Brief Concept of LC-MS

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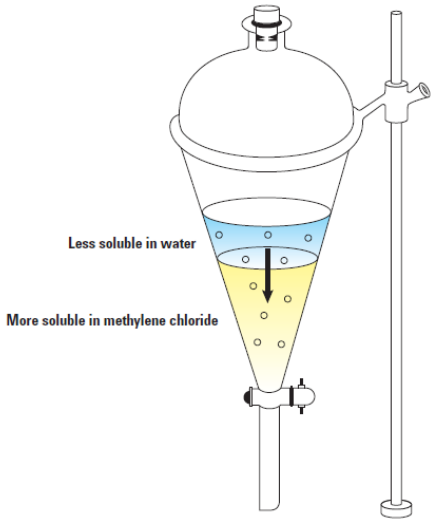
OUTLINE

- How good is your sample preparation
- Quick understand LC
- How to make a good separation
- Understand a short brief of Mass Spectrometer definition
- Connected to Mass Spectrometer
- Type of Mass Spectrometer
- How to choose appropriated Mass Spectrometer

Liquid-Liquid Extraction

- separating analytes from interferences by partitioning the sample between two immiscible liquids
- One phase is aqueous, another is organic
- More hydrophilic goes to aqueous while as more hydrophobic will be found mainly in organic phase



LLE: How to choose a solvent

- A low solubility in water (<10%).
- Volatility for easy removal and concentration after extraction.
- Compatibility with the HPLC or GC detection technique to be used for analysis (avoid solvents that are strongly UV-absorbing or that may cause GC detection problems, such as chlorinated solvents in conjunction with electron capture detector).
- Polarity and hydrogen-bonding properties that enhance recovery of the analytes in the organic phase.
- High purity to minimize sample contamination

LLE: Disadvantage

- Emulsion formation
- Analytes strongly adsorbed to particulates
- Analytes bound to high molecular weight compounds (e.g. protein-drug interactions)
- Mutual solubility of the two phases
- No automate
- High organic consumption
- Not good for complex extraction

Solid-supported Liquid-Liquid Extraction

- Overcome LLE disadvantage with greater benefits
- Diatomaceous earth particle serves as a stationary phase for aqueous phase
- Aqueous-base sample will added to dry sorbent and dispersed through solid support
- Next, a small volume of immiscible organic solvent is added (required gentle pressure or mild vacuum) to allow partitioning

SLE: Benefits

- Greater reproducibility and recoveries compared to LLE techniques
- Prevents emulsification often associated with LLE
- Reduced solvent requirements compared to LLE
- Can be completely automated unlike LLE
- Improved cleanliness of sample extract compared to protein precipitation techniques
- Improved sensitivity compared to protein precipitation techniques

SLE: Disadvantage

- Can be used only aqua-base sample
- Not significant greater than LLE in term of recovery
- Deal with manifold (req. more complicate method development
- Sometimes need pre-buffered (Commercially available)

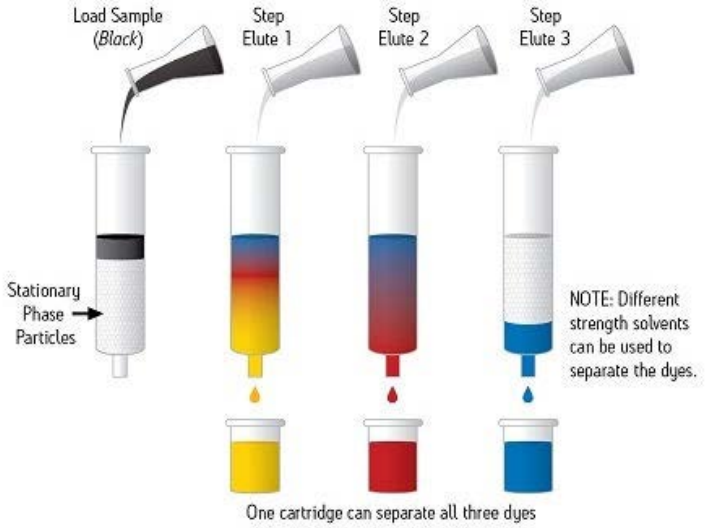
Solid Phase Extraction

SPE is important chromatographic preparation based on chemically different of components in sample.

- Each of components can be eluted by appropriate solvent
- Can be used for gas phase by trapping on sorbent using reactive chemical or

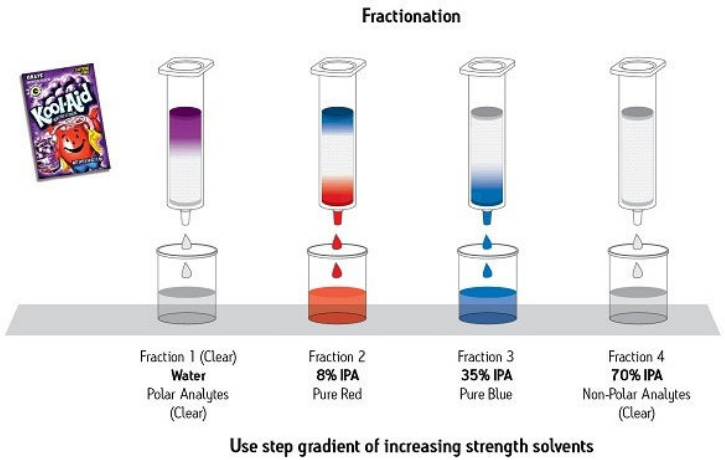
specified with some materials

- Known as “Liquid-Solid Extraction”



SPE Benefits

- Simplification of complex sample matrices along with compound purification
- Reduced Ion Suppression in Mass Spectrometry Technique (Desalting)
- Capability to Fractionate Sample Matrix to Analyze Compounds by Class
- Enrichment of Very Low Level Compounds



SPE: Disadvantages

- Mix Mechanism can be taken place inside cartridge
- Irreversible adsorption of some analytes on SPE cartridges
- More complex method development is required
- Sometimes need evaporating step

QuEChERS

- Quick-Easy-Cheap-Effectiveness-Rugged-Safe ("catcher") is now commonly used in pesticide from FOOD analysis both LC and GC
- 2-Processes; extraction followed by clean-up
- Known as " LL and SPE"

QuEChERS : Extraction Step

Magnesium Sulfate aids the extraction and remove residue water from organic solvent and unwanted contaminants

- Considerations

- Base sensitive compound -> with sodium acetate
- Non-base sensitive compound -> with sodium citrate or sodium chloride

QuEChERS : Clean-up

- Determine the properties of sample matrixes

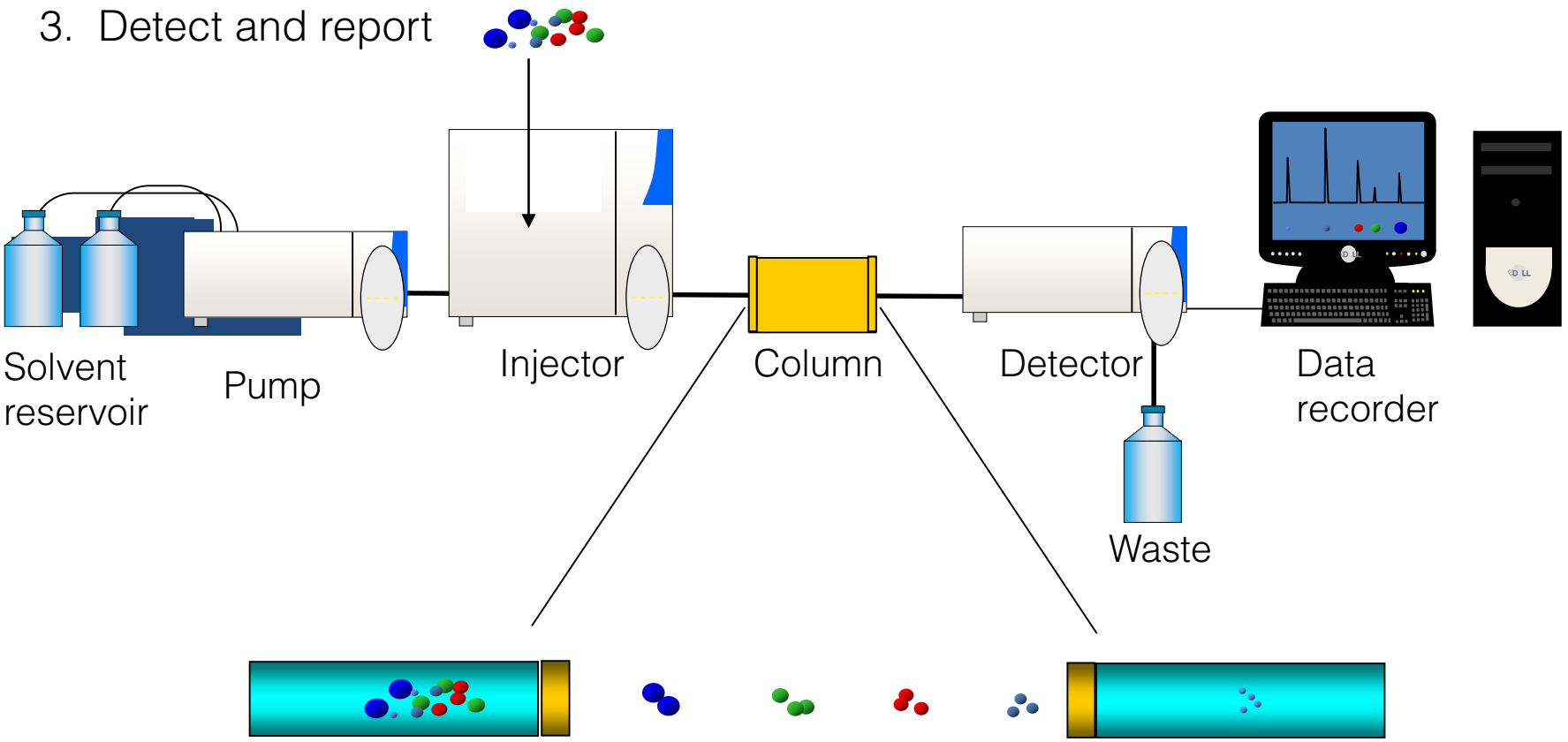
- General
- Fatty
- Pigmented
- Highly Pigmented

- Adsorbents

- C18: REMOVE Low fat interference
- PSA (Primary-Secondary Amine): REMOVE Sugars and organics acid
- GBC (Graphitized Black Carbon): REMOVE pigmented, chlorophyll, carotenoid etc.

Quick Understand LC

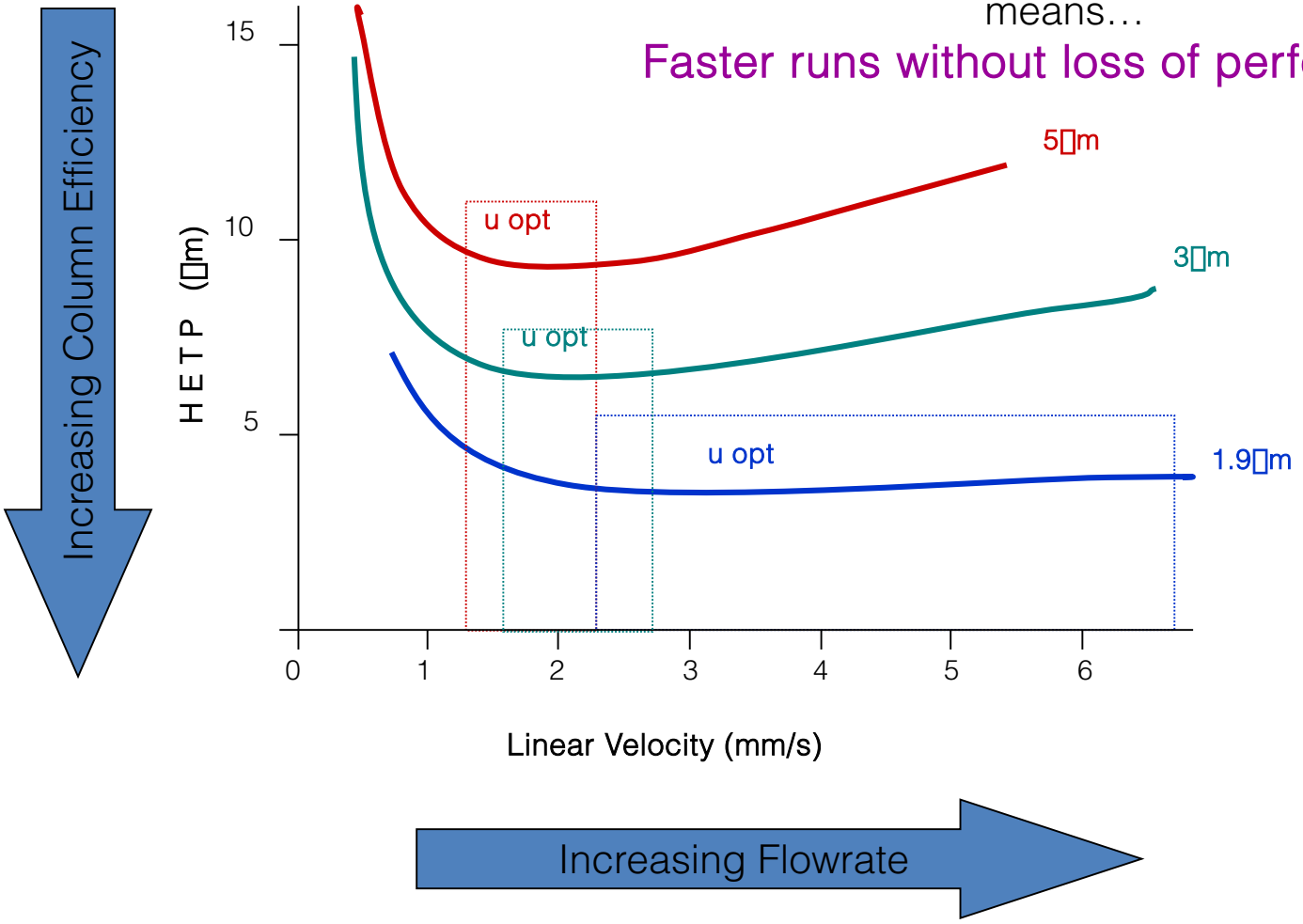
1. Inject sample mixture
2. Separate into individual components
3. Detect and report



What is the Small Particle Advantage ?

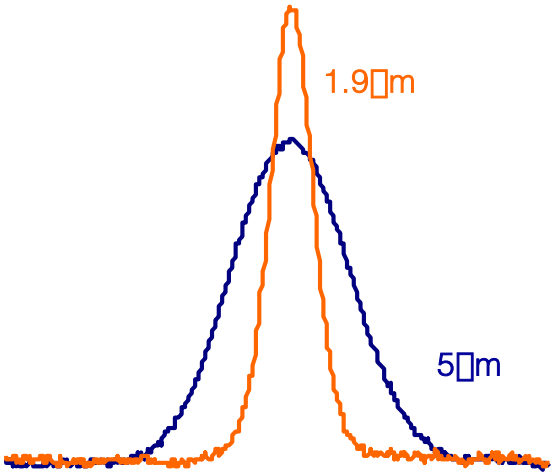
Higher efficiency, independent of flow rate means…

Faster runs without loss of performance



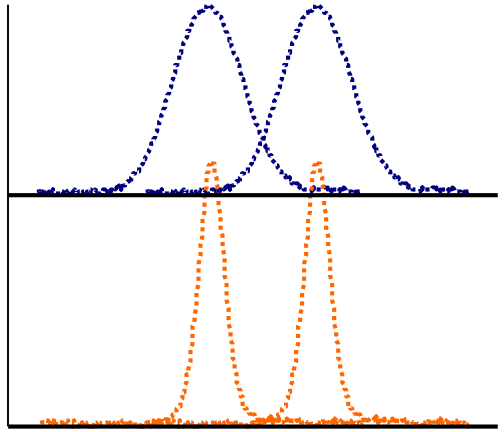
Efficiency is the key!!!

Small Particle Advantage



N = 142,000 plates/m
(189% higher)

N = 75,000 plates /m



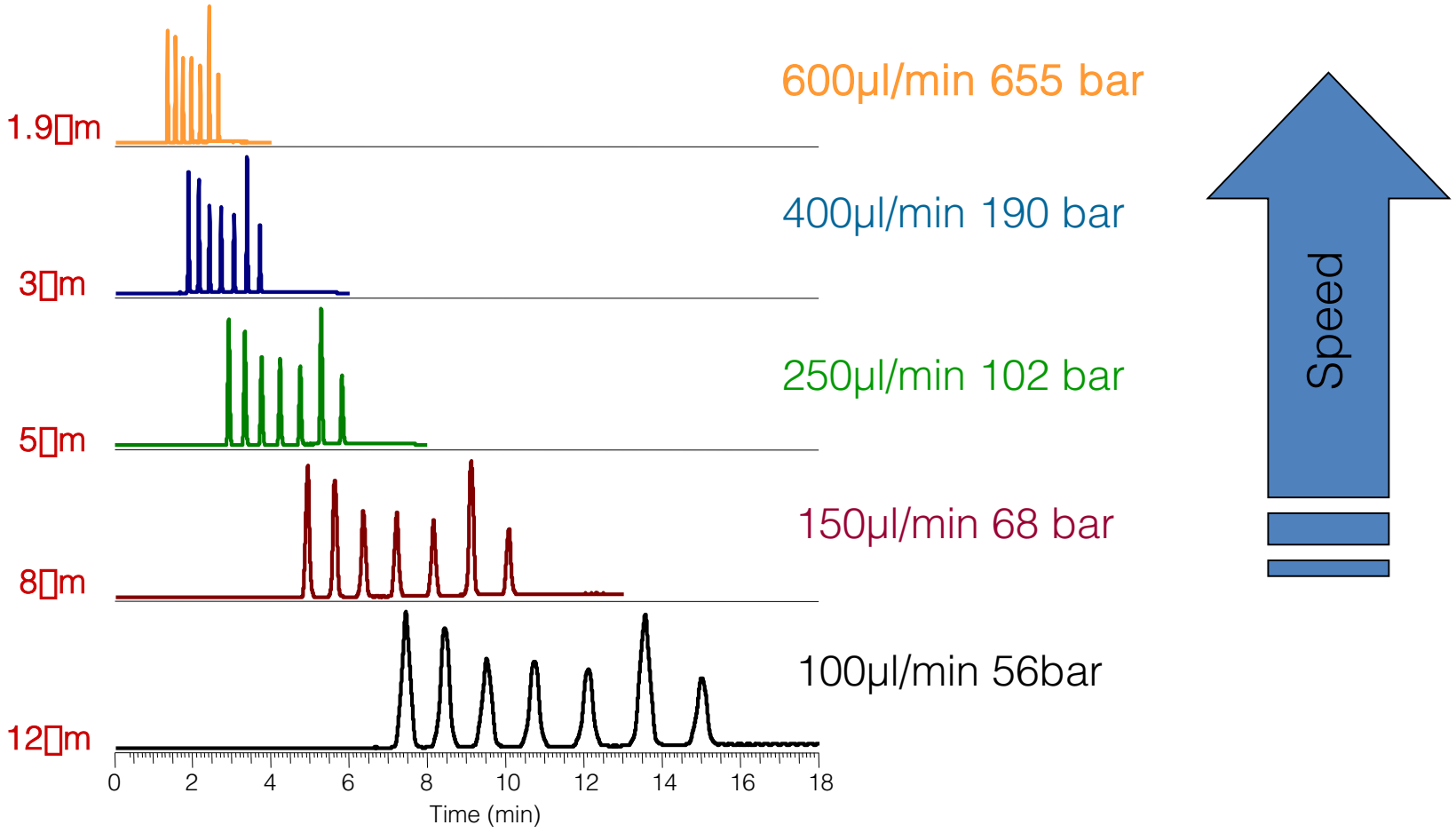
$$R_s = \frac{1}{4}\,\frac{(\alpha - 1)}{\alpha}\,\sqrt{N}\,\frac{k}{1+k}$$

Selectivity | Efficiency | Retention

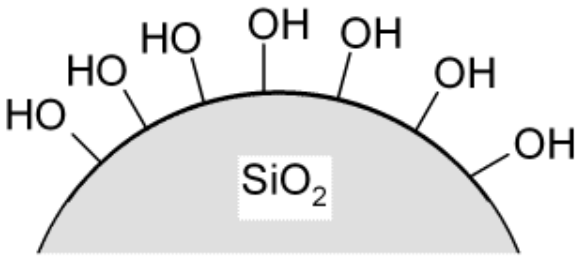
- Higher resolution – narrower peaks
- Higher sensitivity – taller peaks
- Higher peak capacity (more peaks / unit time) – narrower peaks

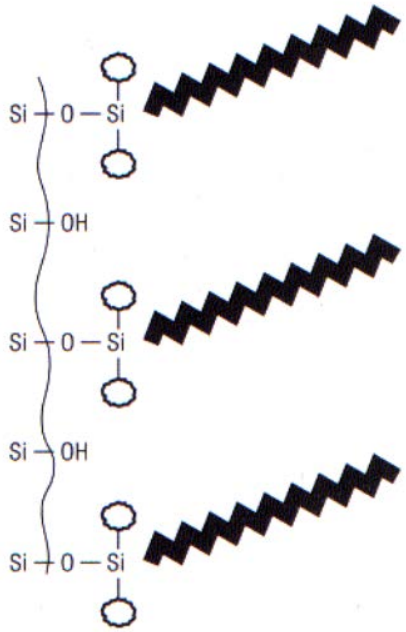
Increase Speed, Maintain Resolution *200x2.1mm*

Speeding up analysis with 1.9 µm Hypersil GOLD



Wait a minute…..what's column chemistry???!!!



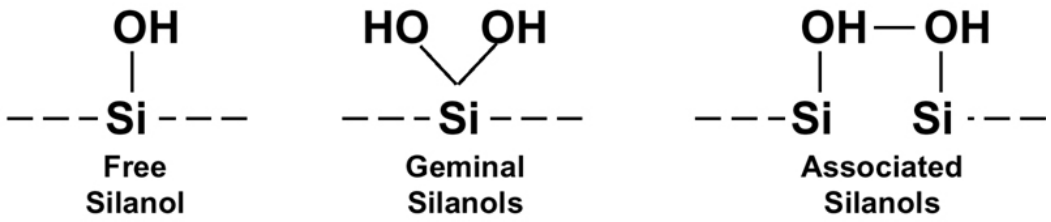


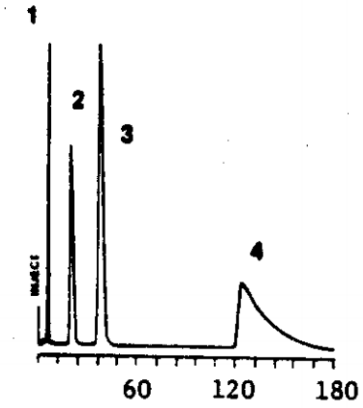
- Diol
- C1, C4, C8, C18
- Aminopropyl
- Nitrile
- Phenyl
- Pentafluorophenyl
- Cation Exchanger
- Anion Exchanger
- etc.

Chemistry is variant. Is all C18 the same ?

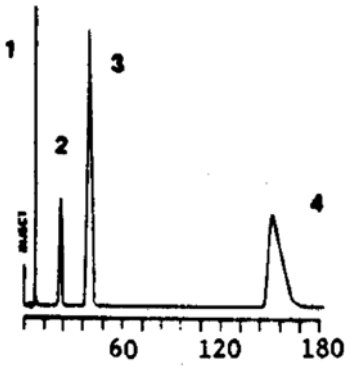
NO !

Un-reactioned: Interaction with Basic Molecules



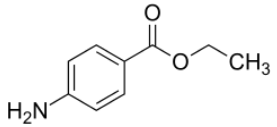


No endcapping



Endcapping

pKA



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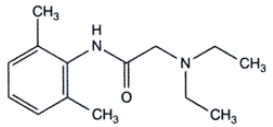

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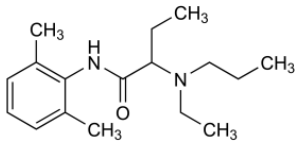

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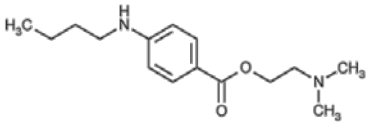
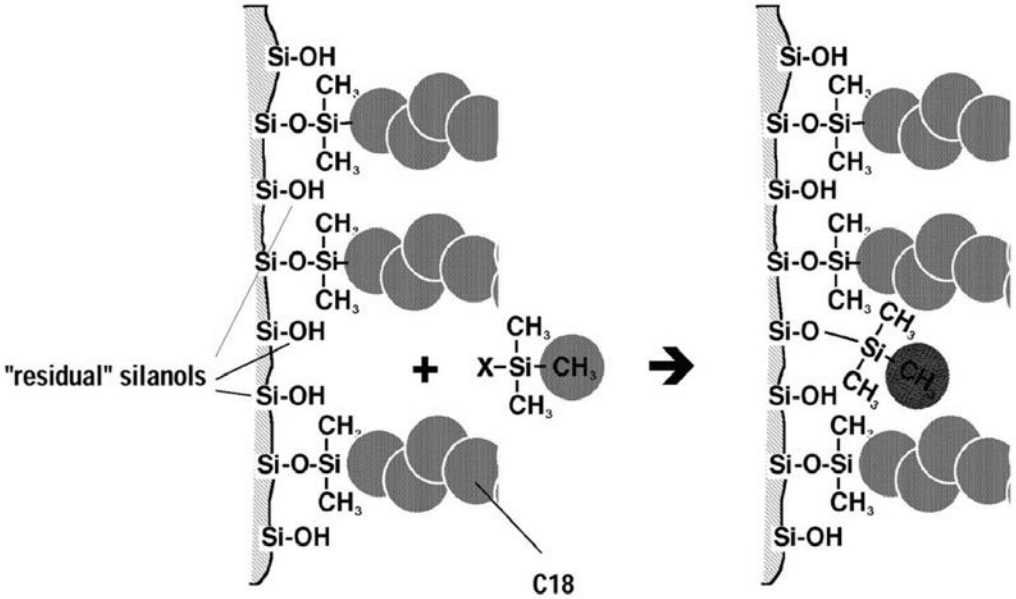
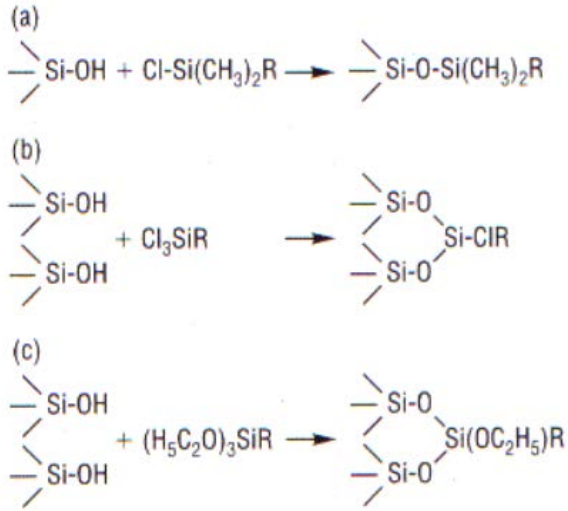


8.5

Endcapping : Prevent peak Tailing & interaction with alkaline



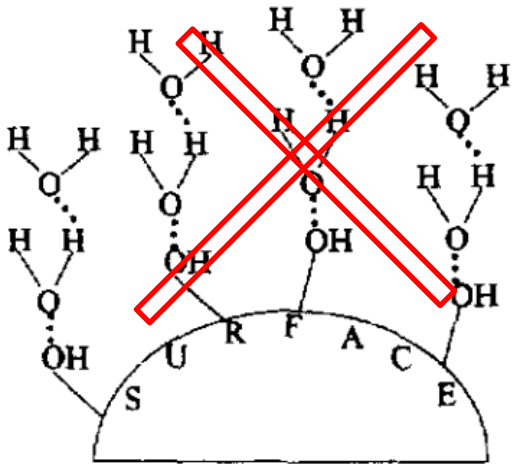


- Polar; amide, urea ,ether
- Hydrophilic
- Trimethylsilyl
- etc.

- Dimethyl silane
- Chloro silane
- Trifunction alkoxysilan
- etc.

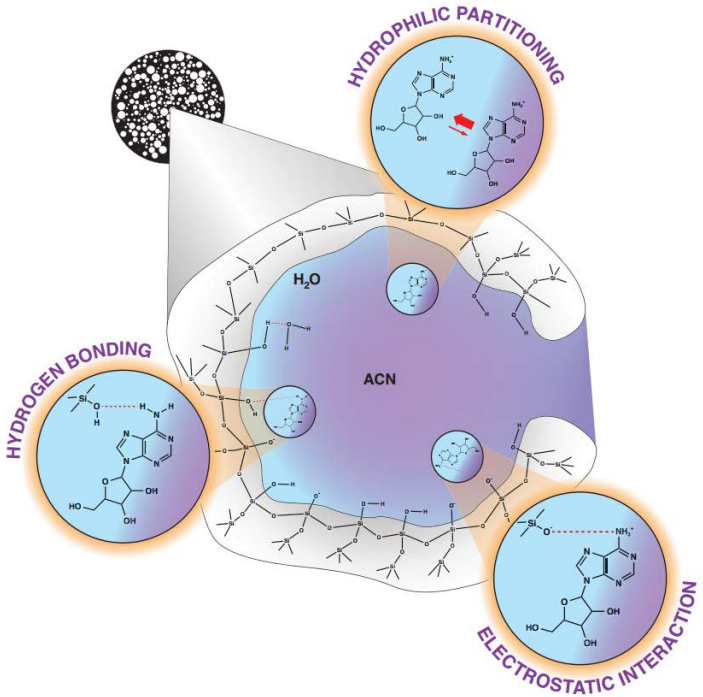
aQ Column and HILIC

aQ



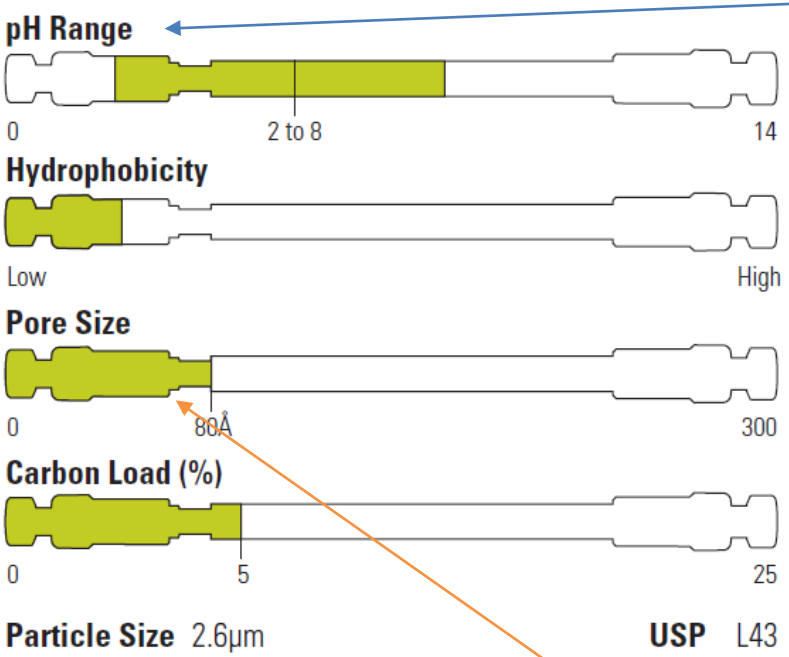
Stable in 100 % Aqueous with polar endcapping,
Enhance retention of polar compounds

HILIC



Retain highly polar and hydrophilic compound, no endcapping → can't use with more than 50% aqueous

Column Properties



Working pH of Mobile Phase

Correlated to % carbon, reflected to polarity of column

US Pharmacopeia defined

% carbon content, higher is not always better resolution. Higher is more hydrophobic surface that resistance to high pH

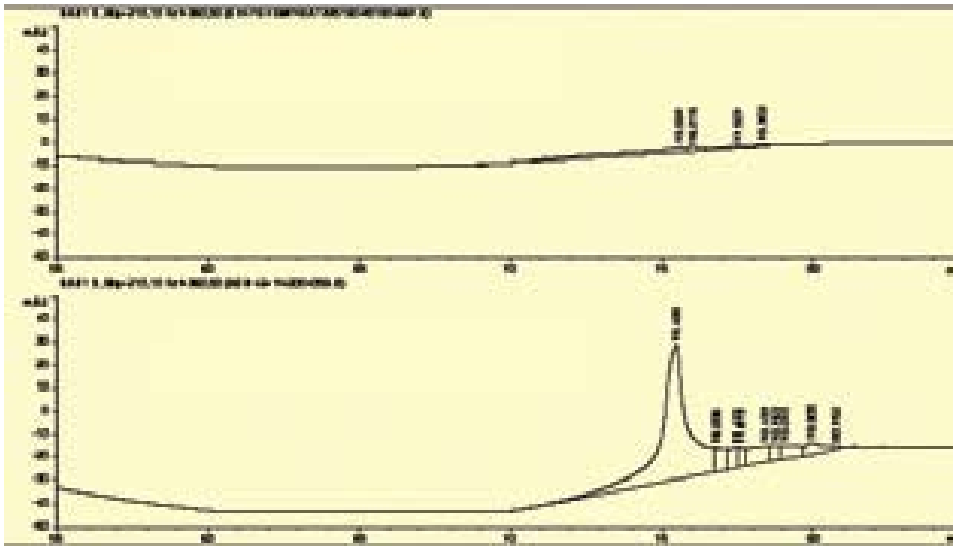
60-90 for small molecule
90-120 for both small molecule and peptide
120-300 for peptide or protein

How to make a good separation

- Test solubility with mobile phase
- Single analyte should started with Isocratic
- More than 2 analytes should started with Gradient
- 0.1% acid help ion pairing separation and enhance ionization step
- DO NOT use phosphate buffer, NaOH, HCl or other non-volatiles buffer in LC-MS system

Acetonitrile Effect

- Very good used with water to facilitated the best separation
- Caused a baseline-shift
- Absorb at 210 nm



Ion Pairing

- Common Ion Pairing NH_4^+, Na^+, Cl^-, H^+
- Increase separation efficiency but affect *m/z* in Mass Spectrometer
- Competitive ion species
- Can be illuminated in fragmentation processes

ppm, ppb, ppt or µg/mL, ng/mL, pg/mL

- Density of matter is NOT EQUAL
- Effect Quantitation

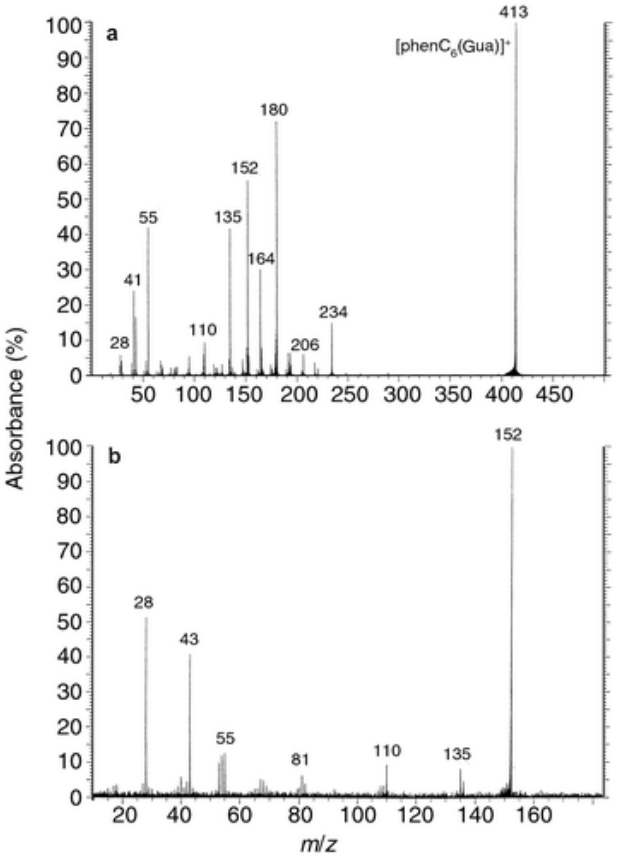
$$d = m/v$$

d=density (gram/mL)

m=mass (gram)

v=volume (mL)

What is a Mass Spectrometer?



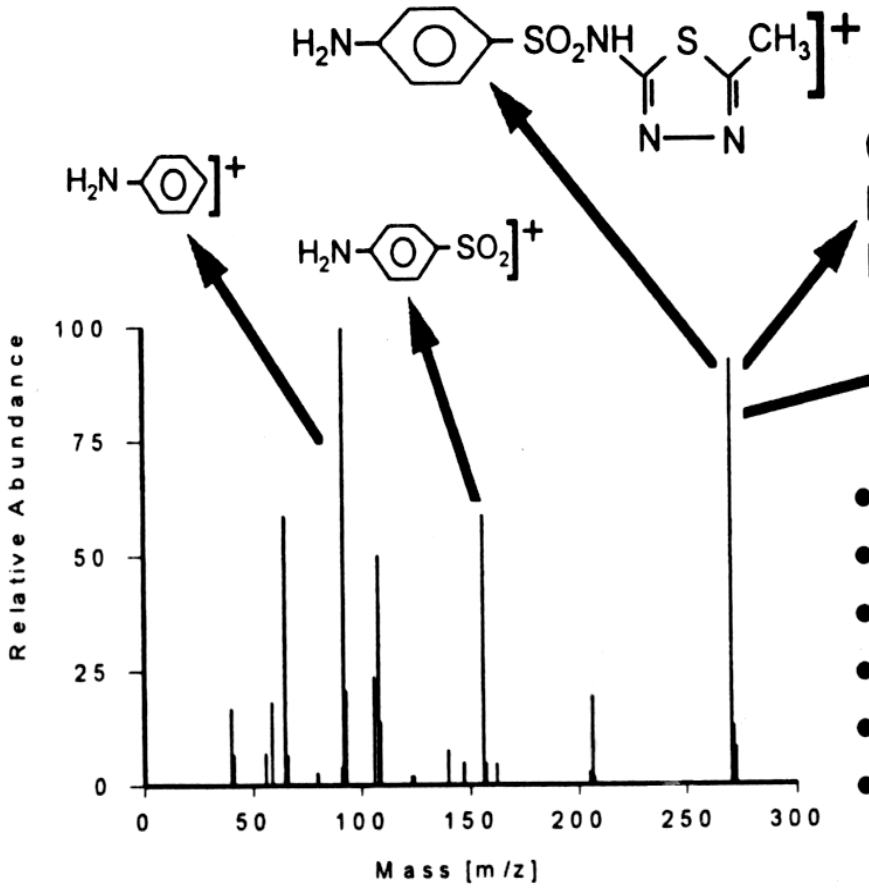
"A device to measure the mass-to-charge ratio of individual molecules that have been converted to ions"

- Mass Spectrum: A plot of mass to charge (m/z) vs. relative or absolute intensity

Welcome to the world of Mass Spectrometer

- All mass analyzers determine the mass of an ion
- All mass analyzers determine the mass-to-charge ratio
- All mass analyzers measure gas-phase ions
- All mass analyzers must operate at very low pressure (a vacuum)

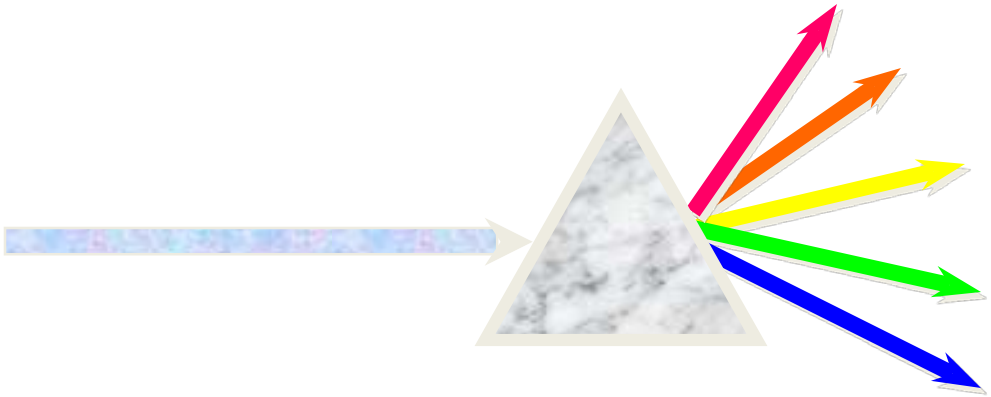
Information Rich Data



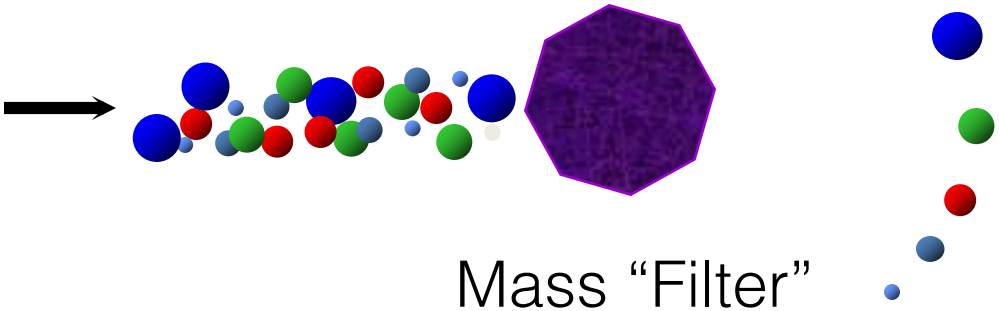
Compound: Sulfamethizole
MW: 270
Empirical Formula:
$C_9H_{10}N_4O_2S_2$

- *Molecular Weight*
- *Elemental Composition*
- *Empirical Formula*
- *Molecular Structure*
- *Unambiguous Identification*
- *Mass Specific Response*

Spectrum Formation

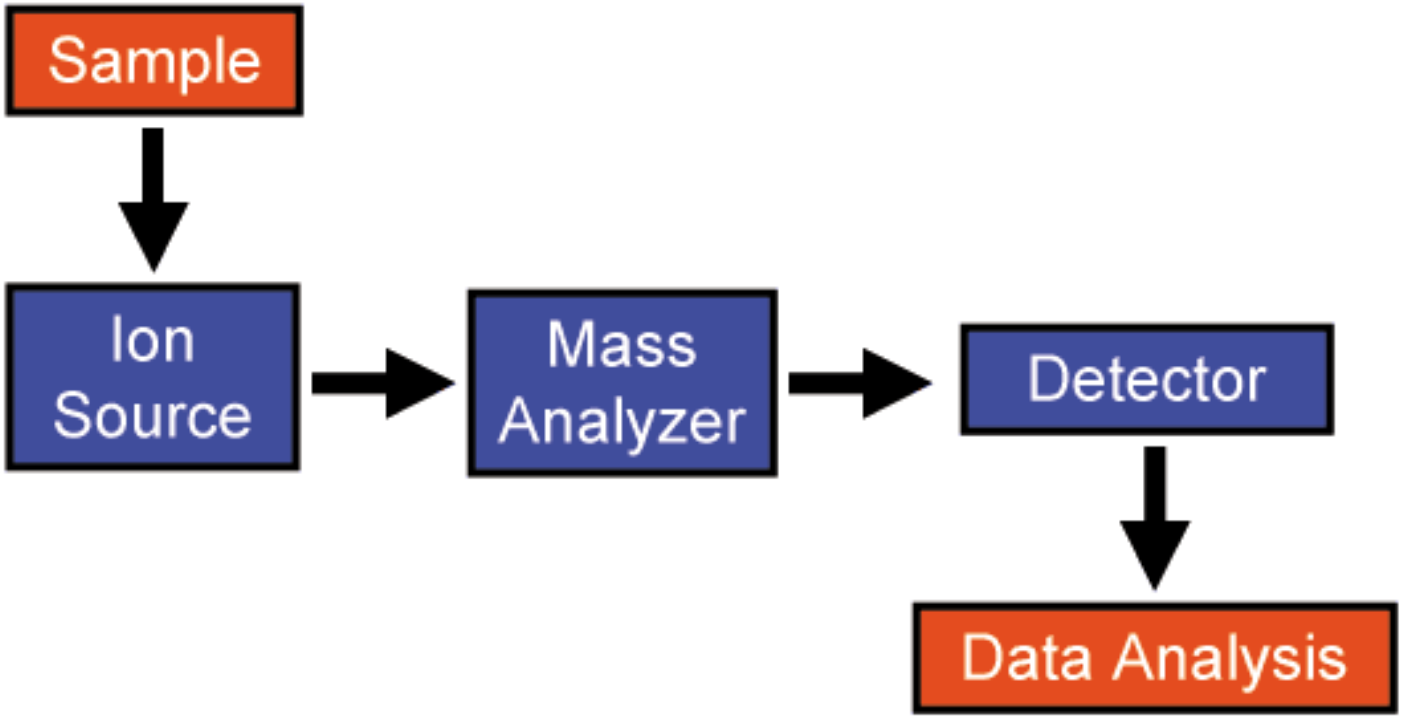


Light “Filter”

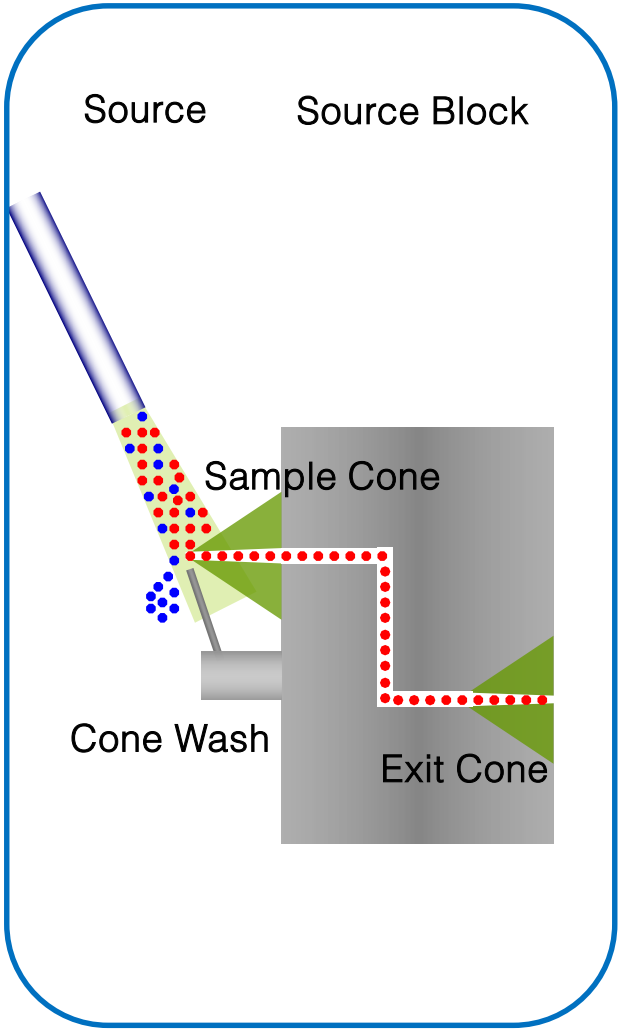


Mass “Filter”

Mass Spectrometer Diagram



Ion Source & Path



Analyzer

RF/DC
Pre-Filter
Lens

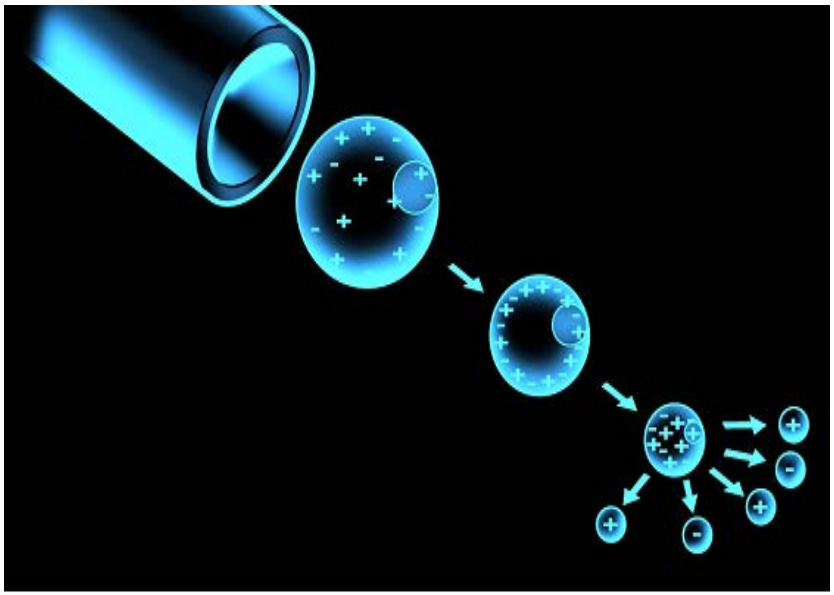
Mass
Analyzer

Molecular Weight Information
Structural Information
Positive Identification
Quantitative Information

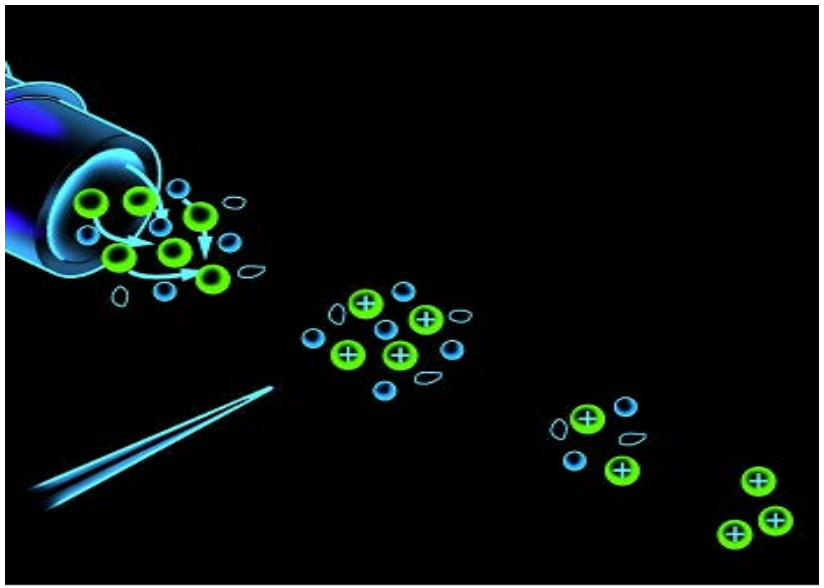
Ion Source

- API; APCI, ESI, APPI
- Fast Atom Bombardment
- Matrix Assisted Laser Desorption/Ionization (MALDI)
- Ion Attachment
- Field Desorption
- Induced Couple Plasma
- Direct Analysis of Real Time (DART)

Atmospheric Pressure Ionization



Electrospray Ionization



Atmospheric Pressure
Chemical ionization

Chemistry Considerations ESI or APCI

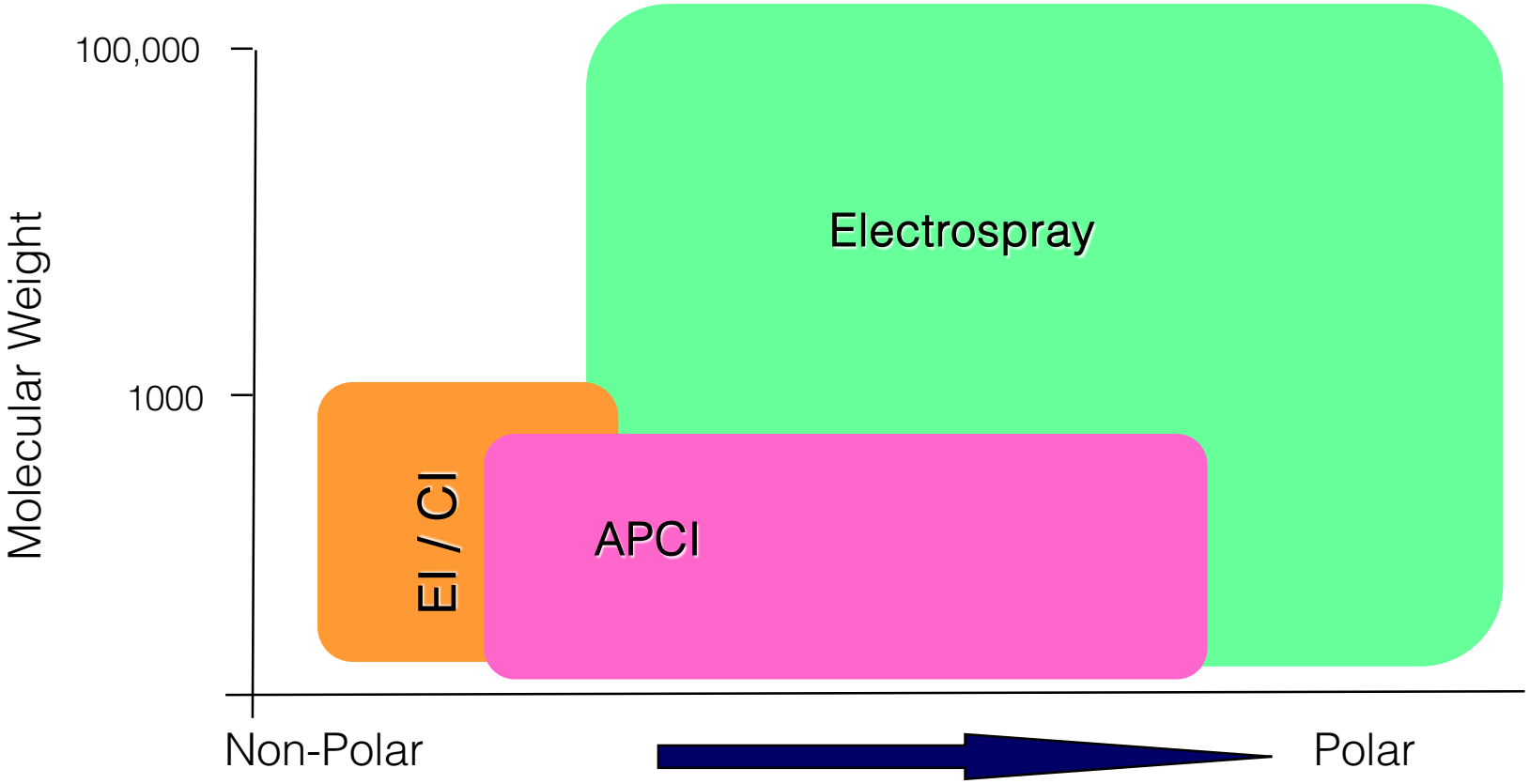
ESI:

- Ions formed by solution chemistry
- Good for thermally labile analytes
- Good for polar analytes
- Good for large molecules (Proteins / Peptides)

APCI:

- Ions formed by gas phase chemistry
- Good for volatile / thermally stable
- Good for non-polar analytes
- Good for small molecules (Steroids)

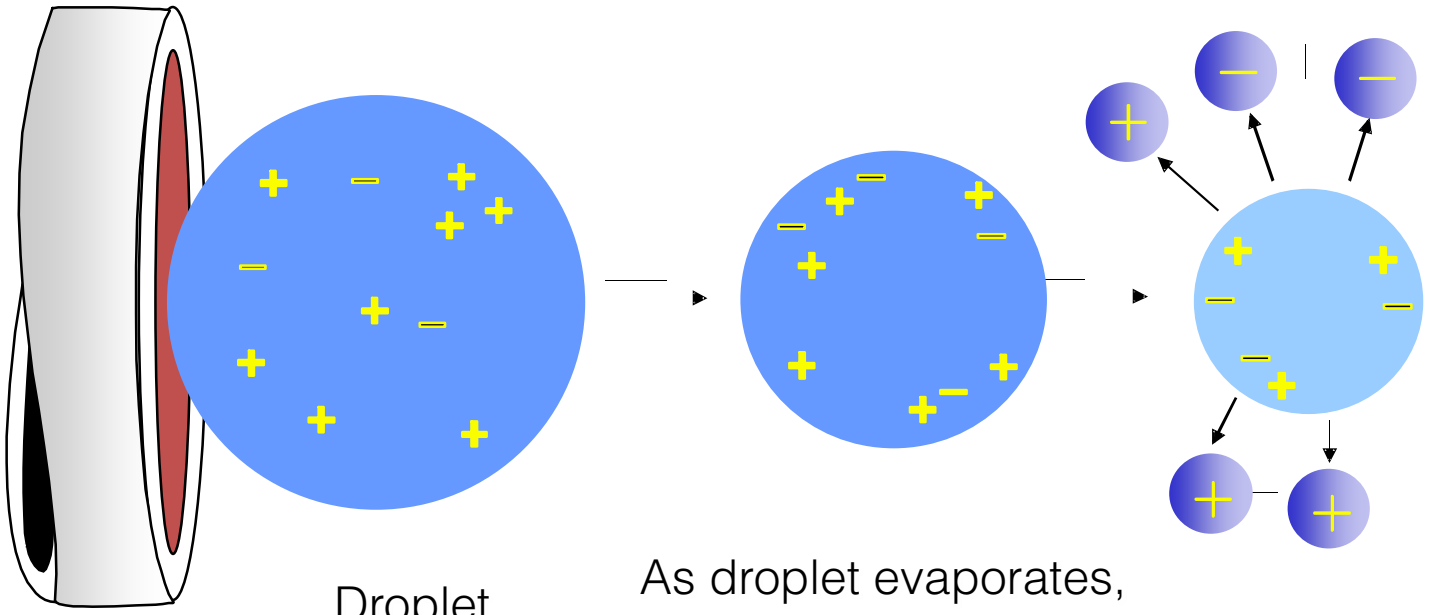
Which Ionization Mode ?



Step in Electrospray

- Production of charged droplets from a capillary tip
  - Under influence of strong electric field
- Reduction of droplet size
  - Rapid solvent evaporation
  - Repeated coulombic explosions (fission)
- Transfer of ions from surface of small droplets to gas phase
  - No heat of evaporation

Ion Evaporation Theory



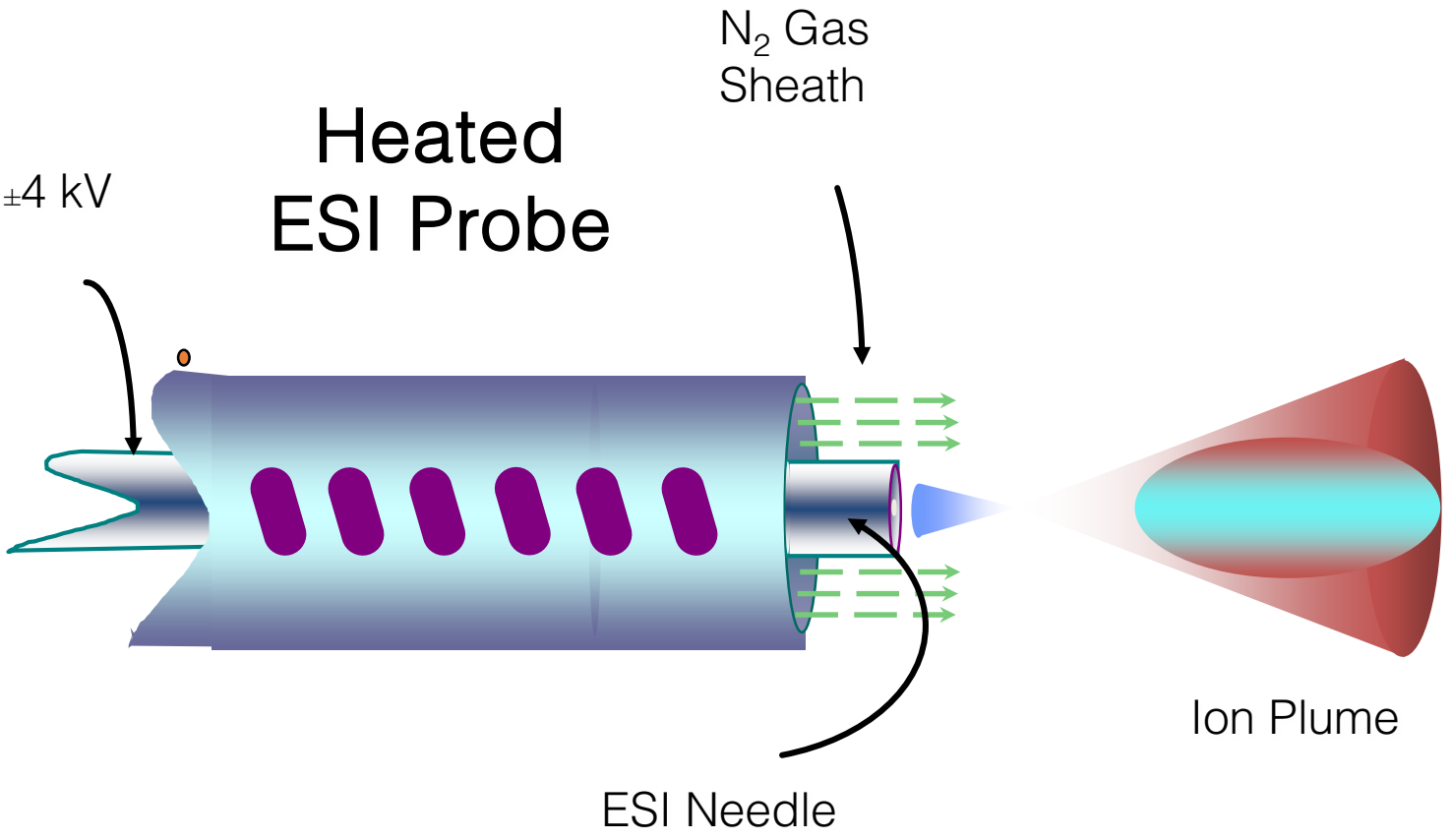
Capillary
+4 kV

Droplet containing ions

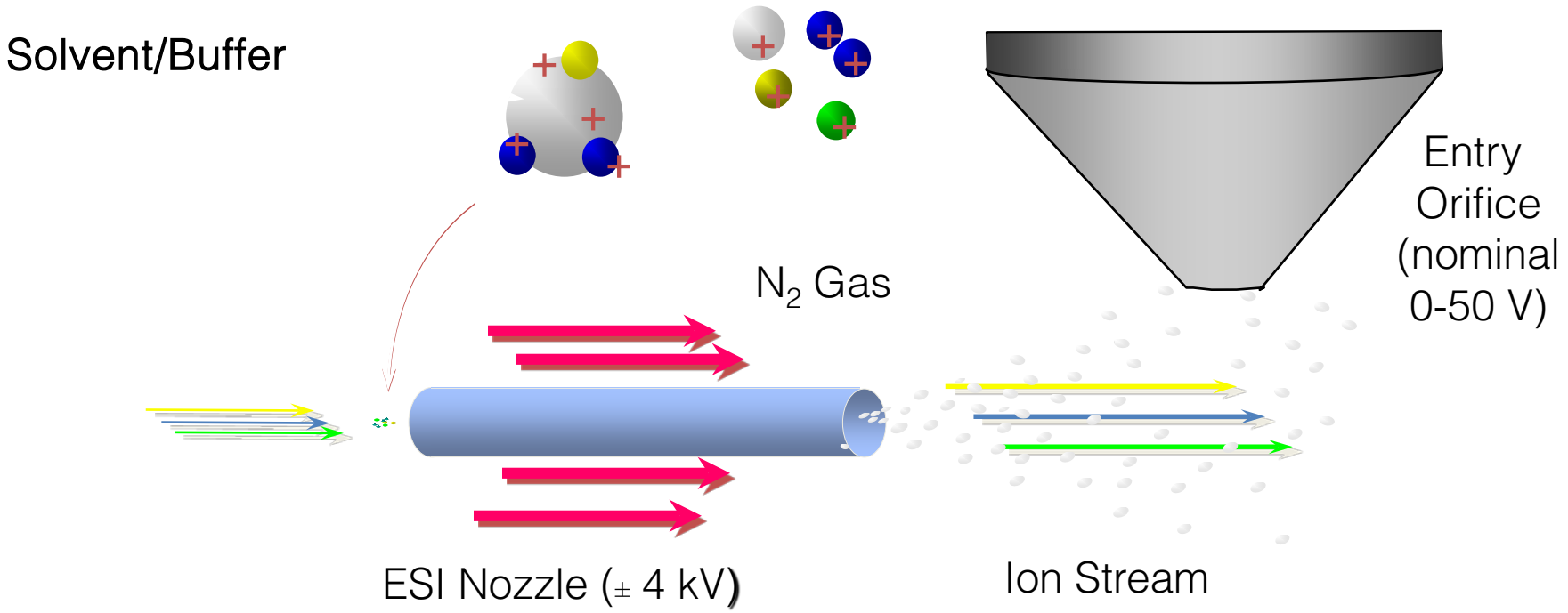
As droplet evaporates, field increases and ions move to surface

Raleigh Limit – Droplet Instability - releases smaller droplets -ions

Electrospray Nozzle Detail



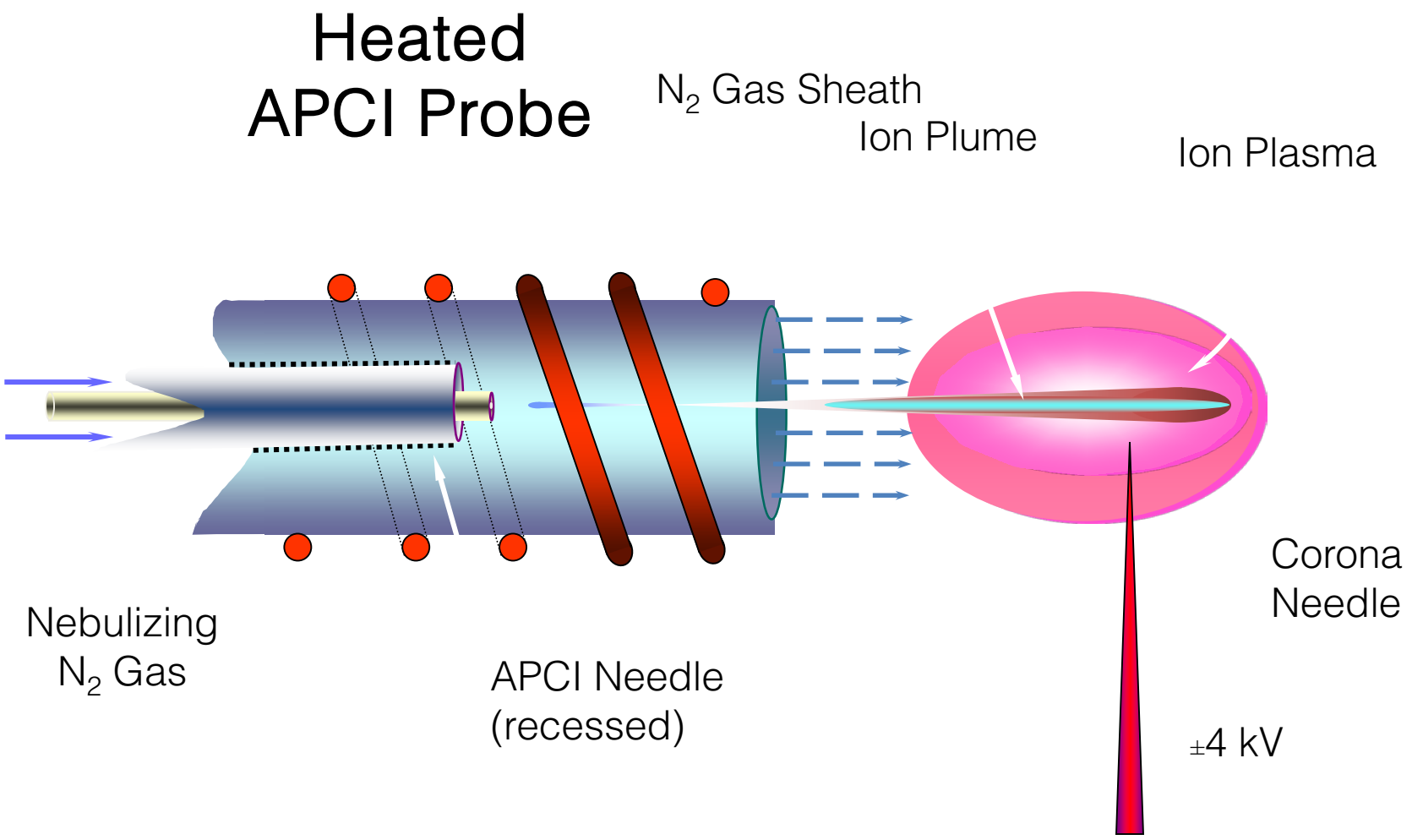
ESI/MS Ion Injection (Desolvating the Spray)



Electrospray Ionization

- Low and high molecular weights
- Singly and multiply charged species
- Very soft ionization
- Mobile phase should have a polar component

APCI Nozzle Detail



Atmospheric Pressure Chemical Ionization (APCI)

- Low molecular weight (<1000)
- Singly charged species only
- Thermal fragmentation may occur
- Mobile phase can be non-polar (normal phase)

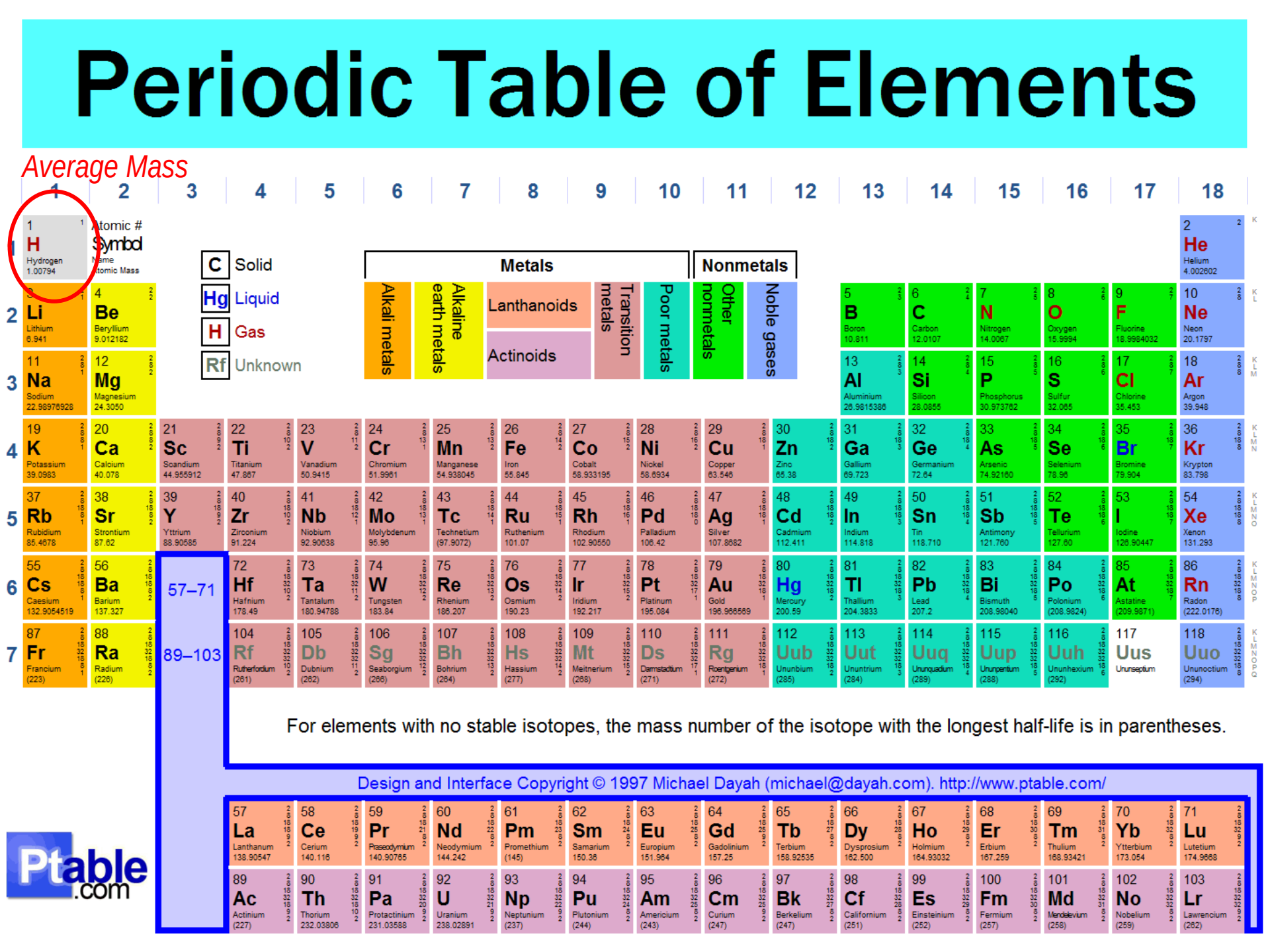
Type of Mass Spectrometer

How to Choose a proper Mass Spectrometer ?

Typical Mass Accuracy

Type of MS	Mass accuracy	Utility for
Quadrupole	0.1 μ	Identify
Traps	0.1 μ	Identify
TOF	0.0001 μ	Empirical formula/ composition
Sector	0.0001 μ	Empirical formula/ composition
FT-MS	0.0001 μ	Empirical formula/ composition

Periodic Table of Elements



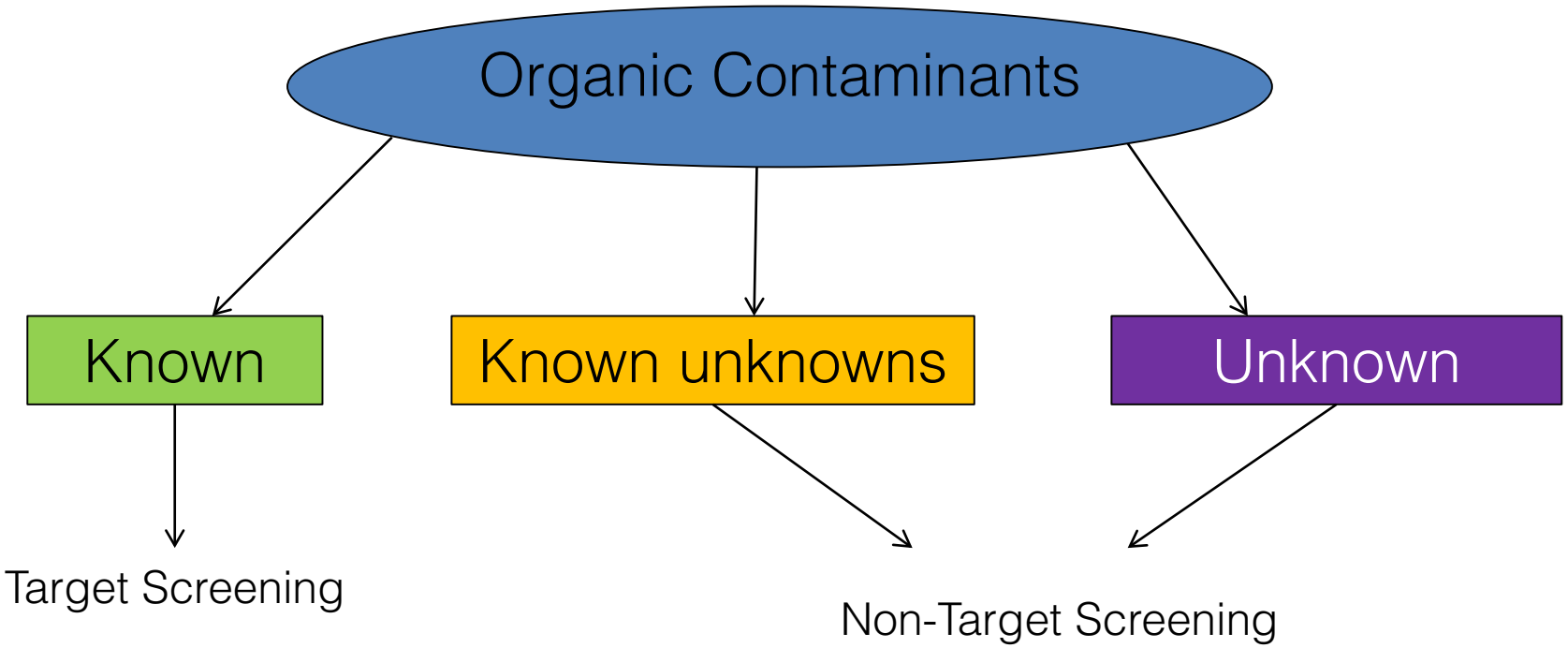
How's About Mass Accuracy

- Average Mass = summing the average atomic masses of the constituent elements, H_2O; 1.00794 + 1.00794 + 15.9994 = 18.01528.
- Exact Mass = summing the masses of the individual isotopes of the molecule, H2O; 1.0078 + 1.0078 + 15.9994 = 18.0106.

The Others Stories;

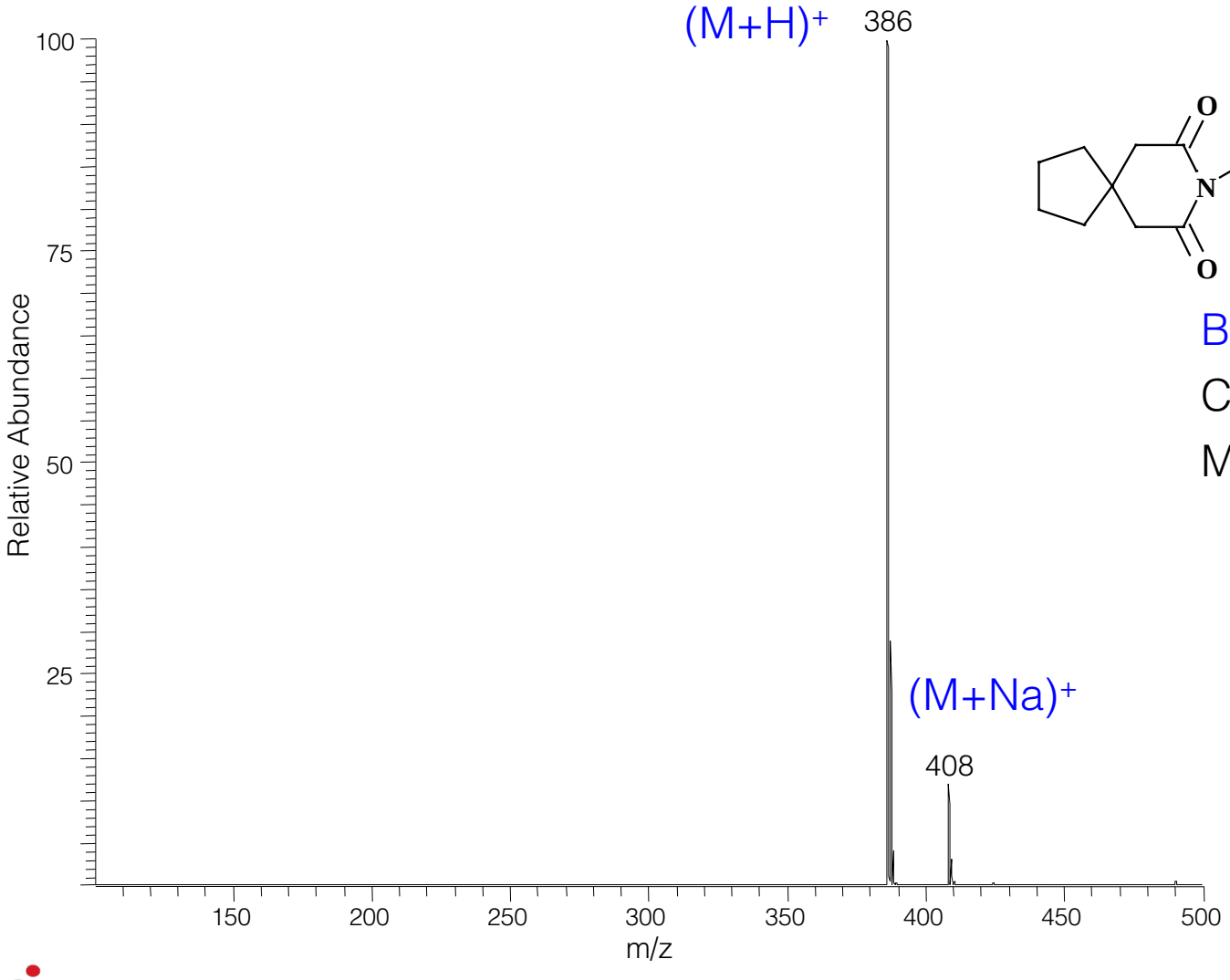
- Isotopomer (Isotopic Isomer) = same type of isotope but difference in position, CH_3CHDCH_3 vs $CH_3CH_2CH_2D$
- Isotopologues = difference in isotope in the molecules, H_2O HOD
- Monoisotopic = sum of masses in molecule. Using of most abundance or stable isotope.

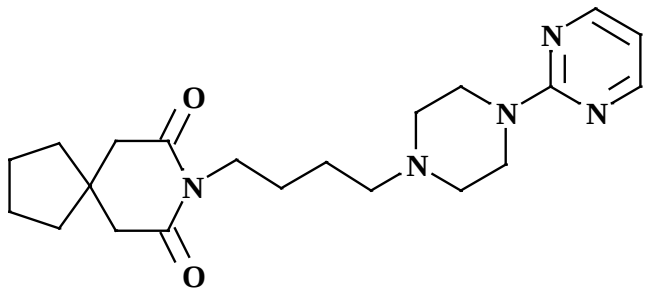
Strategies for screening



Rapid and sensitive screening methods able to assign positive hits undoubtedly to particular organic compounds

Full-Scan MS of Buspirone



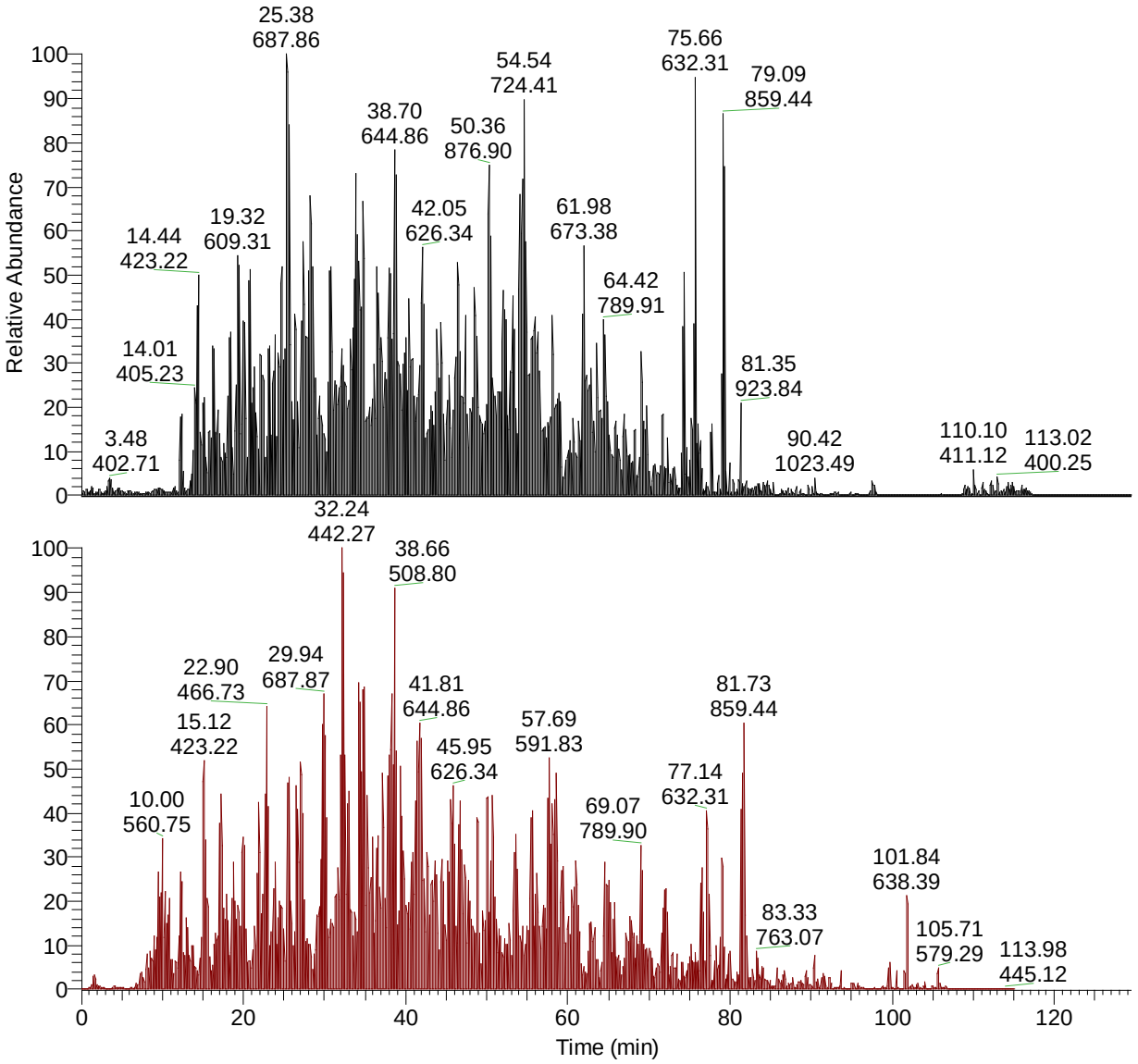


Buspirone

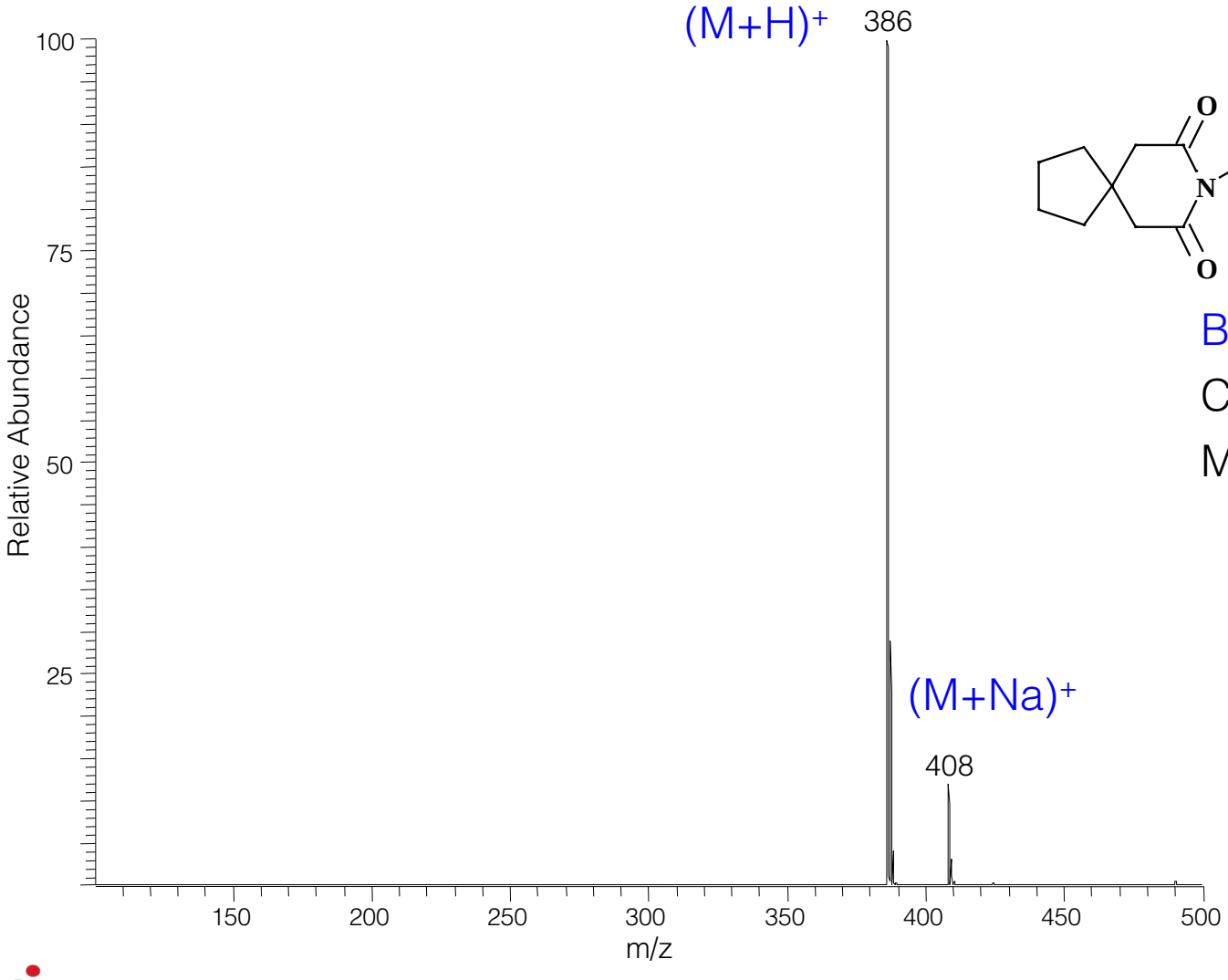
$C_{21}H_{31}N_5O_2$

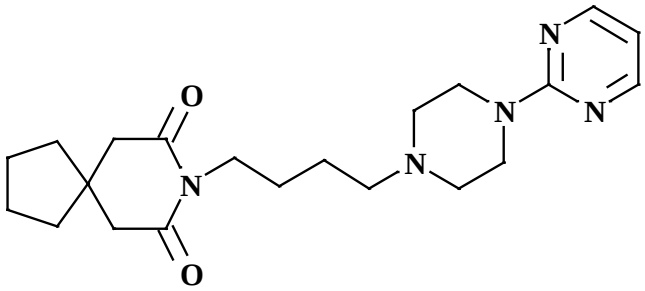
MW = 385

Real-life Full-Scan



SIM MS of Buspirone



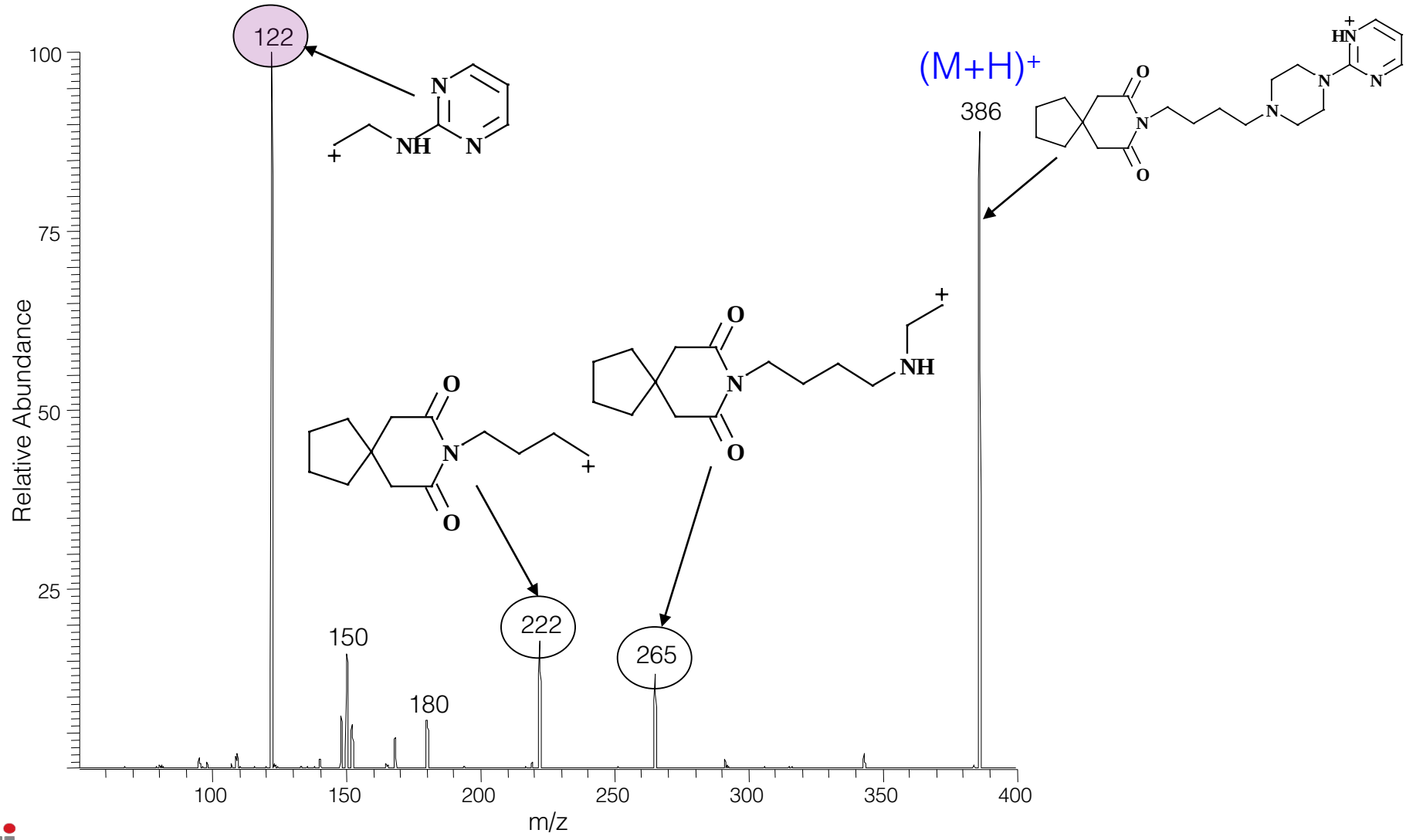


Buspirone

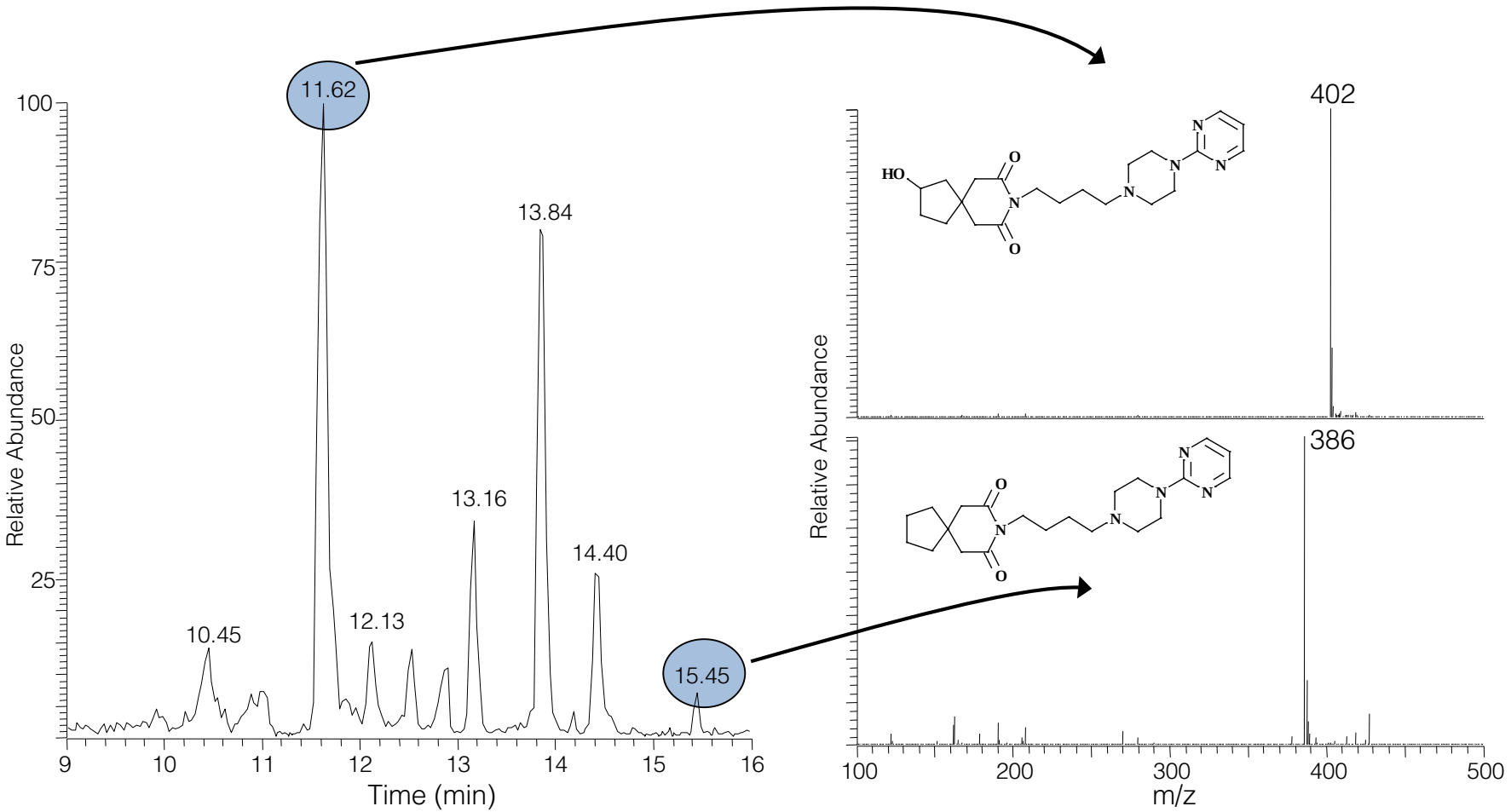
$C_{21}H_{31}N_5O_2$

MW = 385

Product Ion Spectrum of Buspirone

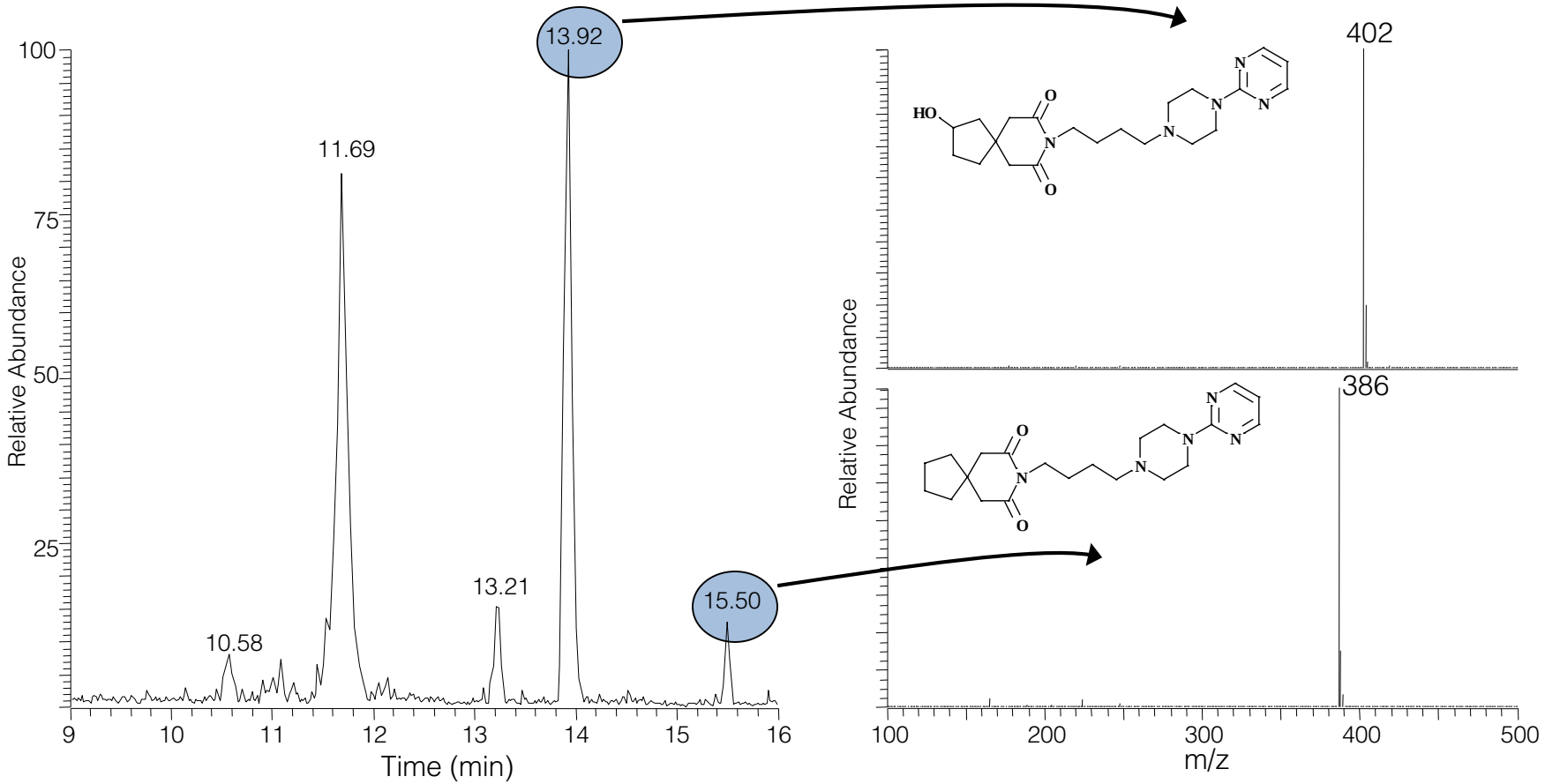


Precursor Ion Scan Mode for Buspirone Metabolites



Precursor Ion Scan: Q3 set to m/z 122

Neutral Loss Scan of Buspirone Metabolites

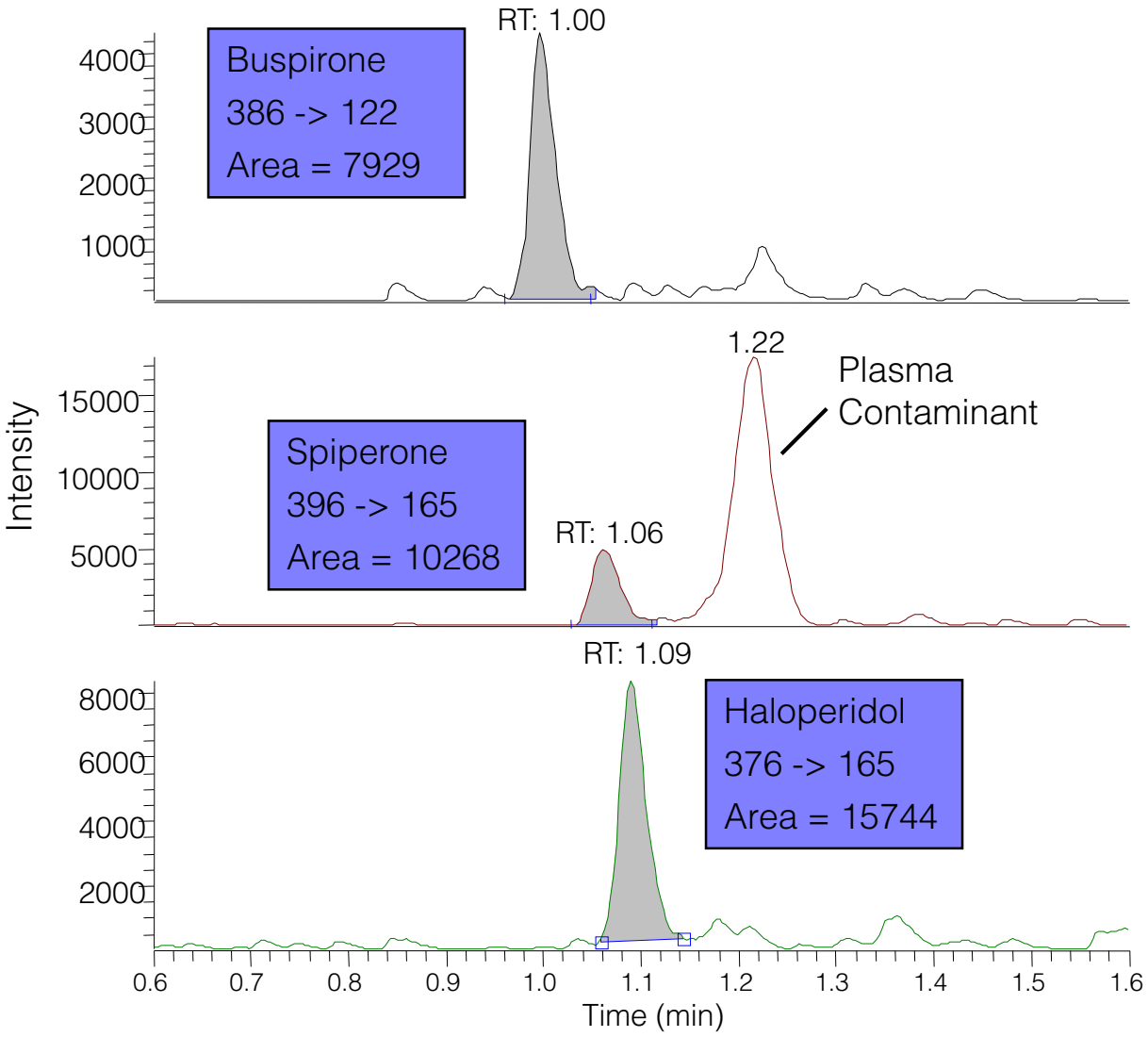


Neutral Loss Scan: Q1/Q3 difference set to 121 Da

Scan Modes

Scan Mode	Purpose
Full-Scan	MW Info.
SIM	Quantitation
Product	Structural Info.
SRM	Targeted Quantitation
Neutral Loss	Analyte Screening
Precursor	Analyte Screening

Quantitation Using SRM Mode on the TSQ Quantum



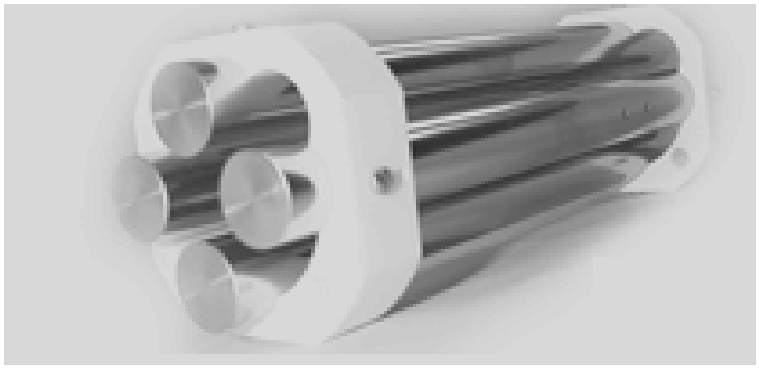
- LC/ESI-MS/MS, SRM Mode
- 10 pg/mL Buspirone, Spiperone & Haloperidol in Bovine Plasma
- 100 fg on column
- Ballistic Gradient Method @ 1 mL/min (no split)
- Total acquisition time = 1.6 min

Targeted Screening Ideal

- High isolation power for higher discrimination
- High precision for accurate mass identification
- Low detection limit
- High mass stability for a long lasting mass calibration
- Highly selective ion monitoring (H-SRM)
- Low dwell time and no cross-talk for no-false interpretation
- Fast polarity switching

Mass Analyzer - Quadrupole

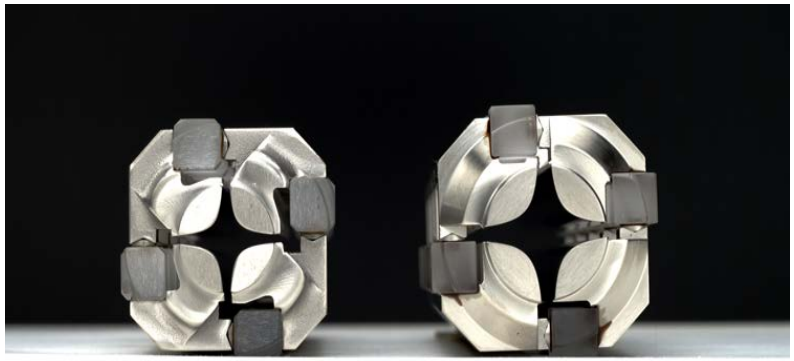
- Quadrupole, comprise of
- Quadrupole pre-filter; Hyperbolic,Round, Square

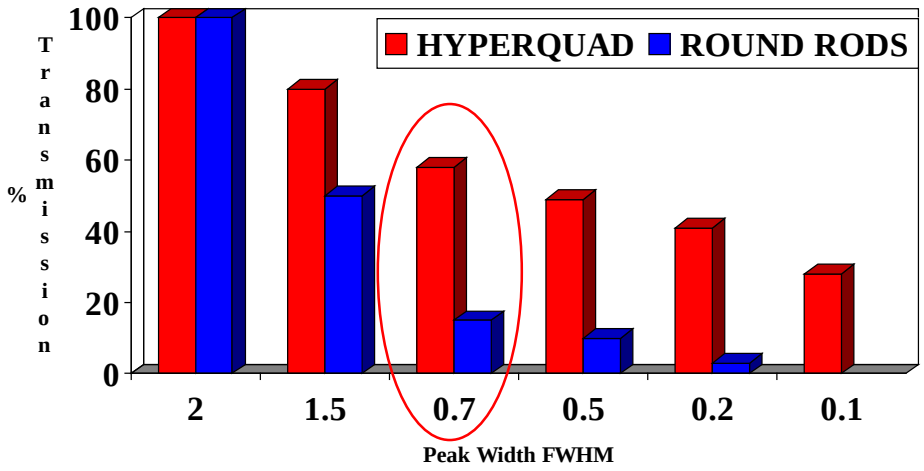


Controlled by RF and DC voltage !
Ion transmitting @ 1 m/z in given time

Advantages Of HyperQuads

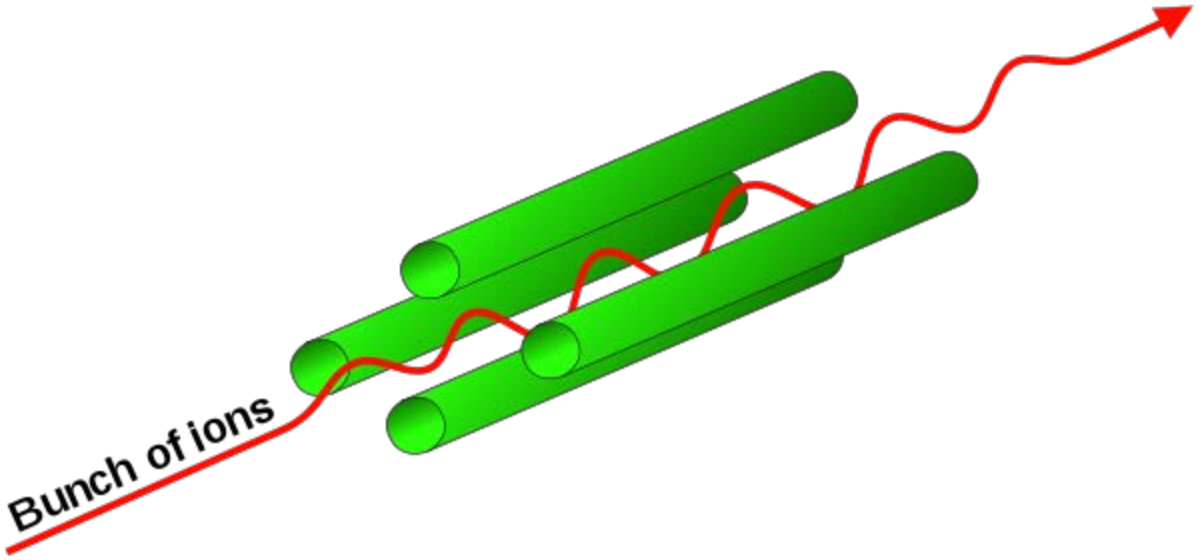
- Forms Pure Quadrupolar Fields (improves peak shape)
- Reduces Fringing Effects (sensitivity enhancement)
- Significantly Improves Resolution
- Improves Transmission (sensitivity enhancement)





Type of Quadrupole Instruments

- Single Quadrupole
- Triple Quadrupole

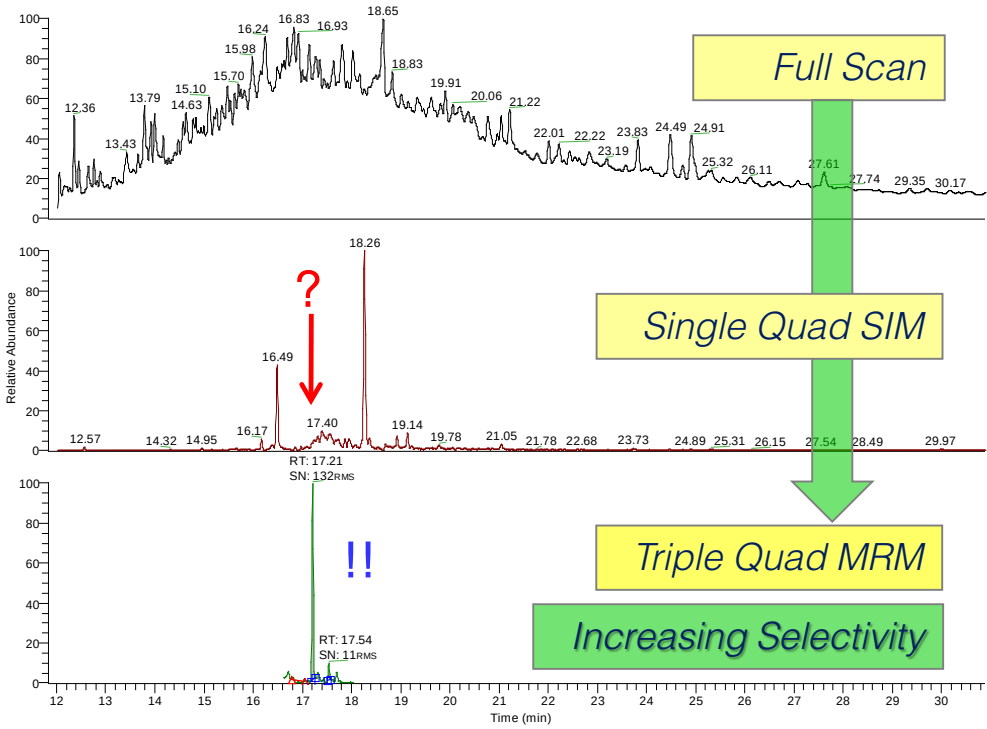


Applications Driver

- High Sensitivity in Matrix Samples
  - Lower levels for increased number of analytes
  - Shorten expensive sample prep
  - Small sample volumes with reduced clean-up

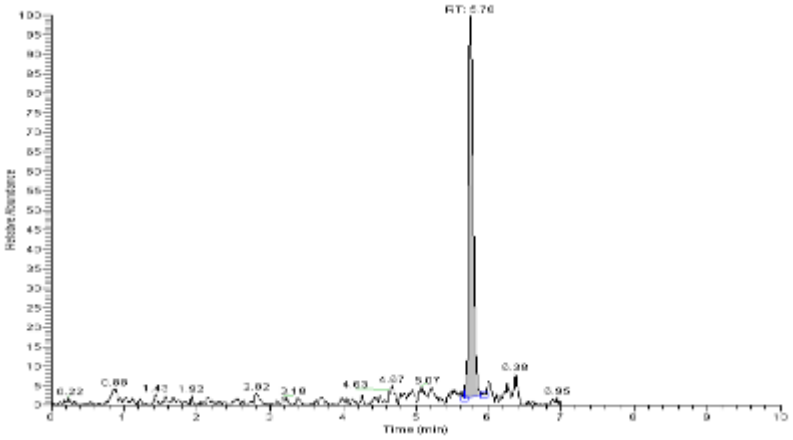
Food Safety

Anabolic steroids in meat production



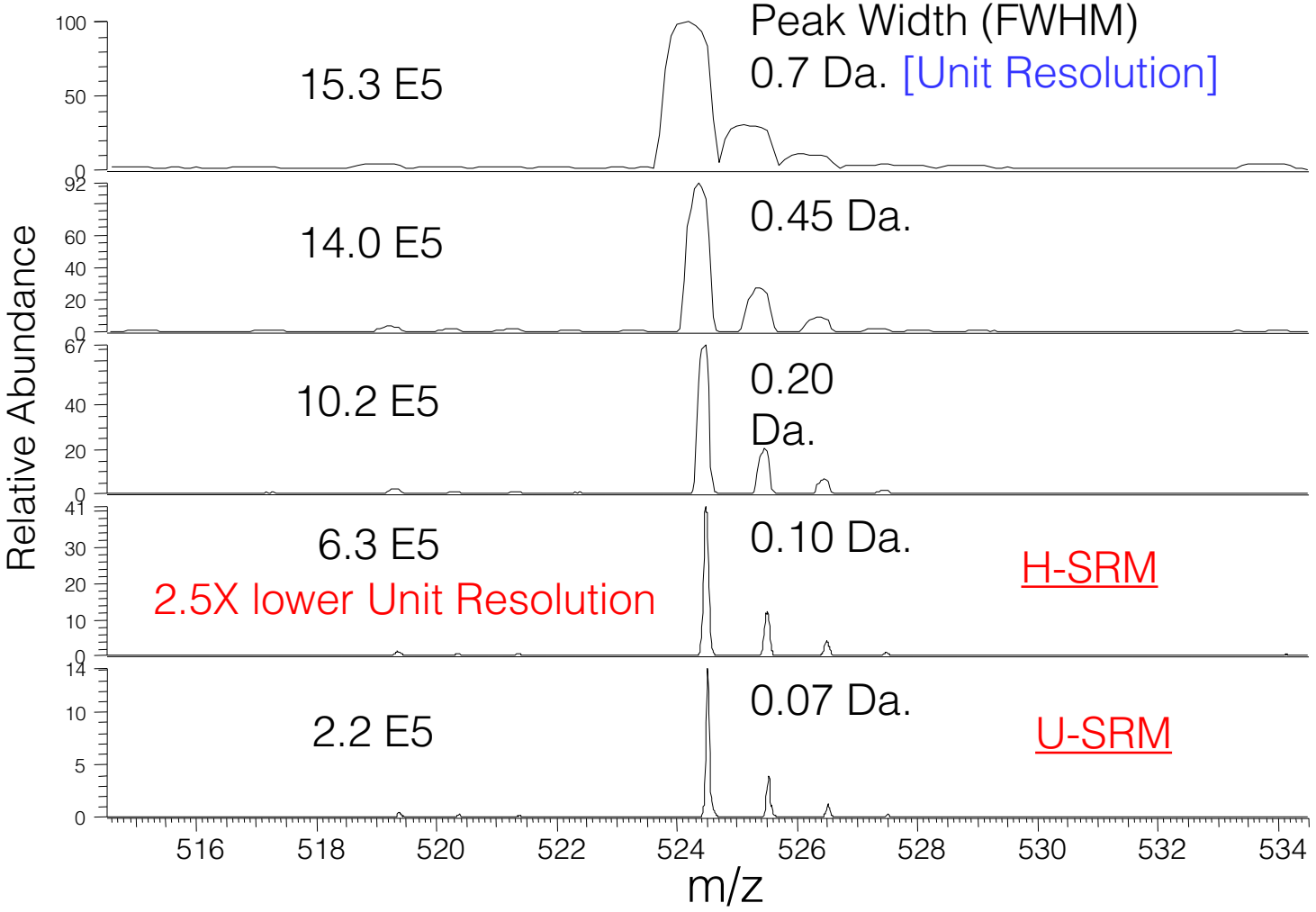
H-SRM Operation – More Method Robustness

✓ *Robust bioanalytical methods*

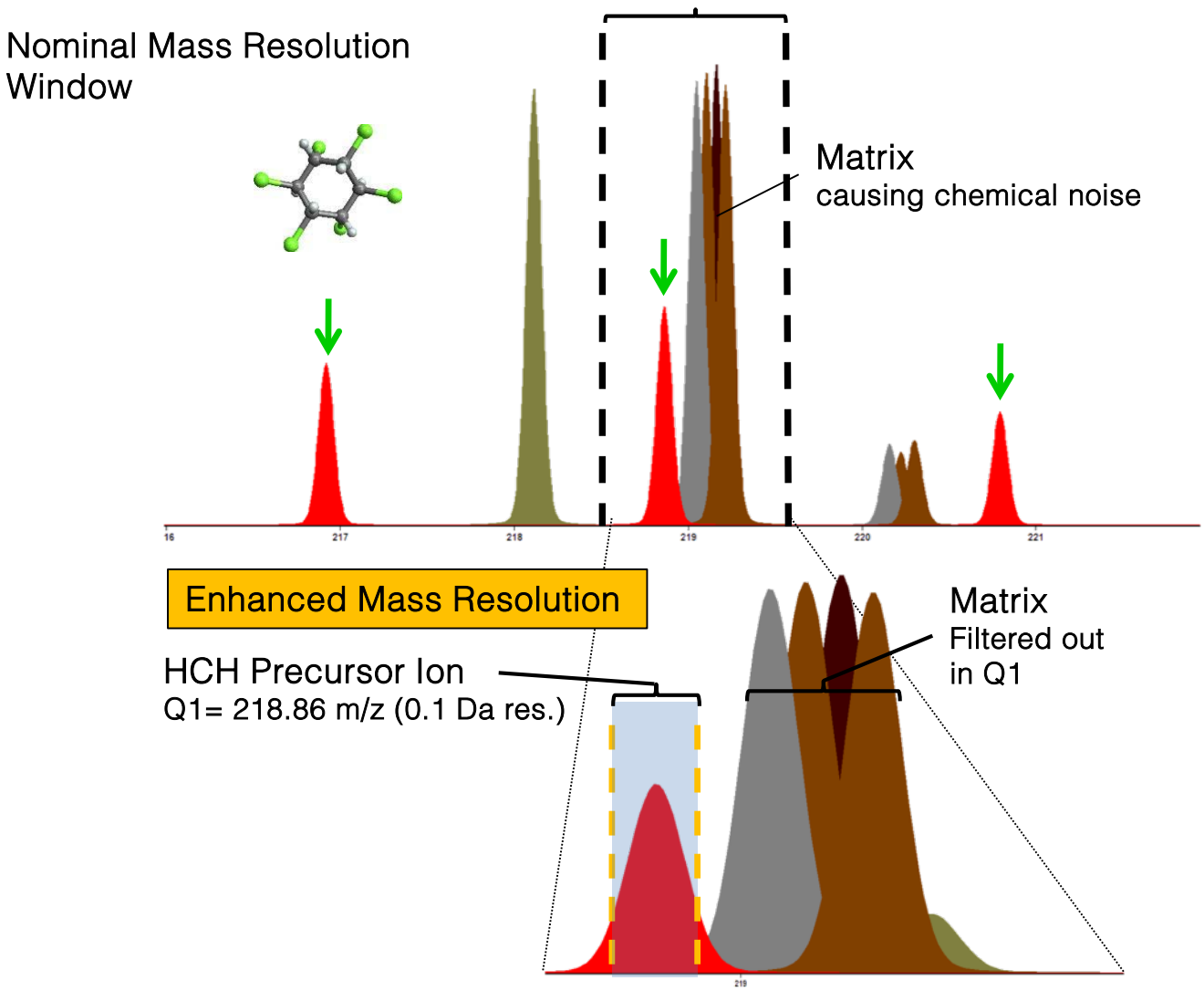


● = only pre-cursor ion transmitted

Transmission Efficiency on TSQ Quantum

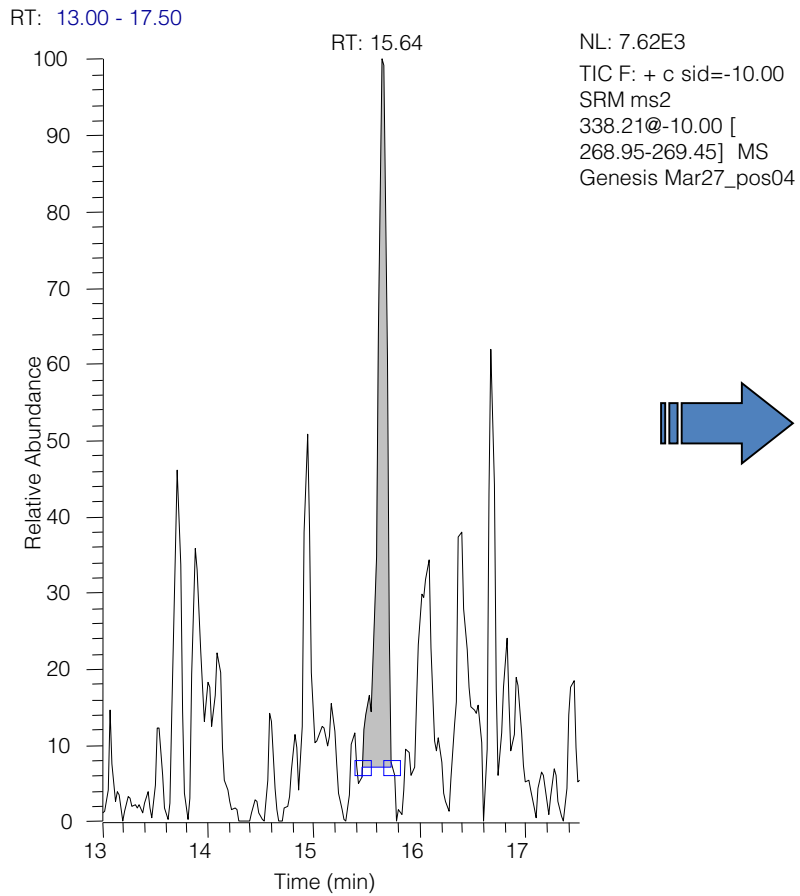


Mass Selectivity - SRM w. Enhanced Mass Resolution

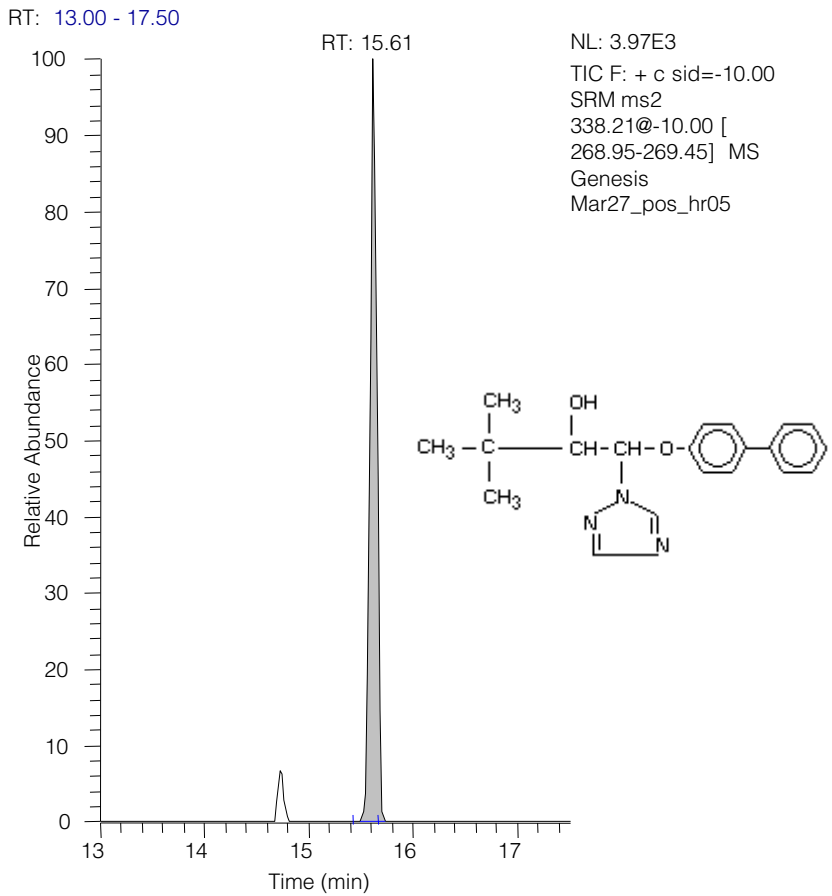


SRM vs H-SRM for analysis of Bitertanol

SRM Mode

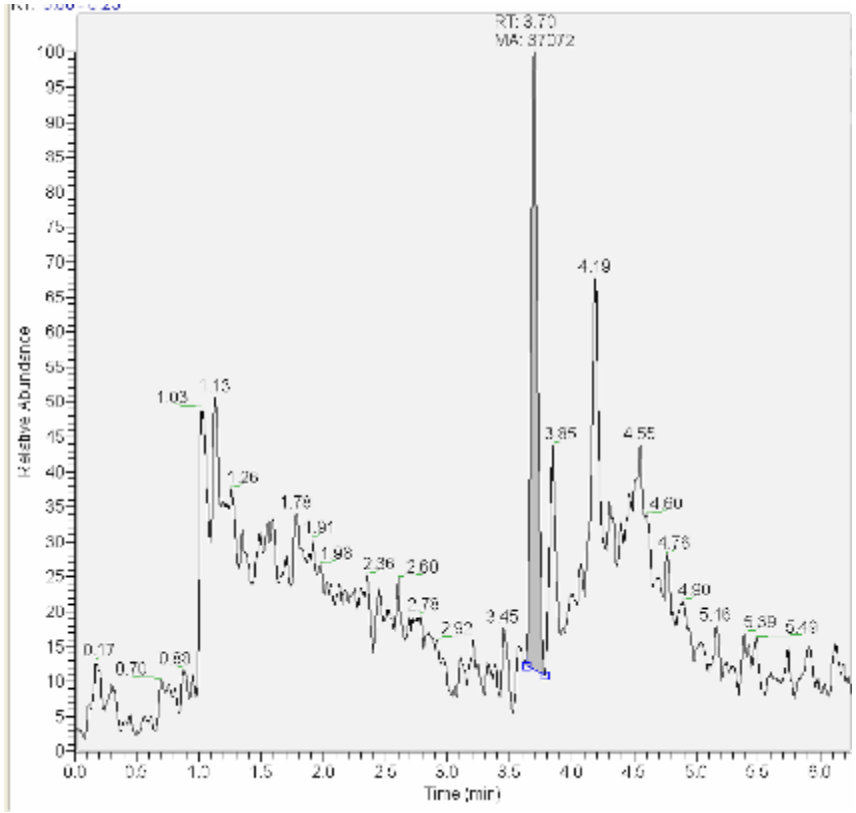


H-SRM Mode

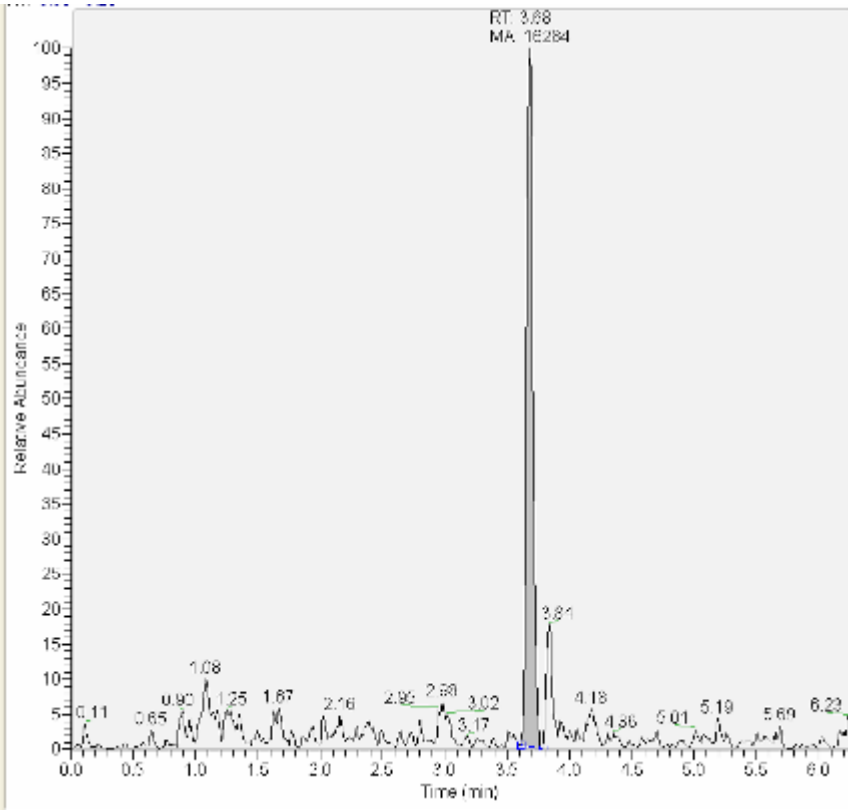


High resolution Comparison

0.7mDa



0.1mDa



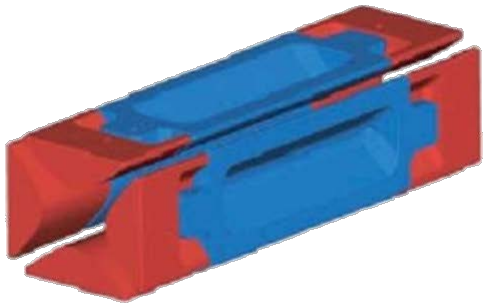
0.5ng/ml hydrolysed Amino Acid standard

Non-Targeted Screening or Newborn Ideal

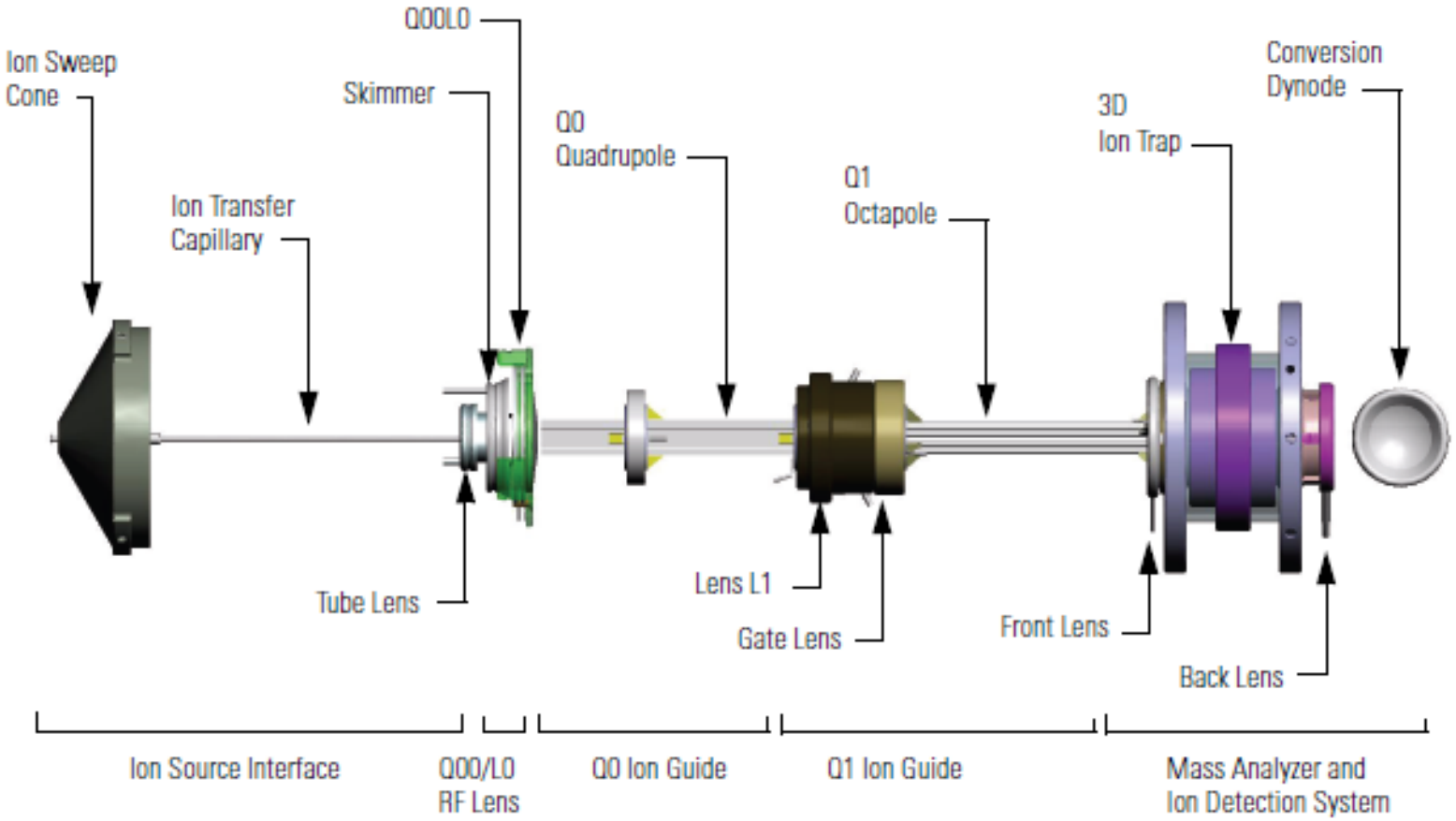
- High isolation power for higher discrimination
- High precision for accurate mass identification
- High resolution for more identification
- High mass stability for a long lasting mass calibration
- MS^n
- Library availability for easy interpretations

Type of Ion Trap Instruments

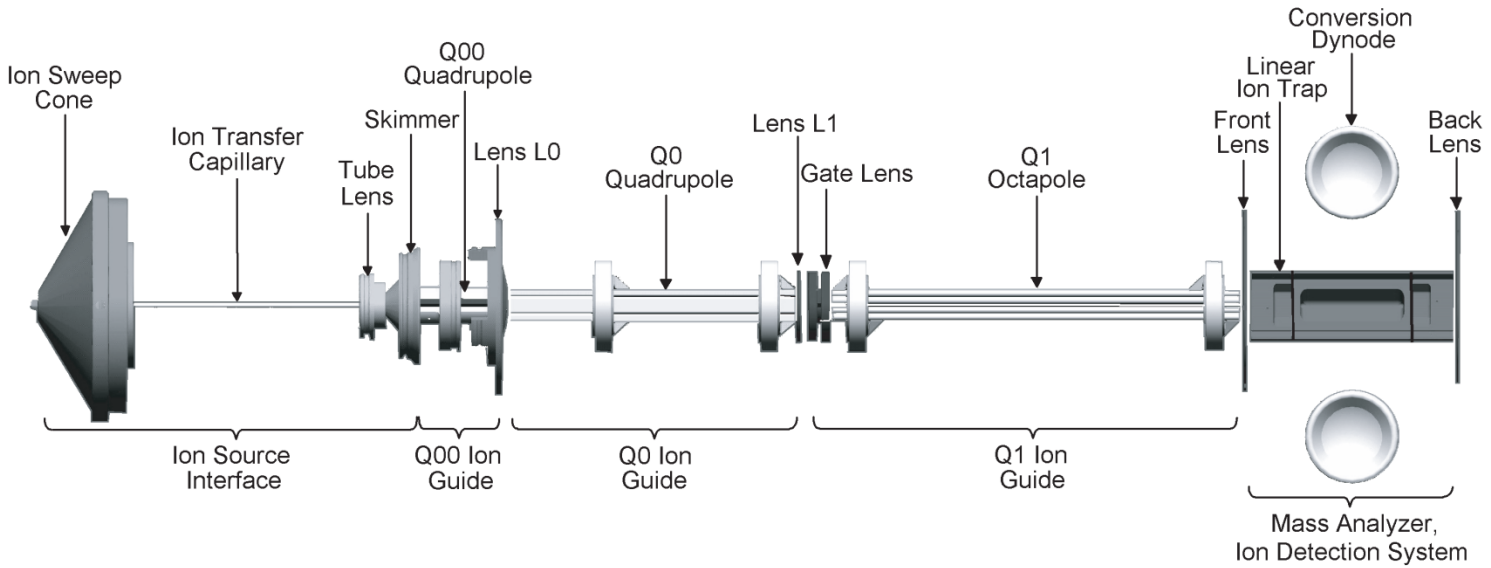
- 3D Ion Trap
- 2D Ion Trap



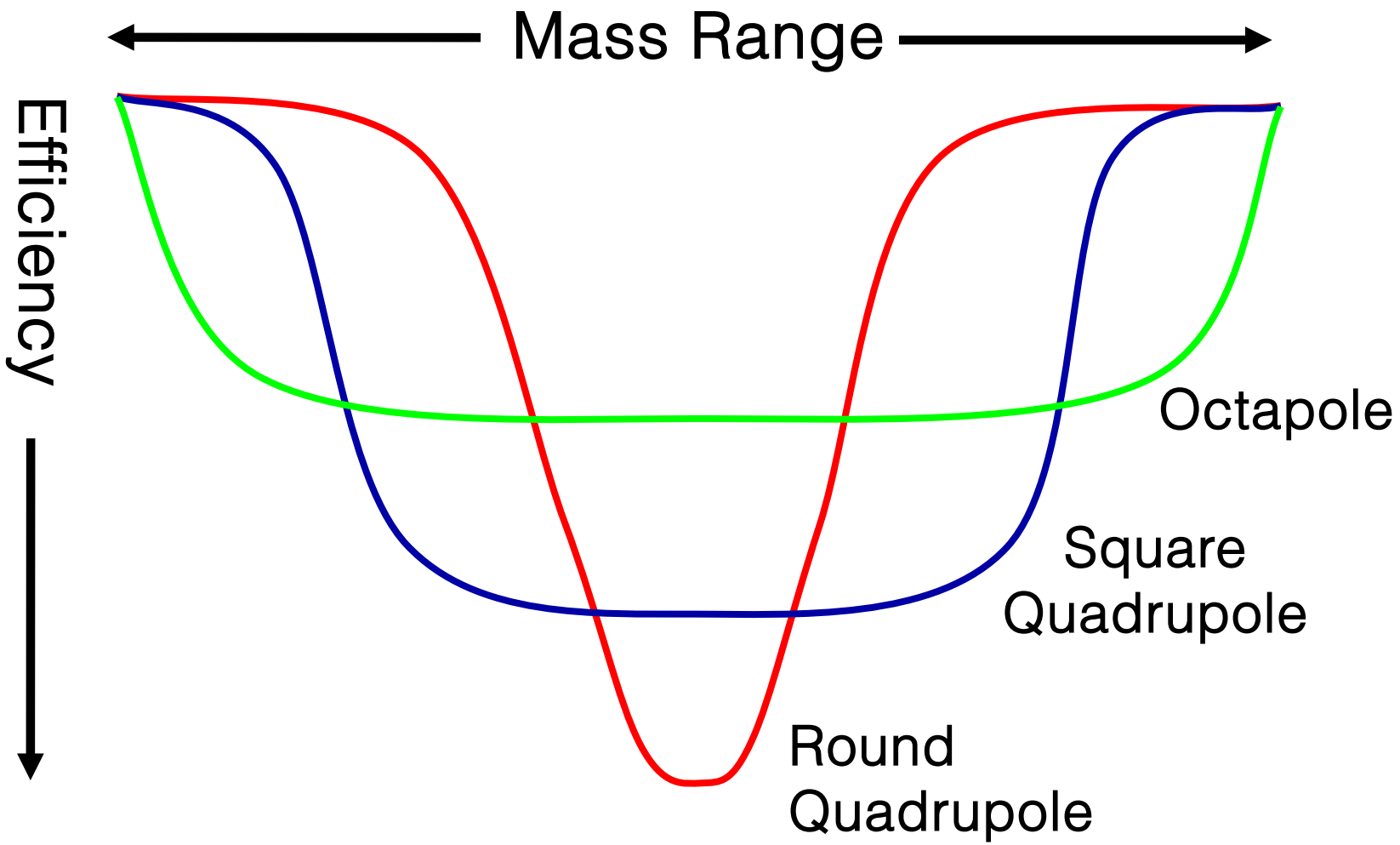
Linear Ion Trap (3D) Mass Spectrometer



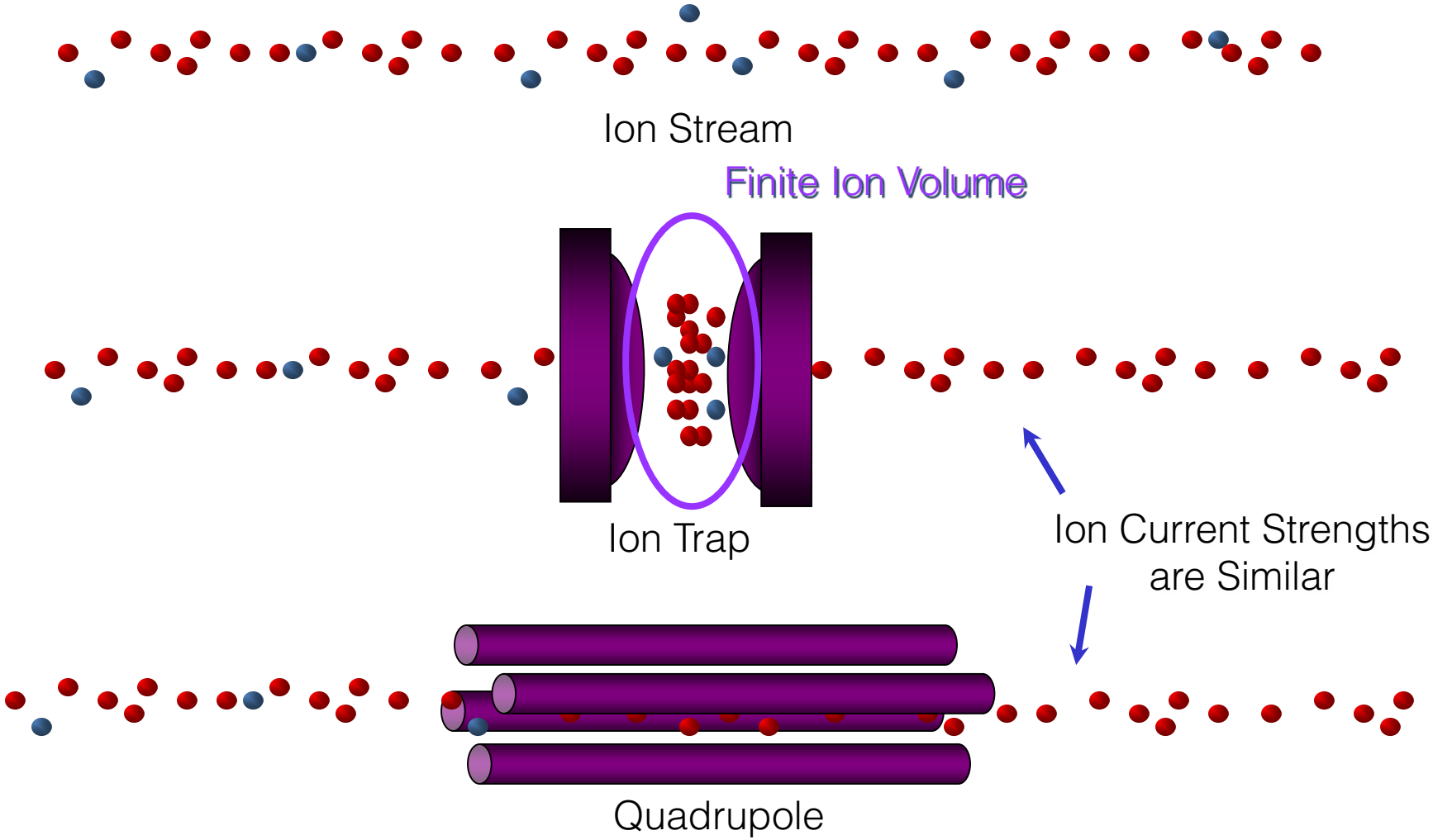
Linear Ion Trap Quadrupole (2D) Mass Spectrometer



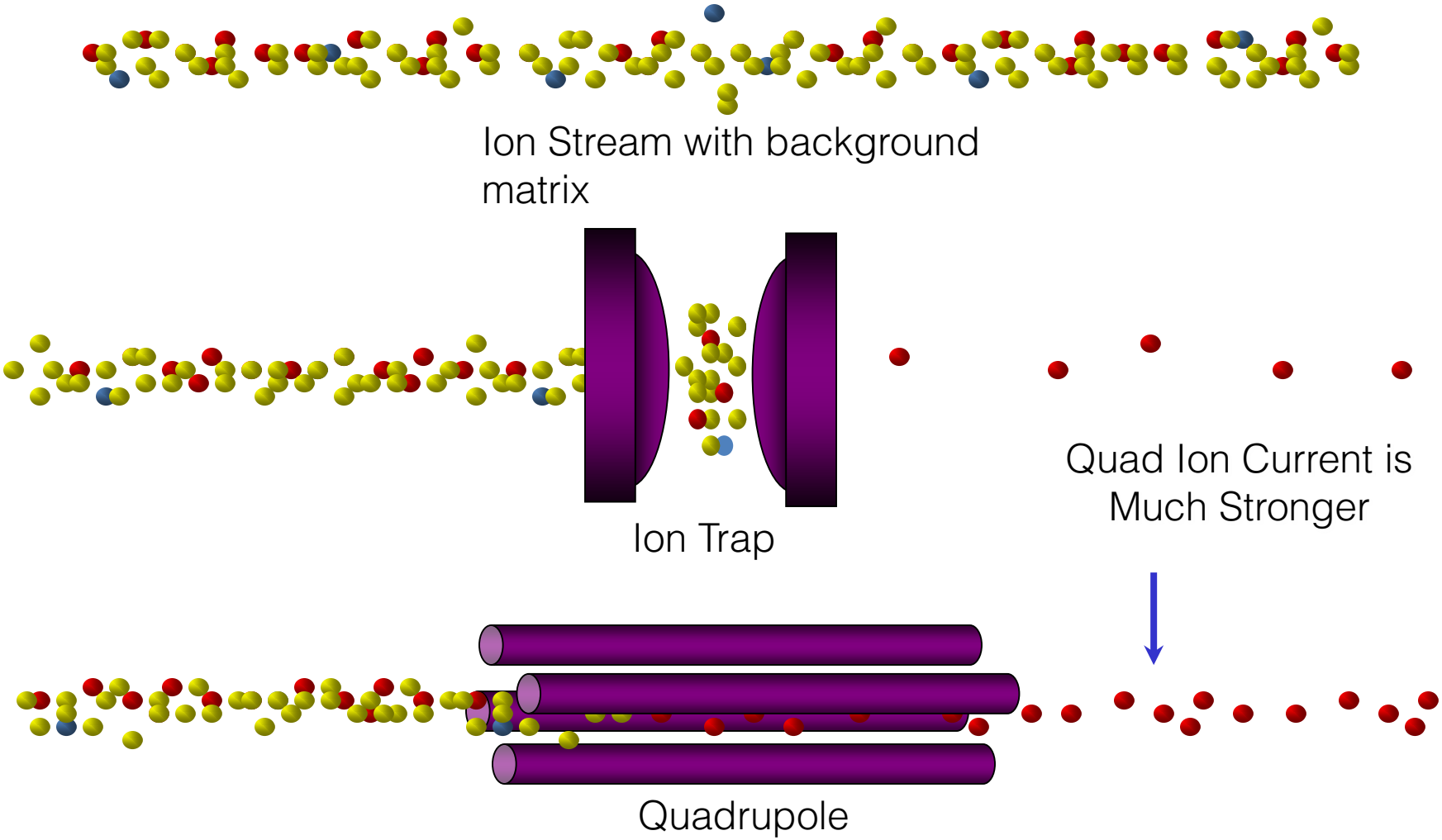
Why Use Square Rods ? – Pre-filter



Trap vs. Quad


Ion Stream
Finite Ion Volume
Ion Trap
Ion Current Strengths
are Similar
Quadrupole

Trap vs. Quad


Ion Stream with background matrix
Ion Trap
Quad Ion Current is Much Stronger
Quadrupole

Advantage of Ion Trap Vs QqQ

Ion Trap	QQQ
Suffered from Matrices	Suitable for high matrices
Target & Non-target	Best for Target, compromise from Non-target
MS^n	MS/MS
Higher LOQ, LLD	Lowest LOQ, LLD

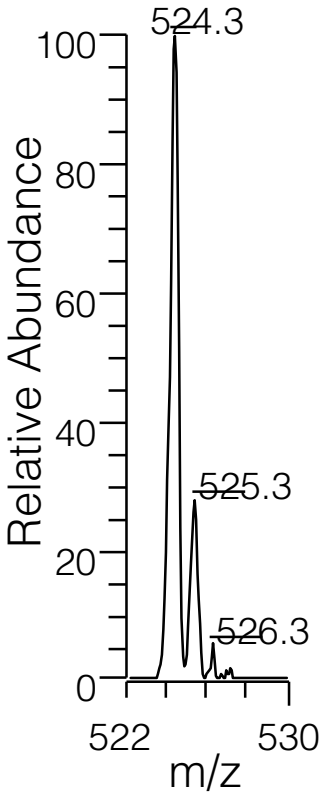
QTRAP, Hybrid MS is the compromised of Ion trap for MS^n & Single Quadrupole for ion transmitting or Trapping without Quadrupole filtered

Ion Trap Scan Functions

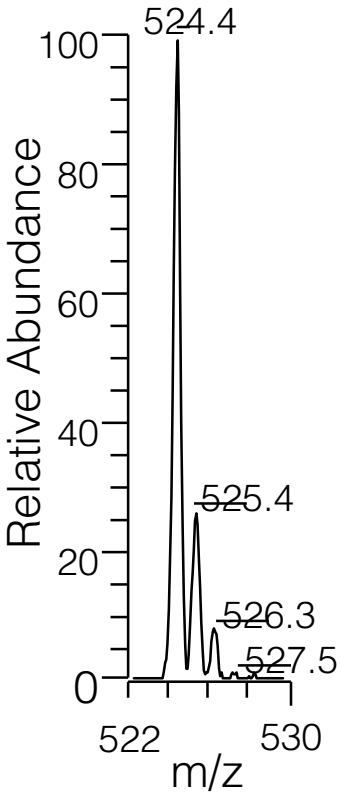
- Trapping - all scan modes
- Isolation - SIM and MS^n
- Excitation - MS^n
- Ejection - all scan modes

Space Charge Effects…

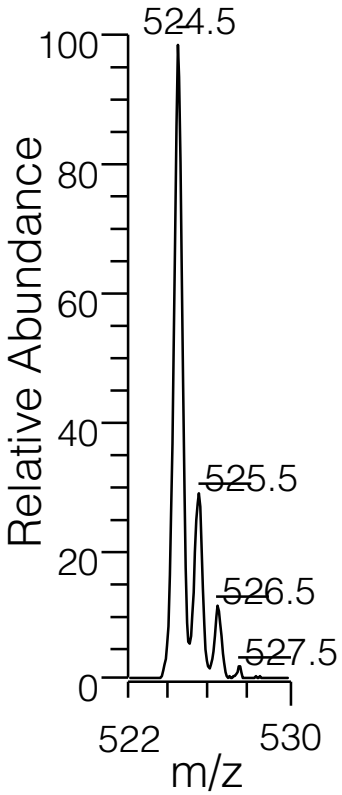
~ 300 Ions



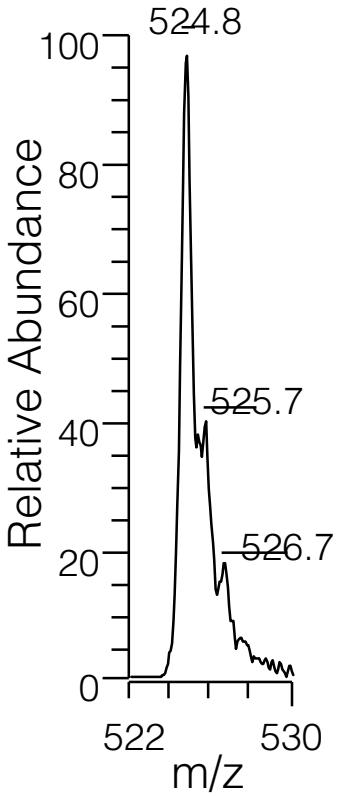
~ 1500 Ions



~ 3000 Ions



~ 6000 Ions

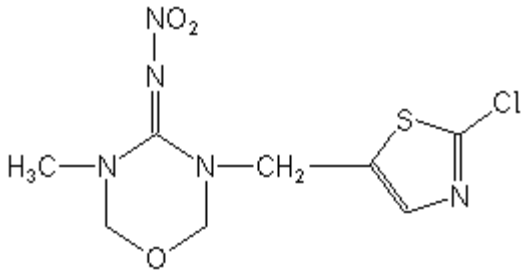


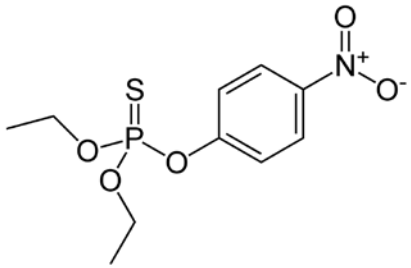
Good Resolution

Poor Resolution

Isobaric Pesticides

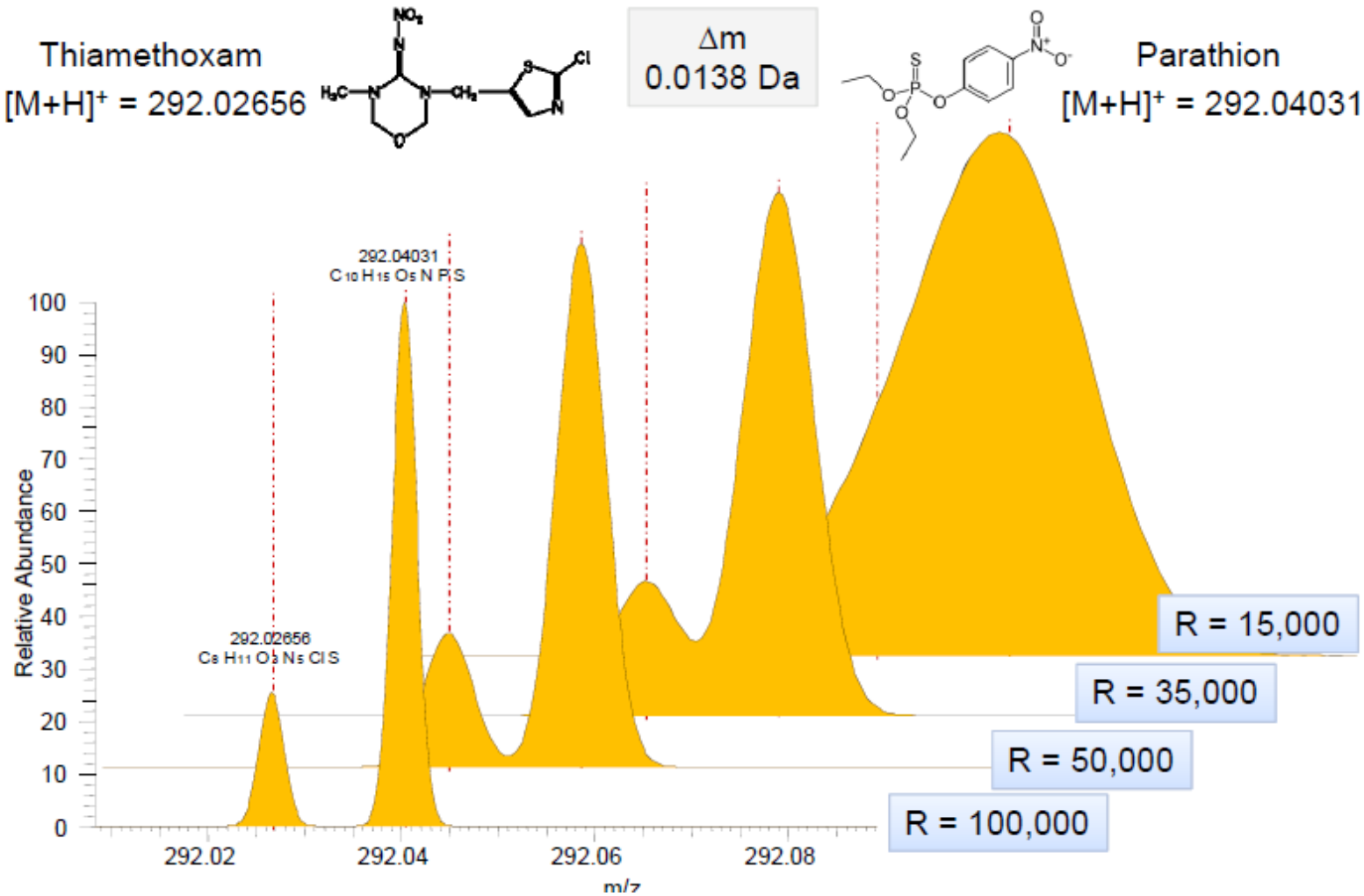
Thiamethoxam: $[M+H]^+ = C_8H_{11}ClN_5O_3S$ (292.02656)





Parathion: $[M+H]^+ = C_{10}H_{15}NO_5PS$ (292.04031)

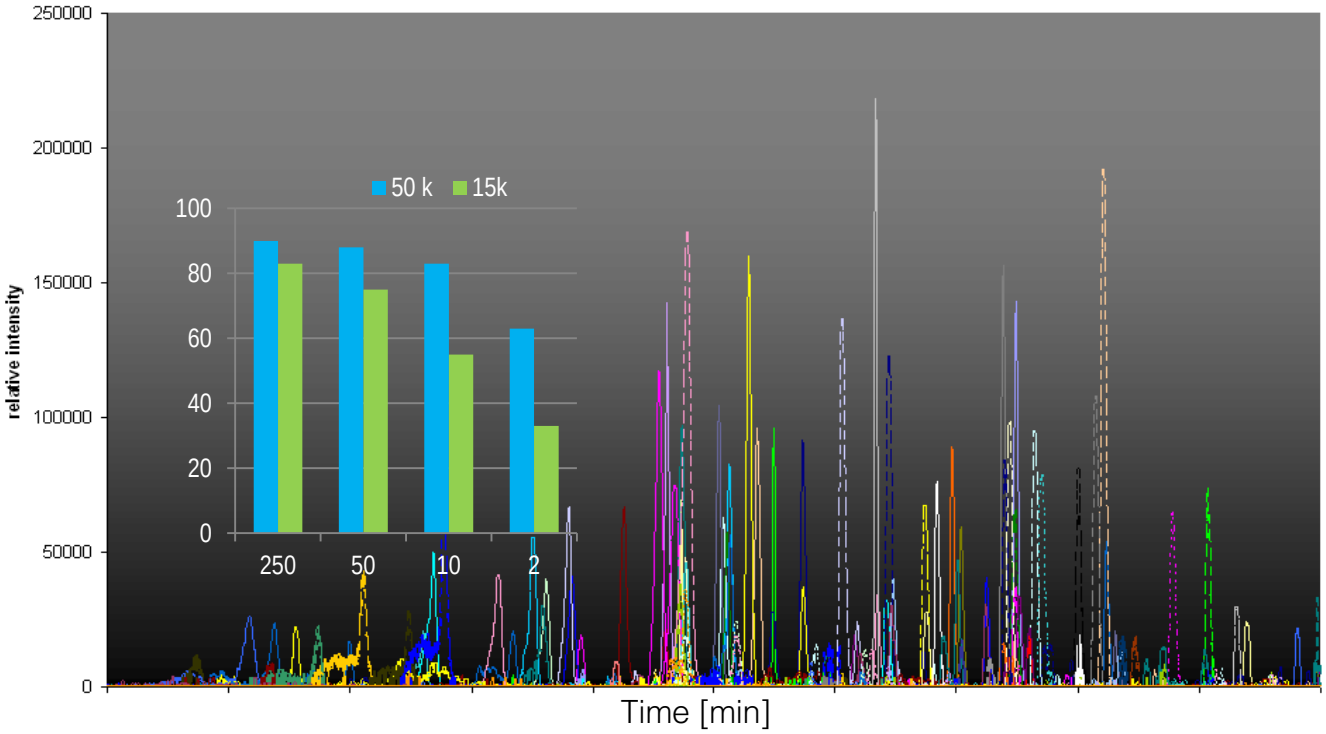
Isobaric Pesticides 3:1 Mix



Pesticide Analysis at different Resolution Settings

Overlaid extracted ion chromatograms from a mixture of 116 pesticides and mycotoxins at a 100ppb level. Extraction was done with 3 ppm mass window. The inset chart shows the number of detected compounds at different concentrations (in matrix) at two different resolution settings

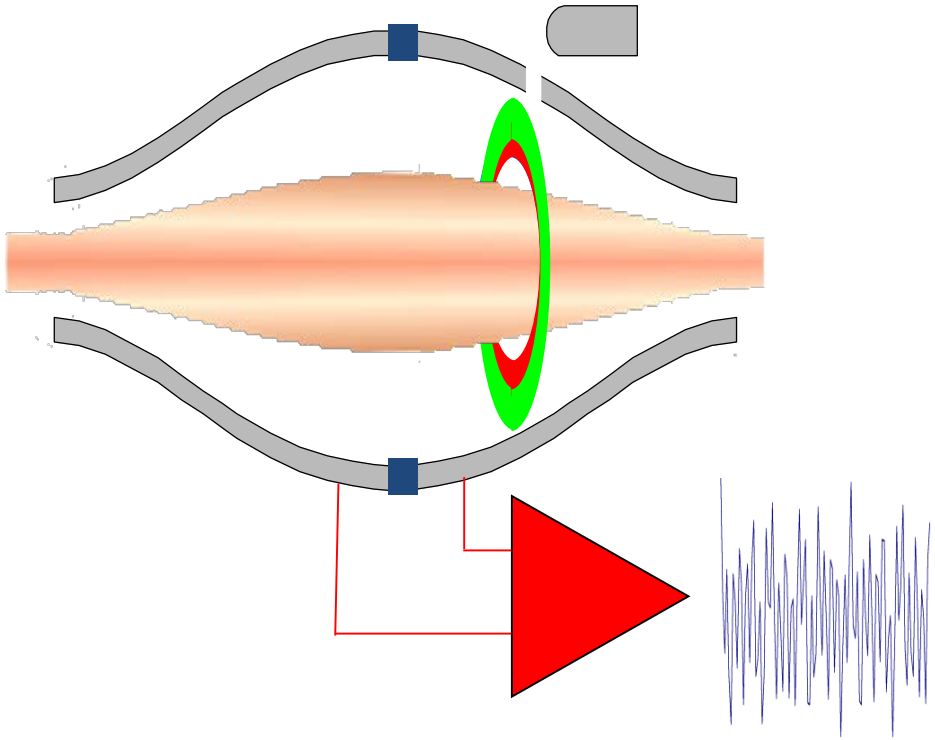
- More Resolution, more Identification !

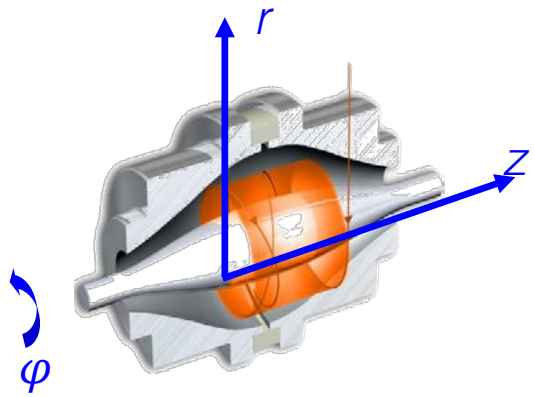


Typical Mass Accuracy

Type of MS	Mass accuracy	Utility for
Quadrupole	0.1 μ	Identify
Traps	0.1 μ	Identify
TOF	0.0001 μ	Empirical formula/ composition
Sector	0.0001 μ	Empirical formula/ composition
FT-MS	0.0001 μ	Empirical formula/ composition

Orbitrap MS – Principle of Operation





Hyper-logarithmic potential distribution:
"ideal Kingdon trap"

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

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

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

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

Technologies Shift - Key Performance of ICRMS

- Very Precise Mass (<1 ppm)
- High Resolution (up to 1,000,000 FWHM)
- Excellent Matrices Elimination
- Isotopic Analysis
- MS^n capabilities – *Alexandre Makarov invented C-Trap for linkage between collision cell and ICR*
- *Step Over TOF/Q-TOF/TOFTOF limitation;* Major drawback – TDC (time to digital converter)
- Although very fast, the TDC is a low cost, ion counting detector – its dynamic range is limited due to its inability to properly count the events, particularly when more than one ion simultaneously hits the detector and also b/c of the deadtime incurred after each count. Additionally, with higher sample concentration, two or more distinct isobaric peaks will not be detected when hitting the detector at the same time, resulting in improper peak height and inaccurate m/z reported

Mass Spectrometer Database

- www.mzcloud.org
- www.chemspider.com
- www.massbank.jp

- Different dataset in each type of instrument