Basic LC-MS and Applications

Rittichai Charoensapyanan

Product Specialist

Agenda

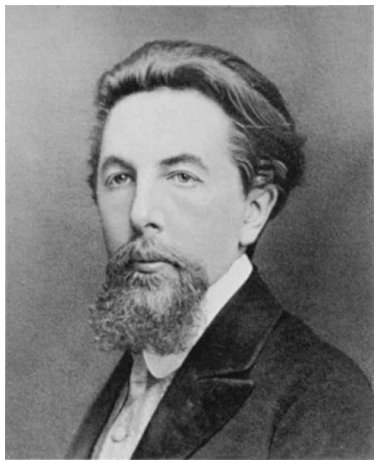
- Principle of Liquid Chromatography
- Principle of Mass Spectrometry
    - Triple Quadrupoles
    - Orbitrap
- Applications of LC-MS

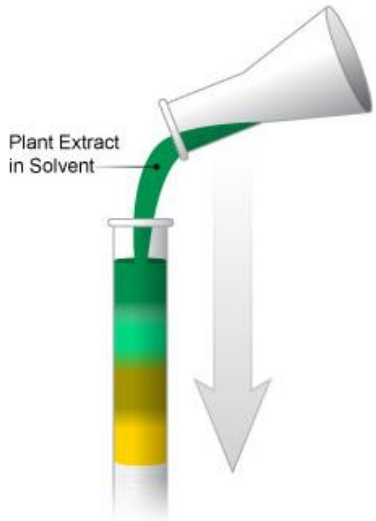
Principle of Liquid Chromatography

Chromatography

"A process by which a mixture is separated into at least two fractions having different compositions"

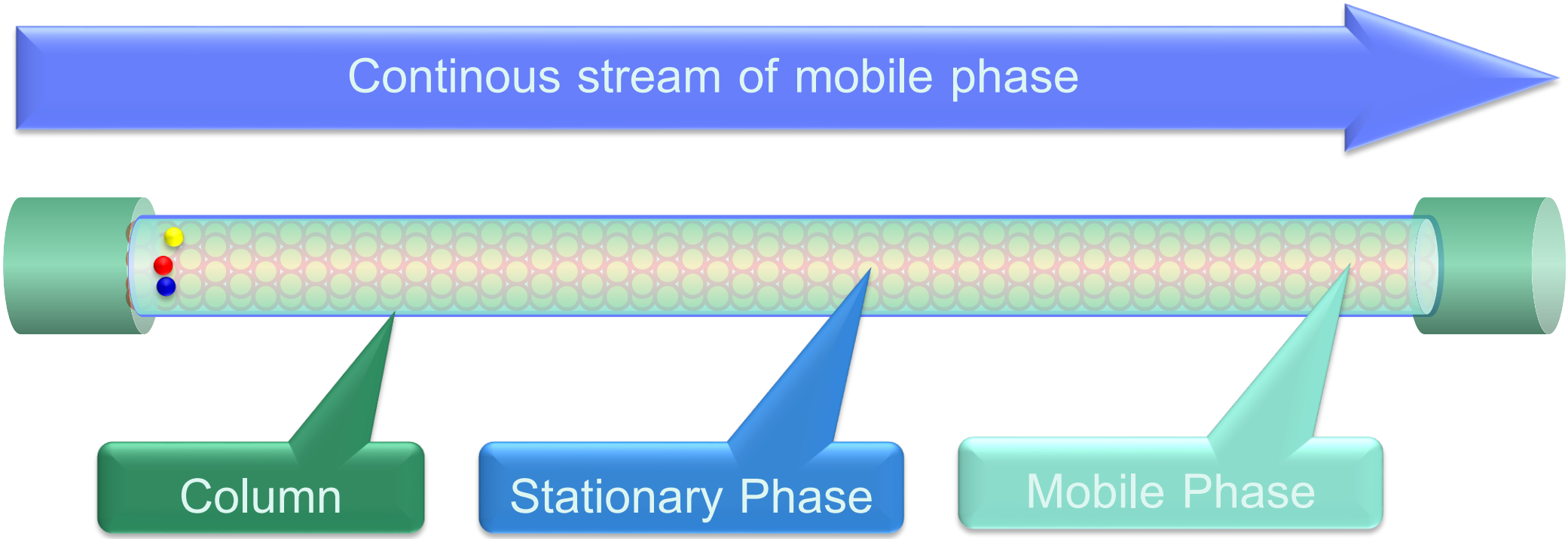
- 1903 - Mikhail Tsvet (Russian Botanist) records a process for the separation of different chlorophylls from plant extract.
  - Coined term "Chromatography" (color writing)





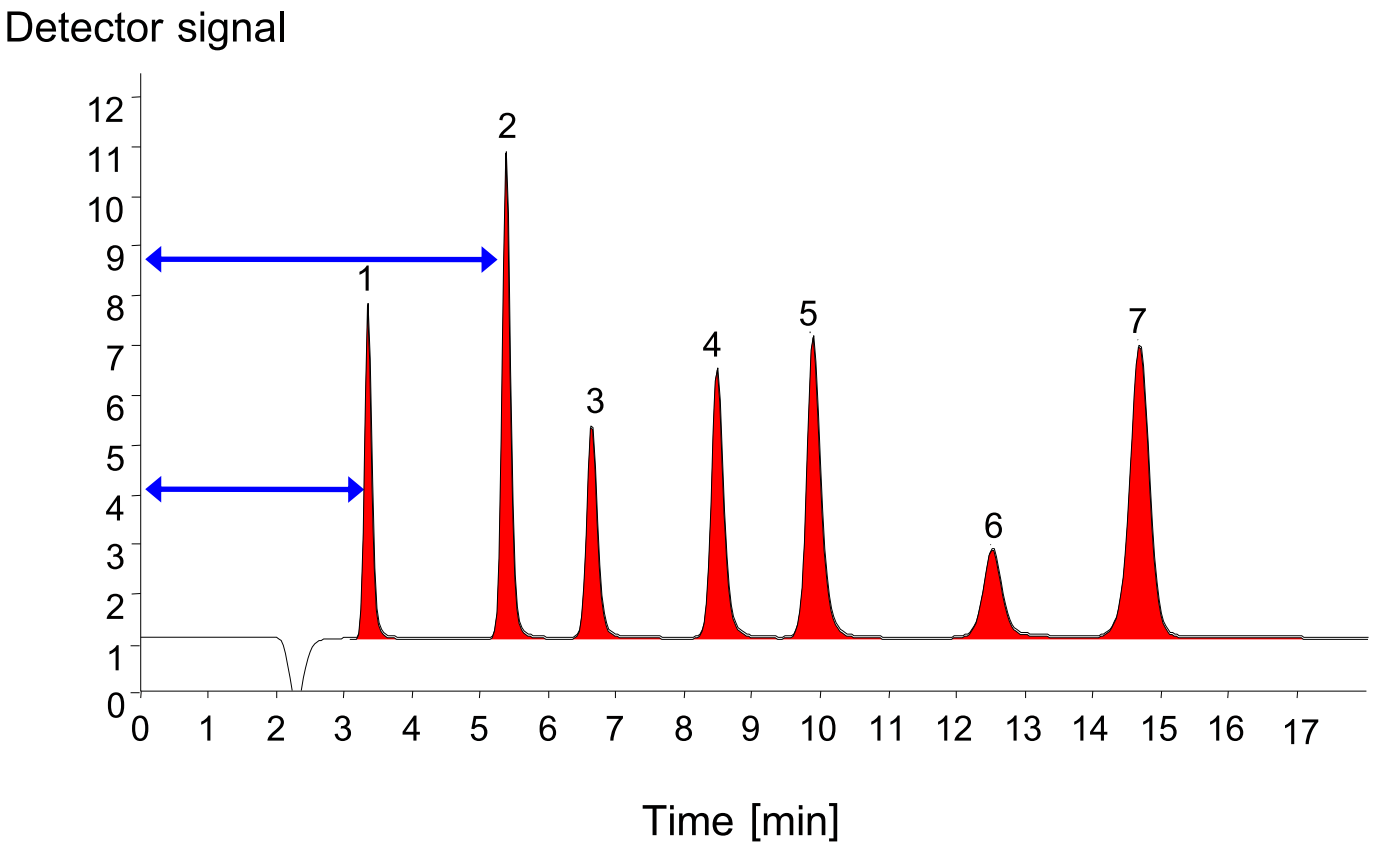
- Why are separations desired?
  - To identify what is present (qualitative analysis)
  - To determine how much is present (quantitative analysis)
  - To purify compounds

How does chromatography work?



- **Separation** : Between two phases (Stationary phase and Mobile phase)
- The components are separate from each other based on difference in affinity for the stationary or mobile phase.
- Separation are achieved based on differences in chemical properties.

Chromatogram

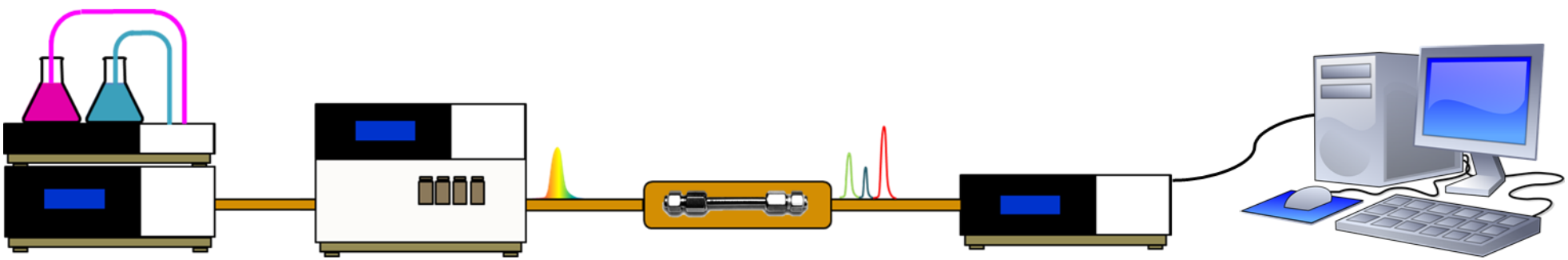


Retention time → **identification**

Peak area → **quantification**

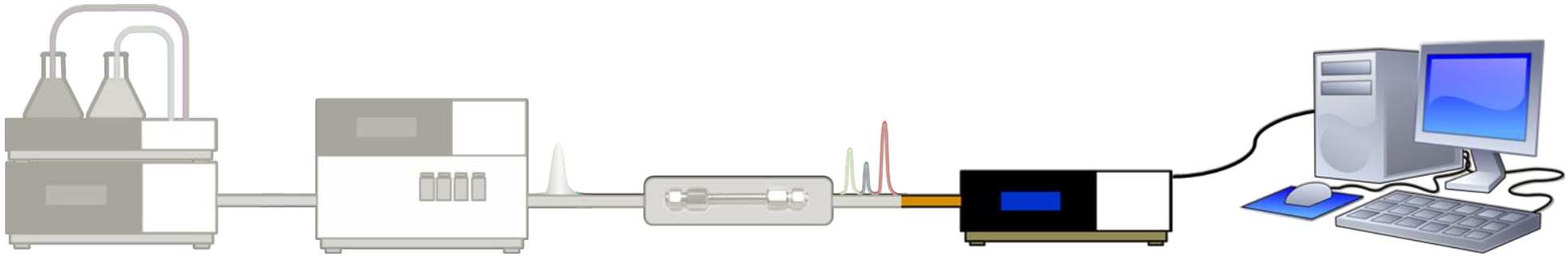
Liquid chromatography

- **Liquid Chromatography (LC)** : Chromatography technique which liquid is used as mobile phase
- High Performance Liquid Chromatography (HPLC)
- HPLC System configuration

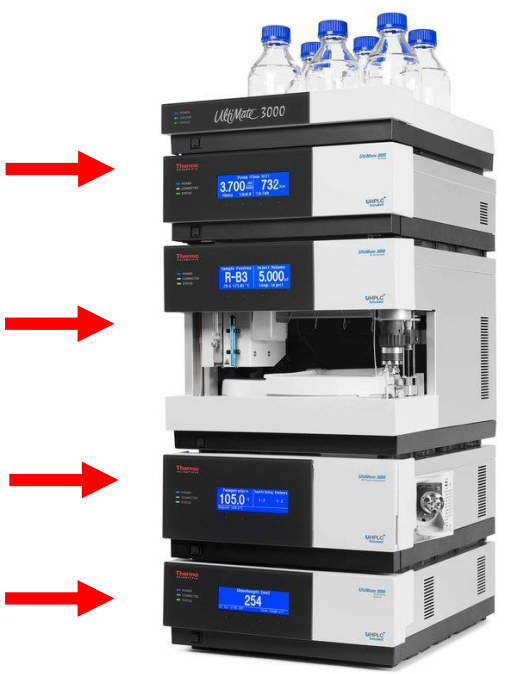


Pump with Degasser | Autosampler | Column (in Column Compartment) | Detector | Computer and Software

HPLC system



- **Pump** : - Control the flow of mobile phase and analyte(s)
  - Mix two or more solvents
- **Degasser** : Remove air bubble in solvents
- **Autosampler** : Inject the sample into a running system
- **Column** : Separate each components
- **Column Compartment** : Control a column temperature
- **Detector** : Detect signal from analyte(s) after seperation
- **Computer and Software** : Control HPLC system and analysis



The UltiMate™ 3000 LC Systems

Pumps


Isocratic


Binary


Quaternary


Dual-Gradient

Autosampler


Basic Automated


Standard


Thermostatted


+ Fractionation

Column Compartments


Standard


With Valves

Detectors


VWD


MWD/DAD


Fluorescence

Corona

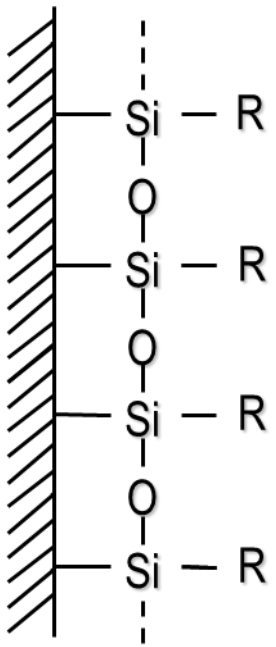

Coulochem

HPLC Modes

- **Normal Phase (NP):** The stationary phase is polar and the mobile phase is non-polar.
  - ➢ Niche mode in modern (prep) LC (e.g. separation of enantiomers)

- **Reversed Phase (RP):** The stationary phase is made of chemically modified silica (normally with non-polar surface) and the mobile phase is polar.
  - ➢ Most common HPLC mode
  - ➢ Good for separation of broad polarity range

Normal Phase Chromatography

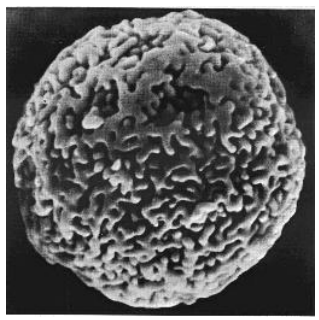


- Common NP stationary phases:

R= OH	Silica
$R=(CH_2)_3NH_2$	Amino
$R=(CH_2)_3CN$	Cyano
$R=(CH_2)_2OCH_2CH(OH)CH_2OH$	Diol

- Common NP mobile phases:

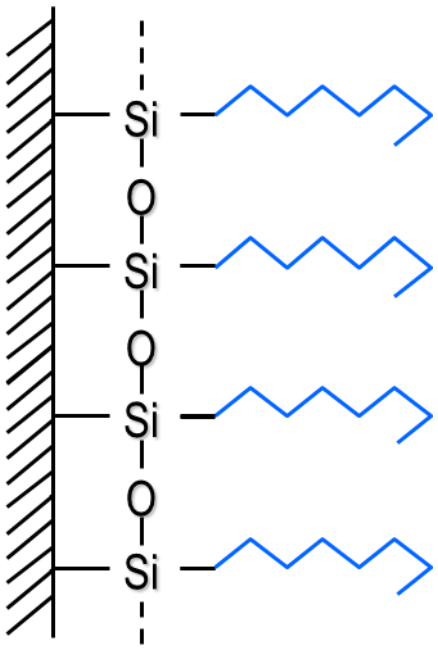
Heptane < Hexane < Toluene < Chloroform < Ethyl acetate < Isopropanol < Acetonitrile < Methanol

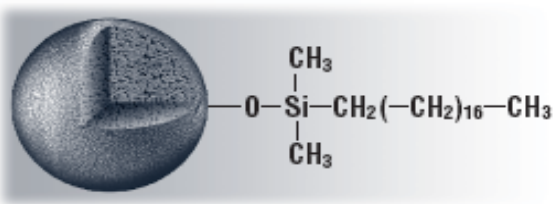


- Summary:

- The least polar component elutes first.
- Increasing the polarity of the mobile phase decreases the elution time.
- Check sample solubility in mobile phase!

Reverse Phase Chromatography





- Common RP stationary phases:

  R=(CH2)17CH3: C18

  R=(CH2)7CH3: C8

- Common RP mobile phases:

  Water < Methanol < Acetonitrile

- Summary:

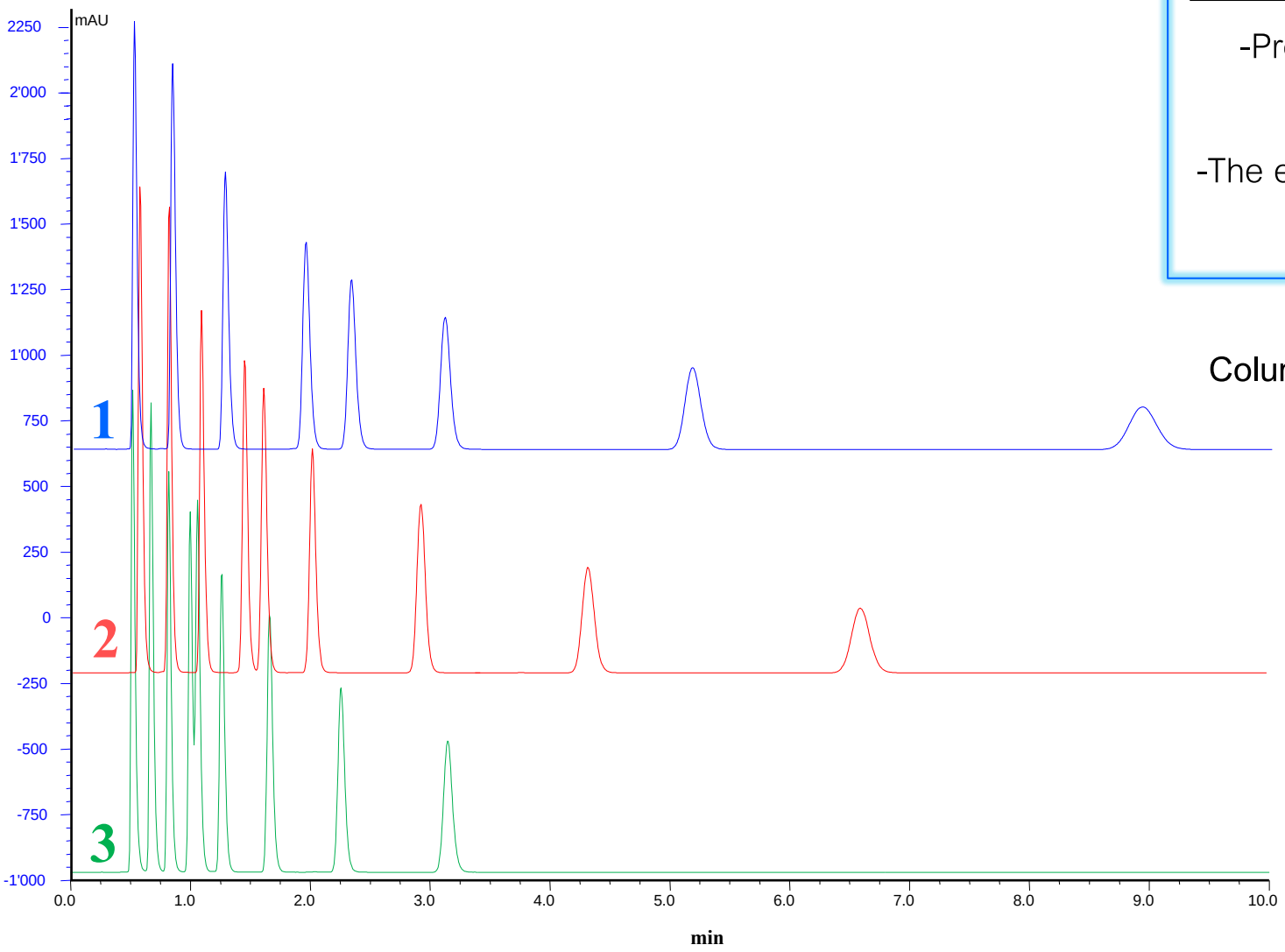
  - The most polar component elutes first.
  - Increasing the polarity of the mobile phase increases the elution time.
  - Most common HPLC mode: 80% RP separation.
  - Good for separation of solutes with broad polar range.

Basic Types of HPLC Solvent Runs

- ISOCRATIC
  - ISO ==> SAME
  - Solvent composition stays the same for the entire run
    (Ex. 60 : 40 Alcohol : Water)

- GRADIENT
  - Gradual change
  - Solvent composition changes throughout the run
  - Gradually changed or Step changes
    (Ex. 100% H_20 / 0% MeOH → 0% H_2O / 100% MeOH)

Isocratic Separation of 9 Alkylphenones



Mobile phase for isocratic separation:

-Pre-mix mobile phase in bottle

or

-The eluents are mixed by the pump:

"dial-a-mix"

Column: Acclaim 3 µm, 3 x 75 mm,

Flow rate 1 mL/min

Mobile Phase: H_2O:ACN

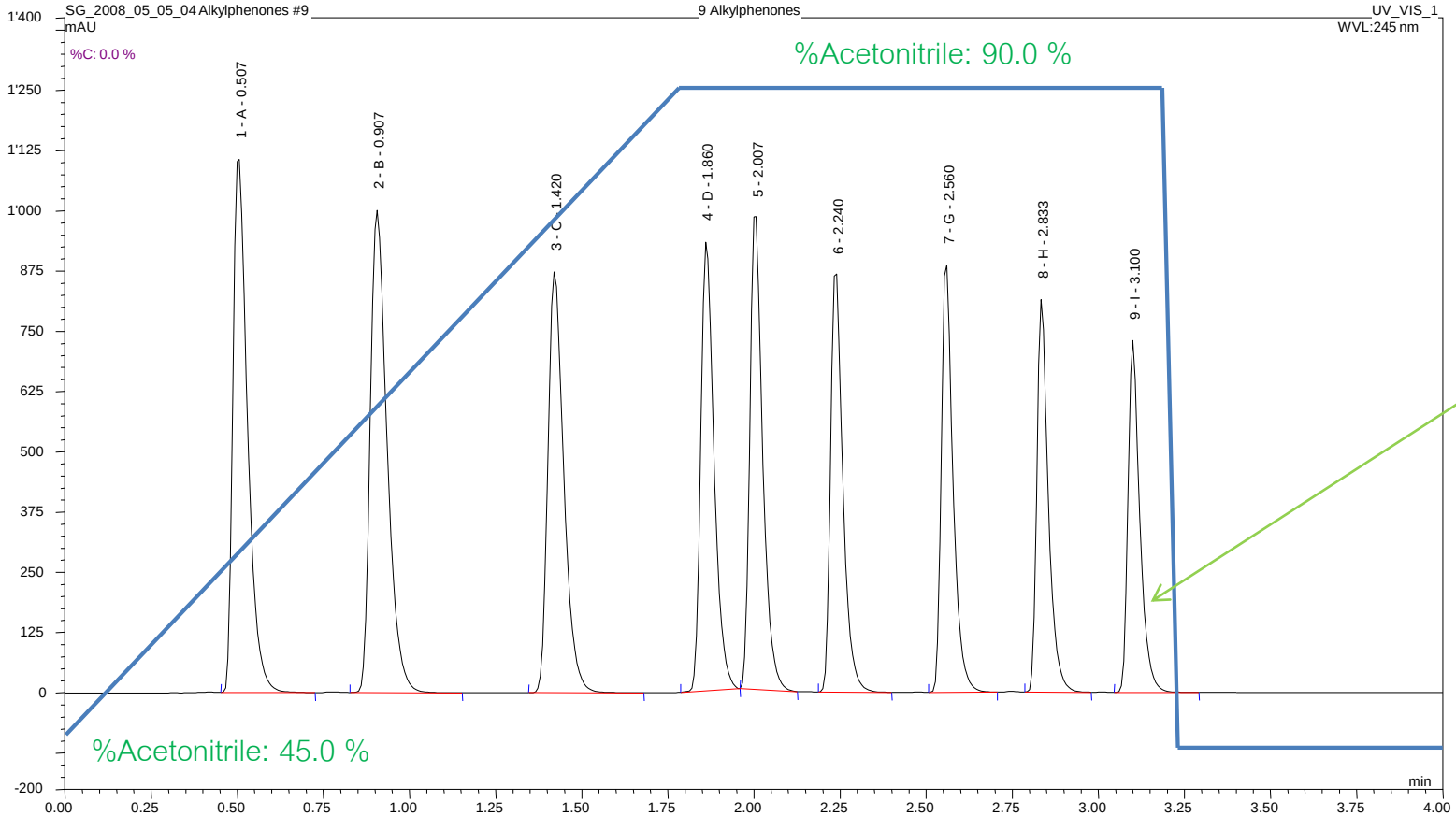
1) 50:50

2) 40:60

3) 30:70

Isocratic flow: mobile phase of constant composition (thermodynamic equilibrium)

Gradient Separation of 9 Alkylphenones



Constant peak width due to increasing elution power by gradient separation

Acclaim 3µm, 3 x 75 mm

Flow rate 1 mL/min

- Gradient elution efficiency is better related to peak capacity
  - a measure of peak number that can be separated in a defined retention period
  - rather than plate number (complex equation for gradient separation).

Gradient flow: composition of mobile phase is time dependent

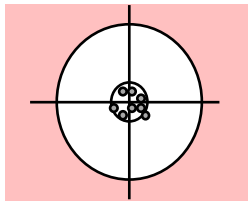
System Contribution – Accuracy and Precision

- Accuracy is the degree of closeness of a measured quantity to its true value

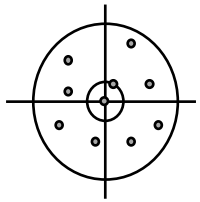
  ⇨ relevance for method transfer

- Precision is the degree of further measurements show the same results (reproducibility) ⇨ deviation of repeated measurements

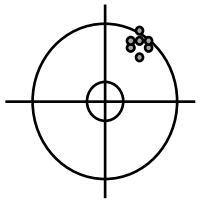
- The target analogy:



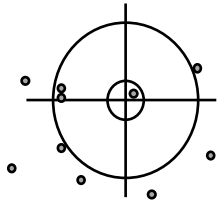
accurate and precise (ideal result)



accurate, but not precise (random errors)



precise, but not accurate (systematic error)



Neither accurate nor precise (useless)

- A valid method or system is accurate and precise!

Principle of Mass Spectrometry

What is Mass Spectrometry?

- The production of ions that are subsequently separated or filtered according to their mass-to-charge (m/z) ratio and detected.
- The resulting mass spectrum is a plot of the (relative) abundance of the produced ions as a function of the m/z ratio."

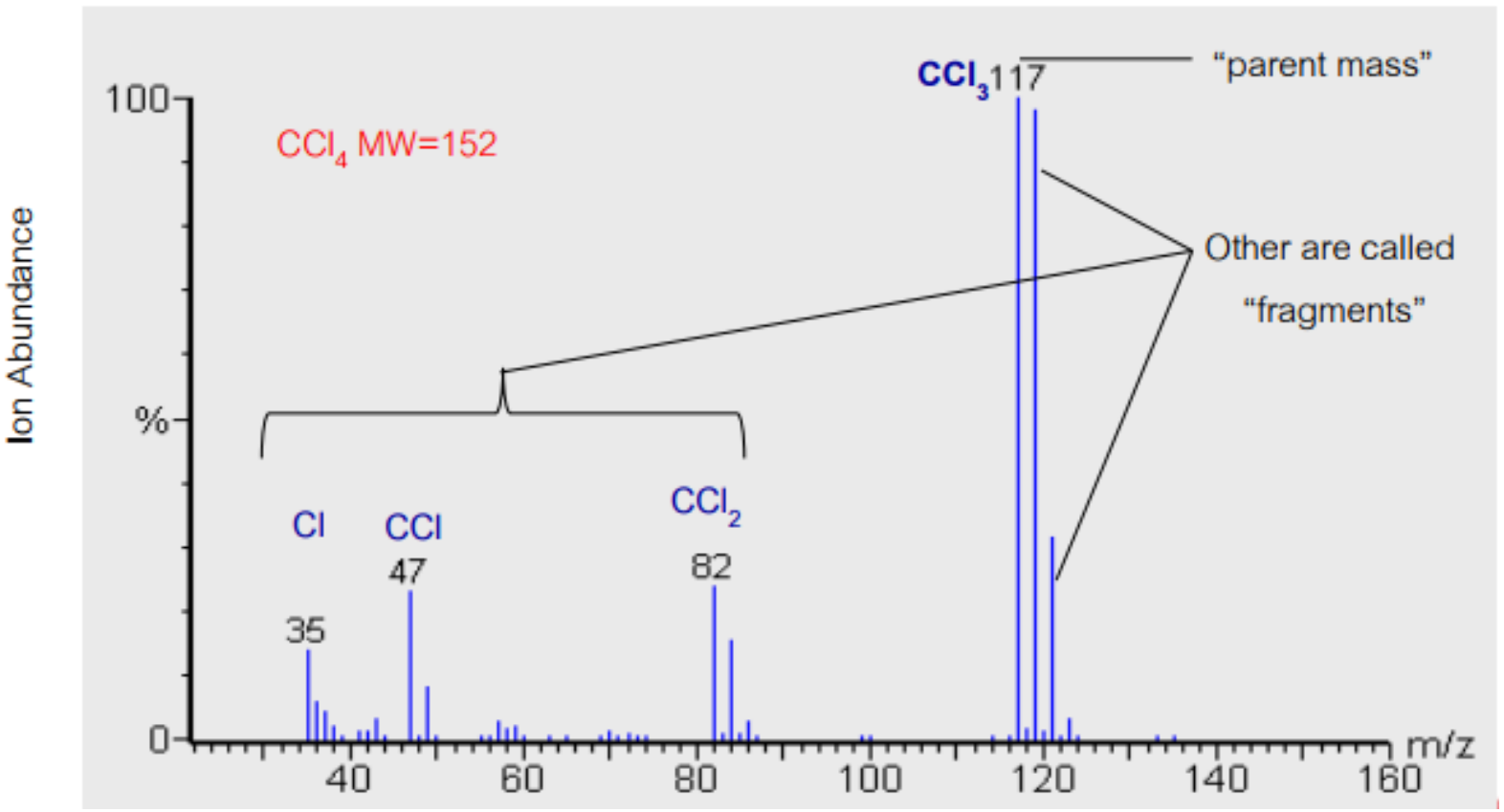
Mass Spectrometry “Simplified”

Generate	→	Ion Production
Move	→	Ion Optics
Select	→	Analyser
Detect	→	Electron Multiplier

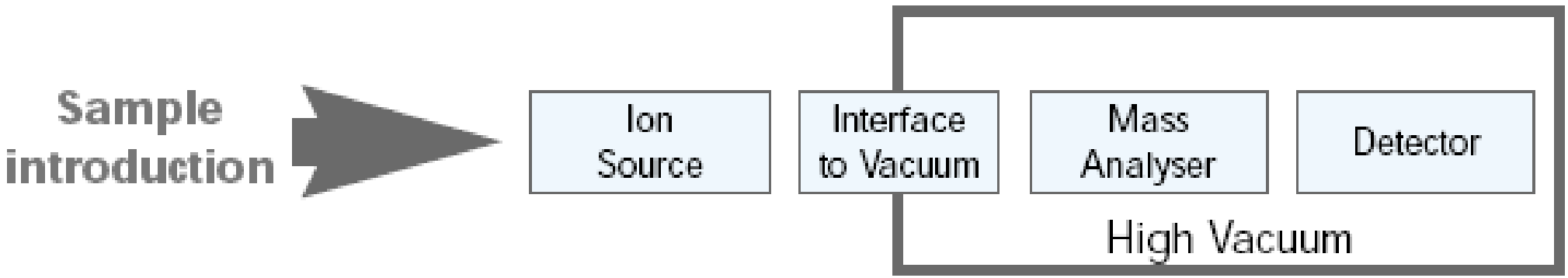
The lifetime of an ion from the point of formation to detection is approximately 50 to 100 microseconds

What is Mass Spectrum?

- Graph of Relative Ion Intensity vs. m/z
- Ion Fragments detail structure and molecular weight of compound



Components in LC-MS



Liquid Chromatography

Ionization

- ESI
- APCI
- MALDI

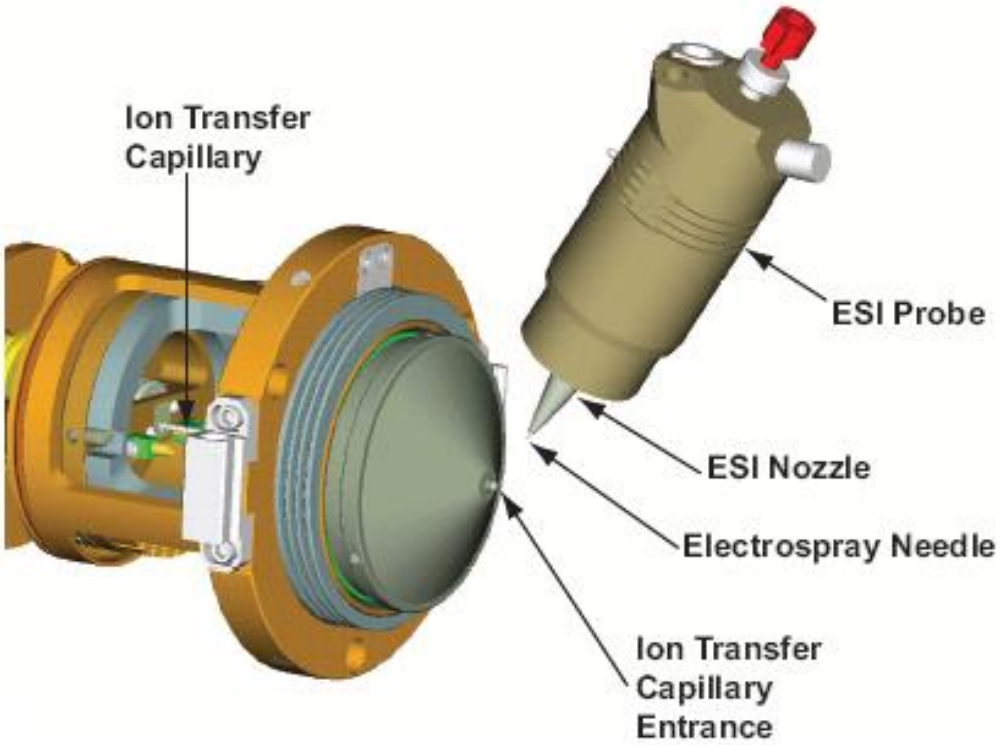
Mass Analyser

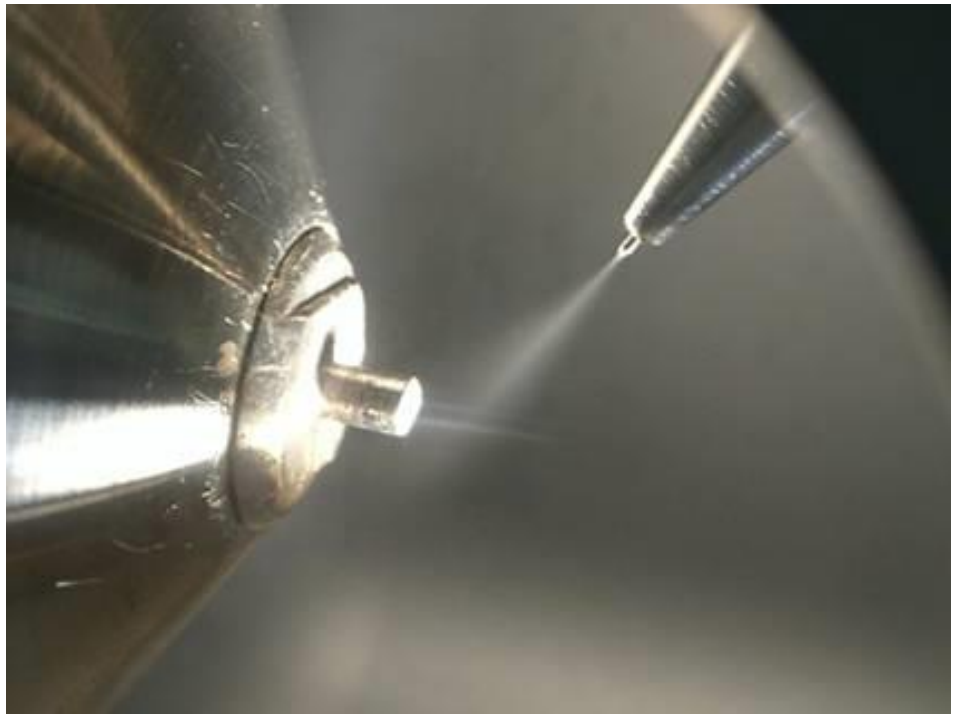
- Triple Quadrupoles
- Orbitrap

Ion Source

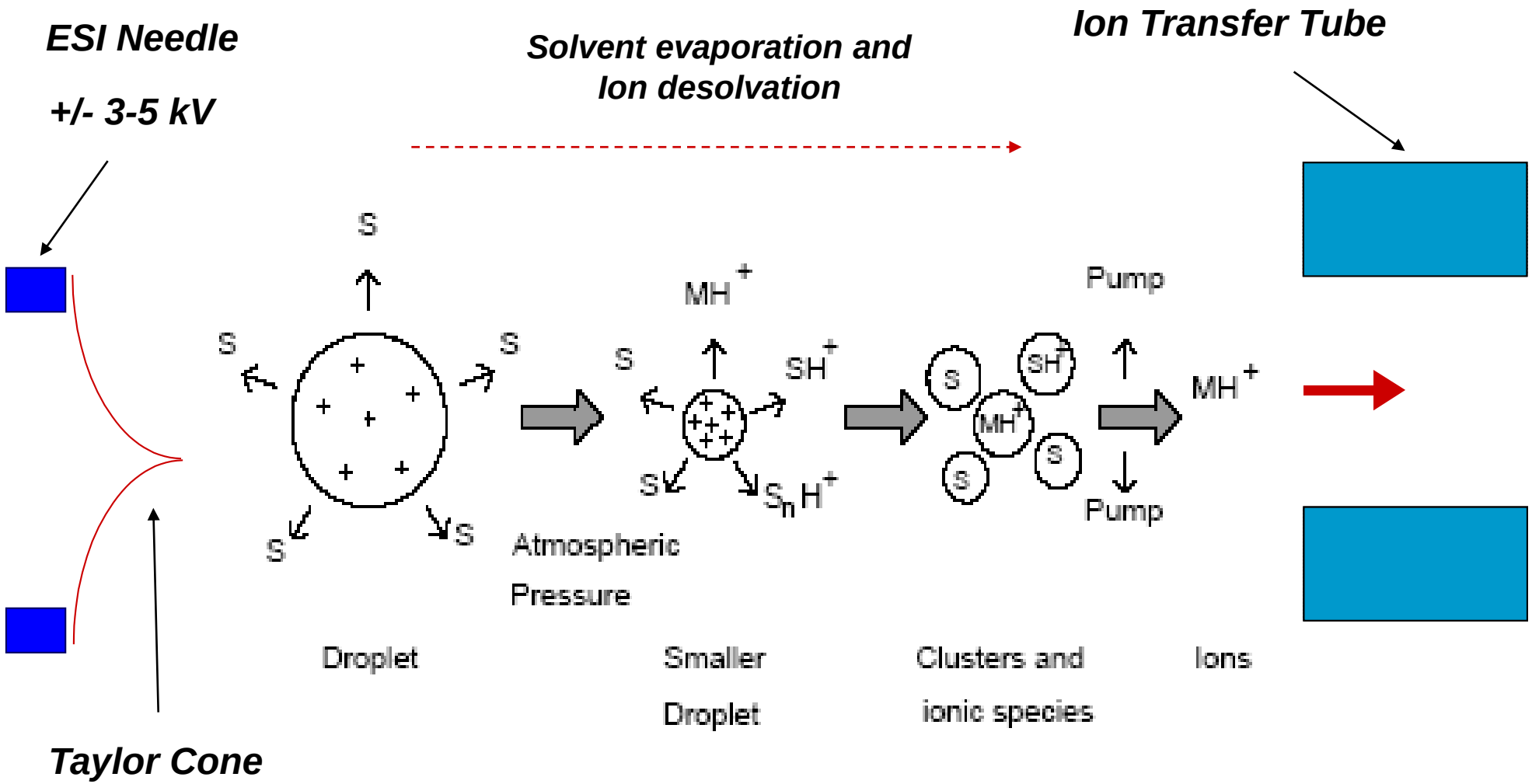
- **Ion source** converts sample molecules (neutral) into charged molecules or molecular ions.
- **Ionization methods in LC-MS**
  - Electrospray Ionization (ESI)
  - Atmospheric Pressure Chemical Ionization (APCI)
  - Matrix assisted laser desorption ionization (MALDI)

Electrospray Ionization (ESI)

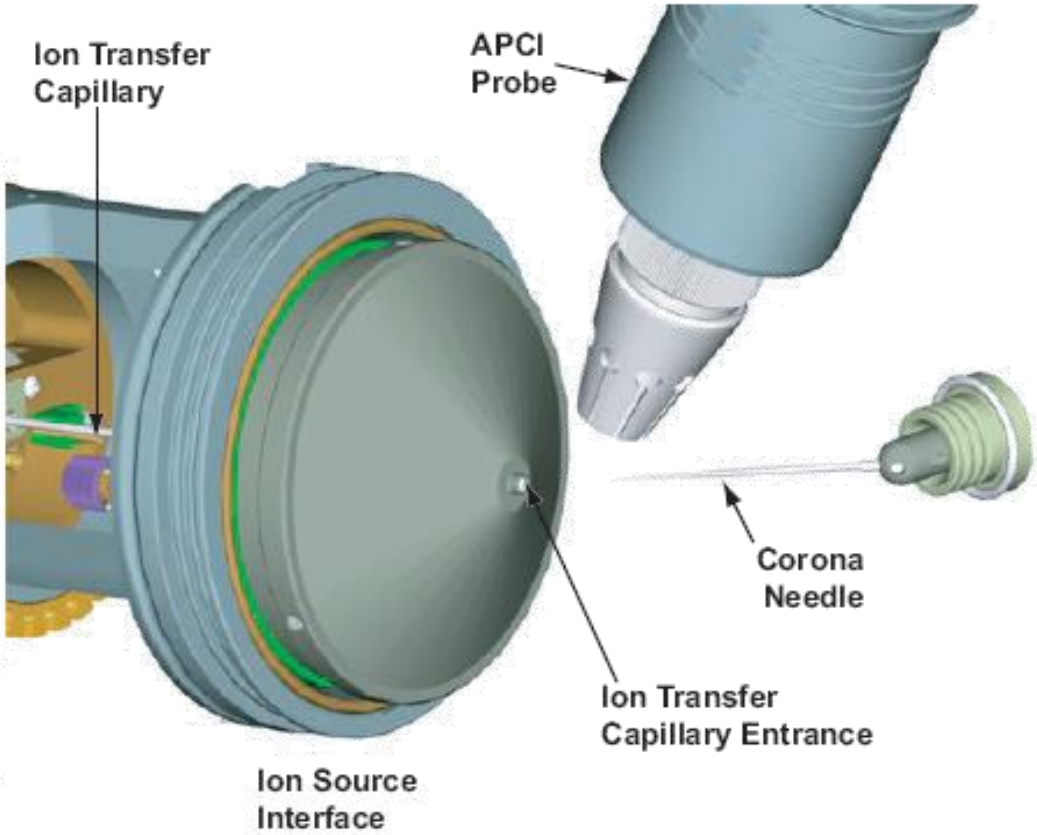


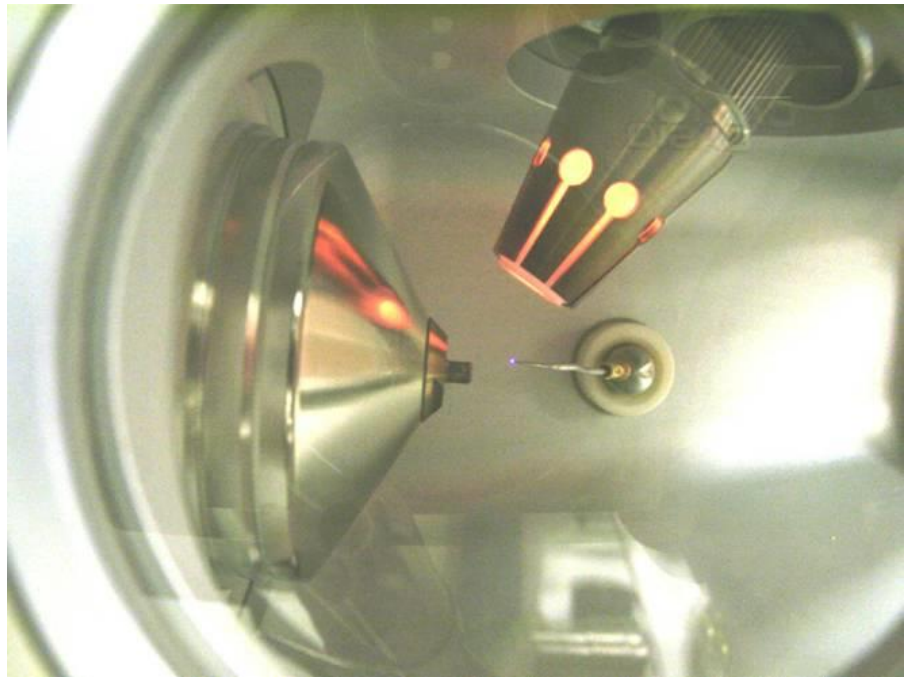


Electrospray Ionization (ESI)

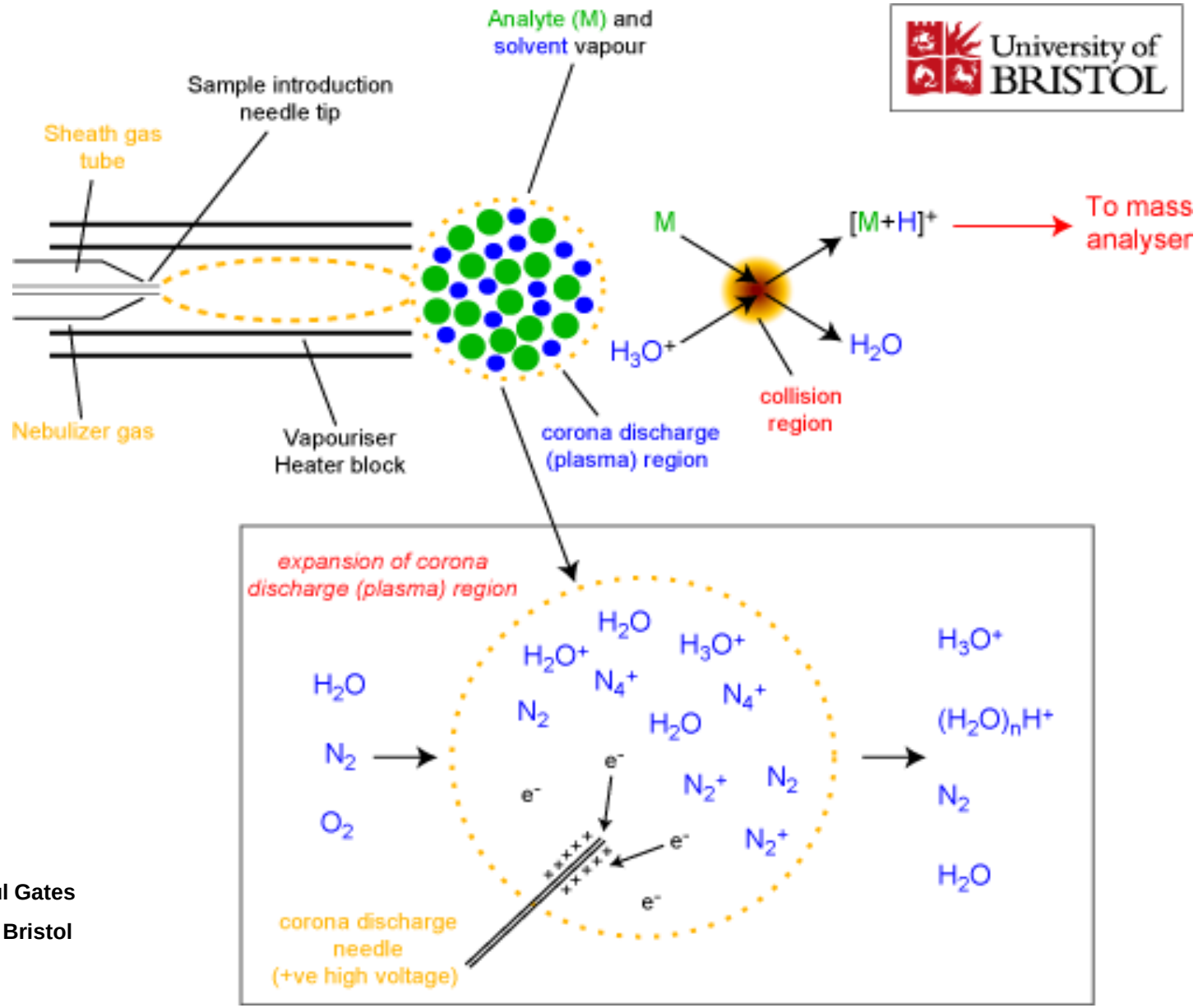


Atmospheric Pressure Chemical Ionization (APCI)





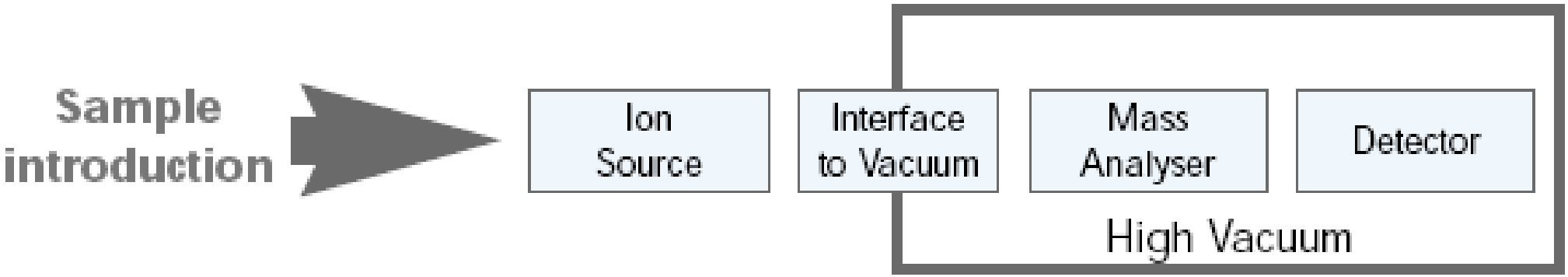
Atmospheric Pressure Chemical Ionization (APCI)

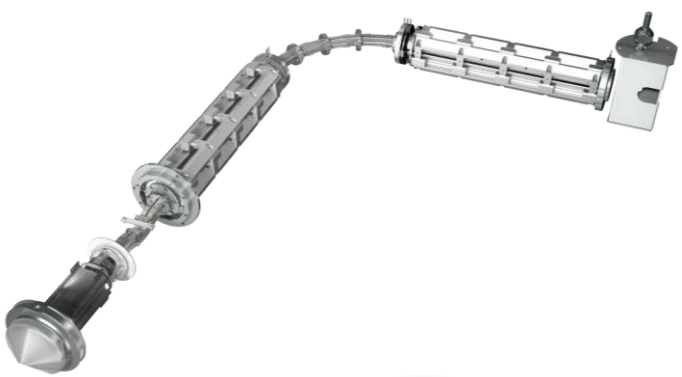


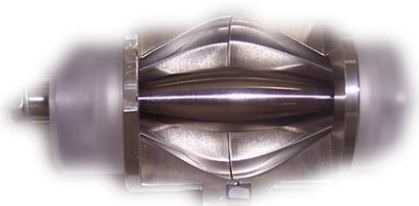
Which is best?

- It depends on the exact application.
- Increasing polarity and molecular weight and thermal instability favors electrospray.
  - Most drugs of abuse are highly polar and are easily analyzed using electrospray.
  - High molecular weight proteins also require electrospray
- Lower polarity and molecular weight favors APCI.
  - Lower background, but compounds must be more thermally stable.

Components in LC-MS



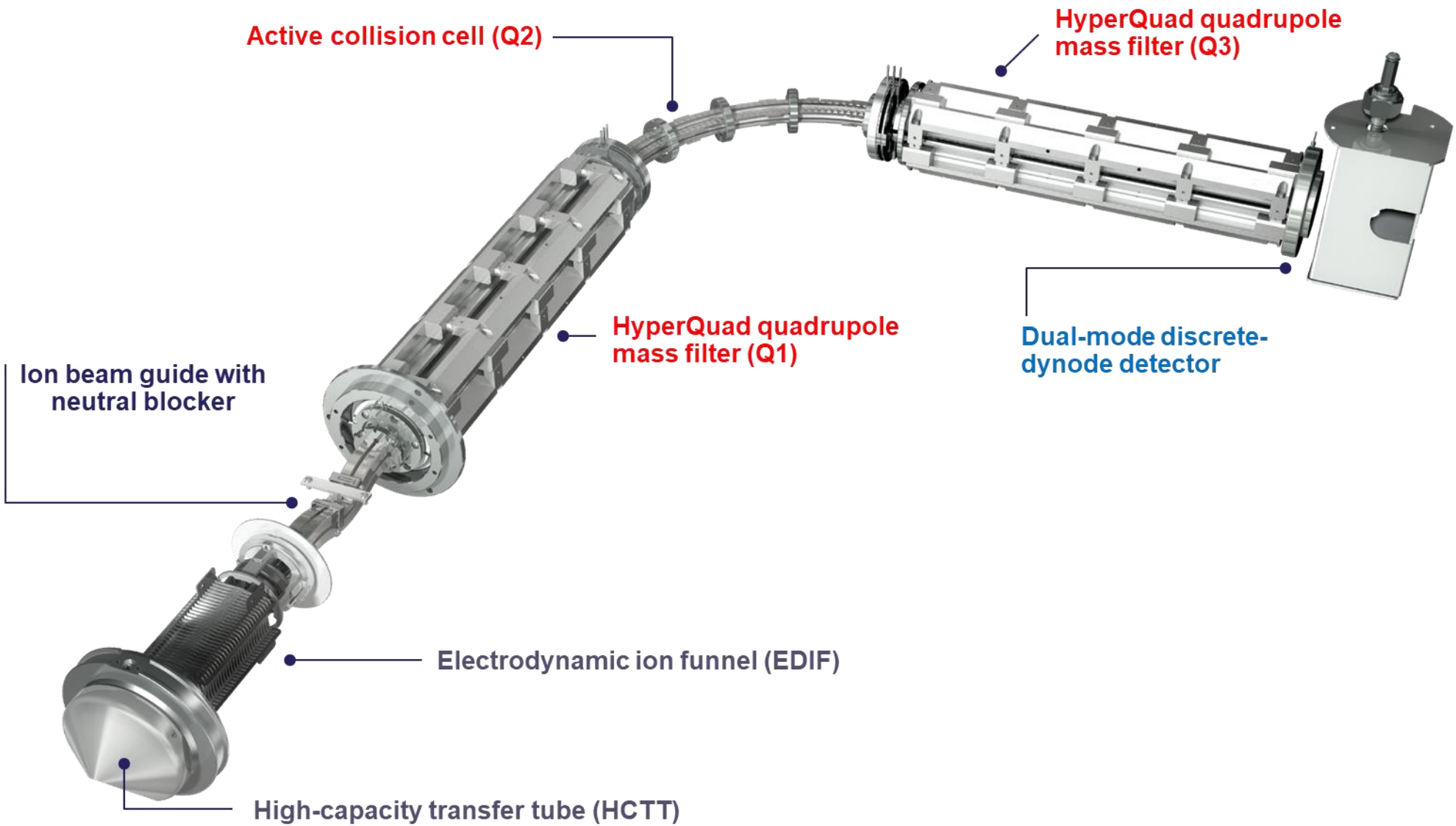




Mass Analyser

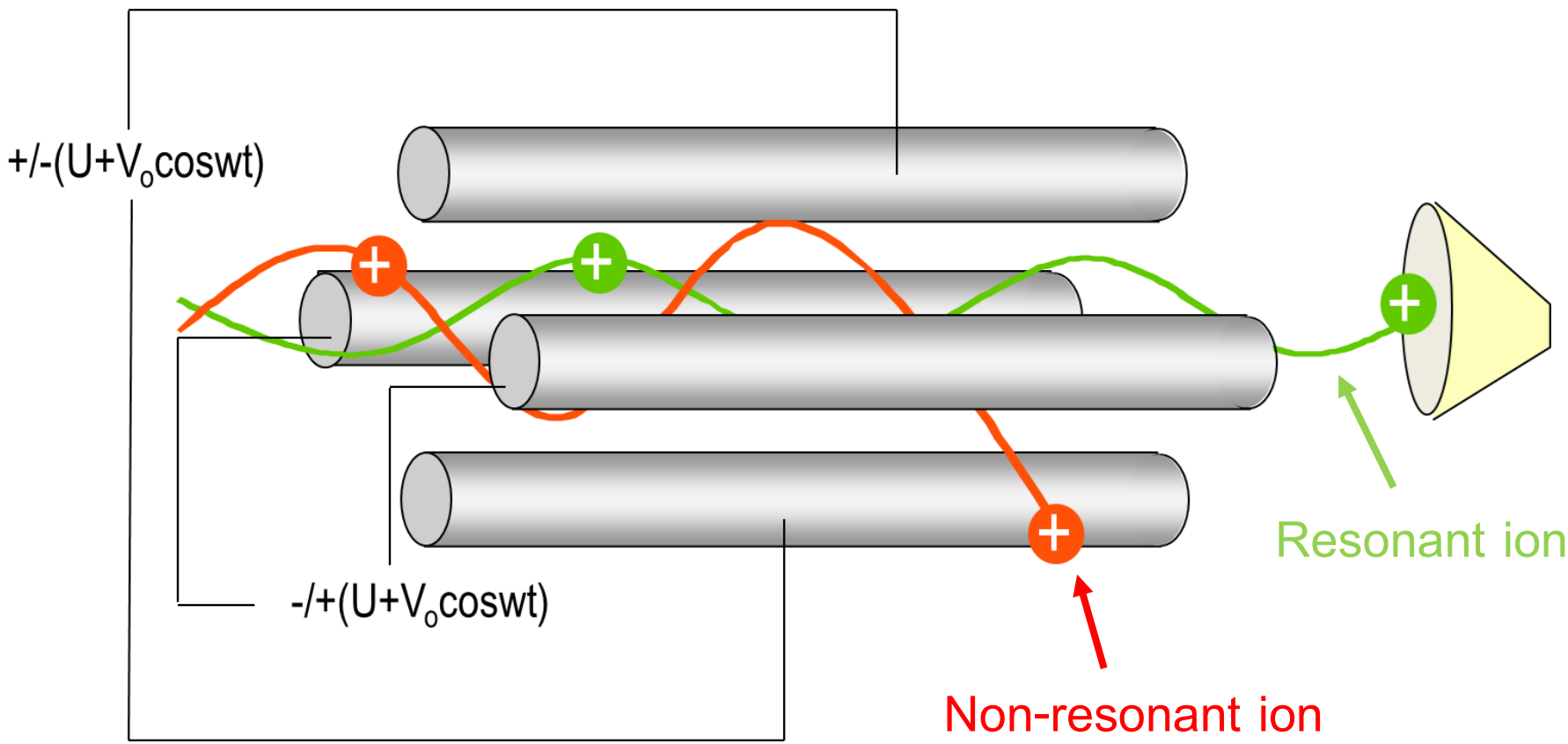
- Triple Quadrupoles
- Orbitrap

Mass Analyzer - Triple Quadrupoles



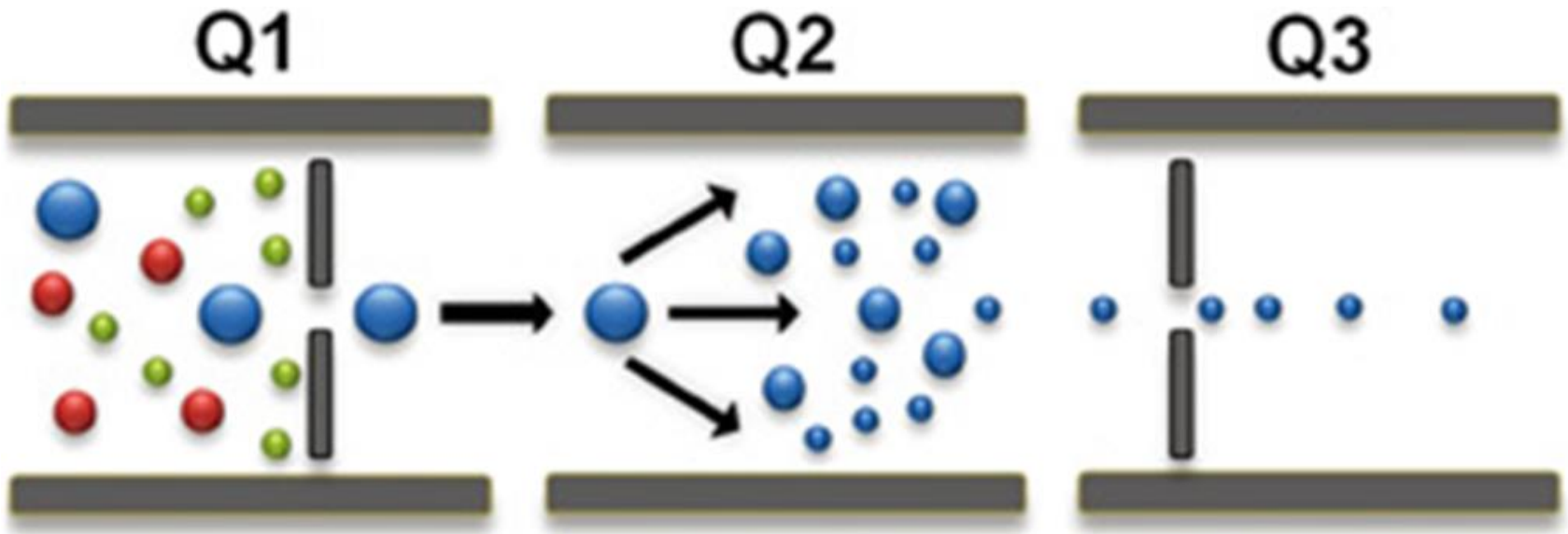
Principle of Operation (Triple Quadrupoles, Q1 & Q3)

- Q1 and Q3 are “mass filter” where ions are scanned by varying the DC/Rf voltage across the quadrupole set.



Principle of Operation (Triple Quadrupoles, Q2)

- Q2 is “collision cell” where precursor ions are fragmented and pass through Q3 for ion sorting again.



Precursor selection

Fragmentation

(Collision gas, AR)

Fragment selection

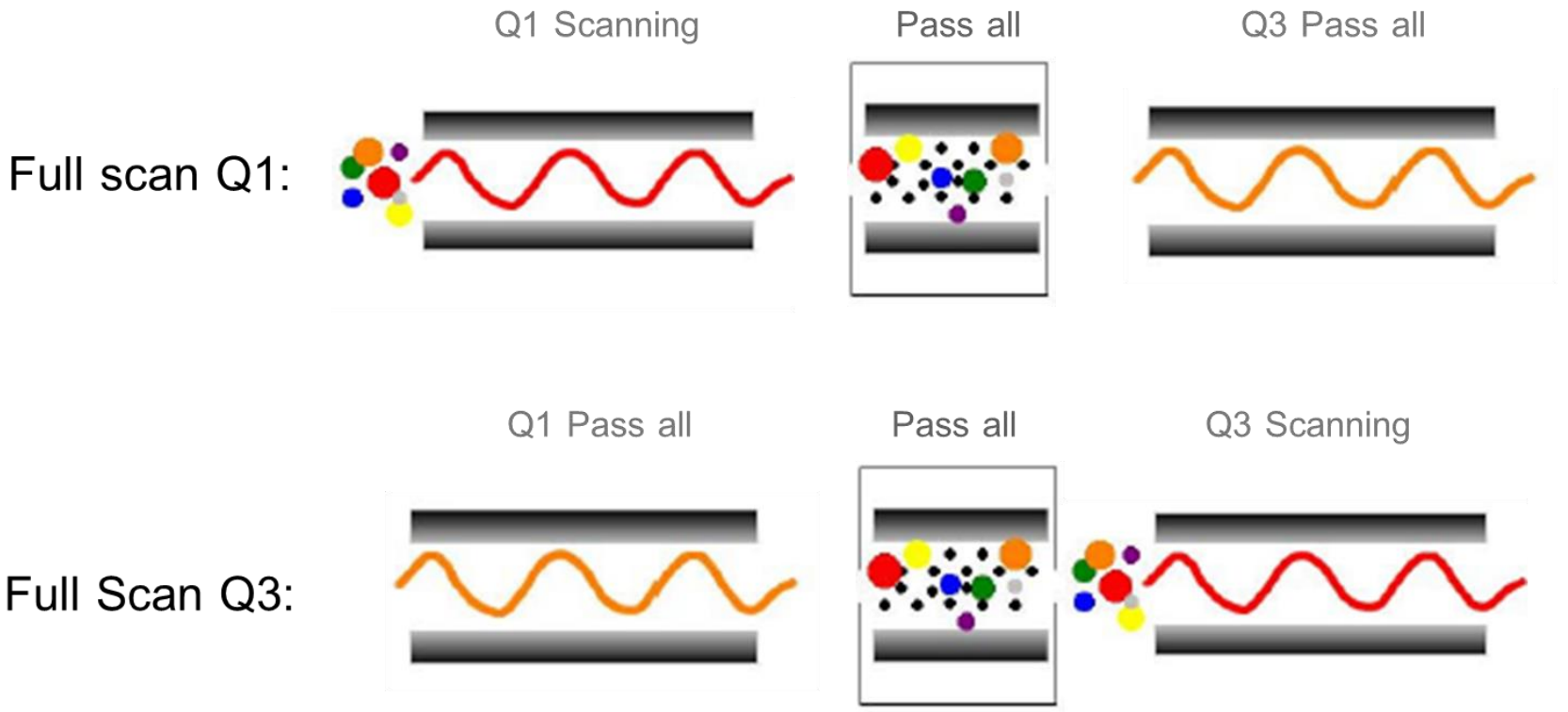
Principle of Operation (Triple Quadrupoles)

http://www.youtube.com/watch?v=LFB14D8pkoc

Scan Modes

Scan Mode	Q1	Q2	Q3	Purpose
Full Scan	Scanning	Pass All	Pass All	MW Info.
SIM	Fixed m/z	Pass All	Pass All	Quantitation
Product	Fixed m/z	Pass All (+ CE)	Scanning	Structural Info.
SRM	Fixed m/z	Pass All (+ CE)	Fixed m/z	Targeted Quantitation
Neutral Loss	Scanning	Pass All (+ CE)	Scanning	Analyte Screening
Precursor	Scanning	Pass All (+ CE)	Fixed m/z	Analyte Screening

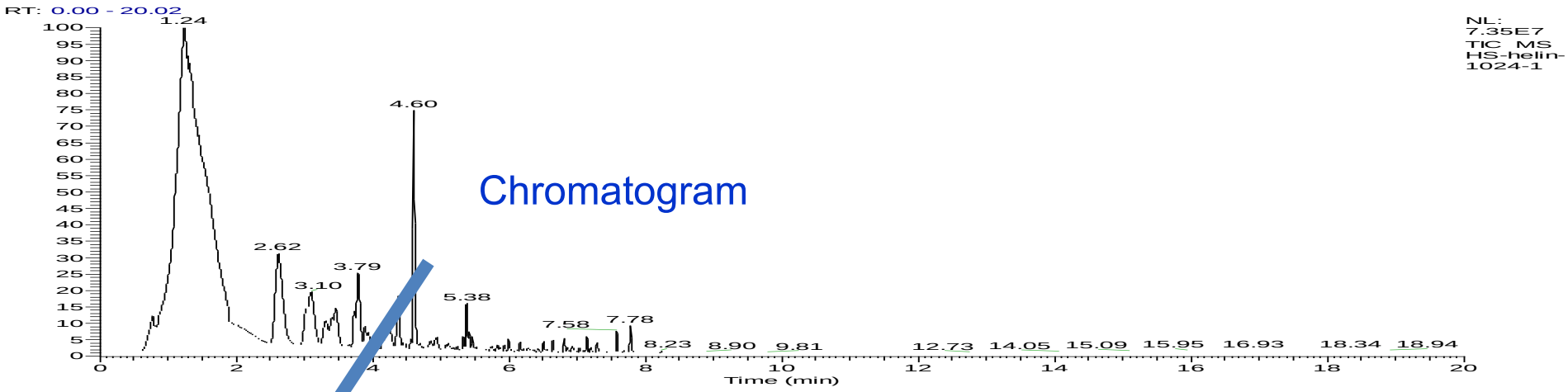
Full Scan Mode (Q1 or Q3)



Purpose: Survey scan of a chromatographic peak

- **Advantages**
  - Provides structure (MW) information
  - Library searchable
- **Disadvantages**
  - Low duty cycle
  - Low sensitivity

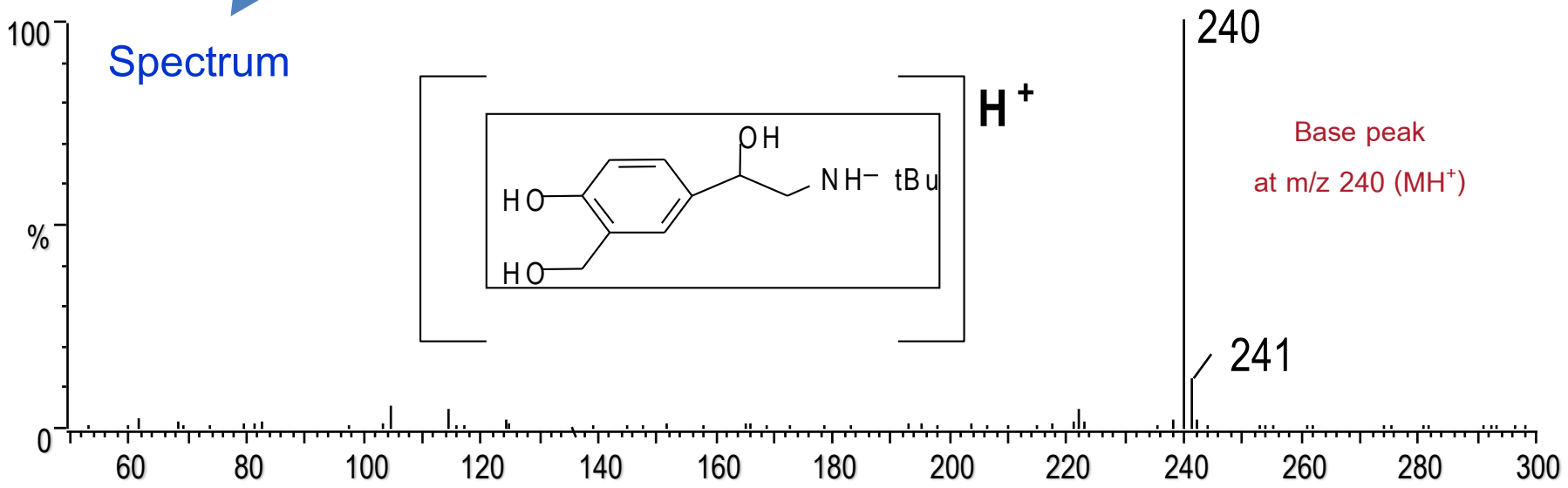
Full Scan Mode



Chromatogram

One Click

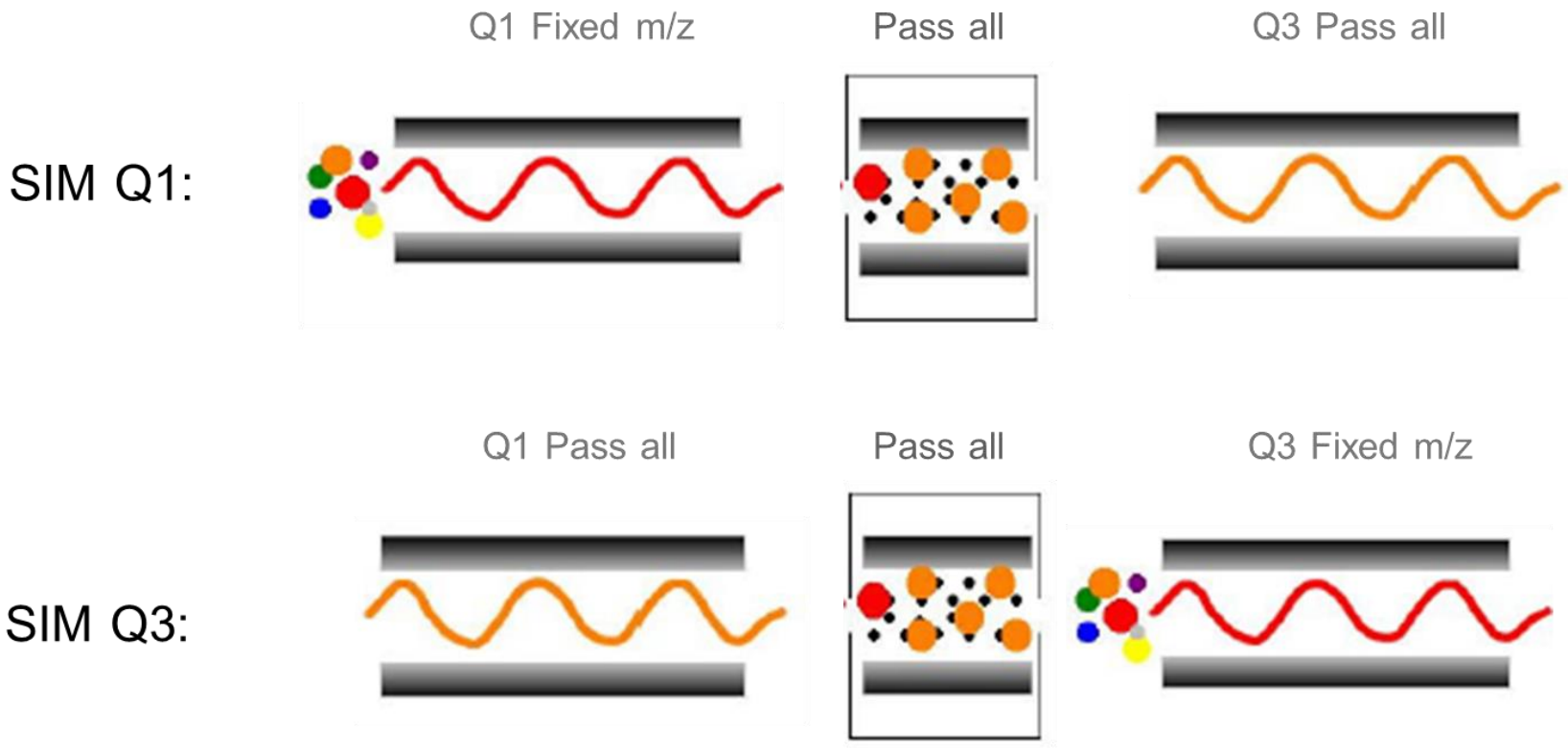
Spectrum



Base peak

at m/z 240 (MH^+)

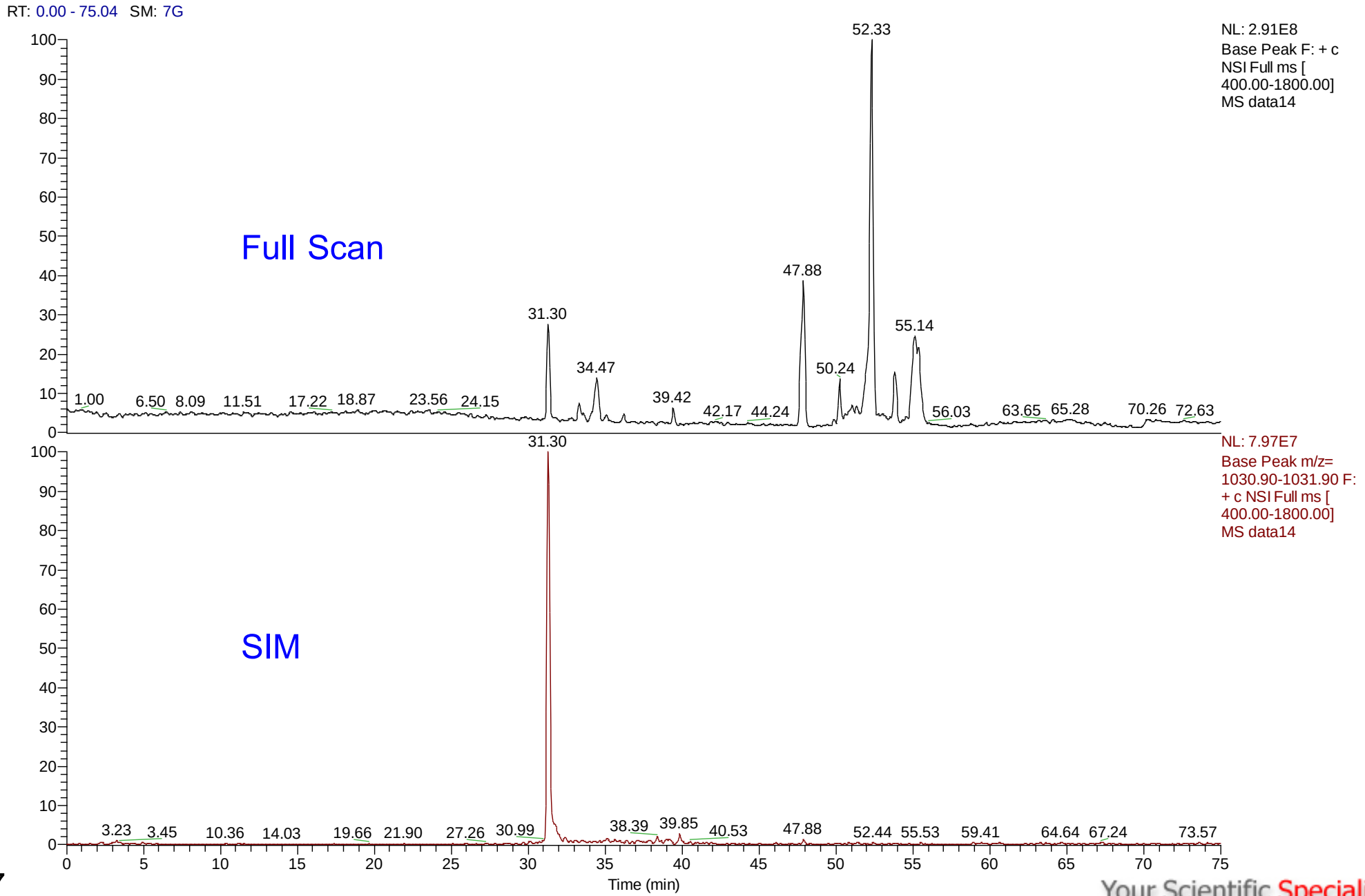
Selected Ion Monitoring Mode (SIM)



Purpose: Quantitation on a specific m/z range of ions

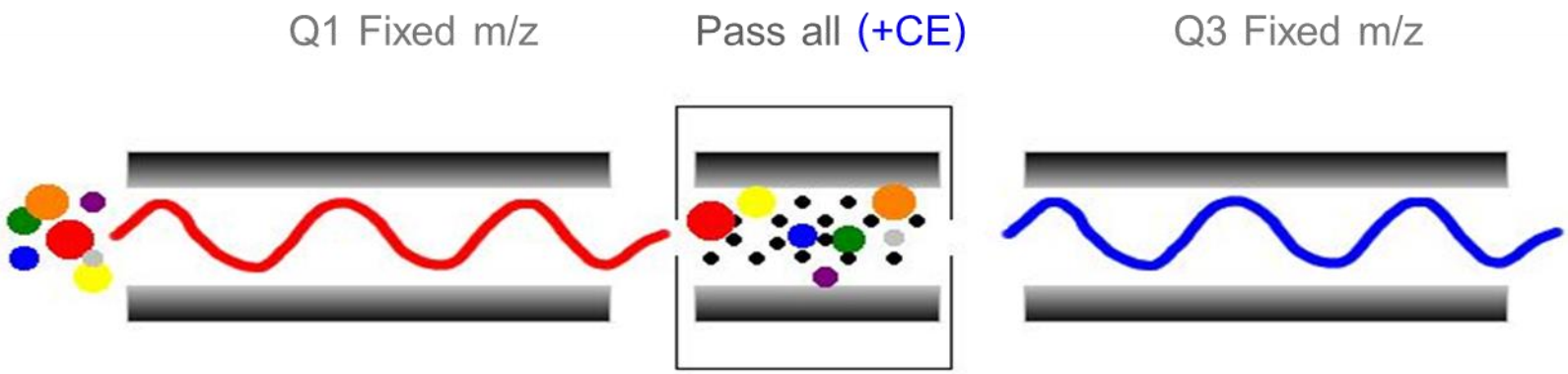
- **Advantages**
  - Targeted analyte monitoring
  - High duty cycle
- **Disadvantages**
  - Can suffer from interferences
  - Not as sensitive or selective as SRM

Full Scan versus SIM



Full Scan versus SIM

Selected Reaction Monitoring Mode (SRM)



Purpose: Targeted quantitation

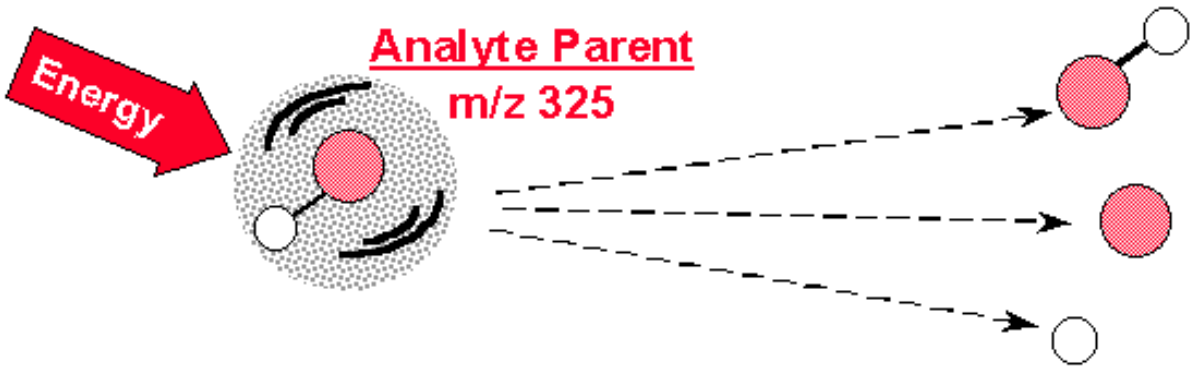
Advantages

- Targeted analyte monitoring
- High duty cycle
- "Simultaneous" monitoring of multiple transitions

Disadvantages

- No structural information

The Need for True MS/MS

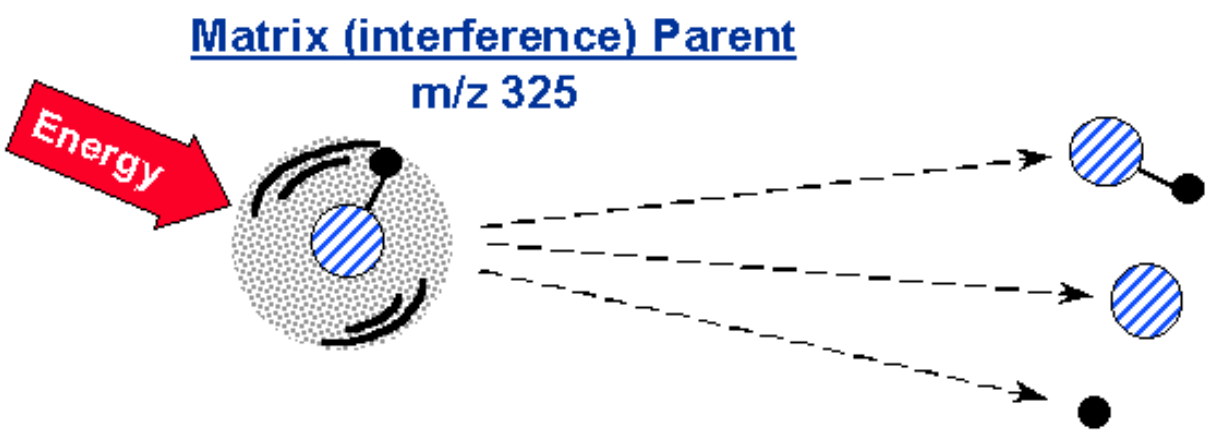


m/z 325 Intact Analyte Parent

m/z 175 Analyte CID Product A

m/z 150 Analyte CID Product B

Different compounds having the same parent m/z have different structures. True MS/MS facilitates Monitoring of a Single Reaction (SRM) for interference free quantitation of analytes in matrix.



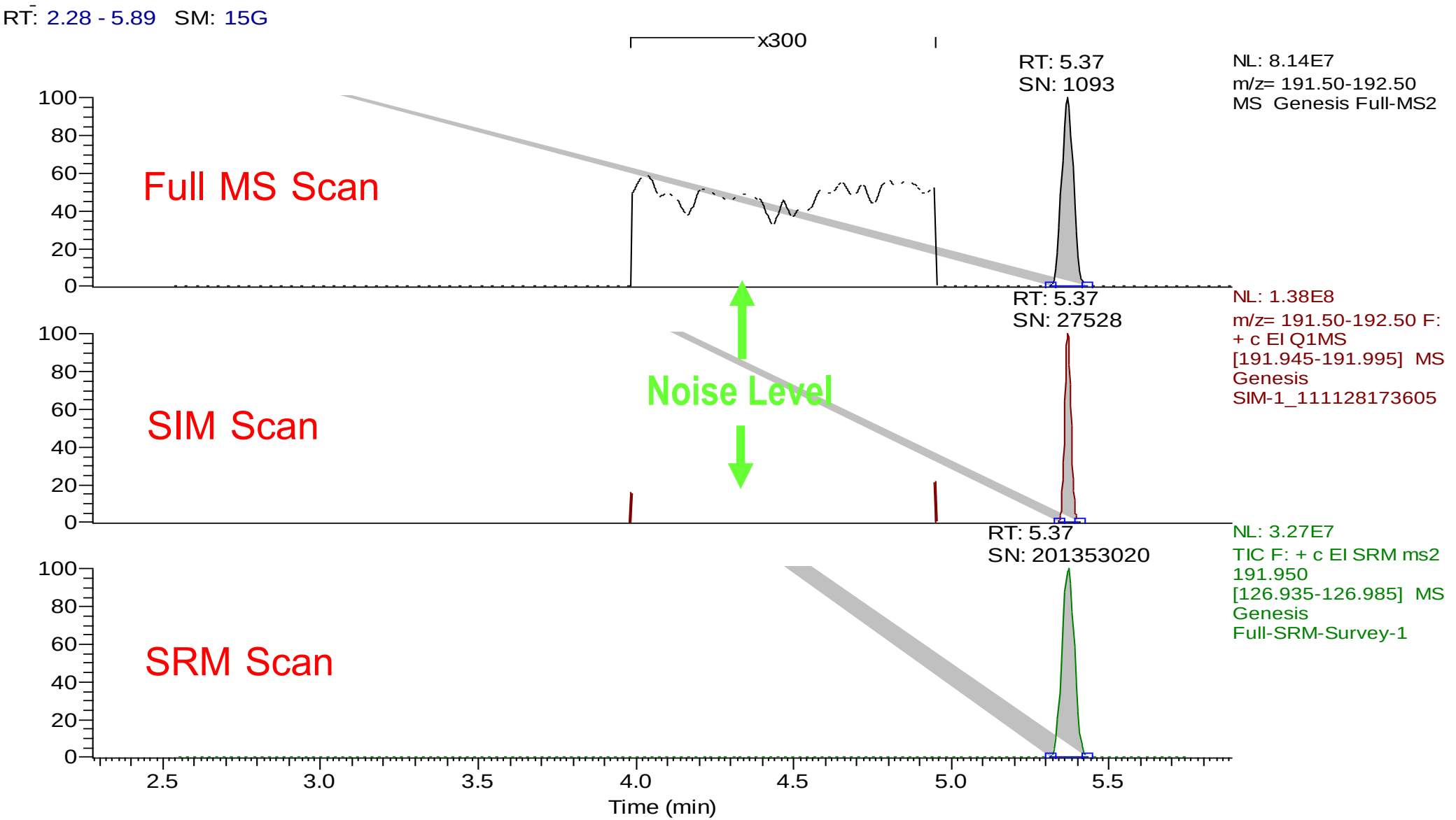
m/z 325 Intact Matrix Parent

m/z 185 Matrix CID Product A

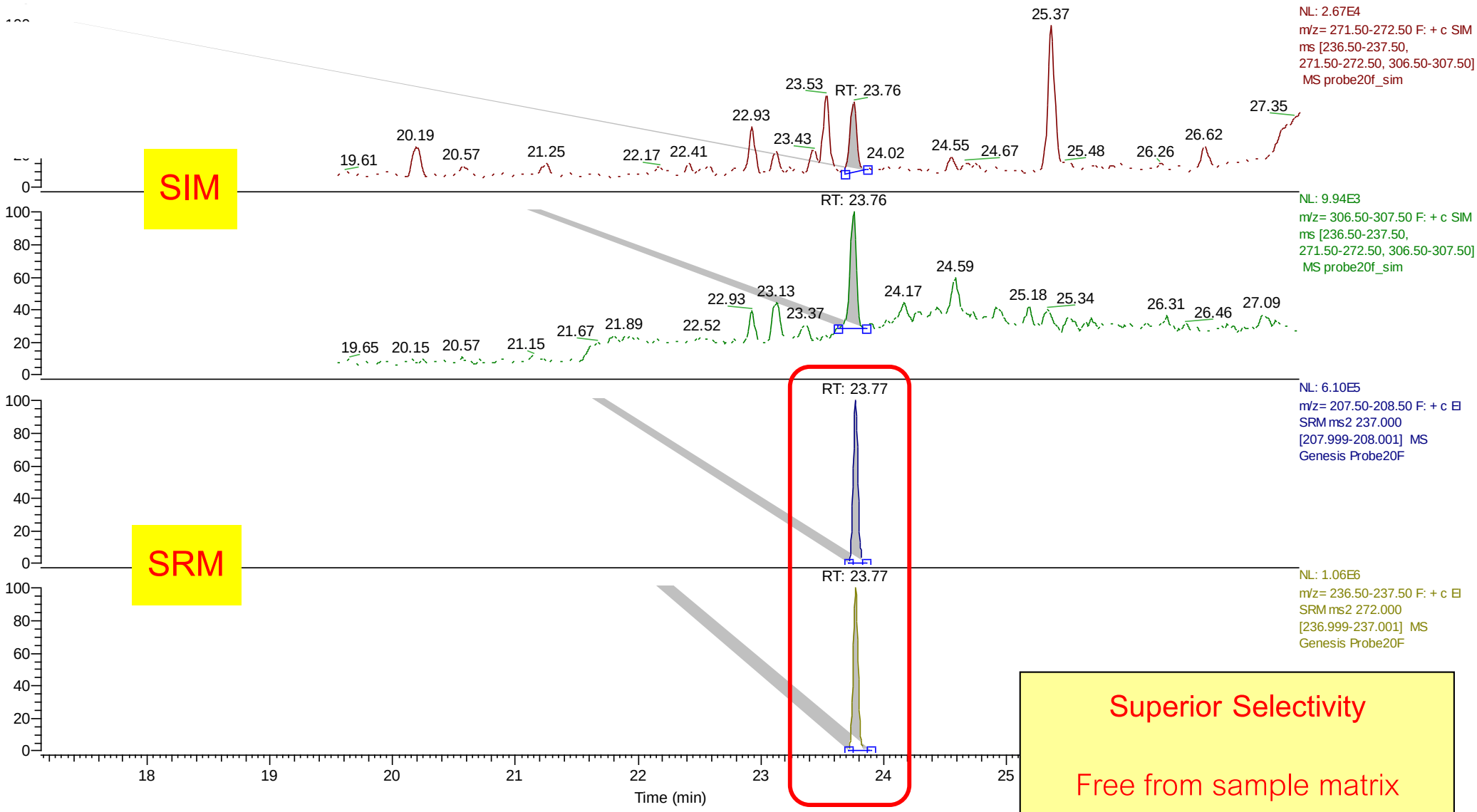
m/z 140 Matrix CID Product B

LC/MS Cross Roads -- Revision 7.5 -- 8/27/98 -- Richard Klein

SRM Selectivity in Complex Matrices



Comparison of SIM and SRM



Superior Selectivity

Free from sample matrix

Anal. Chem. **2000,** *72,* 1156−1162

Electrostatic Axially Harmonic Orbital Trapping: A High-Performance Technique of Mass Analysis

Alexander Makarov*

HD Technologies Ltd., Atlas House, Simonsway, Manchester, M22 5PP, U.K.

This work describes a new type of mass analyzer which employs trapping in an electrostatic field. The potential distribution of the field can be represented as a combination of quadrupole and logarithmic potentials. In the absence of any magnetic or rf fields, ion stability is achieved only due to ions orbiting around an axial electrode. Orbiting ions also perform harmonic oscillations along the electrode with frequency proportional to $(m/z)^{-1/2}$. These oscillations are detected using image current detection and are transformed into mass spectra using fast FT, similarly to FT ICR. Practical aspects of the trap design are presented. High-mass resolution up to 150 000 for ions produced by laser ablation has been demonstrated, along with high-energy acceptance and wide mass range.

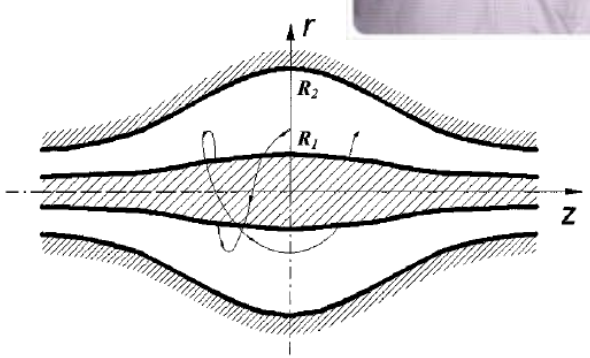
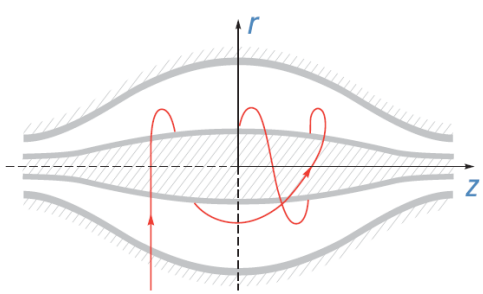


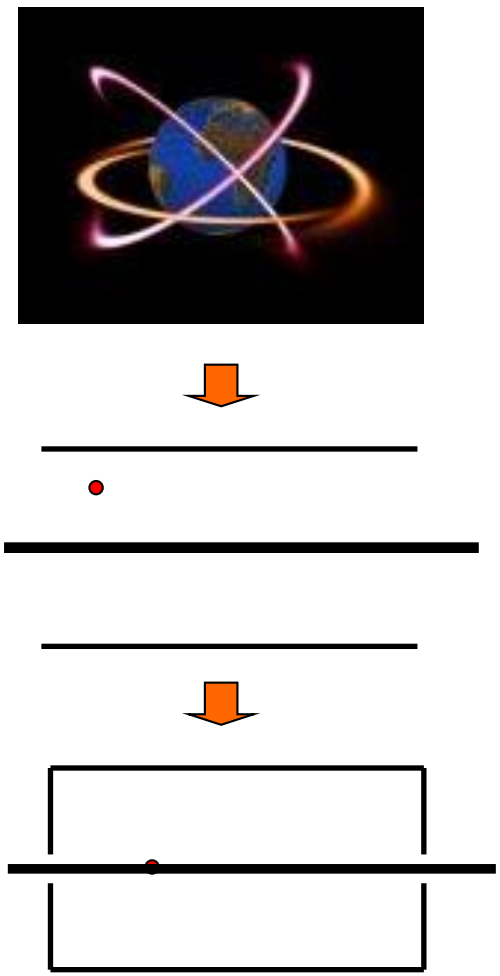
Figure 1. Equipotentials of the quadro-logarithmic field and an example of a stable ion trajectory

Mass Analyzer - Orbitrap

Principle of Operation (Orbitrap)

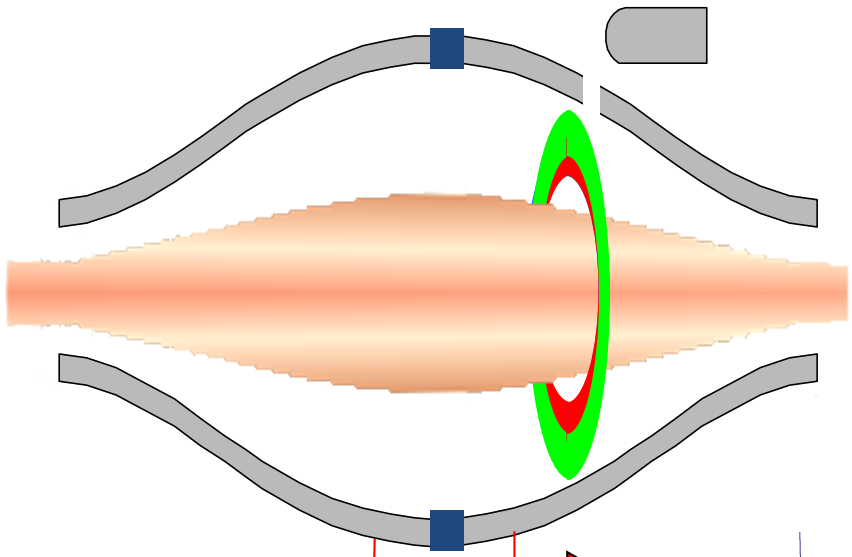
- The Orbitrap is an ion trap – but there are no RF or magnet fields!
- Moving ions are trapped around an electrode
  - Electrostatic attraction is compensated by centrifugal force arising from the initial tangential velocity
- Potential barriers created by end-electrodes confine the ions axially
- One can control the frequencies of oscillations (especially the axial ones) by shaping the electrodes appropriately
- Thus we arrive at …

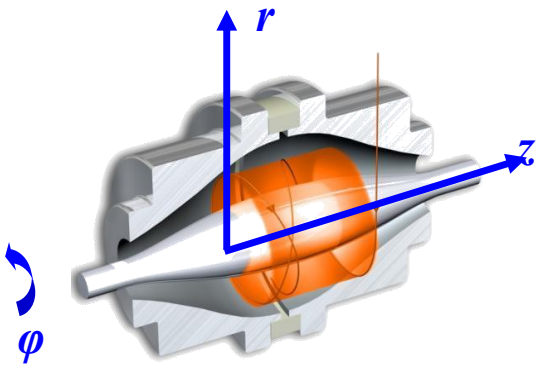




Orbital traps
Kingdon (1923)

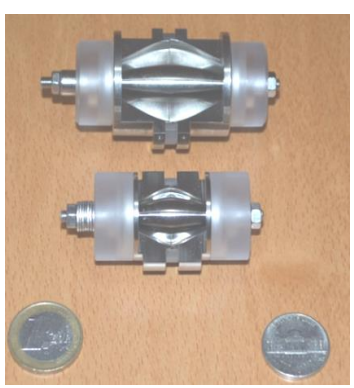
Principle of Operation (Orbitrap)





Hyper-logarithmic potential distribution:
"ideal Kingdon trap"

$$U(r,z) = \frac{k}{2} \cdot \left\{ z^2 - r^2/2 + R_m^2 \cdot \ln(r/R_m) \right\}$$



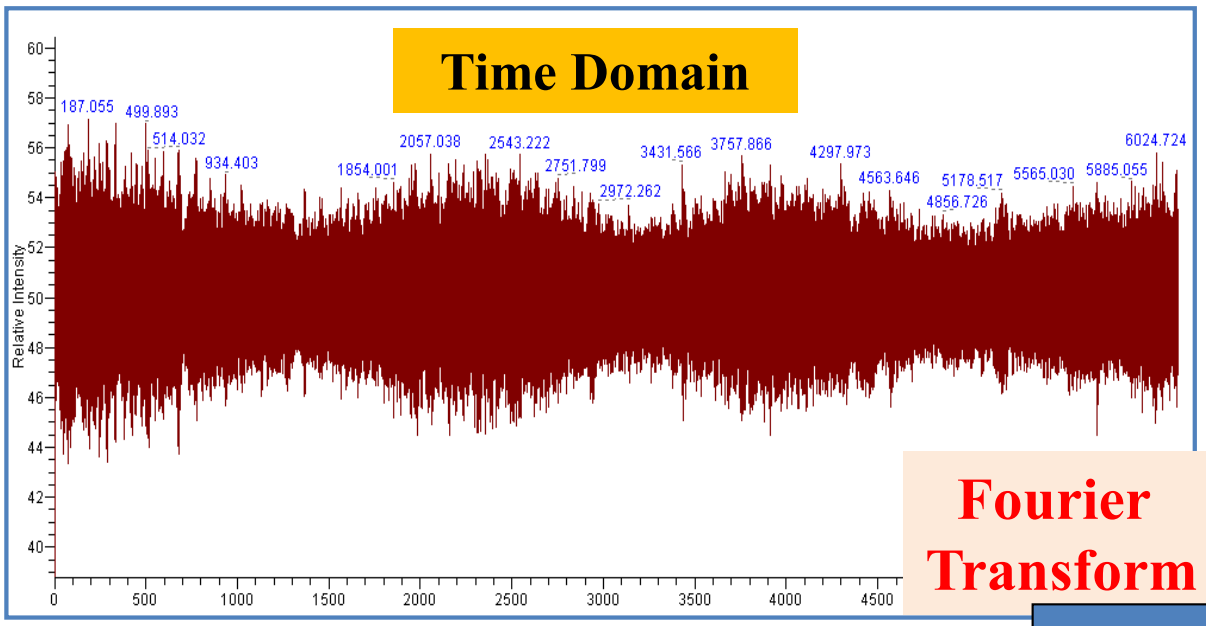
- **Characteristic frequencies:**
  - Frequency of rotation ω_φ
  - Frequency of radial oscillations ω_r
  - Frequency of axial oscillations ω_z

$$\omega_z = \sqrt{\frac{k}{m/q}}$$

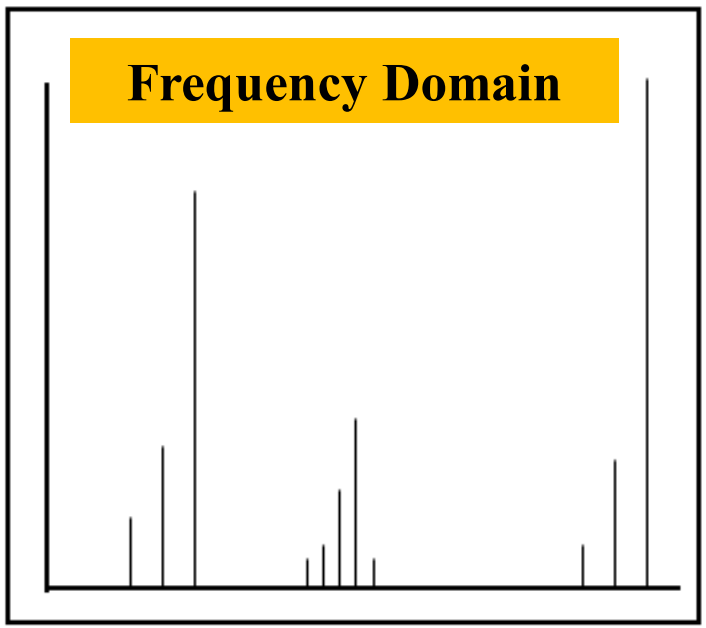
$$\omega_\varphi = \frac{\omega_z}{\sqrt{2}} \sqrt{\left(\frac{R_m}{R}\right)^2 - 1} \qquad \omega_r = \omega_z \sqrt{\left(\frac{R_m}{R}\right)^2 - 2}$$

Makarov A. *Anal. Chem.* 2000, *72*, 1156-1162.

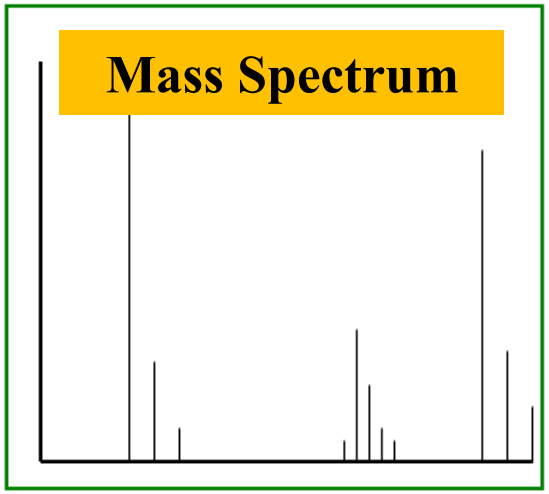
Principle of Operation (Orbitrap)



Fourier Transform



Many Ions Generate a Complex "Transient"

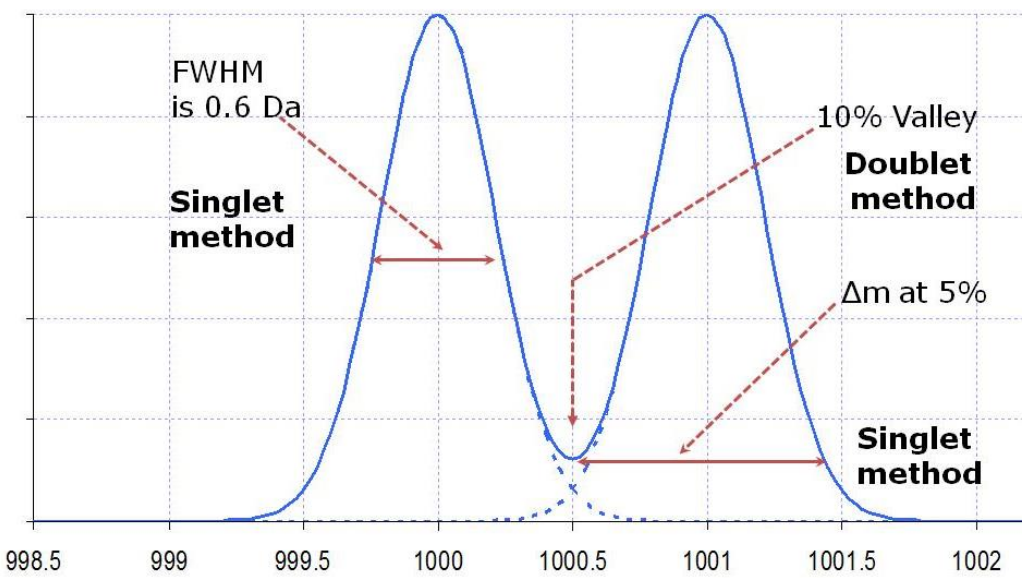


Mass Resolution

- Ability of a mass spectrometer to distinguish between ions of nearly equal m/z ratios (isobars).

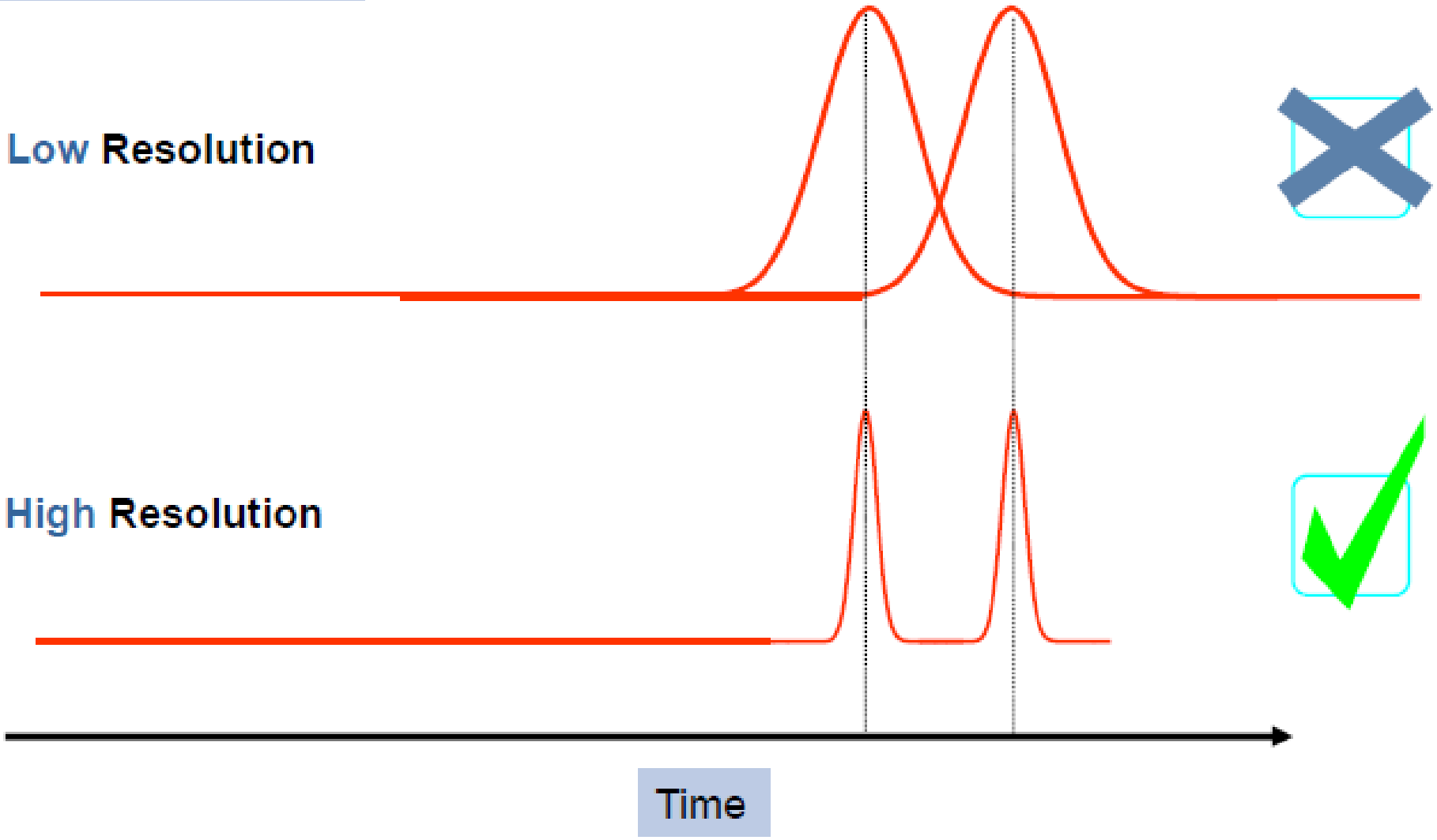
$$R = \frac{m}{\Delta m}$$

- m - measured mass
- Δm - peak width measured at 50% peak intensity (Full Width Half Maximum)
  - or the mass difference between two adjacent peaks of equal intensity, in this case pw @ 10% valley definition is used.



Resolution & Peak Width

Chromatography

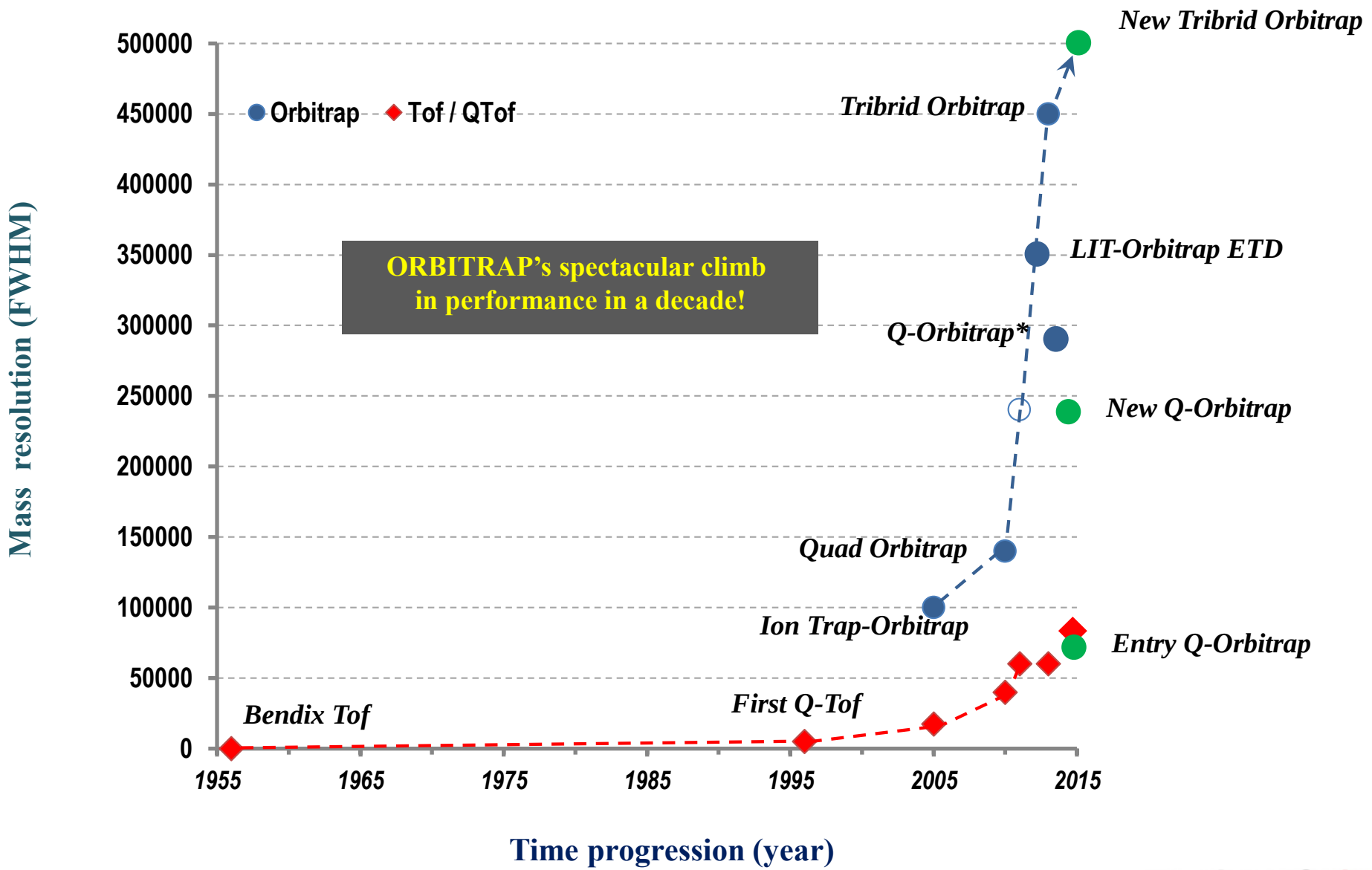


Mass Resolution

- At minimum the resolution of the mass analyzer should be sufficient to separate two ions differing by one mass unit anywhere in the mass range scanned (unit mass resolution).
- Typical values of resolution for low resolution mass analyzers (e.g. quadrupoles and ion traps) are below **5000**.
- High resolution instruments have a resolution exceeding **15000**.

Mass spectrometer	Resolving Power (FWHM)
FT-ICR-MS	1,000,000
Orbitrap	500,000
HR-ToF	60,000
Magnetic Sectors	10,000
Quadrupole / IonTrap in UltraZoom mode	5,000
Quadrupole / IonTrap	1,000

Commercial High Resolution MS Technology Race



Mass Accuracy

- **Nominal Mass**

The mass of an ion with a given empirical formula calculated using the integer mass numbers of the most abundant isotope of each element

Ex : M=249 $C_{20}H_9^+$ or $C_{19}H_7N^+$ or $C_{13}H_{19}N_3O_2^+$

- **Exact Mass**

The mass of an ion with a given empirical formula calculated using the exact mass of the most abundant isotope of each element

Ex : M=249	$C_{20}H_9+$	249.0070
	$C_{19}H_7N+$	249.0580
	$C_{13}H_{19}N_3O_2+$	249.1479

Mass Accuracy

- **Mass accuracy** is the precision of which the mass is measured by the mass spectrometer.
- Typical way of reporting mass error in **ppm (relative mass error)**:

$$mass\ error = \left(\frac{exact\ mass - measured\ mass}{exact\ mass}\right) * 10^6$$

- Absolute mass error can be used (**mDa**).
- **Main advantage:** the possibility to determine the elemental composition of individual molecular or fragment ions, a powerful tool for the structural elucidation or confirmation.

Mass Accuracy

- Accurate mass measurements take advantage of the fact that the combination of elements contained in a molecule have a very specific, non-nominal molecular weight:

  - Carbon has a mass of 12.0000
  - Hydrogen has a mass of 1.0078
  - Oxygen has a mass of 15.9949
  - Nitrogen has a mass of 14.0031

Element	Isotope Nominal Mass	Relative Abundance (%)	Average Mass	Exact mass
H	1	100	1.008	**1.0078**
	2	0.016		**2.041**
C	12	100	12.011	**12**
	13	1.08		**13.0034**
N	14	100	14.007	**14.0031**
	15	0.38		**15.0001**
O	16	100	15.999	**15.9949**
	17	0.24		**16.9991**
F	19	100	18.998	**18.9984**
P	31	100	30.974	**30.9738**
S	32	100	32.06	**31.9721**
	33	0.78		**32.9715**
	34	4.4		**33.9679**
Cl	35	100	35.453	**34.9689**
	37	32.5		**36.9659**
Br	79	100	79.904	**78.9183**
	81	98		**80.9163**
I	127	100	126.905	**126.9045**

Mass accuracy depends on resolution

Higher resolution allows for better mass accuracy

Mass Accuracy

- Typical mass accuracy capability for various MS types:

Type	Mass Accuracy
FT-ICR-MS	0.1 - 1 ppm
Orbitrap	0.5 - 1 ppm
Magnetic Sector	1 - 2 ppm
TOF-MS	3 - 5 ppm
Q-TOF	3 - 5 ppm
Triple Quad	3 - 5 ppm
Linear IonTrap	50-200 ppm (10 ppm in Ultra-Zoom)

Source: Metabolomics Fiehn's lab

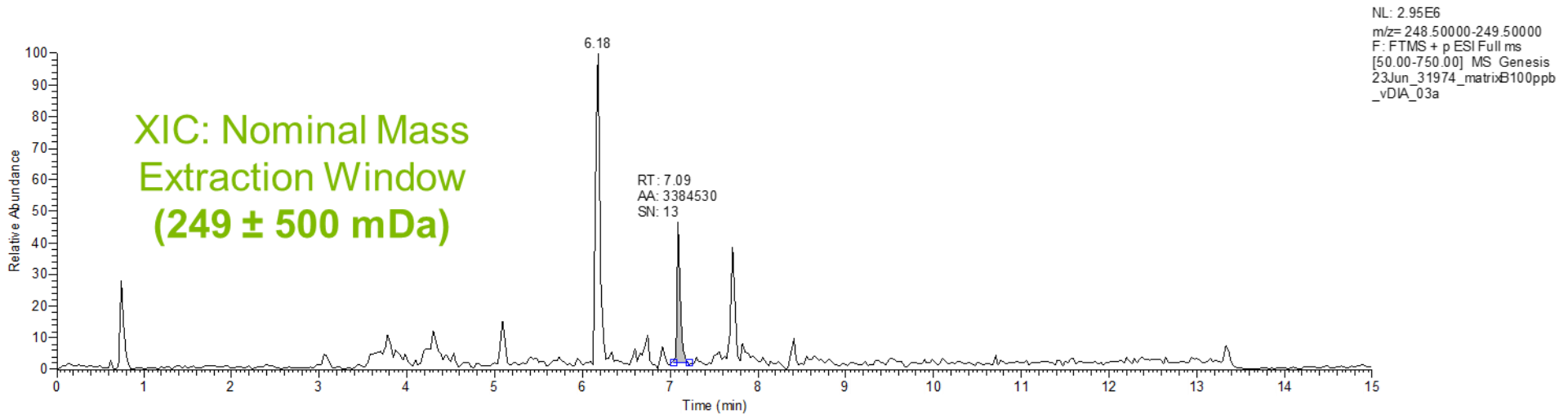
Importance of High Mass Resolution & Accuracy

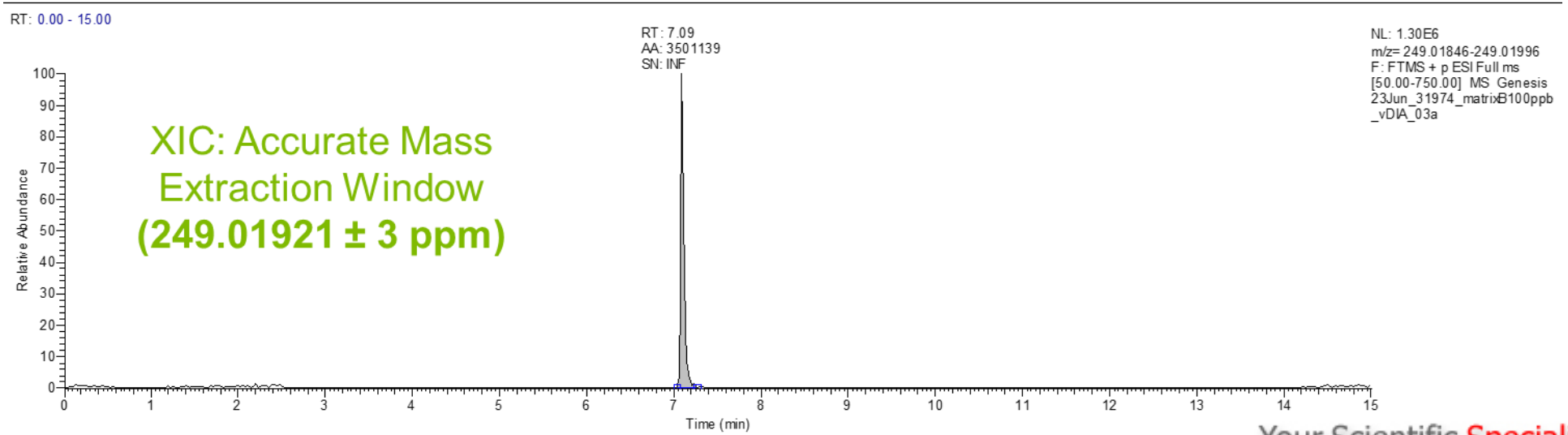
- Selectivity
- Isobaric compounds separation
- Removing interferences
- Unknown compounds identification

Importance of High Mass Resolution & Accuracy

- Selectivity through high resolving power & accurate mass

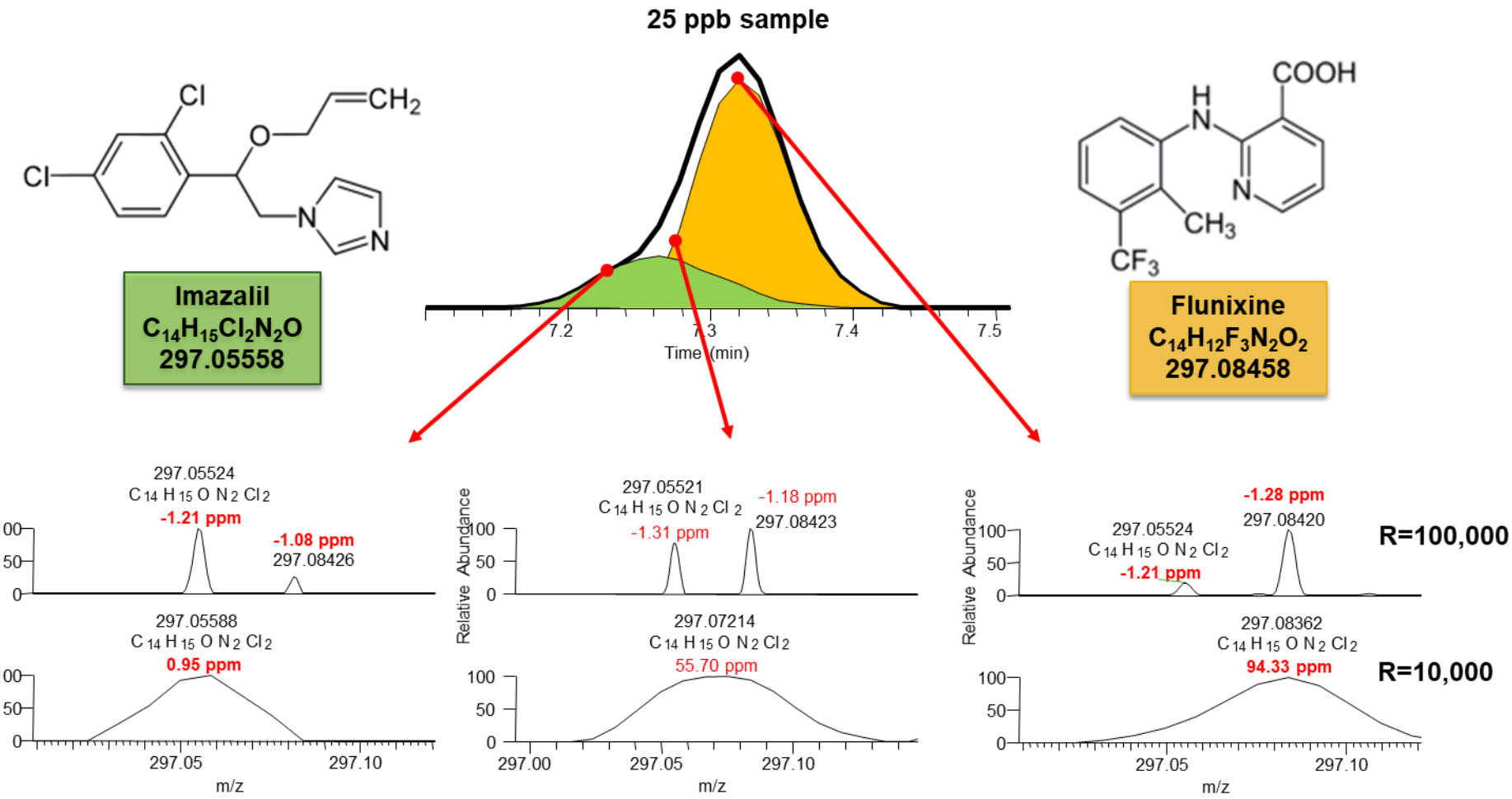
Linuron, m/z 249.01921





Importance of High Mass Resolution & Accuracy

- Isobaric compounds separation

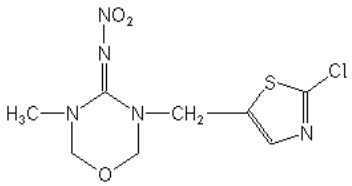


Importance of High Mass Resolution & Accuracy

- Isobaric compounds separation

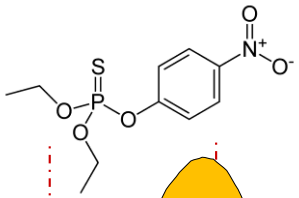
Thiamethoxam

$[M+H]^+ = 292.02656$



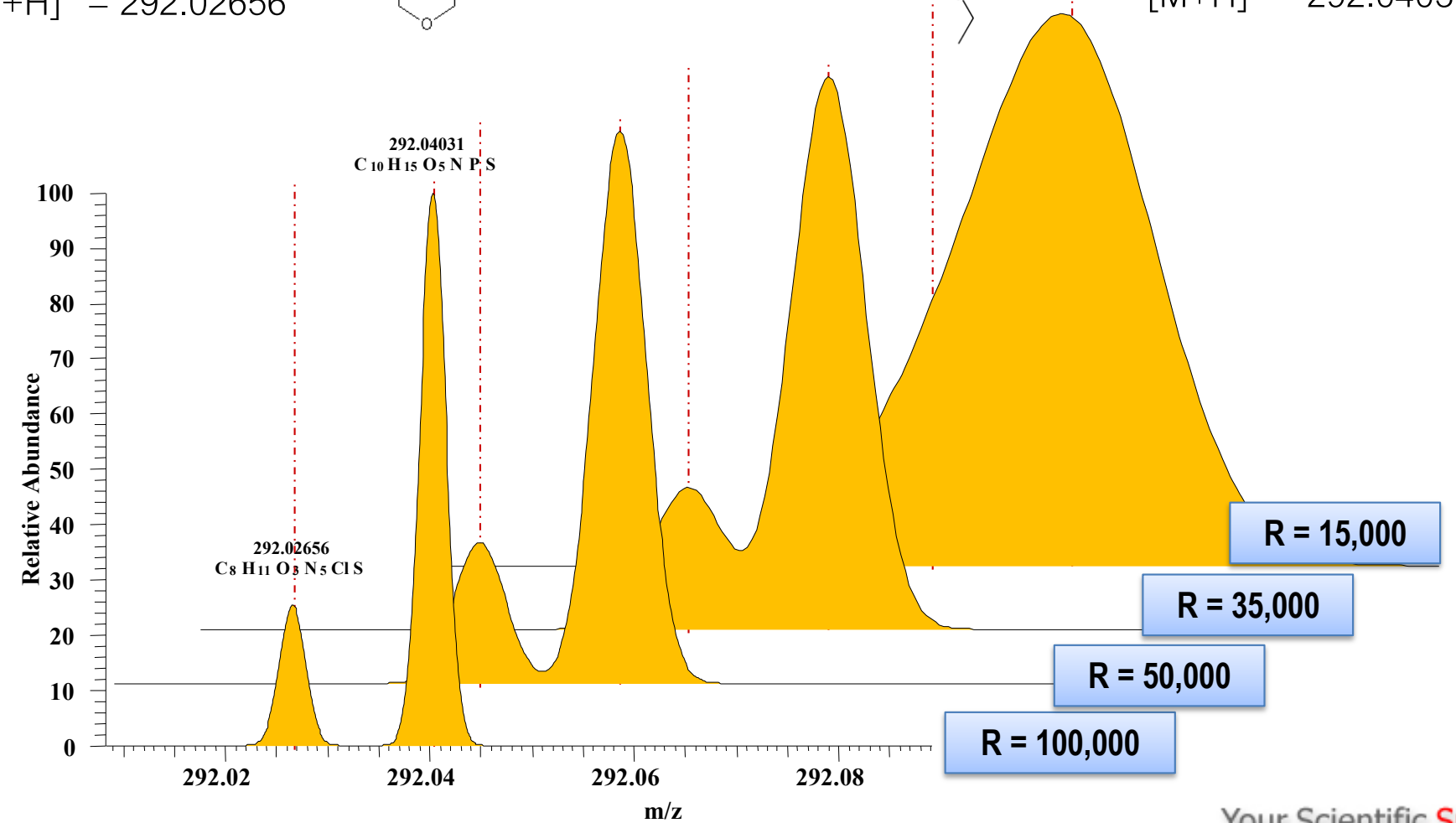
Δm

0.0138 Da



Parathion

$[M+H]^+ = 292.04031$



Importance of High Mass Resolution & Accuracy

- Removing interferences

High resolution is very important for samples with complex matrix (e.g. biological, food), since they will contain a significant

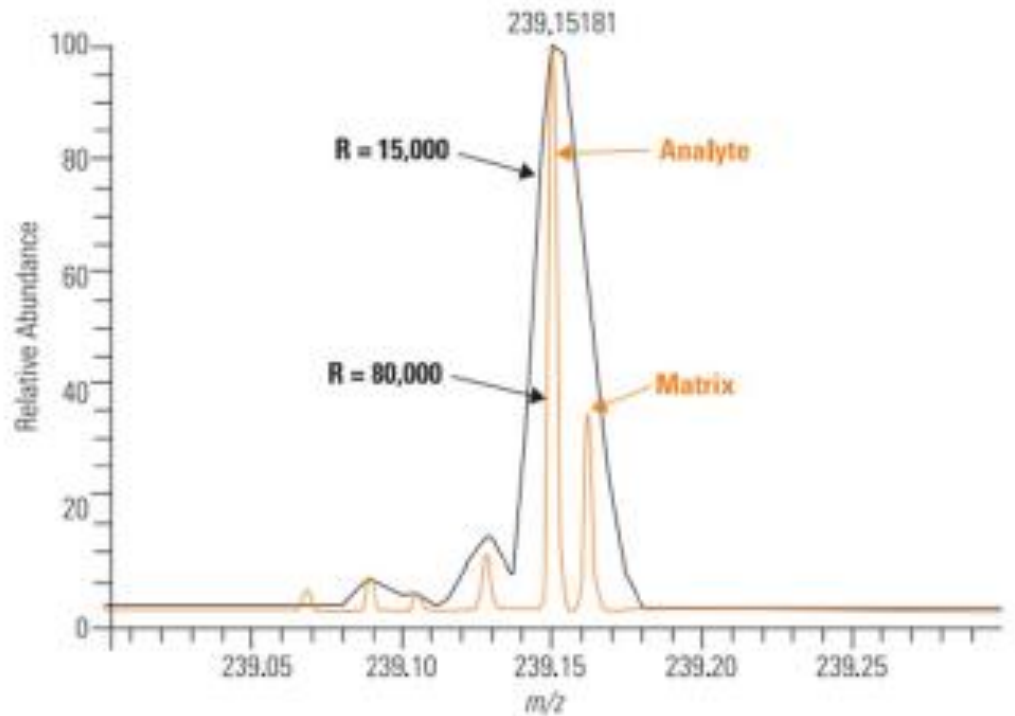


Figure 1: Analysis of the MH+ peak of Pirimicarb at 15,000 and 80,000 resolution.

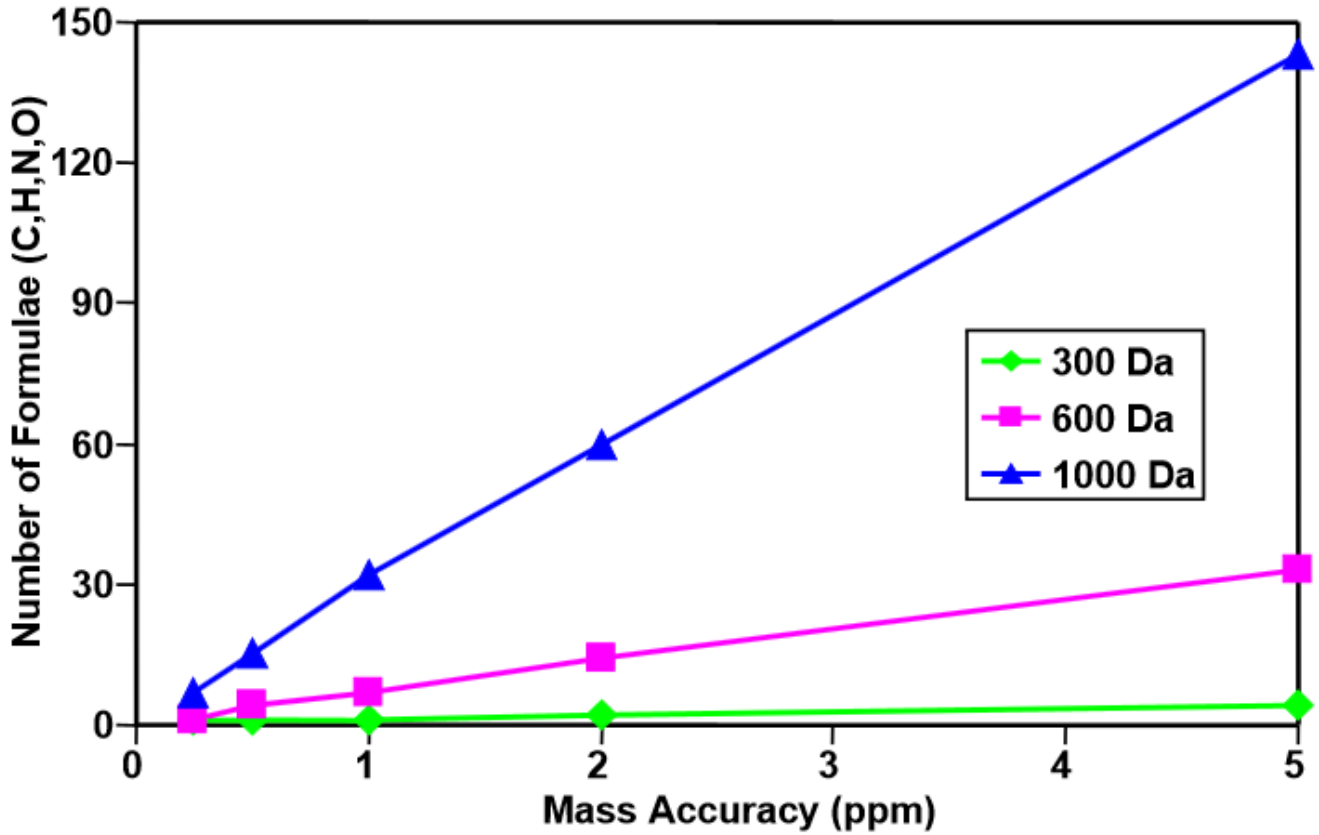
- High resolution is needed for:
  - Separating co-eluting molecules with close masses
  - Mass accuracy
  - Good (semi) quantitation results

Importance of High Mass Resolution & Accuracy

- Unknown compounds identification
  - Accurate mass measurement is the experimentally determined mass measured to an appropriate degree of accuracy and precision (*Gross, J. Am. Soc. Mass Spectrom.,1994*)
  - Accurate mass measurements narrow down the list of possible formulae for a particular molecular weight
  - Mass spectrum and analyst complete the picture:
    - Isotope distributions indicate/eliminate elements (e.g. Cl, Br, Cu)
    - User-supplied info eliminates others (e.g. no F, Co)
    - Suggested formula has to make chemical sense: ($C_6H_{28}O_2$ is not reasonable nor is $Cl_3H_2Co_4$)

Mass accuracy for chemical formulae ID

- The effect of mass accuracy and molecular weight on the number of potential chemical formulae.



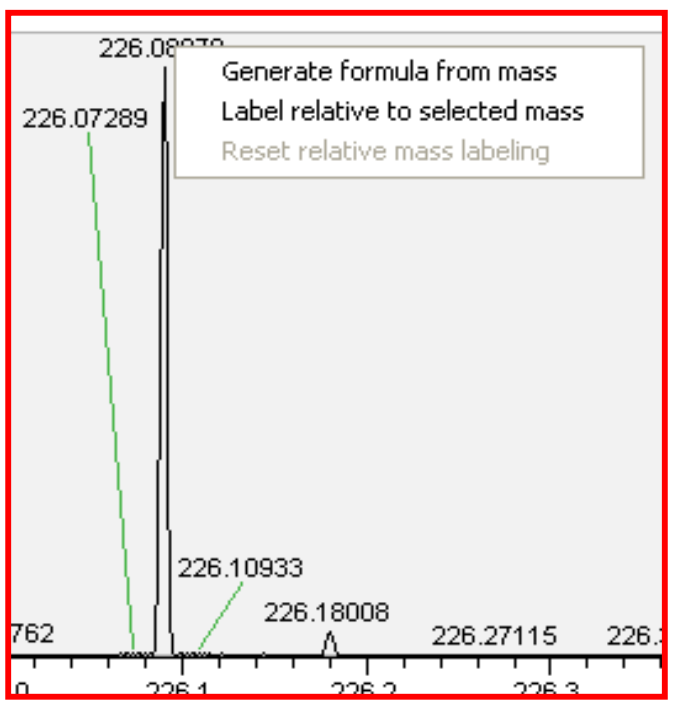
Generate Formula from Monoisotopic Mass

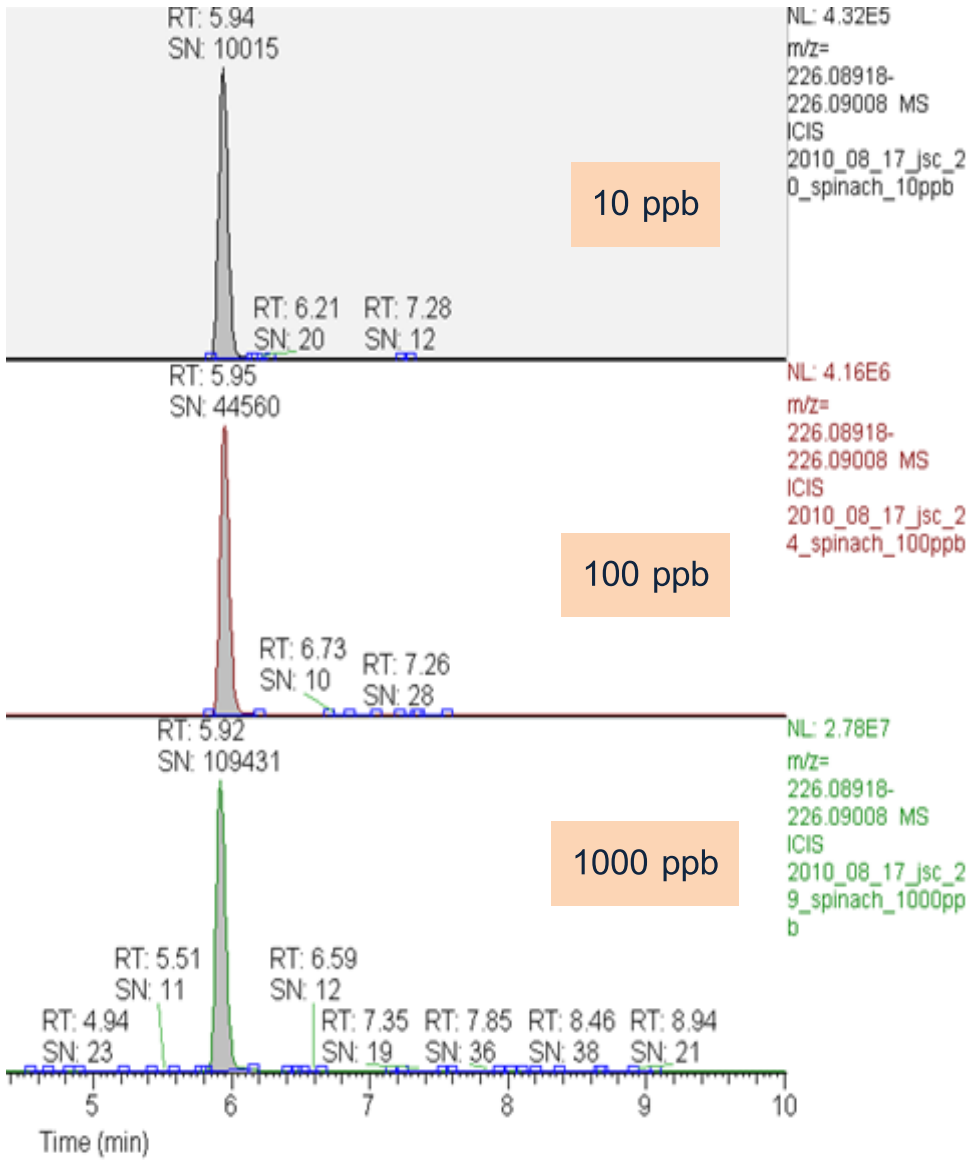
0.549 ppm Mass Accuracy

Right click on mass

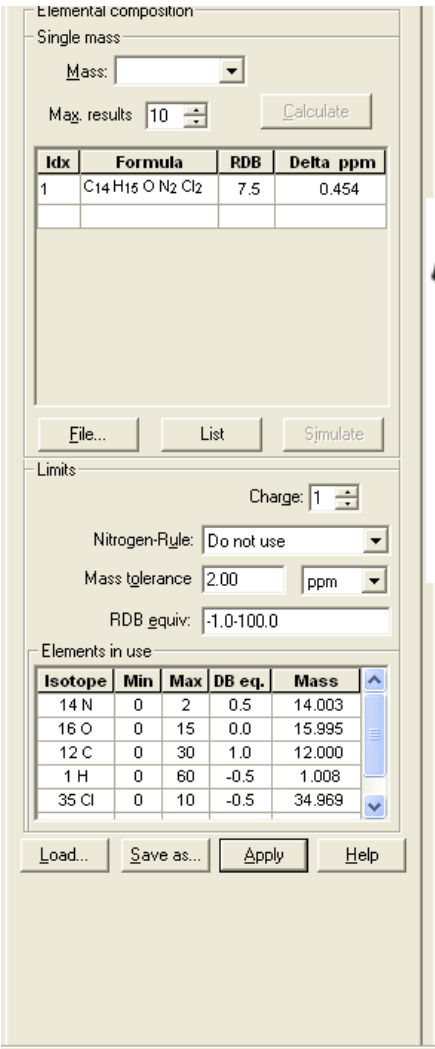
Then select

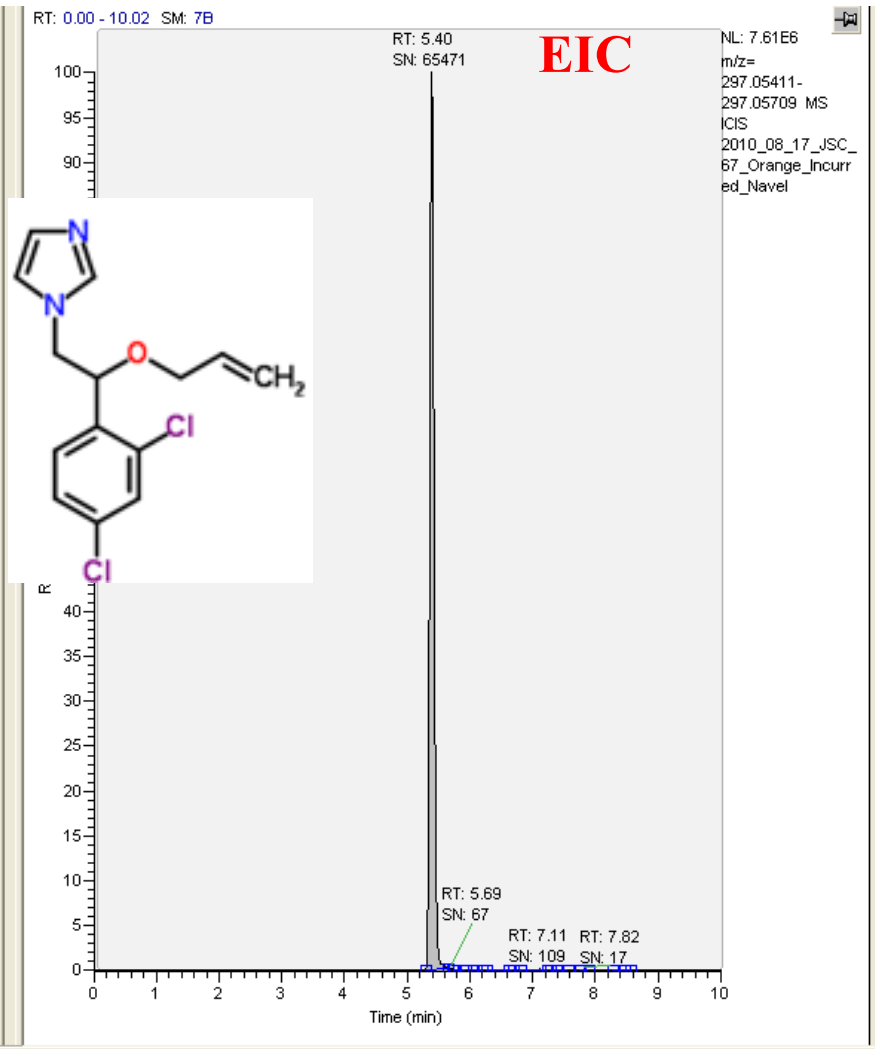
Generate formula form mass

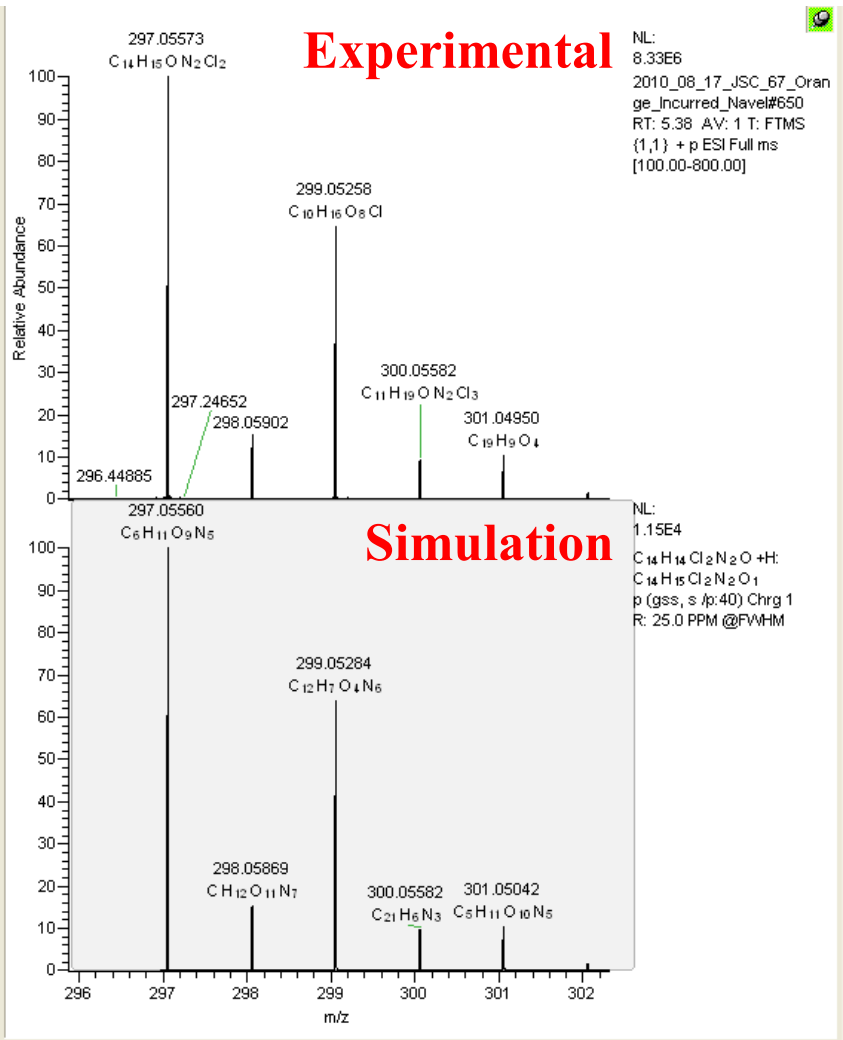




Elemental Composition Generates by Monoisotopic Mass

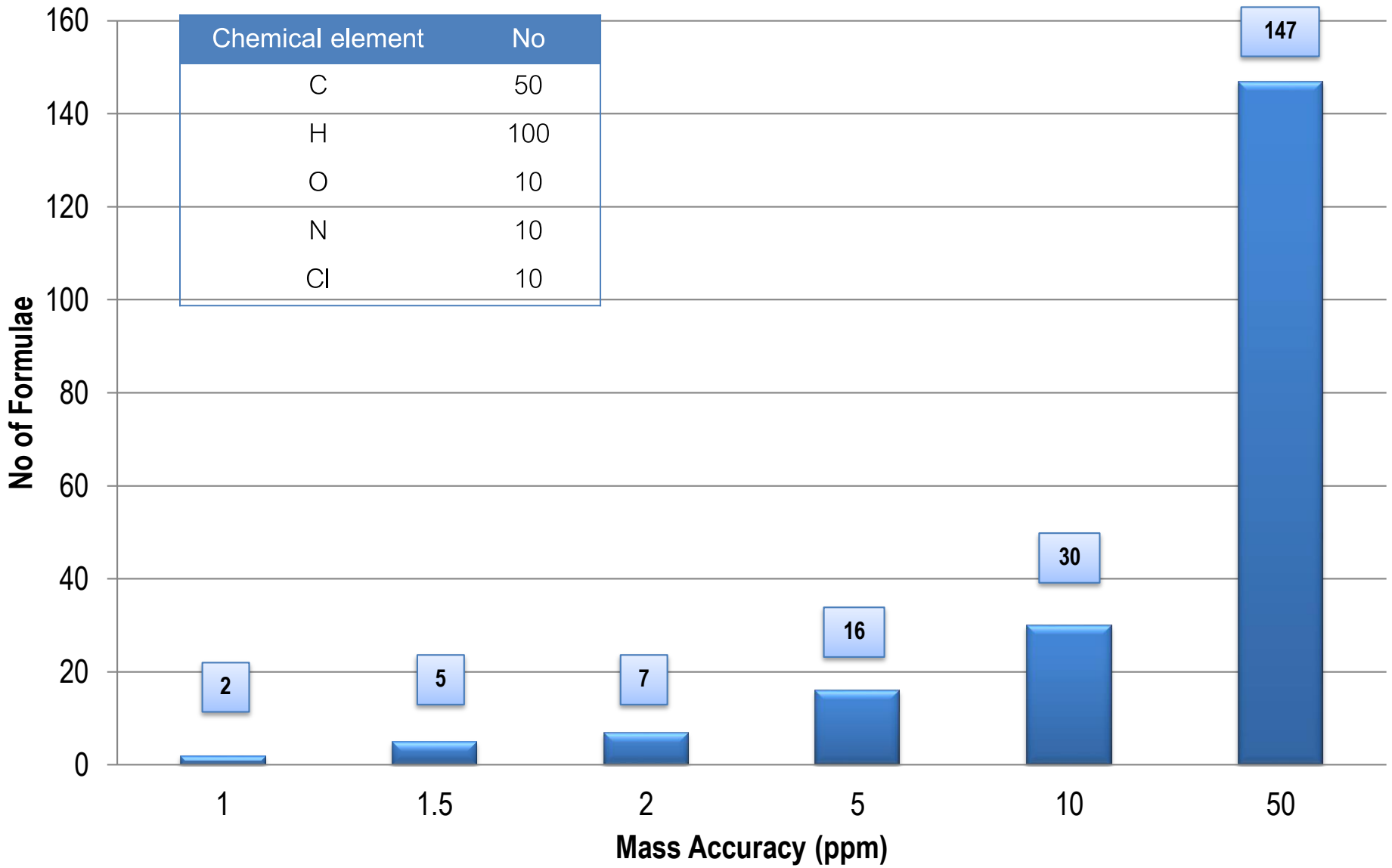




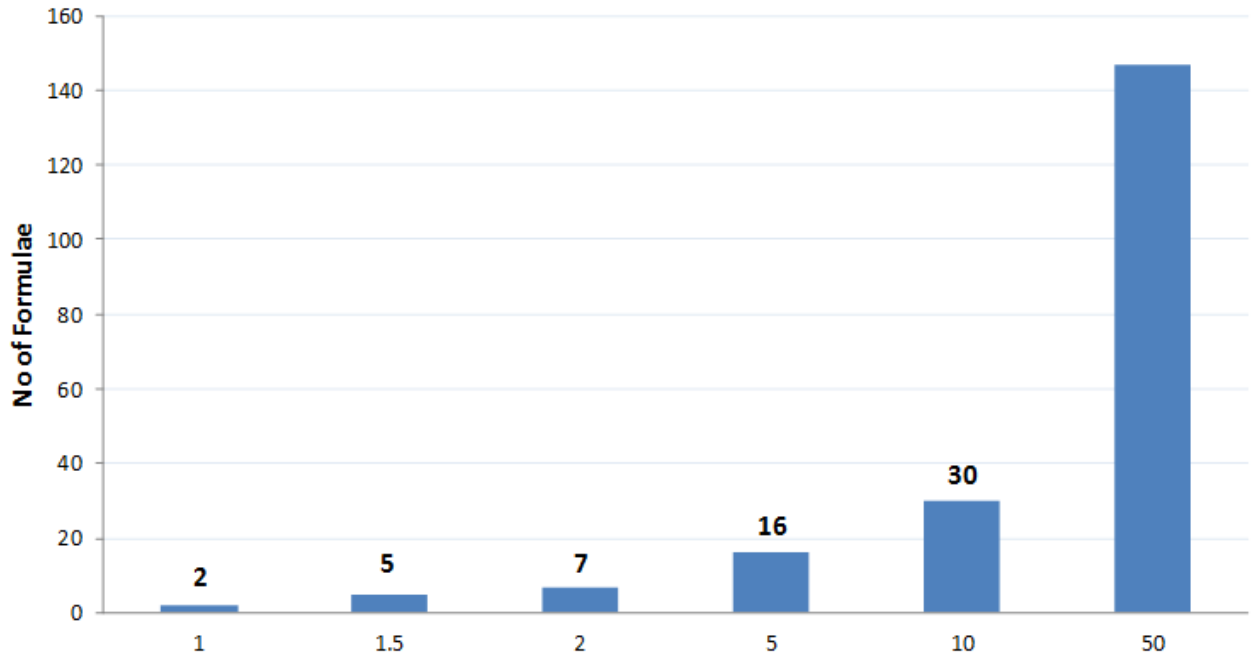


Elemental Composition Statistics

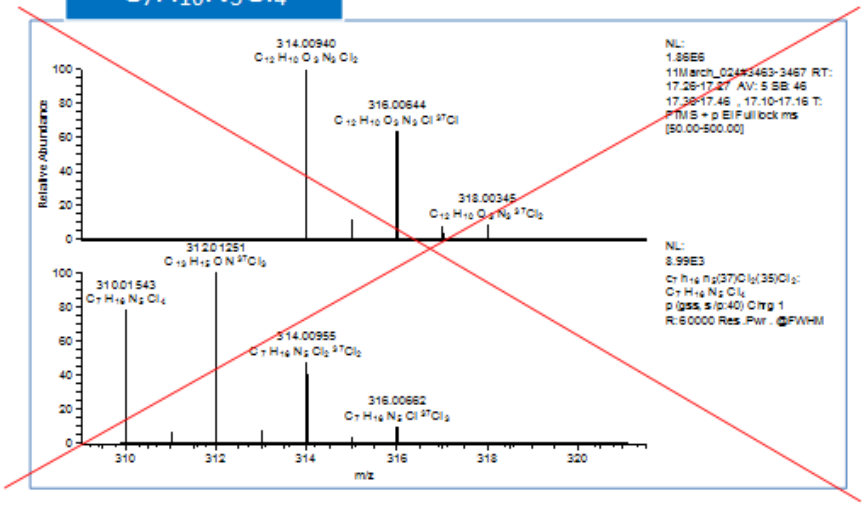
Chemical element	No
C	50
H	100
O	10
N	10
Cl	10



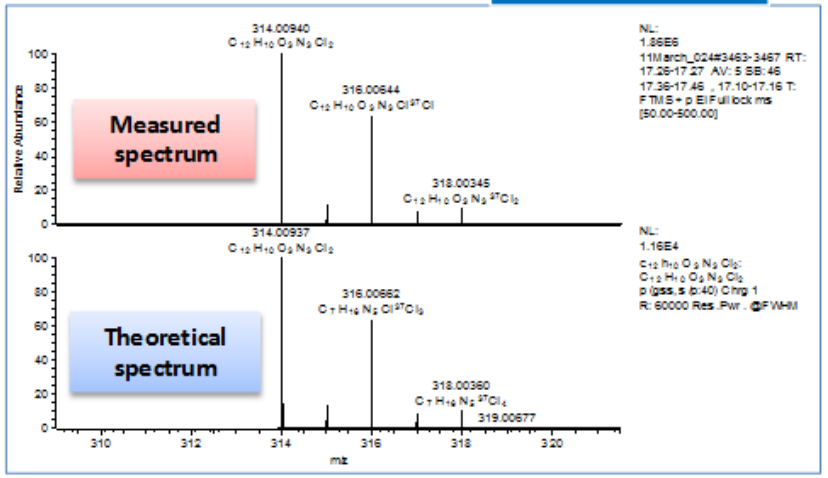
Compound ID confirmation through Isotopic Pattern Match



$C_7H_{16}N_5Cl_4$



$C_{12}H_{10}N_5O_3Cl_2$



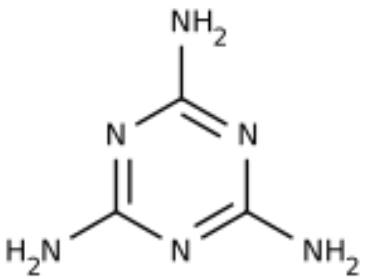
Applications of LC-MS

Applications of LC-MS

- Biomolecule characterization
  - Proteomics
  - Oligonucleotides
- Environmental analysis
  - Pesticides on foods
  - Soil and groundwater contamination
- Forensic analysis/clinical
- Toxicology
- Pharmaceutical analysis
  - Bioavailability studies
  - Drug metabolism studies, pharmacokinetics
  - Characterization of potential drugs
  - Drug degradation product analysis
  - Screening of drug candidates
  - Identifying drug targets
- Etc.

Applications of LC-MS (Triple Quadrupoles)

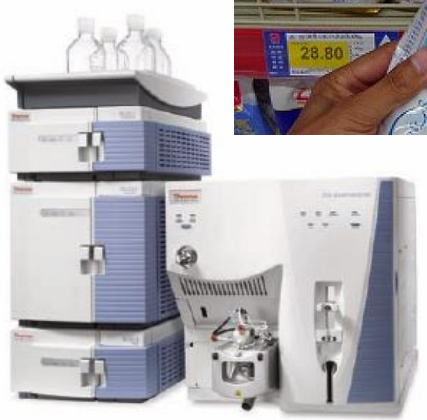
- Targeted analysis (Melamine in milk)

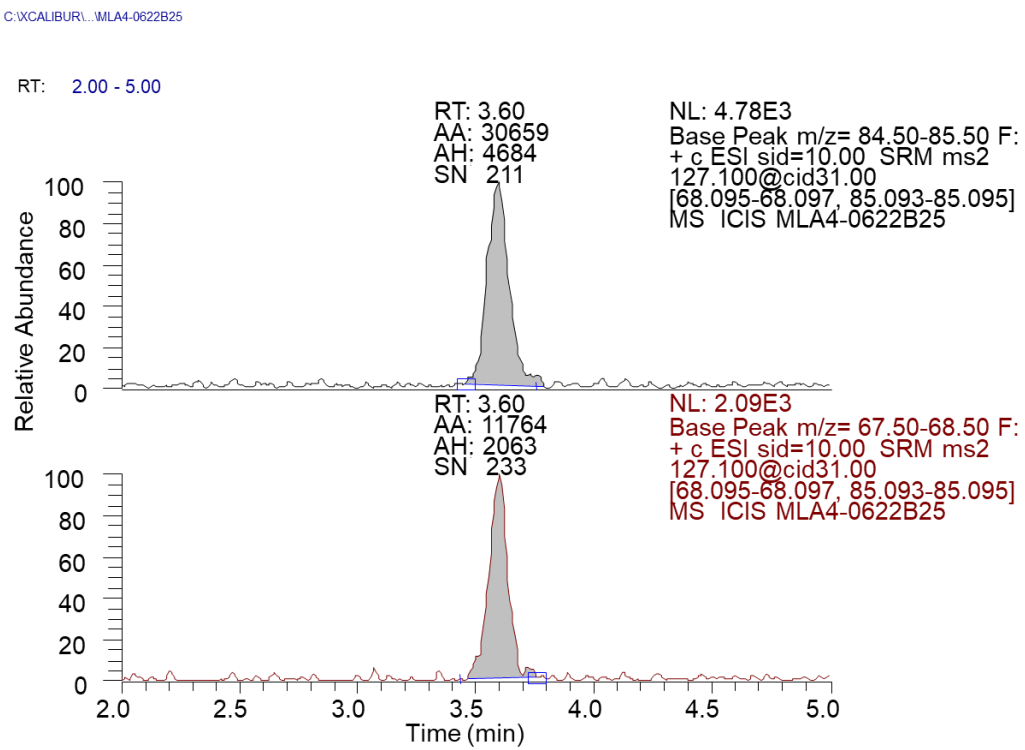


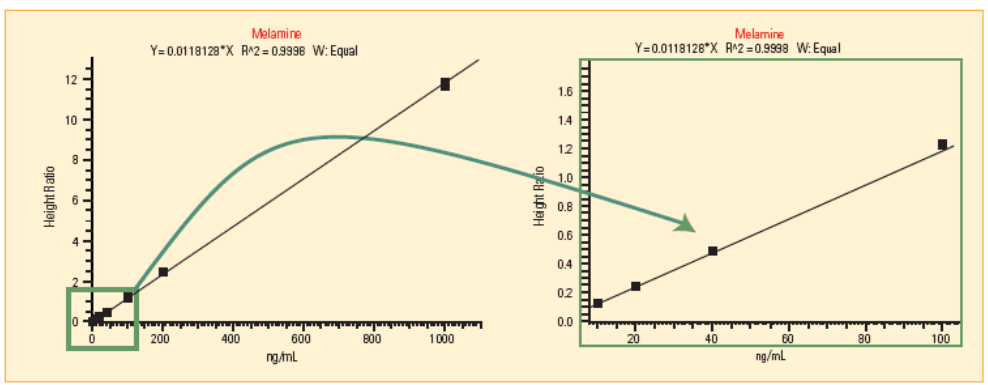
SRM Transitions

127 -> 68

127 -> 85

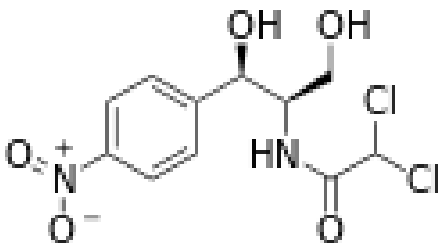




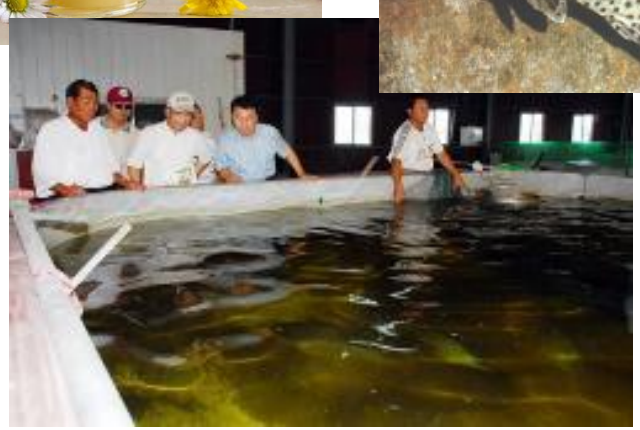


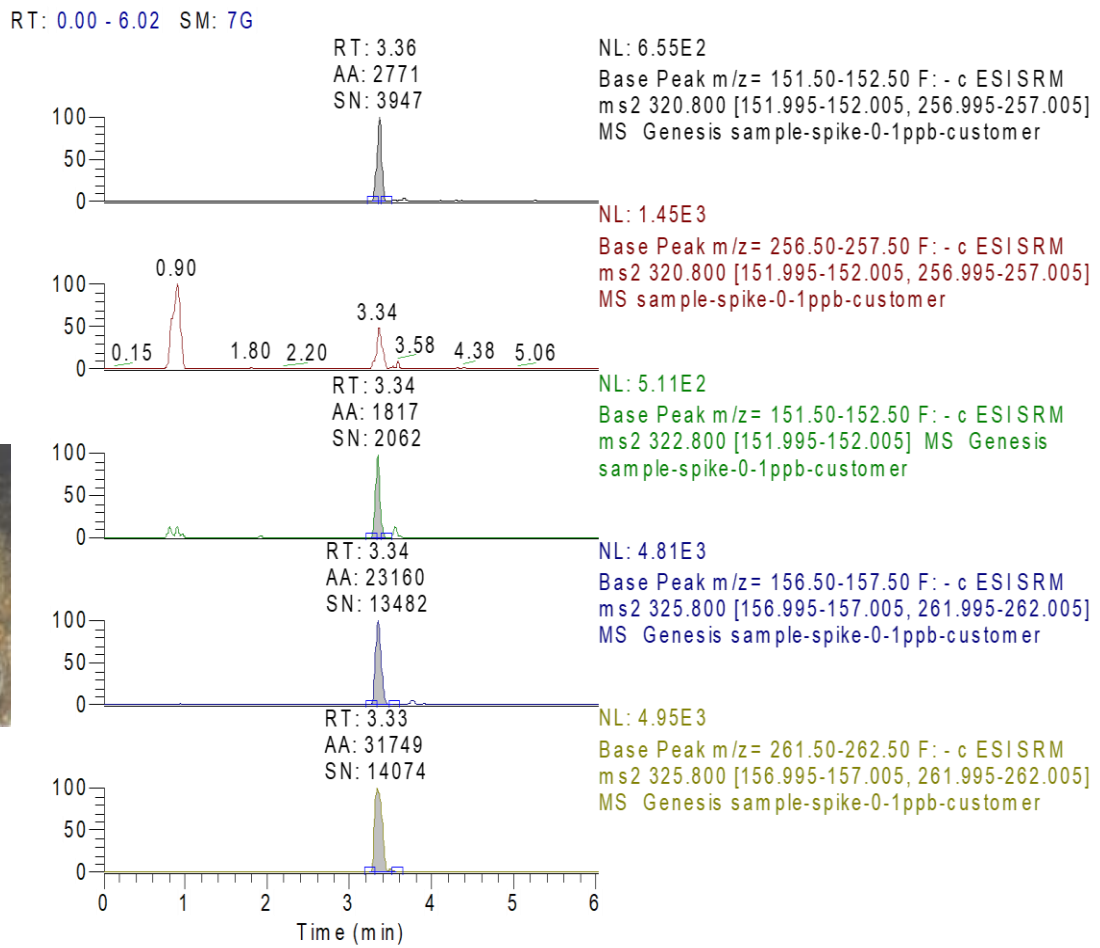
Applications of LC-MS (Triple Quadrupoles)

- Targeted analysis (Chloramphenicol in seafood and royal jelly)



SRM Transitions
321 -> 151
321 -> 262

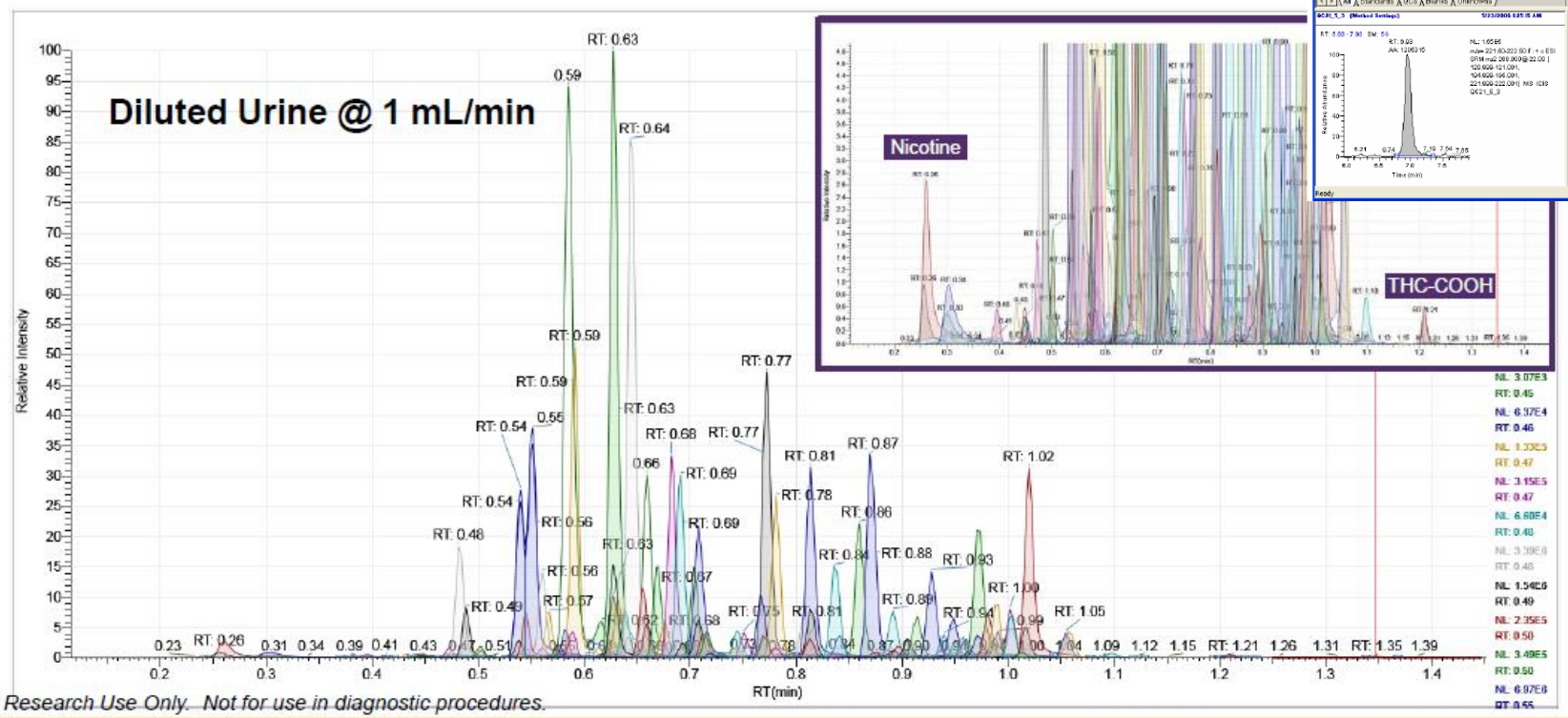




Applications of LC-MS (Triple Quadrupoles)

- Targeted analysis (100 drugs of abuse in urine)

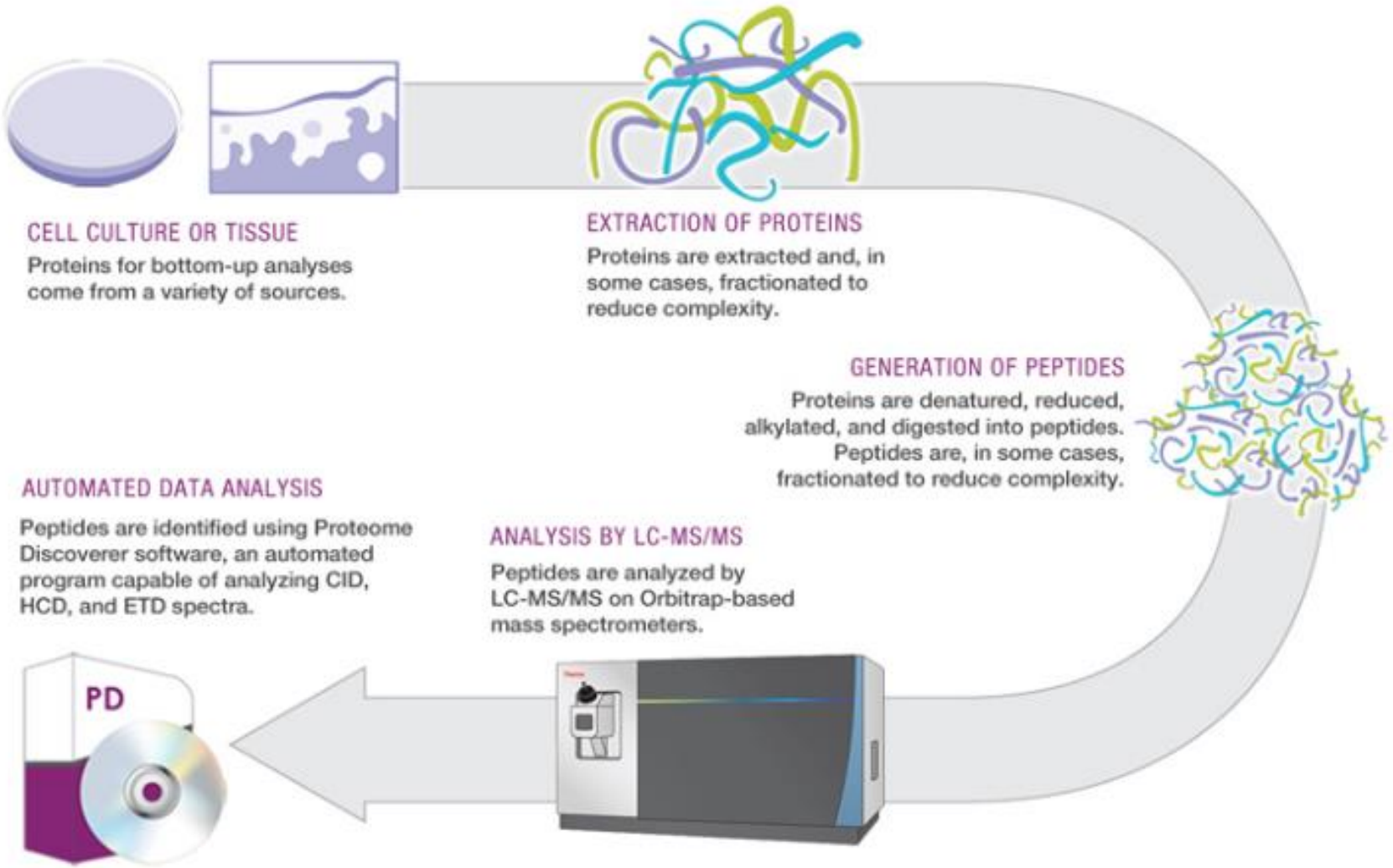
Quantify & Confirm in ONE injection



SRM chromatograms of ~100 drugs of abuse in under 1.3 minutes [THC-COOH elutes at 1.21 min, inset]

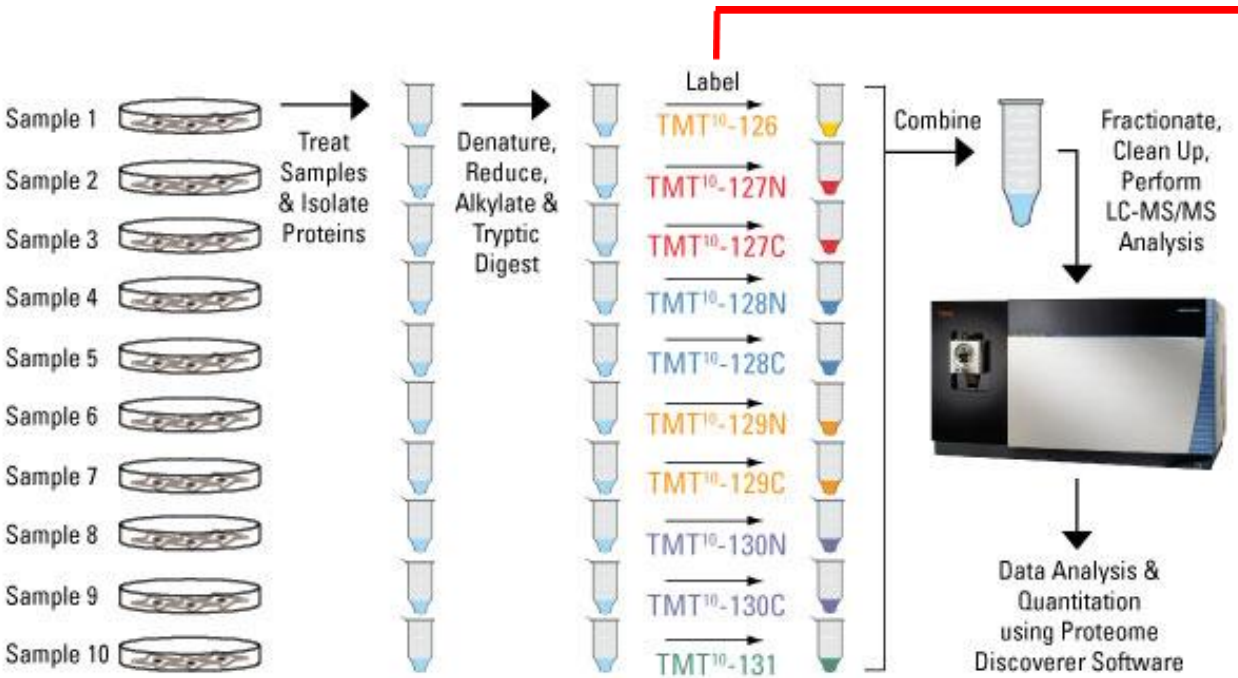
Applications of LC-MS (Orbitrap)

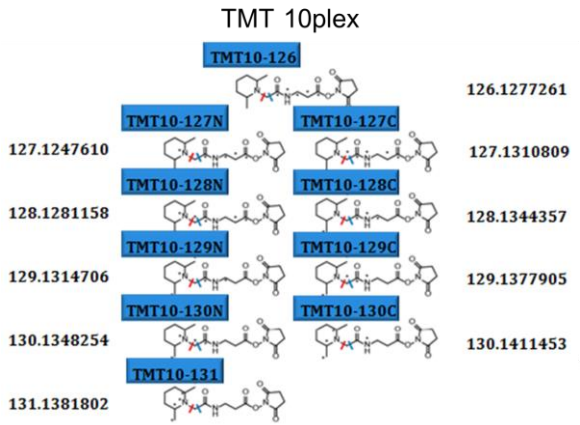
- Non-targeted analysis (protein identification)



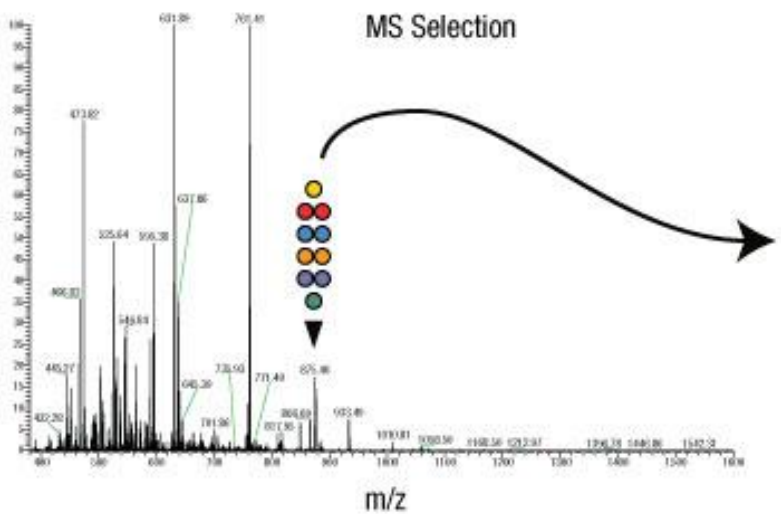
Applications of LC-MS (Orbitrap)

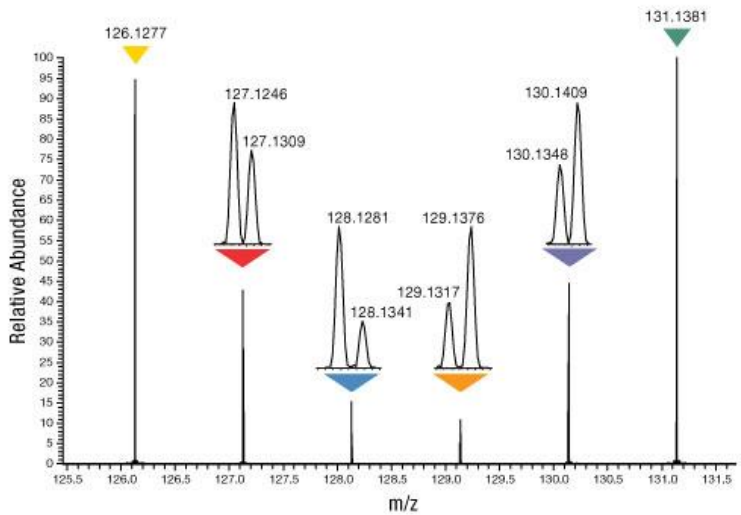
- Peptide quantitation (multiple studies with a single experiment)





Needs high resolution!





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MultiNotch MS3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression across Cancer Cell Line Proteomes

Graeme C. McAlister,[†] David P. Nusinow,[†,§] Mark P. Jedrychowski,[†,‡,§] Martin Wühr,[†,‡] Edward L. Huttlin,[†] Brian K. Erickson,[†] Ramin Rad,[†] Wilhelm Haas,[†] and Steven P. Gygi*[,†]

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Comparison of protein expression ratios observed by sixplex and duplex TMT labeling method

Navin Rauniyar[†], **Benbo Gao**[‡], **Daniel B. McClatchy**[†], and **John R. Yates III**
[†]Department of Chemical Physiology, The Scripps Research Institute, 10550 N. Torrey Pines Rd.,

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Quantitative accuracy in mass spectrometry based proteomics of complex samples: The impact of labeling and precursor interference

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*AnnSofi Sandberg, Rui M.M. Branca, Janne Lehtiö, Jenny Forshed**

Science for Life Laboratory Stockholm and Cancer Proteomics, Department of Oncology–Pathology, Karolinska Institutet, Sweden

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ABSTRACT

Knowing the limit of quantification is important to accurately judge the results from proteomics studies. In order to investigate isobaric labels in combination with peptide pre-fractionation by high resolution isoelectric focusing in terms of limit of detection, quantitative accuracy and how to improve it, we used a human cell lysate spiked with 57 protein standards providing reference points across a wide concentration range. Specifically, the impact of precursor mixing (*isolation interference* and *reporter ion interference*) on quantitative accuracy was investigated by co-analyzing iTRAQ (8-plex) and TMT (6-plex) labeled peptides. A label-free analysis was also performed. Peptides, labeled or label-free, were analyzed by LC–MS/MS (Orbitrap Velos). We identified 3386 proteins by the label-free approach, 4466 with iTRAQ and 5961 with TMT. A linear range of quantification down to 1 fmol was indicated for both isobaric and label-free analysis workflows, with an upper limit exceeding 60 fmol. Our results indicate that 6-plex TMT is more sensitive than 8-plex iTRAQ. For isobaric labels, quantitative accuracy was affected by precursor mixing. Based on our evaluation on precursor mixing and accuracy of isobaric label quantification, we propose a cut off of <30% *isolation interference* for peptide spectrum matches (PSMs) used in the quantification.

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