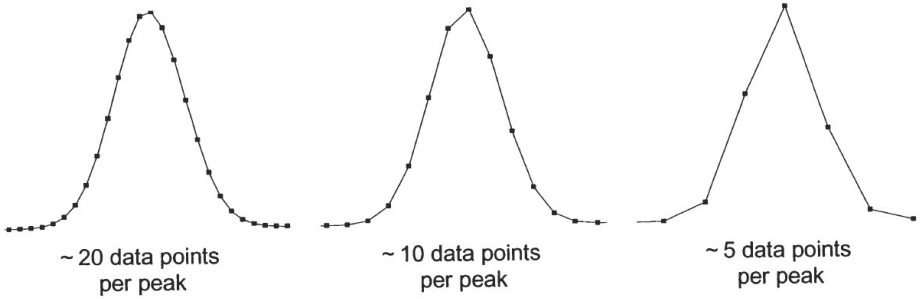
- Accurate mass is always defined as monoisotopic *m/z* value with 4 digits (minimum) .

Factors which affect the success of accurate mass:

- Ion source is not that meaningfull, but MALDI and FAB often result in poor accuracy due to low resolution and large kinetic energy distribution.

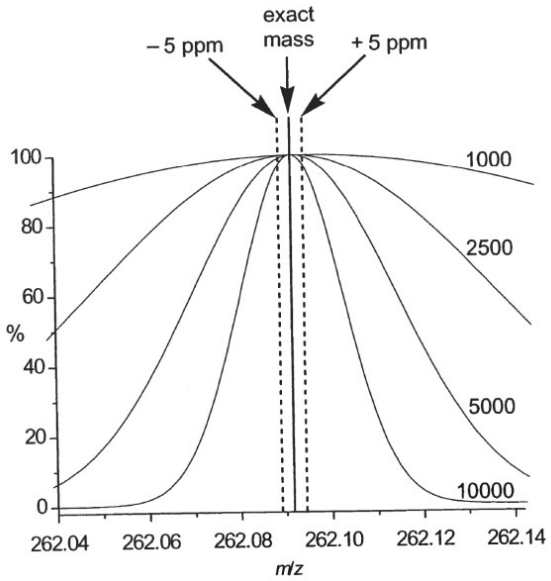
- With ESI, if you infuse too much sample and saturate the detector, your m/z values are no longer reliable (peaks marked with asterisk).

- Optimization and peak shape
  - Peak center tip should be determined as accurately as possible
  - Peak should be symmetric and as narrow as possible
  - Data set should be sufficient (data point density sh



- Mass resolving power
  - Higher the resolution (R_{FWHM}) the narrower the peak and more accurately center tip is defined

- Ion abundance
  - too low → poor shape of the peak
  - too high → detector is saturated → splitting, tailing...



Calibration:

- In calibration the position of *m/z* scale is optimized.
- Known substances (=calibrants) are used for to set the position of the *m/z* scale
- Accurate mass determination can be only as accurate as calibration is
- External calibration (instrument calibration) has to be done at least from time to time
- Internal calibration if needed and usually in accurate mass determination

External calibration:

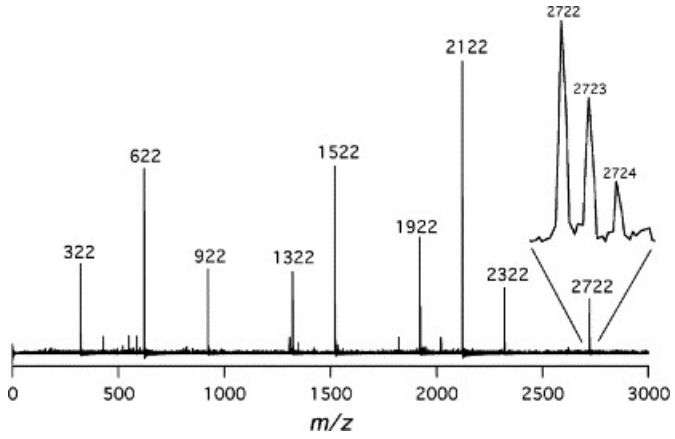
- Is done prior the experiment
- MS spectrum from calibration sample is measured.
- The experimental *m/z* values are set to match with the theoretical ones and *m/z* scale is focused.
- External calibration is especially needed if internal calibration is not used.
- External calibration is most effective when it is done as close to measurement as possible.

- Suitable external calibrant (reference):
  - produces large number of known peaks on required mass range
  - is chemically similar to the samples (charge state etc..)
- Typical external calibrants:
  - NaTFA (*m/z* 100-4000)
  - CsI (*m/z* 20-4000)
  - ES tuning mix
  - PEG and PPG mixtures
  - peptide mixtures

ESI - L Calibration Ions (formerly ESI-TOF)
Empirical Formulas for High Resolution MS

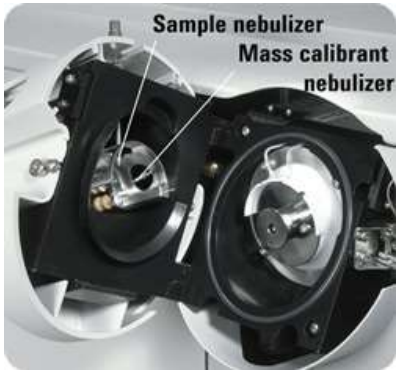
Positive Ion (m/z)	Empirical Formula
118.086255	C5.H12.O2.N
322.048121	C6.H19.O6.N3.P3
622.028960	C12.H19.O6.N3.P3.F12
922.009798	C18.H19.O6.N3.P3.F24
1221.990637	C24.H19.O6.N3.P3.F36
1521.971475	C30.H19.O6.N3.P3.F48
1821.952313	C36.H19.O6.N3.P3.F60
2121.933152	C42.H19.O6.N3.P3.F72
2421.913990	C48.H19.O6.N3.P3.F84
2721.894829	C54.H19.O6.N3.P3.F96

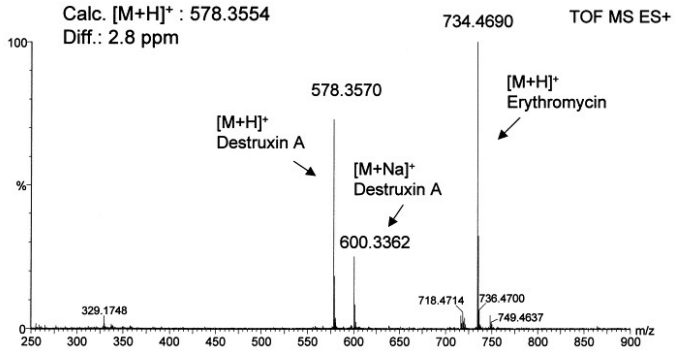
Negative Ion (m/z)	Empirical Formula
112.985587	C2.O2.F3
301.998139	C6.H.O.N3.F9
601.978977	C12.H.O.N3.F21
1033.988109	C20.H18.O8.N3.P3.F27
1333.968947	C26.H18.O8.N3.P3.F39
1633.949786	C32.H18.O8.N3.P3.F51
1933.930624	C38.H18.O8.N3.P3.F63
2233.911463	C44.H18.O8.N3.P3.F75
2533.892301	C50.H18.O8.N3.P3.F87
2833.873139	C56.H18.O8.N3.P3.F99



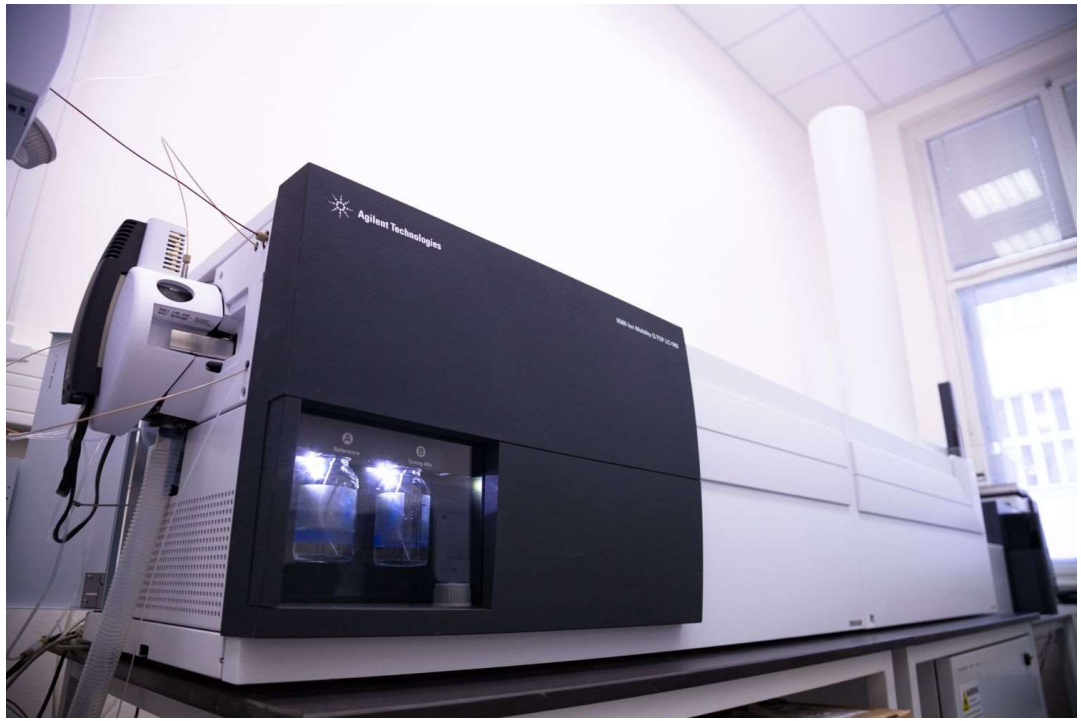
Internal calibration:

- *m/z* scale is calibrated with a reference compound which is infused same time with the sample.
- *m/z* scale is set to match the theoretical *m/z* values
- Only the spectrum is calibrated
- Reference compound can be either:
  - (mixed with the sample)
  - Infused by using a second syringe pump and T-piece
  - Infused automatically from the instrument using separate nebulizer
  - Some instruments use background ions for internal calibration





Modern instruments use automatic infusion of internal calibrant



9. Planning of ESI-MS analysis

= an educated guess for accomplishment of the analysis

- Is based on theory, earlier observations and experience
- Often details on analysis need to be modified during the measurement
- Good planning though saves time, instrument, sample and produces result of good quality

What is the purpose of the analysis?

→ affects on choice of mass range, solvent, possible calibrant, sample concentration and amount, ion source

What is measured?

- Available amount of sample?
- Assumed molecular formula?
- Molar mass, monoisotopic molecular weight?
- Chemical properties?
  - Stability (light, air, thermostability, time, solvents)
  - Reactivity
  - Applicability for ESI-MS analysis
  - Functional groups? Does the compound contain ionizable groups?

How the sample should be prepared?

- Which solvent is chosen for stock solution?
  - In which solvent sample can be dissolved in?
  - *”like dissolves like”*, literature (conditions in synthesis, NMR conditions...)
  - Compound stability in that solvent?

- Which solvent is chosen for sample solution?
  - stability
  - Hydrogen bonding
  - Ionization

- Suitable concentration?

- Are additives necessary?

- What is the amount of the sample?

Which ions are likely observed?

- What functional groups?
  - Acidic groups → deprotonated ions
  - Basic groups → protonated ions
  - Polar groups → adducts

- Can multimers be possibly observed?
- Can multiply charged ions be formed?
- Are the ions positive or negative? → choice of polarization
- Calculate monoisotopic *m/z* values for plausible ions
  → Choice of mass range
  → Choice of the internal calibrant

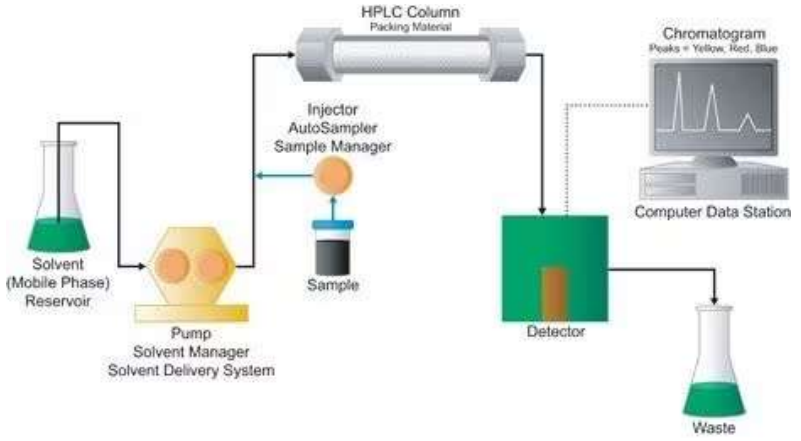
- Do we possibly need additives?

10. Liquid Chromatography

What is chromatography?

IUPAC:

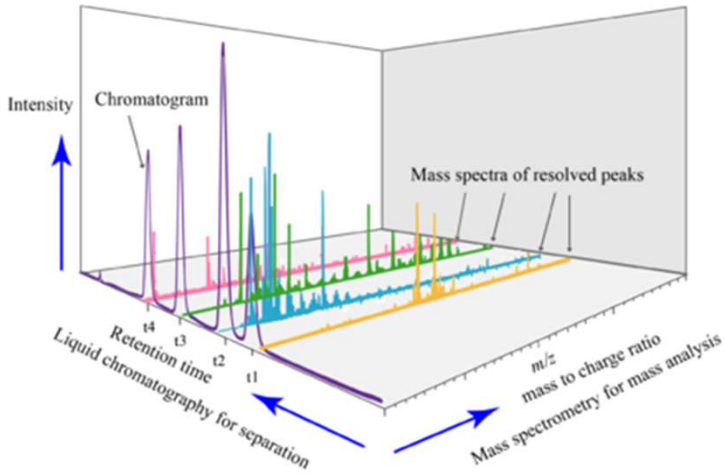
” … physical **method of separation** in which the components to be separated are **distributed between two phases**, one of which is stationary (**stationary** phase) while the other (the **mobile** phase) moves in a definite direction.”



HPLC / UHPLC is commonly used as inlet for mass spectrometry.

Purpose of HPLC is:

1. Separation of compounds in a mixture
2. Define abundance of compounds → mg, ng/ml, nM…



Mass spectrometer is an instrument and it is used for:

1. to ionize sample molecules → gas phase ions
2. to separate ions according to their mass and charge ratio (*m/z* ratio)

The most important single piece of information obtained, is molecular weight for a molecule or atom (ion)

→ elemental composition (chemical formula) for a compound

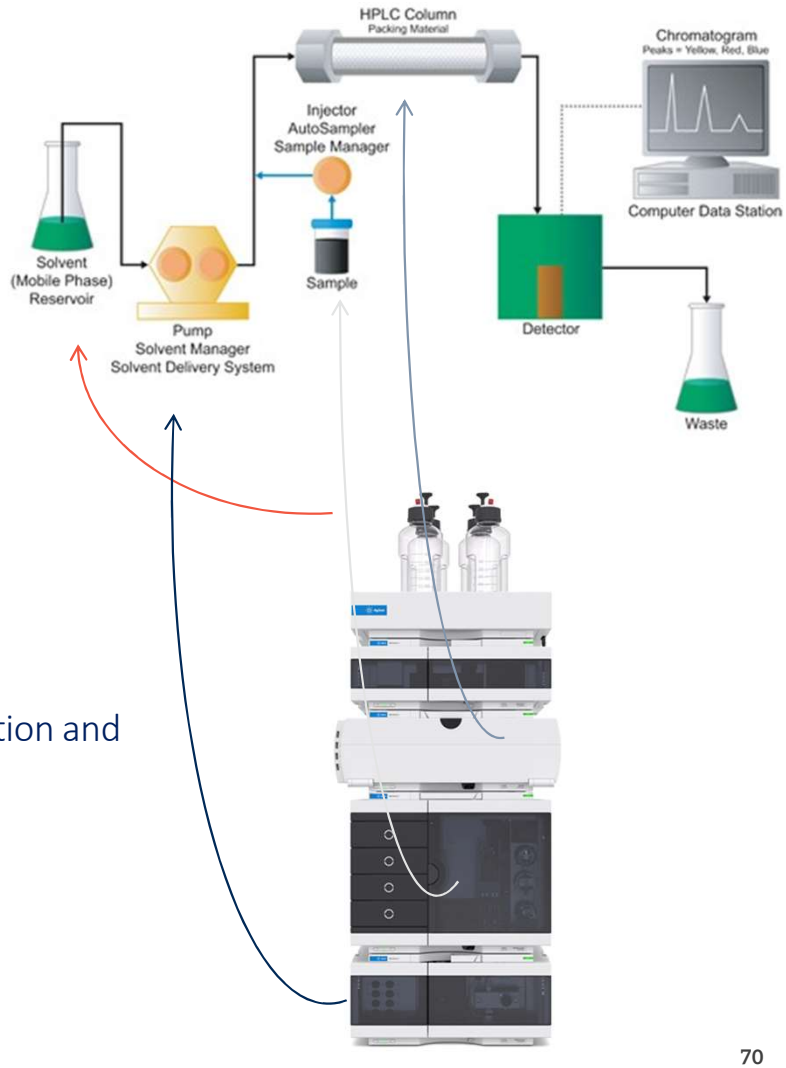
→ identification of the analytes in sample

→ abundance of the compound

Liquid Chromatography

- Liquid chromatography (HPLC, UHPLC) is used to separate, indentify and quantitate variety of compounds of extreme importance:
  - Aminoacids
  - Environmental toxins
  - Drugs (legal and illegal)
  - Carbohydrates
  - Peptides (proteomics)
  - Metabolites (metabolomics)
  - Polar small organic compounds
- Many of the target molecules cannot be studied with GC, because they are not sufficiently volatile or are thermally labile.
- Quality and safety of our daily life is dependent of liquid chromatography.

- HPLC Instruments consists of:
  - solvent reservoir (bottles)
  - pump
  - Injector (autosampler)
  - colum and column oven
  - Detector



- HPLC = high performance (pressure) liquid chromatography
- UHPLC (UPLC) = ultra high performance liquid chromatography
- Often hybrid (hyphenated) instruments HPLC-MS for fast detection and identification.

1. Liquid mobile phase is pumped through a column packed with highly porous particles
2. Mobile phase transports analyte molecules through the column.
3. The surface of the particles interact with the analyte molecules and cause their retention.
4. From column sample components elute at different retention times depending on their interactions with the stationary phase and mobile phase.
5. Elutes are detected and chromatogram created.

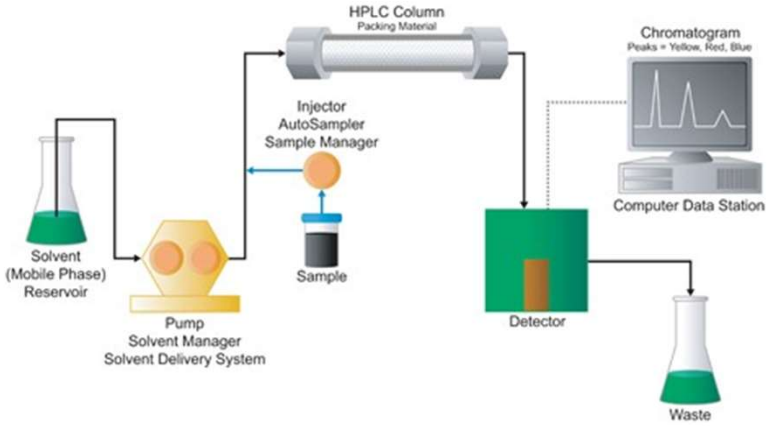


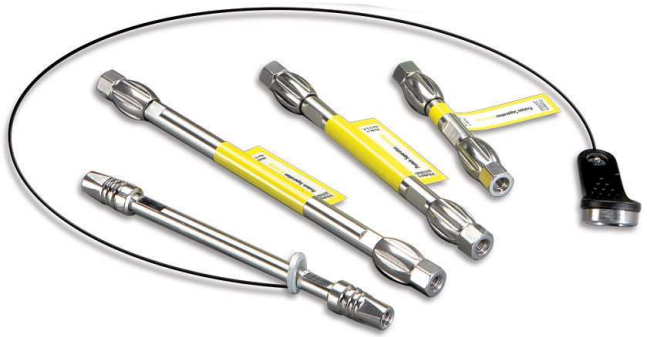
Figure: https://www.waters.com/nextgen/us/en/education/primers/beginner-s-guide-to-liquid-chromatography/how-does-high-performance-liquid-chromatography-work.html

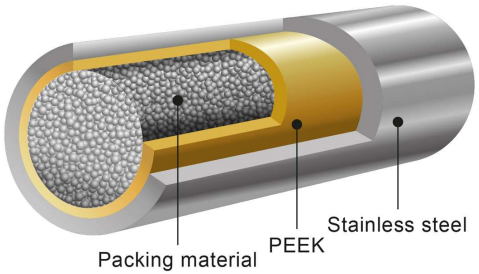
- Both mobile phase and stationary phase interact with analytes, so controlling of retention in LC is different compared to GC.
- Analytes are not evaporated, but are analysed from condensed phase and in athmospheric pressure

→ LC is able to analyse much wider range of compounds (polymers, peptides, proteins, carbohydrates, metal ions, salts…) without thermal decomposition.

Columns

- Most analytical columns are made of stainless steel to resist high pressures (200-1500 bar).
- Diameters range from 1 to 5mm (4.6 mm most common during past years).
- The narrow-bore columns (2.1 mm diameter) require less solvent to achieve same linear velocities, offer increased sensitivities and are easier to couple to mass spectrometers.
- Lengths vary from 3 to 30 cm. Shorter for faster analysis, longer for increased separation.
- Particle sizes vary from 1 to 10 µm in diameter (most common are 3 and 5 µm).
- Smaller particles produce narrower peaks, better resolution, allow higher flow rates → faster analysis.





Modes of liquid chromatography

There are several modes in which liquid chromatography can be used:

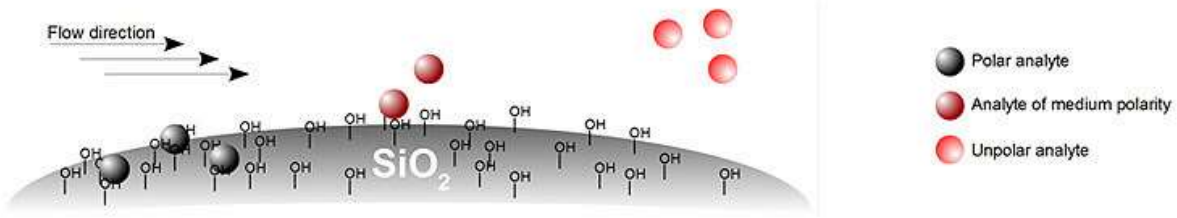
1. Normal phase liquid chromatography (NPLC)
2. Reversed-phase liquid chromatography (RPLC)
3. Ion-exchange chromatography (IEX)
4. Hydrophilic interaction chromatography (HILIC)
5. Size-exclusion chromatography (SEC)
6. Affinity chromatography

1. Normal phase liquid chromatography (NPLC)

- First mode of LC (not most common nowadays)
- Mode of retention is adsorption of solutes to a solid surface
- Separation and adsorption depends on balance between attractive forces of the analyte with surface and with mobile phase.
- The surfaces in NPLC are polar and the mobile phases nonpolar.
- Polar solutes favor adsorption to the surface and nonpolar solutes remain more in mobile phase.

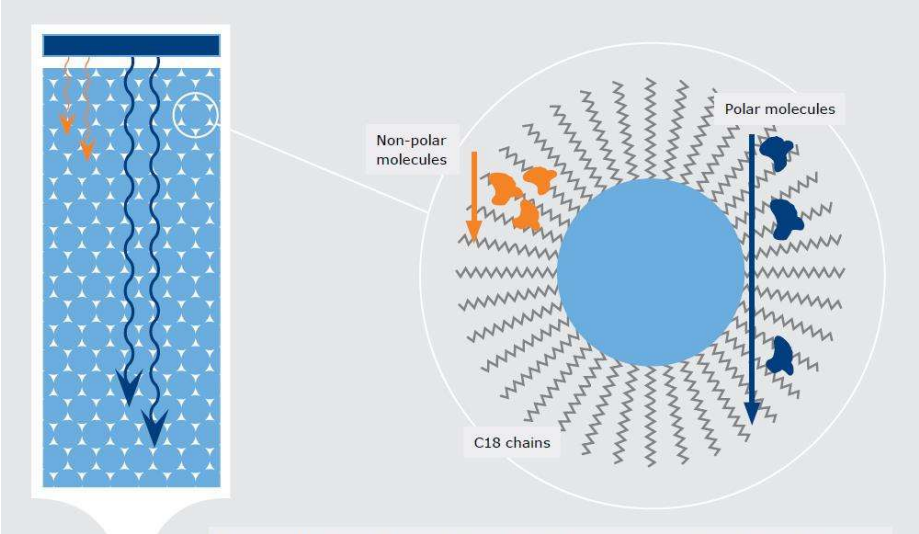
→ **Nonpolar solutes elute earlier than polar solutes**

- NPLC is sensitive for geometric factors and relative positions of functional groups (separation of geometric isomers, polyaromatic hydrocarbons).
- Common mobile phases include hexane and modifiers like alcohols, THF, or dioxane.



2. Reversed phase liquid chromatography (RPLC)

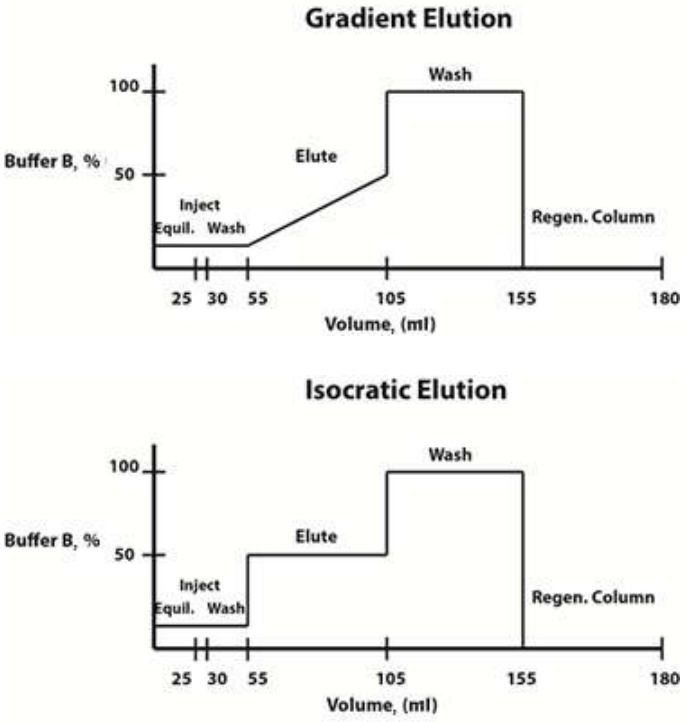
- Most used mode of liquid chromatography
- Stationary phase particles are modified with nonpolar chains like C-8 or C-18 alkyl chains.
- Mobile phase is polar (MeOH, ACN, H_2O). Typically a mixture of water and organic solvent.
- Because system is ”reversed” to normal phase chromatography, this is called reversed-phase liquid chromatography.
- Whereas NPLC is based adsorption, RPLC is based on absorption of molecules into stationary phase.
- Mechanism in RPLC is solute partitioning between mobile and stationary phases.
- *Polar solutes elute before nonpolar.*



Video: difference between normal and reversed phase chromatography https://www.youtube.com/watch?v=MLoitPJQH3g

Isocratic and gradient elution

- Polar molecules elute rather quickly in RPLC. To separate them, mobile phase, which contains mainly water (for example 90% H_2O / 10 % MeOH) should be used.
- When mobile phase contains high precentage of water, nonpolar solutes stay in column.
- → Systematic change in mobile phase composition helps to provide enough retention and still elutes all solutes = **gradient elution**
- If mobile phase composition is remained constant, that is called **isocratic elution**.
- Typically gradient elution starts from mobile phase having more water (80% H_2O, 20% organic) and then is changed over time to mobile phase with more organic (20% H_2O, 80% organic)



Agilent 1290 Infinity II LC System		
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 mm × 150 mm, 2.7 µm, PEEK-lined (p/n 673775-924)	
	Positive Ion Mode	**Negative Ion Mode**
Solvent	A) 10 mM ammonium formate in water with 0.1% FA B) 10 mM ammonium formate in water/ACN 10:90 (v:v) with 0.1% FA	A) 10 mM ammonium acetate in water with 2.5 µM InfinityLab Deactivator Additive, pH = 9 B) 10 mM ammonium acetate in water/acetonitrile 15:85 (v:v) with 2.5 µM InfinityLab Deactivator Additive, pH = 9
Nonlinear Gradient	Time (min) %B 0 98 3 98 11 70 12 60 16 5 18 5 19 98 20 98	Time (min) %B 0 96 2 96 5.5 88 8.5 88 9 86 14 86 17 82 23 65 24 65 24.5 96 26 96
Post Time	4 minutes	3 minutes
Column Temperature	25 °C	50 °C
Flow Rate	0.25 mL/min	
Injection Volume	3 µL	
Autosampler Temperature	4 °C	

https://www.agilent.com/cs/library/applications/application-discovery-metabolomics-hilic-z-5994-1492en-agilent.pdf

Liquid chromatography–mass spectrometry-based metabolomics for authenticity assessment of fruit juices (Metabolomics, **2012**, *8*, 793–803).

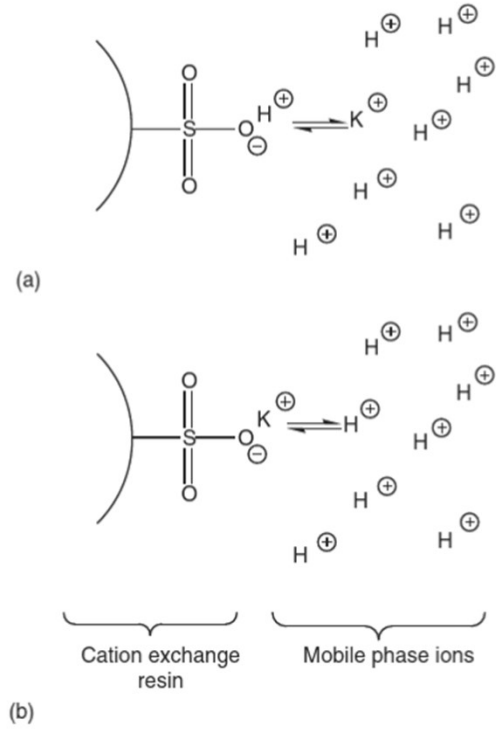
https://link.springer.com/content/pdf/10.1007/s11306-011-0371-7.pdf

”Chromatographic separation was performed using a Restek Ultra II Aqueous C18 reversed phase analytical column (50x2.1 mm i.d., 3 µm particle size, Restek, Bellefonte, Pennsylvania, USA) kept at a temperature of 35C. The injected sample volume was 10 µl. The mobile phase consisted of 5 mM ammonium acetate (eluent A) and methanol (eluent B). The gradient elution was performed as follows: 0–3 min eluent B 10%; 3–5 min eluent B 10–90%; 5–7.5 min eluent B 90%; 7.5–10 min column equilibration-eluent B 10%. A flow rate of 0.4 ml min^{-1} was employed for elution; equilibration of the column was carried out at 0.6 ml min^{-1}”

3. Ion-exchange Chromatography (IEX)

- In IEX, the stationary phase itself is charged to provide retention to charged analytes.
- Column packings have charge-bearing functional groups covalently bonded to polymer matrix.
- Retention mechanism is simple exchange of the sample ions with the counter ions.
- Retention differences are governed by the physical properties:

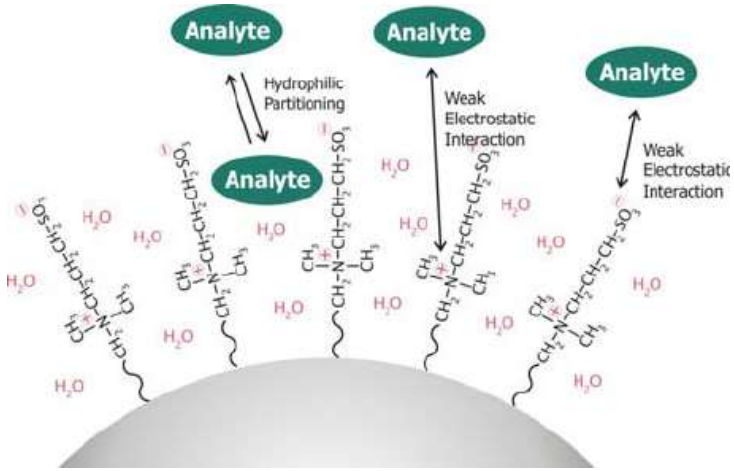
1. Ions with higher charge
2. Ions with smaller solvated radii
3. Ions with greater polarizabilities.



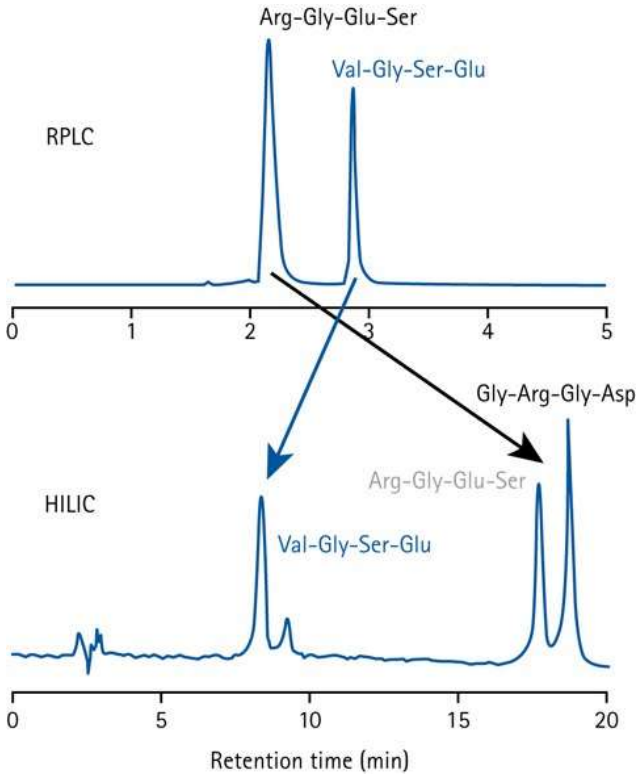
- For example the retention of cations follows the order: $La^{3+} > Al^{3+} > Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cu^{2+} > Zn^{2+} > Mg^{2+} > UO_2^{2+} > Ag^{+} > Cs^{+} > Rb^{+} > K^{+} > NH_4^{+} > Na^{+} > H^{+} > Li^{+}$
- For anions: citrate $> SO_4^{2-} > C_2O_4^{2-} > I^{-} > NO_3^{-} > Br^{-} > SCN^{-} > Cl^{-} > CH_3CO_2^{-} > F^{-} > OH^{-} > ClO_4^{-}$

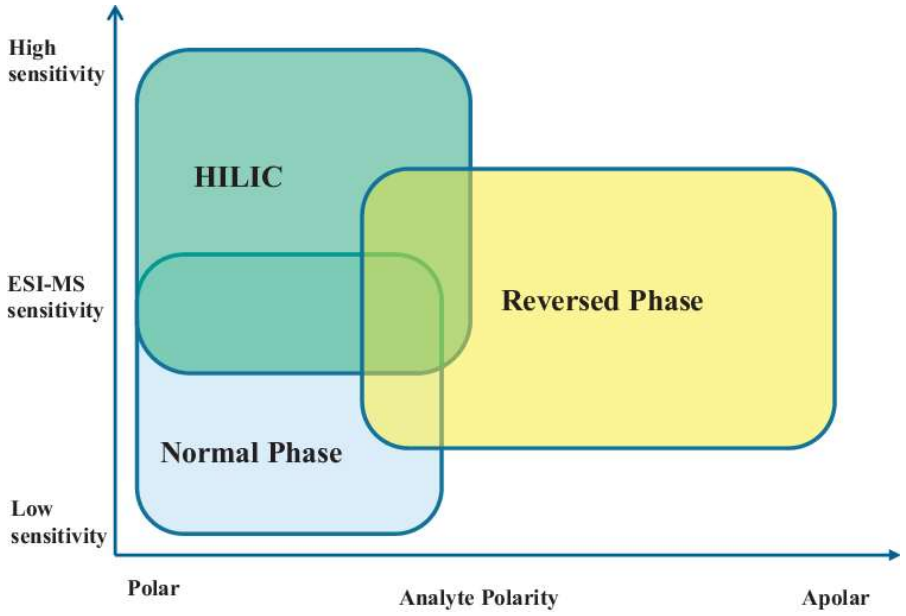
4. Hydrophilic interaction chromatography (HILIC)

- In RPLC small polar molecules elute often too quickly causing overlap (or co-elution) in chromatographic peaks.
- HILIC is an alternative to separate polar and charged compounds.
- HILIC uses stationary phases which are bare silica or short-chain amino or cyano phases.
- Mobile phases contain at least 2.5 % of water and more than 60% of an organic solvent (typically MeCN).
- In HILIC increasing water content in mobile phase causes decrease in retention of organic solutes.



- Retention is based on several modes: partitioning, adsorption and ion exchange.
- In HILIC there is a water layer on silica particles and pores.
- Hydrophilic compounds retain in this water layer → separation is based on relative hydrophilities.

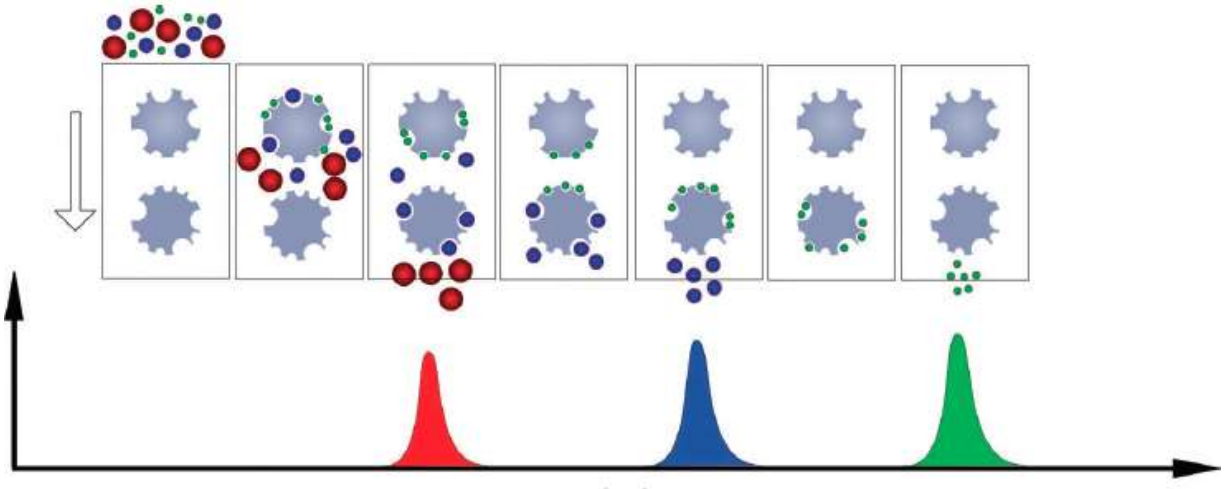




- HILIC is especially attractive for pharmaceutical and biochemical samples, which often contain highly polar groups.

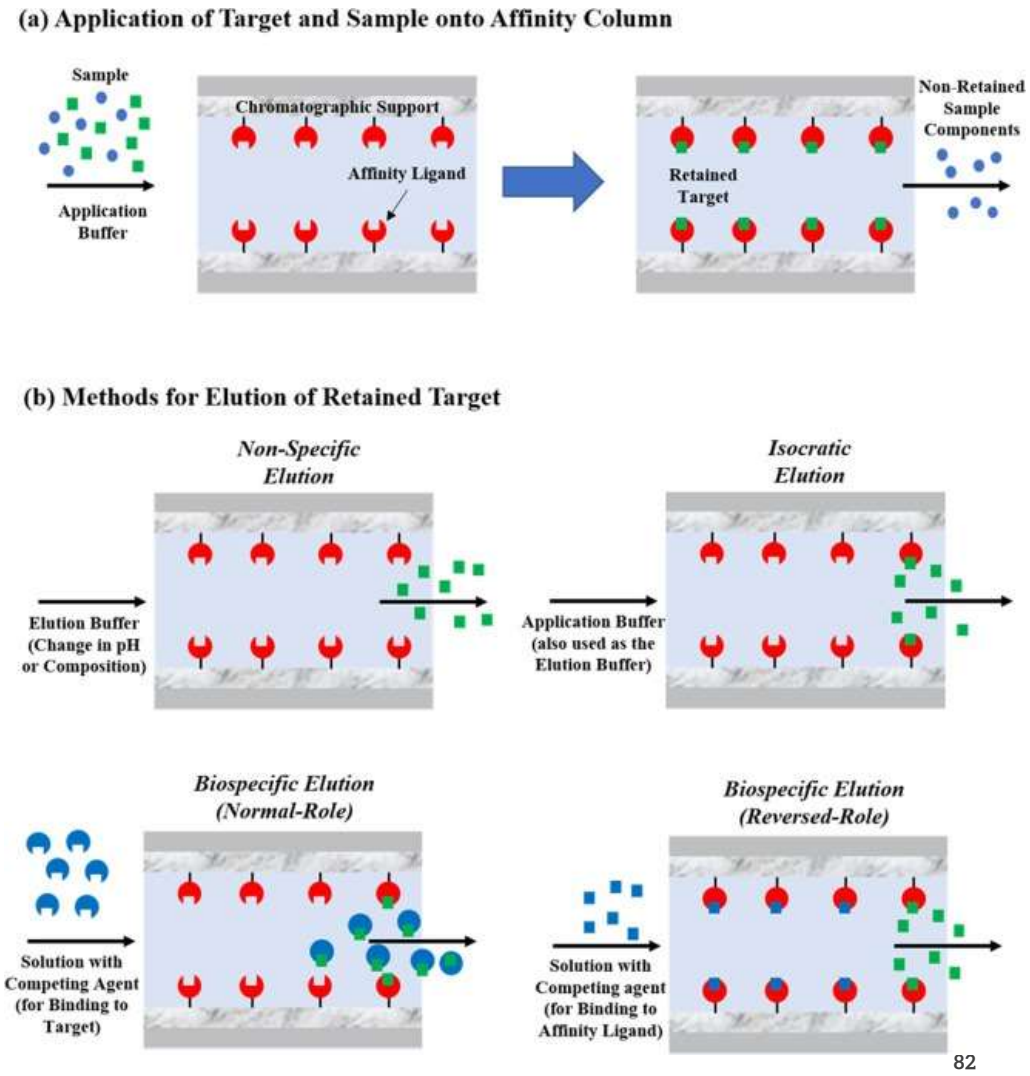
5. Size-exclusion chromatography (SEC)

- In size-exclusion (or gel permeation, GPC) chromatography separation is based on different size of molecules.
- Typically SEC is used to separate macromolecules and polymers of molecular weights from 1000 to 500 million Da.
- The particles in column packing have variously sized pores.
- Very large molecules do not fit to all pores and elute faster. Small molecules have better retention, because they fit to pores better.



6. Affinity Chromatography

- Affinity chromatography is based on specific binding of substrates.
- Based on same ”lock-and-key” principles as high binding in enzymes.
- Stationary phase has been covalently modified with immobilized biochemical (affinity ligand) at solid support.
- Only the solute that spesifically binds at affinity ligand is retained at column.
- Retained solute is then eluted by changing mobile phase contions.

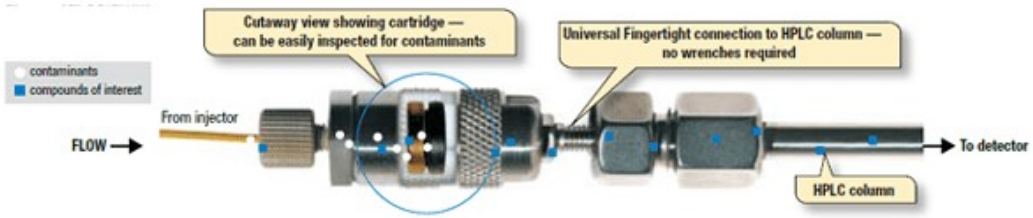


11. Sample preparation for LC-MS

LC-MS is used if:

a) sample is a mixture or has compicated matrix
b) sample has low volume
c) quantitation is needed
d) characterization requires retention time information

- More concentrated samples are often used and concentration depends on individual amounts of analytes in sample
- Guard columns are often used to avoid contamination of analytical column



- Sample is prepared in glass vial (chromatography vial), often with an insert to decrease volume of sample
- Solvent is chosen according to HPLC method and typically same solvent / solvent system is used.
- Sample should not contain any solid particles. Need for filteration (spin or syringe filters) .
- From biological samples proteins are precipitated / removed prior the analysis.
- Injection volumes typically 1-20 ul using autosampler

Agilent 6530 Instrument

Chromatography (HPLC)

Separation
Retention time
(quantitation)

Mass analyzer (TOF, quadrupole)

Mass / Charge *(m/z)*
< m/z 20 000

Collision cell

MS/MS
Fragments

Direct infusion (syringe pump)

Ion Source (ESI)

Ionization for mass analysis