Advanced LC-MS/MS Method Development, Method Troubleshooting and Instrument Operation Needed in Developing Successful Methods for Molecular identification and Quantitation

ASMS on-line course

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Instructor:

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What are your goals for taking this class

1. Improve sensitivity for an analysis?
2. Improve specificity (reduce matrix effects)?
3. Understand the ionization processes and operation conditions compatible with LC/MS?
4. Improve method development?
5. Operational tricks and tips?
6. MS interpretation?
7. Improve quantitative capabilities of a method?

$P_{success} \propto Skill * Knowledge_{sample} * Time$

Discussion time on last day of class

Opportunity for attendees to bring up any questions involving their problems with current LC/MS/MS analysis. This can include but limited to:

1. Method development
2. Trouble shooting method or instrument
3. Improving sensitivity and specificity
4. MS interpretation
5. Quantitative issues like matrix suppression
6. Any aspect of electrospray, APCI or APPI
7. Instrument evaluation- what is best for my needs?

For existing problems for analysis, Ideally have what has been performed already, compound structure and any data that can be shared for the discussion and what byou would like to solve.

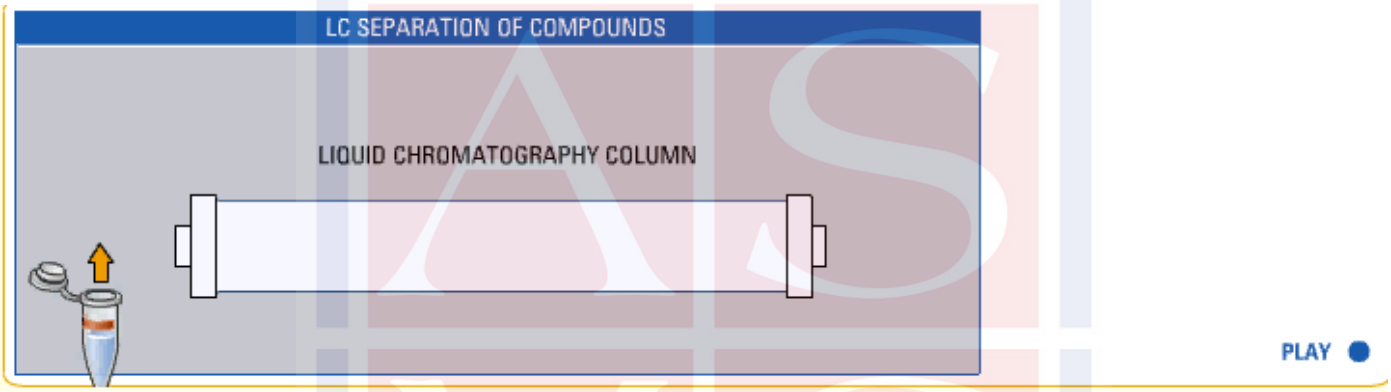
For future analysis that have not been tried yet, ideally have a compound structure and goals for the assay.

Introduction to HPLC Separations and Mass Analyzers

Theory of LC Separations

- **Mode of Operation:**

  Separation based on the partitioning or adsorption of a compound from the mobile phase to stationary phase



- **Advantages:**
  - Applicable to most organic compounds including thermally unstable and nonvolatile compounds
  - Wide range of parameters (stationary phase and mobile phase that can be varied to achieve separation)

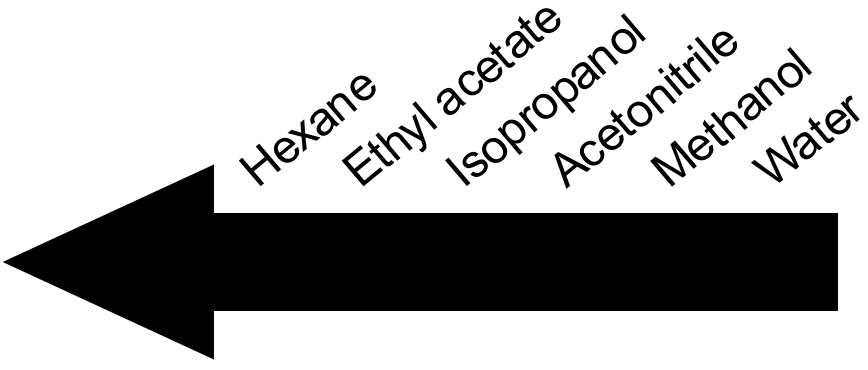
- **Disadvantages:**
  - Lack of universal or specific detectors
  - Separations can be time consuming

Modes of Liquid Chromatography

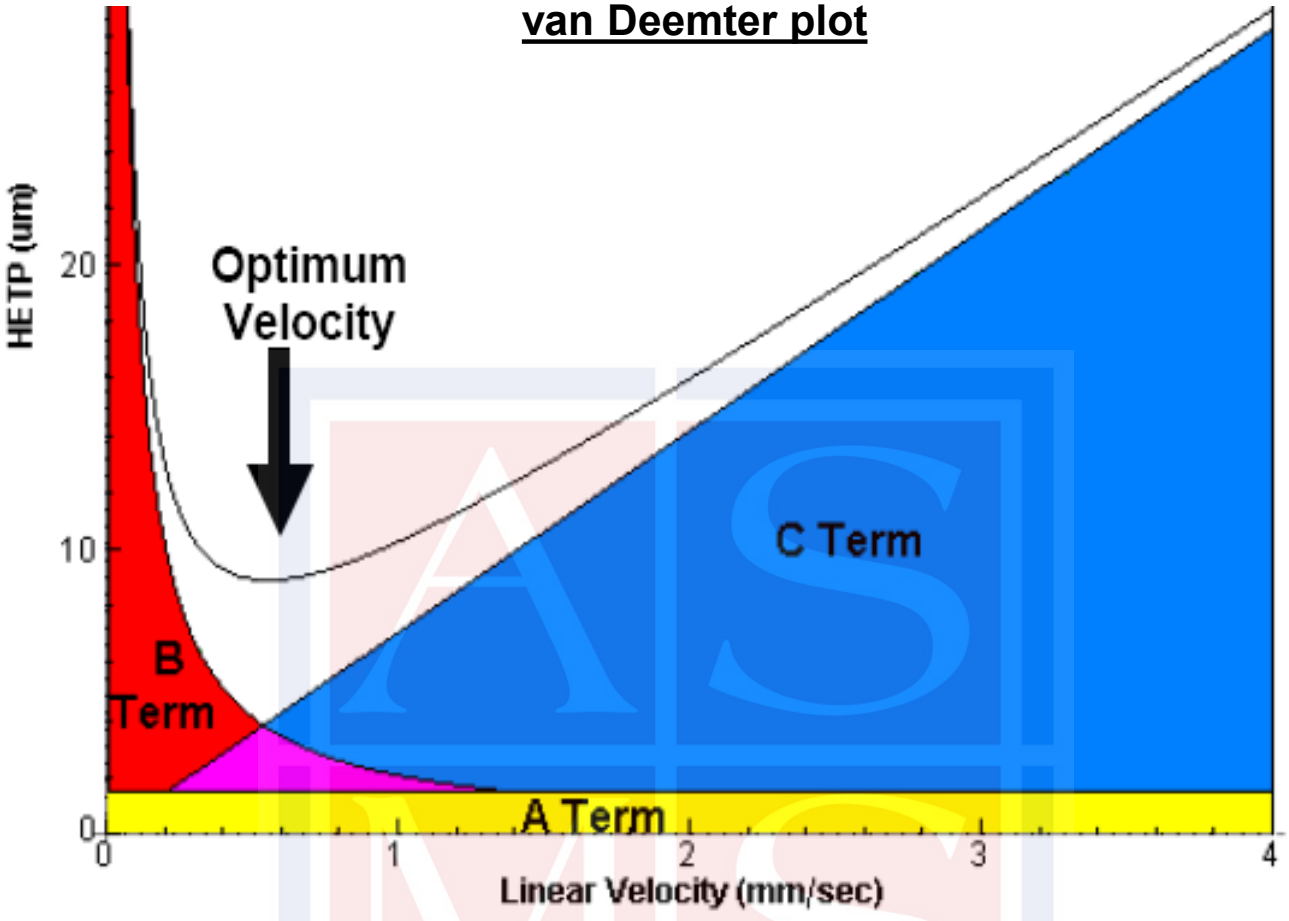
Types of Compounds Separated	Mode	Stationary Phase	Mobile Phase
Neutrals Weak Acids Weak Bases	Reversed-Phase	C-18, C-8, C-4, C-2	Water/Organic Sometimes Modifiers
Bases, Acids	Paired-Ion	C-18, C-8	Water/Organic Ion-Pair Reagent
Compounds Insoluble in Water, Organic Isomers	Normal-Phase	Silica, Amino, Cyan, Diol	Organics
Ionic Inorganic Ions	Ion Exchange	Anion or Cation Exchange Resin	Aqueous/Buffer Counter Ion
High MW Compounds Polymers	Size Exclusion	Polystyrene Silica	Gel Filtration - Aqueous Gel Permeation-Organic

Reversed -Phase

Eluting Solvent Strength, ε°



Chromatography Efficiency



$$HETP = A + \frac{B}{\mu} + C\mu$$

HETP (μm) = height equivalent of a theoretical plate

μ = average linear velocity (mm/s)

A = eddy diffusion ($\alpha\ d_p$)

B = longitudinal diffusion

C = resistance to mass transfer ($\alpha\ d_p^2$)

Why μ-Flow Rate Separations?

Electrospray-MS is a concentration detector, therefore the best detection limits are achieved using chromatographic separations that result in the highest peak concentrations.

Column (mm)	Flow Rate	Peak Volume (μL)	Normalized (1) Concentration
100 x 4.6	1000	360	1.0
100 x 2.1	250	75	4.8
100 x 1.0	50	17	21.2
100 x 0.32	4	1.7	206.6
CE/CEC	0.03	0.002	180,000 (Ideally)
(50 x (50 x 0.05)			

(1) Assumptions: Same volume and amount injected (1 μL x 10 ng/μL). If not sample limited peak concentration can be increased by injecting a larger quantity, based upon column capacity.

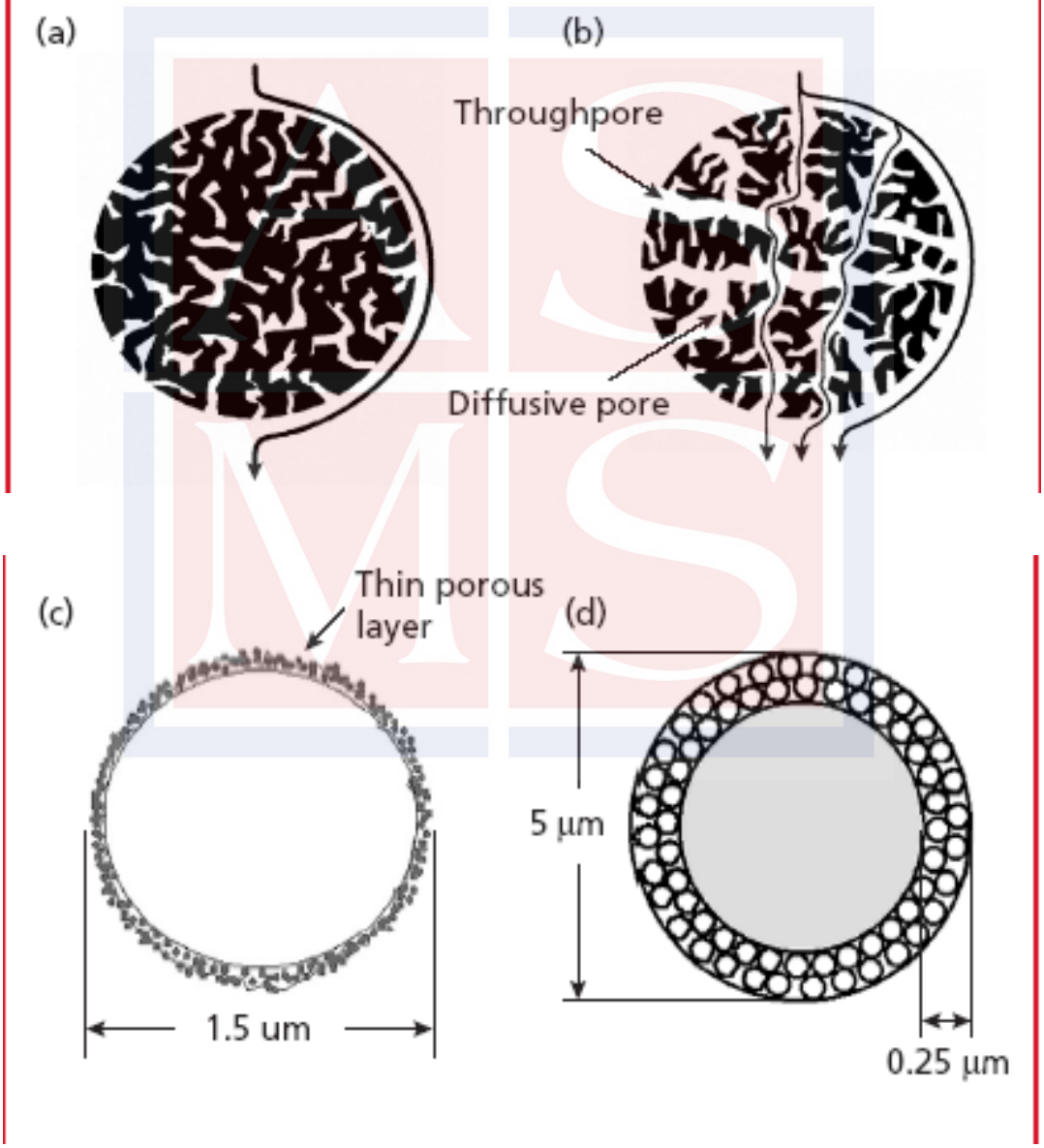
Student Notes: Going to smaller diameter columns increases the peak concentration and therefore improves API-MS detection limits.

HPLC Separation Approaches

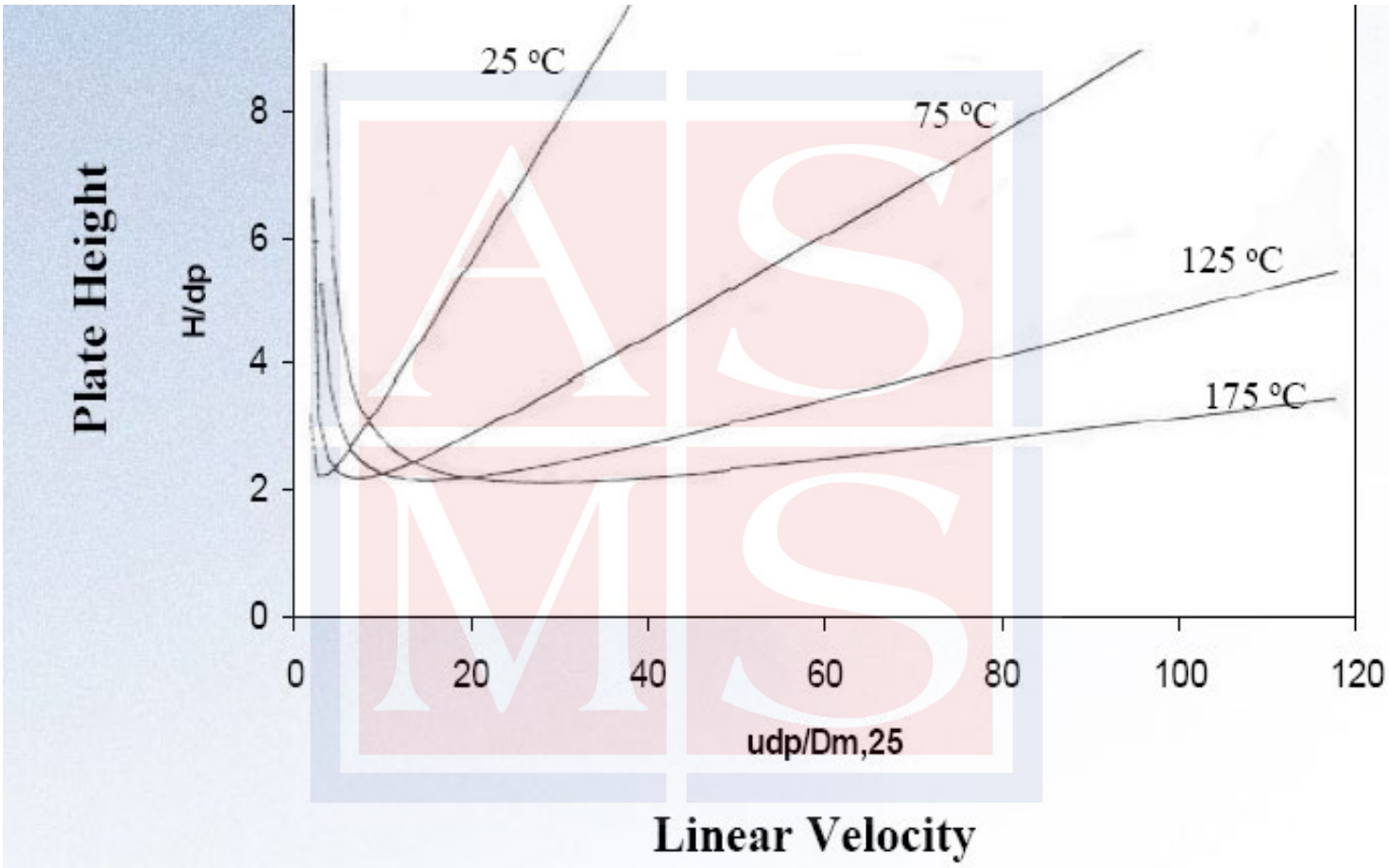
- **Stationary Phases**
  - **Particles:** ***porous vs. non porous***
    - ***size of particle***
    - ***polymeric vs. particles***
  - **Surfaces:** ***CE***
- **Column dimensions**
- **Temperature**
- **Liquid Phase mobility**
  - ***Pressure***
  - ***Electroosomotic***
  - ***Other fields***

Typical Particles used in HPLC

Figure 1: Flow characteristics and design of packing particles in HPLC. (a) totally porous particle; (b) perfusion packing; (c) non-porous silica (NPS) or non-porous resin (NPR); (d) Poroshell particle.



Advantages of High Temperature Separations



F. D. Antia and Cs. Horvath, J. Chromatogr. 1988, 435, 1-15

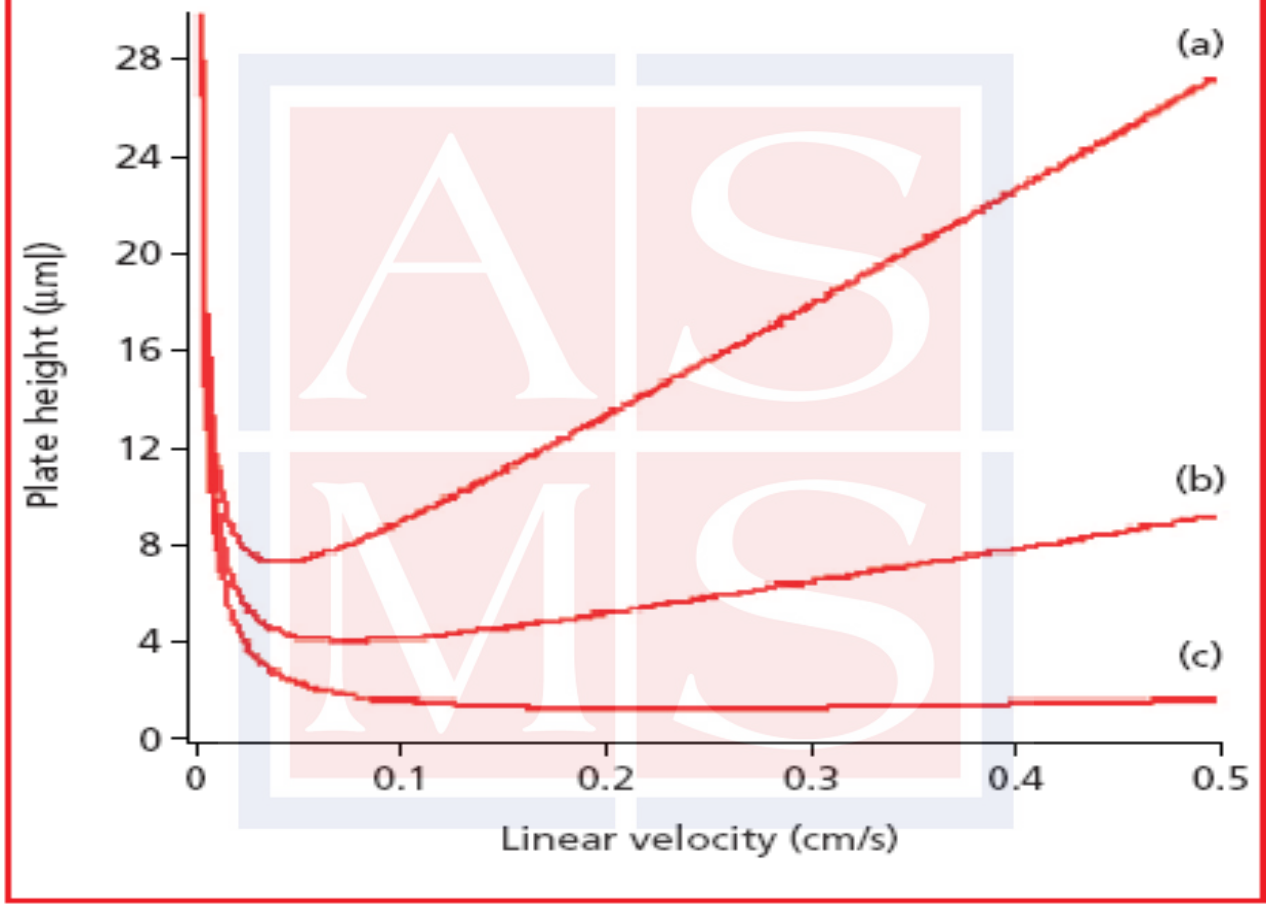
The Increase in Operating Pressure and Separation Performance with Decreasing Particle Size

Table 1: Pressure requirements and performance expected for differing stationary-phase particle diameters in a 25 cm long column. Values calculated for an analyte with $k' = 2$, $D_m = 6.0 \times 10^{-6}$ cm²/s and a mobile-phase viscosity (η) = 1.0 cP.

d_p (µm)	ΔP (psi)	Theoretical plates	Ret. time (min)
5.0	210	25 000	35
3.0	1000	42 000	21
1.5	8000	83 000	10.5
1.0	26 000	125 000	7
0.75	62 000	166 000	5

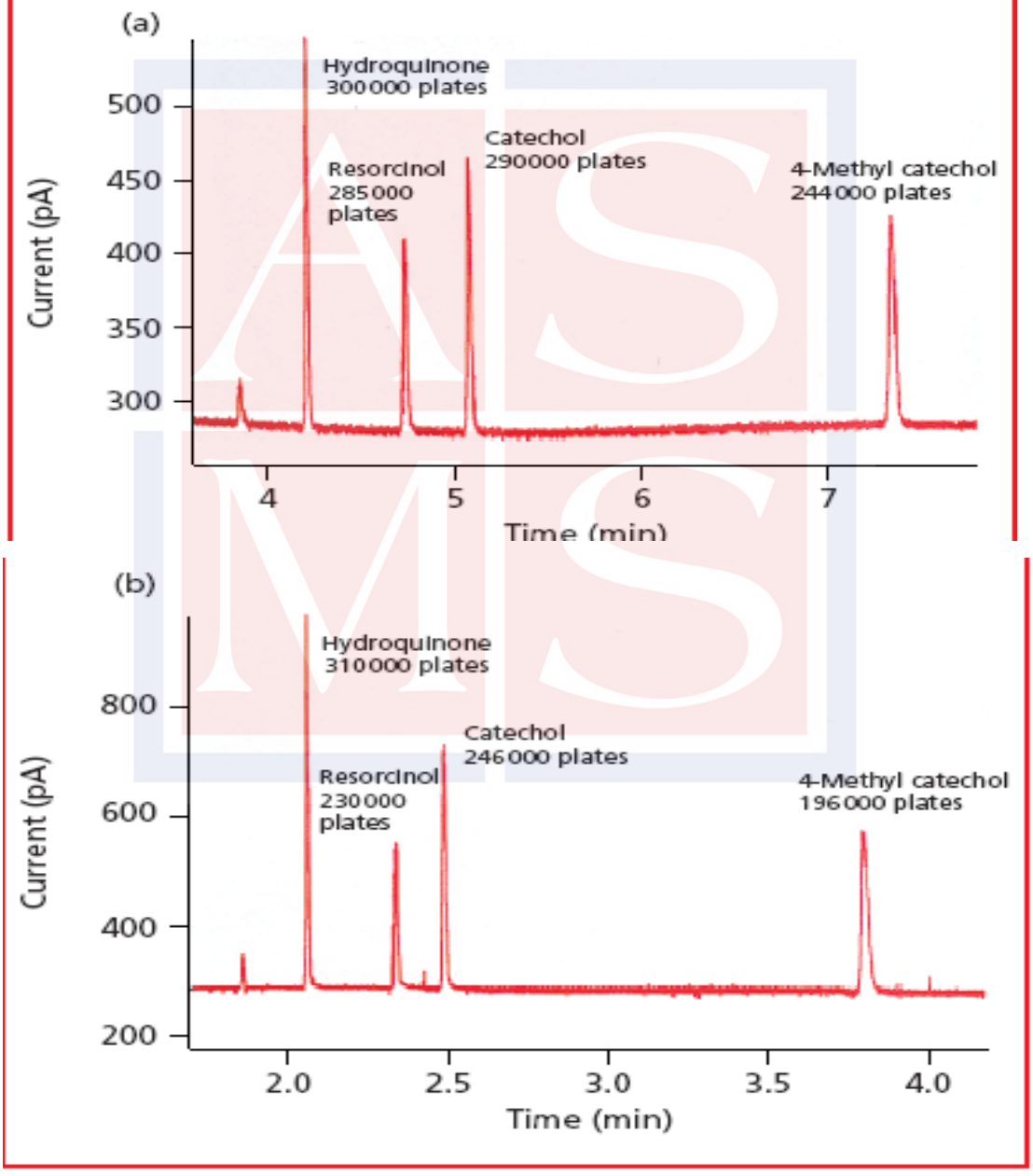
UHP-LC –Why go to smaller Particles?

Figure 1: Theoretical performance of columns packed with (a) 5 µm, (b) 3 µm and (c) 1 µm particles.



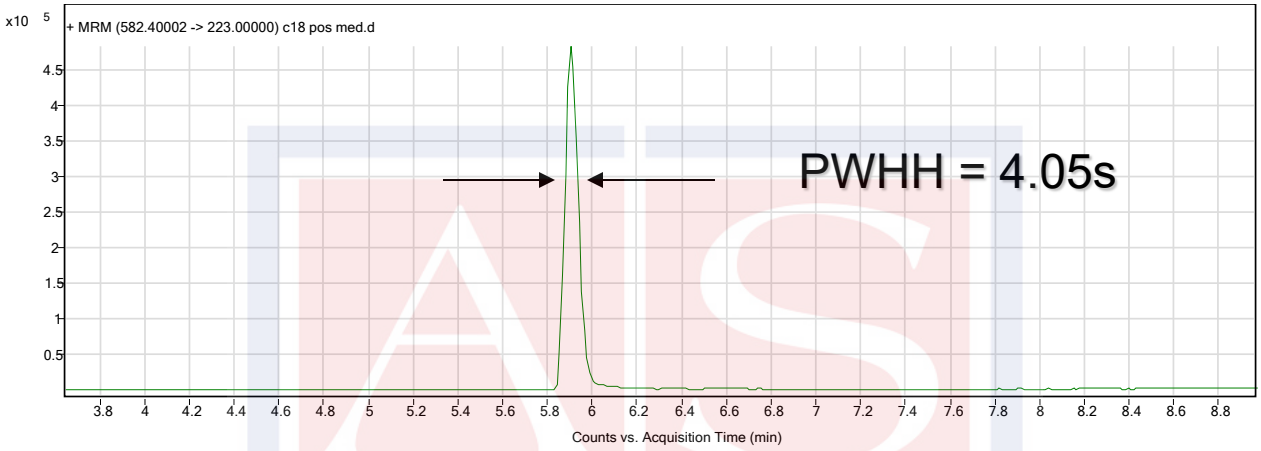
UHP-LC Chromatogram 44K and 103K psi using 1 um particles?

Figure 2: Chromatograms obtained on a column packed with 1.0 µm particles at run pressures of (a) 45 000 psi and (b) 103 000 psi. See text for separation conditions.

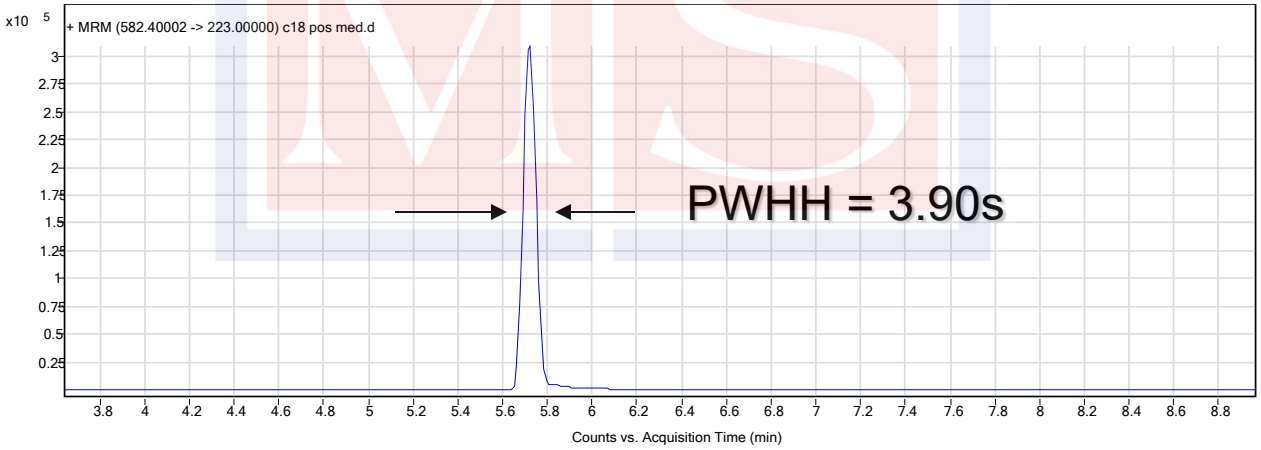


Separations using small particles vs. coated particles under the same LC conditions

- Separation of an ergot alkaloid using a 2.1 mm x 50 mm C18 column with 1.8 um particle



Using a 2.1 mm x 50 mm C18 column with 2.7 um particle coated with a 0.5 um diffuse layer



Advantages of coated particles:
Similar if not better peak resolution
50% lower pack pressure

Disadvantages of the coated particles:
Lower loading capacity
poorer resolution at higher flowrates

What is a HILIC Chromatography?

HILIC = Hydrophilic Interaction Chromatography

- HILIC is a normal phase like method using water miscible solvents
- Stationary phases are polar
- Mobile phases are mostly organic with small % of water- Higher organic results in longer retentions

How Does It Work

- Polar analytes partition into an absorbed water layer on the stationary phase
- Charged polar compounds can undergo cation exchange with the silanol groups of the stationary phase
- Retention is based upon the combination of the above interactions and lack of retention is based on the absence of either interaction

HILIC

Advantages

• Retain Higher polar compounds that would not be retained by reverse phase chromatography

•Complements reverse phase chromatography in selectivity

•Enhances ESI MS sensitivity since higher % organic composition usually results in better sensitivity

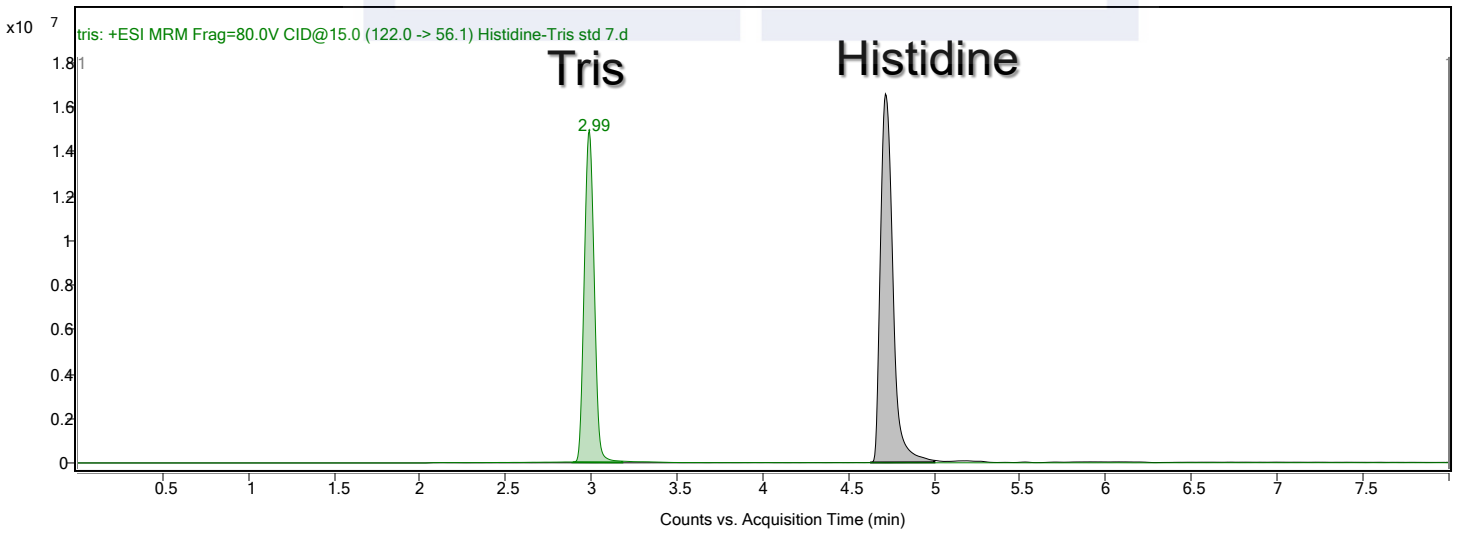
•Allows for sample preparation from liquid phase or SPE to be in organic solvents vs. more aqueous solvents for reverse phase to insure good peak shape and resolution

Disadvantages

• Not as versatile as reverse phase C18, will not work for non-polar compounds.

Example LC/MS/MS separation

100% ACN to 50% ACN in water (0.025% TFA) in 10 min



What is Mass Spectrometry?

- Technique to measure the mass to charge ratio of individual molecules that have been converted to ions

- Components:
  - 1) Ionization Source
  - 2) Mass Analyzer
  - 3) Detector

- Mass Spectrum is a plot of mass to charge (x-axis) vs. relative (to the most intense peak which is called the base peak) or absolute intensity (y-axis)

Ionization Techniques

Gas Phase

- Electron Ionization
- Chemical Ionization

Desorption (ideal for nonvolatiles)

- Secondary Ion MS (SIMS)
- Fast Atom Bombardment (FAB)
- Plasma
- Field
- Matrix Assisted Laser Desorption Ionization (MALDI)
- **Electrospray**

Mass Analyzers

MS in space

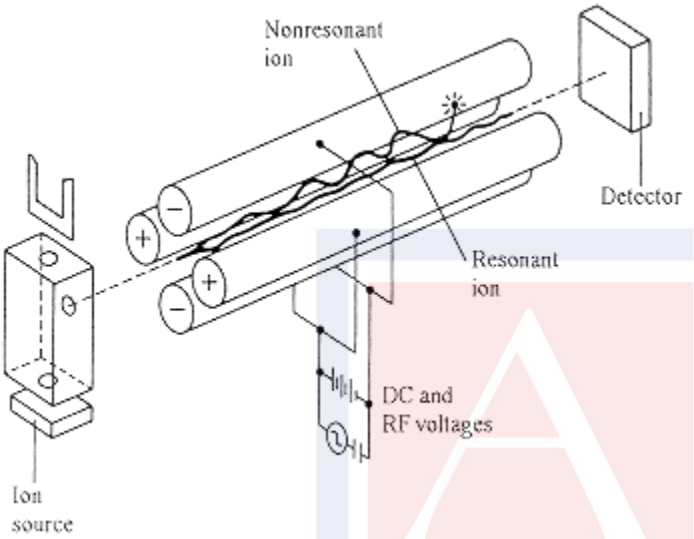
- Electrostatic/Magnetic Sector
- Quadrupole / triple quadrupole
- Time of Flight (TOF)

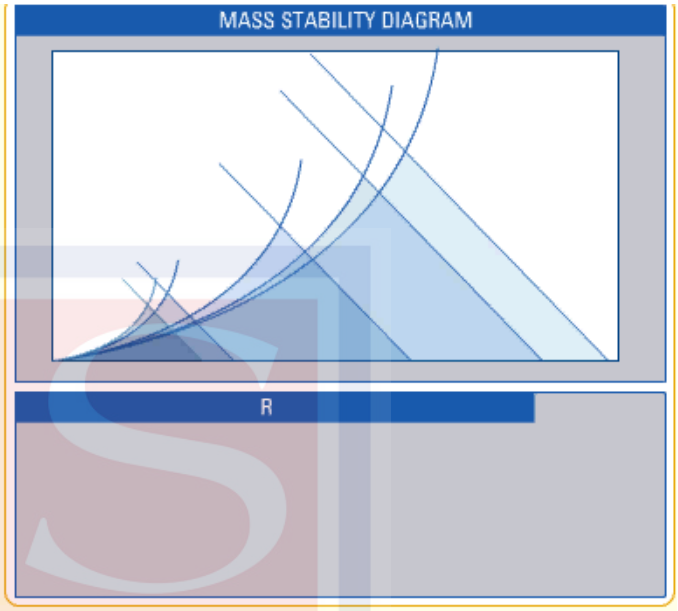
MS in time

- Quadrupole Ion Trap
- Fourier Transform Mass Spectrometry (FTMS)
- Orbitrap

Quadrupole-Triple Quadrupole

A. Quadrupole:

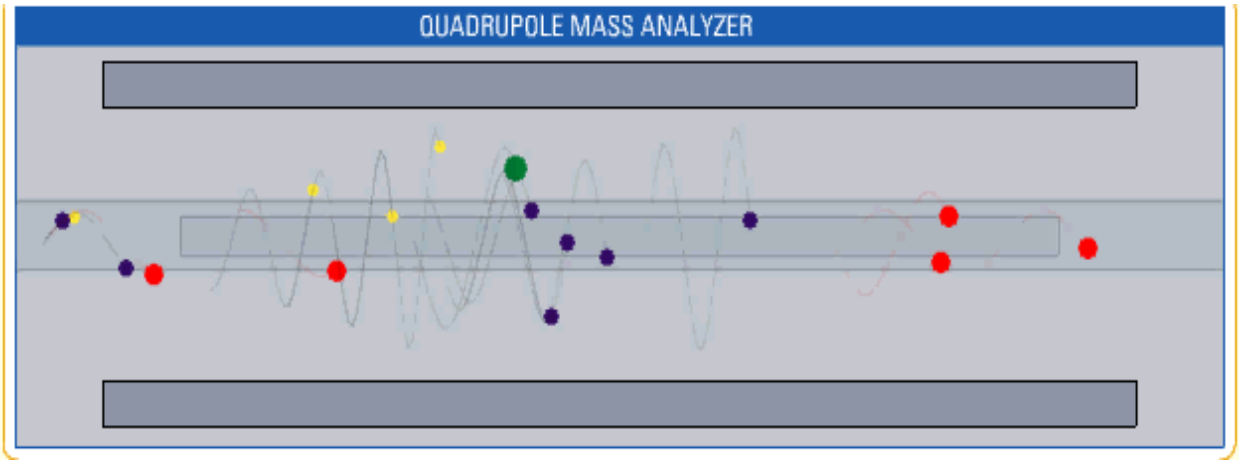




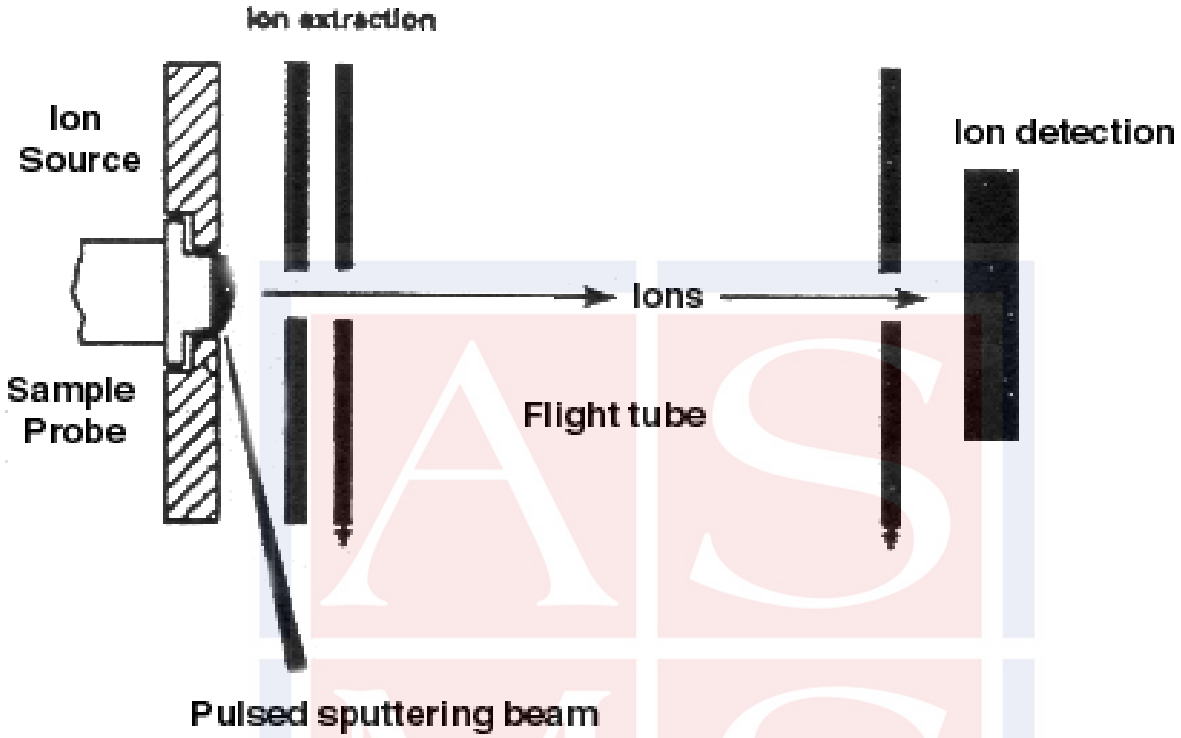
Instrument scanned by scanning the RF and DC while maintaining a constant RF/DC ratio.

$a_y = 4z\ eU/mw^2r_0^2$ (DC) U = DC field

$a_x = 2z\ eV/mw^2r_o^2$ (RF) V = Amplitude of RF field



Time of Flight (TOF) MS



In this time-of-flight mass spectrometer, ions formed from the sample by the pulsed beam arrive at the detector in the order of their m/z value

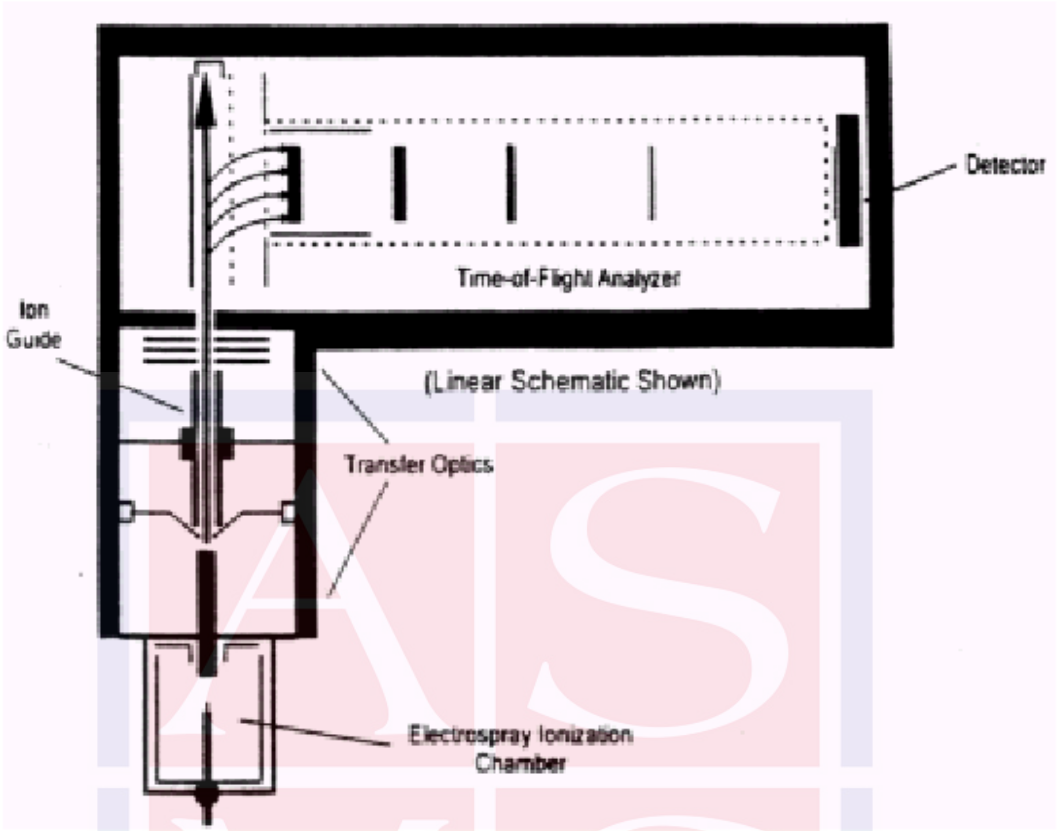
$m/z = (2\ t^2\ KE) / d^2$

t= flight time
d= flight distance
KE = kinetic energy = $0.5\ mv^2$
m= mass
z= charge
v= velocity of the ion

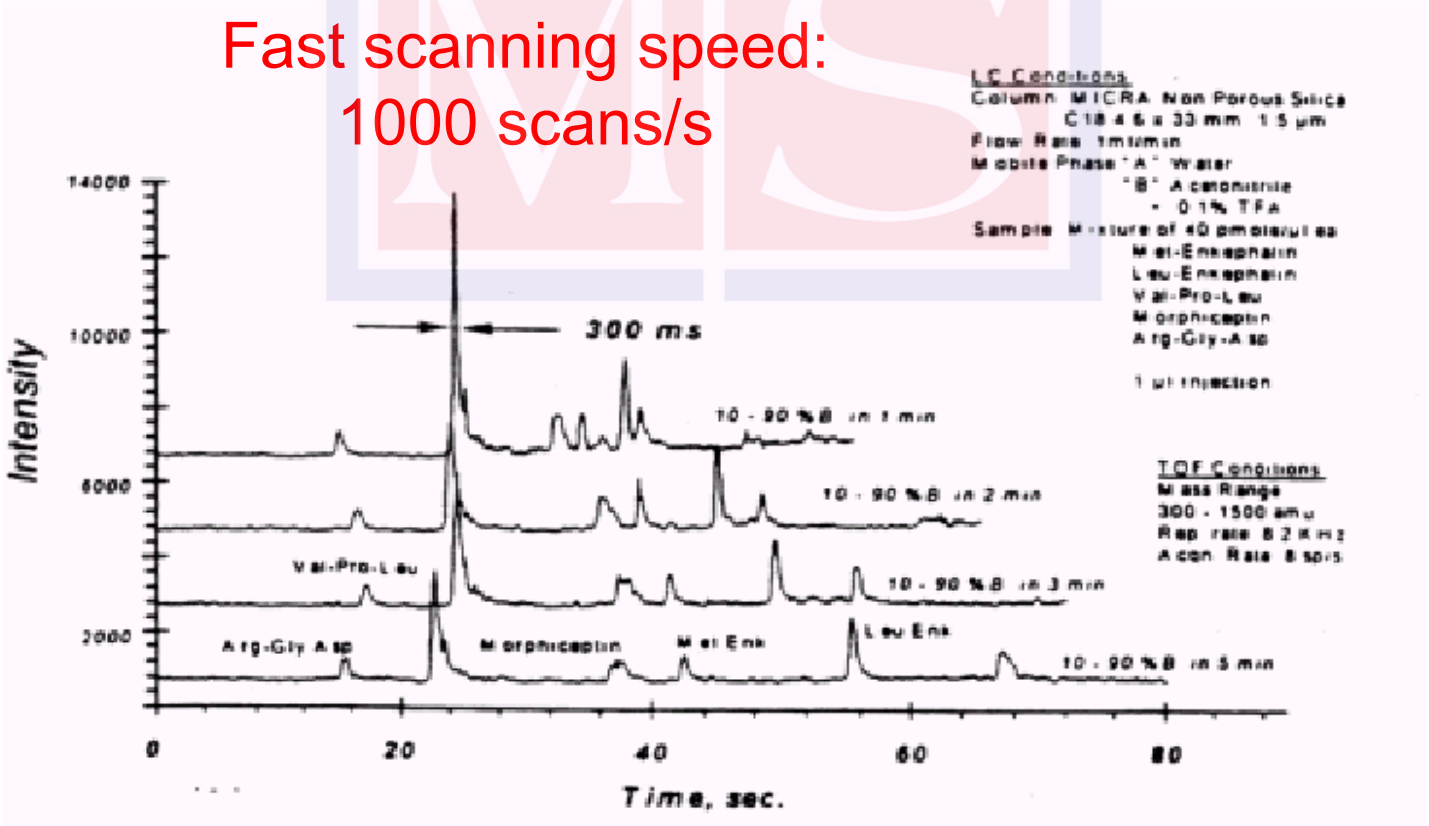
Why TOF-MS?

- **Sensitivity**
  - 50-80% Duty Cycle Using Orthogonal Storage Devices
- **Scan Speed**
  - 50 µs/scan - can easily define 1s wide peaks observed in CE and µHPLC (assume 20 scan per peak therefore need 50 ms cycle times)
- **Specificity**
  - MS/MS using post acceletrating decay
  - Q-TOF
- **Mass Range**
  - No upper mass limit
- **High Mass Resolution (>5000)**
  - Enables accurate mass measurements to determine molecular formulas and charge states
- **High Value/Cost Ratio**

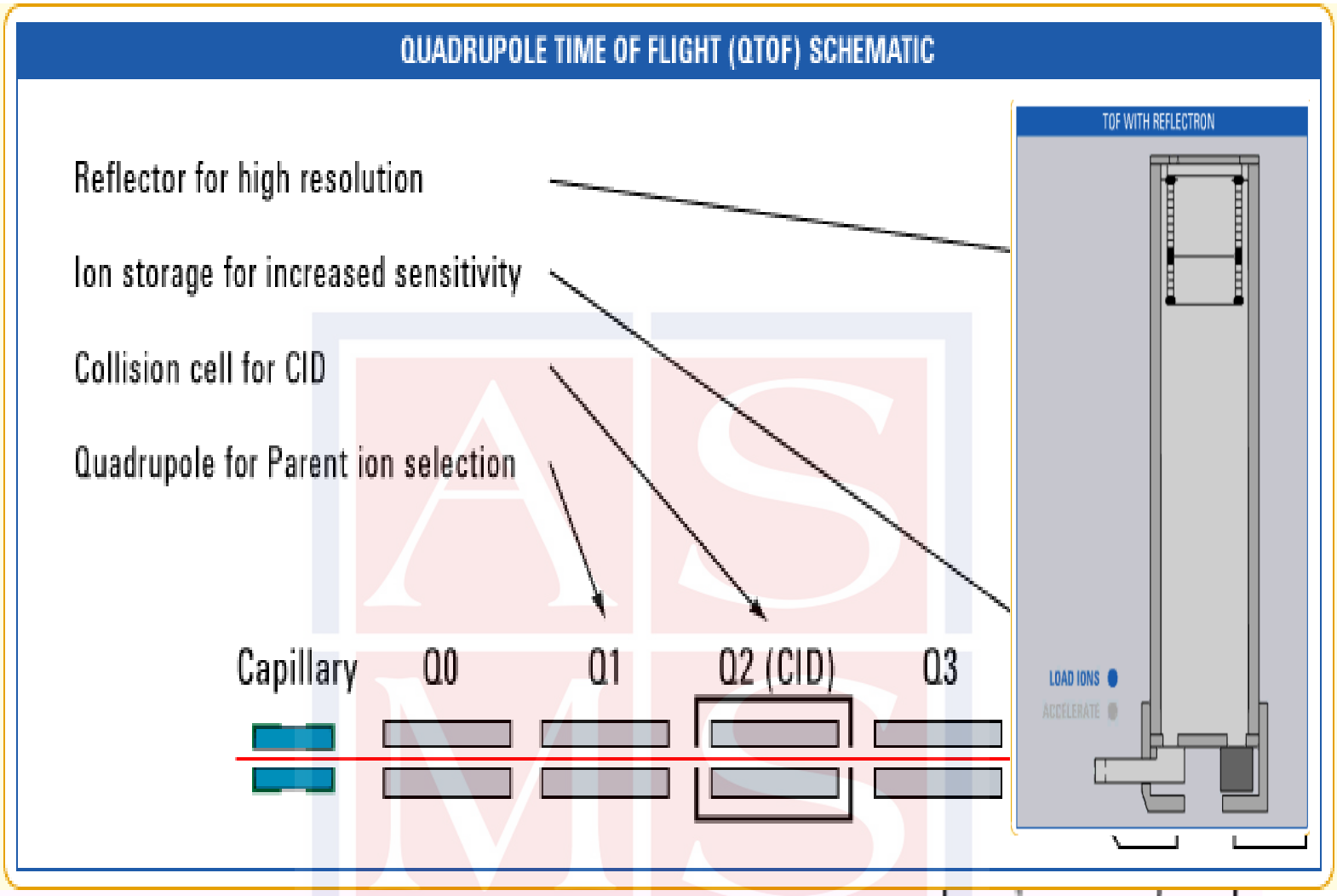
Fast LC Separations of Peptides

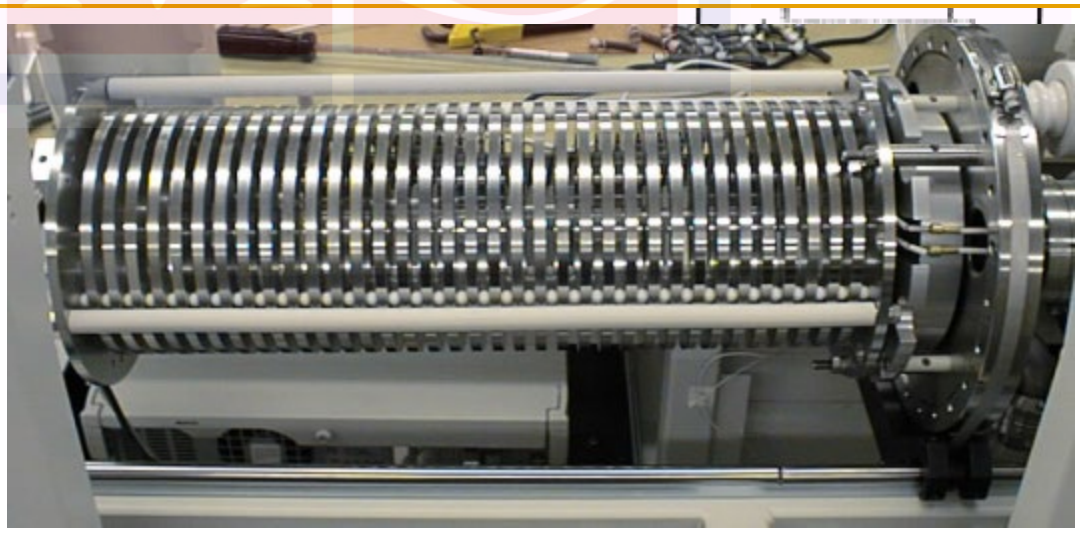


Fast scanning speed:
1000 scans/s

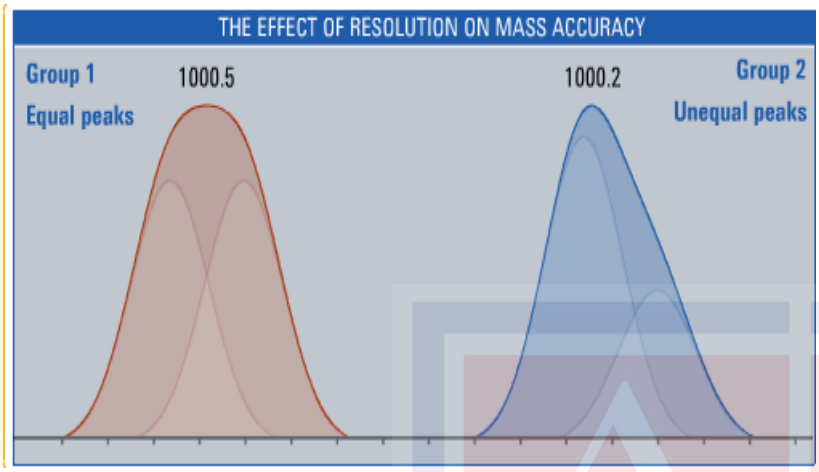


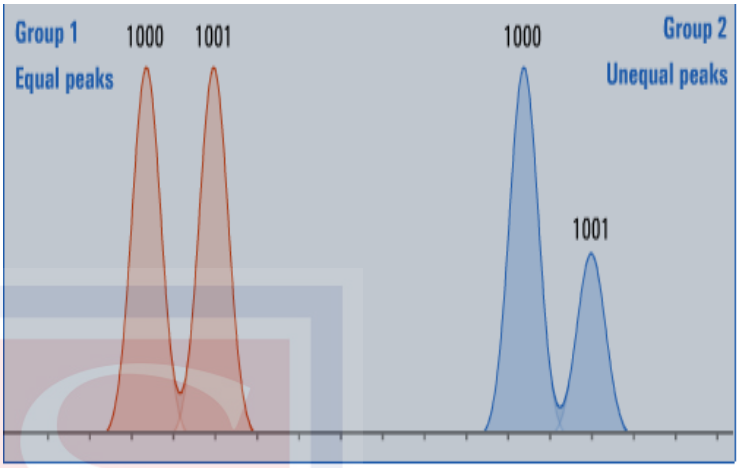
Q-TOF MS Mode

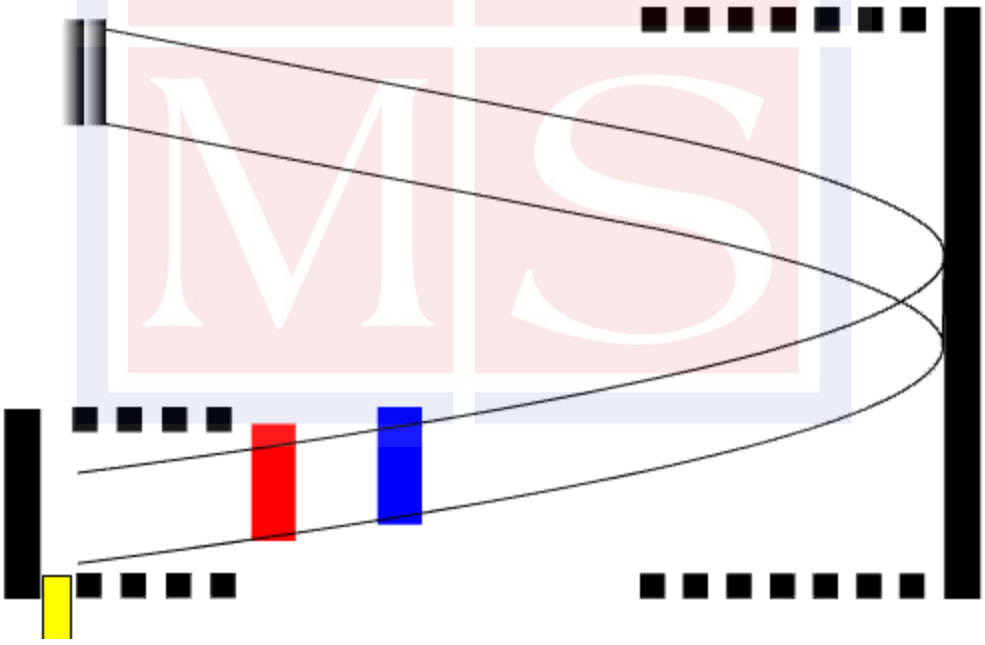

QUADRUPOLE TIME OF FLIGHT (QTOF) SCHEMATIC
Reflector for high resolution
Ion storage for increased sensitivity
Collision cell for CID
Quadrupole for Parent ion selection
Capillary
Q0
Q1
Q2 (CID)
Q3
TOF WITH REFLECTRON
LOAD IONS
ACCELERATE



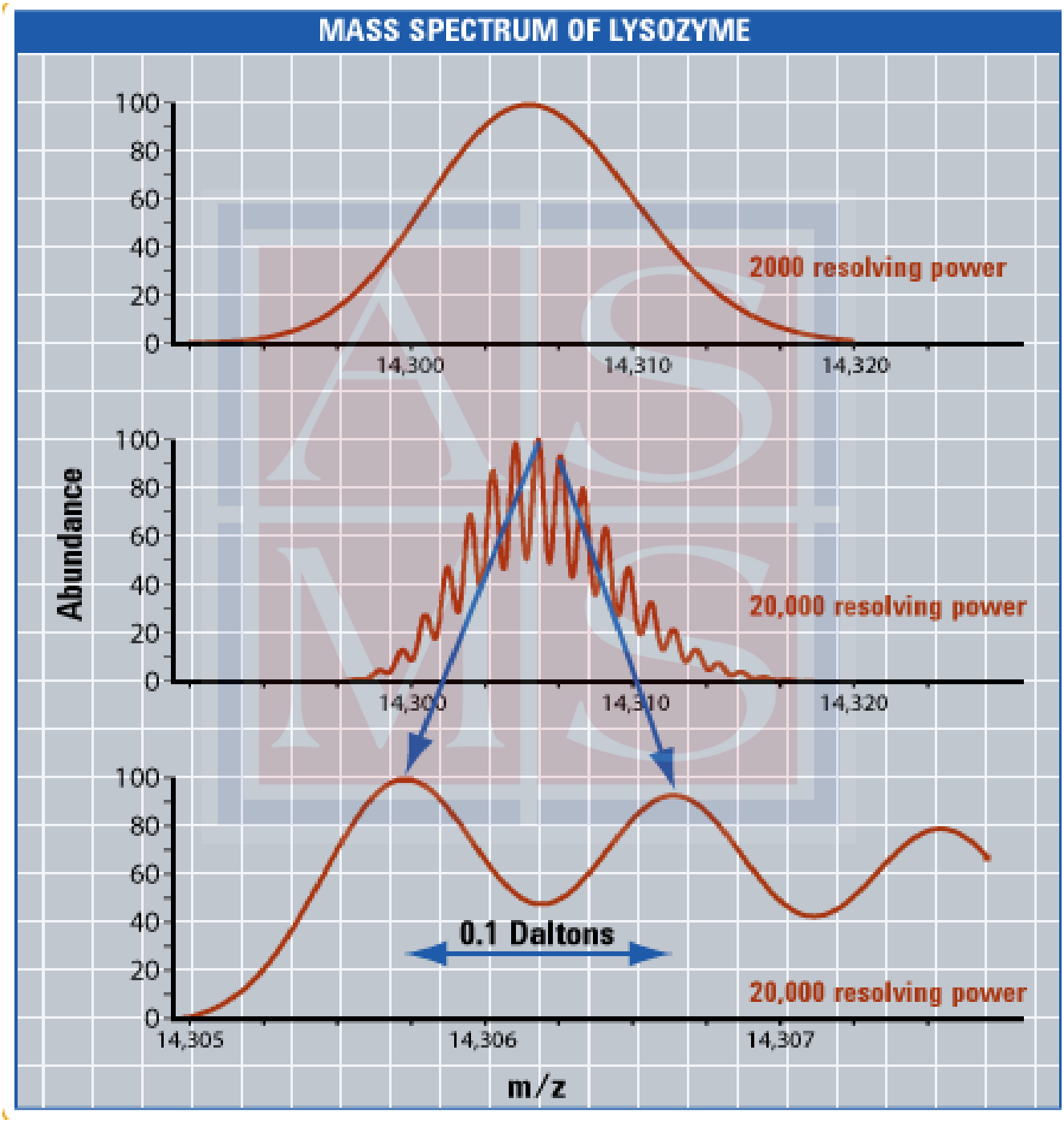
What is mass resolution ?







High Resolution MS techniques to resolve Biopolymers- FTMS and TOF

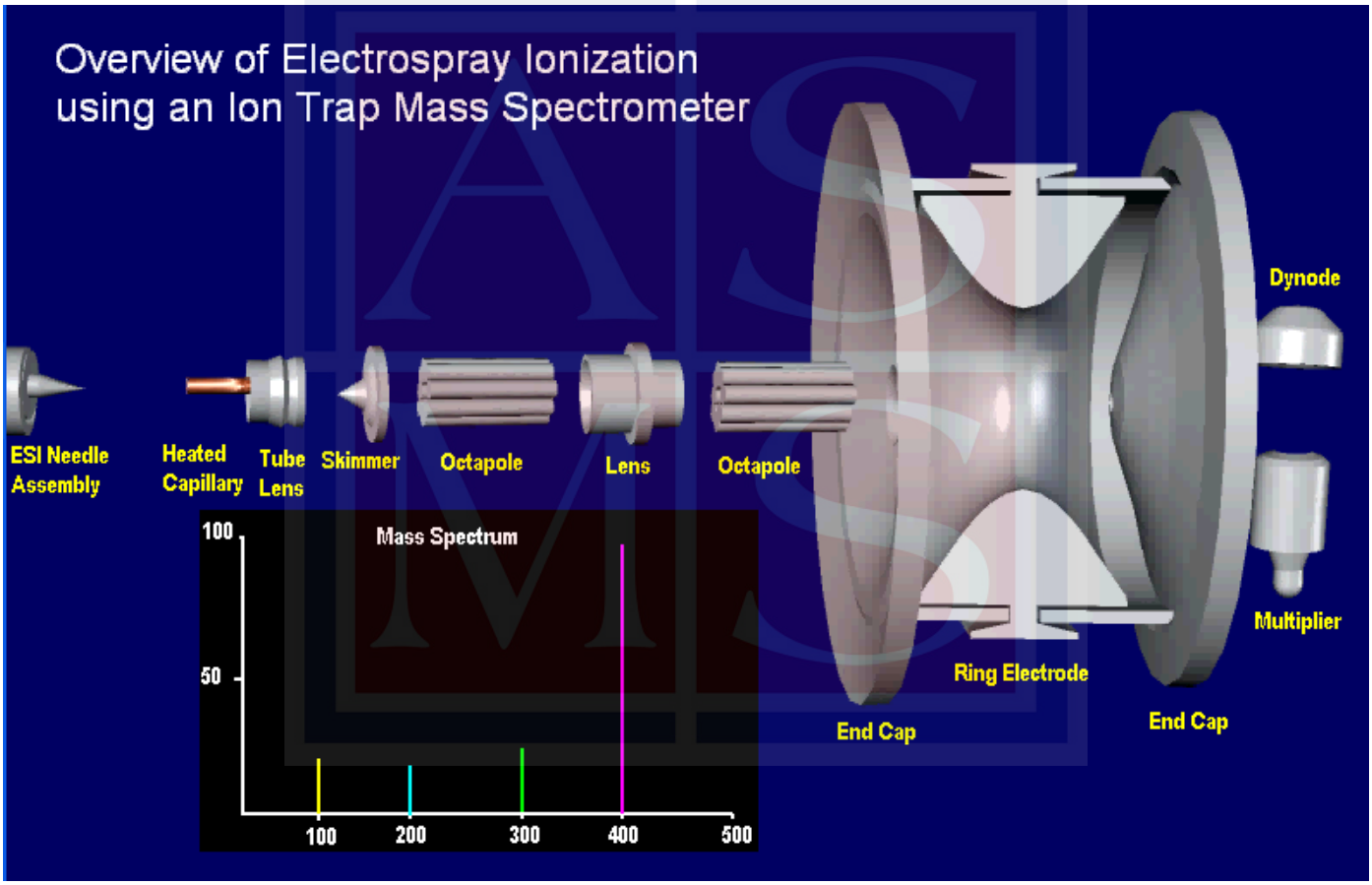


Why Ion Traps

- **Sensitivity:** ion accumulation allows 100x better than a scanning quadrupole)
- **Specificity:** Tandem MS MS^n
- **Mass Resolution:** > 50,000
- **Scan speed:** scans speeds <100 ms
- **Mass range:** can exceed 10,000
- **High value to costs ratio**

Fundamentals of Acquiring a Mass Spectrum on an Ion Trap – Timed Events

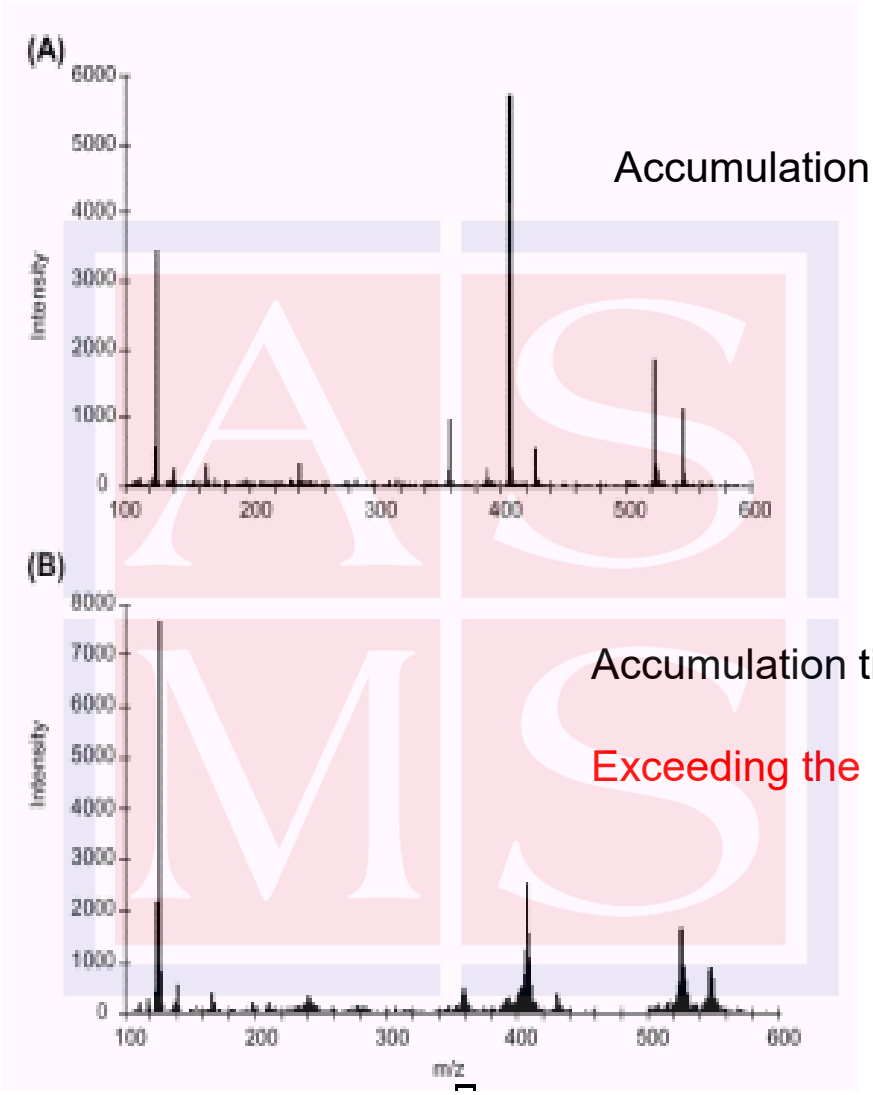
- Ions are accumulated from the API Source in the trap
- The trap is operated in non-linear resonance to eject ions in a sequential fashion



Why Control the Ion Accumulation Process

- API produces numerous charged species which are brought into the ion trap.
- The ion trap can only contain a finite number of ions during mass analysis due to field effects created by ions as the ion population increases.
- Exceeding this number of ions (exceeding the space charge limit) will result in reduced mass resolution, mass accuracy, and linear dynamic range.

Example of What Happens When too many Ions are Accumulated into the Trap



Accumulation time 10 ms

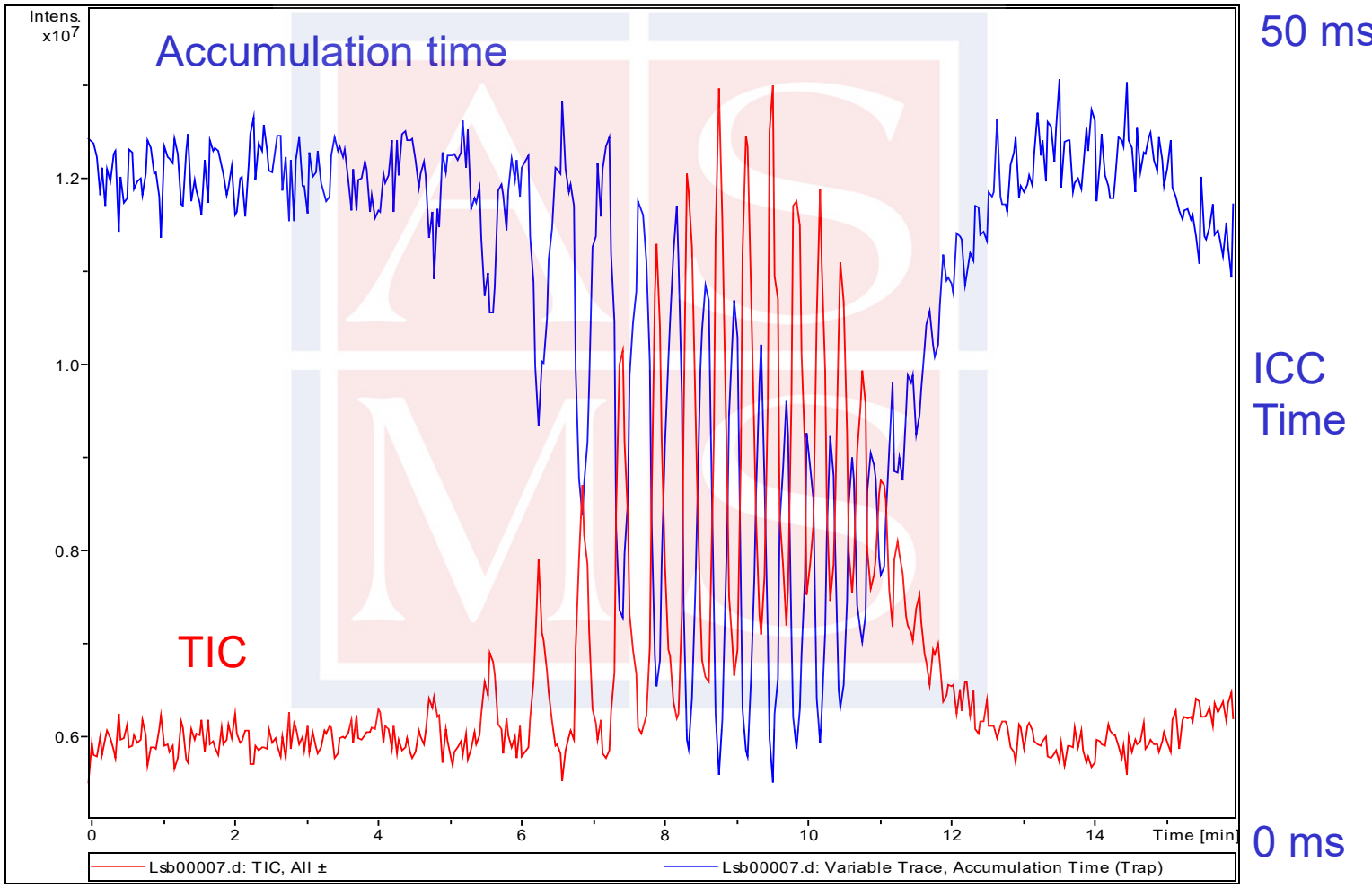
Accumulation time 100 ms

Exceeding the Space Charge Limit

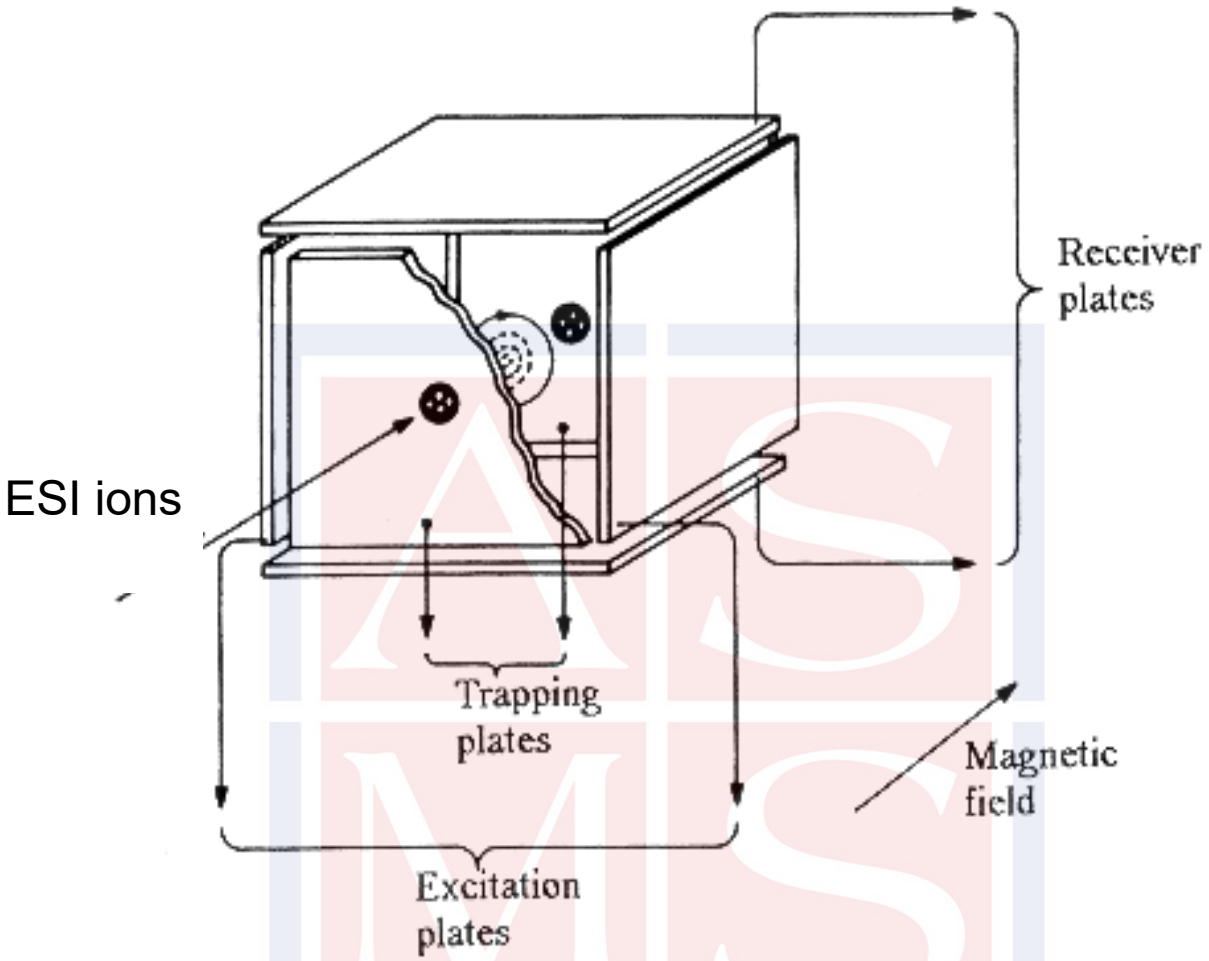
•Space charging –too many ionic species inside the trap distorting the electric fields and impairing the trap performance.

ICC to Remove Space Charge Effects

ICC Time adjusted inversely to TIC

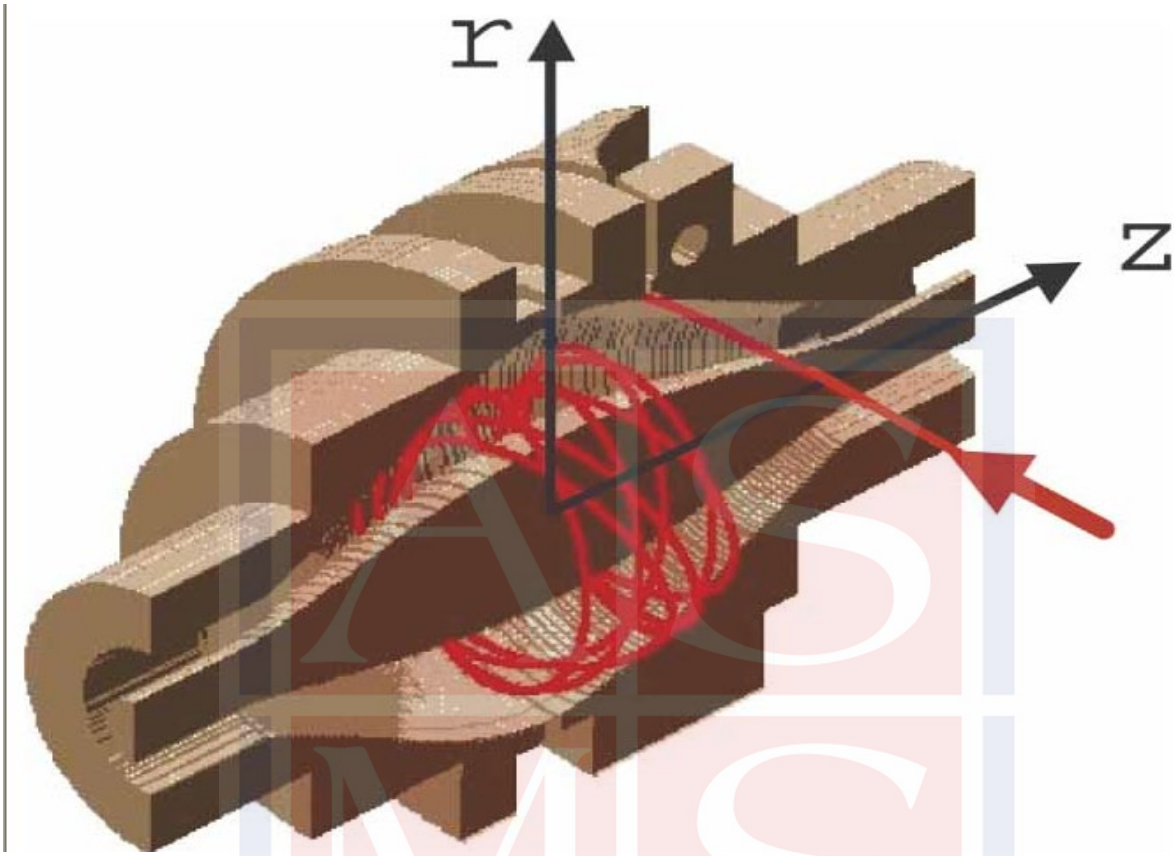


FTMS



Ions are trapped within a cubic cell under the influence of small trapping potentials and a constant magnetic field; the frequency of the signal measured at the detect plate is proportional to ion m/z. This technique offers the highest mass resolution

Orbitrap

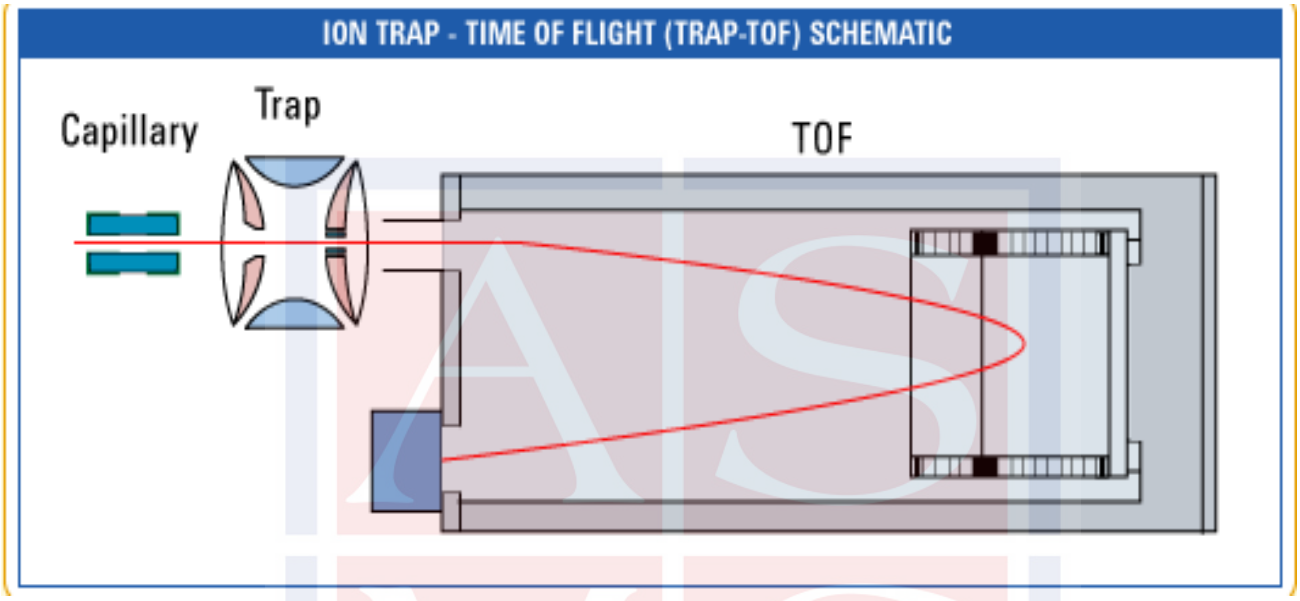


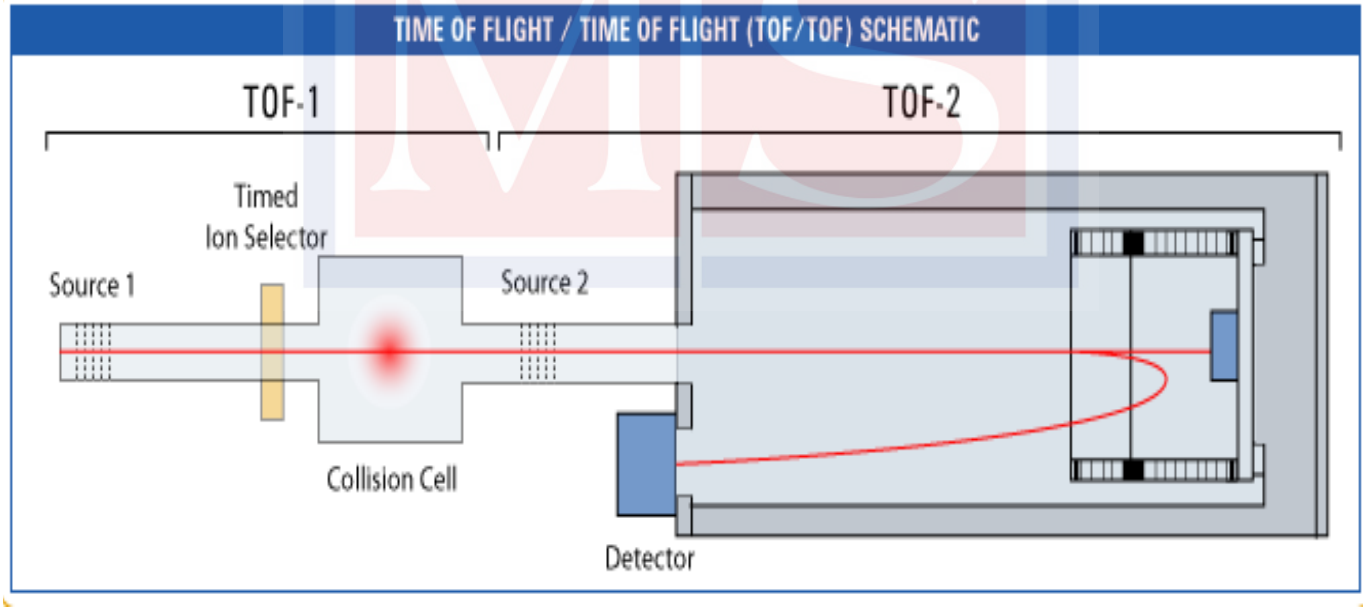
The orbitrap consists of an inner and an outer electrode, which are shaped to create a quadro-logarithmic electrostatic potential. Ions rotate about the inner electrode and oscillate harmonically along the *z*-direction with a frequency characteristic of their *m*/*z* values.

This technique offers advantages from both the ion trap and FTMS and is the newest mass analyzer. Benefits include high mass resolution, MSn, no magnet.

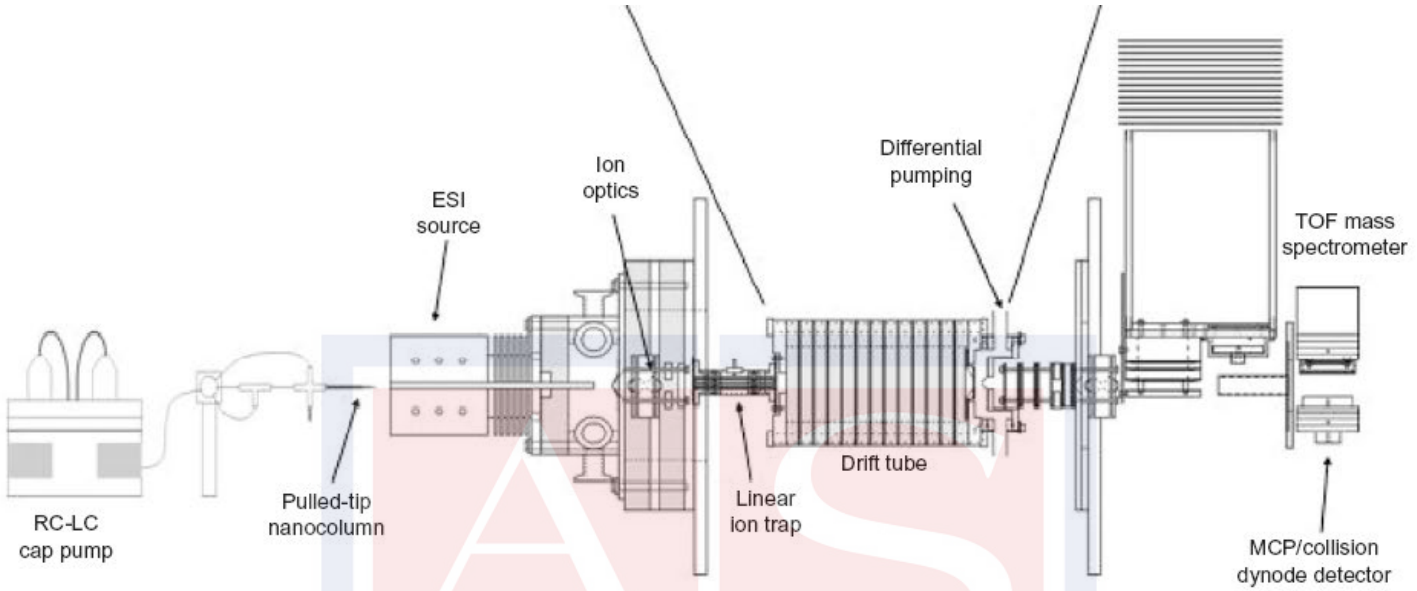
JMS 40, 430-443, 2005 and *Anal. Chem.,* **78** (7), 2113 -2120, 2006

Unique Analyzer Combinations
Trap-TOF and TOF-TOF





Ion Mobility MS



Operating principles

•Ions are separation based upon cross section and charge. (gas phase CE) in an atmospheric pressure drift chamber

Advantages

•Sensitive (allow for a higher sampling of ions from API

•Specificity – it can resolve isomers

•Speed-Separations are fast

•Many variables (to achieve a separation, i.e. gas and voltage)

• Will it replace LC?

Disadvantages

•Limited resolving power- needs a MS analyzer

Differential Ion Mobility

Operating principles:

Separation based on the difference in ion mobility of a monomer vs. the cluster of a compound in an asymmetric field. (AC 78, 5443, 2006) [also known as FAIMS –high-Field Asymmetric –waveform Ion Mobility]

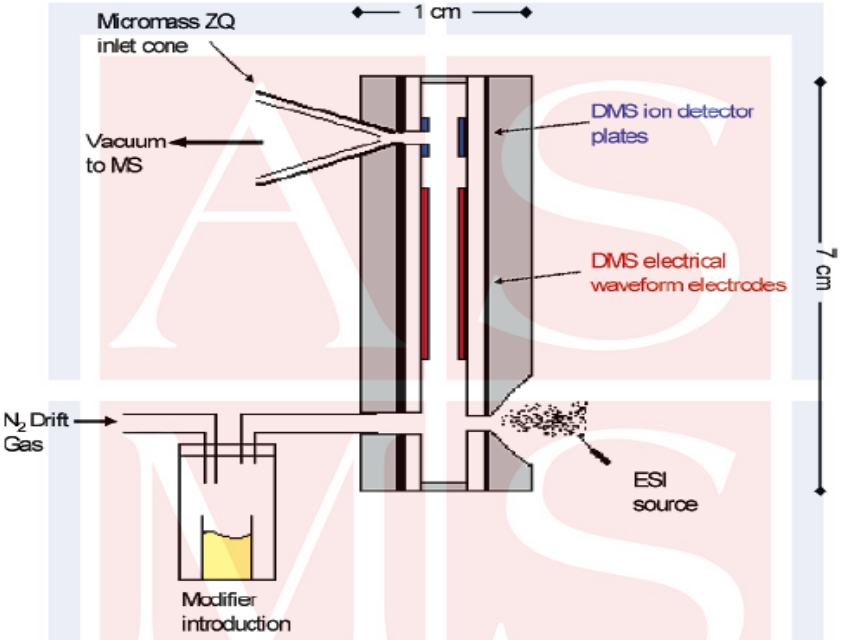


Figure 1. Schematic of ESI-DMS–MS system with approximate dimensions of DMS sensor.

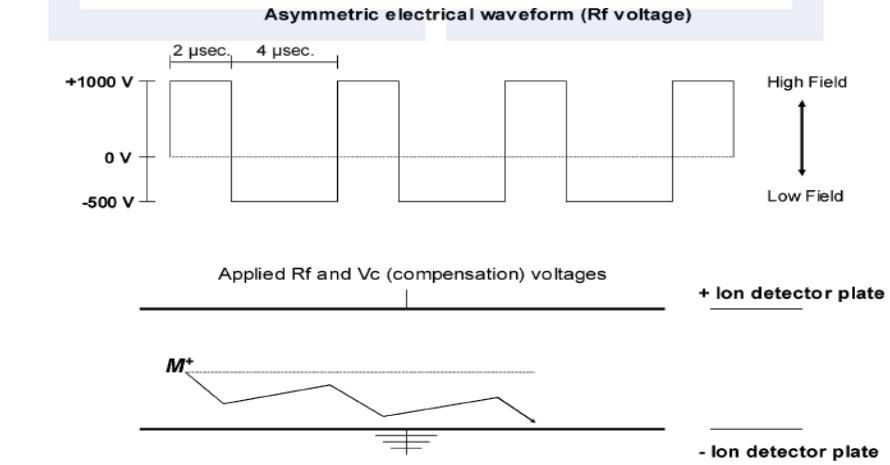


Figure 1. Ion trajectory between two parallel electrode plates as it experiences the asymmetric electrical waveform of +1000 V for 2 μs then −500 V for 4 μs.

When to Use Traps, FTMS and Q-TOF Instruments

- Qualitative Identifications
  - sensitivity
  - MS/MS
- Some quantitation
  - not matrix limited
- When additional full scan sensitivity is required

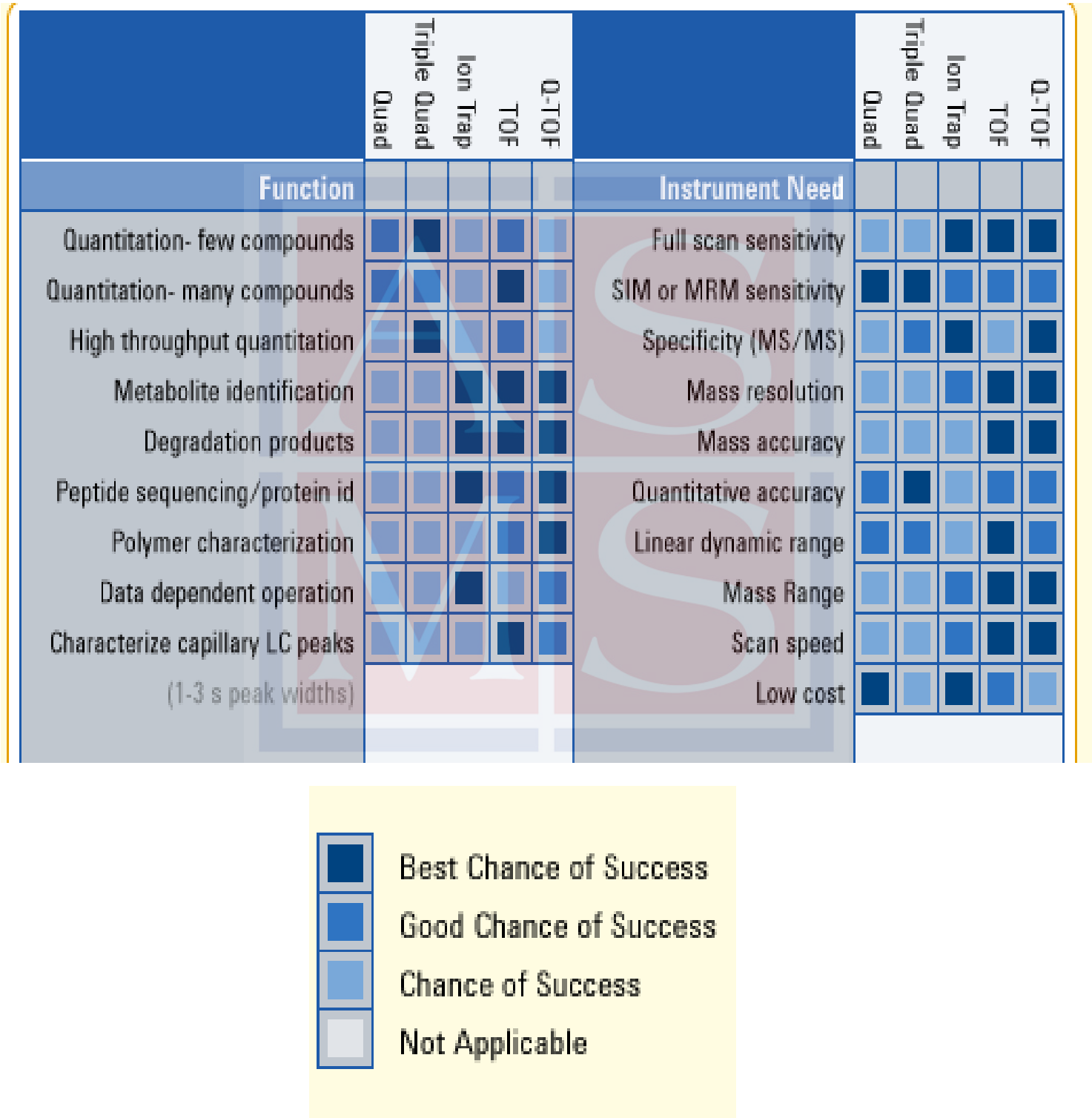
When to Use Single Quadrupoles

- Versatility
  - one instrument for qualitation and quantitation
- Routine/GLP determinations
  - rugged
- Automation
- Molecular weight determinations
  - high molecular weight compounds

When to Use Triple Quadrupoles

- Quantitation
  - Routine/GLP
- High throughput determinations
- Some qualitative work
  - Not sensitivity limited

Choosing the Mass Analyzer to Meet your LC/MS Needs



API –LC/MS Mechanisms and Operations

Search for a LC/MS Interface with All the Desirable Characteristics

Incompatibilities Between HPLC and MS

- MS cannot accept HPLC solvent volume (HPLC 500-4,000 mL/min of gas MS pumps about 10-50 mL/min
- Conventional gas phase ionization in MS not suitable for compounds separated by HPLC which are thermally labile, polar, or high molecular weight.

- Allow efficient sample transfer
- Provide reasonably precise sample transfer
- Permit free choice of LC method
- Permit free choice of MS operating conditions
- Transfer sample without decomposition
- Retain chromatographic peak integrity
- Provide speed, convenience, reliability, and minimal operator skills

How do you achieve the best LC peak capacity and peak concentration – Optimize HEPT

- **Small particles (minimize diffusion) a&b**
  (maximize mass transfer) c

- **Narrow columns (minimize diffusion) a**

- **Short columns (minimize longitudinal diffusion) b**

Note separation is based on gradient conditions for isocratic separations column length does improve resolution

- **Ideal columns for LC/MS**

2.1 x 20 -50 mm 1.8 um particles

Remember electrospray response is concentration dependent, so sharper peaks will generate a larger signal.

Atmospheric Pressure Ionization Mass Spectrometry (API-MS)

WHAT IS IT?

- Processes which lead to the ionization of a compound at atmosphere and followed by mass spectrometric analysis

- API-MS can be operated in the following modes:
  (1) Electrospray
  (2) Pneumatically assisted electrospray (also called ion spray)
  (3) Atmospheric pressure chemical ionization (APCI)
  (4) Atmospheric Pressure Photo Ionization (APPI)

  Each mode has certain advantages for analyzing various compound classes and for handling various inlets as will be explained later

Why Ionize at Atmospheric Pressure vs. Vacuum ?

- **Energy transfer for desolvation and /or vaporization**
  - *e.g. removal of the HPLC solvent in HPLC/MS*
- **Collisional cooling of ions to thermal temperatures**
  - *e.g. maximize the formation of molecular ions and ions from non-covalent complexes*
- **Maximize the yield of reagent ions for gas phase proton transfer reactions**
  - *e.g. Improve sensitivity for gas phase Chemical Ionization (CI)*
- **Minimal contamination within the vacuum system**
  - *e.g. improved ruggedness and ease of cleaning*

Key Characteristics of the 4 Modes of API-MS Operations

ELECTROSPRAY:

Ionization process which uses electrical fields to generate charged droplets and subsequent analyte ions by ion evaporation for ms analysis.

PNEUMATICALLY ASSISTED ELECTROSPRAY:

Same as electrospray (above) except the initial droplet formation is the result of pneumatic nebulization.

APCI:

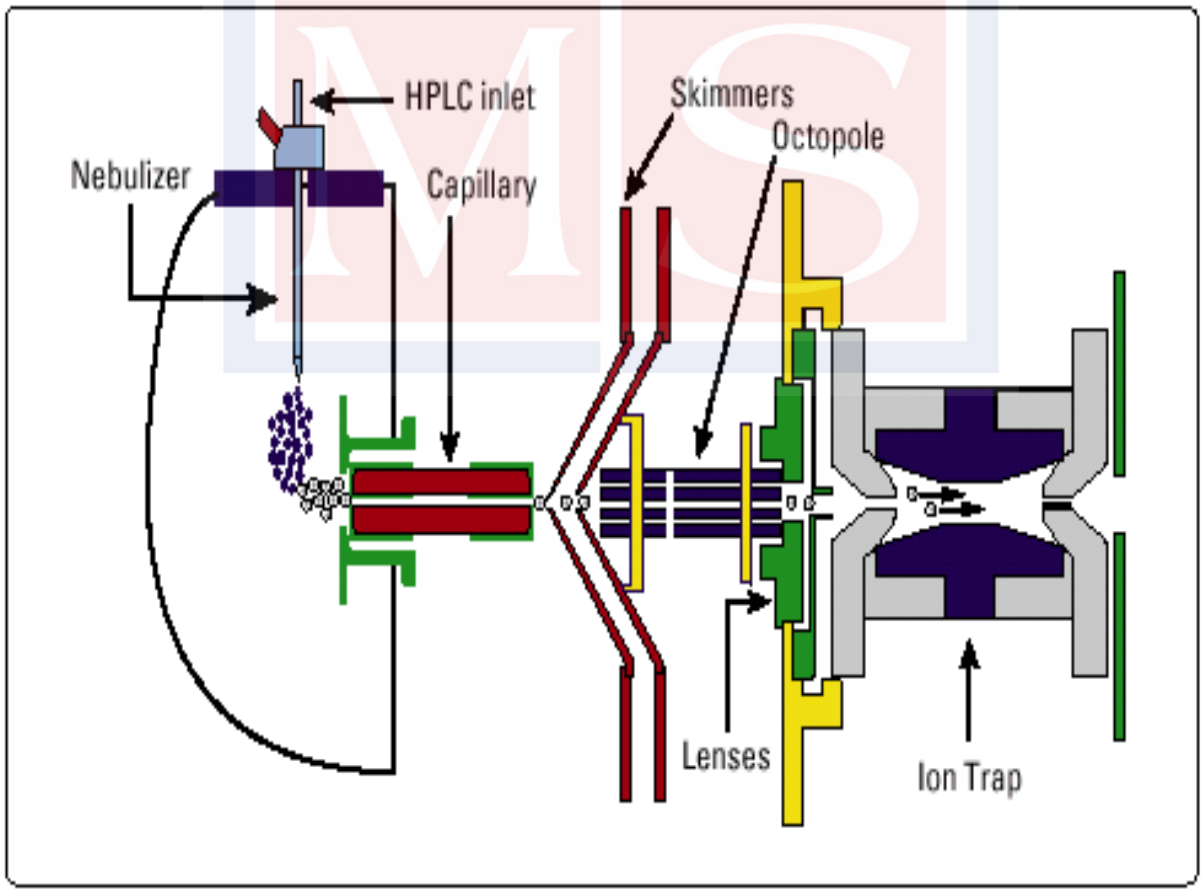
A gas phase chemical ionization (CI) process where the solvent acts as the CI reagent gas to ionize the sample.

Atmospheric Pressure Photoionization (APPI): Krypton lamp producing ultraviolet light ionizes gas phase analytes or dopants with subsequent gas-phase reactions.

API-MS Hardware

GENERAL ASPECTS:

- Atmospheric Chamber
  - Nebulization/desolvation region
  - Ionization region
- Ion Transport
  - Transport ions from atmospheric to vacuum of the MS (~10^{-5} Torr)
- MS Analysis
  - Quadrupole is most common technique but is compatible with most mass analyzers (e.g., magnetic sectors, ion traps, time-of-flight)



Electrospray Ionization or Ion Evaporation

- **Step 1** **Ionization In Solution**
  - **pK_a of sample**
  - **pH of solution**
- **Step 2** **Nebulization**
  - **Surface tension and viscosity**
  - **Pneumatic assistance**
- **Step 3 Desolvation**
  - **Drying gas temperatures and flow**
  - **Heat capacity, H vap**
- **Step 4** **Desorption of Ions From Solution**
  - **Solvation energy**
- **Step 5** **Reactions of Ions in the Gas Phase**
  - **Proton affinity**
  - **Charge exchange**

Pneumatic nebulization and drying on modern instruments, minimize the effect of these parameters

Student Notes: This section of the course will show experimental evidence highlighting these 5 key points for optimization of API-Electrospray Signal Intensity. Examples will be given to demonstrate each sequential step of the ionization mechanism

Why is the Best Electrospray Sensitivity Achieved When the Analytes Exist As An Ion In Solution?

- For a given field strength generated on a charge droplet ionic interactions can be 10^3 to 10^4 times greater than non-ionic interactions (e.g. Van der Waal forces, hydrogen bonding) for neutral molecules
- Therefore, analyte ions can be desorbed from the charged droplets overcoming the solvation energy holding them in the liquid far better than neutral species.
- This desorption process is call Ion Evaporation

Step 1 - How Can Ions Be Created In Solution?

Ionic Species

NH_4^+ PO_4^-

Acid/Base Chemistry

$M - NH_2 + Acid \rightarrow [M - NH_3]^+ + Acid^-$

$M - COOH + Base \rightarrow [MCOO]^- + Base^+$

Association(1) (for neutral species like sugars)

$M^\circ + Na^+ \leftrightarrow [M + Na]^+$

(alkali metal)

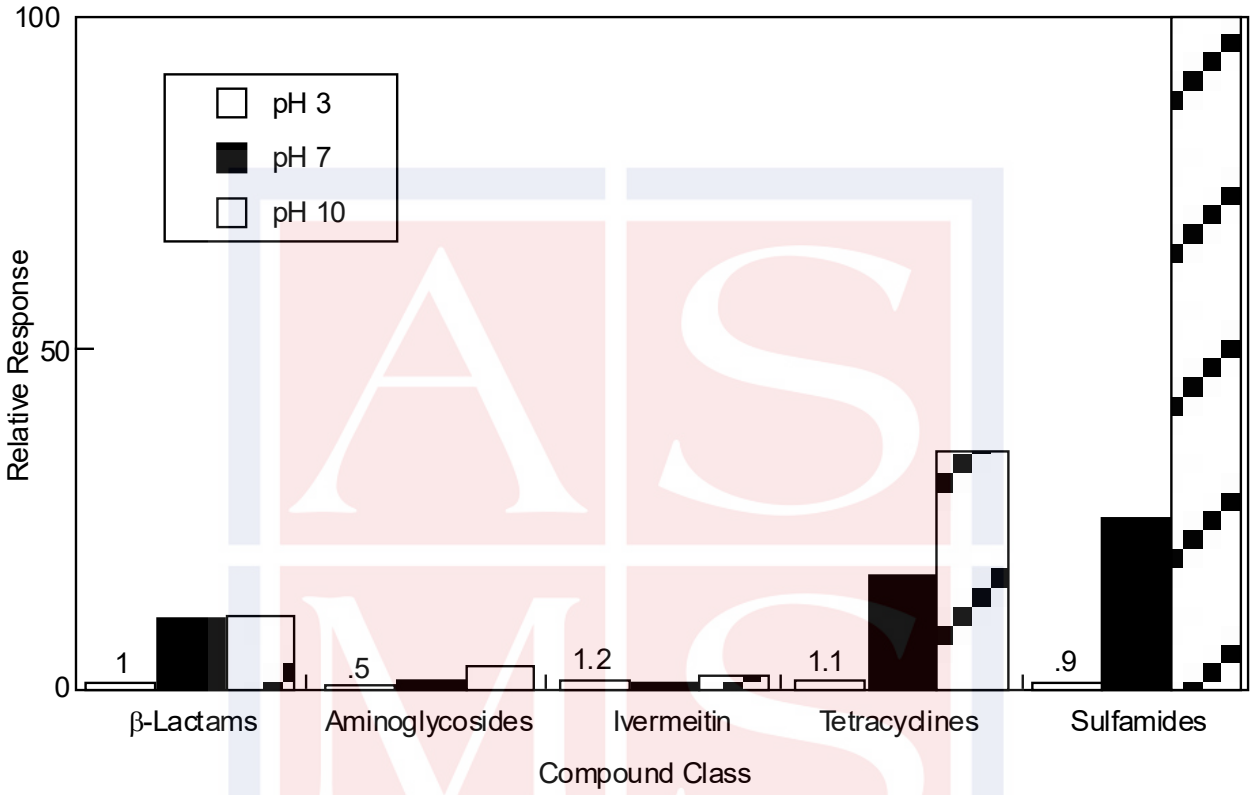
(20 μM sodium acetate)

Derivatization

To form an ion or acid/base product

(1) associations also explain why many common LC additives cannot be used with electrospray-MS

Formation of Ions in Solution: Negative Ion Detection



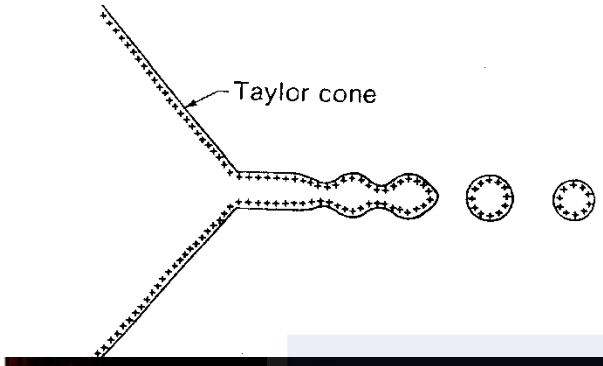
Student Notes: Formation of anions is desired when using negative ion detection. Increasing the pH deprotonates acid sites on the molecule (e.g., carboxylic acid) resulting in improved sensitivity.

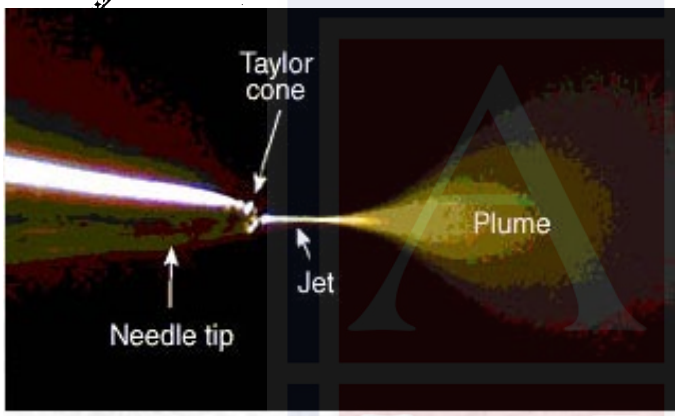
General Rules for Choosing Polarity of Ion Detection and pH

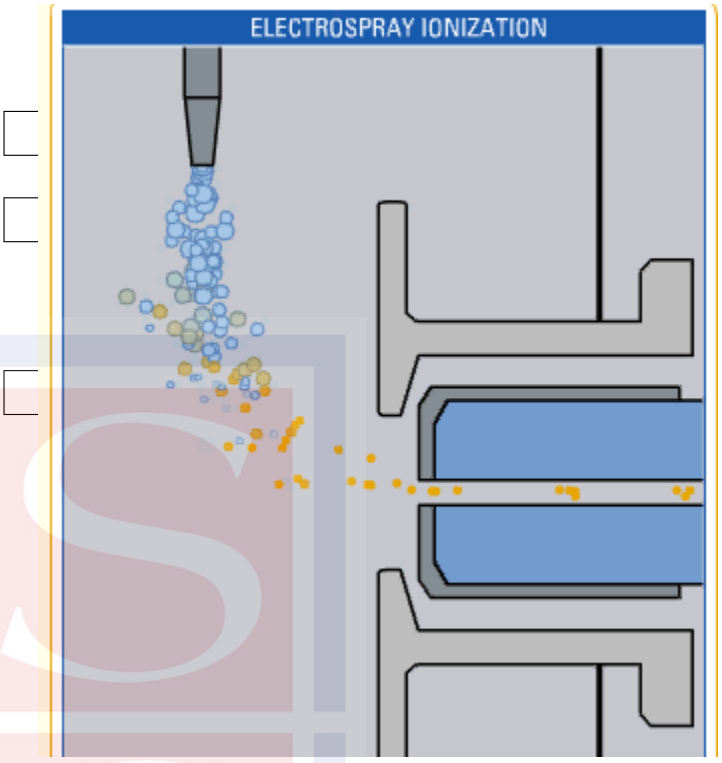
- **Positive Ion Detection:**
  - Basic samples (e.g., peptides containing arginine and/or lysine)
  - Decrease pH
    - Acetic Acid pH (3-4)
    - Formic Acid pH (2-3)
    - Trifluoroacetic Acid (TFA) pH (1-2)
  - pH at least 2 units below pKa of samples
- **Negative Ion Detection:**
  - Acidic samples (e.g. peptides containing aspartate and/or glutamate)
  - Increase pH ammonium hydroxide
  - pH at least 2 units above pKa of samples
- **Neutral Samples:**
  - cationization, charge exchange, APCI

Student Notes: General rule of thumb is that you acidify sample when operating in positive ion detection and basicify sample when operating in negative ion detection. Choose mode of ion formation based on the presence of acidic or basic site in the sample.

Initial Charging of Liquid







$$E_c = 2V_c/[r_c \ln (4d/r_c)]$$

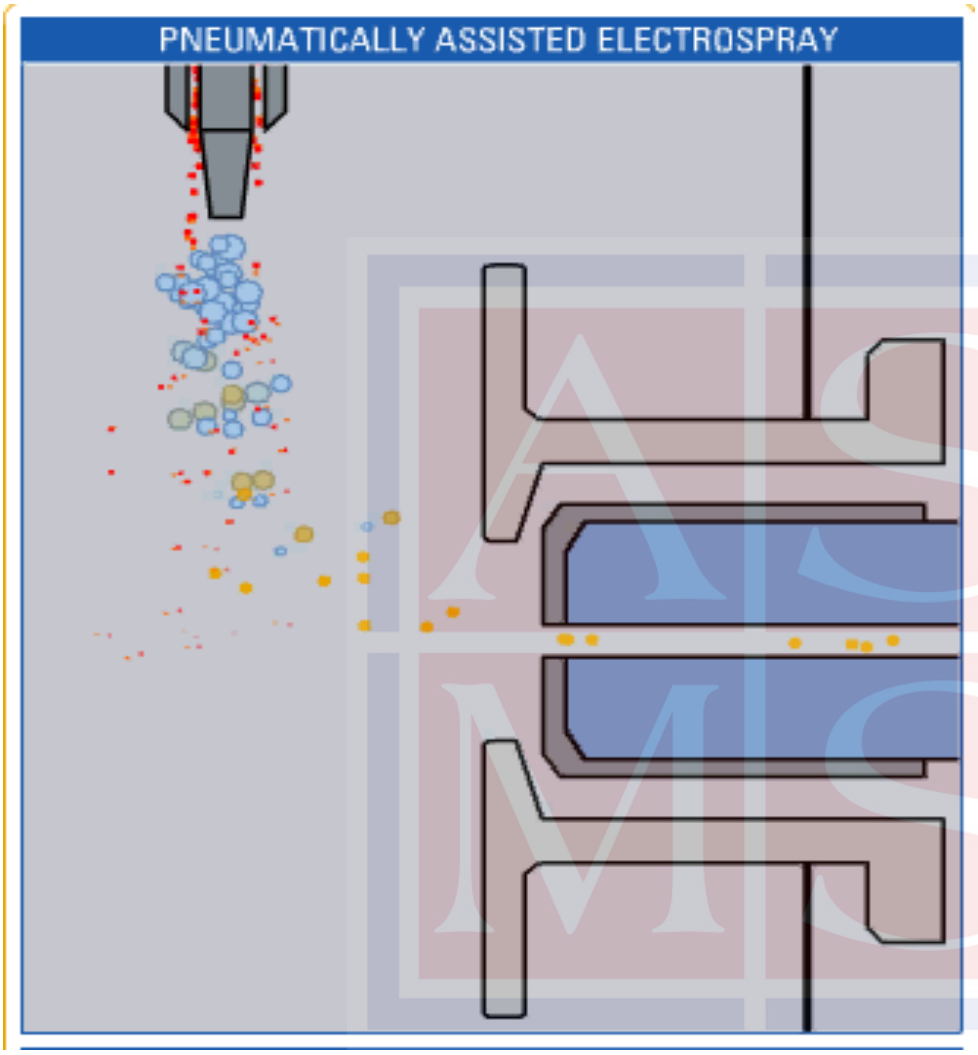
d = distance from capillary to counter electrode

E_c = field
V_c = electric potential
r_c = capillary outer radius

$$I_{es} = h\, v_f^{2/3}\, \sigma_s^{a}\, E_c^{b}$$

I_{es} = electrospray current
V = flowrate
σ_s = conductivity
h = constant
a,b > 0

Step 2 - Pneumatic Assisted Nebulization – To Create Charged Droplets



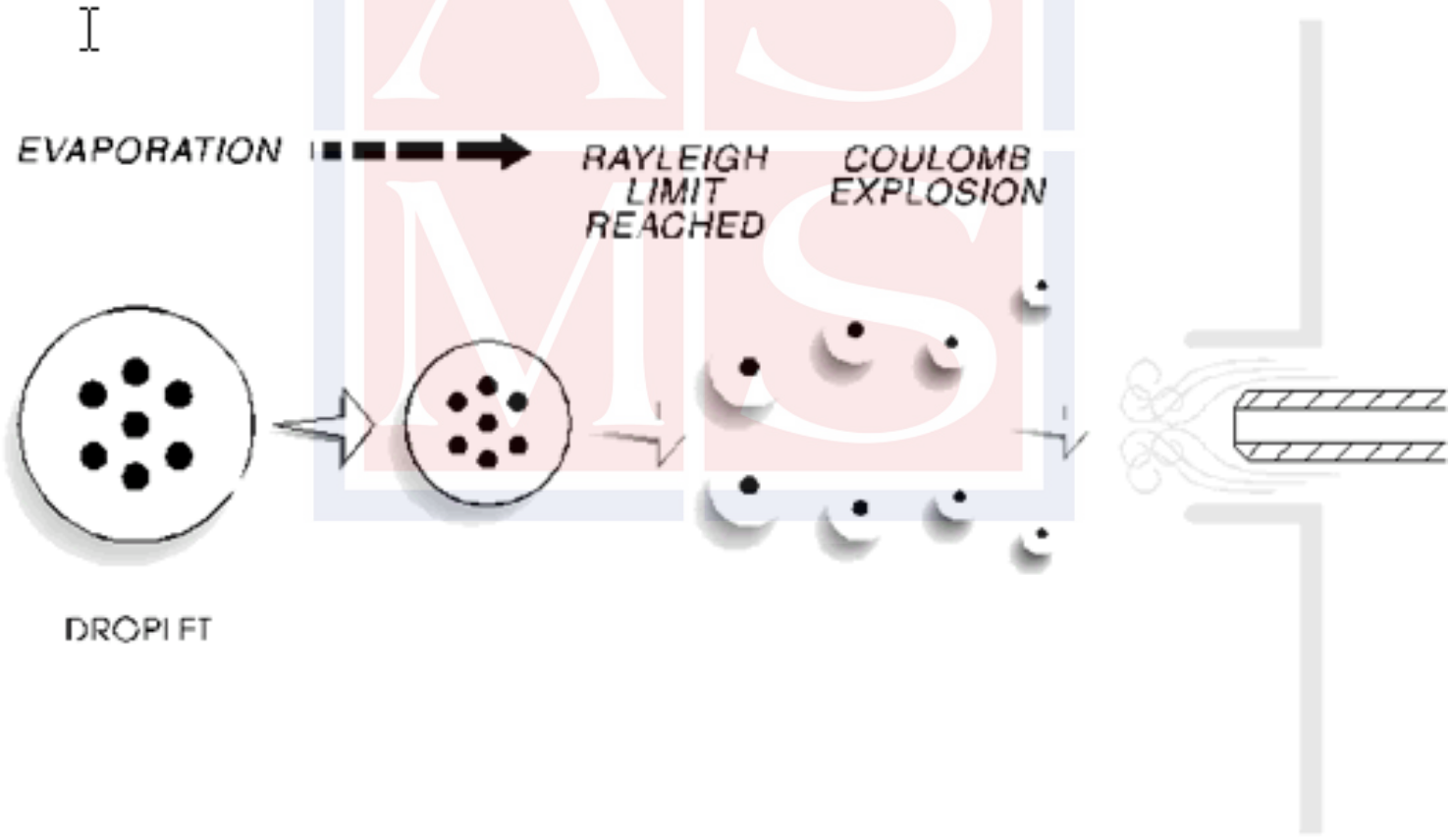
Charged solvent droplets about 2 μm in diameter containing about 100,000 charges

Pneumatic nebulization reduces droplet size variations from viscosity and surface tension due to solvent composition or flow rate

Step 3 - Desolvation of Charged Droplets

Heated Nitrogen evaporates the droplets, increasing the charge to volume ratio.

Rayleigh limit is the maximum charge a droplet can hold and while maintaining its volume. When the charge exceeds this limit, coulomb explosions occur.



Droplet Formation by Coulomb Fission

Rayleigh Stability Limit

$$q_r = [8\pi(e_0\ \gamma\ r^3)]^{1/2}$$

q_r = charge
r = radius of droplet
γ = surface tension
e_0 = permittvity

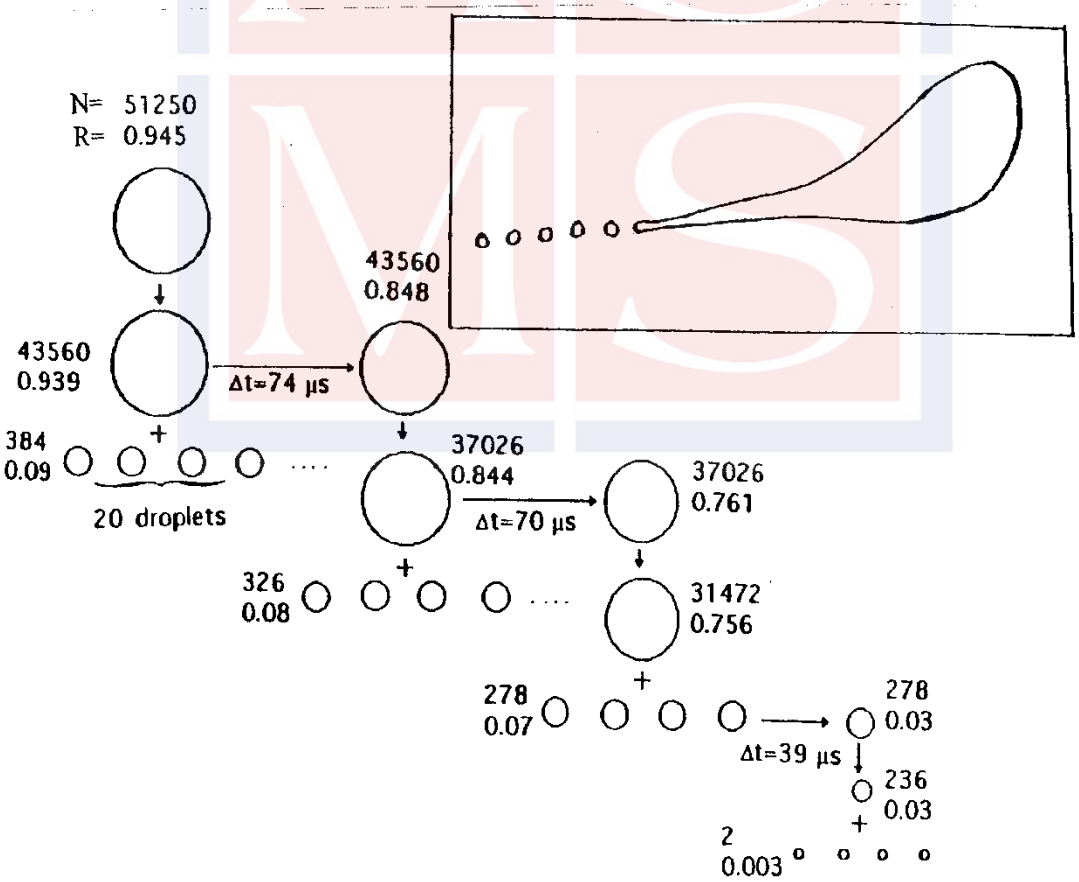


Figure 2. Droplet evolution scheme due to solvent evaporation at constant charge and Coulomb fissions at the Rayleigh limit. First droplet shown is at the Rayleigh limit. The parent droplet produces 20 offspring droplets which carry off 2% of the mass and 15% of the charge. The insert top right, illustrates the fission of one such droplet. Based on data from Gomez and Tang.[19] *N* corresponds to number of elementary charges and *R* is the radius in μ m. Also given is the time Δt(μ s) required to reach the next fission. (From P. Kebarle and L. Tang, *Anal. Chem.* 1993; **64**: 972A.)

Coulomb Fission of Droplets

Phys Fluids 6, 404, 1994

Step 4 - Making Of Ions Ion evaporation Mechanism (IEM)

- Field strength exceeds solvation energy (ΔG_{sol}) of Ions in Solution

$$E_c > \Delta G_{sol}$$

- ΔG_{sol} Hydrophilic > ΔG_{sol} Hydrophobic (solvent choice)
- ΔG_{sol} $[M]^+$ > ΔG_{sol} $[M+(H_2O)_n]^+$ (leads to clusters)
- ΔG_{sol} Large molecules > ΔG_{sol} Small molecules

IEM valid for Mol Wt less than 5000

Electrospray Results in the Lowest Ion Internal Energy Relative to Other Ionization Techniques for MS

- Ion energy < 0.1 eV
- Enables detection of non-covalent complexes

Making Of Ions
Charge Residue Mechanism (CRM)

- **Rayleigh fissions desolvates large molecules**

$$\Delta G_{sol} > E_c$$

(typically Mol Wt > 100,000)

-

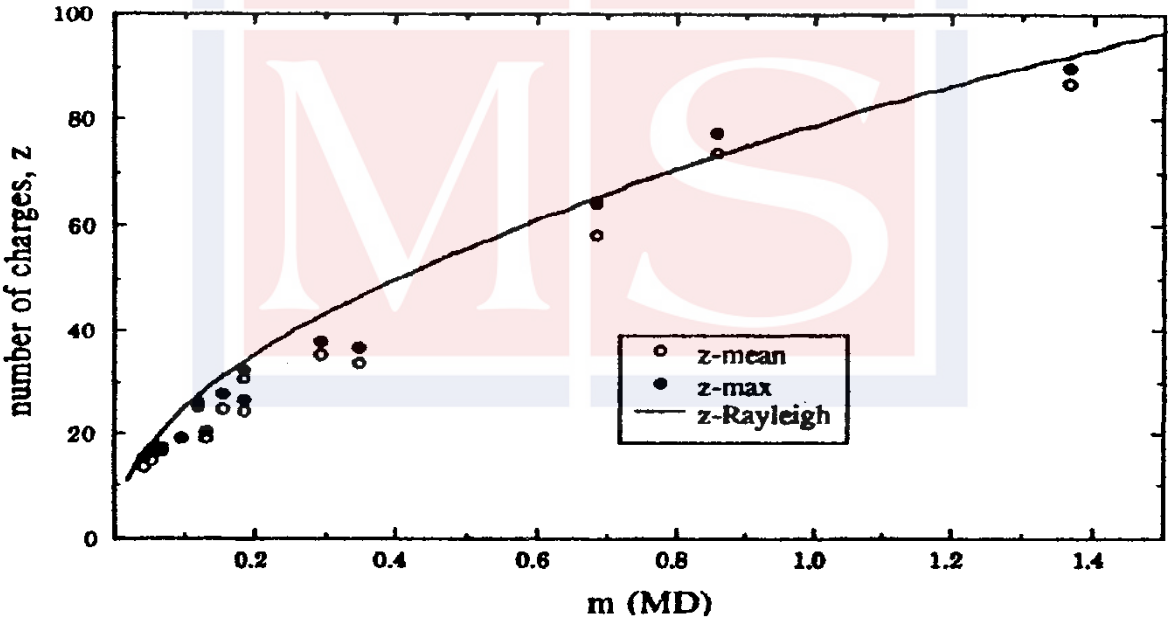


Figure 3. Maximum and mean charge on various native proteins compared with the Rayleigh limit (continuous curve). Molecular weights of the proteins given on the *x*-axis are in units of 10^6 Da. Data points are taken from Tolic *et al.*[80] Reprinted from Analytica Chimica Acta, **406**, J. Fernandez de la Mora, "Electrospray ionization species proceeds via Dole's charged residue mechanism", p. 97, © 2000 Elsevier Science Ltd.

Step 5 - Gas Phase Reactions for Electrospray

Proton transfer and charge exchange reactions can occur from reaction in the atmospheric chamber through the ion transport region. This high pressure region permits 1000's of ion/molecule reactions to occur.

- Proton Transfer

  Samples with lower proton affinities than NH_3 or triethylamine (206 and 232 kcal/mole respectively) can lose a proton and become neutralized or form adductions

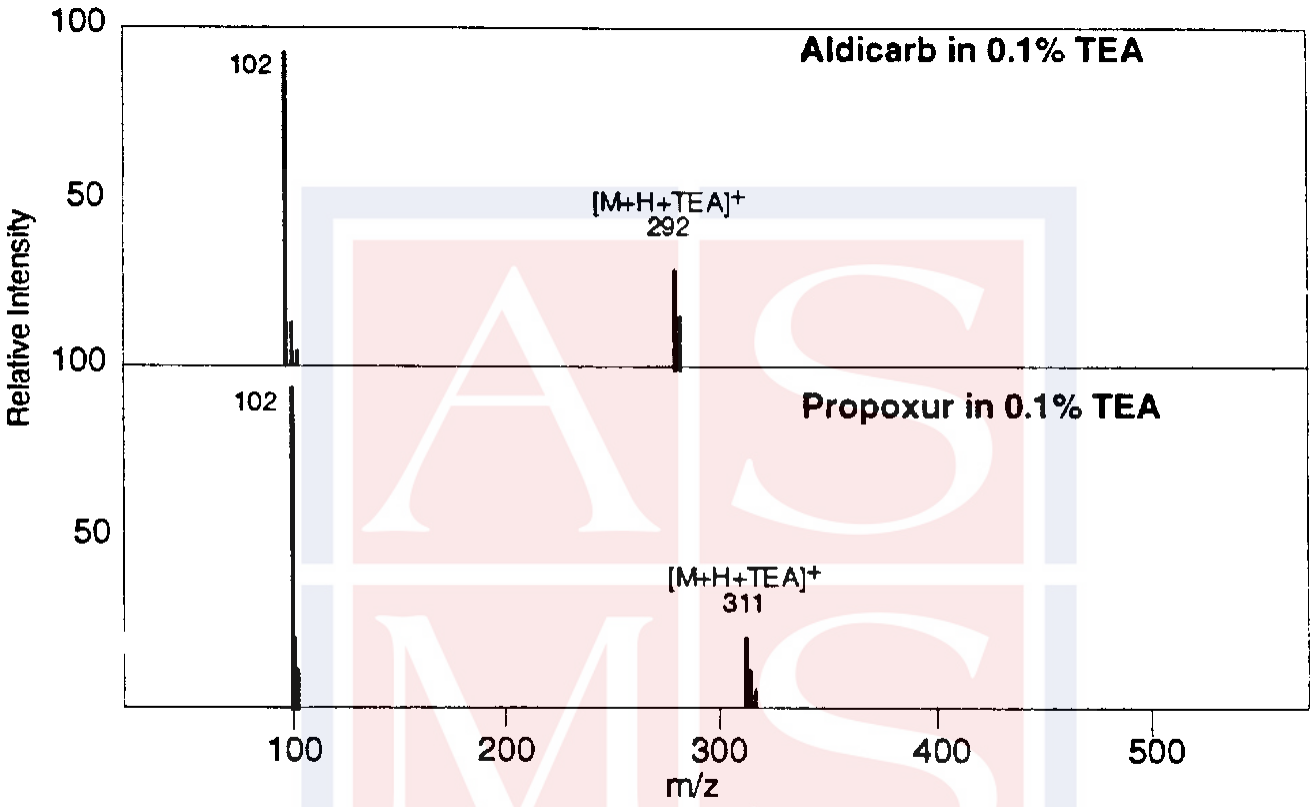
$$CH4 < H_2O < MeOH < EtOH < IPA < NH3 < TEA$$

134 173 185 190.3 191.4 206 232

Kcal/mole

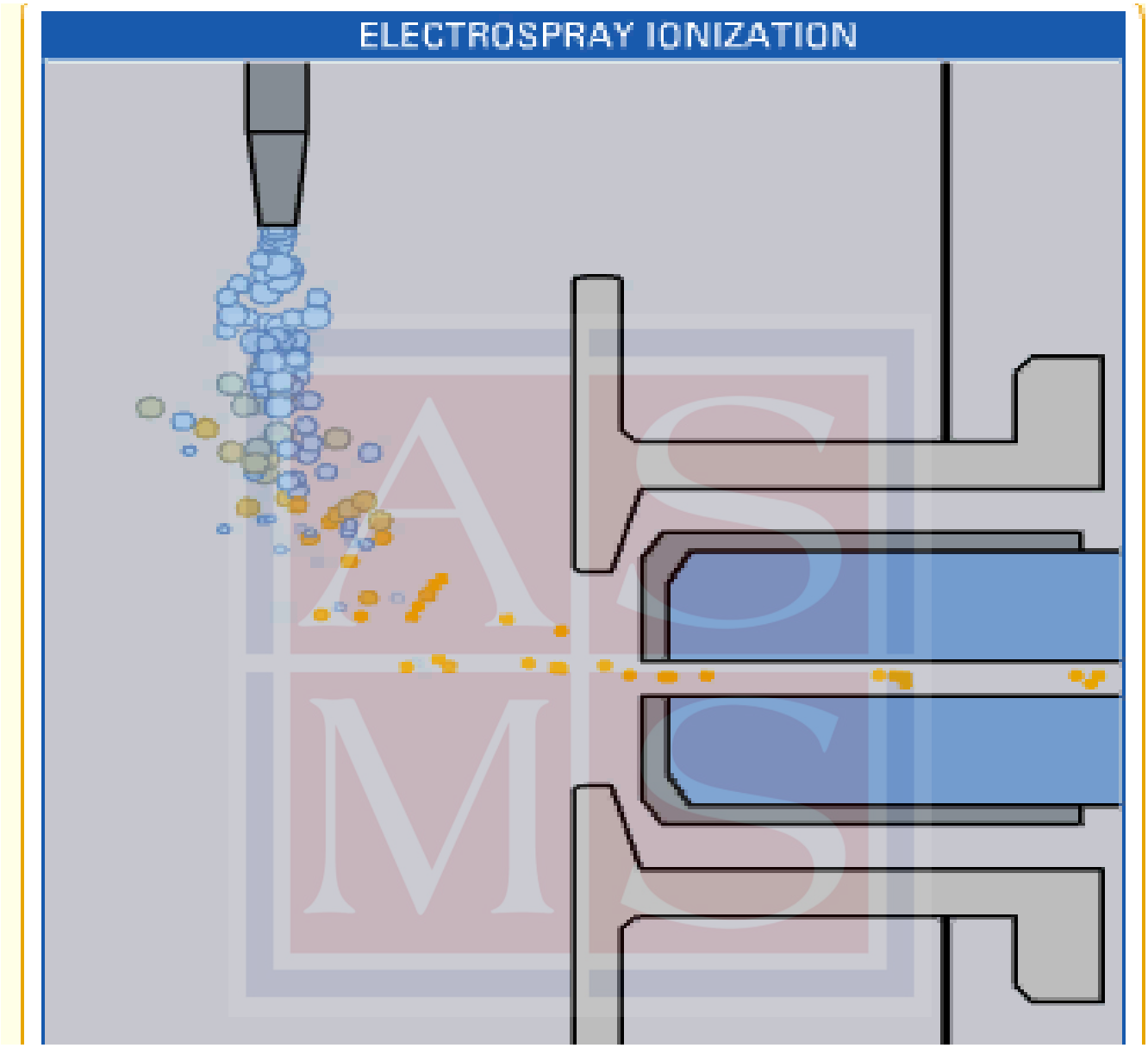
Student Notes: Often gas phase reactions are not considered in electrospray, but often account for loss of signal intensity even when pH, solvent and ion evaporation conditions are optimized. The ions detected by MS will be the result of any gas phase reactions that occur in the system.

Gas Phase Reactions - Electrospray



Student Notes: TEA has a very high PA (232 Kcal/mole) and will deprotonate most organics resulting in the formation of $[TEA+H]^+$ at m/z 102. In most cases TEA cannot be used with positive ion electrospray-MS.

API-Electrospray Mechanism

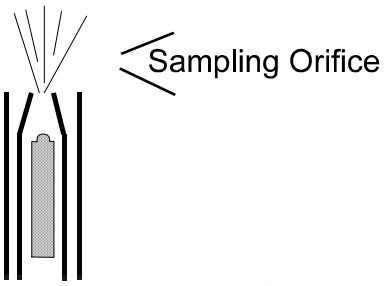


Mechanisms of Ionization

- Ion Evaporation:
- Electric field charge liquid
- Charged droplets
- Desolvation to reach high field strengths (10^8 V/cm^2)
- Gas Phase Ions

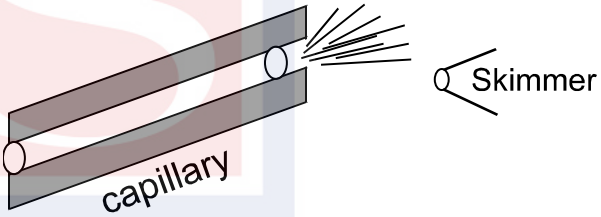
Ways to Reduce Background Caused by Charged Particles

- Orthogonal Spray



- Z-Spray

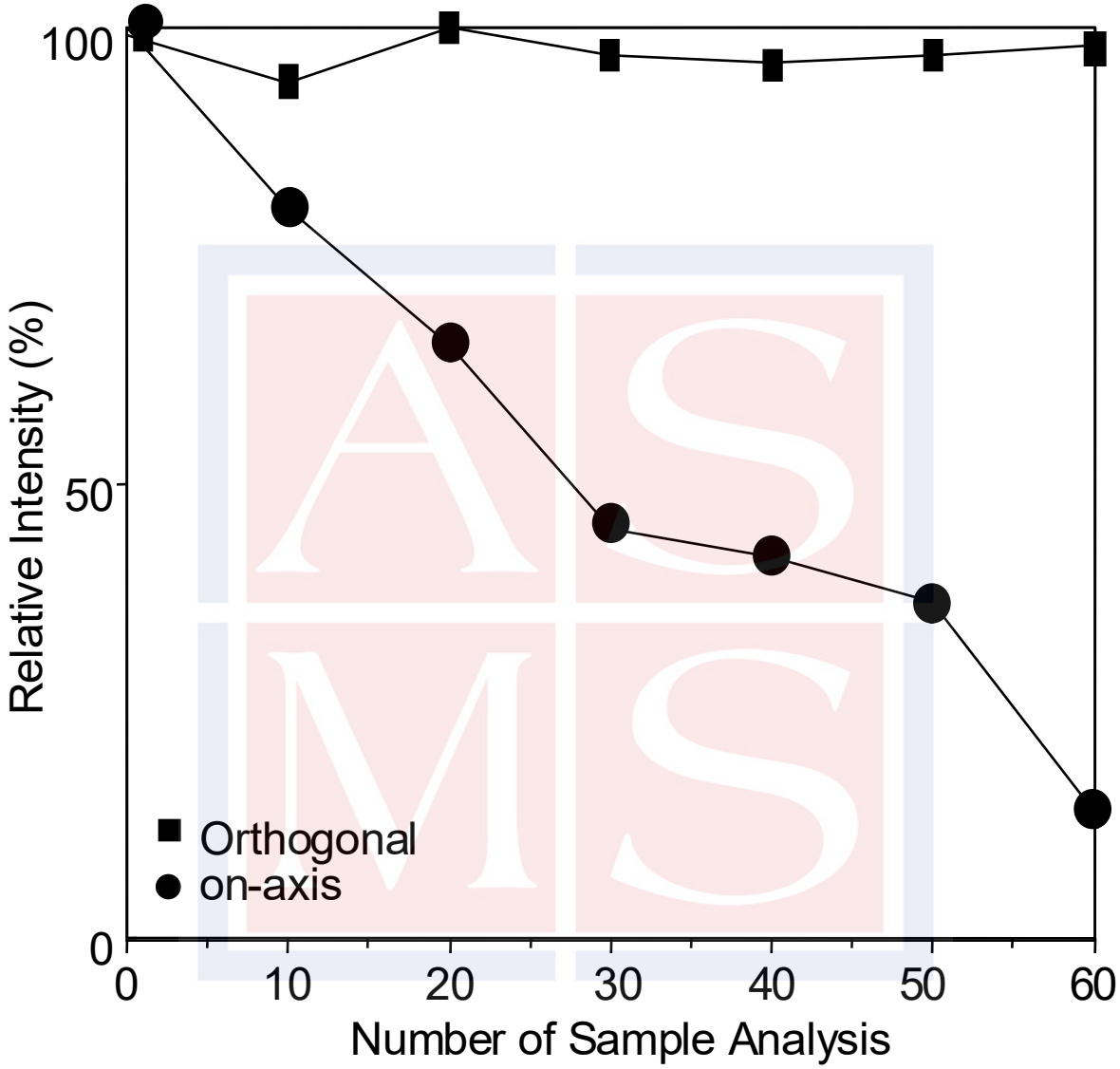
- Off axis
  Capillary-Skimmer



- Pulse Counting
  1 droplet = 1 ion
  (multiplier 1 droplet >>> 1 ion)

Current manufactures use off axis (80-100 degrees nebulizer-sampling orifice design

Comparison of Orthogonal vs. On-axis for Repetitive Analysis of Plasma Extracts



How are Ions Sampled at Atmospheric Pressure?

- Electrostatic focusing not effective (MFP < 0.1 μm)
- **Viscous Gas Drag**

  Flow of nitrogen nebulization or drying gas from atmosphere to the vacuum through the sampling orifice pulls analyte ion into system
- Is this efficient? **No**
  - ES yields about 100% ionization efficiency

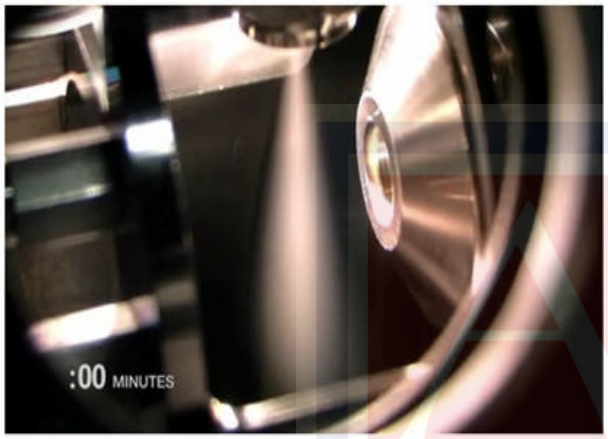
    (EI ~ 0.01 - 0.001%)
  - Only 0.01% of the ions are sampled.

    (EI 5-50%)

How can ion sampling efficiency be improved?

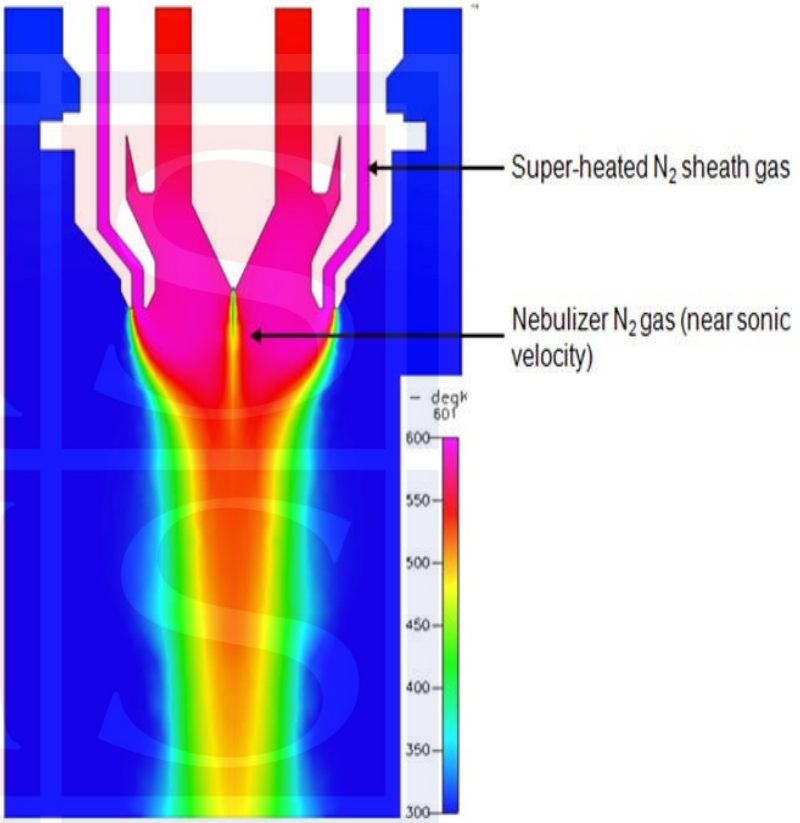
- **Improved vacuum capacity $$$**
- **Focusing of spray to increase ion density at sampling orifice**
- **Ion funnels (focusing of ions at high pressures)**
- **Nanospray**

Focusing of Spray to Improve Sensitivity

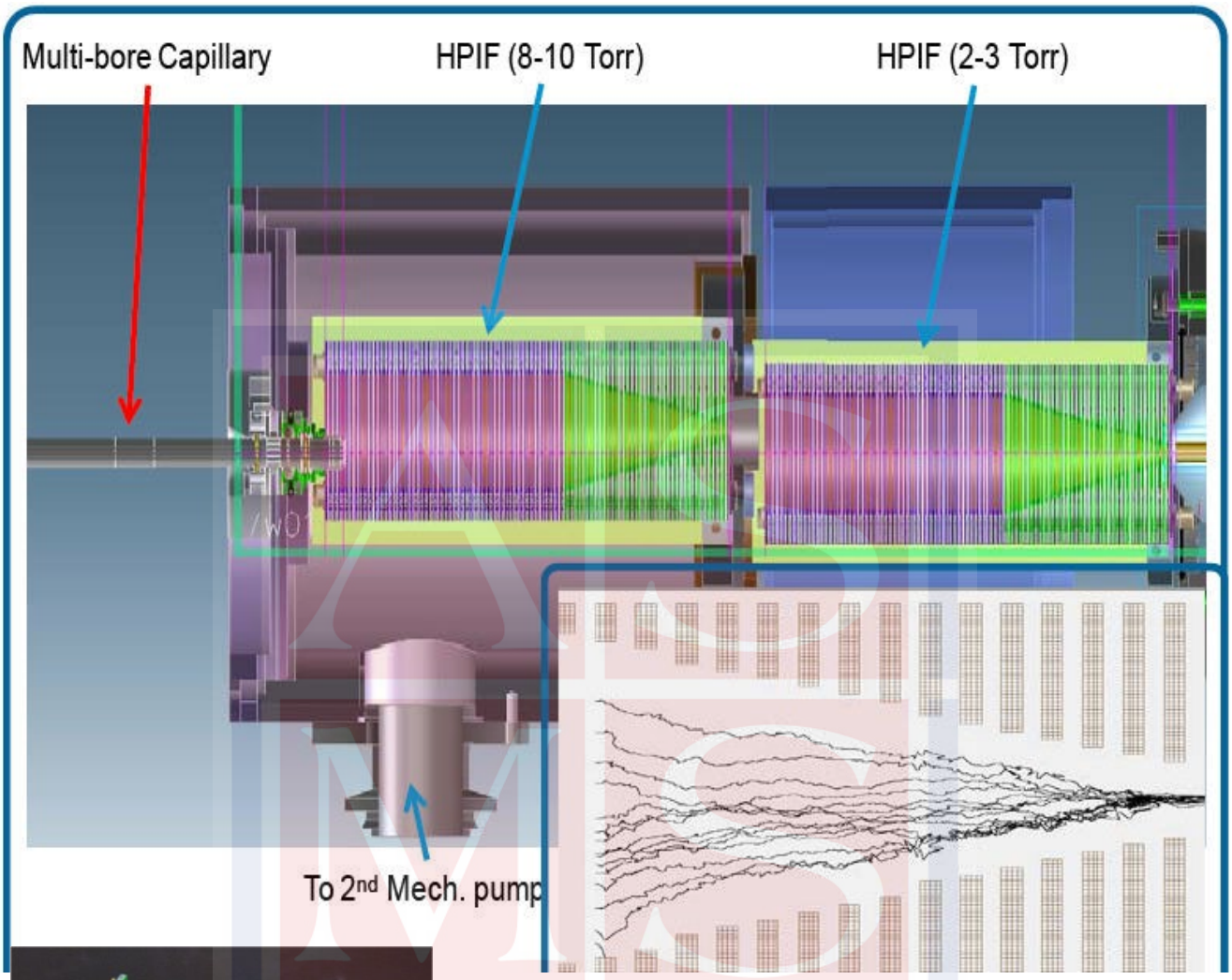


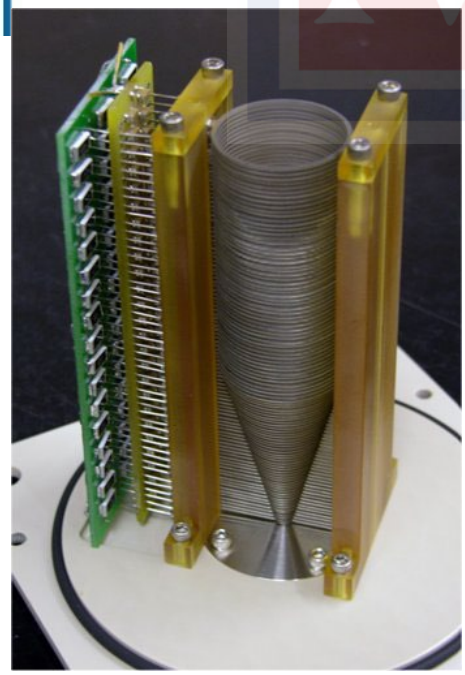
Start temperature = 25 °C

Stop temperature = 200 °C



Ion funnels





•100's of circular thin ring electrodes that are taper at a 25-35 angle
• rf voltage 180 out of phase applied to adjacent electrodes
•dc voltage gradient directs ion axially through funnel

•*Ion yields decrease with increasing pressure*

Highest Sensitivity Achieved at Lowest Flow Rates and Narrow diameter Capillaries

- Lower flow rates and smaller diameter capillaries result in smaller droplets (< 200 nm) and increase in the charge/volume ratio. Ions can be desorbed from initial droplet.

→ Near 100 % ionization efficiency

→ Minimal dispersion of droplets leading to increased sampling efficiency

→ Sensitivity increased by 2-3 orders in magnitude when going from high uL to nL flow rates

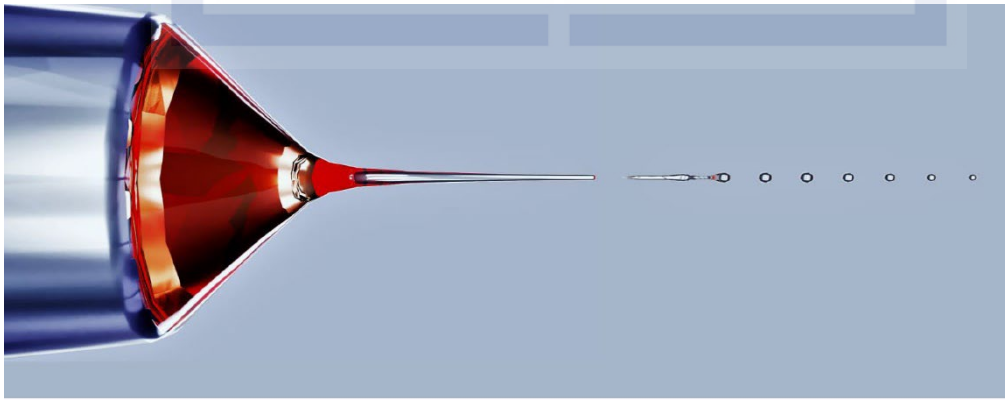
→ Sensitivity is mass flux dependent at nL compared to concentration dependent in the uL range of flow rates

MicroTip Electrospray Needles

- Micro Tips allow for stable electrospray operation down to 10 nL/min.
- **Hardware**:

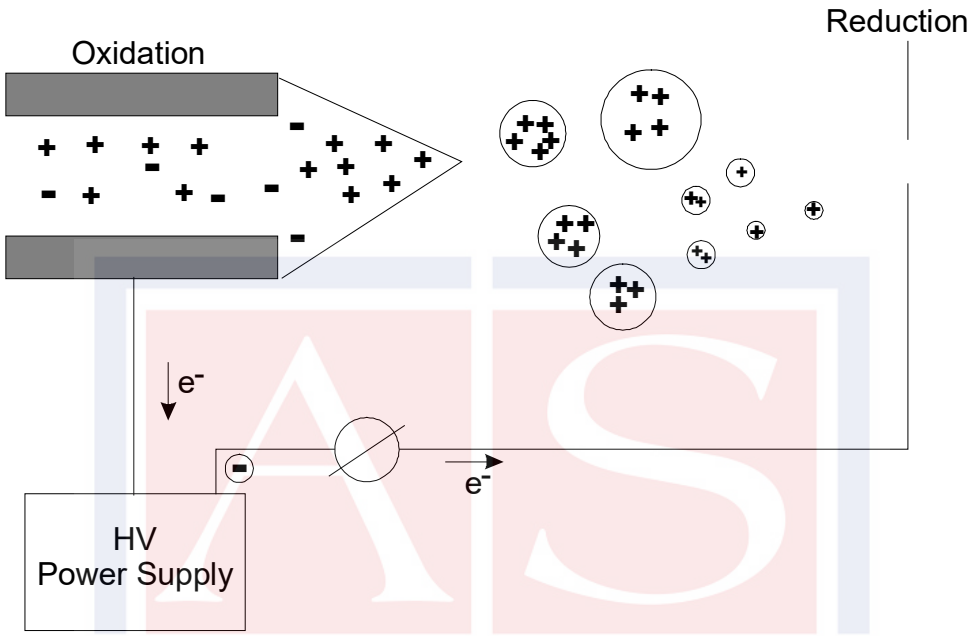
Tapered Needle

- **Advantages**
  - Provide best absolute sensitivity
  - Ideal when sample limited
  - Direct CE/MS coupling (discussed later) and capillary LC/MS
- **Disadvantages**
  - Plugs
  - Fused silica tip short lived



Student Notes: Micro Tip Electrospray is a relatively new technique and information is provided for those who have applications that demand maximum sensitivity or minimal sample consumption. See Anal. Chem., 68, 1-8 (1996) for further information.

Electrochemical Nature of Electrospray



Oxidation at the liquid/metal interface at the electrospray needle

$$Fe_{solid} \longrightarrow Fe^{+2} + 2e-$$

Reference:

Anal. Chem. 63, 2109-2114, 1991.

Why Positive Ion Electrospray Signals are Detected at High pHs

- Electrochemical oxidation in needle lowers pH by 3-4 units

$$4OH^- \longrightarrow 2H_2O + O_2 + 4e^-$$

(in neutral solution $2H_2O \longrightarrow 4H^+ + O_2 + 4e^-$

- $[M+NH_4]^+ \xrightarrow[\text{in declustering region}]{\text{CID}} [M+H]^+ + NH^3$

References:

Anal. Chem., 66, 712-718, 1994
Rapid Comm. Mass Spectrum, 11, 1120-1130, 1997
Anal. Chem., 71, 769-776. 1999

Solvents Compatible for API-MS

Suitable for ES and APCI	Suitable for only APCI
Methanol	Toluene
Ethanol	Benzene
Propanol	Hydrocarbons (e.g., Hexane)
Isopropanol	Styrene
Butanol	CCl_4
Acetonitrile	CS_2
Water	Cyclic Hydrocarbons (e.g., Cyclohexane)
DMF(1)	
DMSO(1)	
Acetic Acid	
Formic Acid	
Acetone	
CH_2Cl_2	
$CHCl_3$	

(1) At lower solvent percentages (~10% or less) under ES conditions

Student Notes: Solvents suitable for electrospray will permit the formation of ions in solution, easily nebulized and desolvated and have minimal solvation energy. Solvents like H_2O easily support the formation of ions in solution but it's solvation energy makes ion desorption more difficult. Isopropanol is a good solvent since it can support ions in solution but solvation energy is much lower than water. Toluene will not support the formation of ions in solution (precipitation will occur).

High Polarity Solvents Leads to Higher Charge States in Multiple Charged Ions

Polarity increases, surface tension increases resulting

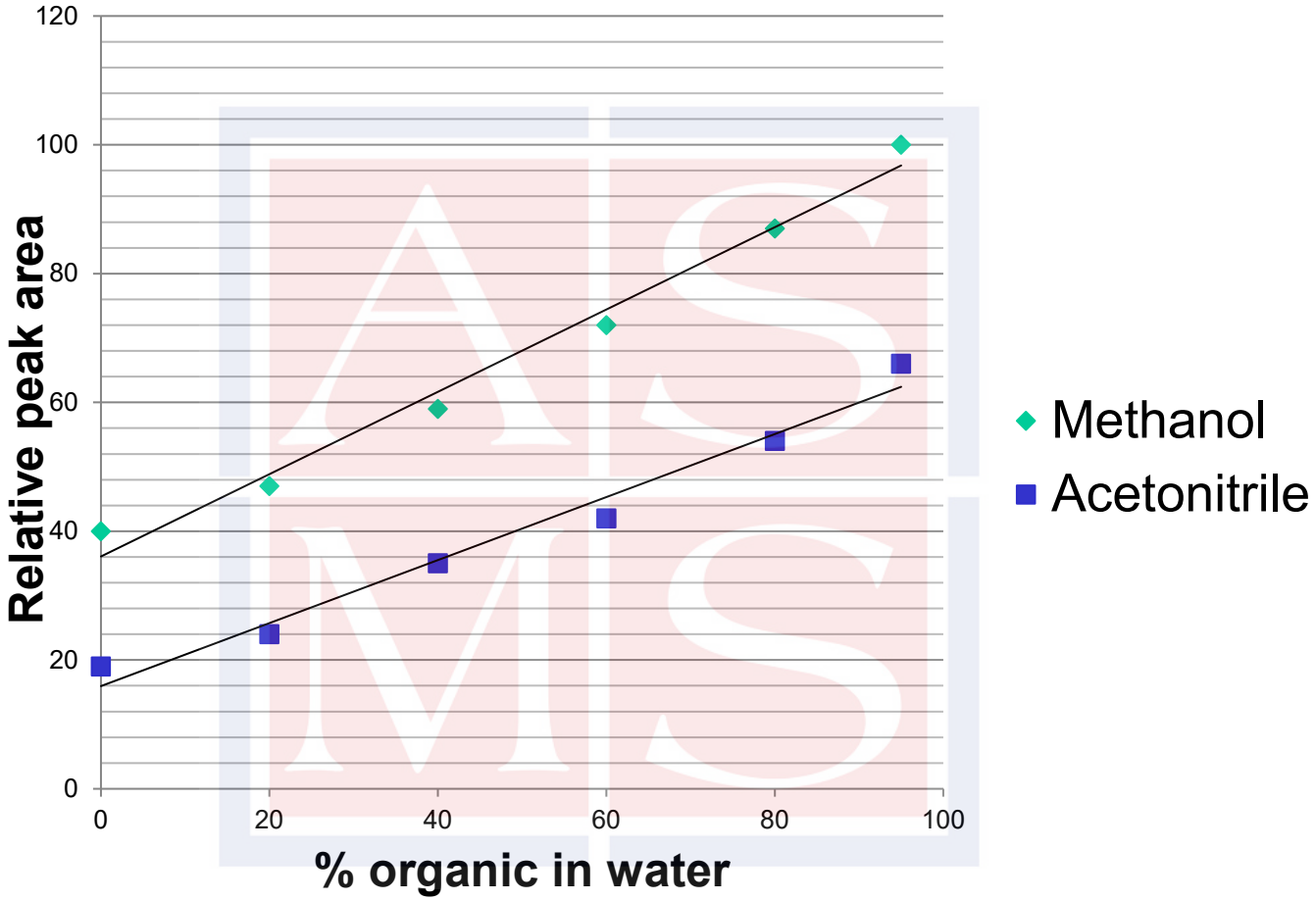
an increase of charge (q_r) on the droplet

As q_r increases the charge state increases

H_20 > MeOH > EtOH > IPA > BuOH

More charging----------Less charging

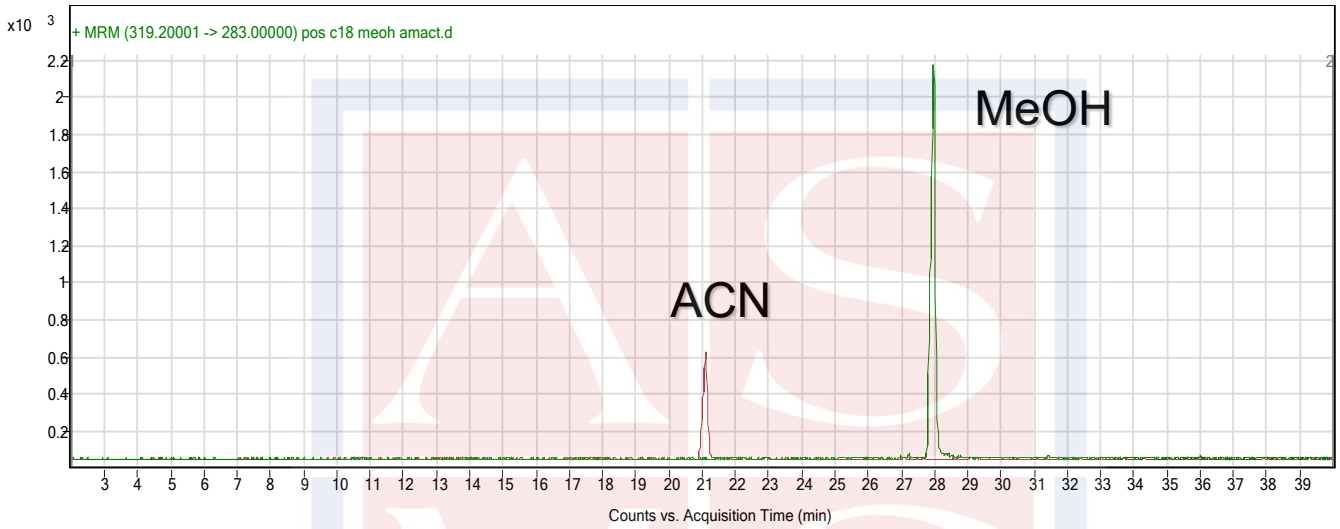
Response for Penicillin G in Various Percentages of organic



Student Notes: Higher percentages of organics usually result in better ES sensitivity due to easier desolvation and weaker solvation energy. Also response does not vary drastically over a range of organic percentages, an advantage when performing a gradient LC/MS analysis.

Improving sensitivity in LC/MS by eluting the target peak in a more organic mobile phase

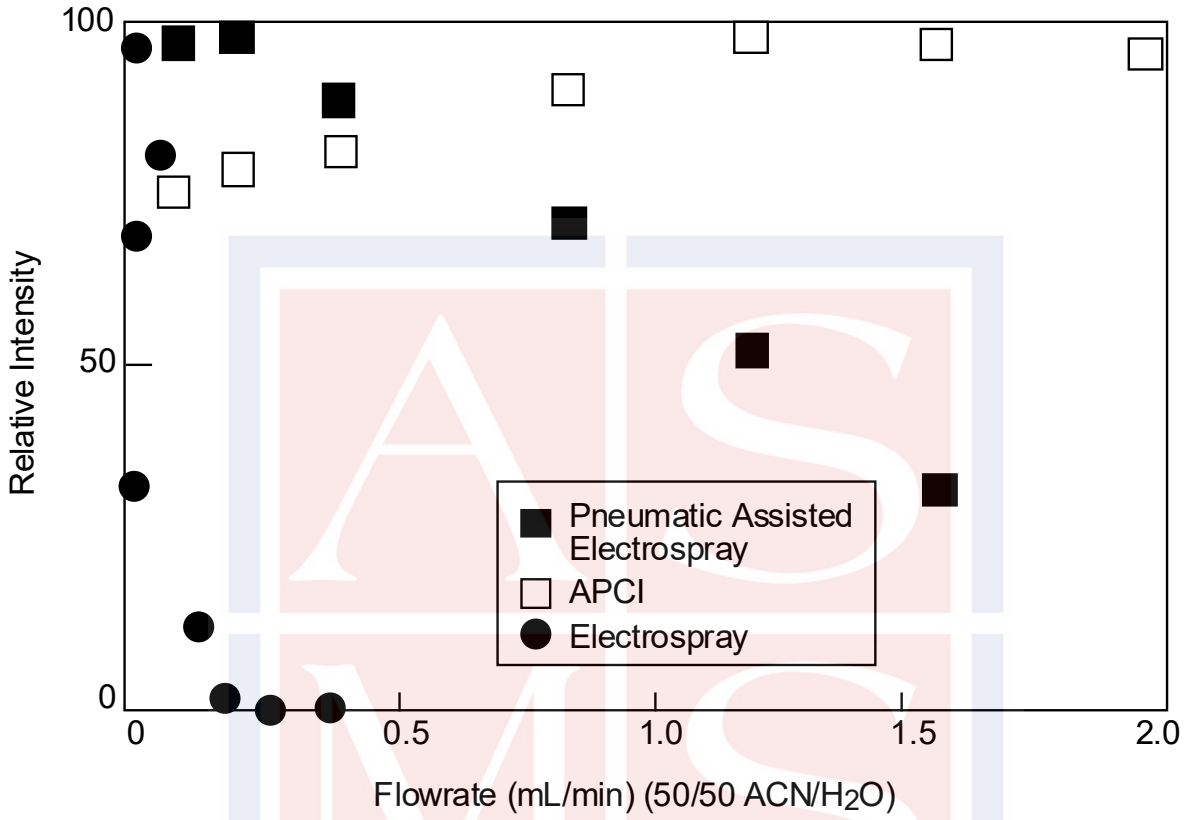
Separation of zearalenone (Mycotoxin) using a c18 column under identical conditions except for the organic modifier (ACN vs. MeOH)



How to achieve elution at higher percent organic

- Change stationary phase (e.g. C18, phenyl, CN) to more closely match sample
- Choose weaker organic mobile phase (e.g. MeOH)
- HILIC separations
- Post column addition of organic (does gain out weigh the loss from dilution)

API-MS Flow Rates



- Higher flow rates are achieved using pneumatic assistance or thermal nebulization (APCI)
- Electrospray is limited to 1-10 μL/min due to limitations in nebulization brought about through charging a liquid

Student Notes: Using API-MS techniques of electrospray, pneumatic assisted electrospray and APCI allows most flow rates used in LC/MS and CE/MS to be achieved. Post-column addition or splitting can be used to give further versatility and will be discussed shortly.

API-MS Additives

- **pH**

  Acetic acid, formic acid, TFA, ammonium hydroxide

- **General Buffers/Ion Paired Reagents**

  Ammonium acetate/ammonium formate

  Triethylamine (TEA)

  Heptafluorobutyric acid (HFBA)

  Tetraethyl or tetrabutylammonium hydroxide (TBAH)

- **Cationization Reagents**

  Sodium or potassium acetate at the 20-50 μM level

- **General Considerations**

  1. **Ion pairing** (neutralize precharged ion when desorbed into gas phase)
  2. **Volatility** (contaminate spray chamber, plug orifice)
  3. **Conductivity** (reduces formation of small droplets for ion evaporation)

- **Cannot use phosphate, sulfate, or borate buffers typically used in HPLC**

Problems in Using Typical Buffers for Electrospray

$$[M+H]^+ + A^- \rightleftharpoons [M+H + A]^o$$

A = Sulfate, Phosphate, Borate ⇌ Favors Neutral Product (ideal for LC)

A = Acetate, Formate ⇌ Favors Charged Species (ideal for electrospray)

Ionic Pair Strength

sulfate, borate, phosphate > TFA > formate, acetate

Problems in Using Typical Buffers for Electrospray

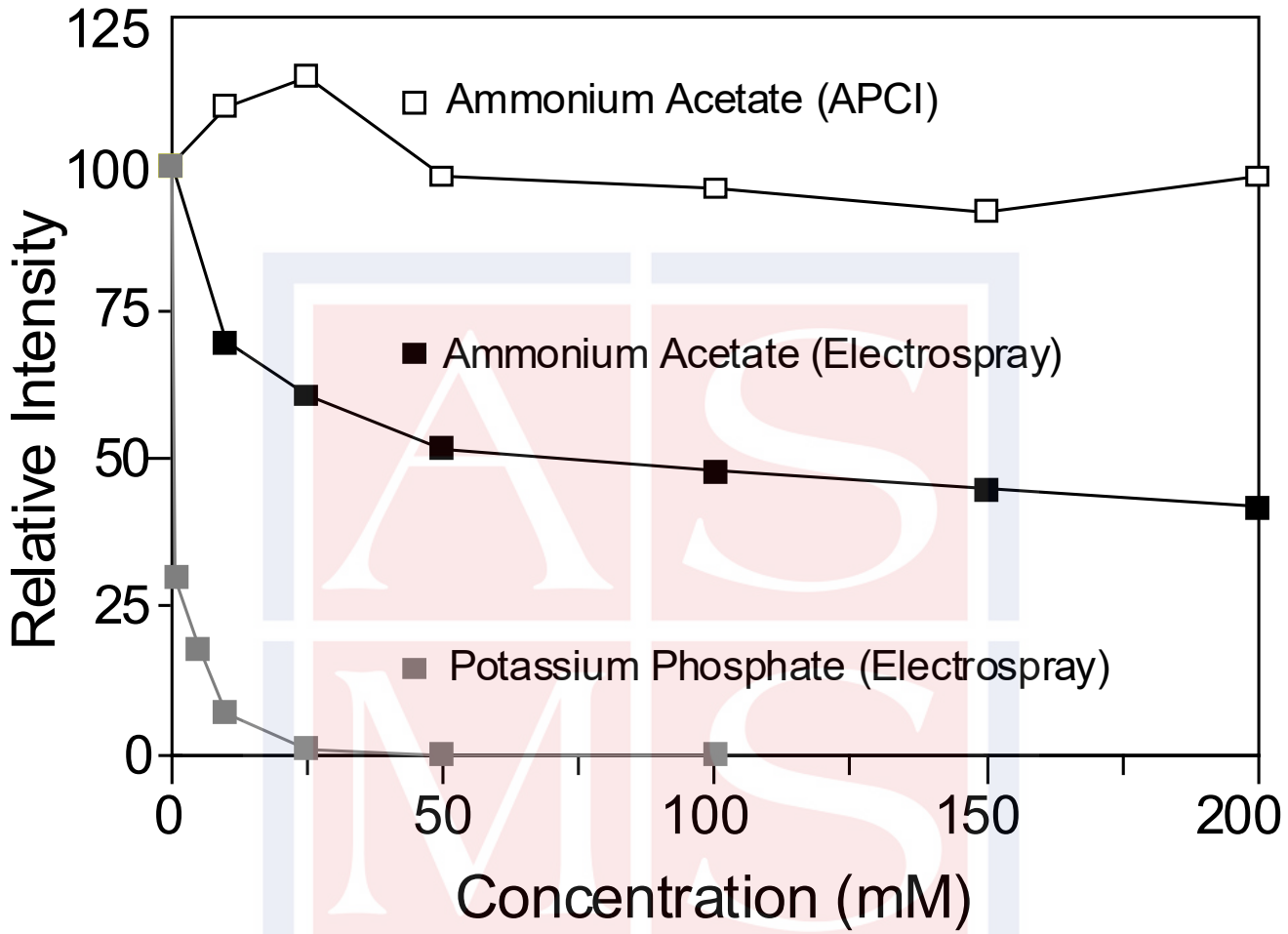
$[M\text{-}H]^- + C^+ \rightleftharpoons [M\text{-}H + C]^o$

C = Na, K, Li ⇌ Favors Neutral Product

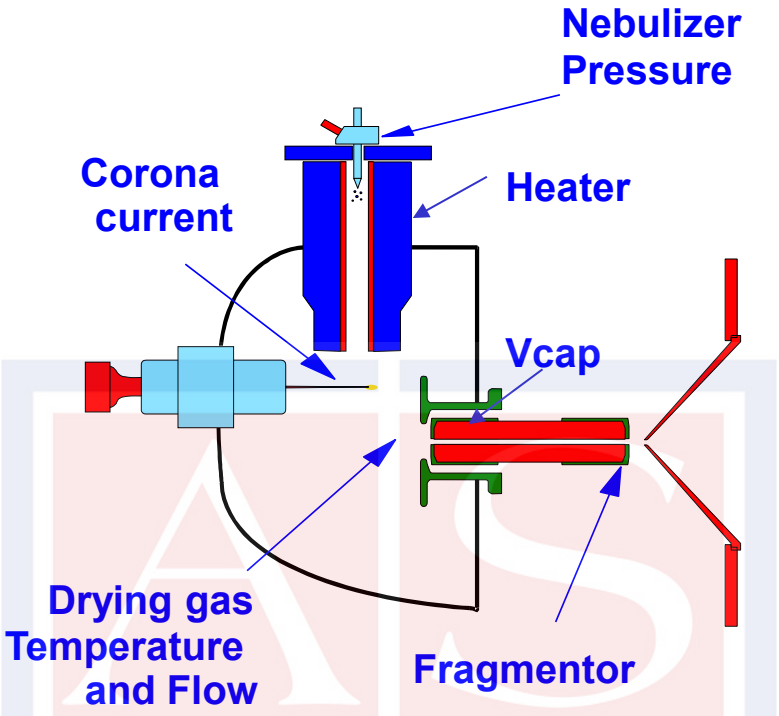
C = NH_4^+ ⇌ Favors Charged Species

Volatility only an issue due to atmospheric chamber contamination and electrical shorting

Effect of Weak and Strong Ion Pair Additives on Signal Intensity



APCI Source and Conditions

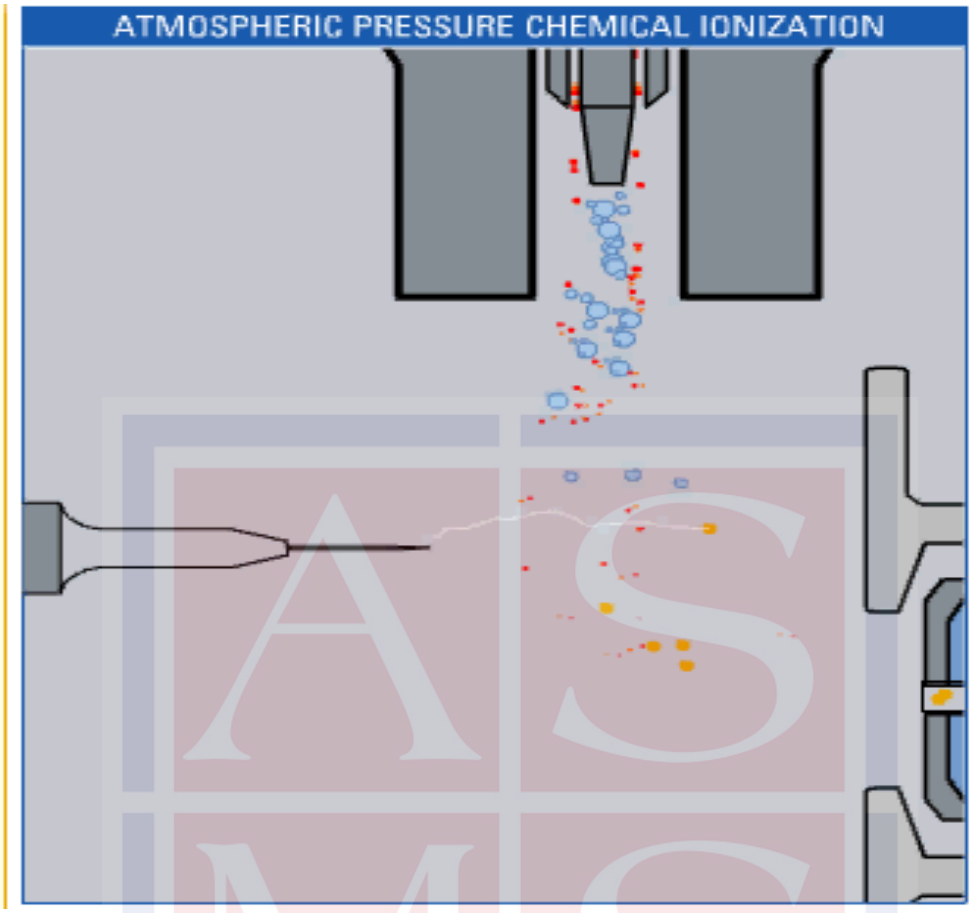


HPLC Flow Rate >100µL/min

Initial settings:

Nebulizer pressure	60 psig
Drying Gas Temperature	start with 350° C
Drying gas flow	4 L/min
Vaporizer temperature	optimize with FIA
Vcap	start with 2500 V
Corona current	optimize with FIA start with 25 µA (neg) or 4 µA (pos)

APCI Mechanisms



Mechanisms of Ionization

- Vaporization Þ Solvent ionized Þ Charge transfer to analyte

$$N_2 + e^- \longrightarrow N_2^{+\bullet} + 2e^-$$

$$N_2^{+\bullet} + 2N_2 \longrightarrow N_4^{+\bullet} + N_2$$

$$N_4^{+\bullet} + H_2O \longrightarrow H_2O^{+\bullet} + 2N_2$$

$$H_2O^{+\bullet} + H_2O \longrightarrow H_3O^+ + OH\bullet$$

$$H_3O^+ + M \rightleftharpoons [M+H]^+ + H_2O$$

APCI

Key Parameters

- **Ionization**

  Gas Phase CI

  Protonation (e.g., H_3O^+) (Bases)

  Charge exchange

  Deprotonation (acids)

  Electron Capture (halogens, aromatics)

- **Probe Temperature**

  Higher temperatures to desolvate and vaporize sample.

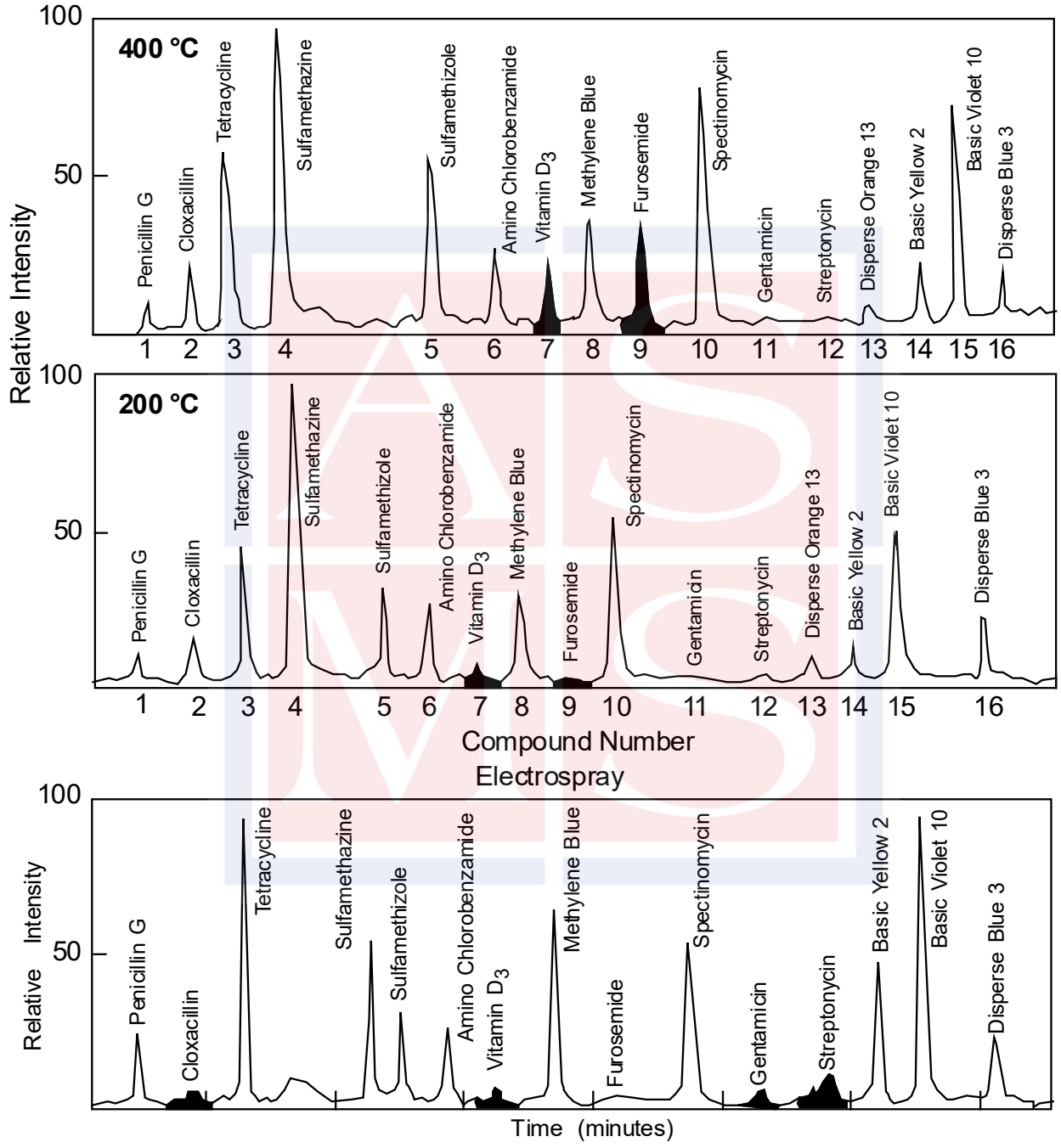
  Too high temperatures lead to sample decomposition.

- **General:**

  APCI less dependent on solvent choice, flow rates, or additives compared to electrospray.

Student Notes: APCI is a gas phase ionization technique. The probe temperature is the most important parameter to achieve good sensitivity and minimal decomposition.

APCI Evaluation of 16 Compounds at Probe Temperatures of 200 °C and 400 °C



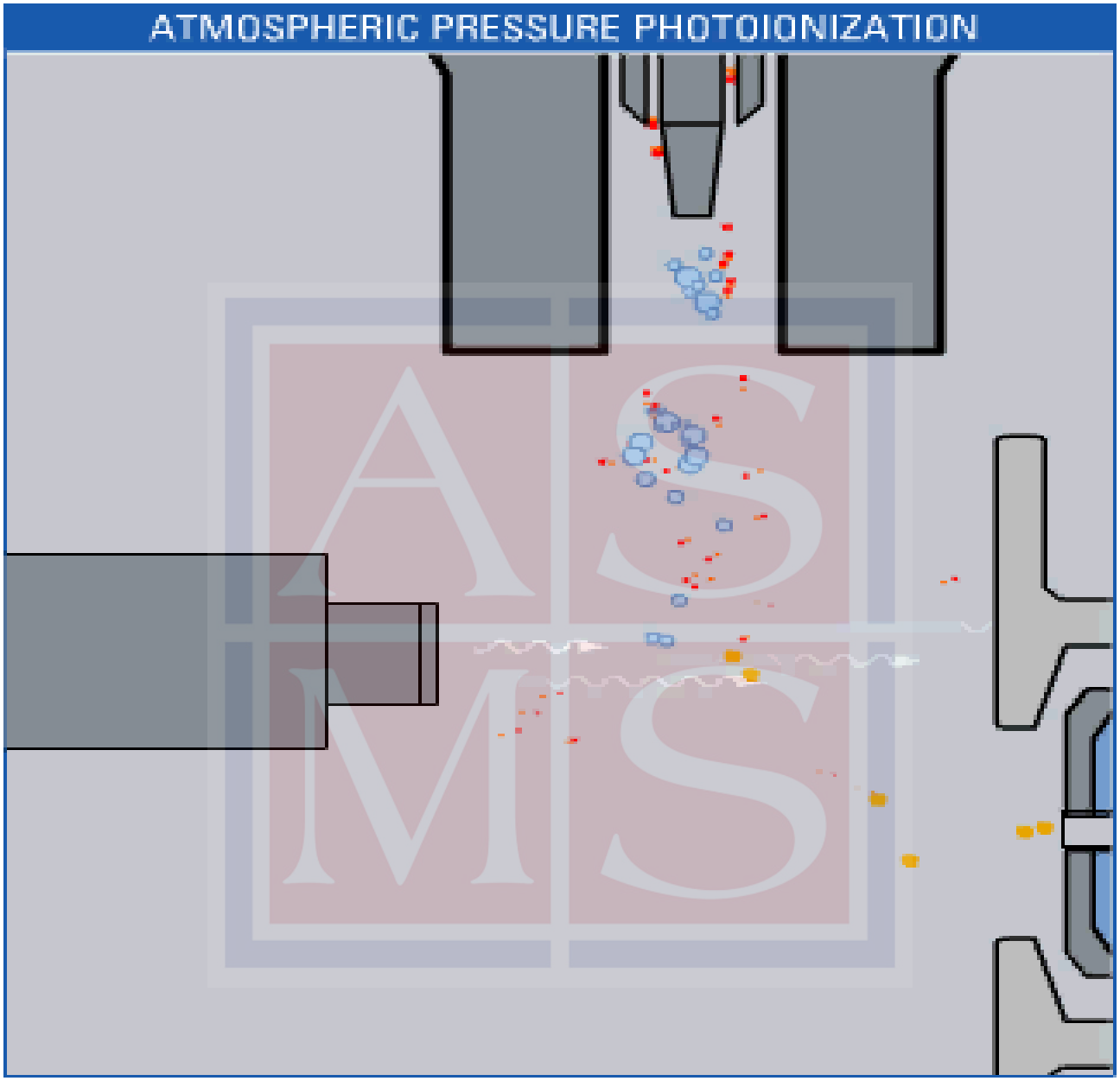
Student Notes: Higher probe temperatures (200 → 400 °C) where necessary for the analysis of furosemide and vitamin D3. Also, comparing APCI to electrospray, compounds that are charged in solution (e.g., penicillin, methylene blue, basic yellow 2, basic violet 10) or nonvolatile (e.g., gentamicin, streptomycin) are favored for electrospray analysis.

When to Use APCI

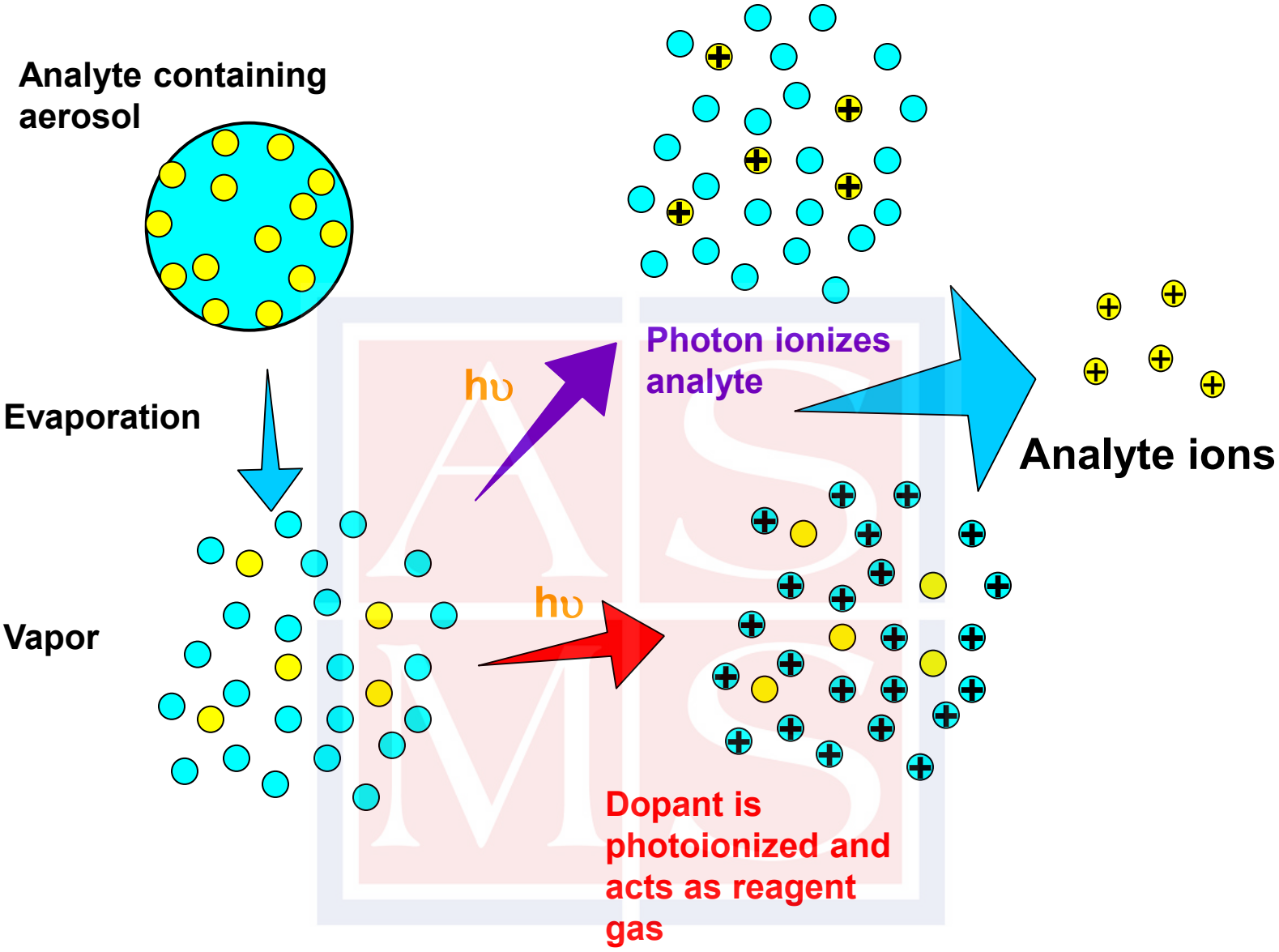
- Sample contains no acidic or basic sites (e.g. hydrocarbons, alcohols, aldehydes, ketones, esters).
- Sample can be vaporized.
- Flow rates, solvents or additives not compatible with electrospray.
- Sample exhibits a poor electrospray response
- Ease of operation/ruggedness of API-MS method.

Student Notes:The above bullets highlight some of the key reasons to use or evaluate APCI for the analysis of a sample.

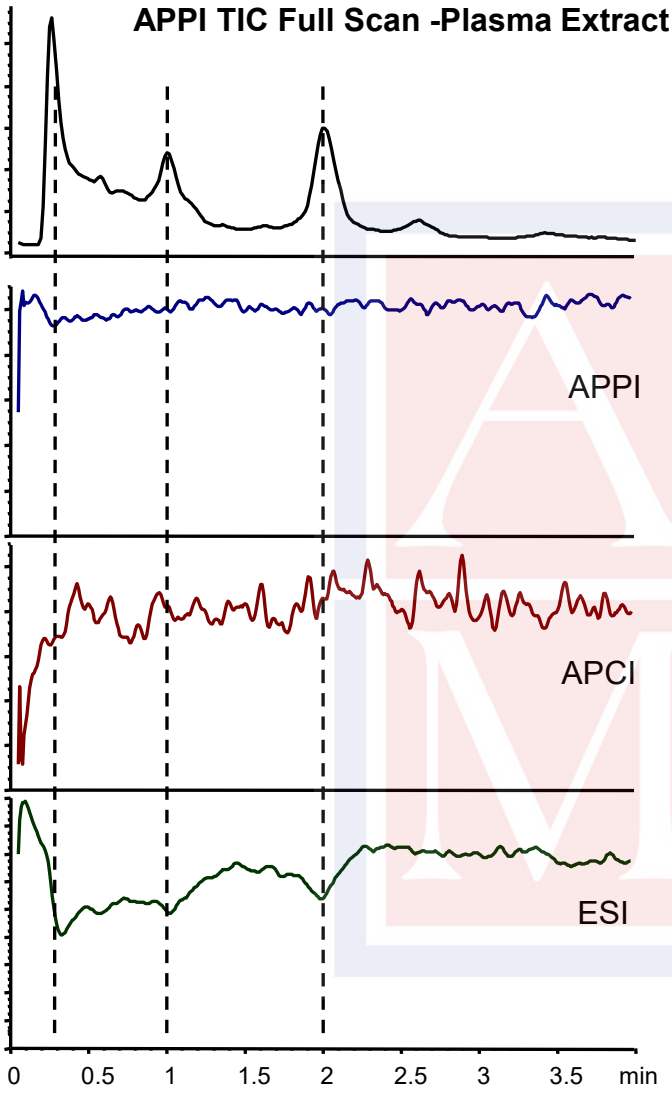
APPI Source



APPI Mechanism



APPI Very Resistant to Ion/Matrix Suppression



Determination of ion suppression susceptibility for APPI, APCI, and ESI by post-column addition and detection of fluphenazine while running an LC/MS chromatogram of rat plasma.

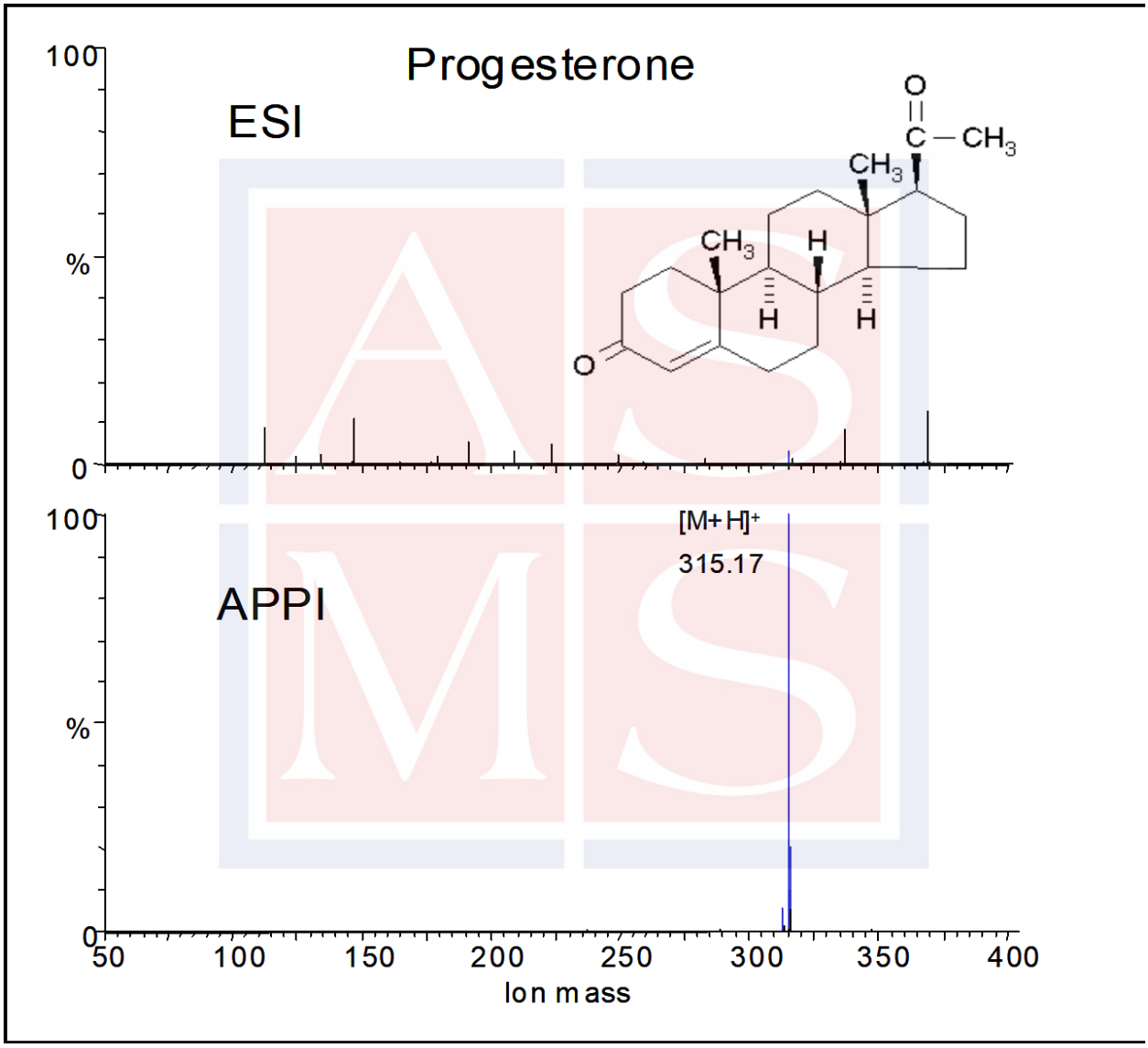
Ratio of signal for FIA/MS vs. LC/MS

	APPI	APCI	ESI
Average deviation	74%	57%	23%
Median deviation	68%	52%	22%

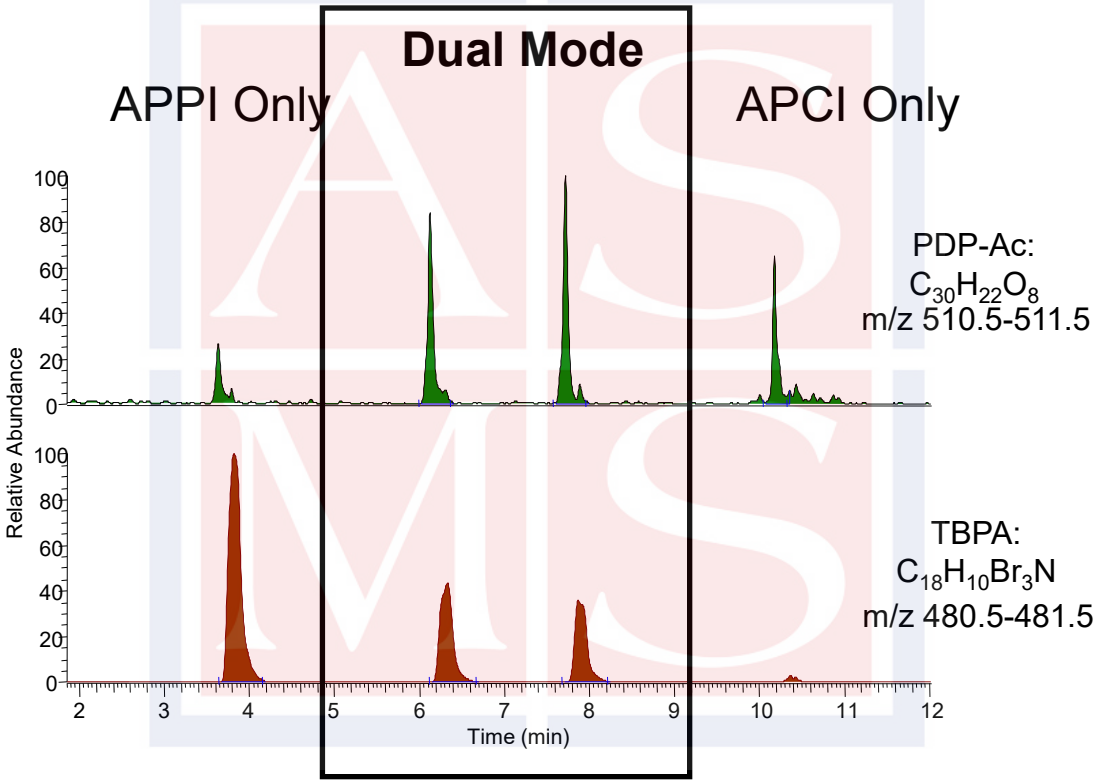
Measurement performed on a 29-compound library of unknown purity

These results show minimal ion suppression by APPI. APCI shows less ion suppression than ESI, but APCI signal is noisier than by APPI

APPI and ESI of Progesterone

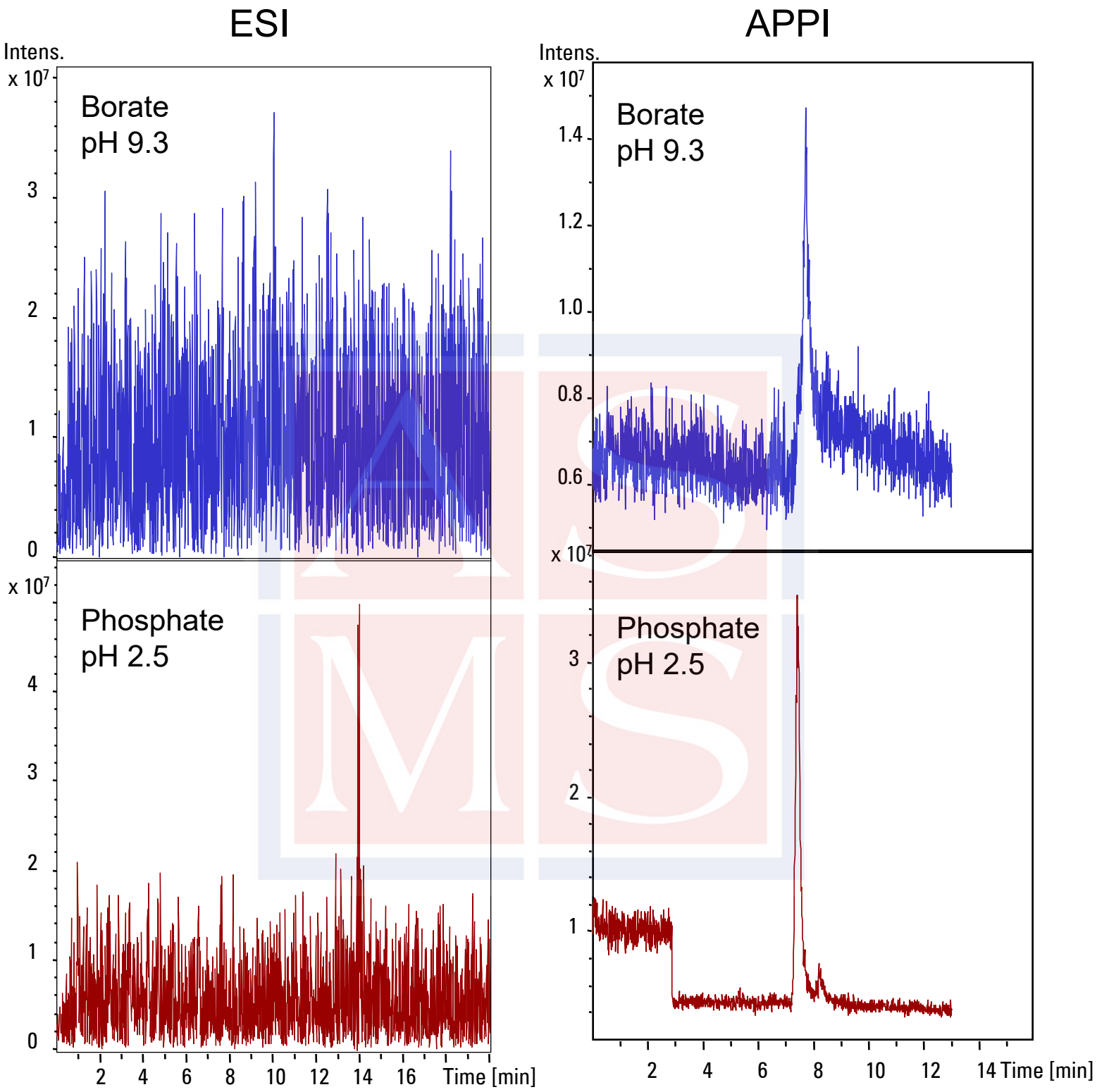


Dual APPI and APCI mode of Operation



EIC traces for PDP-Ac and TBPA for APPI only, dual mode, and APCI only mode This example shows the utility of the dual mode for simultaneously detecting both compounds.

Using Common buffers with APPI

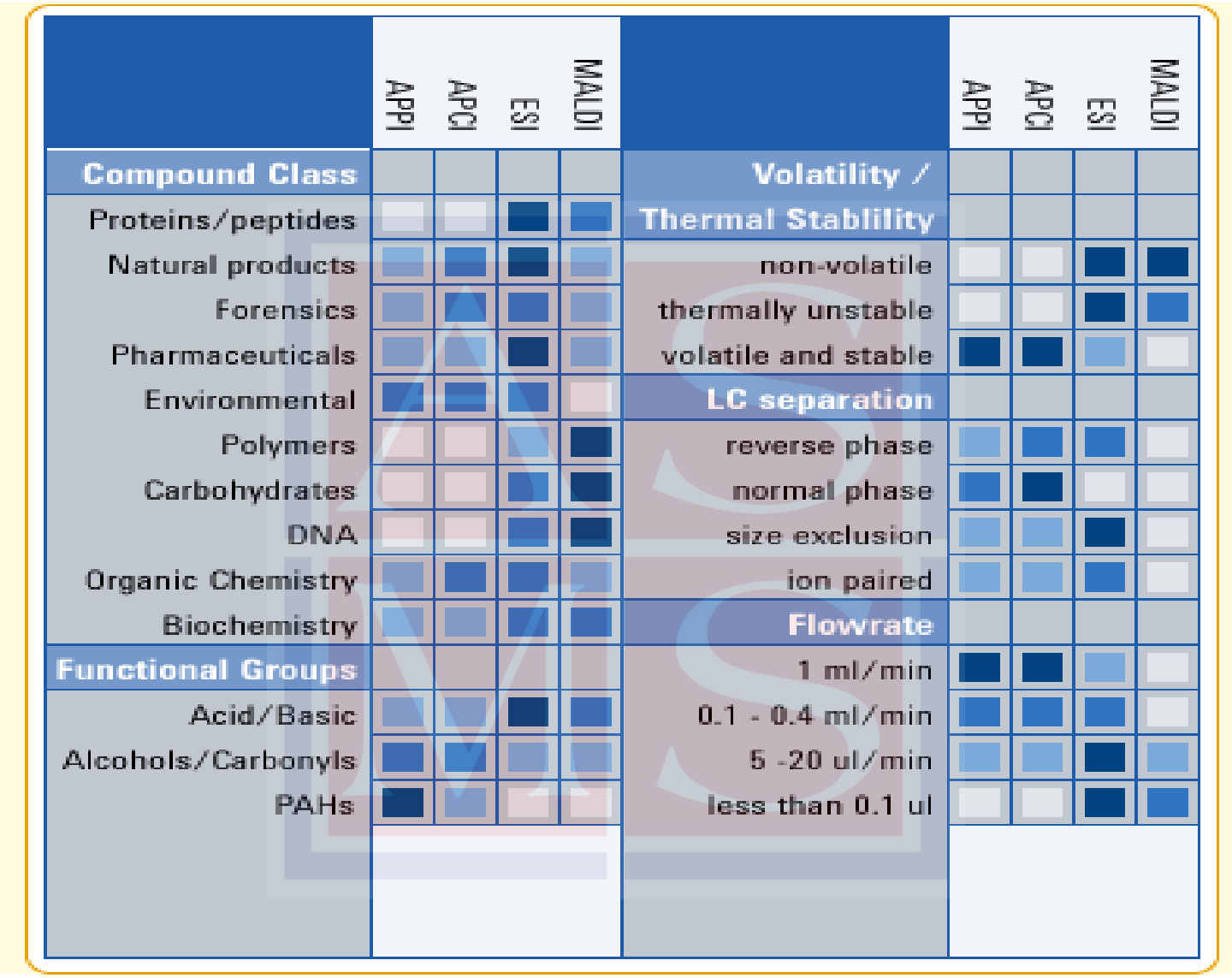


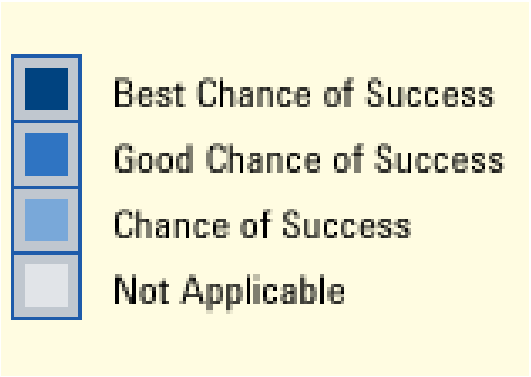
When to Use APPI

- Samples that are aromatic or have conjugated double bonds (direct ionization)
- Sample contains no acidic or basic sites (e.g. hydrocarbons, alcohols, aldehydes, ketones, esters)- dopant ionization
- Sample can be vaporized.
- Flow rates, solvents or additives not compatible with electrospray (e.g. normal phase separations).
- Sample exhibits a poor electrospray response
- Sample is masked by LC solvent background (Direct APPI does not ionize common solvents)

Student Notes:The above bullets highlight some of the key reasons to use or evaluate APCI for the analysis of a sample.

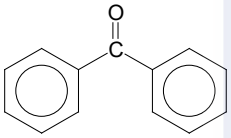
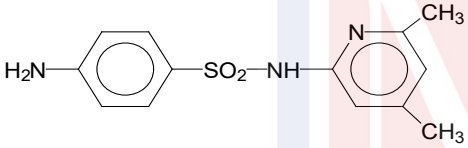
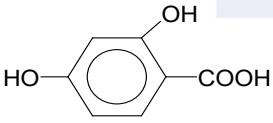
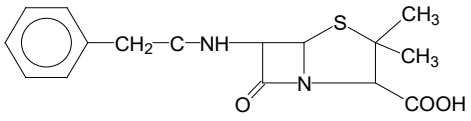
Choosing the Right API Technique





Quiz

Decide on the probable mode of API-MS operation, polarity of detection and any mobile phase changes that should be performed to analyze the following samples: ***(Choose one response in each column)***

Compound	Mode (Electrospray APPI or APCI)	Polarity (+ or - ion)	Mobile Phase (pH, post column addition)
1. Peptide: TYR-GLY-PHE-LYS-MET	APCI ES	+ -	Increase pH Decrease pH
2. Benzophenone 	APCI ES	+ -	Increase pH Decrease pH
3. Sulfamethazine 	APCI ES	+ -	Increase pH Decrease pH
4. Dihydroxybenzoic Acid 	APCI ES	+ -	Increase pH Decrease pH
5. Penicillin G. 	APCI ES	+ -	Increase pH Decrease pH

Coupling API to Liquid Phase Separations

Initial Considerations

- **Goals of the Analysis:**
    - Qualitative
    - Quantitative
    - Target compound
    - Screening
    - GLP
- **Sample Concentration and Matrix:**
    - Define sample preparation procedures that should be employed
    - Define method of sample introduction (e.g., HPLC, CE, Flow Injection)

Student Notes: Items that need to be thought out prior to performing an API-MS analysis to insure success of the determination.

Sample Preparation

- **Why?**

  Often API-MS techniques fail due to inadequate sample preparation resulting in signal suppression or common ion interferences. The main issues that need to be considered prior to API-MS, that can mean the success or failure of the analysis are:

  1. Salt
  2. Matrix components
  3. Concentration

- **Methodology:**

  The main sample preparation methods to be covered include:

  1. Ultrafiltration
  2. Solvent extraction/desalting
  3. Liquid-liquid extraction
  4. Solid phase extraction (SPE)
  5. Immunoaffinity
  6. On-column concentration
  7. Column switching (LC/LC)

Student Notes: Often sample preparation procedures are needed to provide the specificity or sensitivity to achieve a successful API-MS determination. The following pages will highlight some sample preparation procedures used for API-MS.

Liquid-Liquid Extraction

Mechanism:

- Selection of immiscible solvents and conditions (e.g., pH, ion pair reagent) to promote the partitioning of the analyte from the aqueous phase (sample) into the selected organic solvent phase. The organic phase is removed from the extraction container and concentrated for analysis.

Applications:

- Collection of lipophilic or neutral (at the pH selected) analytes in the presence of polar organics
- Concentration of analyte

Advantages:

- Many choices of solvents, pH and ion pair additives to perform the extraction
- Widely used
- Provides enrichment

Disadvantages:

- Off-line process
- Often leads to large volume of solvent usage and disposal
- Sometimes difficult to obtain high recoveries
- Time consuming

Solid Phase Extraction (SPE)

Mechanism:

- Analyte of interest eluted from solid support while interferences are retained
- Interferences eluted while analyte is bound to solid support then subsequently eluted

Applications:

- Impurity removal/desalting
- Concentration of analyte
- Phase exchange
- In-situ derivatization

Advantages:

- Many choices of cartridge sizes and solid supports
- Quick and easy
- Enrichment

Disadvantages:

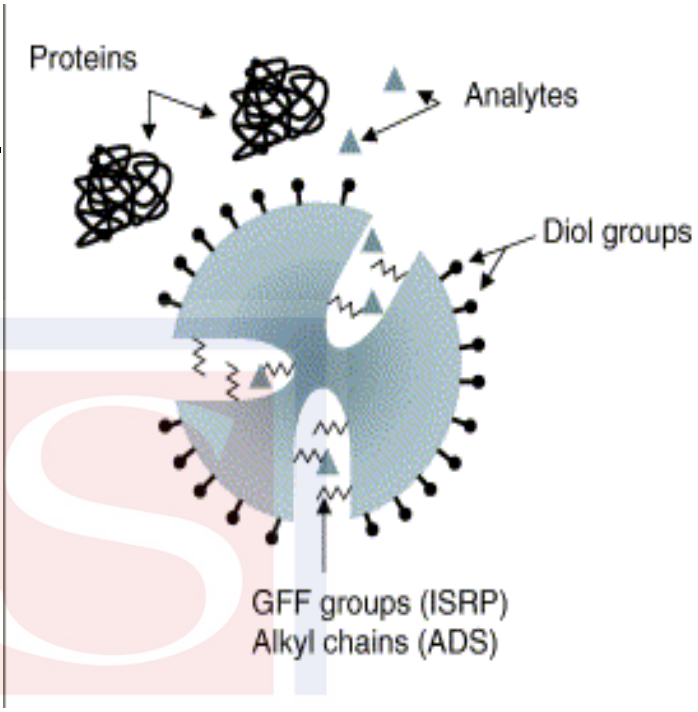
- Usually an off-line step
- Requires solvent removal for concentration
- Requires cartridge conditioning
- Dependent on loading and wash solvent, sample volume loaded and mass loading

Student Notes: Solid phase extraction methodology is briefly highlighted. This is one of the most common cleanup procedures currently used.

SPE-Restricted Access Media

- Mechanism:

  Restricted Access Media (RAM)has two distinct surfaces. The outer surface offers selectivity by size exclusion. Only small molecules reach the inner surface, which offers selectivity and trapping of the analyte by conventional partitioning (e.g., C_{18}).



- Applications:
  - Separation and enrichment of small molecules in the presence of large molecules.
- Advantages:
  - Can handle matrices such as blood, urine, serum
  - Removes the need to perform protein, precipitations, solvent extracts and solvent removal
  - One step clean-up offering both size exclusion and C_{18}.
  - Sample concentration.
- Disadvantage:
  - Most work with pH and solvent system to prevent protein precipitation and plugging of cartridge or column.

Immunoaffinity

- Mechanism

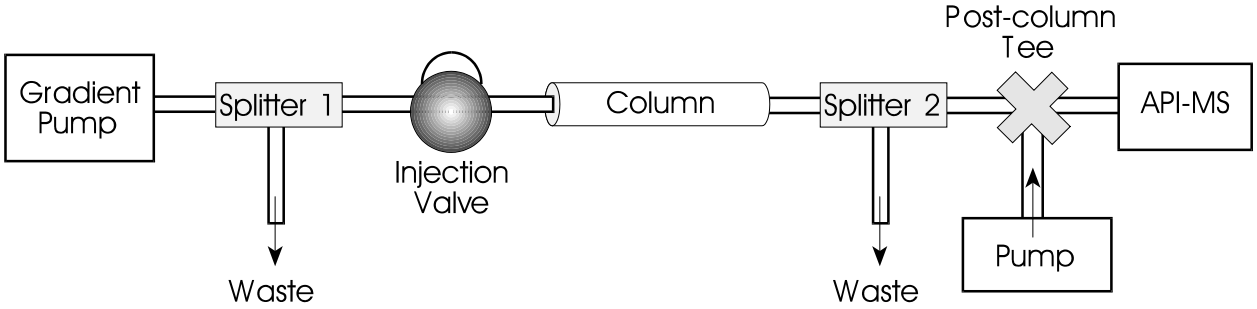
  Highly specific biochemical interaction between a bound ligand and the sample. This interaction is broken when pH or ionic strength of solution is changed.

- Advantages:
  - High degree of sample cleanup
  - Concentrates sample (200x)
  - Automation
  - Some commercial sources/also activated columns available for immobilization of user generated antiserums
- Disadvantages
  - Few affinity column commercially available
  - Often requires user to produce specific antiserum, purify the antiserum, immobilize the antiserum to sepharose and pack the column
  - Affinity columns can be deactivated if exposed to harsh conditions (e.g., organic, high temperature, pH)

Student Notes: Immunoaffinity methods offer the highest degree of sample cleanup and sample concentration. Each affinity reagent is specific for a particular site on a compound. Reagents from different manufacturers raised to be active for a given compound may exhibit different cross reactivity due to differences in the sites of recognition.

HPLC/MS

- **Hardware:**



Pump mL/min	Splitter 1	Loopsize (uL)	Column i.d (mm).	Column Flow Rate (mL/min)	Post Column Splitter 2	Post-column Tee Pump (mL/min)
0.5 - 1.0	yes	0.2 - 10	0.32	0.004 - 0.008	No	0.002 - 0.005
0.5 - 1.0	yes	1 - 20	1.0	0.04 - 0.06	No	0.02 - 0.04
0.2 - 0.3	no	5 - 50	2.1	0.2 - 0.3	Yes (1)	0.1
1.0	no	20 - 100	4.6	1.0	Yes (1)	0.1 - 0.2

(1) Post column splitting for ES operation

Use When:

- Complex matrices
- Numerous components
- Low-mid sample concentration

- **Advantages:**
  - Fast/simple
  - Handles salts and complex matrices
  - Automation
  - Sample concentration

- **Disadvantages:**
  - Complexity and capacity are HPLC limited
  - Mobile phase compatibility between HPLC and MS.

Student Notes: Characteristics of HPLC/MS for API-MS are explained.

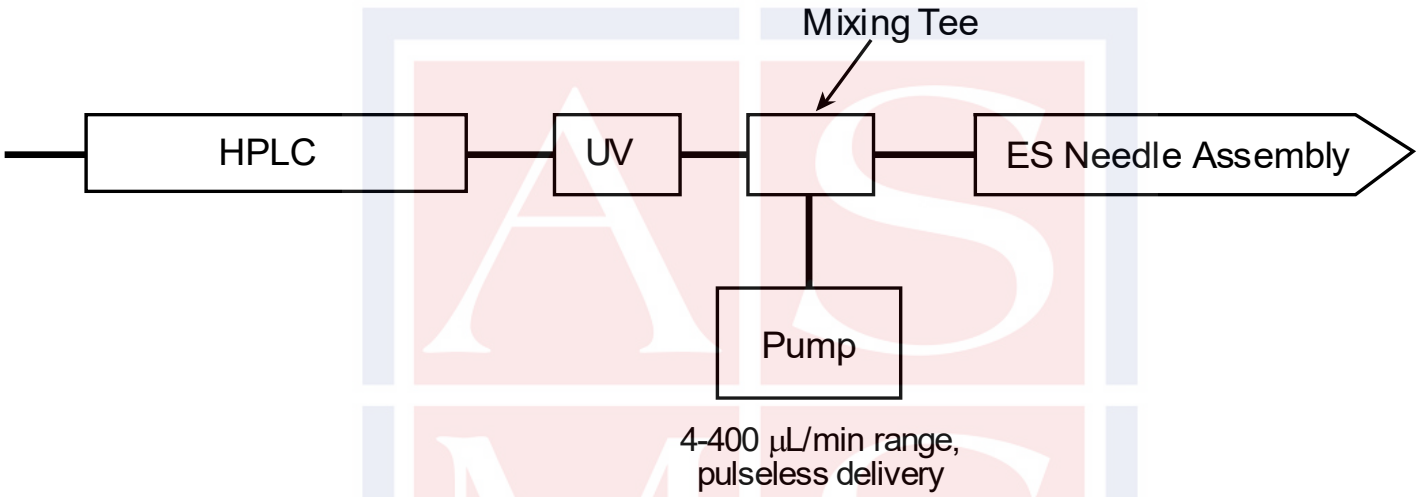
Adapting Existing LC Methods to LC/API-MS

- **Buffers**
- Sulfates, phosphates and borates need to be substituted to more volatile salts such as ammonium acetate, formate, trifluoroacetic acid (TFA), Heptafluorobutyric acid (HFBA), tetrabutylammonium hydroxide (TBAH)
- ***Ionic strength of buffer is often more important than buffer choice to achieve a separation***
- **pH**
- pH should be kept the same using volatile additives such as formic acid, acetic acid, TFA and ammonium hydroxide
- **Ion Pair Reagents**
- Volatile ion pair reagents should be employed such as HFBA and TBAH. It is difficult to achieve the same retention times with ion pair reagent substitutes. Often larger ion-pair reagents resulting in m/z interferences.
- **Organics**
- Most organics typically used in LC are compatible for LC/MS
- **Columns**
- Most columns are compatible with LC/MS. Note: Columns used with ion pair reagents for LC may exhibit contaminations from alkali salts for a long time.

Student Notes: Discussion adapting existing LC conditions for LC/MS using API-MS.

Creating the Optimal Chemistry for Electrospray Through Post-Column Addition

- **How:**



Student Notes: Schematic and hardware needed for post-column solvent modification

Post-Column Modification of LC Solvent to Optimize API-MS Response

(1) pH adjustment to optimize for positive or negative ion detection.

(2) Addition of isopropanol to aid in the desolvation of aqueous solvents and to dilute ionic buffers to achieve acceptable API-MS performance.

(3) Addition of Lithium or sodium acetate post-column (50 μM) to aid in cationization of samples. (Used for samples that lack or have weak sites for protonation). *

(4) Using the "TFA FIX" to improve sensitivity for more ionic buffers. *

(5) Increase flow rate to achieve a suitable ES current (e.g., for capillary electrophoresis separation).

(6) Reduce flow rate to achieve suitable ES operation.

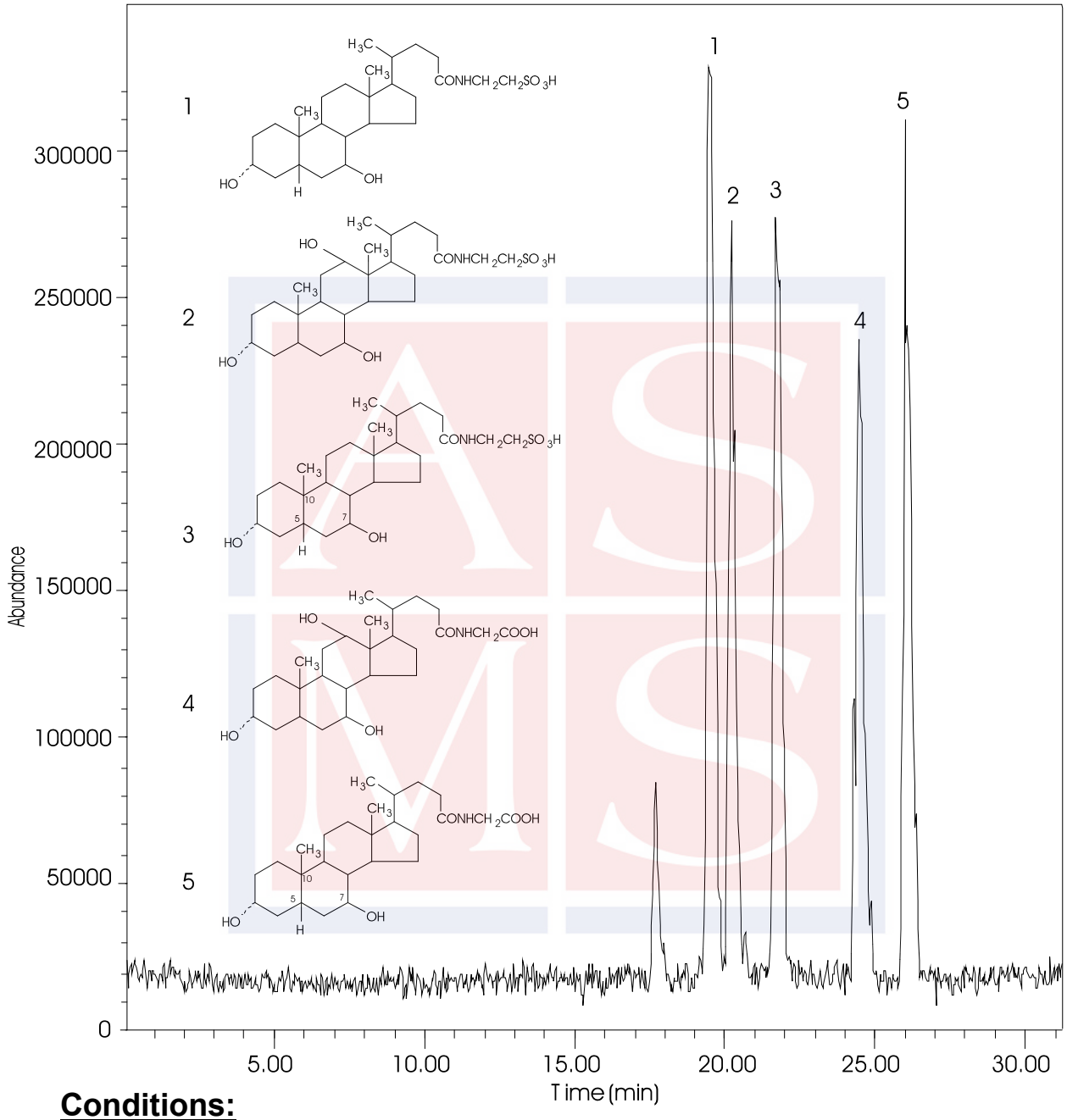
(7) Derivatization to improve electrospray sensitivity e.g.: cationic species, amine.

(8) Add alkyl ammonium salts to reduce the formation of alkali metal adducts

* Details found in following pages

Student Notes: There are at least 8 situations where post-column addition could be beneficial for an electrospray-MS determination

LC/MS Detection of Bile Acids

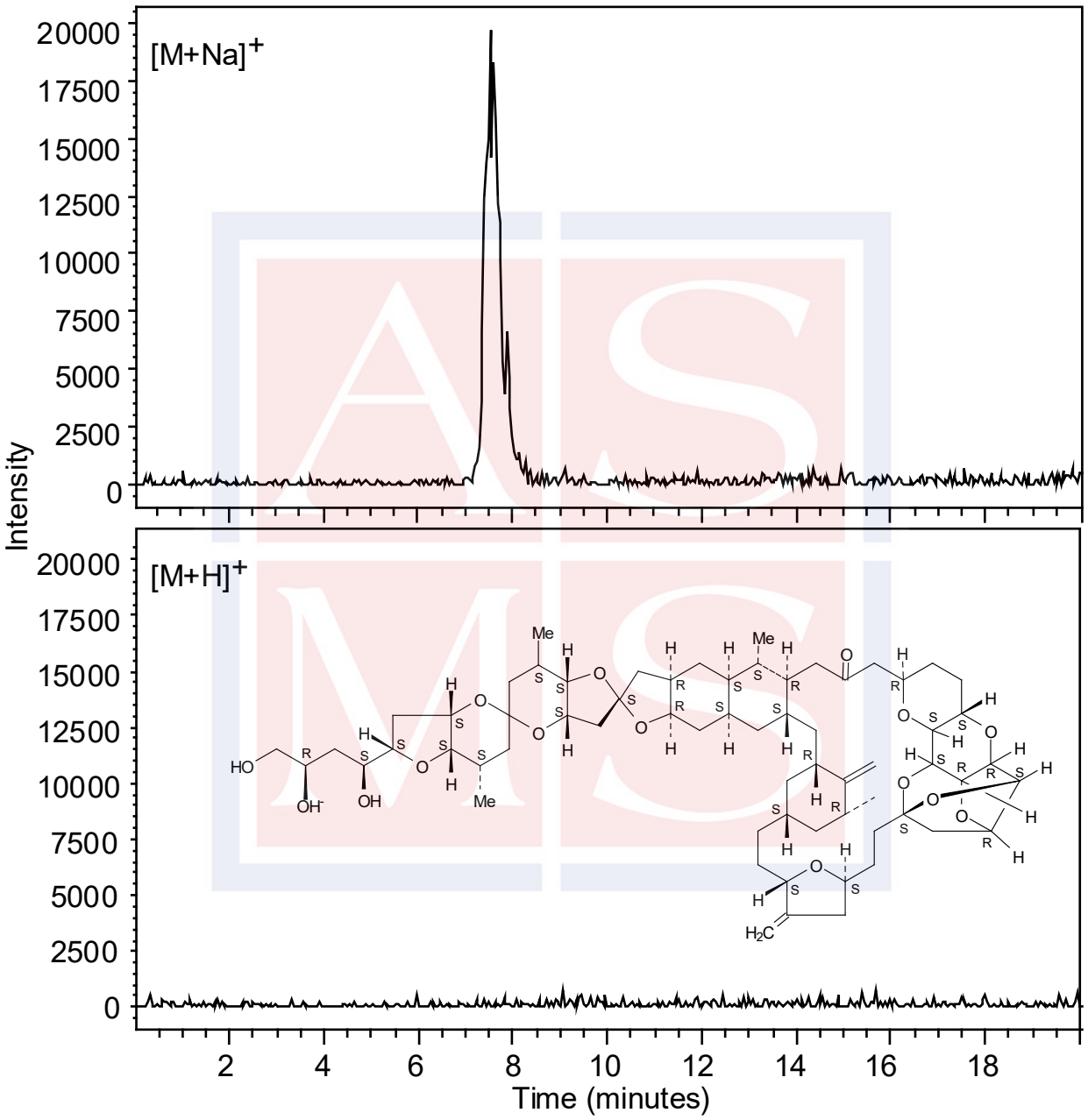


Conditions:

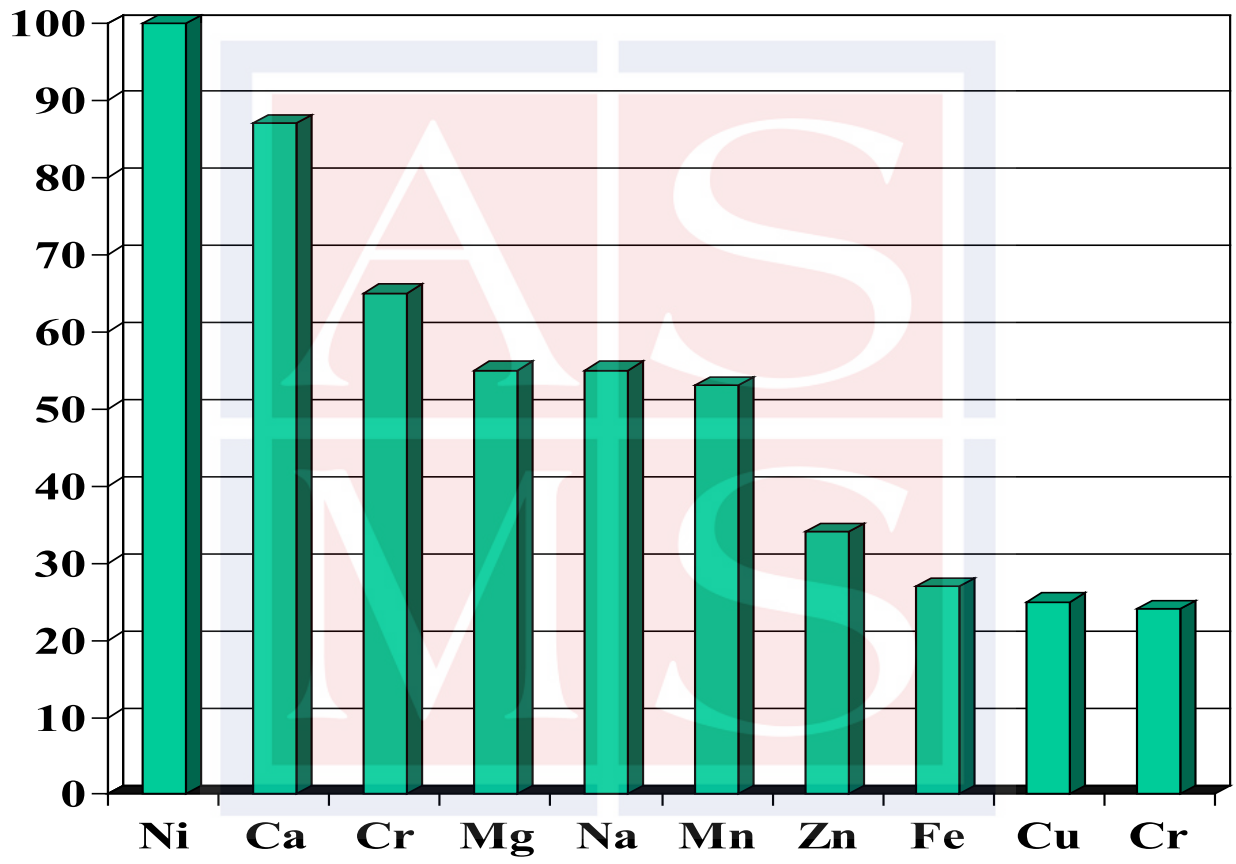
Separation on a C_{18} 100 x 4.6 mm column 0 to 100% MeOH in 30 min at 1 mL/min. pH was increased post column using ammonium hydroxide: water:isopropanol 10:40:50 at 25 μL/min ES-MS operated using negative ion detection.

Student Notes: Example of a LC/MS separation using a 4.6 mm i.d. column and post column addition of ammonium hydroxide to increase pH.

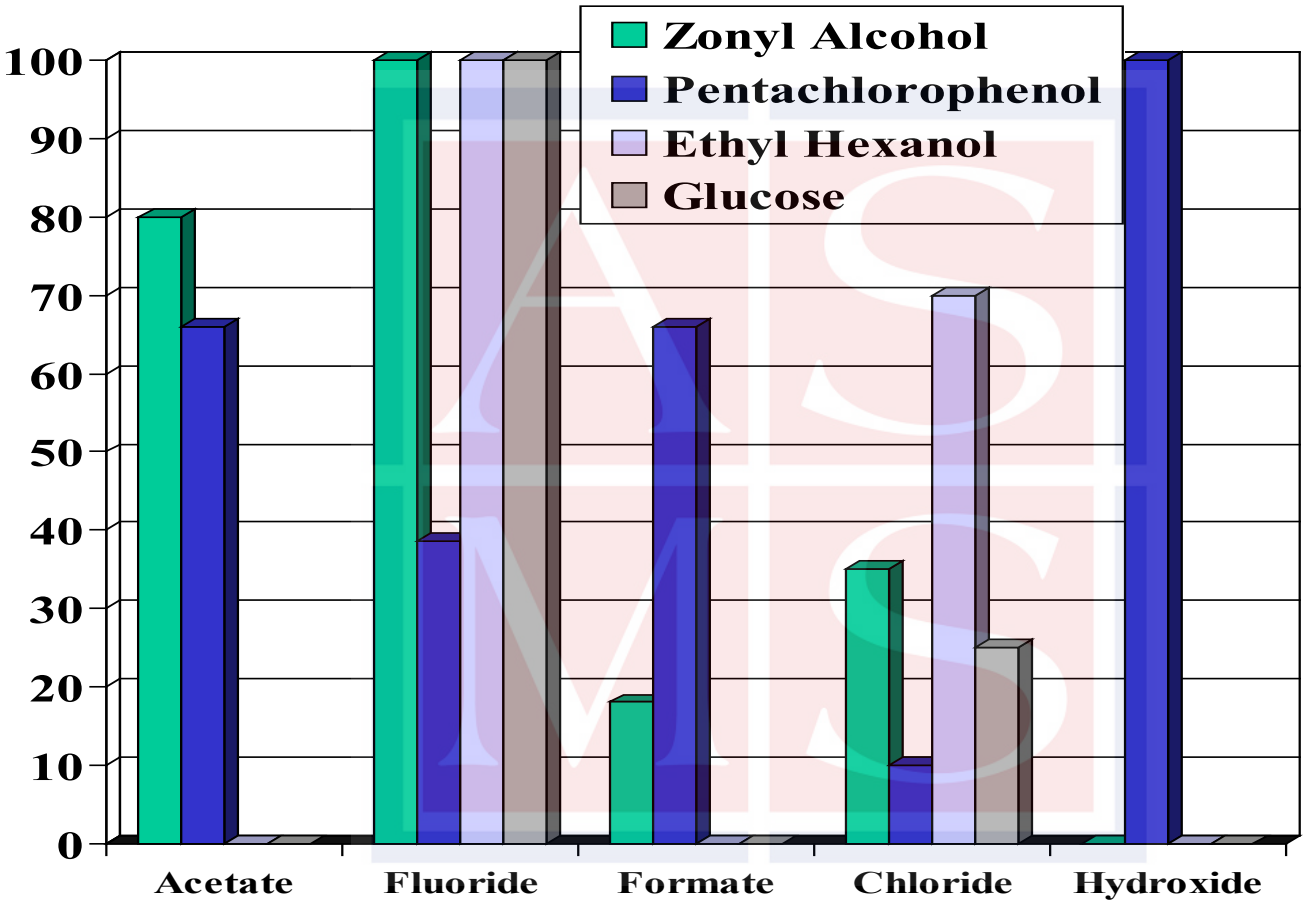
Improving Electrospray Response for Halicondrin (mw 1114) by the Formation of the $[M+Na]^+$ Ion



Metal Adducts with Small Acids to form Positive Ions By Electrospray MS



Response of Alcohols Using Various Adducts for Negative Ion Formation



TFA-Fix

Normal

$[M+H]^+ + [CF_3COO]^- \rightleftharpoons [M+H\ CF_3COO]^\circ$

(Favored)

Strong ion pairing with acid anion

TFA Fix

Solution RCOOH (propionic acid) displaces CF_3 COOH based on volatility (distillation).

- RCOOH added post-column (e.g., 20% acid, 80% isopropanol at 0.1 mL/min).

$[M+H]^+ + [RCOO]^- \rightleftharpoons [M+H\ RCOO]^\circ$

(Favored) Weak ion pair with propionic acid

Will Work When:

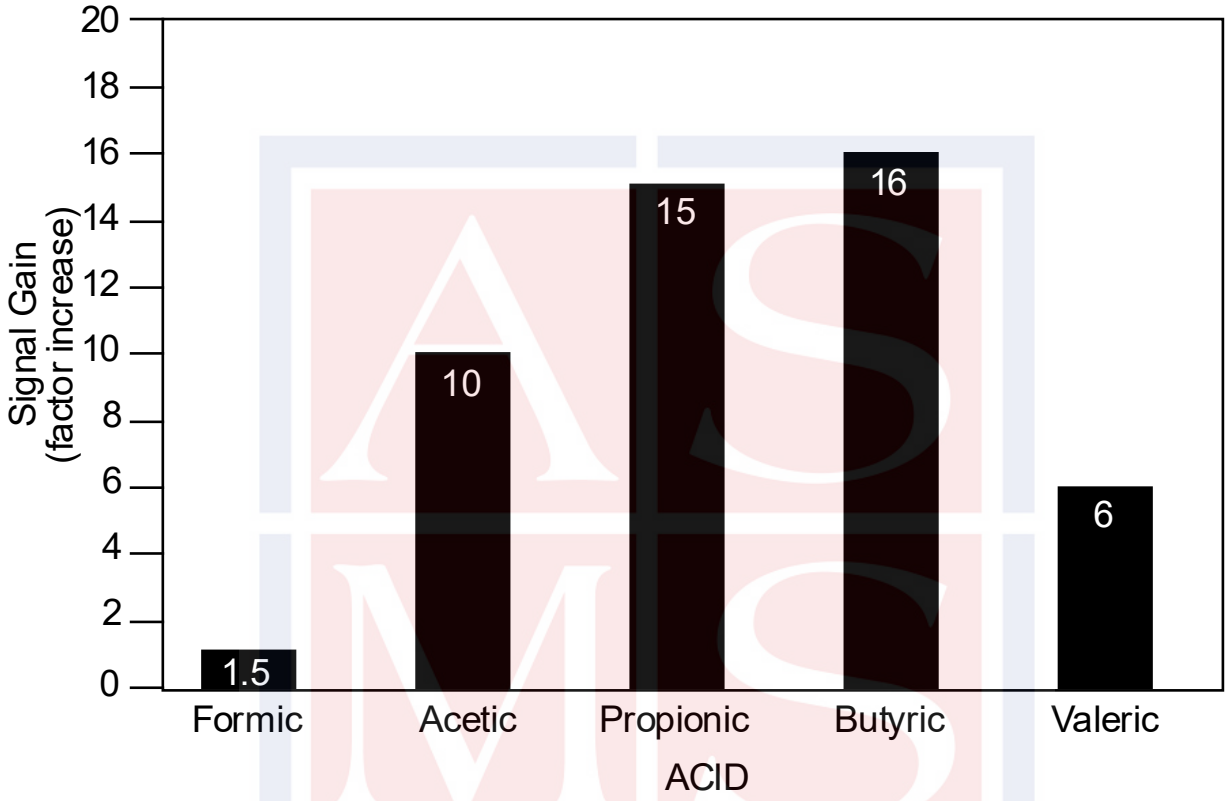
bp Weak ion-pair ion > bp Strong ion-pair ion

e.g., bp propionic acid > bp HFBA > bp TFA

Student Notes: The TFA-Fix substitutes an acid that forms a much weaker ion pair with the analyte, resulting in an increased response for the analyte ions.

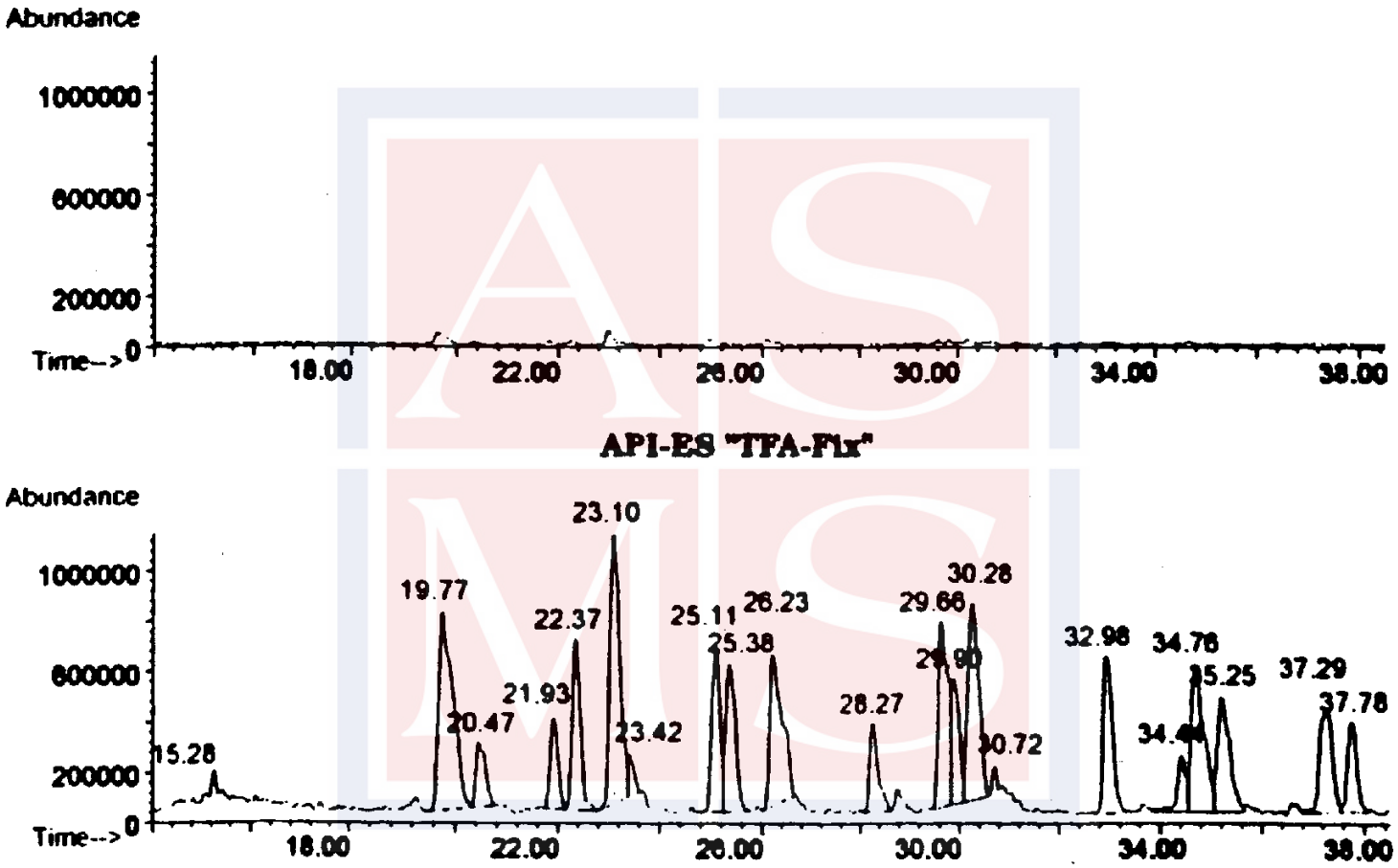
Why Propionic Acid?

- Signal Increase (20% in final solution)



Student Notes: High percentages of most weak acids will increase signal intensity in the presence of HFBA and TFA. Propionic acid offers the best compromise in sensitivity gain and laboratory compatibility (smell).

1 nmol Chicken Lysozyme Tryptic Digest Map by On-line HPLC/API-Electrospray MS



HPLC Conditions

Column: 2.1 x 250 mm Vydac C-18
Flow Rate: 200 ul/min
Solvent A: Water, 0.1% TFA
Solvent B: Acetonitrile, 0.1% TFA
Gradient: 0-60 min 0-60% B
Temperature: 50 °C

Is The TFA-Fix Needed?

$[M+H]^+ + [CF_3COO]^- \rightleftharpoons [M+H\ CF_3COO]^\circ$

↓

TFA gas

If TFA level is low enough and enough heat is applied, TFA is removed from the solution reducing the TFA concentration causing the equilibrium to favor free vs. bound

$[M+H]^+ + [CF_3COO]^- \rightleftharpoons [M+H\ CF_3COO]^\circ$

Will work at TFA levels below 0.025% (depending on the heating)

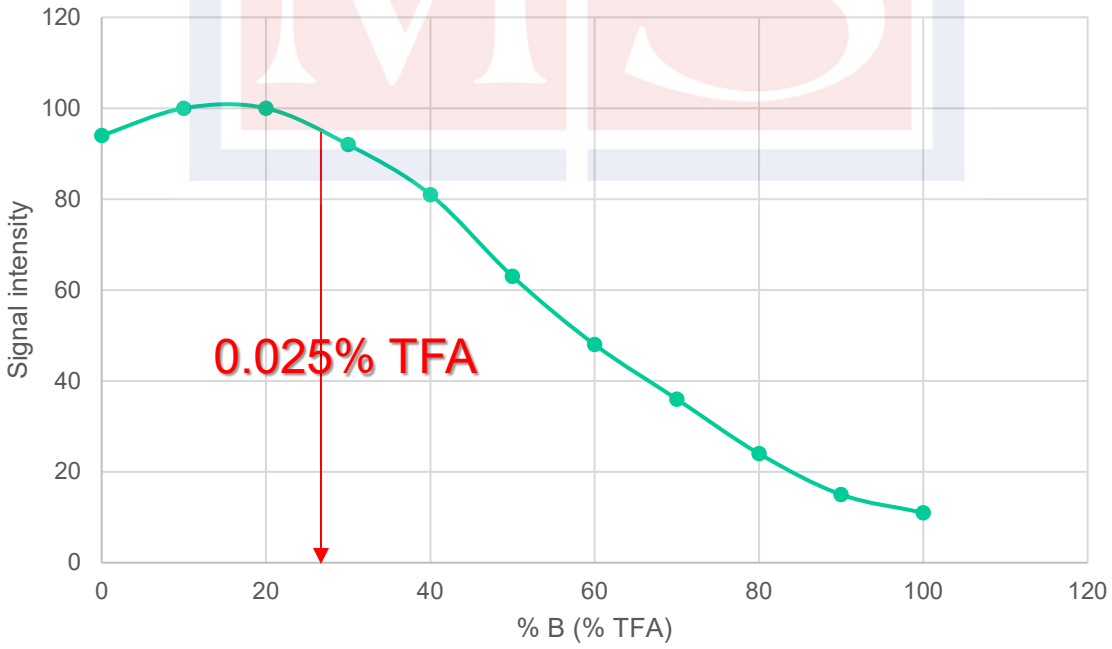
Levels approaching 0.1 % may need to be used in some cases

How Much TFA can you use without signal suppression

Different manufactures LC/MS systems apply different amounts of heat, so the amount of TFA you can use has to be determined.

How to determine:

- Run with conditions “hot” for the drying and nebulizing gas.
- Prepare mobile phases A= 50/50 ACN/H2O with 0.001% TFA. B= 50/50 ACN/H2O with 0.1% TFA.
- Flow inject the same amount of sample going from 100% A to 100% B at 10% increments
- Observe where the signal roll-off begins and this is the optimal maximum level of TFA that can be used.



High-Speed LC/MS - Turn & Burn

Mechanism:

Develop rapid separators (1-3 min) to separate target species from components that suppress API performance (e.g. salts) and rely on MS/MS - MRM for specificity.

Equipment:

- Columns

  2.1 - 4.6 mm id x 33-50 mm long columns with 1-3 µm particles operated at 1-5x above optimal flowrate.

  Tandem MS

Advantages:

- Sample throughout (1-2 minutes per analysis)
- Sensitivity-specificity is maintained
- Great for target compound analysis

Disadvantages:

- Limited clean-up interferences
- Not suitable for characterizing numerous components in a complex matrix
- Usually requires MS/MS
- Speed can be limited by LC equipment (e.g. injection rinse cycle time)

Improving LC/MS Sensitivity

- Decrease column i.d. and particle i.d.

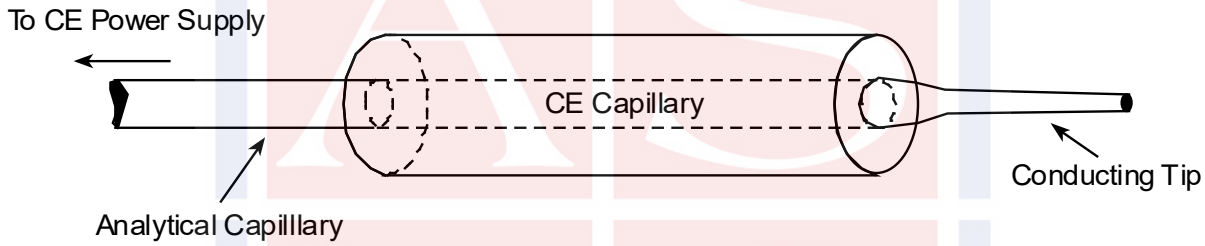
→ *5-10 μm chip technology and 1-2 um particles, nl-ul flowrates*

- On-Column concentration
- Trapping Column
- Sample preparation

Direct Coupling Capillary LC/MS

Requires

- 10-20 μm capillaries with the end tapered to electrospray at < 1 μL/min
- Gold plating to achieve electrical contact



- Special considerations in HPLC hardware and dead volumes of pump and plumbing
- Limited sample injection volumes
- Plugging of nanospray trip
- Ruggness of column and nanospray needle

Best approach when sample limited

On-Line Column Concentration

- **Equipment:**

  Conventional HPLC Column 0.32 - 4.6 mm i.d.

- **Mechanism:**

  Injection of large volume of sample onto a column in a weak mobile phase to generate a narrow plug at the front of the column. Sample plug is eluted and resolved chromatographically as solvent strength is increased (gradient).

- **Advantages:**
  - On-line/minimal sample handling/automation
  - Concentrates sample 100 x
  - Cleanup (based on chromatography), desalting
- **Disadvantages:**
  - Concentrates interferences
  - Limited to column capacity for total sample (e.g., dirty samples quickly exceed column capacity)
  - Can be time consuming

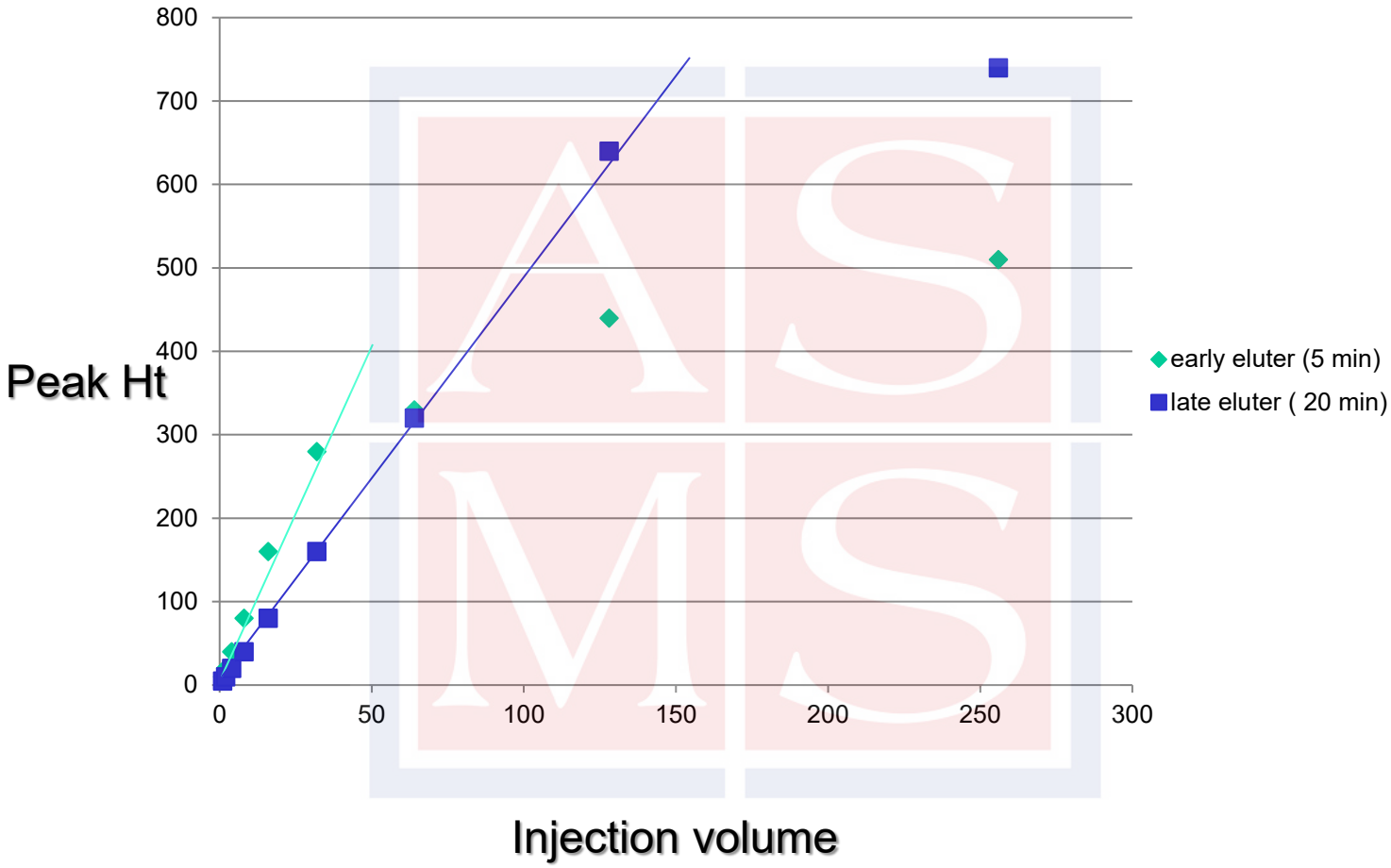
Student Notes: On-column concentration for LC/MS is covered.

On-line Column Concentration

- Procedure:
  - Equilibrate column in **weak mobile phase** such as water.
  - Load sample (0.1 - 10 mL) onto column. **Sample should be dissolved in a weak mobile phase** and may require sample cleanup (e.g., ultrafiltration or SPE for serum, urine, milk, tissue).
    - ***NOTE: If sample is in organic, solvent must be evaporated and sample reconstituted in an aqueous solvent system.***
  - Change mobile phase to chromatograph the analyte (either step to elution mobile phase or start gradient).
  - Note the maximum loading volume is determined when the peak height is no longer linear with injection volume (peak broadening occurs).
  - In general the longer the retention time, the weaker the LC solvent and the weaker the dissolving solvent, the larger the volume of sample can be loaded.

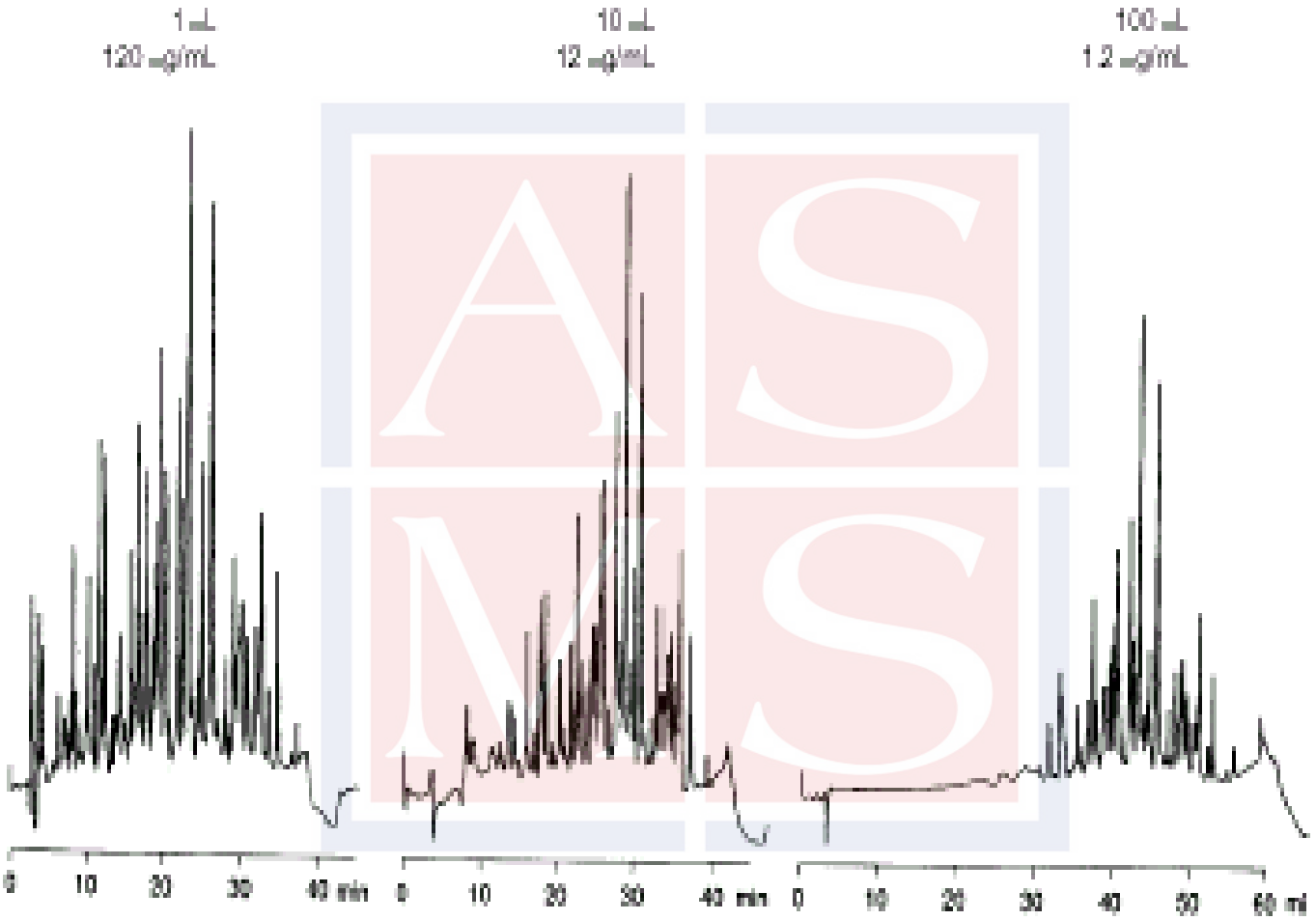
Student Notes: Procedure for on-column concentration is provided

On-line Column Concentration: Elution time affect on maximum injection volume



- LC conditions: 0-100% MeOH in 30 min

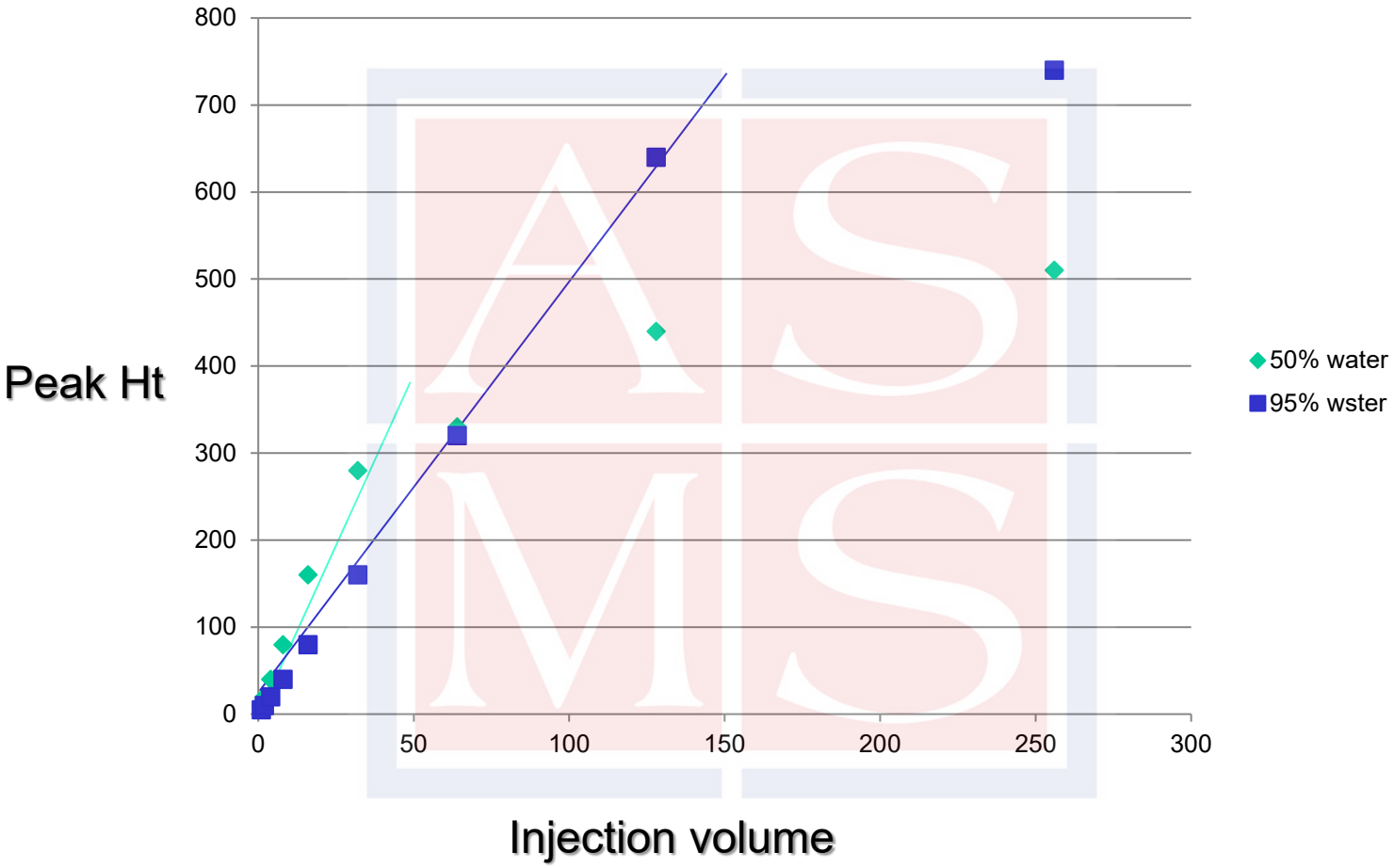
Large Volume Injections (On-Column Focusing)
Injection of 6.6 Pmole Digested β-Lactoglobulin A



From J. P. Salzmann of L. C. Packings

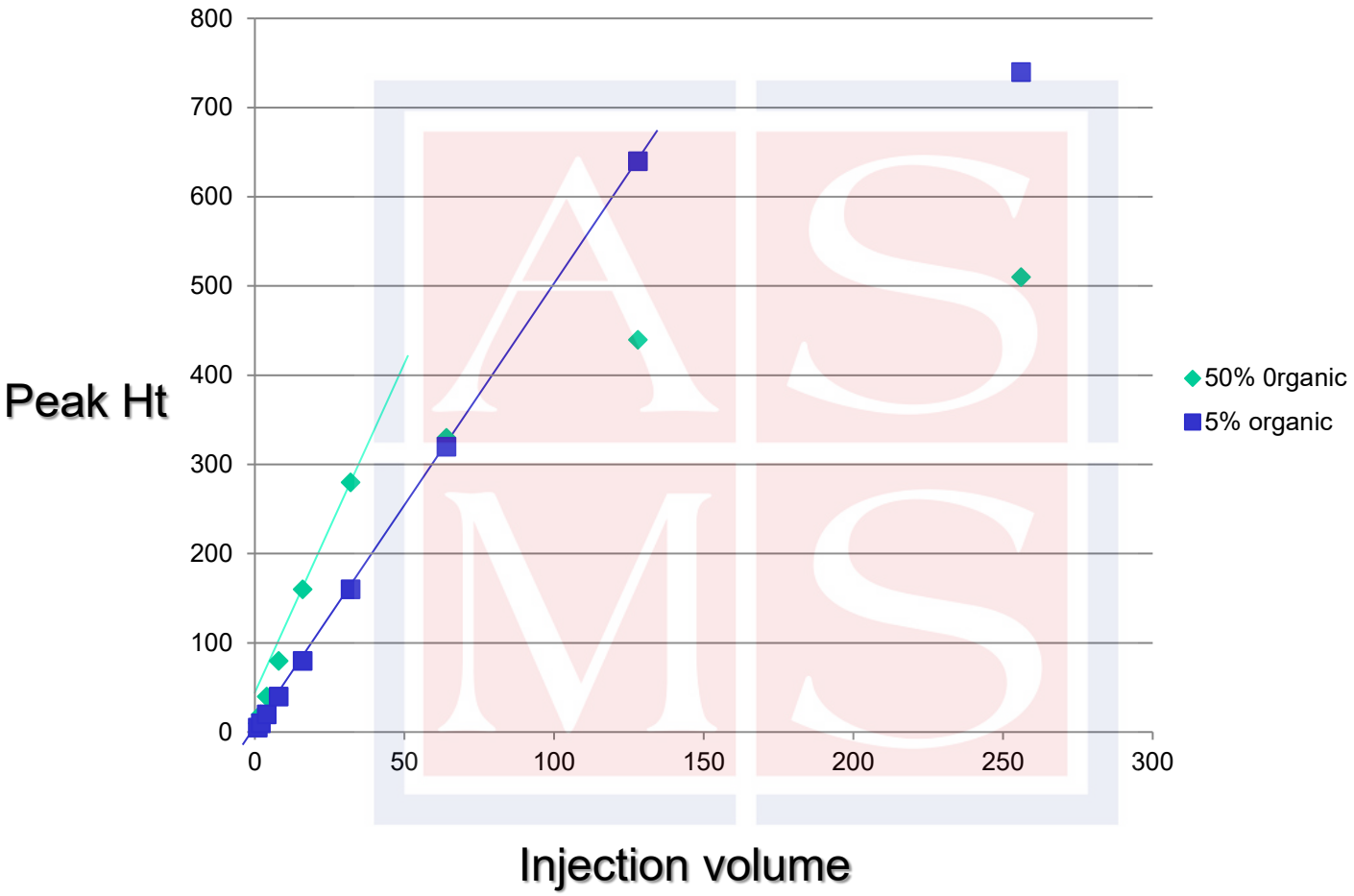
Student Notes: Example of on-column concentration for a capillary (0.32 mm i.d.) column

On-line Column Concentration: Initial LC % organic affect on maximum injection volume



- Initial LC conditions:
  - 50/50 water /MeOH
  - 95/5 water/MeOH

On-line Column Concentration: % organic in recon solvent affect on maximum injection volume



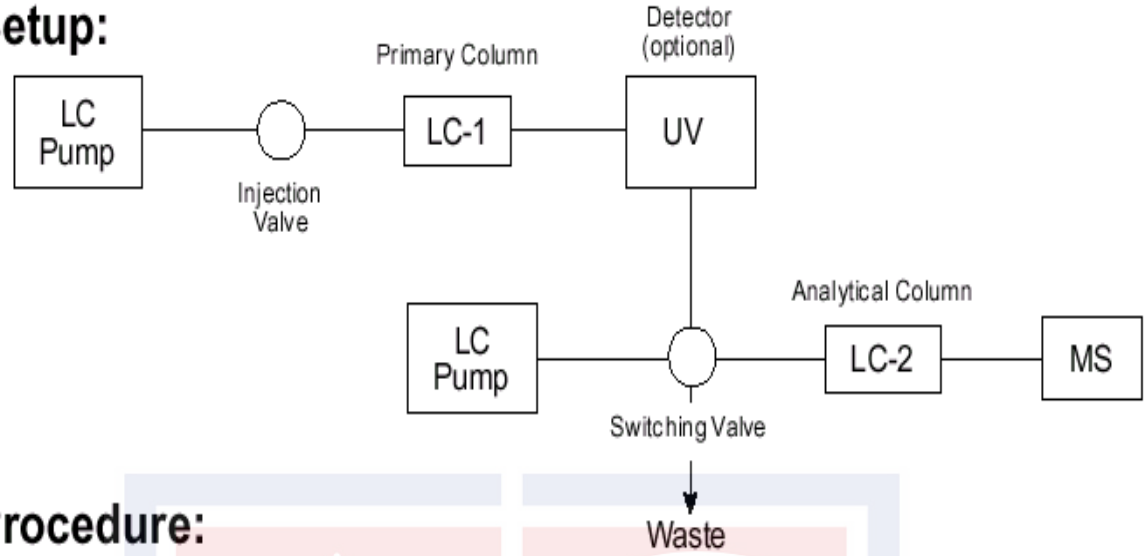
Column Switching (LC/LC)

- Mechanism
  - Sample applied to column and fractionated into analyte(s) and matrix.
  - Analytes transferred to analytical column
  - Analytical column resolves analyte for detection
- Application:
  - On-line sample cleanup and concentration
- Advantage:
  - Automation
  - Minimal sample loss and handling
  - Decrease analysis time
  - More reproducible
  - Customize sample preparation
- Disadvantages:
  - Requires more complexity in pumps and switching valves.
  - More expense in equipment
  - Solvents must be compatible with both stationary phase and one another.

Student Notes: Column switching methodology is briefly highlighted

Column Switching (LC/LC)

Setup:

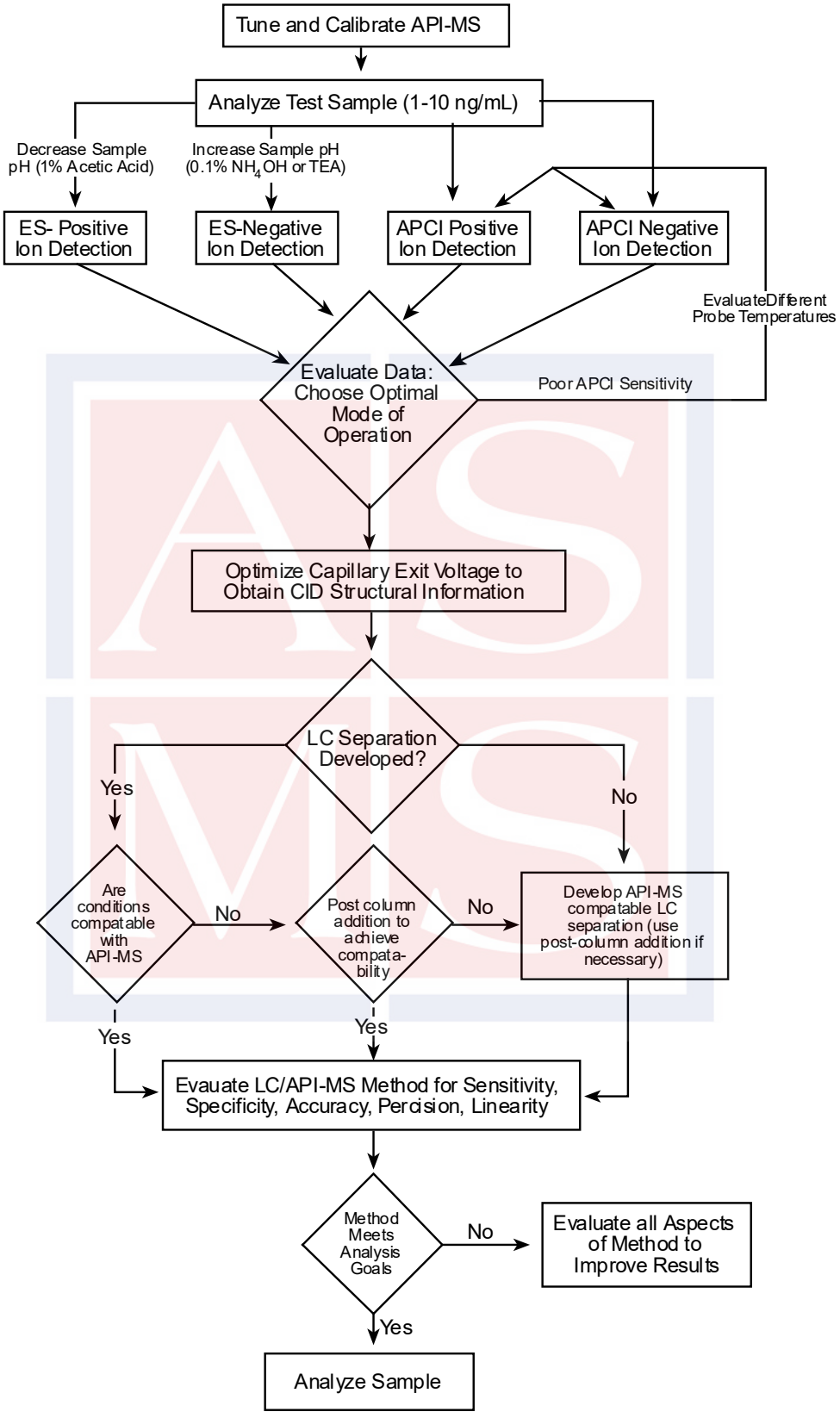


Procedure:

- Setup system and select column and mobile phase to separate analyte from matrix (LC-1) and chromatograph analyte (LC-2).
- Decide on mode of operation based upon cleanup and concentration offered by LC-1.
  - Heartcut (narrow retention window loaded to LC-2)
  - Backflush (sample concentrated and only retain material loaded to LC-2)
  - Frontcut (early eluting material loaded to LC-2)
  - Endcut (late eluting material loaded to LC-2)
- The optional detector (e.g., UV) can aid in selecting switching valve times.
- Load and inject sample.
- Conditions chosen for LC-1 to cleanup and/or concentrate analyte.
- Analyte transferred to LC-2
- Different elution system is used to further resolve analyte from matrix components for LC/MS detection.

Student Notes: Column switching methodology is briefly highlighted.

Optimization Scheme for API-MS



Student Notes: Flowchart describing optimization of API-MS analytical methodology.

Interpretation of Mass Spectra Generated by API-LC/MS/MS

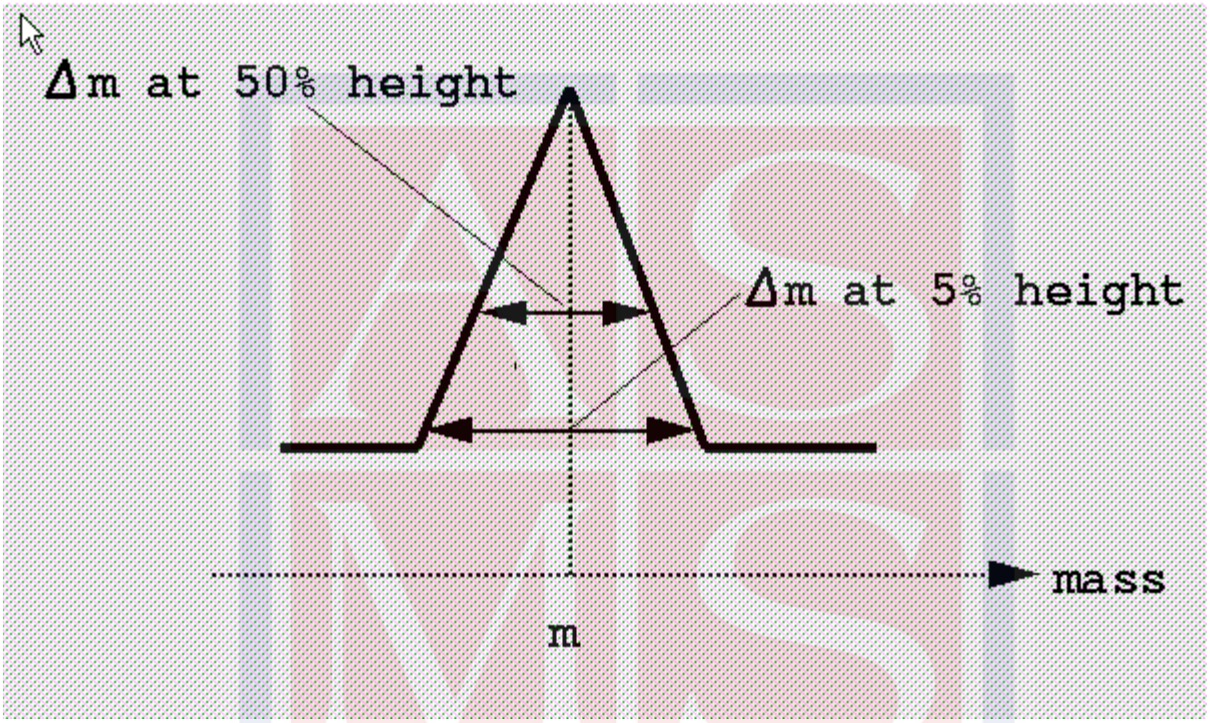
Interpretation of API Mass Spectra

Electrospray and APCI are soft ionization techniques providing molecular weight information. The molecular species detected depends on the solvent additives and conditions used for the analysis.

- Positive Ion Detection
  - $[M+H]^+$ acid conditions
  - $[M+Na]^+$, $[M+K]^+$ (presence of salts)
  - $[M+NH_4]^+$ ammonium buffer present
  - $[M+X]^+$ X = solvent or buffer cation
  - $[2M+H]^+$ dimer formed at higher concentrations
  - $[M+H+S]^+$ solvent adducts
- Negative Ion
  - $[M-H]^-$ basic conditions
  - $[M+X]^-$ X = solvent or buffer anion
  - $[M-H+S]^-$ solvent adduct

Define Mass Resolution and Mass Accuracy

- Resolving Power: M / △ M



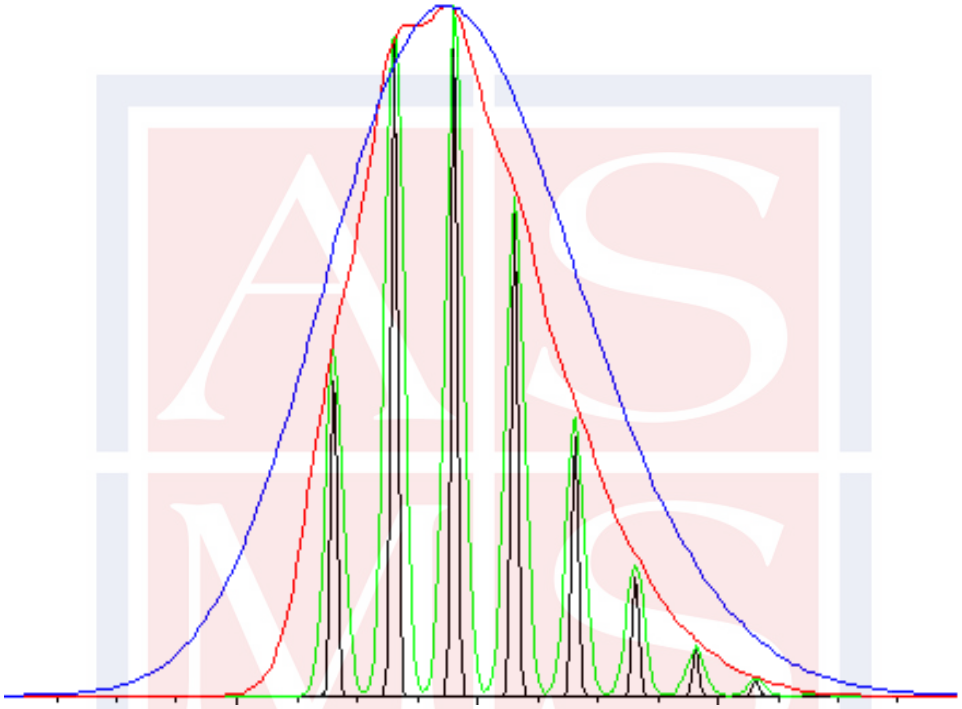
Mass accuracy:

- **ppm = 10+6 (measured mass - theoretical mass) / theoretical mass**

- Millimass unit (MMU) = **measured mass - theoretical mass**

  High resolution does not imply good mass accuracy

Example of Increasing MS Resolving Power



1000 resolving power

3000 resolving power

10,000 resolving power

30,000 resolving power

Value of Accurate Mass Determinations

Determining the molecular formula for the measured mass of 348.924988

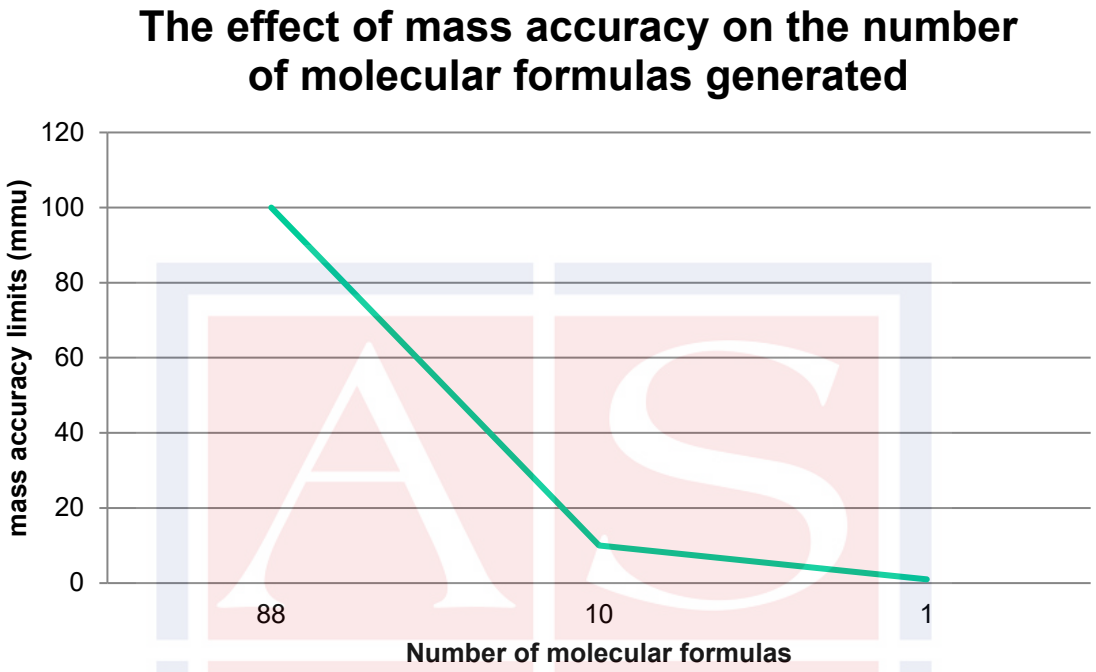
12 ppm mass accuracy

	-2.89	9.0	C8 H5 N5 O3 C11 P1 S2
	-6.56	9.0	C8 H7 N5 O1 C11 P3 S1
	0.35	4.0	C8 H9 N1 O6 C12 P0 S2
	-8.66	4.0	C8 H10 N3 O2 C13 P0 S2
	-3.24	4.0	C8 H11 N1 O4 C12 P2 S1
	-12.33	4.0	C8 H12 N3 O0 C13 P2 S1
	-1.57	3.0	C8 H13 N1 O2 C11 P1 S4
	-6.91	4.0	C8 H13 N1 O2 C12 P4 S0
	-5.25	3.0	C8 H15 N1 O0 C11 P3 S3
	-3.41	9.0	C9 H5 N5 O2 C12 P0 S2
	2.01	9.0	C9 H6 N3 O4 C11 P2 S1
	-7.08	9.0	C9 H7 N5 O0 C12 P2 S1
	-1.57	9.0	C9 H8 N3 O2 C11 P4 S0
---CORRECT---->	-3.67	4.0	C9 H11 N1 O3 C13 P1 S1
	-2.10	3.0	C9 H13 N1 O1 C12 P0 S4
	-7.43	4.0	C9 H13 N1 O1 C13 P3 S0
	10.67	9.0	C10 H5 N1 O7 C11 P1 S1
	1.57	9.0	C10 H6 N3 O3 C12 P1 S1
	6.91	9.0	C10 H7 N1 O5 C11 P3 S0
	3.15	8.0	C10 H8 N3 O1 C11 P0 S4
	-2.19	9.0	C10 H8 N3 O1 C12 P3 S0
	-4.29	4.0	C10 H11 N1 O2 C14 P0 S1
	-7.87	4.0	C10 H13 N1 O0 C14 P2 S0
	10.06	9.0	C11 H5 N1 O6 C12 P0 S1
	1.05	9.0	C11 H6 N3 O2 C13 P0 S1
	6.47	9.0	C11 H7 N1 O4 C12 P2 S0
	-2.62	9.0	C11 H8 N3 O0 C13 P2 S0
	8.05	8.0	C11 H9 N1 O2 C11 P1 S3
	4.46	8.0	C11 H11 N1 O0 C11 P3 S2
	5.95	9.0	C12 H7 N1 O3 C13 P1 S0
	7.70	8.0	C12 H9 N1 O1 C12 P0 S3
	5.42	9.0	C13 H7 N1 O2 C14 P0 S0

4 ppm mass accuracy

Diff. ppm	Unsat.	Compositions
-3.67	4.0	C9 H11 N1 O3 C13 P1 S1 <--
1.05	9.0	C11 H6 N3 O2 C13 P0 S1
-2.62	9.0	C11 H8 N3 O0 C13 P2 S0

Value of Accurate Mass Determinations



10 mmu mass accuracy

Formula (M)	Diff (ppm)
C22 H28 N2 O	0.12
C11 H28 N8 O4	9.6
C17 H28 N4 O3	-11.85
C12 H24 N12	13.58
C16 H32 O7	-15.82
C15 H32 N2 O6	17.59
C13 H24 N10 O	-19.83
C16 H28 N6 O2	21.57
C12 H28 N6 O5	-23.81
C20 H32 O4	29.55

1 mmu mass accuracy

Formula (M)	Diff (ppm)
C22 H28 N2 O	0.12

Changing a molecular formula into a structure

Search molecular formula C22 H28 N2 O using Chem Spider

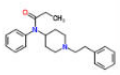
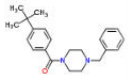
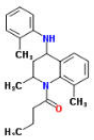
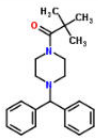
http://www.chemspider.com/FullSearch.aspx

Note: there are 1722 structures for that molecular formula

Found 1722 results

Search term: **MM >= 336.2197 AND MM <= 336.2207 AND abs(Monoisotopic_Mass - 336.2202) as mass_defect**

1 2 3 4 5

ID	Structure	Molecular Formula	Molecular Weight	# of Data Sources	# of References	# of PubMed	# of RSC	Mass defect
3228		$C_{22}H_{28}N_2O$	336.4705	63	7530	12402	32	0.0000
630541		$C_{22}H_{28}N_2O$	336.4705	28	32	0	0	0.0000
534936 - 0/2 defined		$C_{22}H_{28}N_2O$	336.4705	26	30	0	0	0.0000
627291		$C_{22}H_{28}N_2O$	336.4705	22	22	0	0	0.0000

What is Collision Induced Decomposition (CID)?

A process where energy is transferred to an ion through collision with neutral molecules. The energy transfer is sufficient to result in bond cleavages and rearrangements of the selected ion.

Why is it Important?

In the early 70's McLafferty (JACS, 95, 3886, 1973) demonstrated the bond cleavage and rearrangements observed for the ion that undergoes CID is representative of the molecular structure of the neutral molecule.

Structural elucidation

We will cover key fragmentation mechanisms used for the interpretation CID Mass Spectra

CID Energies

Collisions of polyatomic molecules are inelastic.

$V_i > V_f$ (where V_i is the relative initial velocity of the ion and V_f is the post collision velocity of the products) vs elastic where $V_i = V_f$

In inelastic collisions, translational energy is converted into internal energy as represented by q'.

$q_{max} = [N/(N + Mp)] E_{lab}$

N = mass of neutral target molecule

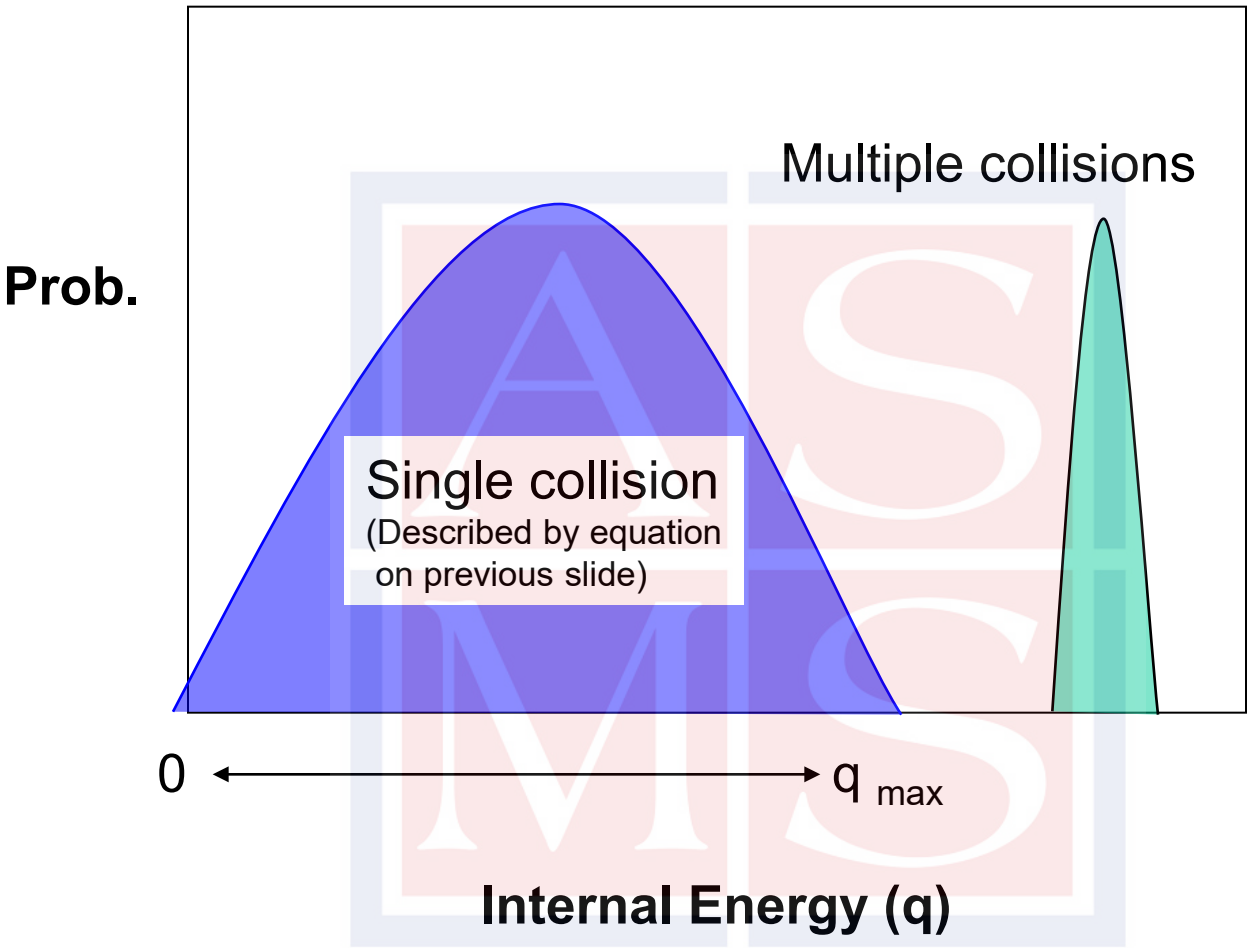
Mp = mass/charge of parent ion

E_{Lab} = laboratory kinetic energy

The maximum energy (q_{max}) converted from translational to internal energy increases when the operator increases the ion energy (E_{Lab}) and the collision gas mass.

For CID in Electrospray Transport	N = 28 (nitrogen)
For Triple Quadrupole	N = 28 or 40 (N_2 or Ar)
For Ion Trap	N = 4 (helium)

CID Energy Distributions

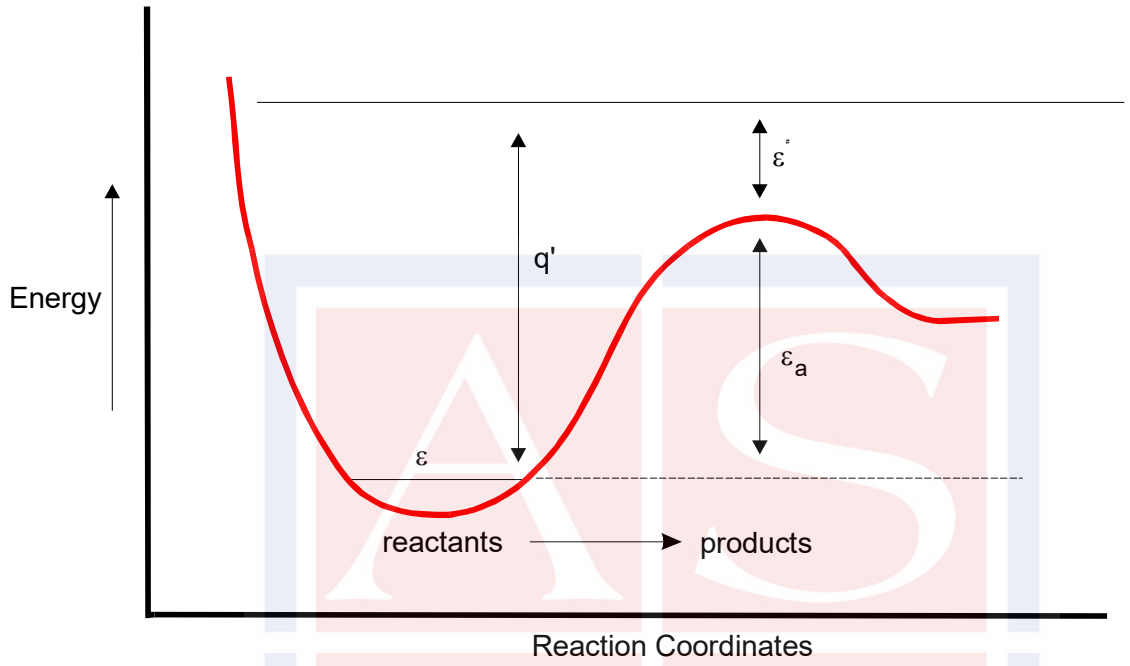


Single collision- Internal energies range from 0 (no collision) to q_{max} for a perfect collision.

Multiple collisions- The energy distribution is narrower and the average internal energy is higher.

CID Reactions

CID Reaction energies are shown below:



q' = amount of energy converted from translational to internal from the CID process

ε' = excess energy

ε_a = activation energy

Total internal energy = ε + q'

ε = reactant initial energy

CID reactions will be most favored at higher q' and low ε_a and when lower energy products are formed. The operator can try to increase q' by increasing the energy of collision (E_{lab})and collision frequency (pressure).

CID reactions- Rate of reaction

- **As the molecular weight increases, the number of oscillators (S) in the molecule increase. A large number of oscillators dissipate the internal energy in the molecule, reducing the rate of fragmentation [k(ε)]. The relationship between rate of fragmentation k(ε) and S is given below - (V = frequency factor):**
- $k(\varepsilon) = V\,[(q' - \varepsilon)\,S^{-1}]\;x\,(E_{lab})$

- **To increase energy transfer and k(ε), the operator can increase ion energy (E_{lab}), collision gas pressure, and collision gas (e.g., He → Ar).**
- **Practical considerations in amount of transfer energy limits the acquisition of CID spectra to molecular weights in the 2000-3000 dalton range or lower. This is a direct results of the increasing number of oscillators with increasing mass.**

Do CID Mass Spectra Differ Depending on How the $[M+H]^+$ Species Were Generated?

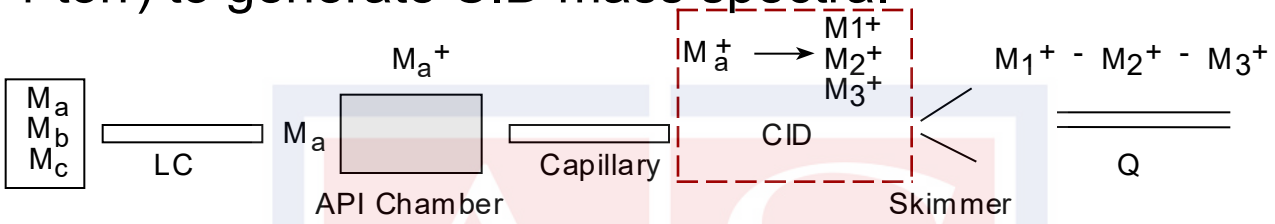
- **No; CID spectra are independent of how the $[M+H]^+$ species was generated.**

  - Different ionization techniques (e.g., methane CI vs. electrospray vs. APCI) can produce the same $[M+H]^+$ ion, but with slightly different internal energies (ε). Note, however, that the changes in ε are small compared to q'.

CID in the API Transport Region (In-source CID)

- What is it?

  Use of electric fields in the API-MS transport region to collisionally activate ions at a mid-pressure region (~0.01 - 1 torr) to generate CID mass spectra.



- Characteristics of API Transport CID
  - N_2 drying gas serves as collision gas
  - Collision energy controlled by the potential difference from capillary exit (fragmentor) to skimmer. Usually skimmer voltage is constant so collision energy is directly proportional to fragmentor voltage.
  - N_2 pressure is set by interface design. Ions undergo multiple collisions over the 3-4 mm distance between capillary exit to skimmer. Typically there could be hundreds of collisions. Multiple collisions can impart more energy and reduce losses due to scatter.
  - Mass spec. operation (resolution, mass assignment and transmission) is nearly independent of fragmentor voltage.

CID in the API Transport Region

This can be confusing due to the number of names this process is called.

- **Cluster buster**
- **Orifice potential**
- **Cone voltage**
- **Fragmentor**
- **Capillary exit voltage**
- **Up-front CID**
- **Transport CID**
- **In-source CID**
- **Poor-person MS/MS**

Advantages/Disadvantages of CID in Transport Region

- Advantages
  - Near 100% efficiency; sensitive
  - Simple (do not need to know in advance what ions are present)
  - Wide range of energies; fragments can be produced from even the most stable compounds
  - Similar CID spectra to those obtained by tandem MS
  - Cost; less expensive than tandem MS

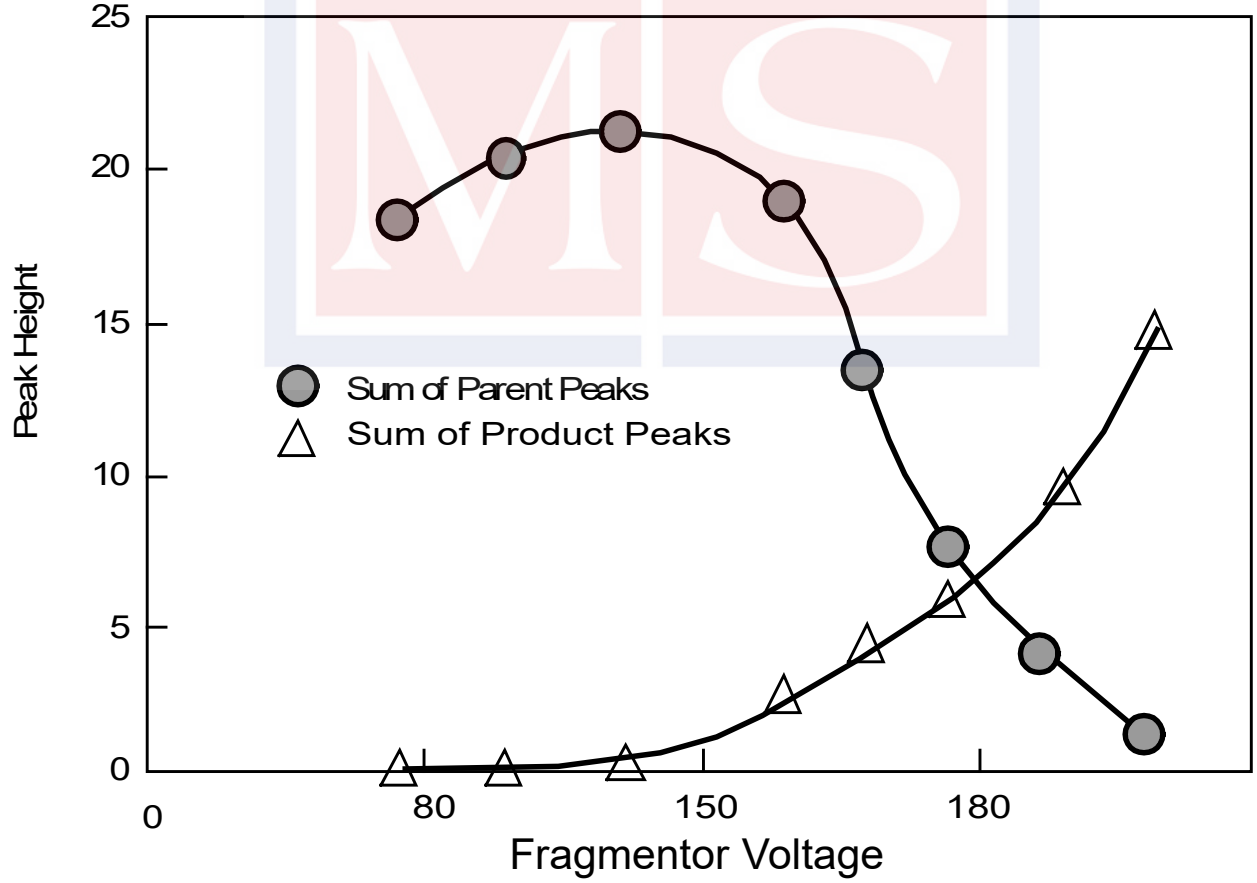
- Disadvantages
  - Impurities or coeluting peaks will result in complex spectra
  - Must have chromatographic resolution; increased analysis time
  - Lack of libraries (i.e.. EI); manual interpretation

CID in API Transport for Sulfamethazine

- Keypoints:

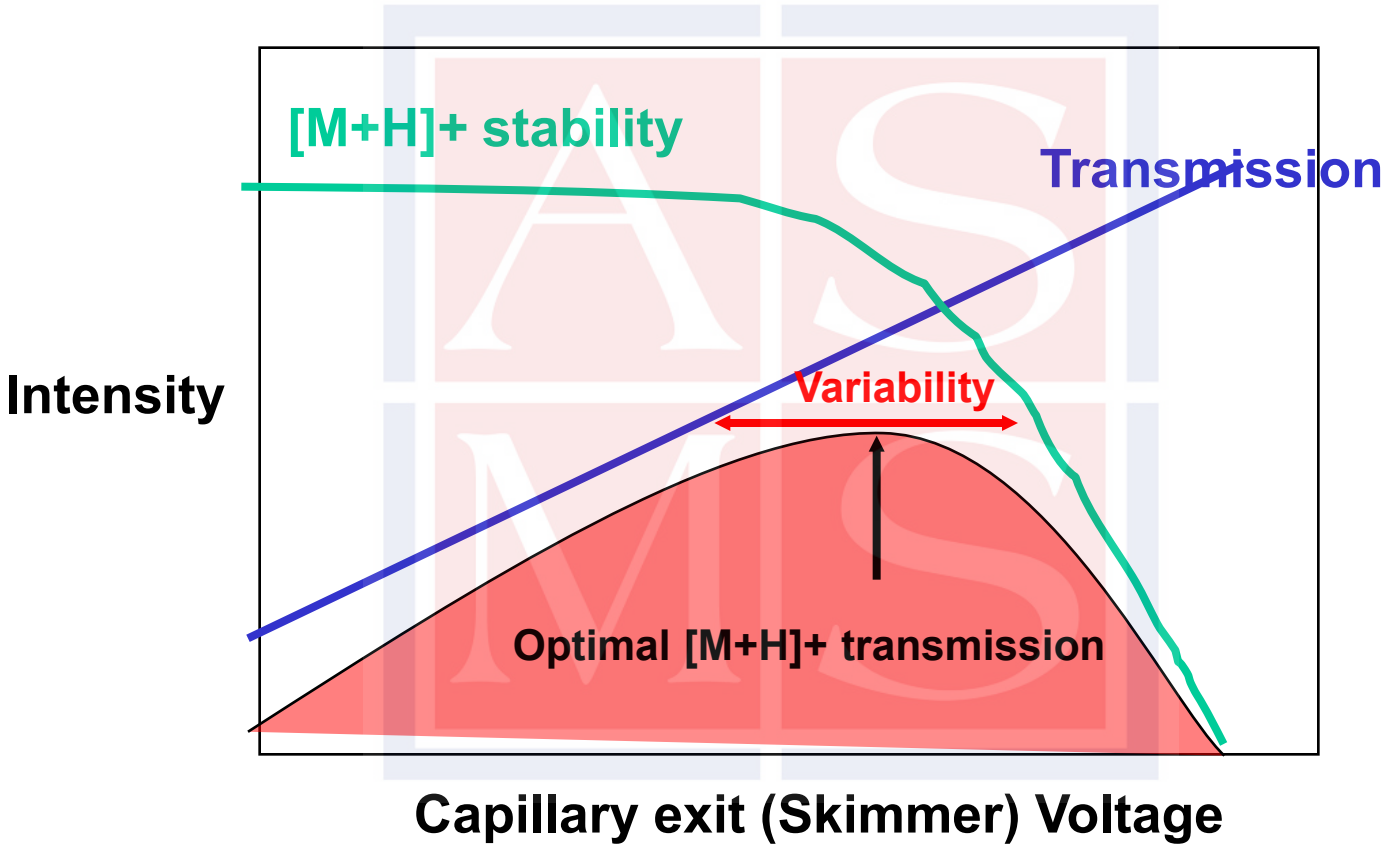
This figure shows how the ratio of product ions, relative to the $[M + H]^+$ ions of sulfamethazine, increases as fragmentor potential increases.

Furthermore, note that there is little loss (<20%) of overall ion current (parent + product ion currents) for all potential differences evaluated. This is not the case for CID on sector or triple quadrupole instruments.



Optimization of Sensitivity in the API Transport Region

Each compound has an optimal voltage for transmission of the [M+H]+ ion based on the bond strength and m/z



Variability in the optimal voltage results from:

1. **Compound stability (weaker bonds move optimal voltage lower.**
2. **Energy transfer (q) into the molecule (higher m/z compounds have less conversion of kinetic to internal energy so voltage is increased. Lower m/z compounds have more kinetic energy is converted into internal energy so the voltage is decreased.**

What is Tandem MS?

A process in which an initial mass analyzer selects a parent ion or precursor (from all ions present in the system) for CID. The product ions formed are then mass analyzed by the second mass analyzer. Tandem MS can be tandem in space (triple quadrupoles, sector instruments) or tandem in time (ion trap instruments).

Definitions:

- Experiments performed using Tandem instruments are termed MS/MS experiments
- Parent Ion or Precursor Ion

  ion selected by initial mass analyzer for CID (this term applies only to MS/MS experiments)

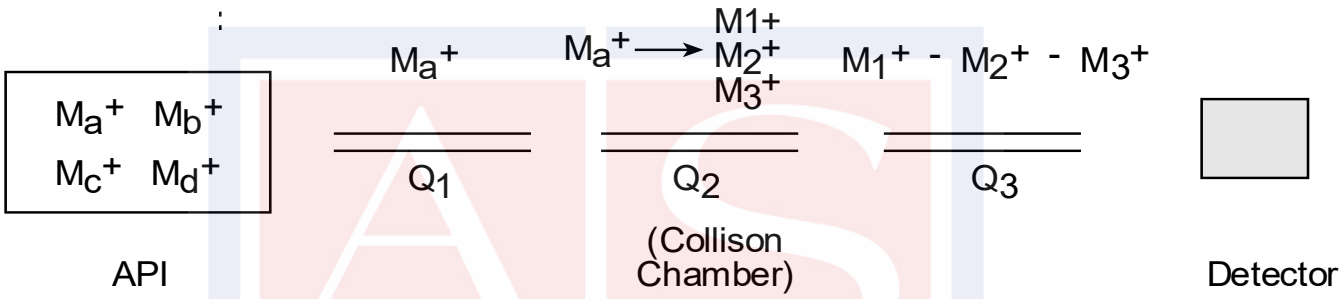
- Product Ions

  fragment ions formed after CID (same term is used for in source and tandem CID experiments)

CID in Tandem MS

- What is it?

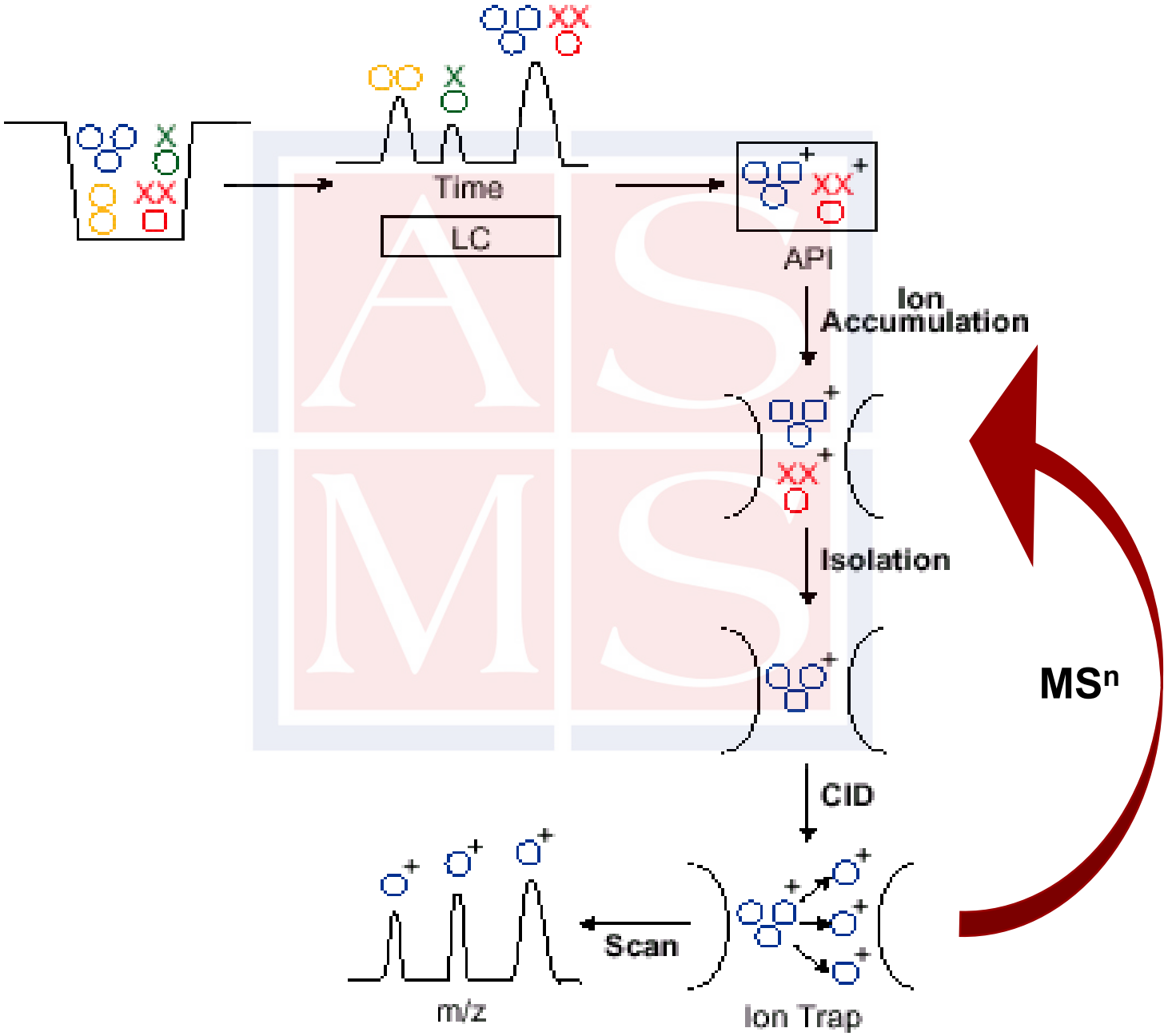
  A method that uses the first mass analyzer (Q1) to select a particular m/z for introduction into a collision chamber (Q2). The selected ion undergoes CID with a gas (e.g., argon) in the chamber and product ion (fragments) are mass resolved by a second mass analyzer (Q3).



Triple quadrupoles are the most common MS/MS instruments, however sectors, ion traps, and time-of-flight analyzers can also be used.

- Characteristics of Tandem MS
  - Ions are mass selected prior to CID
  - Operator chooses collision energy, collision gas and collision gas pressure (3 factors which require optimizations)
  - Mass analyzers can be linked to perform numerous types of MS/MS experiments
    - product ion scan
    - neutral loss scan
    - precursor ion scans
    - multiple reaction monitoring (MRM)
  - Typical complexity far exceeds CID in API transport region
  - CID conditions can influence MS resolution and transmission

Tandem MS in Time – Ion Trap



When to use MS^n and What Should One Use for the Value of n

- Often low energy CID in a trap leads to one rearrangement product ion (e.g. loss of H_2O), therefore, additional stages of MS/MS can offer more structural information and specificity.

- n can be from 2-11 on the commercial Traps (record is 17 for a trap). (what was the product ion spectrum after MS^{17} ?)

- If there are numerous product ions formed after each stage of MS/MS going beyond n=4 can result in significant loss of sensitivity. Remember the charge will not exceed the initial parent ion. The more product ions formed the further the initial charge is divided.

Advantages/Disadvantages of CID in Tandem MS

- Advantages
  - Chemical specificity (components do not need to be chromatographically resolved)
  - Structural elucidation
  - Reduced analysis times
  - Sensitivity (gain in S/N through noise reduction)
  - Chemical background is reduced by parent ion selection
  - Screening: (neutral loss, parent ion scans)

- Disadvantages
  - Cost
  - Interpretation of spectra
  - Complexity of instrument operation

Comparison of CID Characteristics for Various MS Instruments

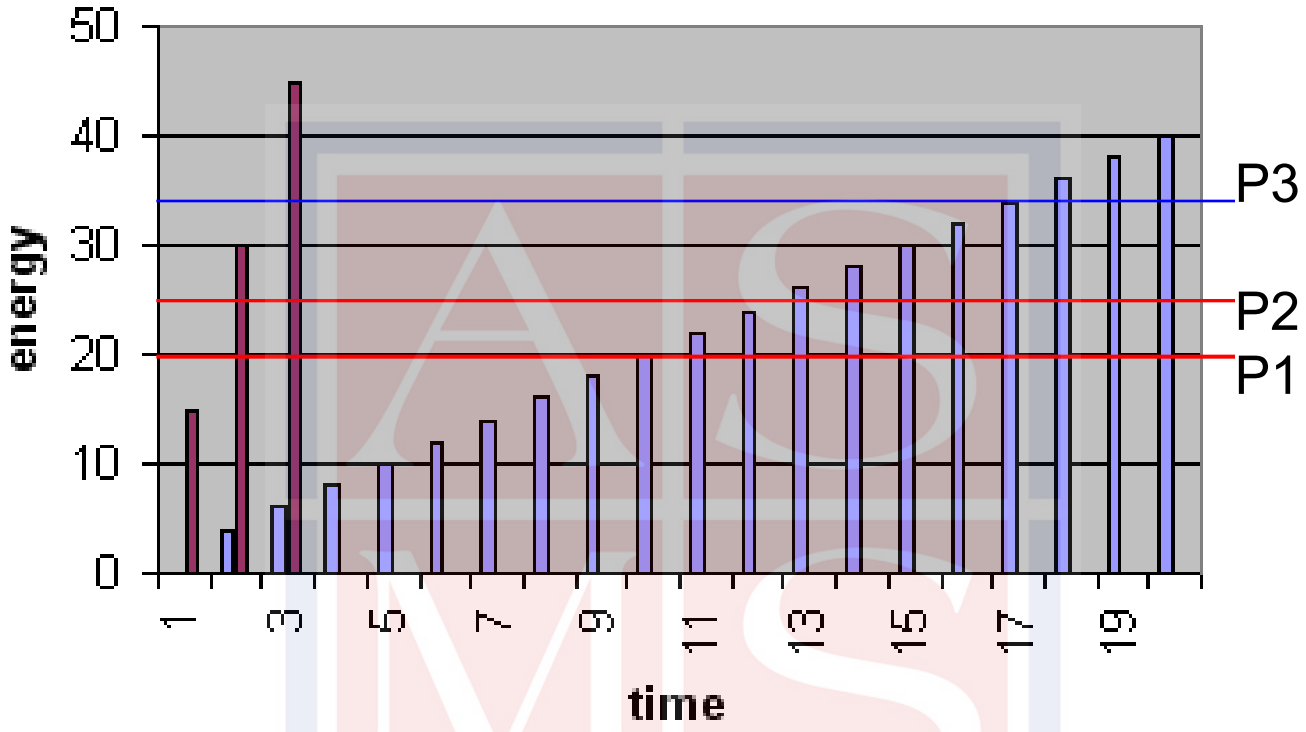
MS Instrument	Typical Gas	Single or Multiple Collision	Ion Energy (eV)	Collision Time (μs)
Quadrupole	Ar, Xe, N_2	M	10-100	5-50
TOF/TOF	He, N_2	S	2,000 - 8,000	<5
Ion Trap	He	M(1)	1-2	20,000 - 40,000
CID in API Transport	N_2	M(1)	10-400	5-50

(1) *In general, multiple collision conditions (achieved by operating at higher pressures or having the collision process occur over a long time) results in a much higher energy transfer to the ion and produces more intense and numerous product ions.*

Understanding CID Mass Spectra from a Triple Quad vs. an Ion Trap

Triple Quad

Ion Trap



•Rise(y axis) proportional to E_{lab}

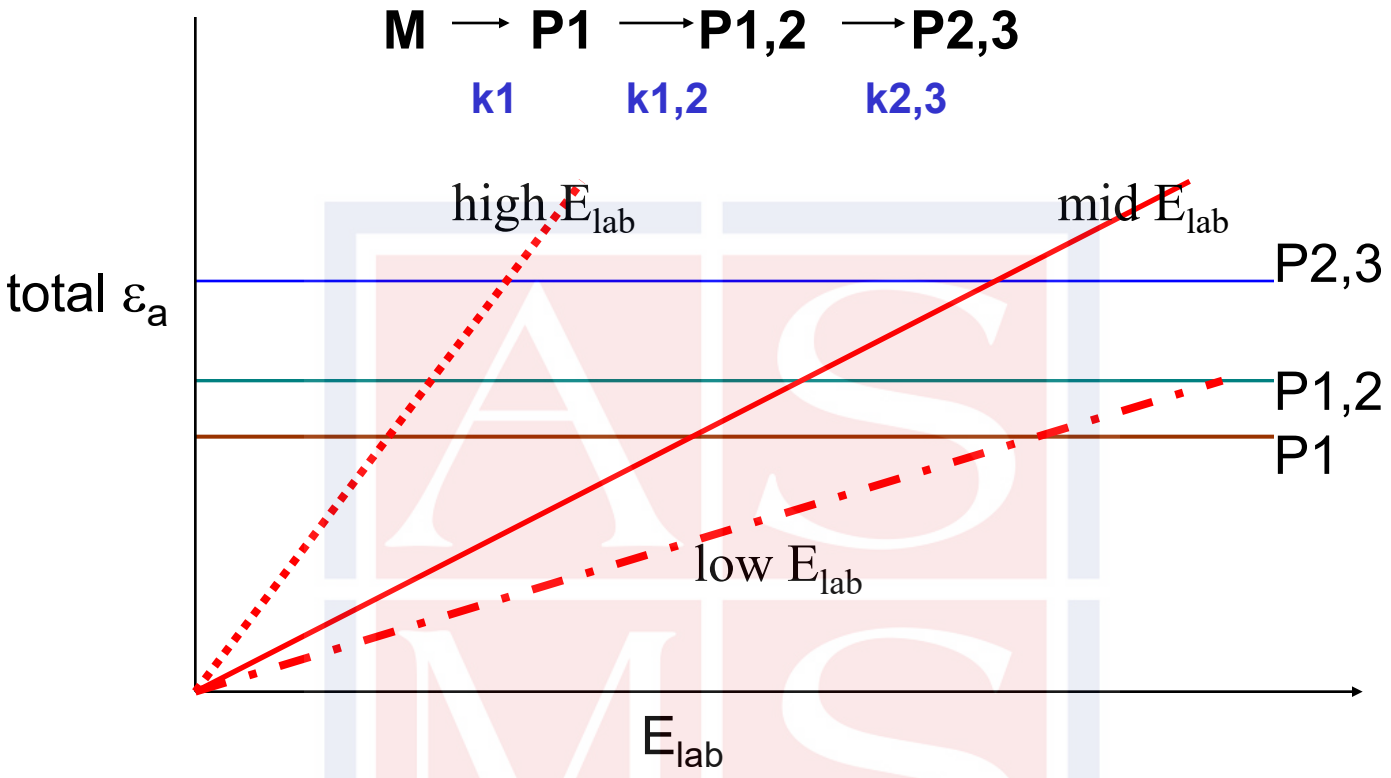
•X-axis proportional to collision frequency and time

Traps show single ion spectra if lower energy processes have reaction rates equal or faster than higher energy products e.g. k(P1) > k(P2) > k(P3)

The triple quad would activate many pathways together with the intensities related to the rates of reaction

Understanding CID Mass Spectra from a Triple Quad

Triple quads can generate product ions from other products in a sequential manor in the collision cell



- The red lines are showing the rate the ion is gaining internal energy at a low mid and high E lab voltage

- At higher E lab, it is possible for P1 to continue to gain energy from collisions to form P1,2 (and likewise P1,2 to form P2,3)

Traps do not show this sequence since only M is excited, Products do not gain any energy after formation unless broad band excitation is used

Interpretation of CID Mass Spectra

- Variability of Spectra

  The fragmentation patters of CID spectra are affected by several variables. The most significant of these are type and pressure of collision gas, energy of the ions, instrument configuration, and charge state. There is no standard set of conditions under which all CID experiments are performed and as a result the CID spectra generated on any given compound will be different. Manual interpretation of CID spectra appears to be the only choice for identifying unknowns.

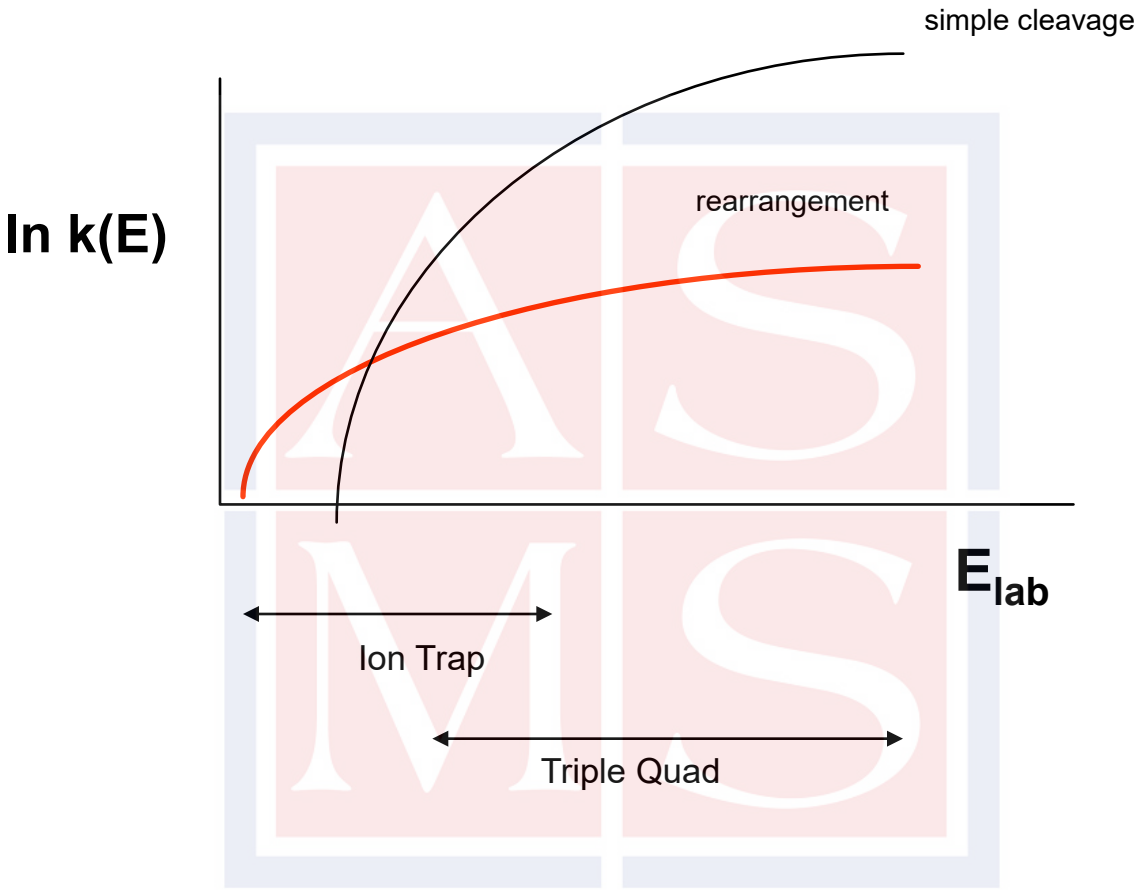
- Fragmentation Mechanisms

  CID fragmentation occurs through unimolecular decomposition of the internally excited even-electron ion. The basic energetic considerations that are used for odd-electron interpretations (i.e.. from EI) still apply to even-electron interpretation; the stability of the product ion formed, stability of the leaving group and bond liability are important driving forces.

  Keep in mind that the decomposition pathways overwhelmingly favor formation of another even-electron ion and even electron leaving group.

  Radical losses can be observed is resonance stabilized conjugated systems (aromatic compounds) when the energy to break a resonance system exceeds the energy to form a radical loss

Rearrangements vs. Simple Cleavages - Which Will Occur?



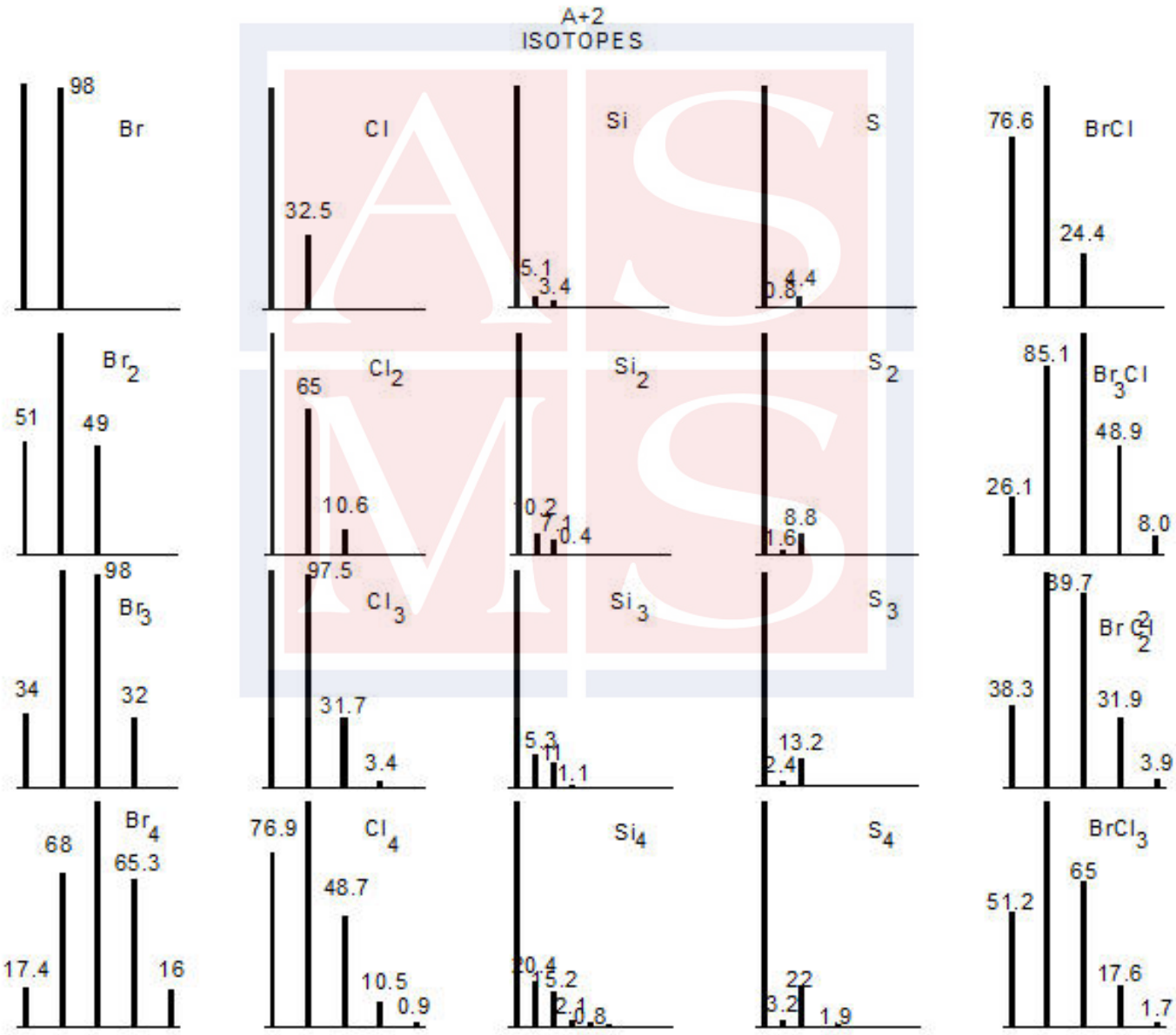
• in general, cleavages have higher energies of activation and are therefore favored at a higher E_{lab} (higher fragmentor voltage)

Table of Atomic Masses and Isotopic Ratios for Common Elements

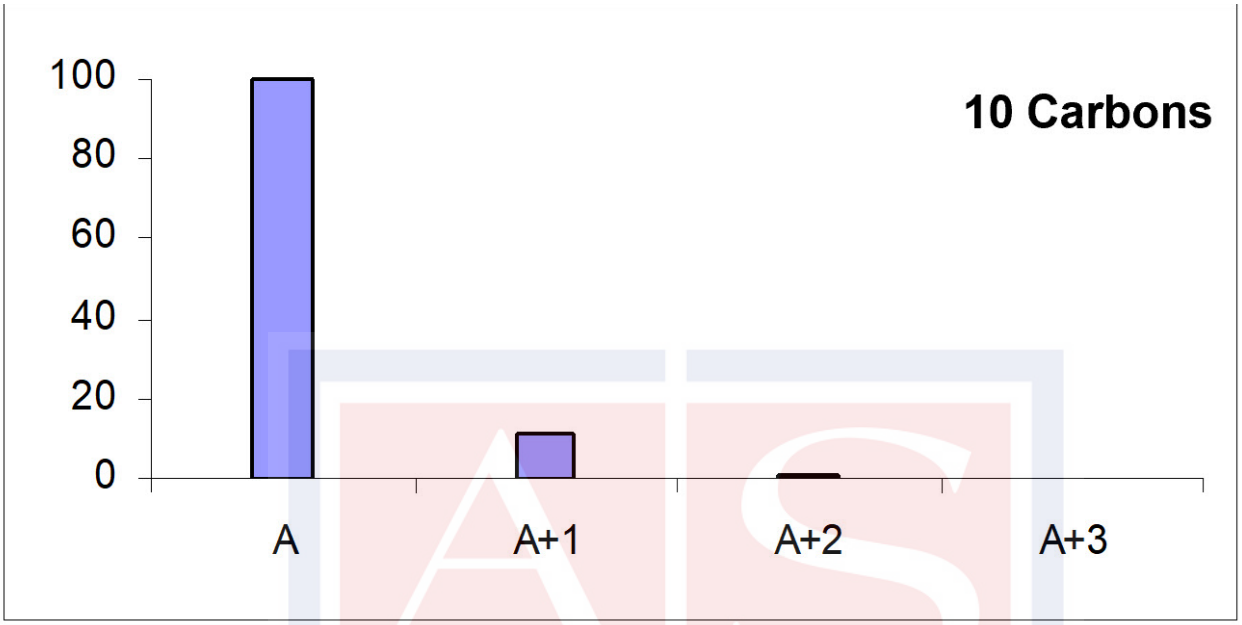
Atom	Mass (A)	Rel. Abund.	A+1 Rel. Abund.	A+2 Rel. Abund.
Hydrogen	1.0078	100	-	-
Carbon	12.0000	100	1.1	-
Nitrogen	14.0031	100	0.37	-
Oxygen	15.9949	100	-	0.2
Fluorine	18.9984	100	-	-
Silicon	27.9769	100	5.1	3.4
Phosphorous	30.9738	100	-	-
Sulfur	31.9720	100	0.8	4.4
Chlorine	34.9989	100	-	32.5
Bromine	78.9183	100	-	98
Iodine	126.9045	100	-	-

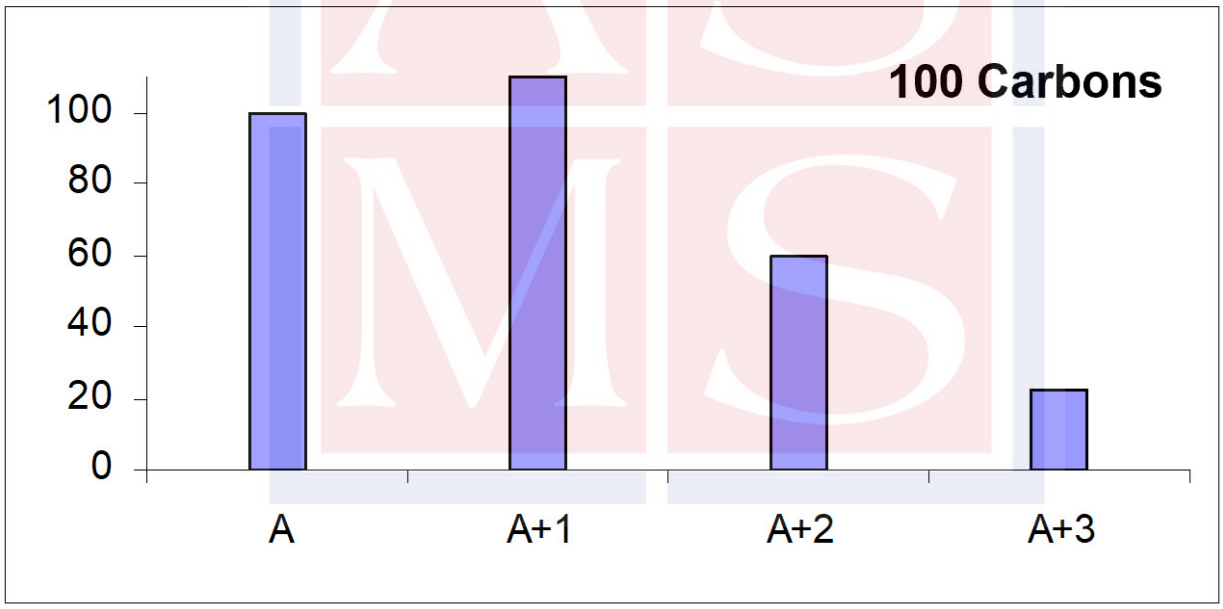
A = API generated ion (e.g., $[M+H]^+$, $[M-H]^-$, $[M+Na]^+$, etc.)

Isotopic Abundances for Combinations of “A + 2” Element Compositions



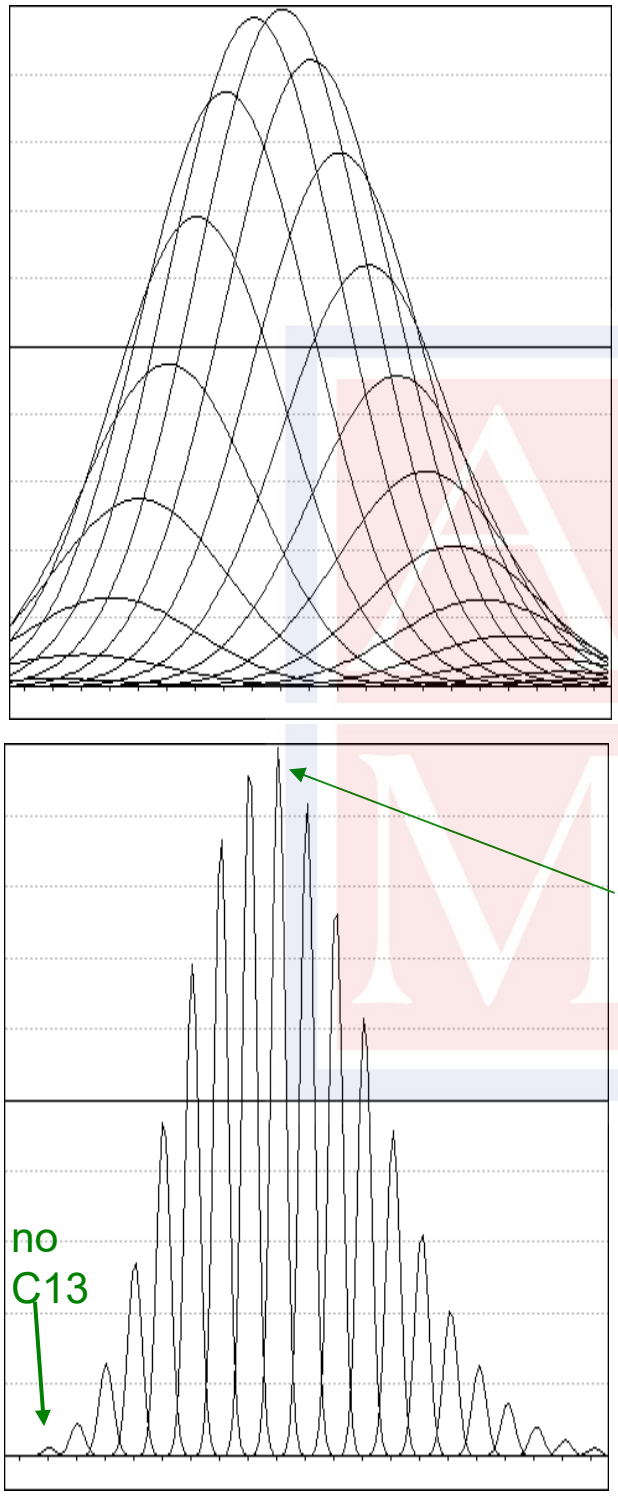
Isotopic Clusters for Large Molecules





Unlike small molecules, the largest mass peak in the isotopic cluster is not the lowest mass ion.

Mass Peak of a Large Molecule



2000
Resolution

PW=7.46

Lysozyme
$C_{613}\ H_{950}\ N_{192}\ O_{186}\ S_{10}$
Most abundant MW:
14305.79
Average MW: 14306.18
Monoisotopic MW:
14290

25,000 Resolution

Nominal vs. Monoisotopic vs. Average Molecular Weight

- **Nominal mass:** The integer mass of the most abundant isotope. This is the sum of nominal mass of the elements composing the compound.
- **Monoisotopic mass**: The exact mass of the most abundant isotope. This is the sum of exact mass of the elements composing the compound.
- **Average mass:** The calculated mass based on the atomic weight. This is the sum of atomic weight of the elements composing the compound. The atomic weight is the weighted average of the naturally occurring isotopes in an element

Nominal, Monoisotopic and Average Mass for Common Elements

Element	Symbol	Nomical Mass	Monoisotopic Mass	Average Mass
Hydrogen	H	1	1.0078	1.0080
Carbon	C	12	12.0000	12.0110
Nitrogen	N	14	14.0031	14.0067
Oxygen	O	16	15.9949	15.9993
Fluorine	F	19	18.9984	18.9984
Sulfur	S	32	31.9721	32.0647
Chlorine	Cl	35	34.9688	35.4610
Bromine	Br	79	78.9183	79.9035
Iodine	I	126	126.9045	126.9045

Nominal vs. Monoisotopic vs. Average Molecular Weight

Example: dichloro- amino toluene ($C_7H_7NCl_2$)
- the 3 different masses calculated are:

Nominal mass:	175
Monoisotopic mass	174.9955
Average mass:	176.0617

Typically:

<u>nominal mol wt</u> or <u>monoisotopic mol wt</u> is used for compounds below 2000-3000 daltons (must resolve isotopic cluster)

<u>average molecular weight</u> is used for compounds above 3000 daltons

Nitrogen Rule for $[M+H]^+$ Ions

- **Even number of nitrogen's then even number molecular weight, odd number of nitrogen's then an odd molecular weigh *(this applies to neutral molecules)***

- **For even m/z ($[M+H]^+$ ions); the molecule has an odd number of nitrogen's.**

  e.g. $[M+H]^+$ ion at m/z 300

  mw = 299

  - compound has an odd number of nitrogen's

- **For odd m/z ($[M+H]^+$ ions); the molecule has an even number of nitrogen's.**

  e.g. $[M+H]^+$ ion at m/z 301

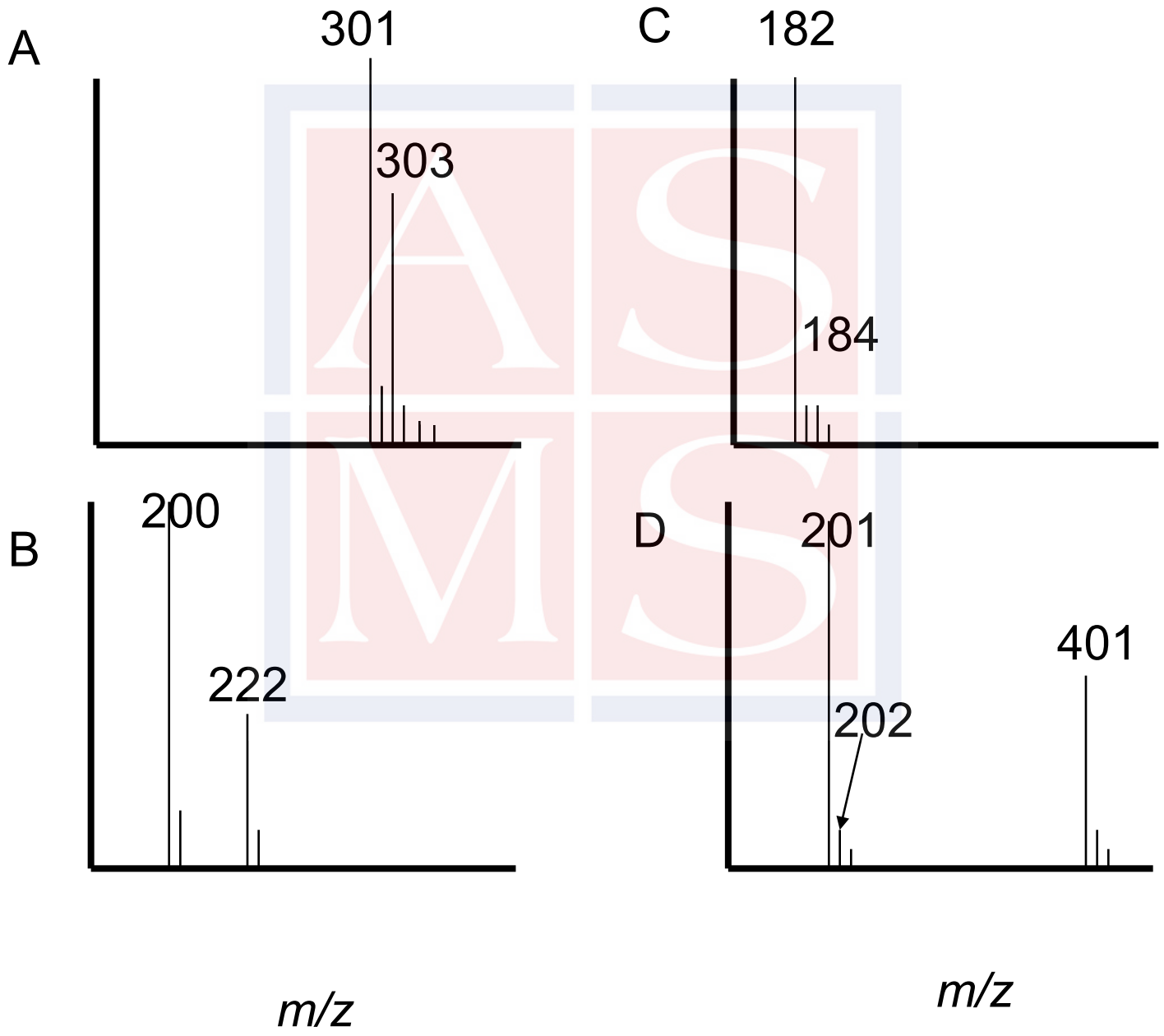
  mw = 300

  - compound may have 0, 2, 4 … nitrogen's

Problem:

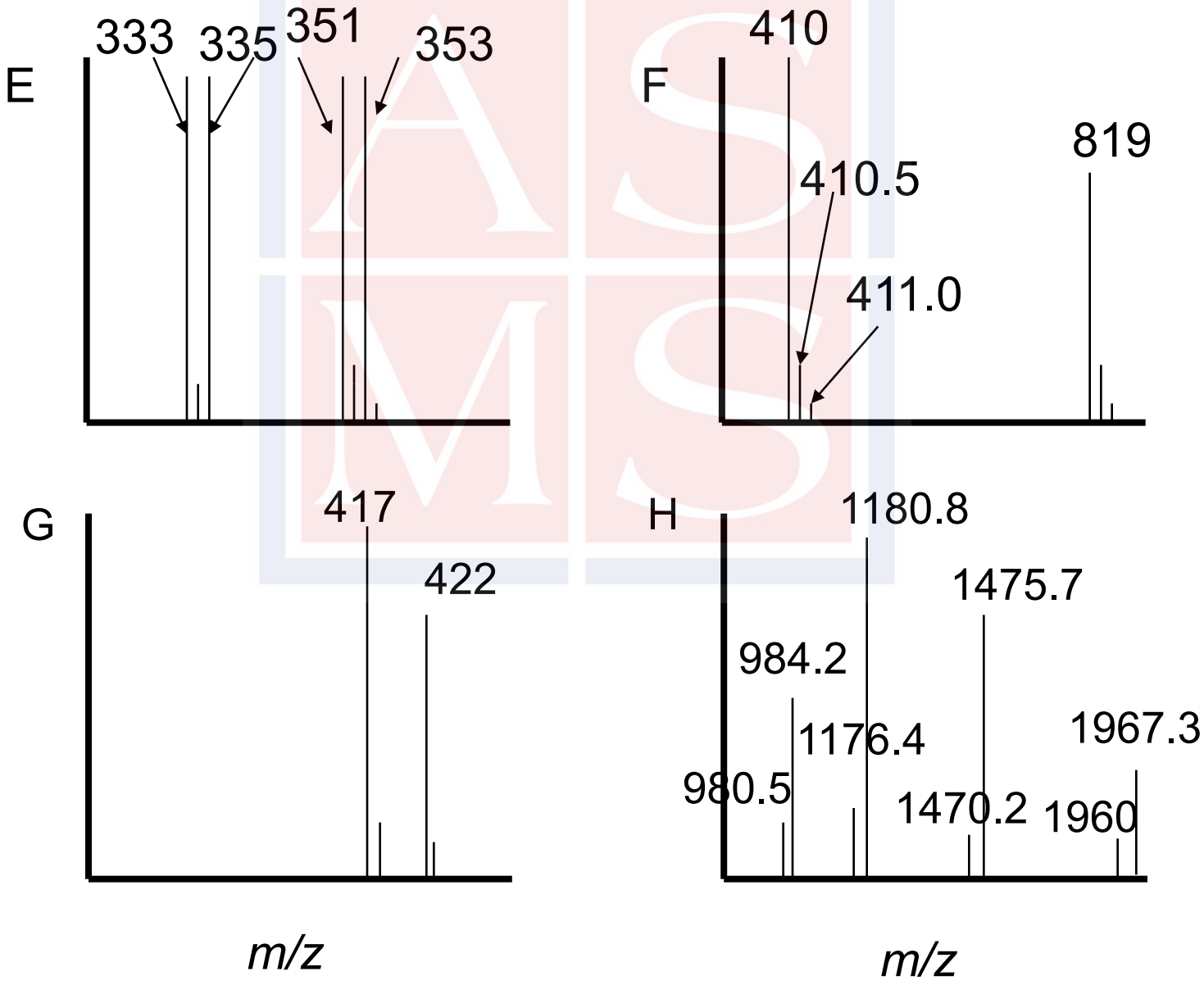
Identify the molecular weight.

Determine if there are any A+2 elements and comment on the number of nitrogen's for the positive ion electrospray mass spectra below.



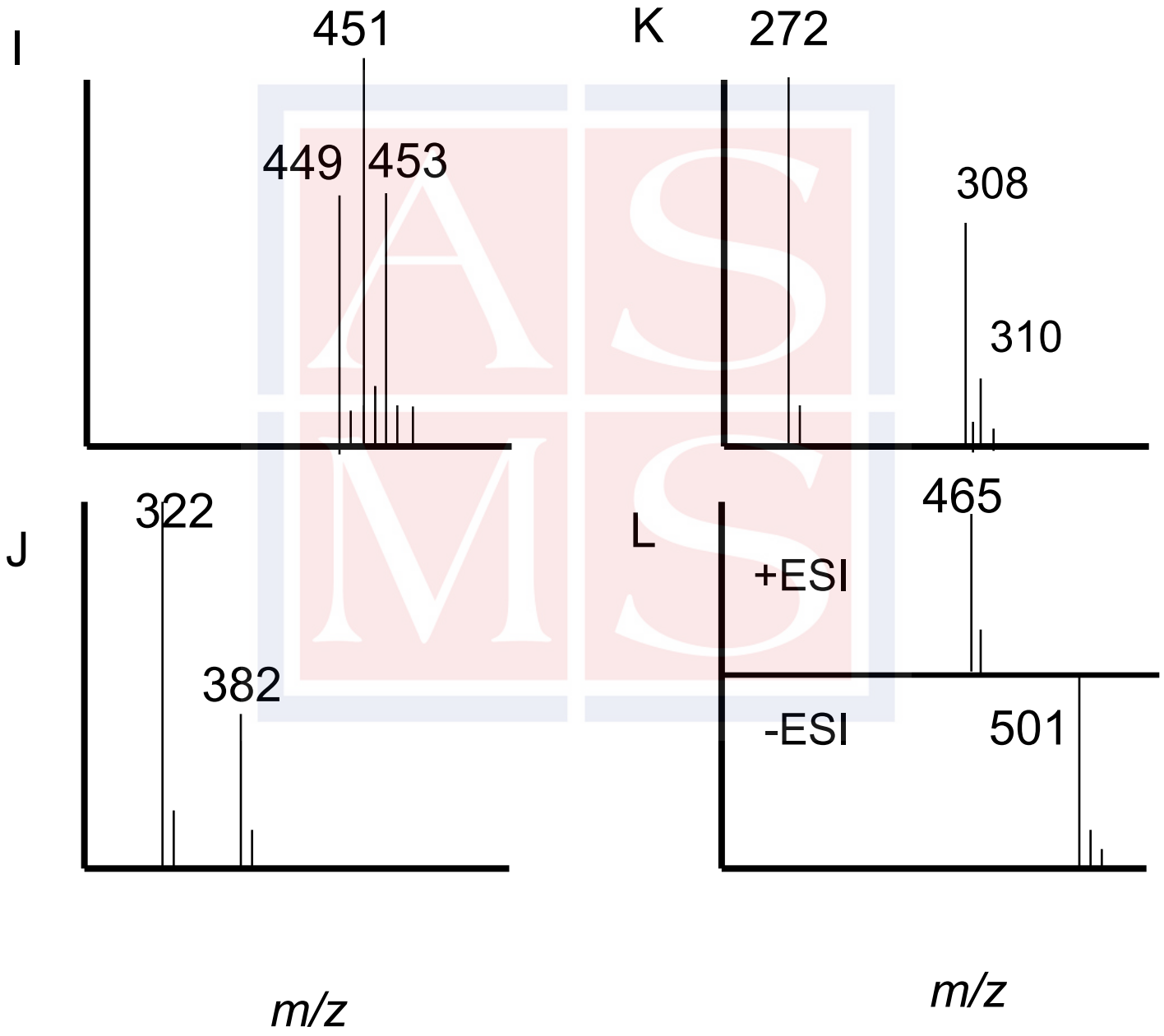
Problem Con't :

Identify the molecular weight. Determine if there are any A+2 elements and comment on the number of nitrogen's for the positive ion electrospray mass spectra below.



Problem:

Identify the molecular weight. Determine if there are any A+2 elements and comment on the number of nitrogen's for the negative ion electrospray mass spectra below.



Answers

A

- **MW a) 300 b)302 C) 278**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

B

- **MW a) 199 b)200 C) 221**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

C

- **MW a)180 b)181 C) 160**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

D

- **MW a) 200 b)400 C) 378**
- **A+2 Isotopes Yes NO**
- **# of Nitrogens Odd Even**

E

- **MW a) 332 b)334 C) 350**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

F

- **MW a) 409 b)818 C) 795**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

G

- **MW a)416 b) 421 C) 399**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

H

- **MW a) 5877 b)5900 C) 2938.5**
- **A+2 Isotopes Yes NO**
- **# of Nitrogens Odd Even**

I

- **MW a) 448 b)450 C) 452**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

J

- **MW a) 323 b)321 C) 381**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

K

- **MW a)309 b) 273 C) 309**
- **A+2 Isotopes Yes NO**
- **# of Nitrogen's Odd Even**

L

- **MW a) 442 b)500 C) 464**
- **A+2 Isotopes Yes NO**
- **# of Nitrogens Odd Even**

Questions

(1) Why do compounds 2000-3000 in molecular weight show little CID fragmentation?

a (number of oscillators) b dissipate internal energy C) take more energy to fragment d) all of the above, e none of the above

(2) Name at least three variables the operator can change to increase the extent of CID fragmentation in triple quadrupoles

A Collision energy, b pressure, c mw of gas, d time, e all of the above

F a, b, c

(3) What is the main difference between electrospray transport CID and triple quadrupole MS/MS?

A energy, b collision gas , c parent ion selection

(4) Will rearrangements be more favored at higher or lower ion energies (E_{lab})?

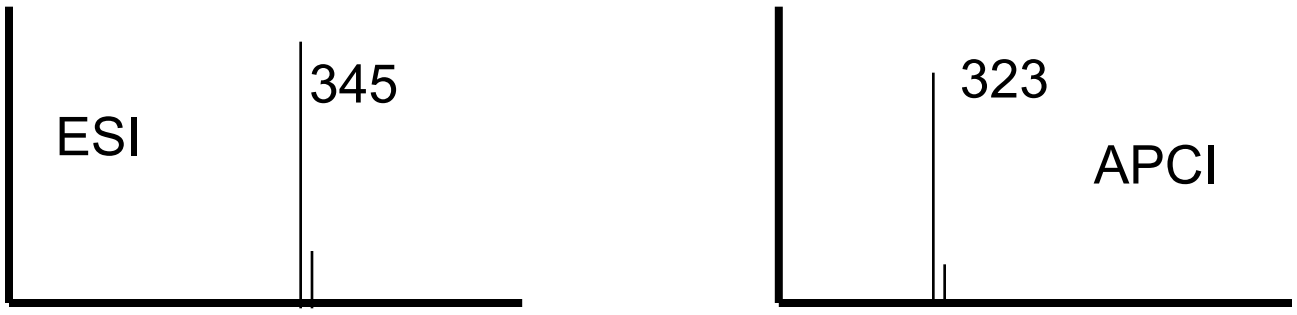
A higher, b lower, c makes no difference

(5) In inelastic collision, what is conserved?

(a) velocity of species?
(b) kinetic energy?
(c) internal energy
(d) total energy?
(e) all of the above

(6) Identify the molecular weight for this compound analyzed by APCI and ESI positive ion detection

A 322, b 344, c 346



Questions (continued)

(7) **What does tandem in space mean?**

- Give examples of tandem in space instruments.

a triple quad, b q-tof, c ion trap

(8) **What technique results in the highest CID efficiencies (low vs. high pressure) ? Why?**

A low pressure b high pressure c does not matter

(9) **List three reasons why one would perform MS/MS**

A sensitivity, b specificity, c speed, d complexity, e all a-d, f only a-c

(10) **What are 3 major driving forces that influence the m/z and intensity of the CID ion(s) detected?**

N

A Ion stability, b rate of reaction, c collision energy, d Instrument choice, e all of the above, only a, b, c

(11) **What would typically be added to an acetonitrile/water mobile phase to increase positive ion electrospray sensitivity?**

A Acid b base c NaCl d nothing

(11) **To increase negative electrospray sensitivity?**

(12) **A Acid b base c NaCl d nothing**

Questions (continued)

(12) Would positive ion or negative ion detection be used to detect the following compounds by ESI-MS (assume proper pH adjustment will be made)

(a) R-COOH

A positive ion b negative ion c either

(b) $R\text{-}NH_2$

A positive ion b negative ion c either

(c) $R\text{-}PO_4H$

A positive ion b negative ion c either

(d) $NH_2\text{-}R\text{-}COOH$

A positive ion b negative ion c either

(e) R-OH

A positive ion b negative ion c either

(13) Why are sulfate, phosphate or borate buffers not used in electrospray-MS?

A ion pairing, b volatility, c conductivity, d all of the above, e only b and c

(14) Name a compatible ion pair reagent (for cation) with electrospray.

a Heptafluoro butyric acid, b TFA, C tetra butyl ammonium hydroxide d Ammonium acetate, e all of the above

(15) Why do fragment ions observed in electron ionization (EI) differ from CID fragment ions generated from electrospray?

A collision energy, b radical ions vs even electron ions c both a and b, D none of the given answers.

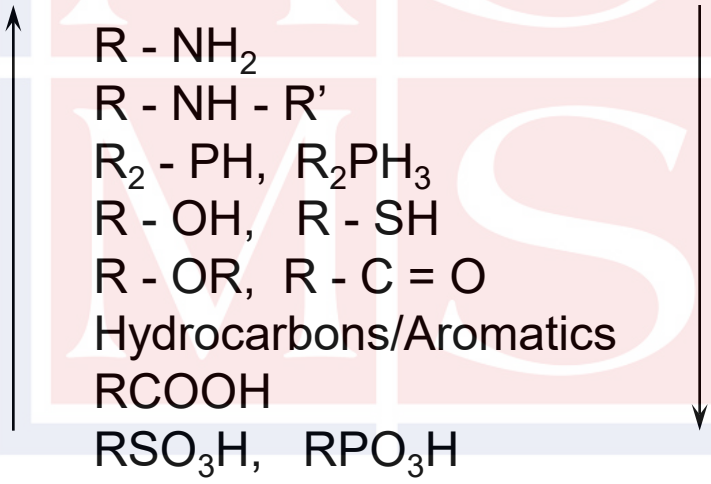
(16) Explain when odd electron CID fragments can be detected from even electron ions.

A when the compound is aliphatic , b at high collision energies, c when the compound is aromatic , d when it is energetically favored, e none of the above, f) c and d , g) a and d

Determining the Site of Ionization

Recognizing the site of ionization in API techniques is important when postulating inductive cleavages and rearrangement processes. For positive ion detection the site of protonation must be postulated. The ease of protonation depends on the compounds basicity and follow this general order

Favorable for Protonation
(+ Ion Formation)

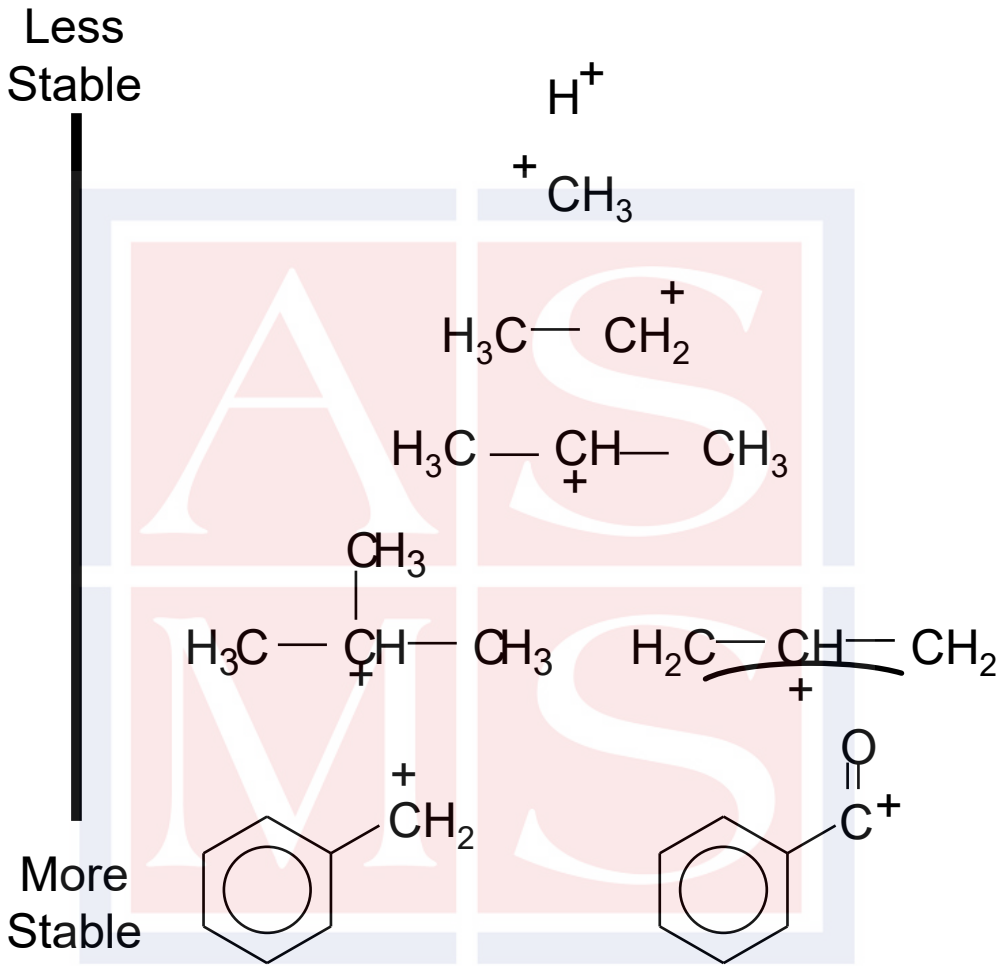


(- Ion Formation)
Favorable for Deprotonation

Common Neutral Losses

Mass	Fragment
2	H_2
17	NH_3
18	H_20
20	HF
27	HCN
28	CO/C_2H_4
30	CH_2O
34	H_2S
36	HCl
44	CO_2
74	$C_3H_6O_2$
80	HBr

Carbocation Ion Stability



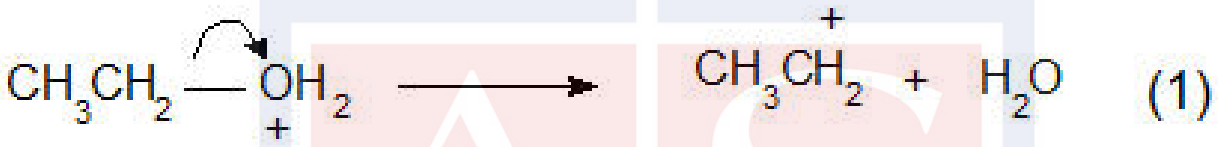
Types of Fragmentations

There are several fragmentation mechanisms common to even-electron ions:

- Single bond cleavage with charge migration
- cyclization with charge migration
- Multiple cleavages
  - with charge migration
  - with charge retention
  - ring fragmentations
- Rearrangements
  - hydrogen rearrangements
- Homolytic cleavages
  - charge site remote fragmentation

Single Bond Cleavage

The simplest of all cleavage mechanisms. This pathway involves the movement of two electrons from one bond toward the charge. Migration of the charge is typical of single bond cleavages. An example is shown in Equation 1.

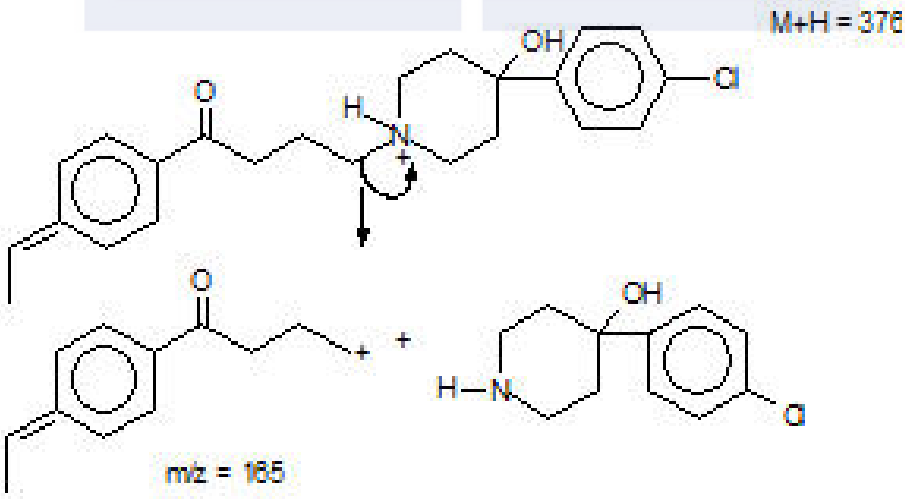


(1)

The protonated molecular ion of ethanol can undergo a single bond cleavage to form an ethyl carbocation and a neutral molecule of water. The charge has moved from the oxygen to the carbon. The formation of a stable neutral molecule is the important factor affecting this reaction pathway. The stability of the product ion formed is also another important factor.

Example of Single Bond Cleavage:

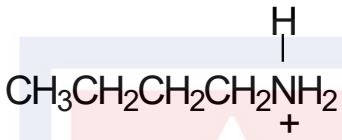
Haloperidol



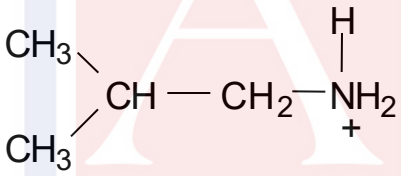
Question 1

Which of the following three compounds would have the highest abundance of signal correspondence to the single bond cleavage product? Why?

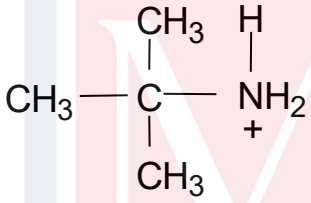
A.



B.

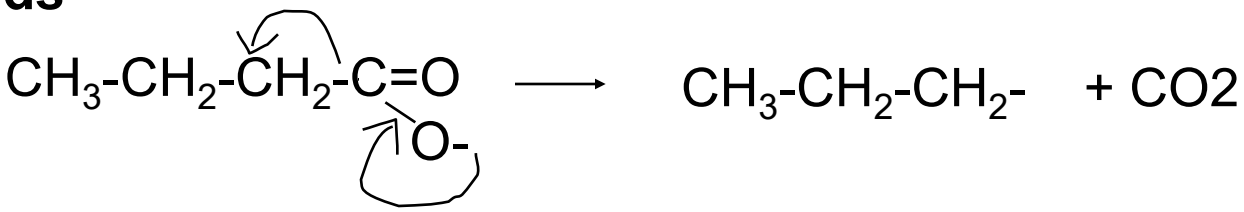


C.



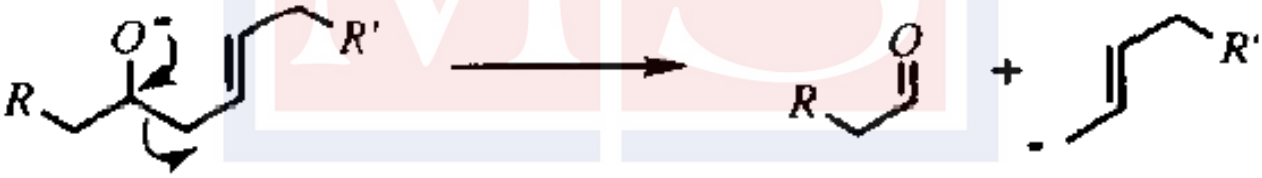
Negative Ion simple cleavage

Acids



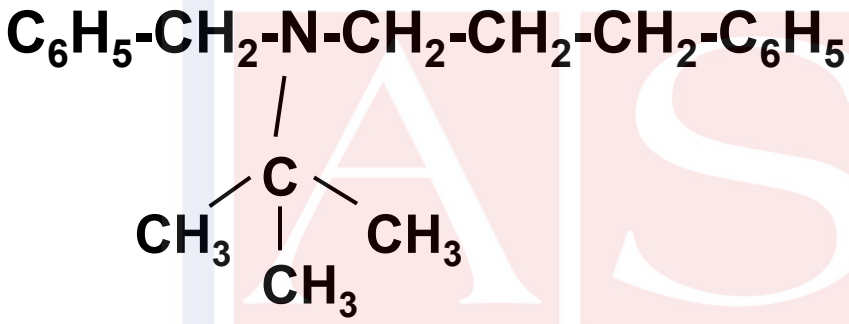
The formation of a stable neutral molecule and stability of the product ion are the important factor affecting this reaction pathway.

Alcohols

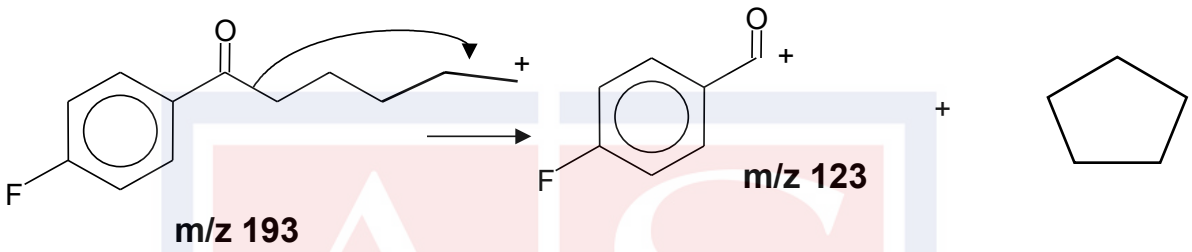


Single bond cleavage-Problem

- List simple cleavage products for the compound below and order in the products based on the relative ion intensity.



Example Cyclization



Note: Cyclization forms a stable leaving group

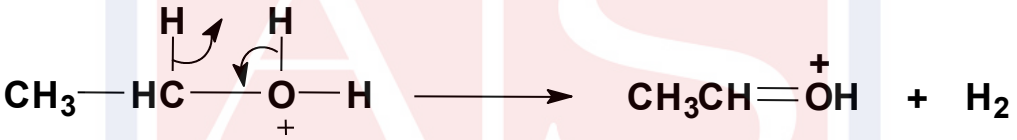
Cyclization is very common in a transition state as Prerequisite to fragmentation via a 3, **4 (most common),** 5 and 6 member ring.

They can be charge driven or charge remote

Multiple Cleavages

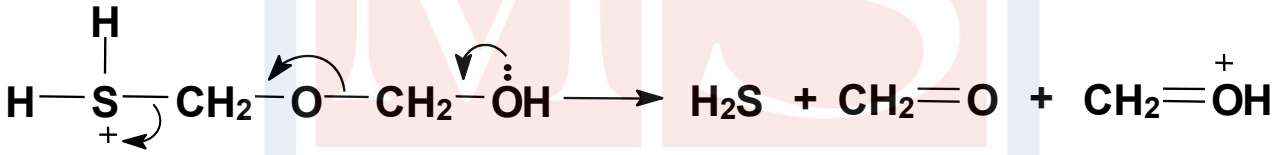
When more than one bond is broken during fragmentation, the charge may move from the initial site or remain localized. In most instances, a neutral molecule is eliminated as shown in Equation 3

with retention



(3)

with migration

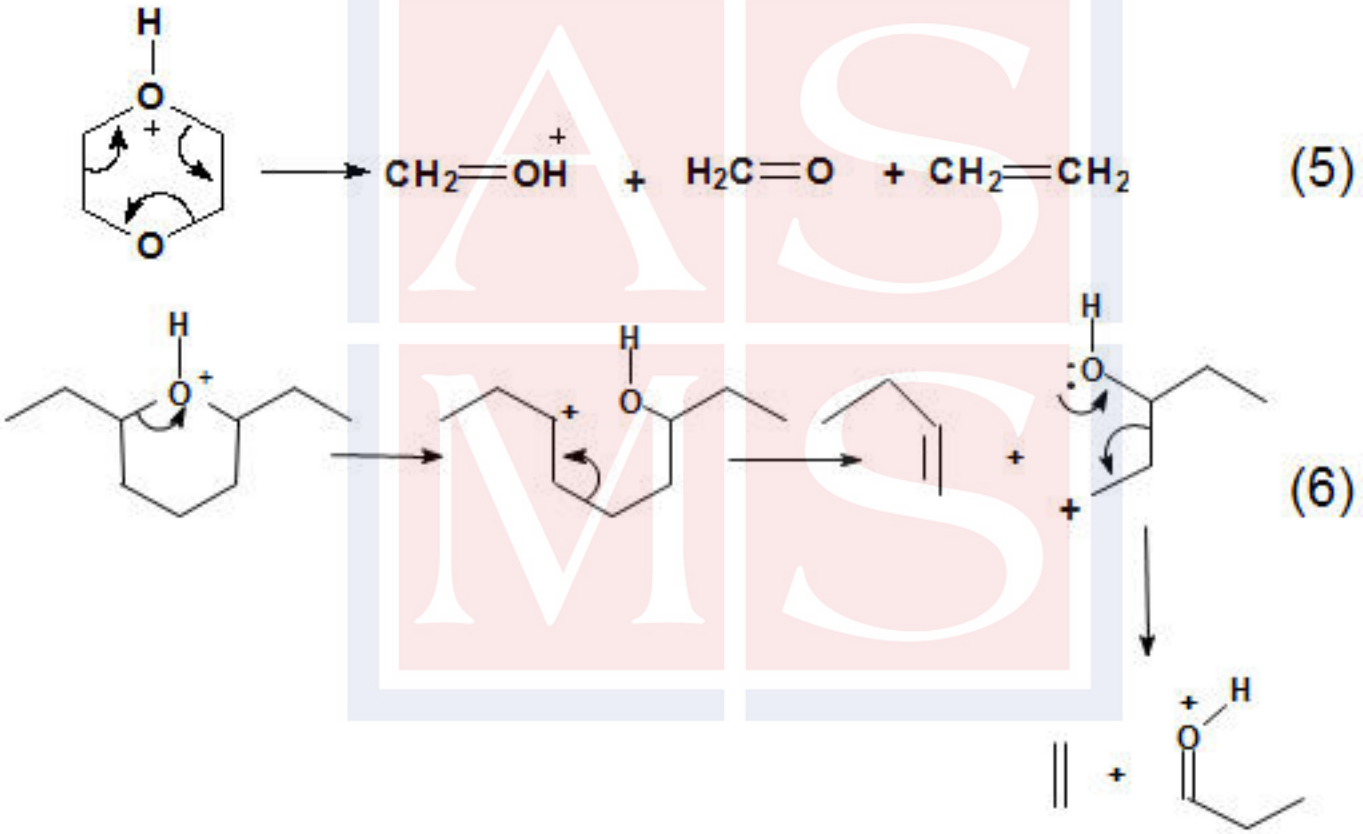


(4)

McLafferty states that concerted H transfer and 1,2 hydrogen shifts are common in ee fragmentations.

Ring Opening and Fragmentation

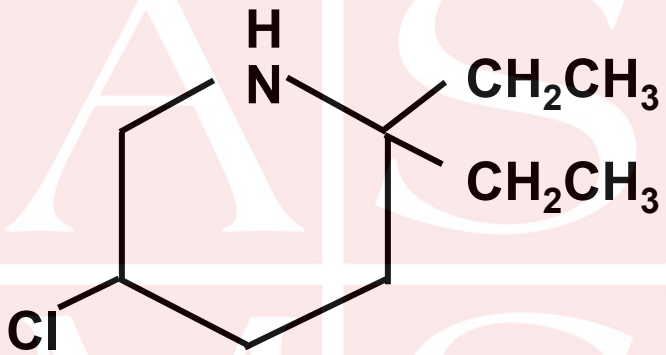
Ring fragmentations require the cleavage of multiple bonds. With even-electron ions this results in charge retention. Hydrogen rearrangements are also typical of even electron fragmentations.



Ring Opening--Problem

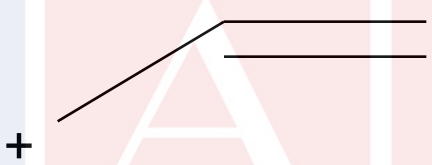
Draw ring opening fragmentation pathways for compound below:

Which ring opening pathway is favored (opening on the left or right of N)?



Rearrangements to form Allyl Carbocations

Reactions involving H-rearrangements, ring opening and intermediate 4,5 or 6 member complexes are common in ring systems. The driving force is the formation of more thermodynamically favorable allyl carbocation ions.

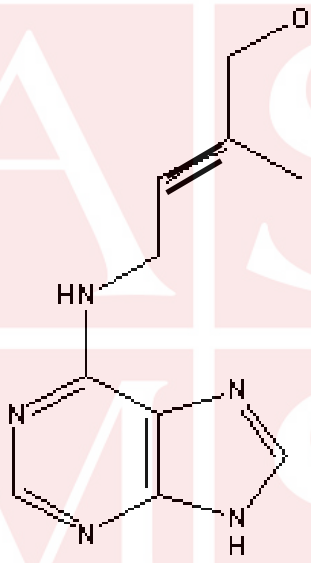


Multiple bonds are involved and charge can be either retained or moved.

Problem

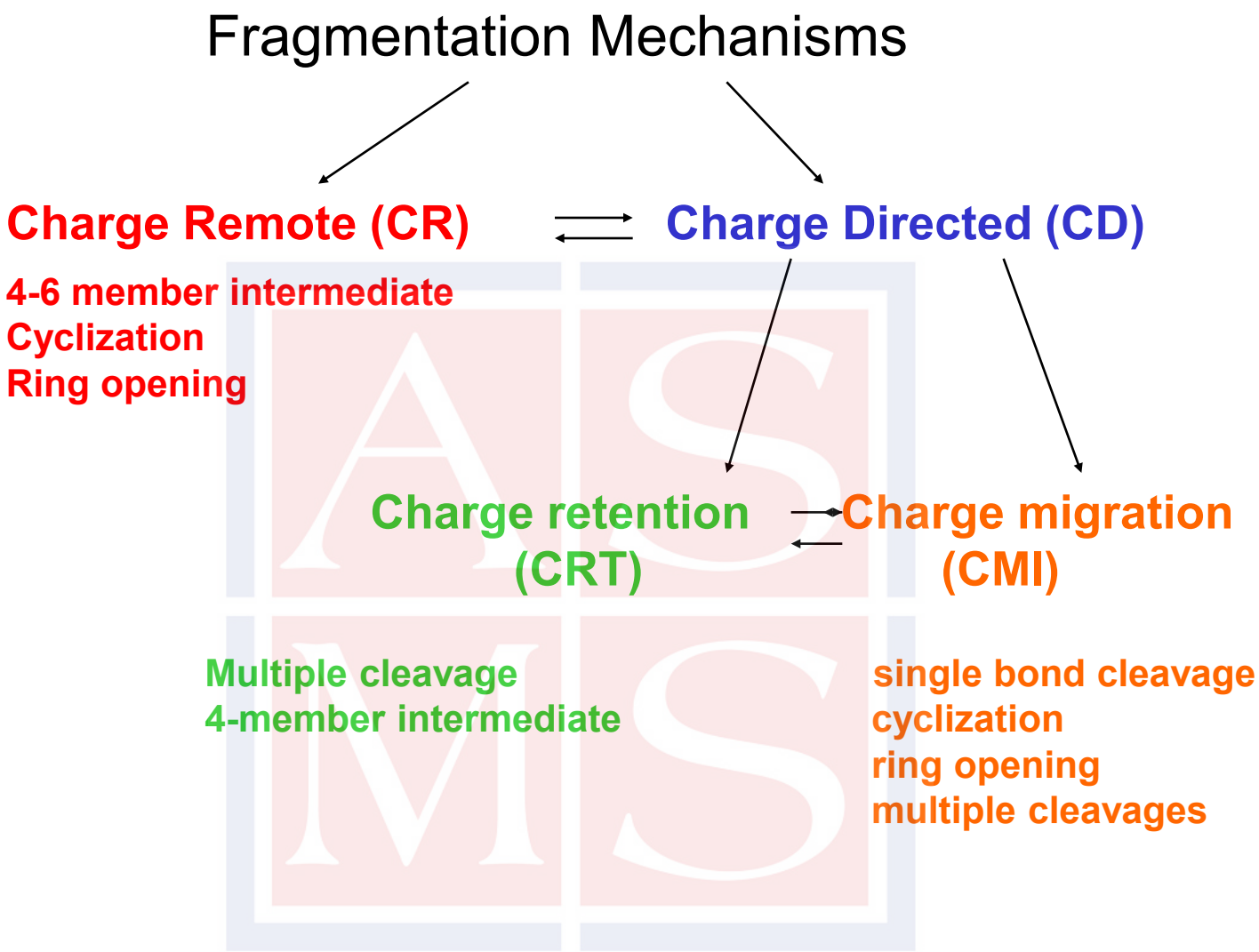
Explain the peaks in the MS/MS spectrum at m/z 85 and 136 for the compound shown below:

positive ion detection electrospray



MW 219

Summary of Fragmentation Types



Attachment Processes

• Cations or anions can attach via lone pair electrons on N, S or O to form clusters in ESI (Only gas phases clusters are formed in APCI).

•These cluster ions can offer better sensitivity and different CID fragmentation information than protonated or deprotonated molecular ions.

•Typically only charge remote processes will result in useful information from these cluster ions

•Generally best sensitivity is observed from the smallest anions or cations

Li>Na>K>Rb>Cs

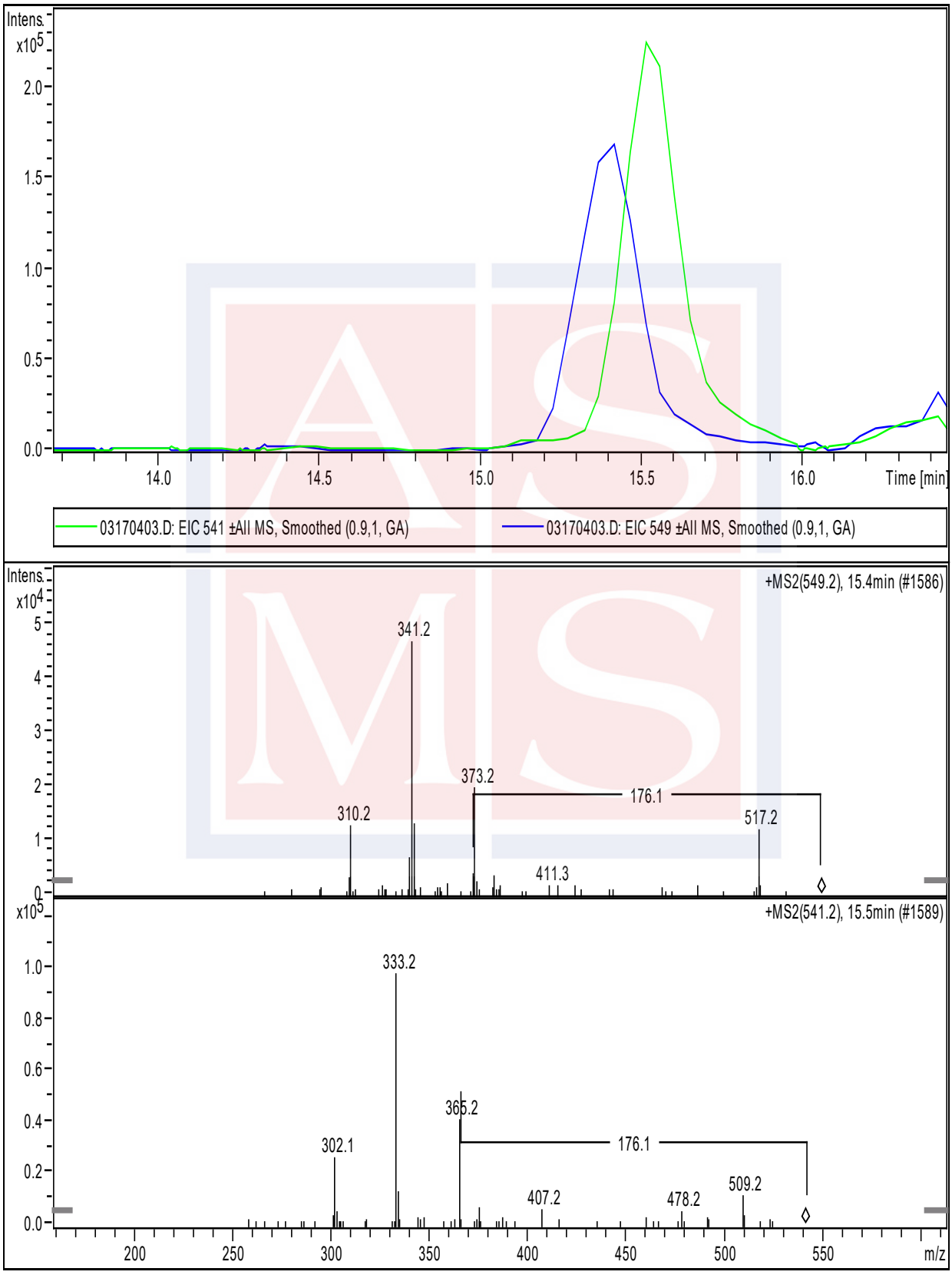
F>Cl>Br>I

•Structural information can vary with size depending on the overlap of the anion or cation with the organic molecule orbital.

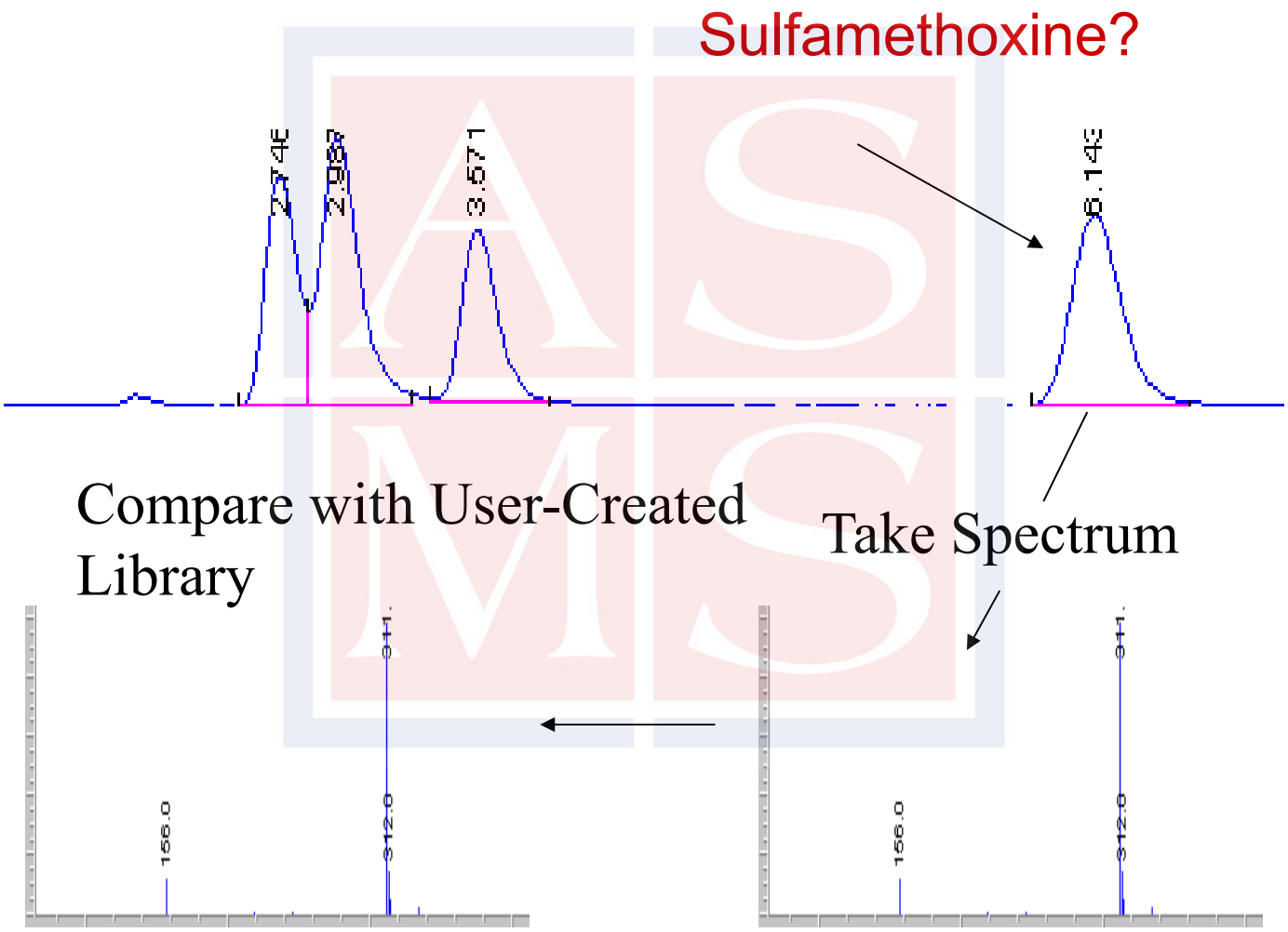
Use of Isotopic Labeling to aid in CID Interpretation

- **Labeling with stable isotopes (e.g. deuterium, 13C, 15N or 18O can greatly aid in finding metabolites and postulating their structure. (e.g equal molar dosing of labeled and unlabeled and searching for equal intensity peaks at mass difference for labeled and unlabeled).**
- **Use of naturally occurring isotopes from A+2 elements can also aid in finding metabolites and elucidating their structure.**
- **Exchange experiments with D_2O can aid in the determination of exchangeable protons and in CID structure elucidation. Exchangeable protons are commonly on N, O, and S. These exchange experiments need to be performed in the absence of solvents with exchangeable protons**
- **Care must be taken in the choice of isotopes, mass increase, placement of the isotopic labeling and solvents in exchange experiments,**

LC/MS/MS Glucuronide Metabolite d0/d8



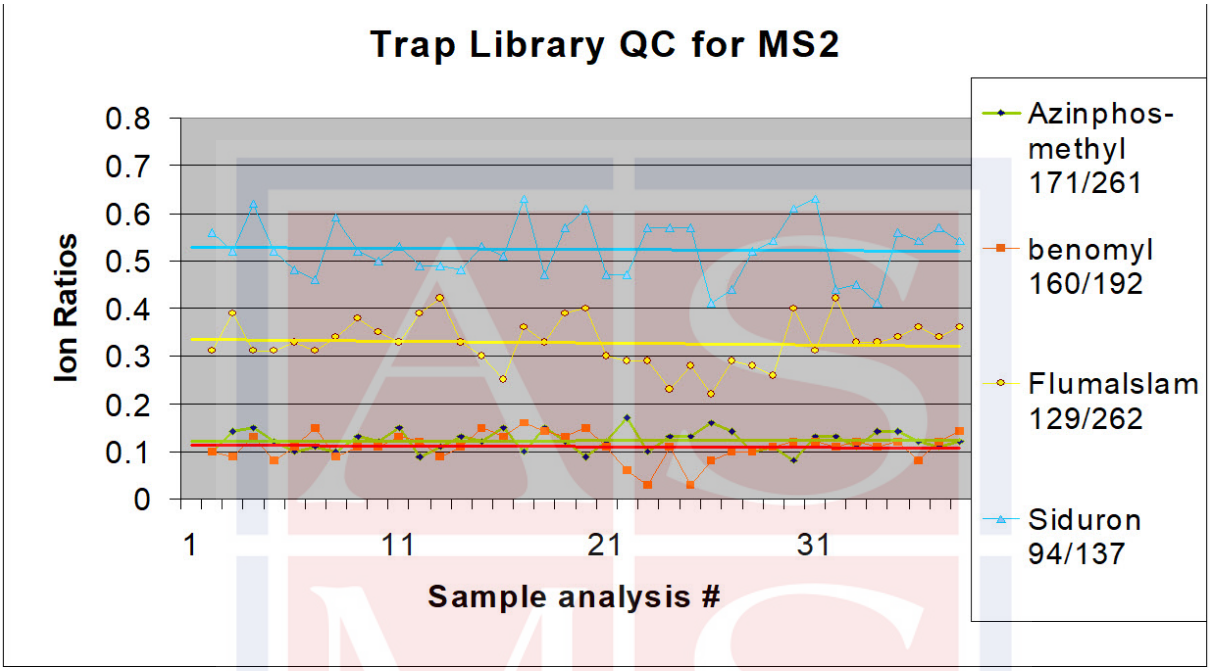
Spectral Libraries: Principle

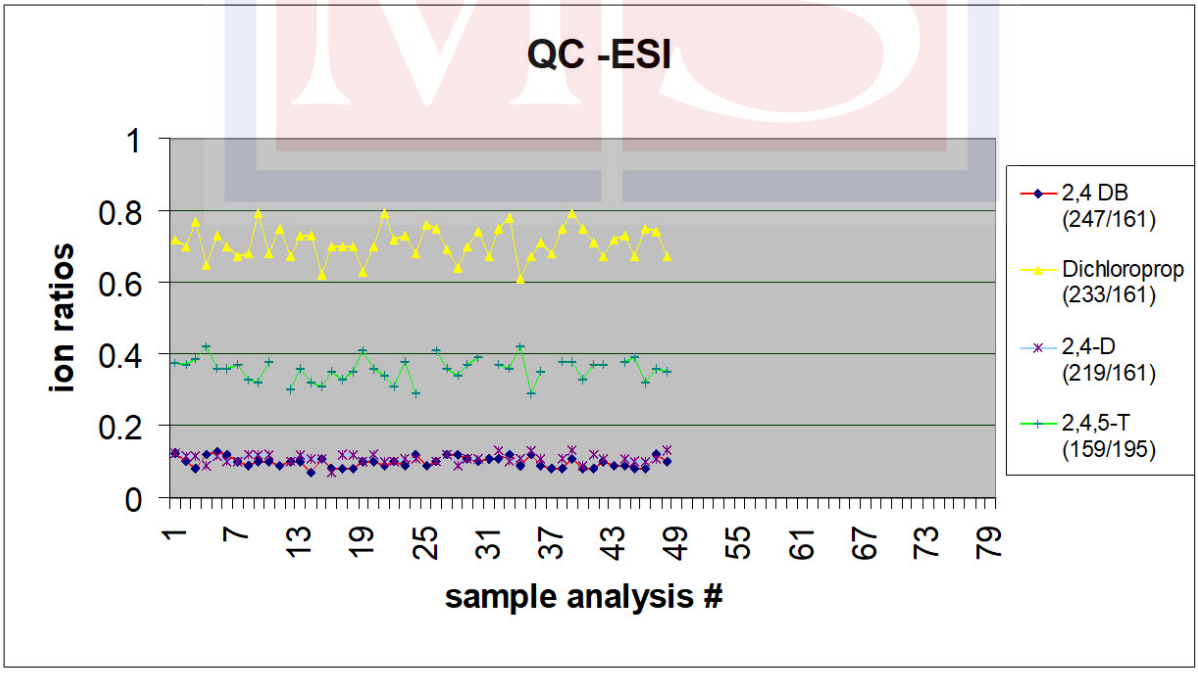


Generating Reproducible CID Spectra (for Library Searching)

- Check that the CID reference compound shows same m/z and relative intensity (±10%) used to generate library or spectrum on a different time or instrument.
- Only search a library that uses the same CID energy that was used to generate the unknown CID mass spectrum.
- It is often advantageous to create two libraries (higher energy and lower energy CID library) to enable generation of molecular species and fragmentation from a broader range of compounds.
- CID libraries created on the same instrument type should match if the reference compound tune is met. However, different instrument types (e.g., ion trap and triple quadrupole) may not generate CID spectra that are equivalent. This is due to fundamental differences in operation, collision time, collision efficiencies, collision gases and ion energies, potentially resulting in different m/z ion and relative intensities. The instrument variables that can be changed may not be sufficient to achieve an equivalent CID mass spectrum of the reference compound.

Generating Reproducible CID Spectra (for Library Searching)

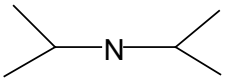


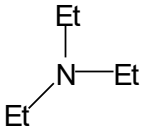


Questions (continued)

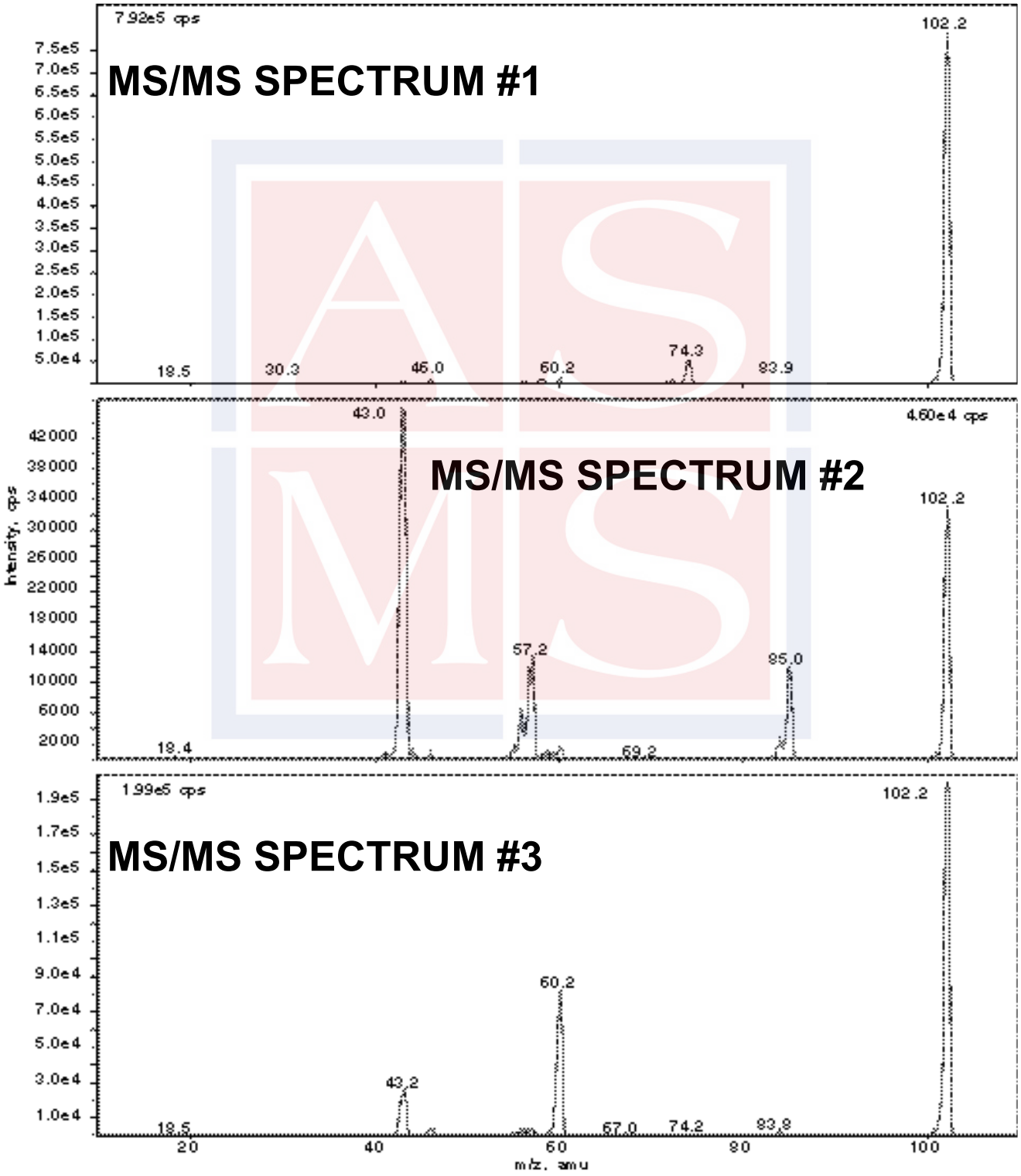
1. Match each spectrum with a structure. Collision energy 15 eV.

STRUCTURES



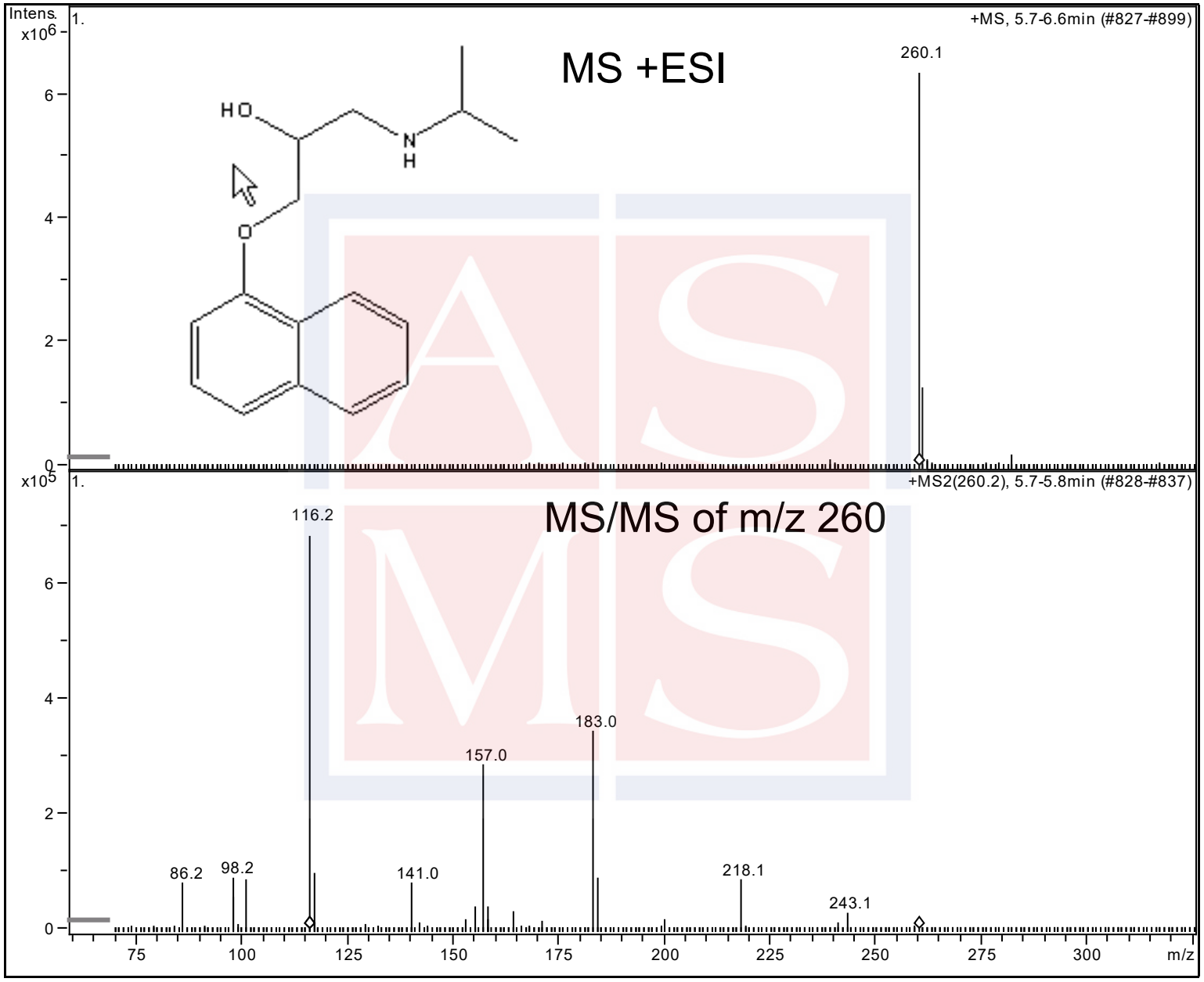


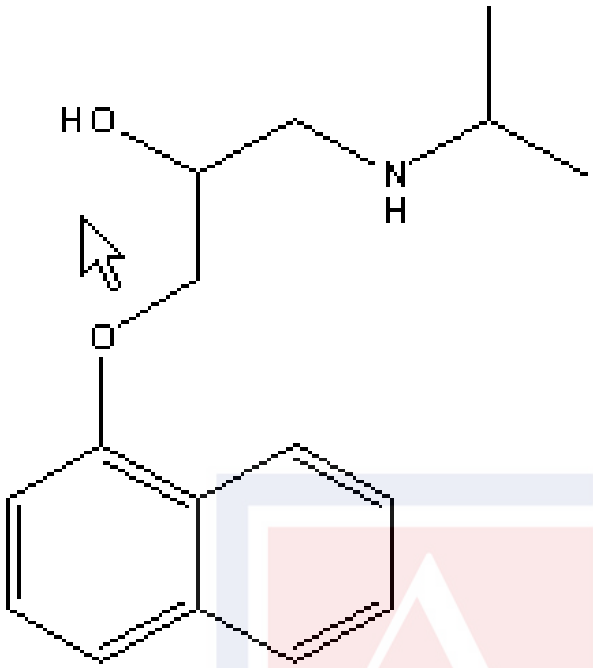
$CH_3(CH_2)_5NH_2$

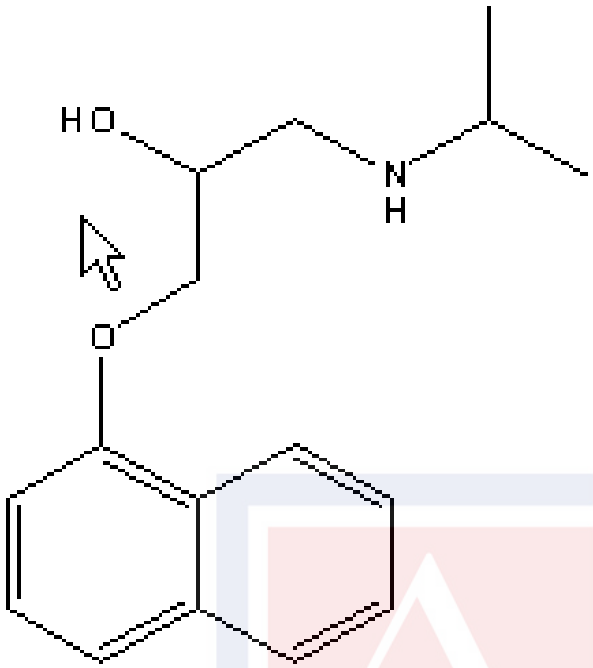


#2 Problem

Identify the ions at m/z 116, 157, 183 and 218 in the MS/MS spectrum below:




HO
N
H
O


HO
N
H
O

Quantitative LC/MS/MS

Sensitivity- Maximization of the quantifying ion current

- Sample Preparation
  - –Clean up sample
  - –Concentrate sample
  - –Final solution in weak LC solvent (on-column concentration)
- LC Separation
  - –Sharp peaks (maximize peak concentration,1-3 um particles)
  - –Fast (short columns)
  - –Minimize additives that cause ion suppression
- API Source
  - –Acid/base chemistry
  - –Optimize capillary exit/cone/orifice voltage
  - –All ion current in one m/z ion vs. adducts
  - –+/- ion detection
  - –Post column addition
  - –Optimize all lenses for ions of interest
  - –Divide chromatogram into segments
- MS Analyzer
  - –Tandem MS
  - –Optimize product ion formation
  - –High resolution
  - –SIM or MRM
  - –Optimize dwell times to get sufficient points across peak
  - –Divide MS acquisition into segments

Sensitivity based upon ionization mode and polarity of detection

1. Evaluate different modes if ionization APPI, APCI and ESI for a target compound. Compare s/n not absolute area since chemical noise will vary the mode of ionization.

2. Compare negative and positive ion detection. Compare s/n not absolute area since chemical noise will vary the polarity of detection

Usual observation: Negative ion is less sensitive than positive for my compound even though it is a zwitterion and should form positive and negative ion equally well.

Reason for this observation: negative ion current amplification is different than positive, resulting in lower intensity peaks. However the noise is lower as well.

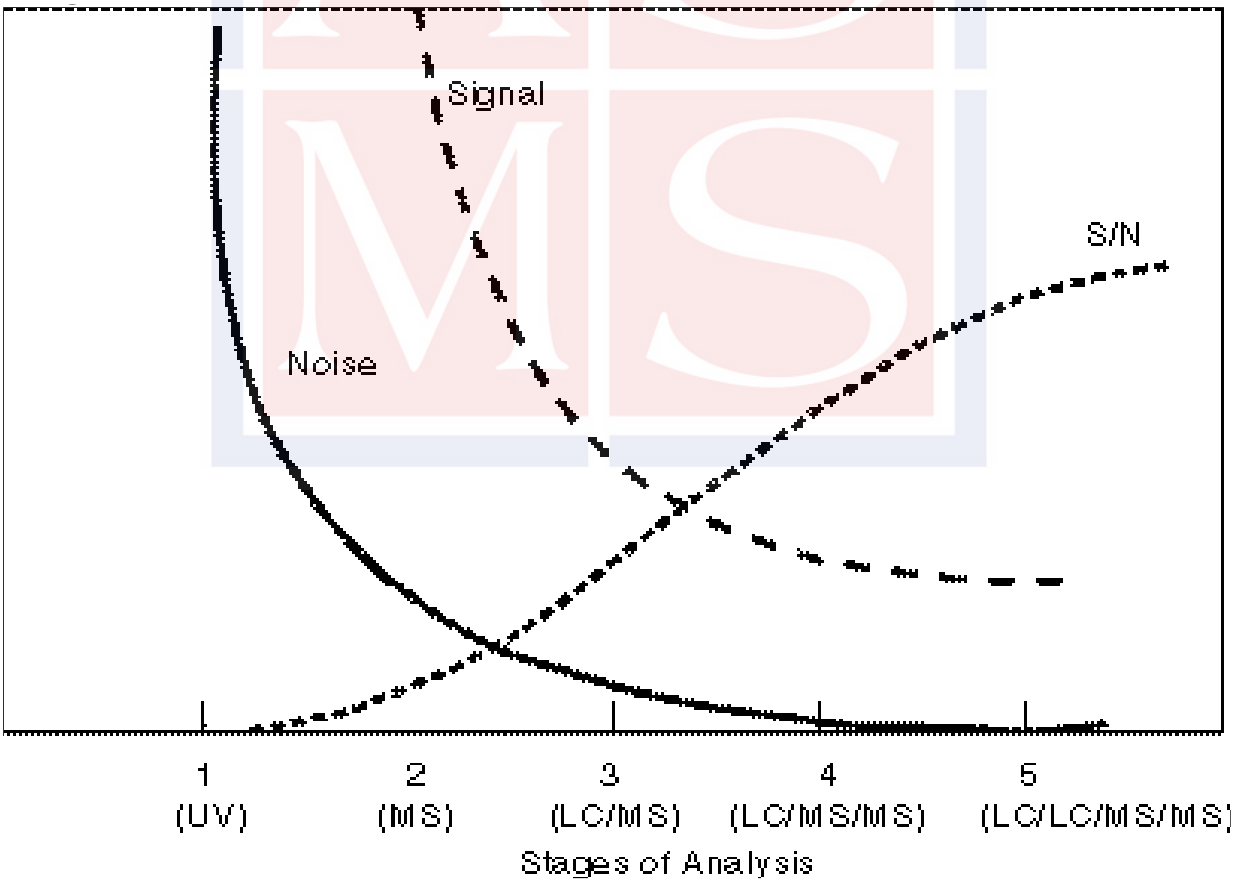
Conclusion: base comparison on s/n

General observation: There appears to be less chemical noise from a biological sample in negative ion detection

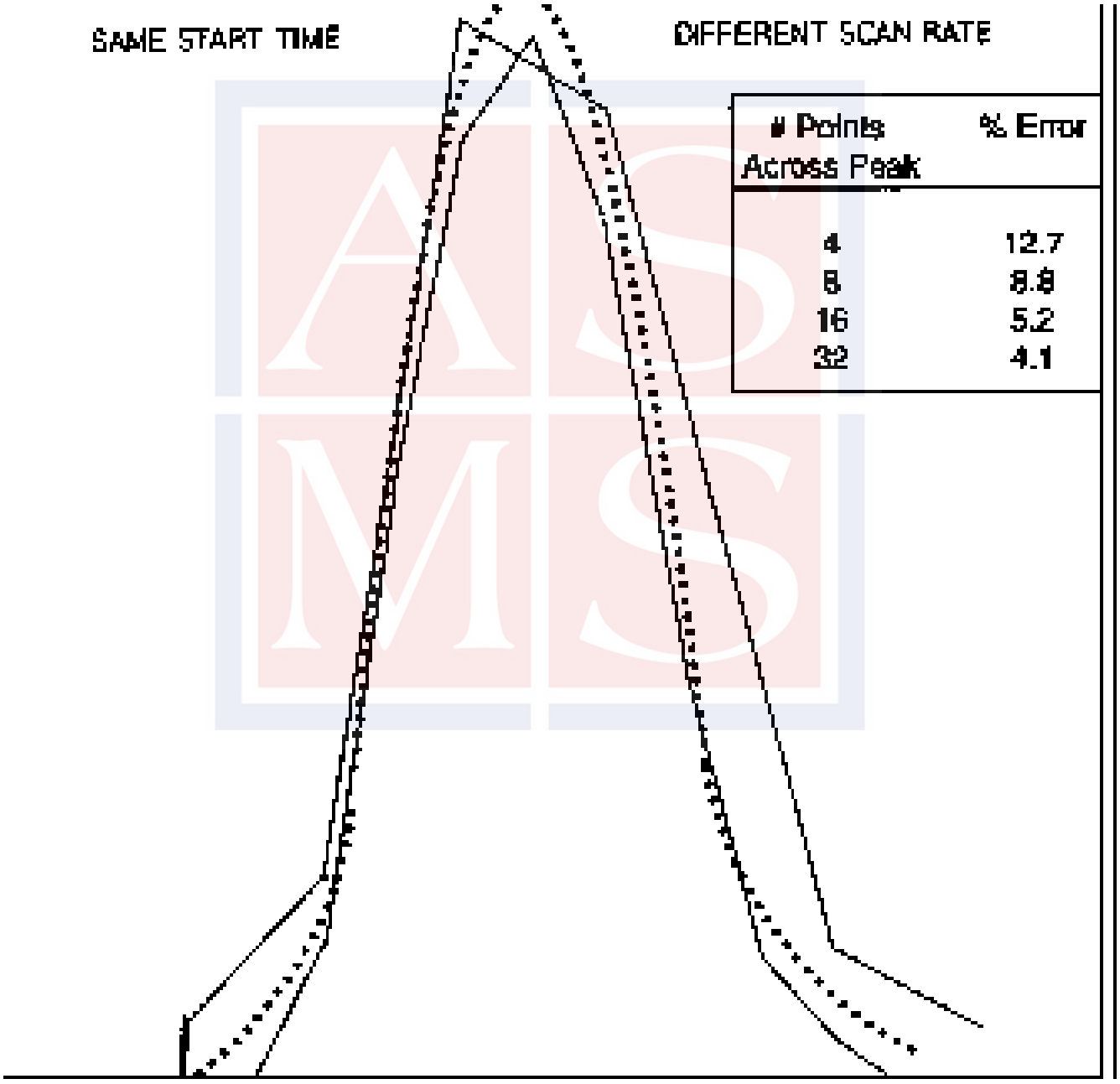
Increase in S/N with Increasing Stages of Analysis

- Keypoints:

  The use of multiple stages of analysis can greatly decrease the chemical noise of the analysis resulting in an increase in signal to noise (S/N) ratio. If the measurements are not limited by chemical noise then subsequent stages



Quantitative Analysis: Precision and Accuracy With Different Scan Rates



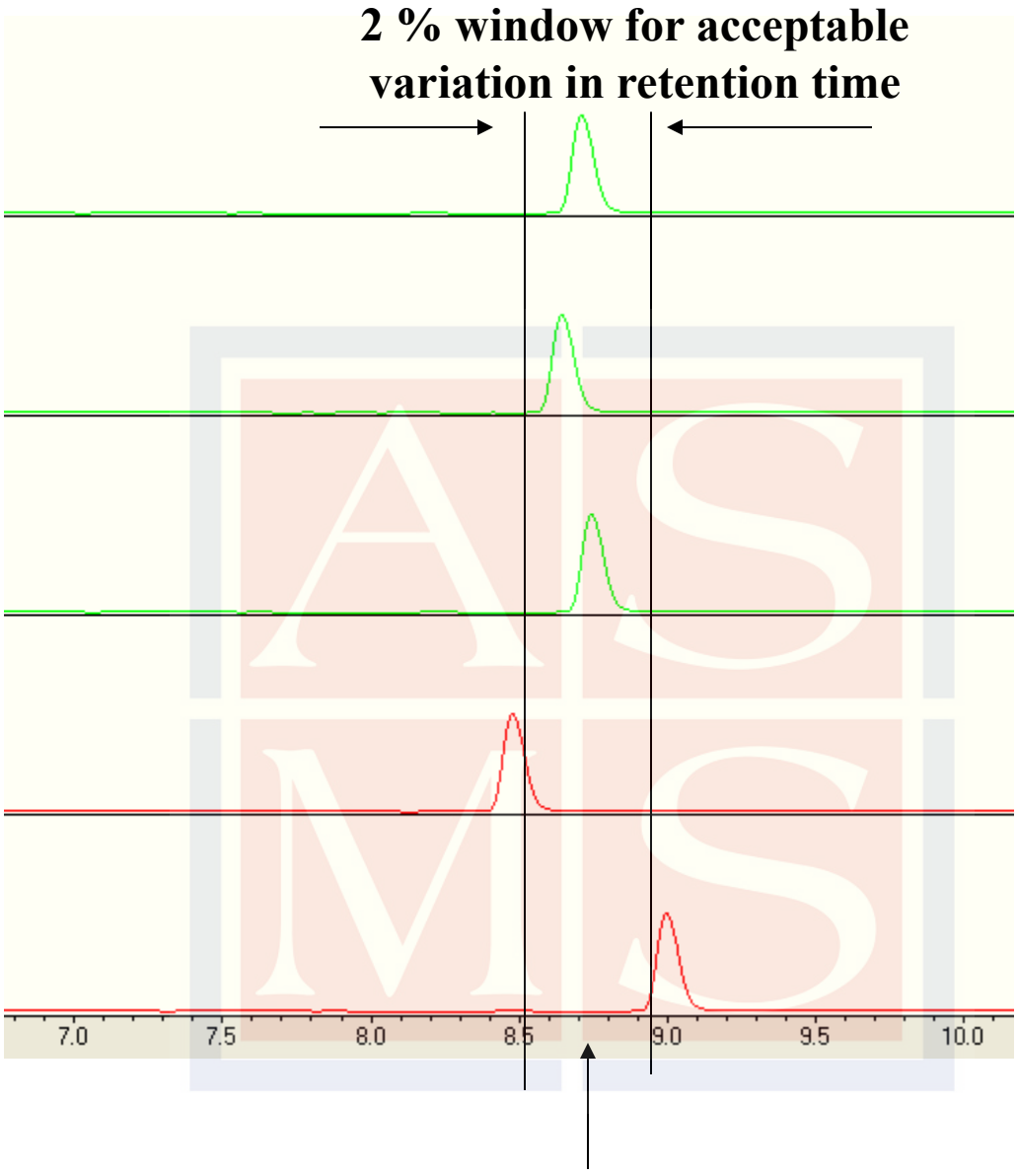
Specificity

- Detection of 3 or more ions at the proper retention time, proper m/z and at the proper ratios of intensity. J. Sphon; J. Assoc. Off. Anal. Chem., 61(5), 1247-1252 (1978).

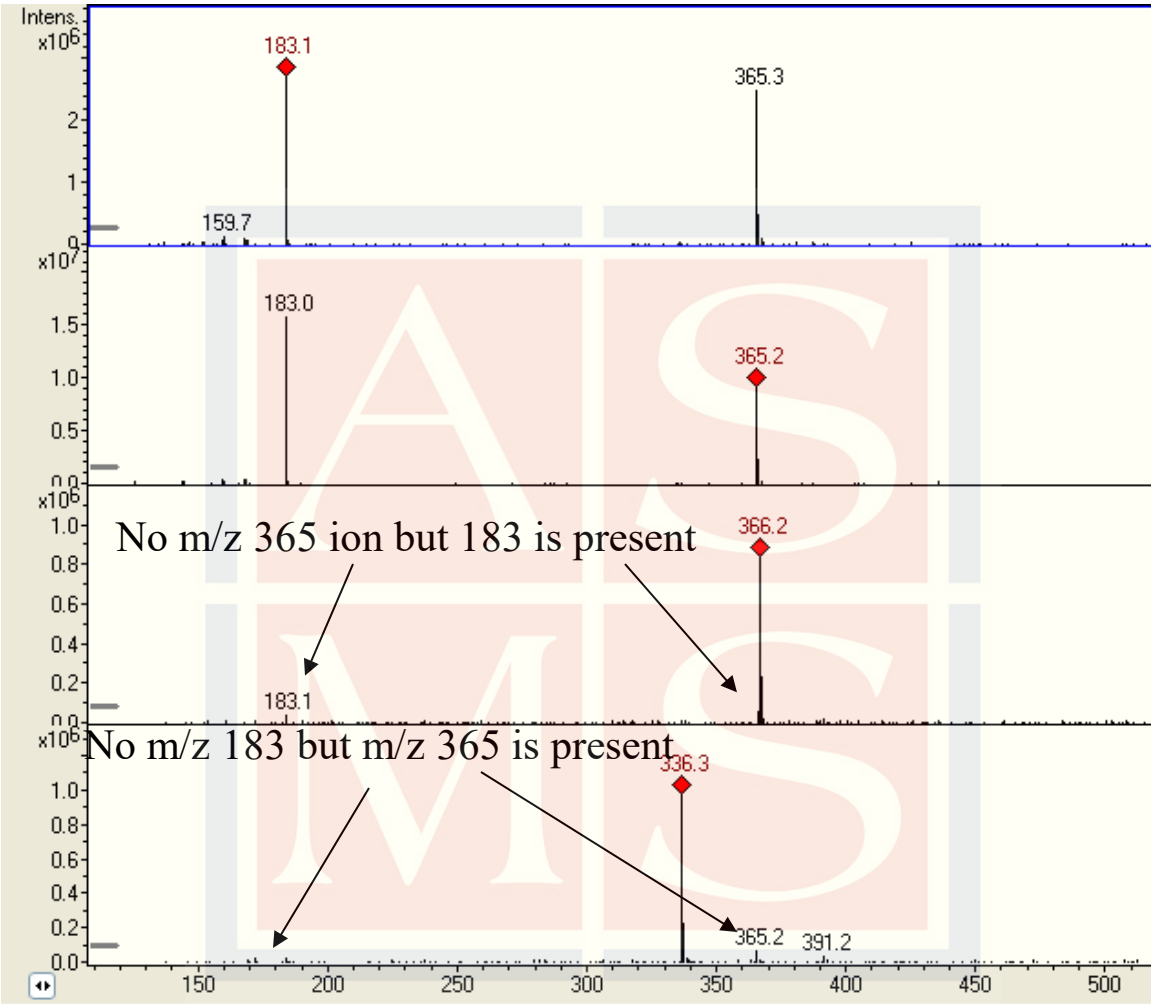
- Matrix does not interfere with target ions (<30% response of matrix to LOD).

- Ways to improve specificity:
  - sample clean-up
  - separation
  - different ionization techniques (e.g., negative vs. positive ion detection
  - MS/MS, high resolution MS

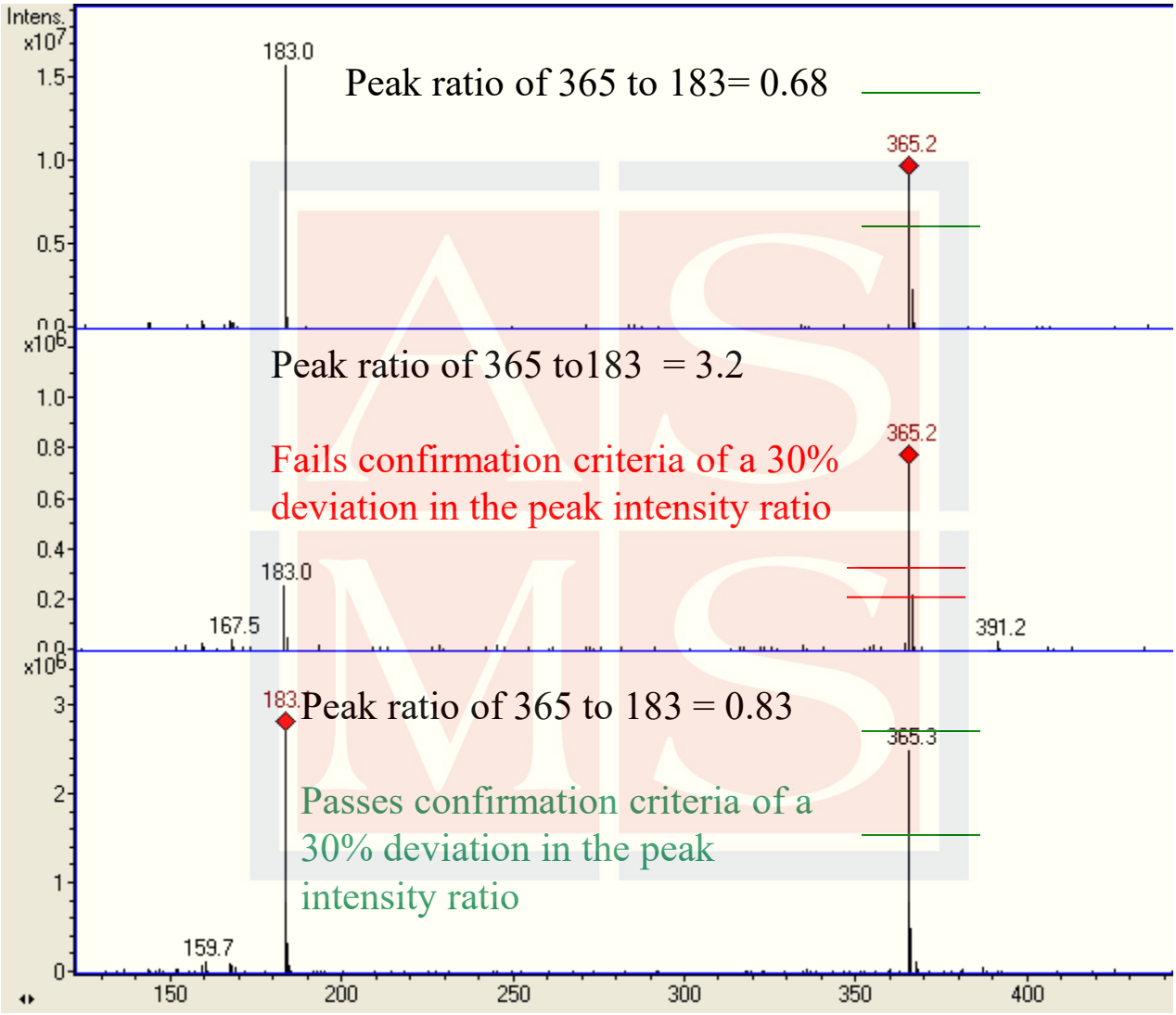
Retention Time Matching



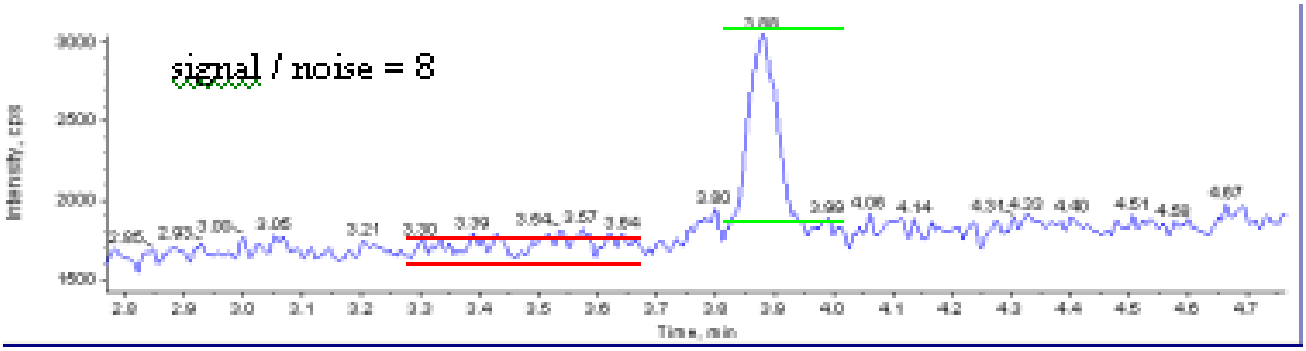
Monitoring 2 or more ions

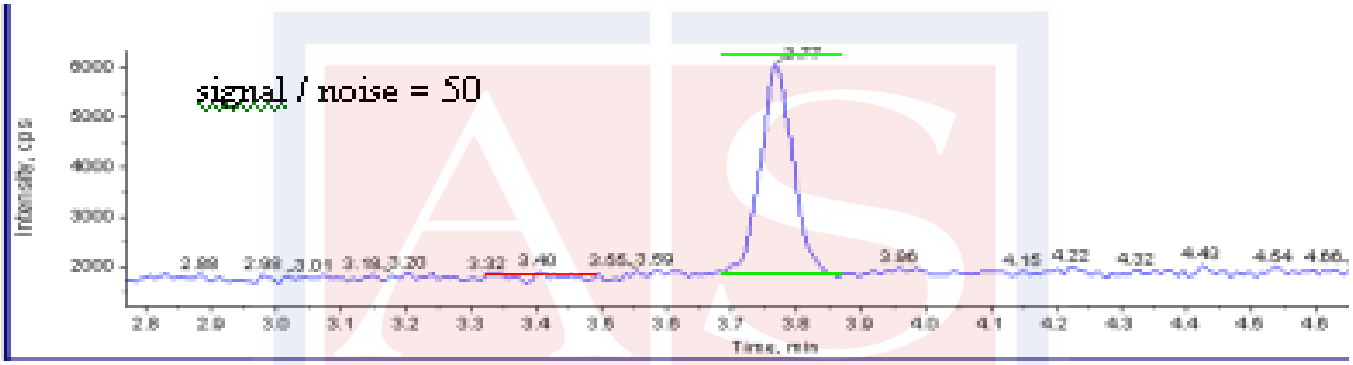


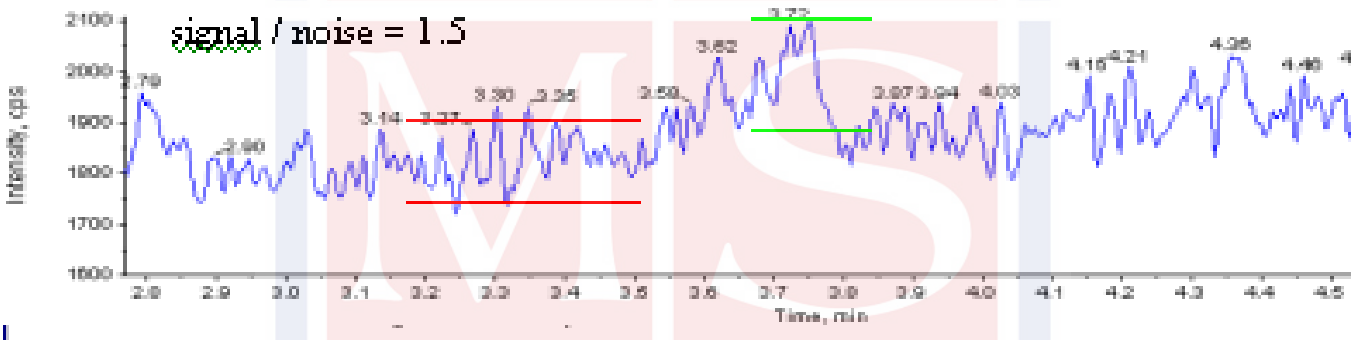
Ion Ratios

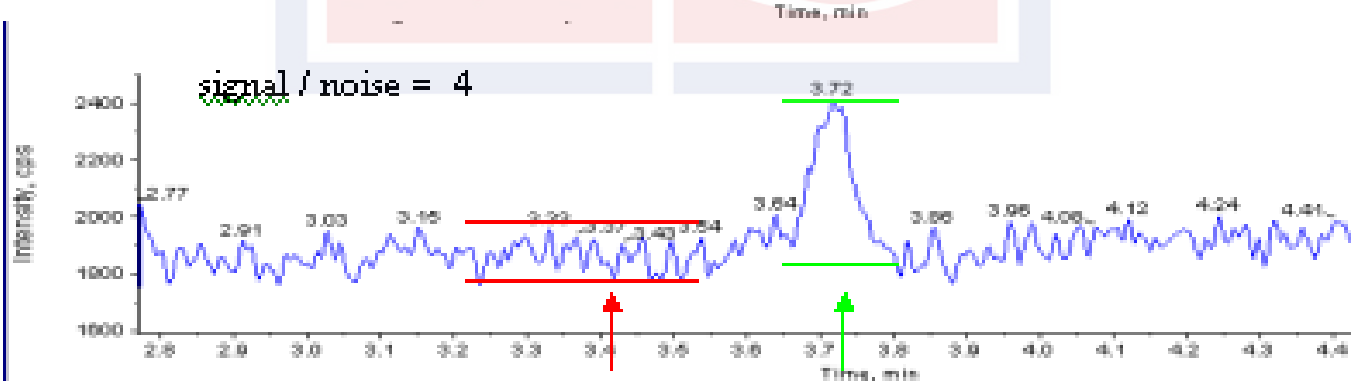

Intens.
x10^7
1.5
1.0
0.5
0.0
183.0
Peak ratio of 365 to 183= 0.68
365.2
x10^6
1.0
0.8
0.6
0.4
0.2
0.0
Peak ratio of 365 to183 = 3.2
Fails confirmation criteria of a 30% deviation in the peak intensity ratio
365.2
183.0
167.5
391.2
x10^6
3
2
1
0
183.
Peak ratio of 365 to 183 = 0.83
365.3
Passes confirmation criteria of a 30% deviation in the peak intensity ratio
159.7
150
200
250
300
350
400
m/z

Signal / Noise



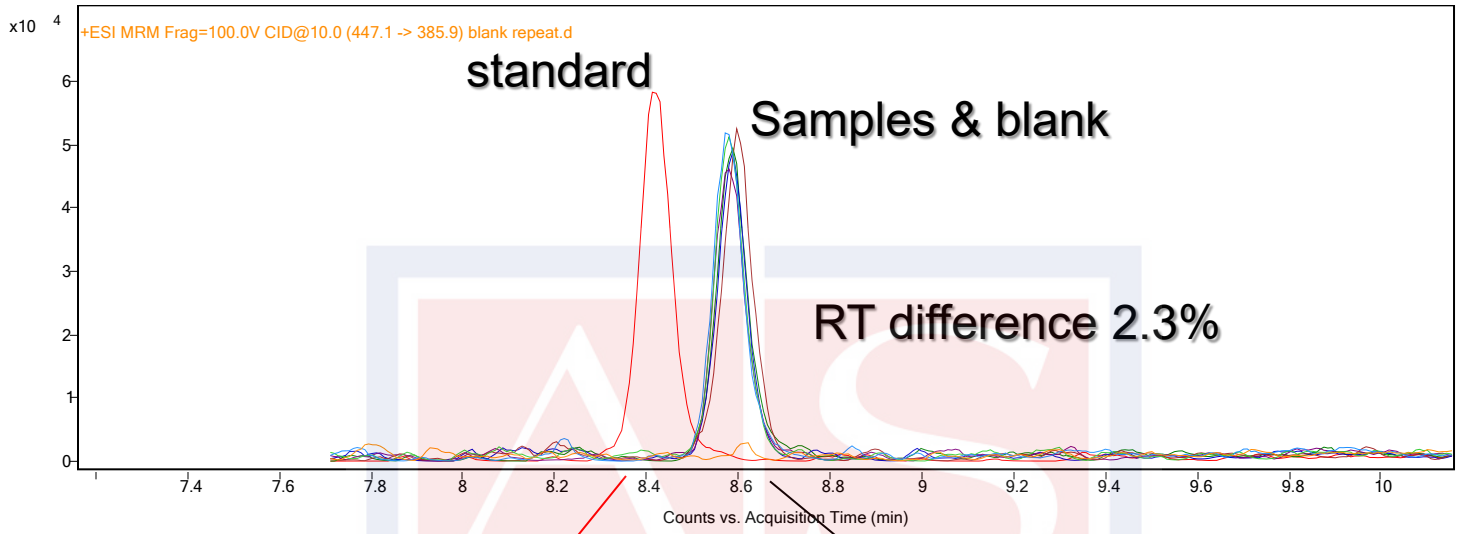




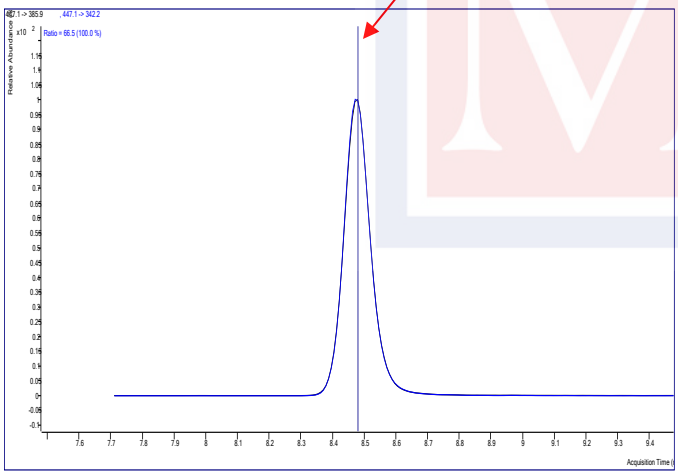


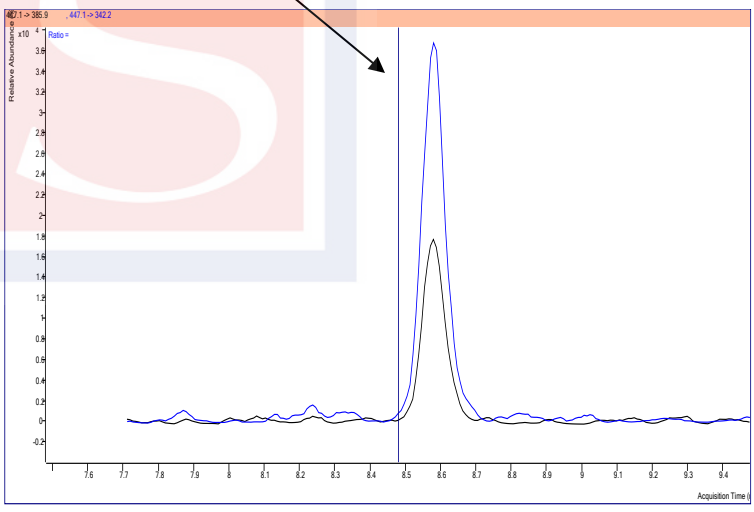
Noise Signal

Example of an interference that closely matches the target analyte



Ion Ratios





Precision/Accuracy

To obtain the best precision random errors must be reduced

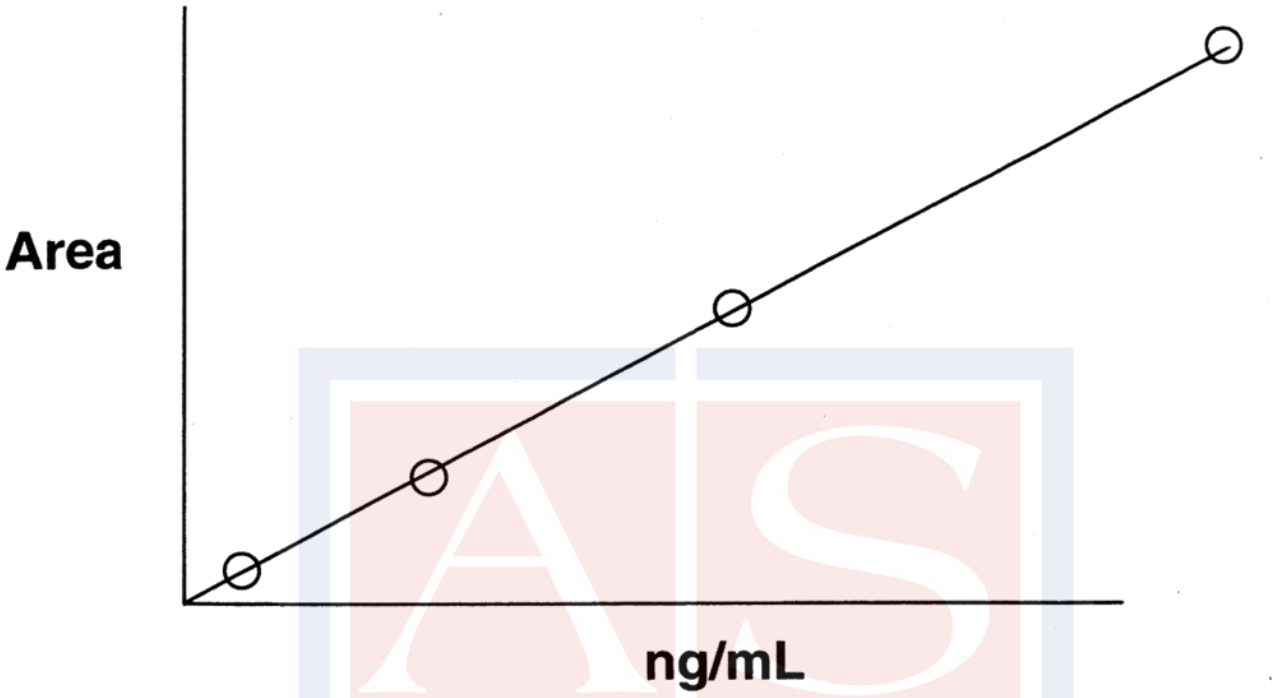
e.g., sample degradation, extraction variability noise from a matrix, reading scale errors, fluxion in balance or pipette

(Note: good accuracy can be obtained if enough measurements are averaged.)

To obtain the best accuracy systematic errors must be reduced.

e.g., uncalibrated pipette or balance, error in preparing a stock standard used for calibration, consistent dilution to wrong final volume.

Calibration



Features:

- Should bracket analyte concentration range
- Typically 6 points plus blank
- Standards are prepared in matrix
- QC's at different levels should be analyzed to evaluate curve
- Use same criteria for analysis and peak integration
- Weighted regressions or non-linear regressions can be used to span wide concentration ranges and reduces effects of less precise points of one end of the calibration curve

Calibration (continued)

Internal Standards

Why:

Reduce errors due to:

1. Dilutions
2. Evaporation
3. Degradations*
4. Variability in recovery*
5. Derivatization*
6. Adsorptive losses*
7. Instrumental variability (injection, chromatography MS response)

* IS choice is important in reducing errors

Choices:

- close to chemical behavior to analyte
- unique ions (no interference or common ions to analyze under MS or MS/MS conditions)
- Purity (no contribution to target analyte)

1. Isotopically labeled (2H, 13C, 15N, 18O)
2. Homologues (methyl, benzyl)
3. Structural analogs

Considerations for Isotopically Labeled internal standards

- **Choice of Label:**

2H will show a slightly earlier elution time from the LC compared to 13C, 15N or 18O. Thus 2H does not exactly compensate for matrix effects.

Location of labels:

Non exchangeable sites and sites that do not undergo scrambling in MS/MS

- **Number of Labels:**

Must have no overlap in m/z with the natural isotopes from the unlabeled compound.

- **Purity of Internal std:**

no unlabeled contribution to the internal std.

- **Common ion interferences:**

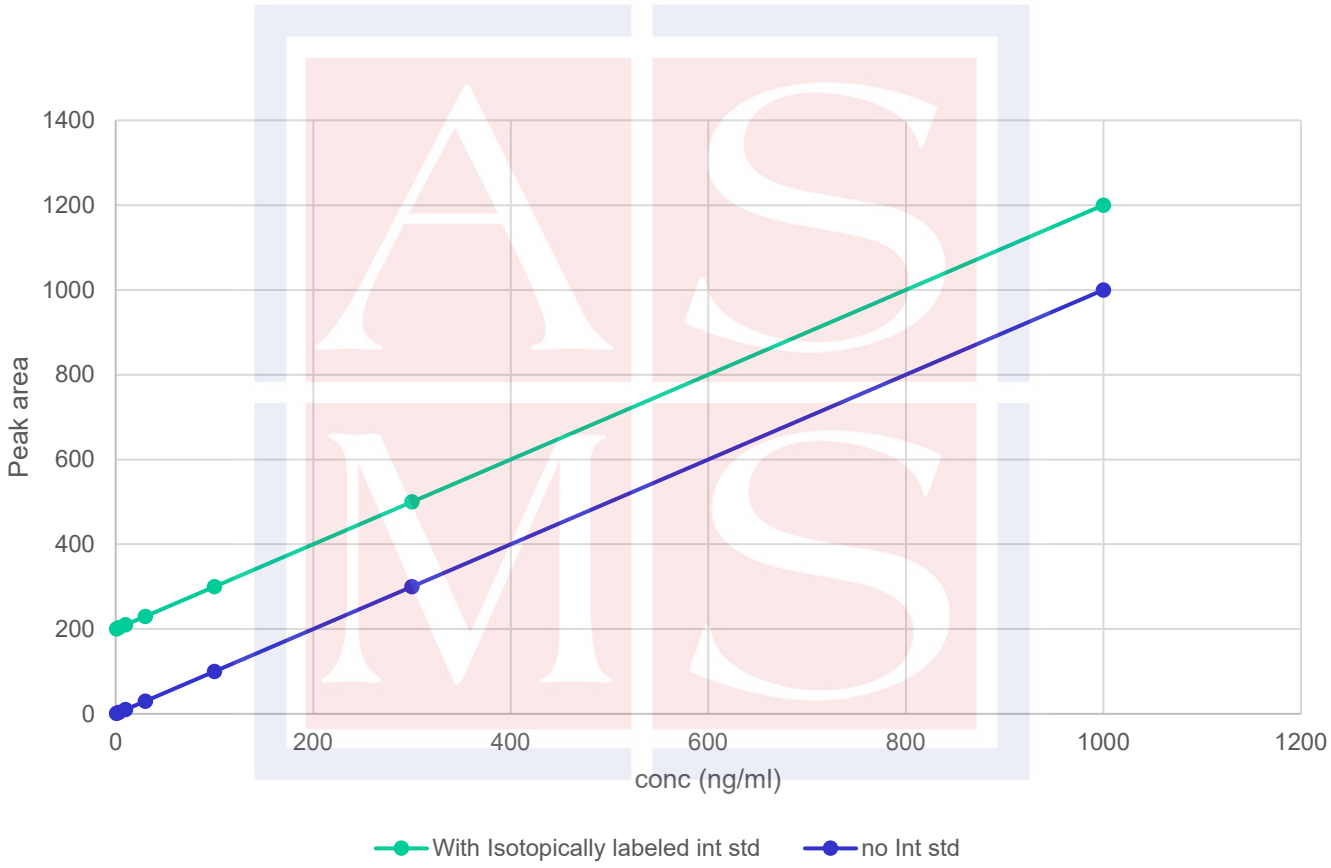
this comes from crosstalk which is rare in newer instruments and mostly from H2 scrambling under CID conditions in MS/MS.

- **Concentration of internal std:**

Usually at a concentration that is 25-33% of the calibration range. This prevent suppression of the target compound by the internal std.

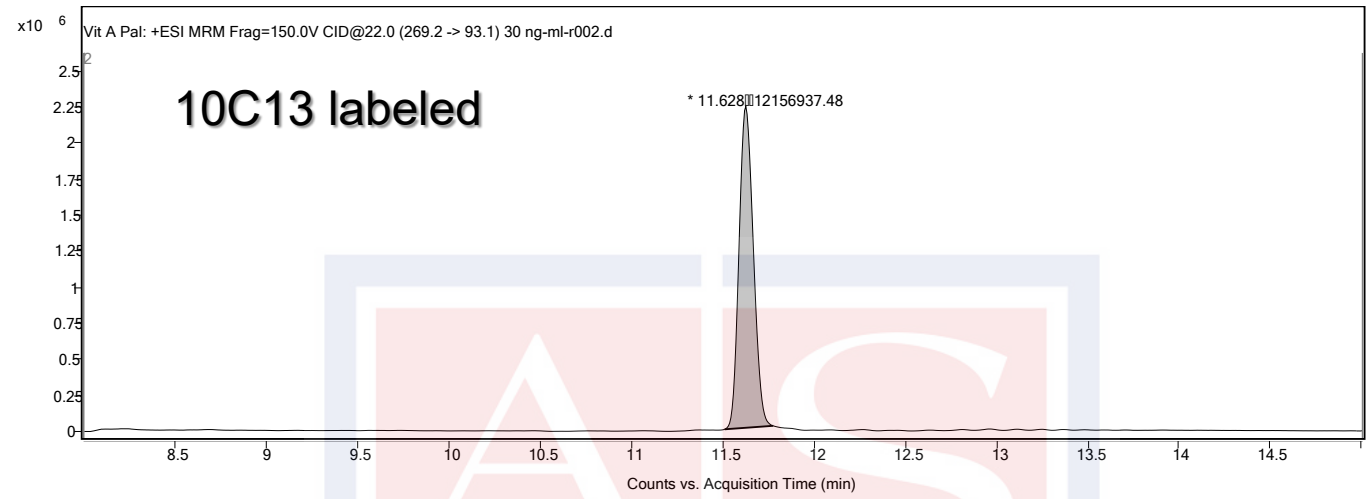
Considerations for Isotopically Labeled internal standards

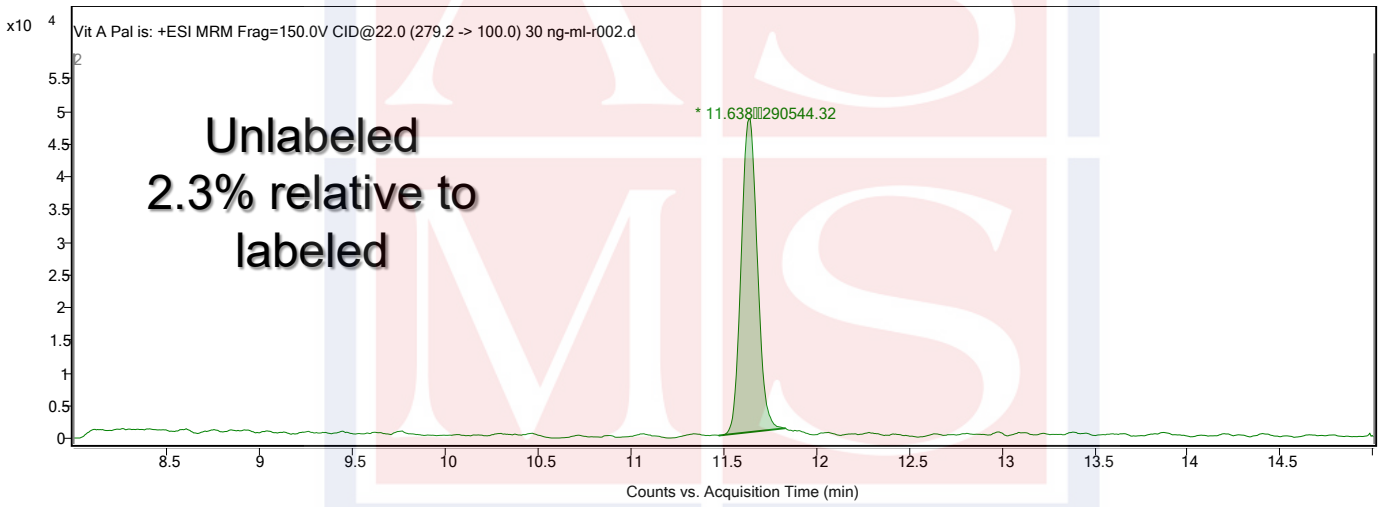
Explain where there is a positive Y intercept in the calibration using an isotopically labeled internal standard.

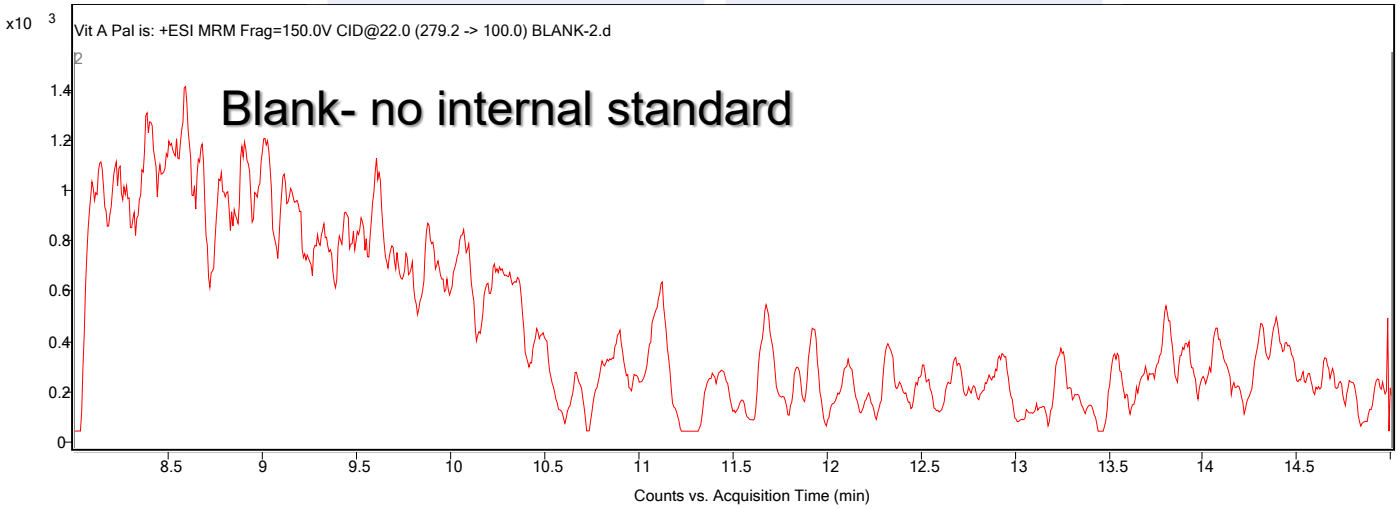


What else could cause a positive Y intercept ?

Contamination of the unlabeled material in an Isotopically Labeled internal standards







How much Isotopically labeled internal standards should be added?

Considerations:

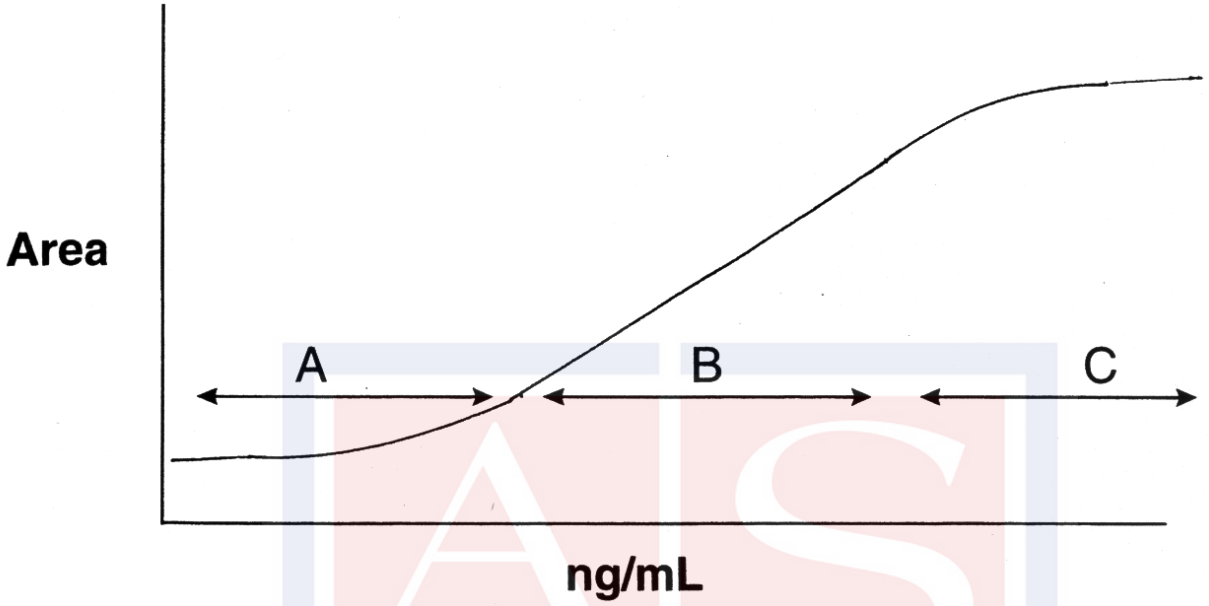
1. **Often quantity is mid point of calibration range.**
2. **Lower levels avoid suppression from ionization saturation**
3. **Lower levels minimize interferences form unlabeled compound in the internal standard.**
4. **Higher levels result in better signal reproducibility**

Question:

Desired calibration range is 1-1000 ng/ml (LOQ = 1ng). The internal standards has a 1% contribution with the unlabeled compound. Non-linear ESI response occurs starting at at 1200ng

What concentration of internal standard should be used for the analysis?

Real World Calibration



Ideally would look to work in linear range (B)

- Typically B range can be 3-4 orders in magnitude

Reasons for A Range

- common ion interference (matrix background)
- contamination from IS

Reasons for C:

- Detector saturation
- Ionization saturation

To increase dynamic range on high end of curve, dilute sample and reanalyze.

Ratio of Ions/Droplets

Conc. (mw500)	Ions[1]	Ions/Droplets
50 fg	1×10^{7}	2×10^{-6}
50 pg	1×10^{10}	2×10^{-3}
50 ng	1×10^{13}	2
50 μg	1×10^{16}	2×10^{3}

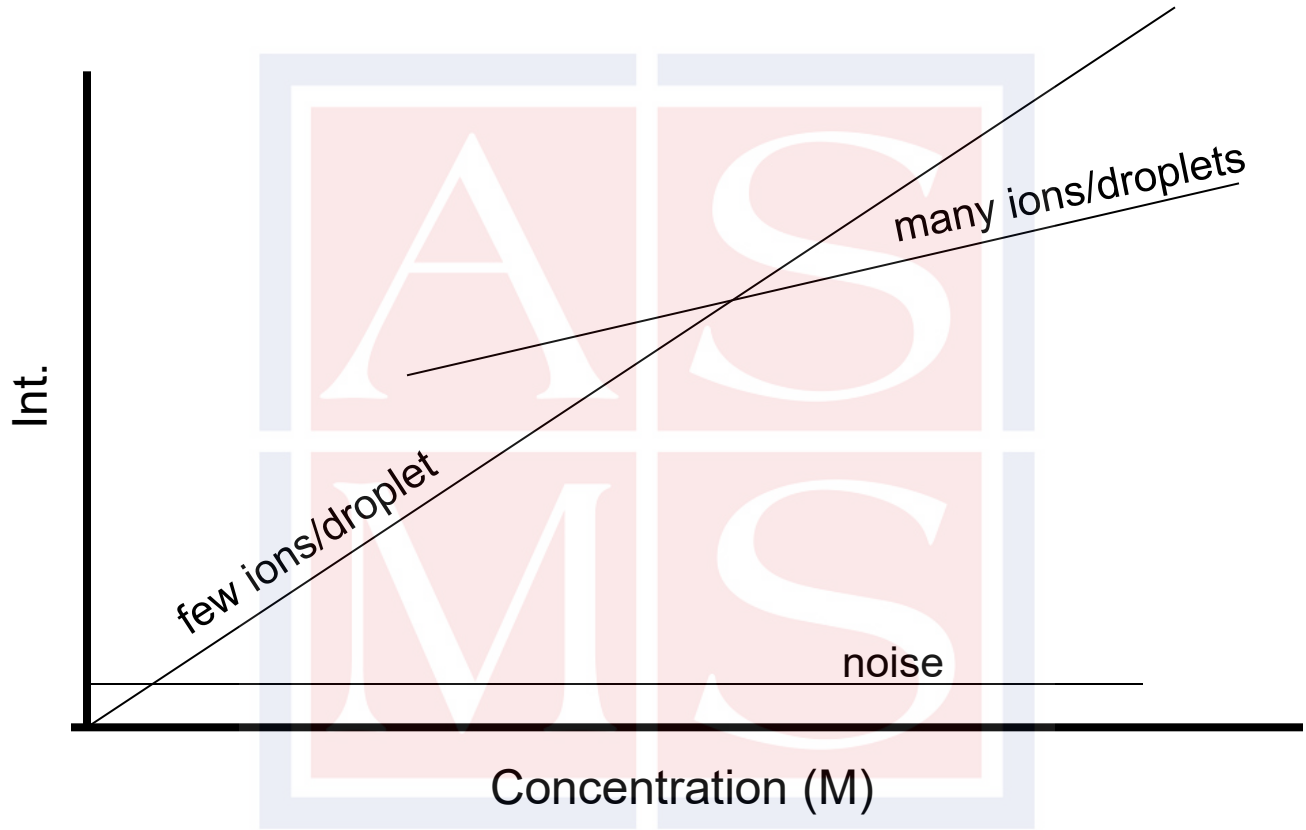
[1] Assuming equal distribution over a 10s wide peak

Assumptions:

1 mL/min (2 μm droplets) → 5×10^{9} droplets/s

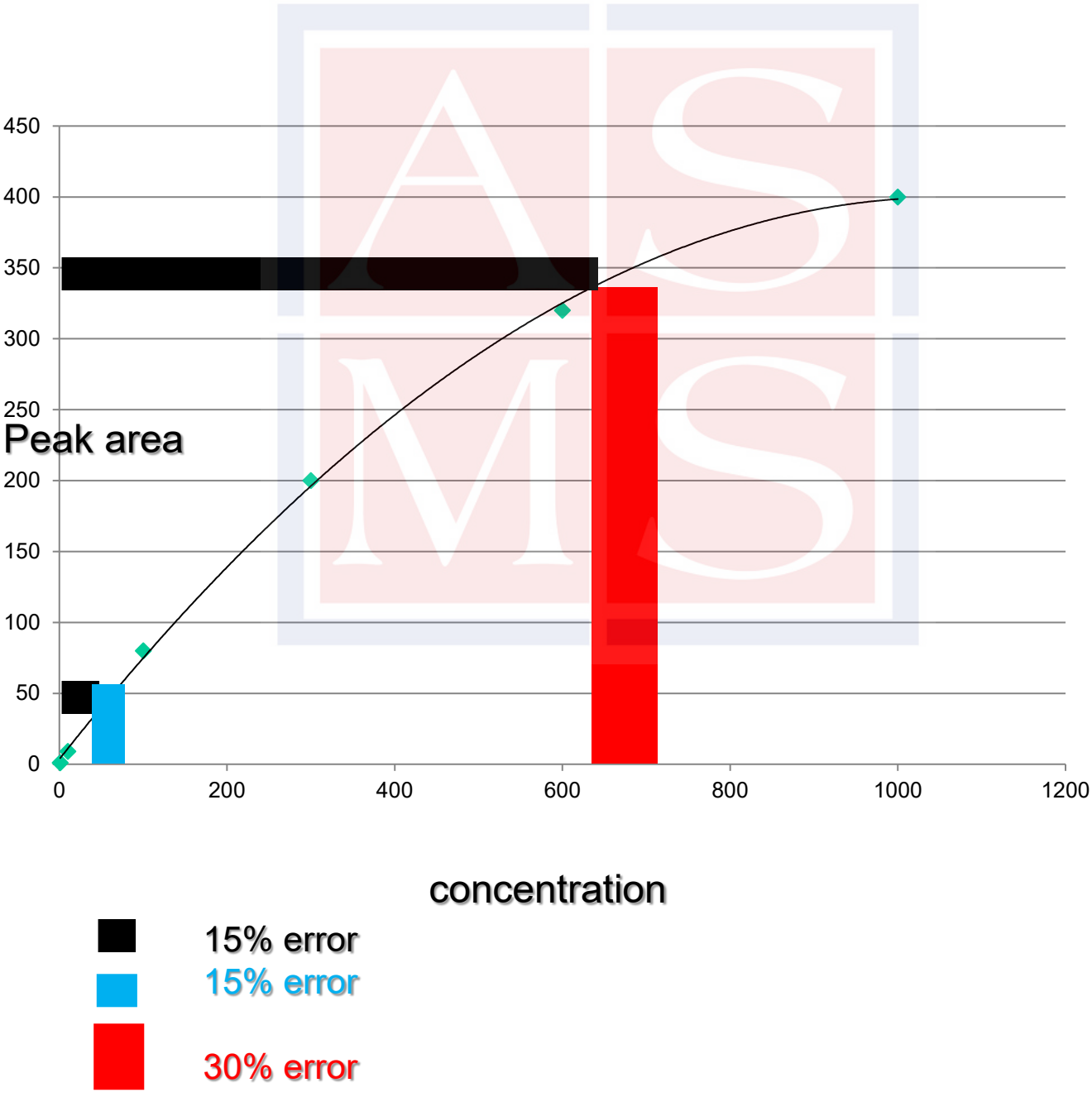
Each 2 um droplet produces about 1000, 50-100 nm droplet

Explanation for Non-Linearity in Real World Calibrations



Problems with accuracy with Non-linear calibrations

For a given error in peak area measurement the concentration error increases as the slope decreases



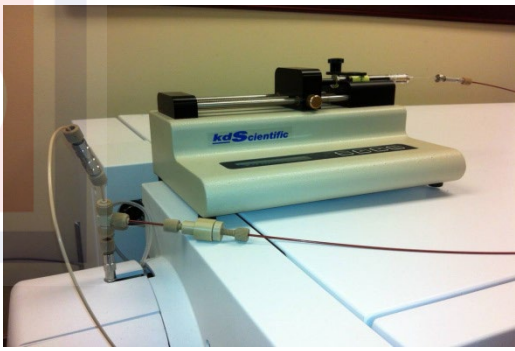
Understanding Matrix Effects

Matrix effects in LC/MS/MS include:

- *Ion suppression in the ion source (usually lower signal but could also result in enhancement) MS/MS or high resolution MS will not help*
- *Common ion interferences (false positives) different MRM transitions in MS/MS and high resolution MS can reduce*
- *Adsorption or matrix binding (false negatives) need changes in extraction to reduce*

Recognizing matrix effects

- *Compare solvent standard to matrix extract spiked with the same level of standard*
- *Add post column the standard while performing an LC/MS/MS analysis on a blank extracted matrix (shows matrix effects over entire run)*

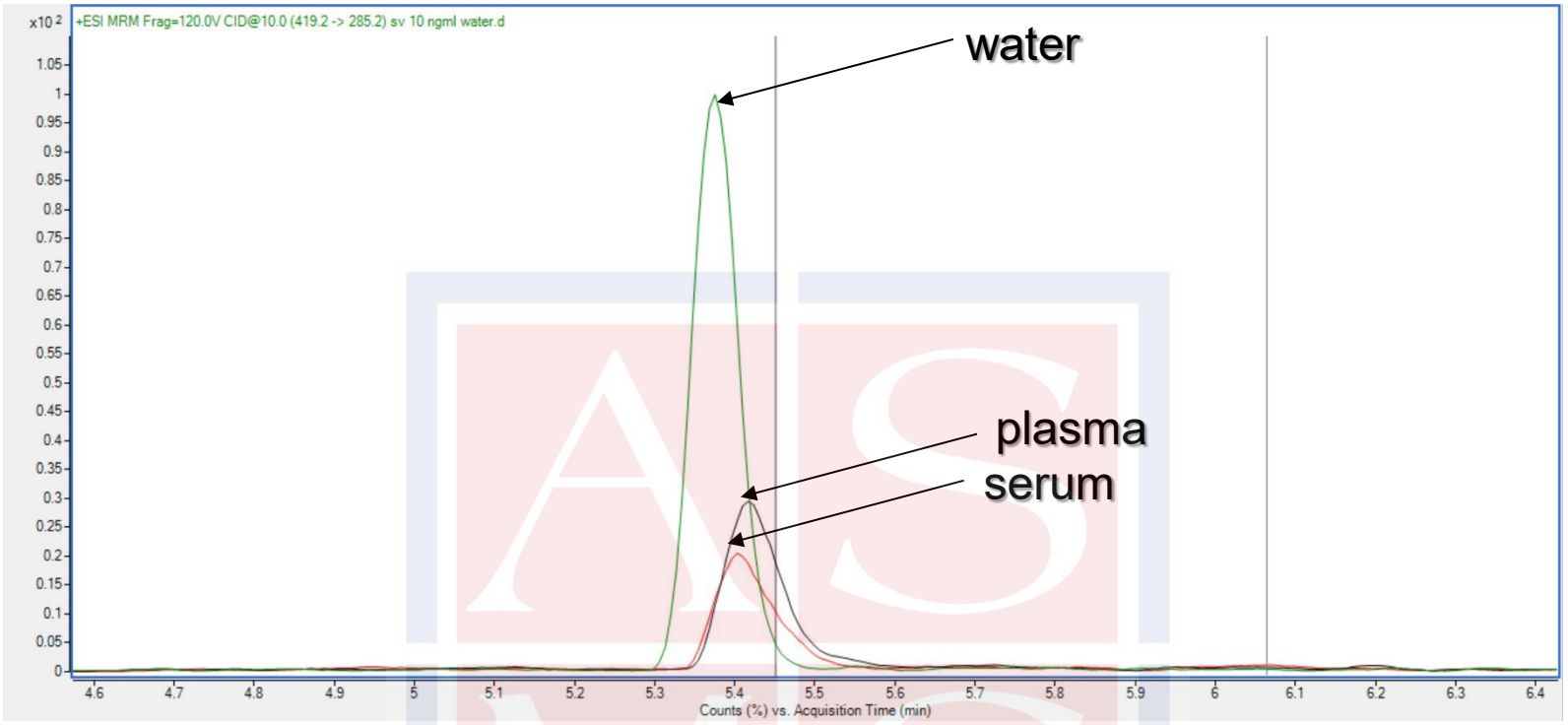


Reducing Matrix Effects

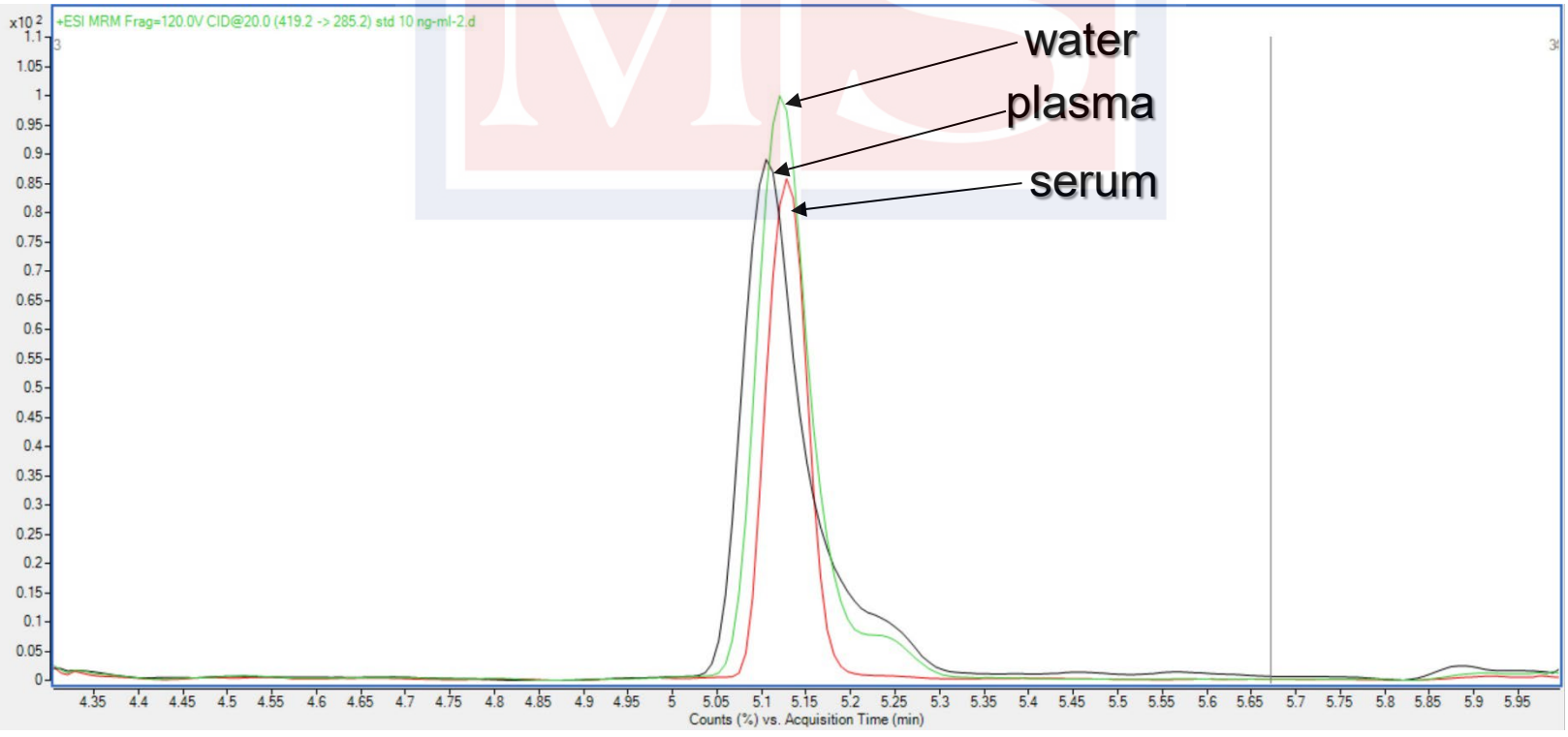
- *Improving/change the extraction*
- *Changing chromatography- longer analysis*
- *More MS power-different MRM transitions, more MS resolution*
- *Matrix matched calibrations*
- *Isotopically labeled internal standards -13C, 15N 18O labeled standards coelute with unlabeled analyte (2H and others do not)*
- *Spiked samples (standard addition)*
- *Try different API technique) APCI or APPI vs. ESI*

Matrix Effects comparison between protein precipitation and SPE

Protein precipitation of Simvastatin :



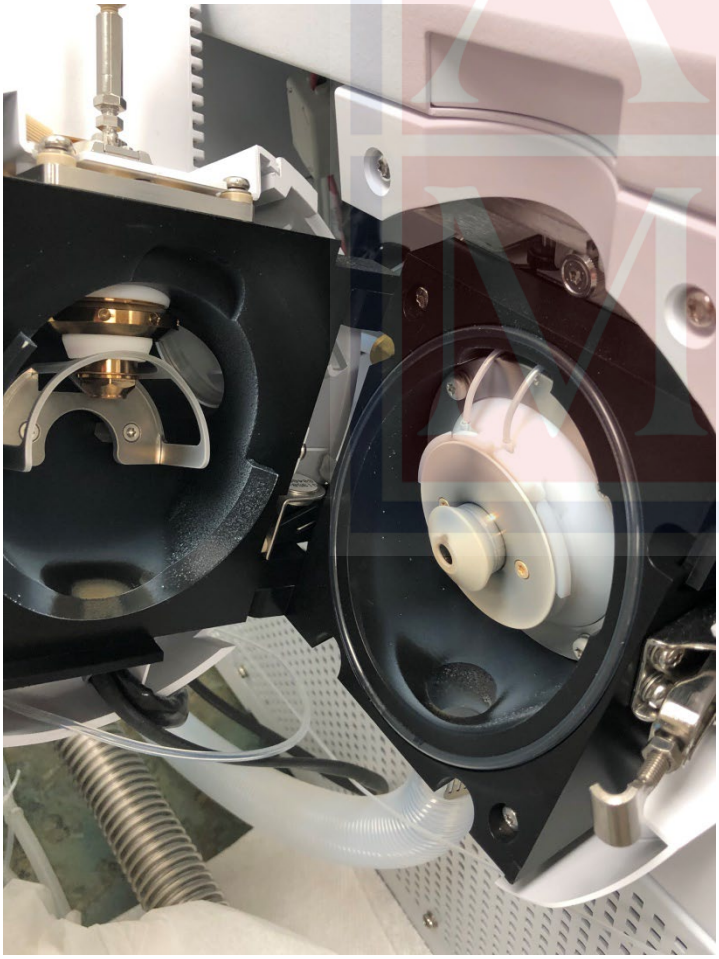
SPE of Simvastatin :



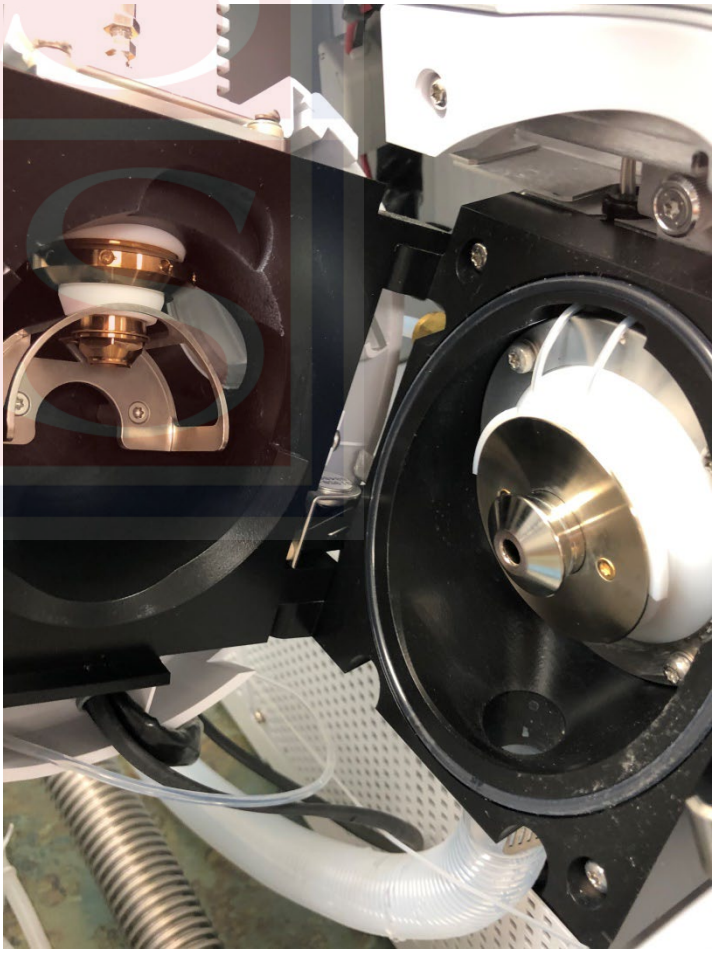
Avoiding unnecessary contamination from LC run

Use the Divert valve in your system to keep the LC flow from entering the API source for the portions of the LC chromatogram which do not have target analytes.

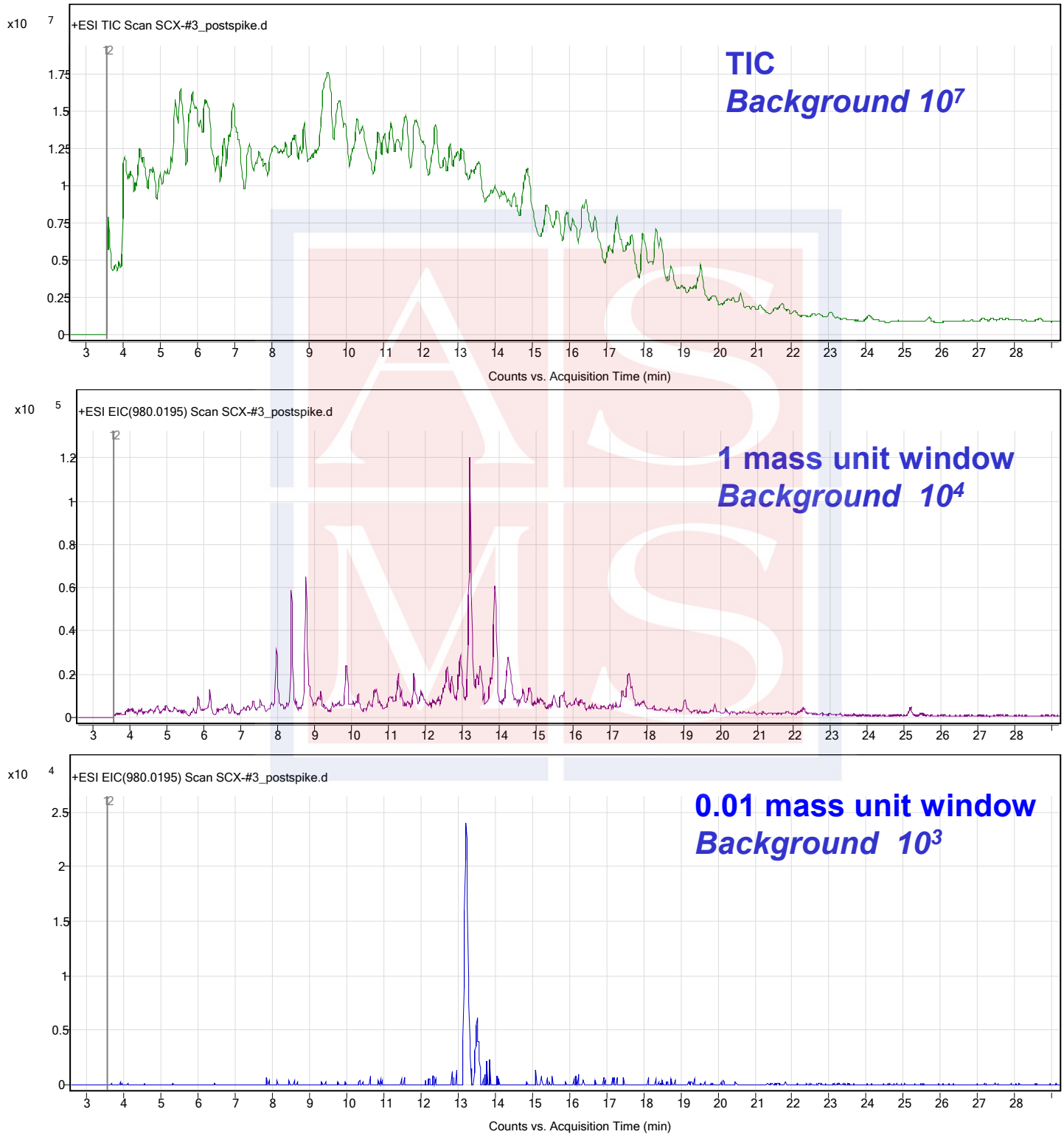
No Divert



Solvent front diverted

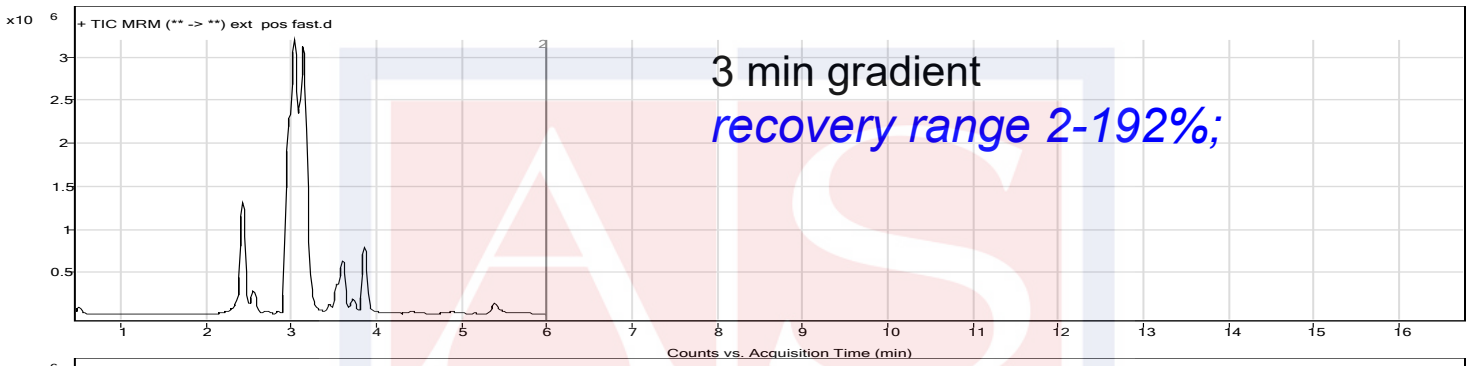


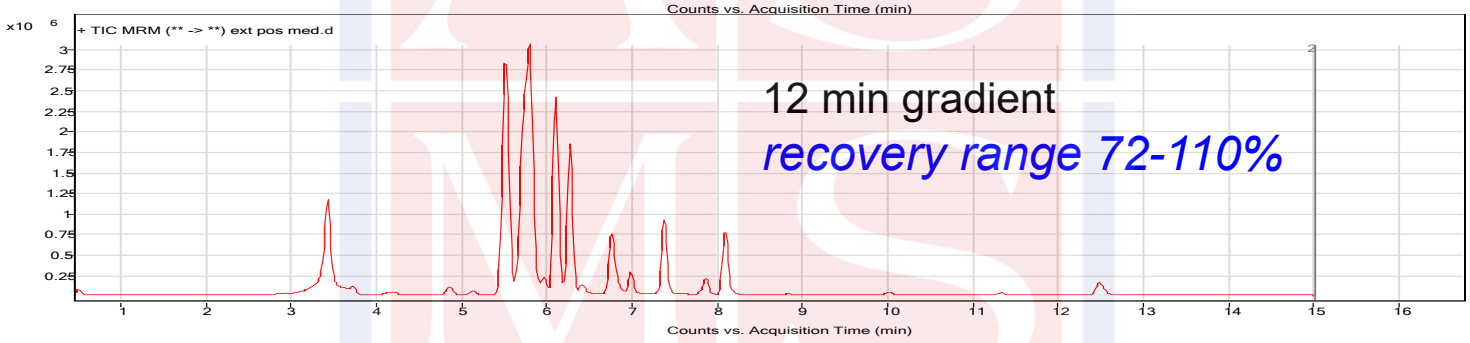
Reducing common ion interferences by increasing mass resolution

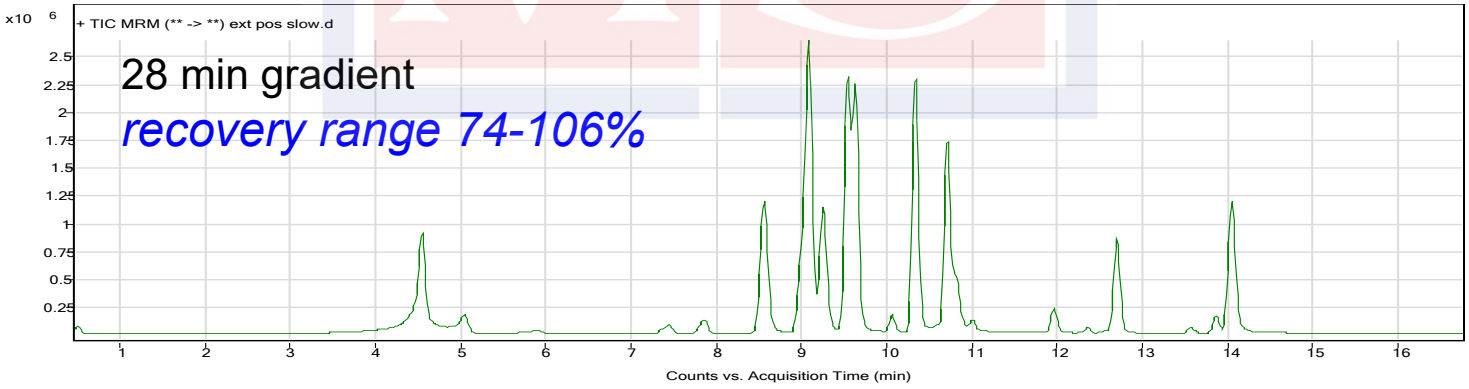


LC/MS/MS Analysis of mycotoxins extracted from spiked corn using 3 different gradient elution times

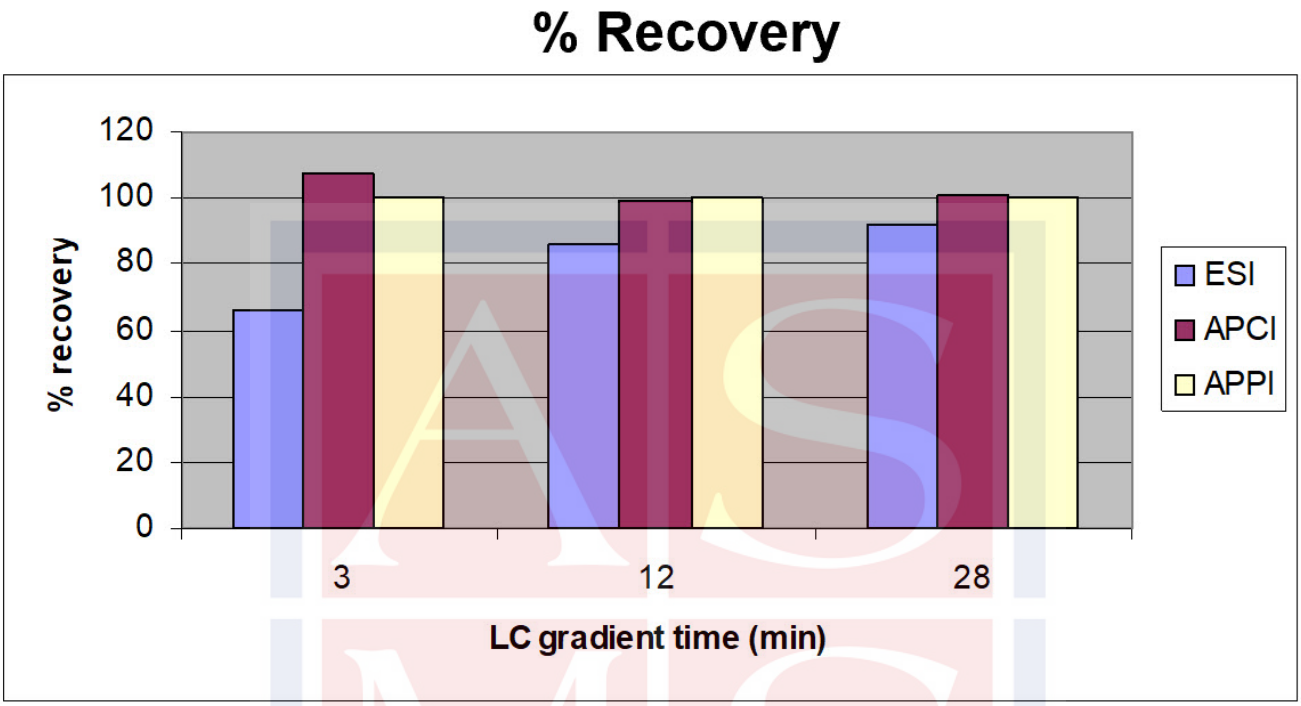
- *Conditions: poroshell 120 2.1x50 mm 2.7 um particles gradient 5-95% ACN in 3, 12 and 28 min, 0.3 ml/min, ESI pos ion detection*

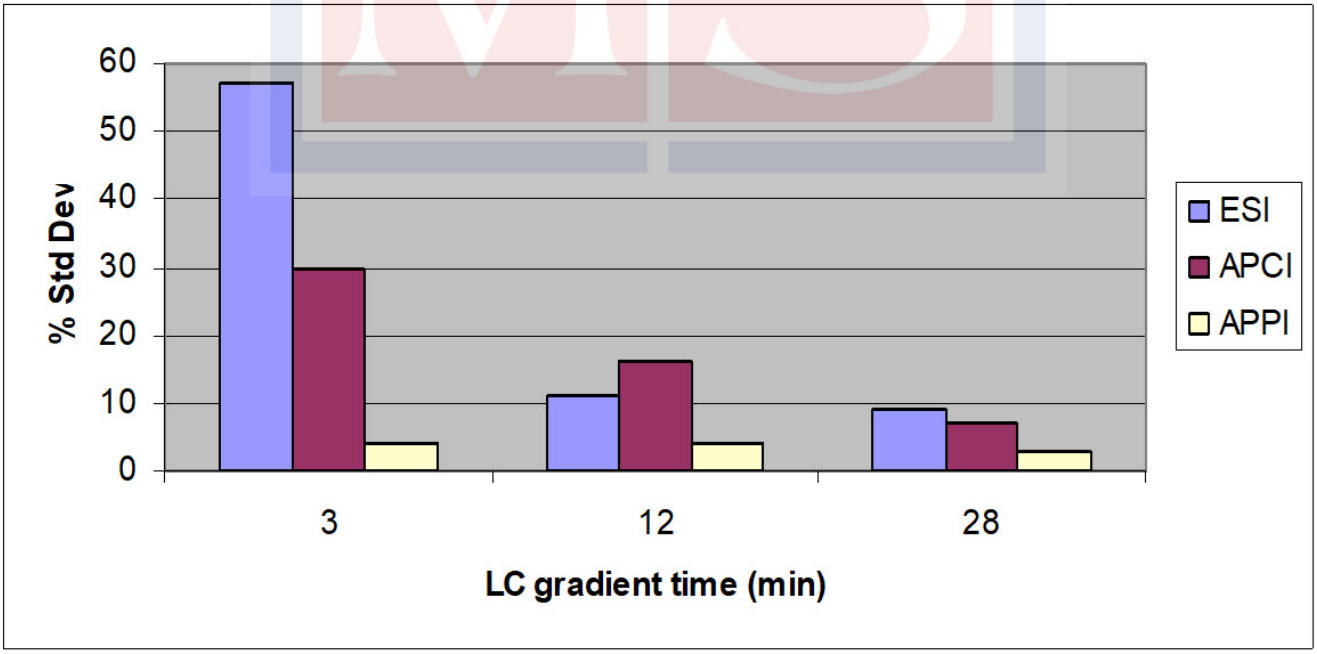






Comparison of average %recovery and % std dev using ESI, APCI and APPI for mycotoxins in a corn extract





Peptide Quantitation – Know your amino acids

Name	3-Letter Symbol	1-Letter Symbol	Molecular weight	Molecular Formula	Residue Formula	Residue Weight (-H_2O)	pK_a [1]	pK_b [2]	pK_x [3]	pI [4]
Alanine	Ala	A	89.10	$C_3H_7NO_2$	C_3H_5NO	71.08	2.34	9.69	—	6.00
Arginine	Arg	R	174.20	$C_6H_{14}N_4O_2$	$C_6H_{12}N_4O$	156.19	2.17	9.04	12.48	10.76
Asparagine	Asn	N	132.12	$C_4H_8N_2O_3$	$C_4H_6N_2O_2$	114.11	2.02	8.80	—	5.41
Aspartic acid	Asp	D	133.11	$C_4H_7NO_4$	$C_4H_5NO_3$	115.09	1.88	9.60	3.65	2.77
Cysteine	Cys	C	121.16	$C_3H_7NO_2S$	C_3H_5NOS	103.15	1.96	10.28	8.18	5.07
Glutamic acid	Glu	E	147.13	$C_5H_9NO_4$	$C_5H_7NO_3$	129.12	2.19	9.67	4.25	3.22
Glutamine	Gln	Q	146.15	$C_5H_{10}N_2O_3$	$C_5H_8N_2O_2$	128.13	2.17	9.13	—	5.65
Glycine	Gly	G	75.07	$C_2H_5NO_2$	C_2H_3NO	57.05	2.34	9.60	—	5.97
Histidine	His	H	155.16	$C_6H_9N_3O_2$	$C_6H_7N_3O$	137.14	1.82	9.17	6.00	7.59
Hydroxyproline	Hyp	O	131.13	$C_5H_9NO_3$	$C_5H_7NO_2$	113.11	1.82	9.65	—	—
Isoleucine	Ile	I	131.18	$C_6H_{13}NO_2$	$C_6H_{11}NO$	113.16	2.36	9.60	—	6.02
Leucine	Leu	L	131.18	$C_6H_{13}NO_2$	$C_6H_{11}NO$	113.16	2.36	9.60	—	5.98
Lysine	Lys	K	146.19	$C_6H_{14}N_2O_2$	$C_6H_{12}N_2O$	128.18	2.18	8.95	10.53	9.74
Methionine	Met	M	149.21	$C_5H_{11}NO_2S$	C_5H_9NOS	131.20	2.28	9.21	—	5.74
Phenylalanine	Phe	F	165.19	$C_9H_{11}NO_2$	C_9H_9NO	147.18	1.83	9.13	—	5.48
Proline	Pro	P	115.13	$C_5H_9NO_2$	C_5H_7NO	97.12	1.99	10.60	—	6.30
Pyroglutamatic	Glp	U	139.11	$C_5H_7NO_3$	$C_5H_5NO_2$	121.09	—	—	—	5.68
Serine	Ser	S	105.09	$C_3H_7NO_3$	$C_3H_5NO_2$	87.08	2.21	9.15	—	5.68
Threonine	Thr	T	119.12	$C_4H_9NO_3$	$C_4H_7NO_2$	101.11	2.09	9.10	—	5.60
Tryptophan	Trp	W	204.23	$C_{11}H_{12}N_2O_2$	$C_{11}H_{10}N_2O$	186.22	2.83	9.39	—	5.89
Tyrosine	Tyr	Y	181.19	$C_9H_{11}NO_3$	$C_9H_9NO_2$	163.18	2.20	9.11	10.07	5.66
Valine	Val	V	117.15	$C_5H_{11}NO_2$	C_5H_9NO	99.13	2.32	9.62	—	5.96

[1] pK_a is the negative of the logarithm of the dissociation constant for the -COOH group

[2] pK_b is the negative of the logarithm of the dissociation constant for the $-NH_3^+$ group

[3] pK_x is the negative of the logarithm of the dissociation constant for any other group in the molecule

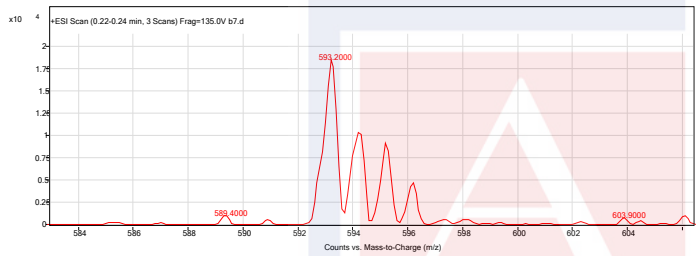
[4] pI is the pH at the isoelectric point

References: D. R. Lide, *Handbook of Chemistry and Physics, 72nd Edition*, CRC Press, Boca Raton, FL, 1991.

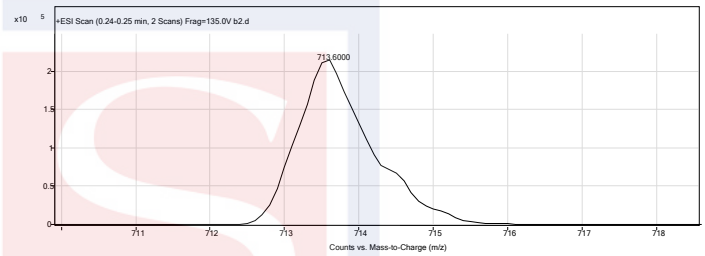
Quantification of peptides

Peptides can be treated like small molecules but offer additional challenges:

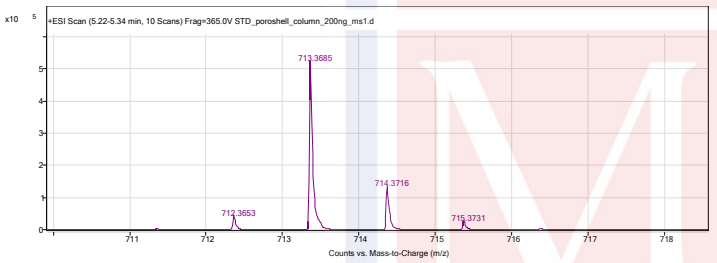
1. Peptides often give multiple charged ions so one observes the [M+H]+ [M+2H]+2 [M+3H]+3 etc.



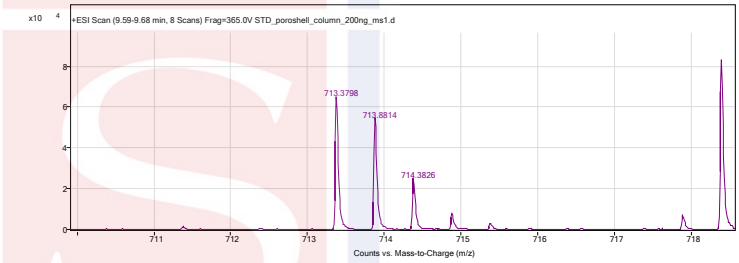
quad +1 charge



quad +2 charge



TOF +1 charge



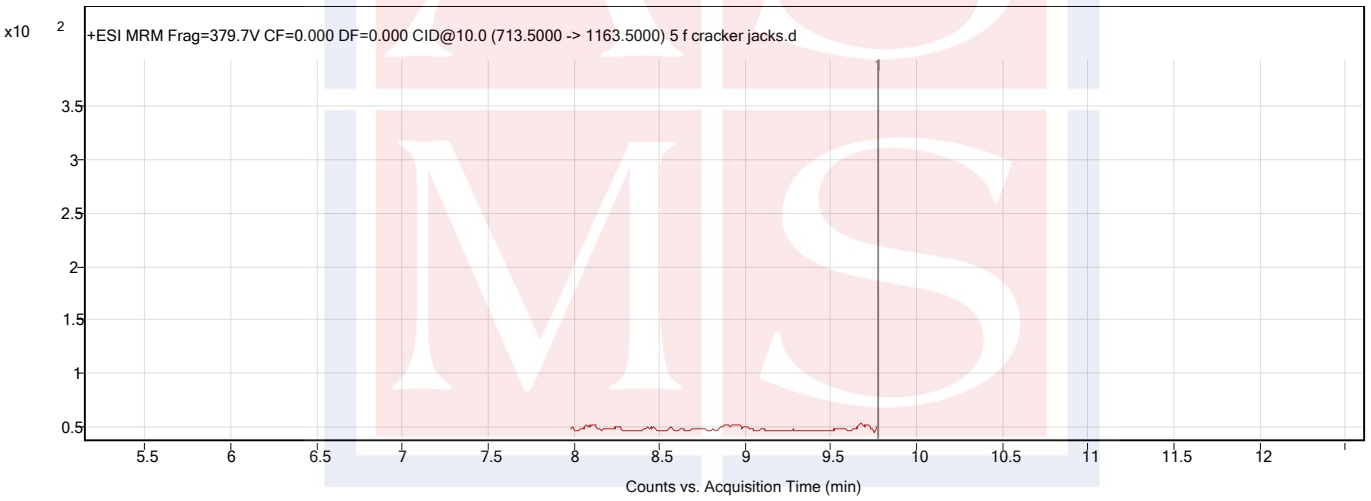
TOF +2 charge

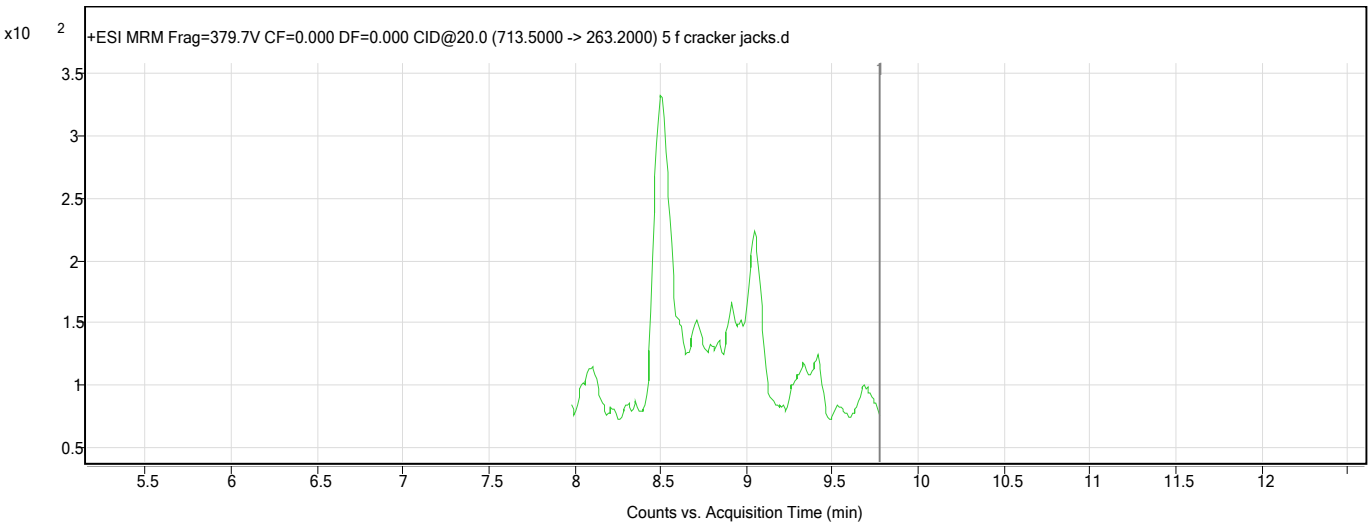
- The relative intensities of these ions can vary with conditions (for example, reduction of pH will increase the higher charged species)
- The most intense charge state is not always the best for MS/MS sensitivity – evaluate several of the charge states detected for product ion s/n

Quantification of peptides
Choice of MRM ions

Working with +2 or greater charge states allows for the unique ability to choose product ions above the parent ion m/z.

This often results in the reduction of chemical noise and removal of any potential interference form single charged compounds

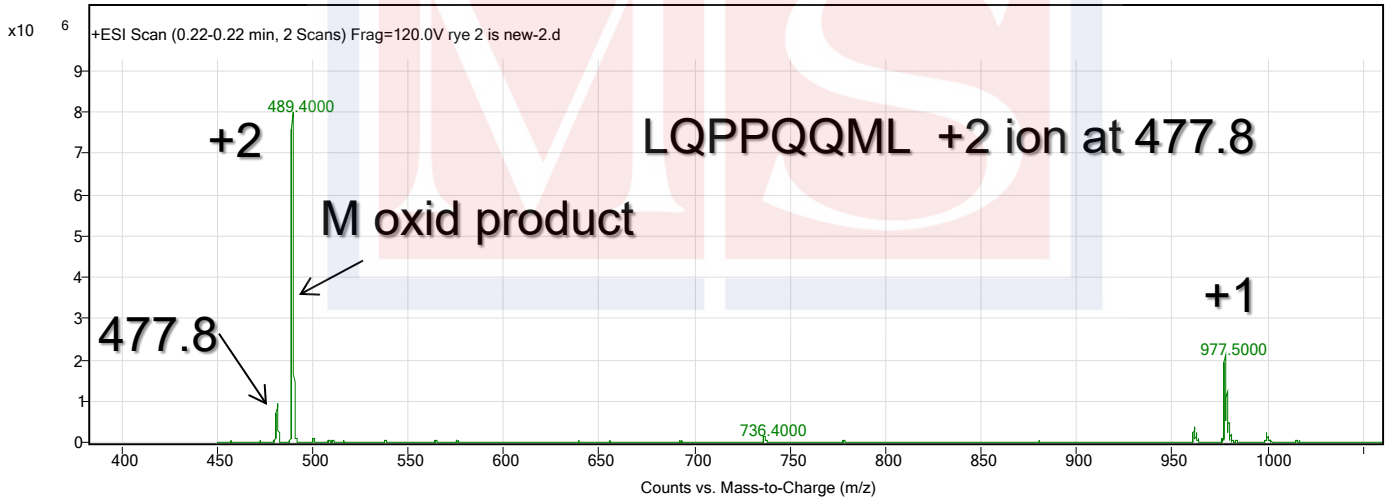




Peptide stability

Peptides with reactive or labile amino acid residues should might result in the detection of a reaction product.

1. Methionine, cysteine and tryptophan are likely to be oxidized.
2. Asp-Pro and Asp-Gly peptide bonds are unstable
3. Asparagine and glutamine are subjected to deamidation.
4. N-terminal glutamine can undergo cyclization
5. Phosphorylation or glycosylation products can form



Protein Quantification

Absolute protein quantification is based on two main workflows:

1. Extract protein from sample – digest -analyze target peptides my LC/MS/MS
2. Digest sample – extract peptides- analyze target peptides by LC/MS/MS

Protein extraction is done using: C18, gel electrophoresis , ion exchange or using specific antibodies. Often affinity removal of high level plasma proteins (e.g. IgG) is done prior to digestion.

Peptide extraction can be done by C18, ion exchange and has been demonstrated using affinity approaches specific for the target peptide (SISCAPA Stable Isotope Standards and Capture by Anti-Peptide Antibodies).

Protein Quantification

Digestion of proteins:

Digestion breaks the protein into LC/MS/MS measureable peptides and makes standards more affordable. Common enzymes used for digestion are:

Name	Cleave	Don't cleave	N or C term
Trypsin	KR	P	C
Trypsin/P	KR		C
Arg-C	R	P	C
Asp-N	BD		N
Asp-N_ambic	DE		N
Chymotrypsin	FYWL	P	C
CNBr	M		C
Lys-C	K	P	C
Lys-C/P	K		C
PepsinA	FL		C
TrypChymo	FYWLKR	P	C
V8-DE	BDEZ	P	C
V8-E	EZ	P	C

Other considerations include:

- Deglycosylation of the protein prior to digestion
- Breaking the disulfide bonds with dithiothreitol (DTT) and capping with iodoacetamide (addition of 43 in mass).

Method Validation

- **Calibration Curve**

  6 Replicates of minimum of 6 levels plus blank
  - (Replicate = extraction from matrix an analysis)
  - (Duplicate = analysis of same extract)

- **Precision**
  - 6 Duplicates at 3 different levels (low, medium, and high)
  - 3 Replicates of above

- **Accuracy**

  Duplicates of a minimum of 6 levels throughout the calibration range

- **Recovery**

  3 Replicates at low, medium, and high levels

- **Specificity**

  Demonstration that target analyte free matrix from a minimum of 3 different sources shows <30% response of the LOQ response

Method Validation (continued)

- **LOQ**
  - Ideally set to meet detection goals of analysis
  - General considerations for LOQ:
    - 3-10x LOD
    - recovery: relative yield > 70%, <120%
    - precision: CV < 30%

Stability

Matrix (pre-extraction) Stability

3 replicates at low, medium and high levels over length of proposed storage (e.g., 0-6 months)

Dry Extract Stability

5 replicates at low, medium and high levels

1) immediately after extraction
2) immediately 1 day after extraction (room temp/freezer)
3) immediately 5 days after extraction (room temp/freezer)
4) immediately 14 days after extraction (room temp/freezer)

Method Validation (continued)

Reconstituted Stability

5 replicates at low, medium and high levels

1) immediately after reconstitution
2) 3 hours after reconstitution (room temp)
3) 6 hours after reconstitution (room temp)
4) 24 hours after reconstitution (room temp)
5) 72 hours after reconstitution (room temp)

Freeze/Thaw Cycles

Check stability after at least 3 freeze/thaw cycles at the low, medium, and high QC levels of:

1) matrix (pre-extract)
2) extract-dry
3) extract-reconstituted?

GLPs

- Main source of regulatory requirements used by FDA/FIFRA

- All instruments and equipment and for analysis of drug products be calibrated at suitable intervals in accordance to a written protocol (code of Federal regulations (CFR) 211.160(b) (4). The written protocol will include:
  - directions/instructions
  - schedules of performances of various functions
  - limits on accuracy/precision
  - remedial actions if conditions are not meet

- Complete records of the calibrations will be maintained> (CFR 211.194(d) $\rightarrow$ Standard Operation Procedures SOPs) define maintenance, testing, calibration an procedures.

Instruments that fail in any point, mentioned above shall not be used.

SOP Composition

- Introduction
- Scope/Implementation
  - Introduction/Definitions
  - Procedures (can make references to instrument manuals)
    - general operation
    - calibration
    - non-routine calibration
    - data collection
    - data handling/storage
    - general record keeping
    - maintenance/service
- Training of responsible persons
- Documentation/Records
- Revisions $\rightarrow$ living document

Daily Tuning Consideration for LC/MS

Daily Checks

- Check mass accuracy +/- 0.1 amu
- Check mass width (0.5 amu at half height)
- Check relative ion ratios
- Check absolute abundances

Corrective Actions

- Clean source including spray shield and capillary cap or cone
- Confirm flow through spray needle
- Check electrospray needle and replace if damaged
- Increase gain setting to raise absolute ion abundances
- Adjust ion mass and peak widths

Method Assessment

A LC/MS/MS assay was developed by MIOP Labs for the parent drug and 1 metabolite in plasma. The method uses isotopically labeled internal standards, a liquid phase extraction, reverse phase LC and positive ion electrospray MS/MS detection.

Parent drug	Int. std Parent drug	Met 1	Int. std Met 1
C24H15NO3	C24H2 D13NO3	C24H13N2O4	C24H11D2N2O4
MW= 366	MW= 378	MW= 393	MW= 395

Extraction: 100ul of plasma is added to a 96 well plate 1 ul of internal standard in 1M HCL is added followed by chlorobutane and vortexed. The supernatant is taken from the bottom and is dried at 80C with N2 till dry. Samples are reconstituted in 25 ul of acetonitrile and 25 ul are injected for the analysis.

Chromatography: The Pluto UHPLC system was used with a mars C18 column (2.1mm x 250mm x5 um) operated at room temperature. A gradient of 0-100% B in 3 min with a 12 min hold at 100% B. (A= water with 1% formic acid and B= 95.25% acetonitrile 4.75% water). The flowrate was 80 ul/min and the column equilibration time to initial conditions was 2.5 minutes.

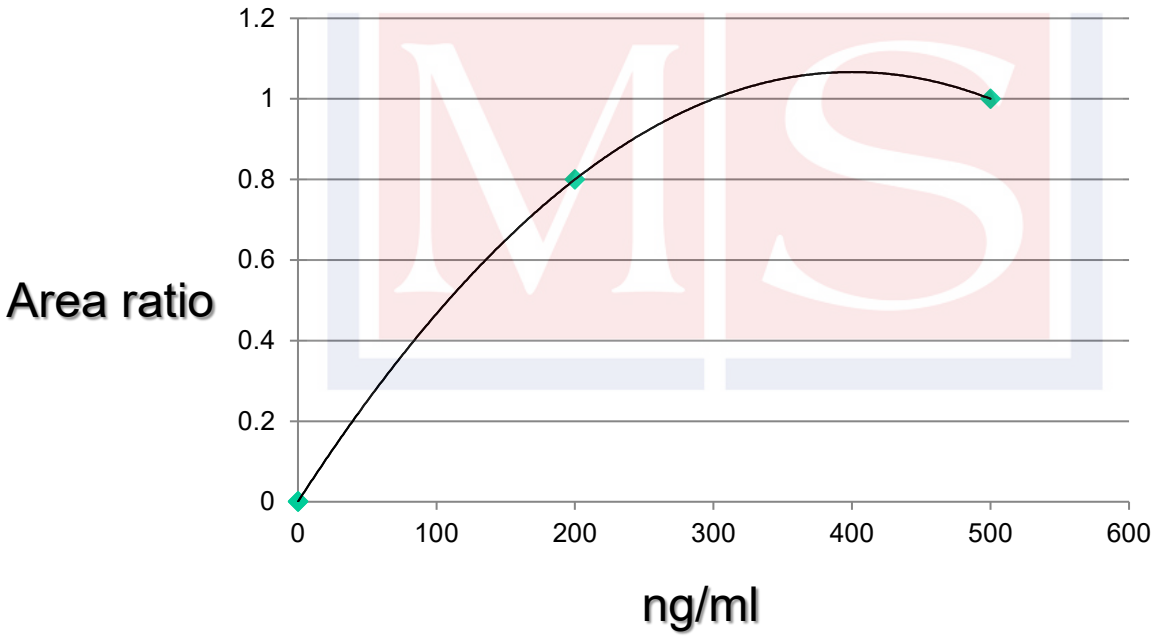
MS/MS: The Saturn V penta-quad was operated in triple quad mode and was calibrated by infusion of the parent drug. The source and MS conditions (MRM transitions and collision energy) were optimized also by infusion of the 2 compounds The MRM transitions monitored are shown below (**bold** ions used for quant. the other used for confirm):

Parent drug		Int. std Parent drug		Met 1		Int. std Met 1	
Q1	Q3	Q1	Q3	Q1	Q3	Q1	Q3
366	**348**	**379**	**361**	**394**	**372**	**396**	**374**
366	149	379	121	394	187	396	127

Method Assessment continued

Method performance and calibration:

The parent drug eluted at 14.7 minutes and metabolite 1 at 10.7 minutes. The internal standard for the parent drug eluted earlier at 14.3 minutes while metabolite 1 internal standard coeluted with the unlabeled metabolite. The calibration performed using 0.1, 200 and 500 ng/ml solvent standards with an example calibration curve for the parent drug shown below. The calibration curve was forced through zero since there was no background detected in the solvent standards. The typical range measured for the parent drug and metabolite was 450-550 ng/ml. The calibration was checked weekly by running the 500 ng/ml calibration standard. Records for the work were written in the lab book after the client paid for the project



Discuss reasons and possible solutions for the observation below

Problem:
The assay using positive ion ESI LC-MS/MS results in calibration standard recoveries increasing throughout the analysis.

There is no internal standard used in the analysis.

What is the possible explanation and solution to this problem?

Discuss reasons and possible solutions for the observation below

Problem:

Trying to develop a 1 minute analysis for a drug in plasma using C18 2.1 x 50 mmm column and electrospray positive ion MS/MS but been having problems with precision and sample recovery due to matrix suppression.

Any suggestions what can be done to achieve a 1 minute analysis?

Discuss reasons and possible solutions for the observation below

Problem:

The MRM peak in electrospray positive ion MS/MS is trailing. Checked the chromatography using a UV detector in line and the peak is symmetrical.

Why is the peak tailing in the MS and can this be eliminated?

Discuss reasons and possible solutions for the observation below

Problem:

The calibration curve for my drug in plasma shows a positive Y intercept and I cannot achieve the sensitivity required for the assay. This assay uses an isotopically labeled internal standard.

What should be done to improve sensitivity and reduce the positive Y intercept?

Discuss reasons and possible solutions for the observation below

Problem:

I am using a [M+Na]+ adduct of the target drug in MRM for quantification using electrospray positive ion LC-MS/MS. The calibration are non-linear and have to use a quadratic fit.

Why is this the case and what can be done to improve linearity?

Discuss reasons and possible solutions for the observation below

Problem:

The response for the QC for the target drug being monitored by electrospray LC-MS/MS decreases over the day. There is no internal standard in the analysis.

What are the possible reasons for this and what can be done?

What would the use of an isotopically labeled internal standard offer?

Discuss reasons and possible solutions for the observation below

Problem:

The [M+H]+ ion is very weak and can not meet the desired LOQ for the analysis of 1 ng/ml. The base peak in the full scan mass spectra is the [M+H-H2O]+ ion.

What can be done to improve the abundance of the [M+H]+ ion?

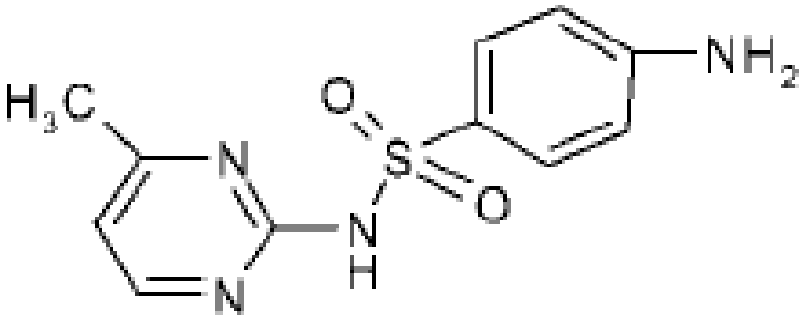
Can a quantitative LC-MSMS assay be based on the [M+H-H2O]+ ion, a decomposition product from the parent?

Discuss reasons and possible solutions for the observation below

Problem:

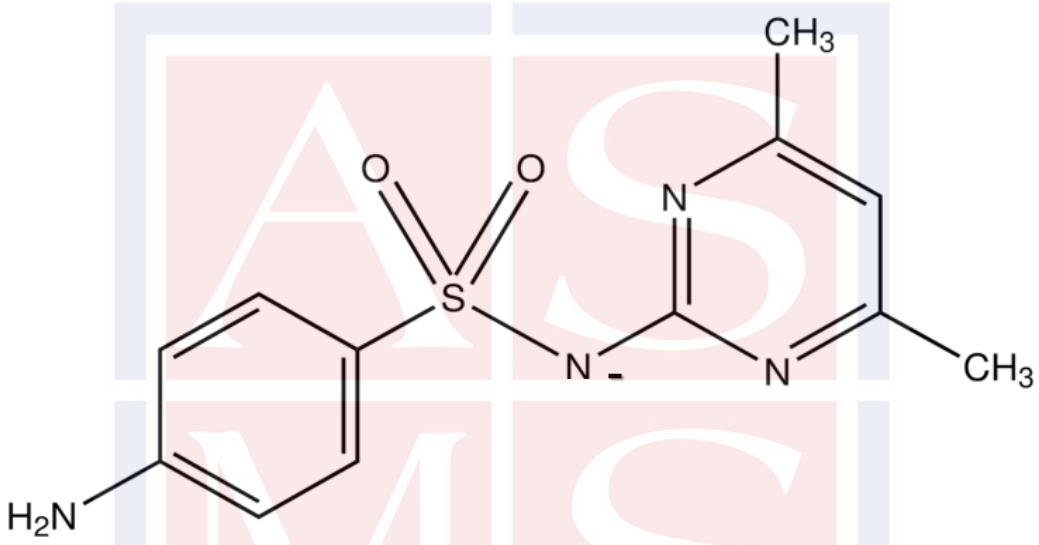
I was surprised that sulfamethazine produced a strong [M-H]- anion at m/z 263. Why does that occur?

In the optimization of collision energy for product formation the transition of m/z 263- 199 was predominate. The loss of 64, believed to be SO2 was observed. How does that occur?



Discuss reasons and possible solutions for the observation below

Explaining the loss of 64 for SO2



Questions

(1) What is indicated when a calibration shows:

(a) A slope less than 1

(b) A non linearity, b different Reponses between analyte and int std, c adsorption d all of the above

(b) A negative y-intercept

A non linearity, b different Reponses between analyte and int std, c adsorption d all of the above

(c) Non-linearity on the low concentration end

A saturation, b noise, c adsorption d ion pairing

(d) Non-linearity on the high concentration end

(e) A saturation, b noise, c adsorption d ion pairing

(2) What should be done if your sample exceeds the calibration range on the upper concentration end?

A extrapolate calibration, b nothing, c dilute and reshoot, d make a new calibration

(3) Why are isotopically labeled standards favored for IS in LC/MS

(4) A always available, b inexpensive, c best match between physical and chemical chariacteristics of the analyte, d all of the above

(5) Why are they not always used?

A, cost, b availability, c both a and b, d neither a or b

(4) What is the problem of using a deuterium label in substitution for an hydroxy proton?

(5) A Nothing, b cost, c exchange in solution, d sample suppression

Questions

(5) Predict what will be observed in precision an accuracy for the following situations:

(a) dilution to the wrong volume after extraction

A Precision change, b accuracy change, c both a and b , d no change

(b) error in making the IS stock solution for generating the calibration curve

A Precision change, b accuracy change, c both a and b , d no change

(c) fluxions with the pipette for diluting the sample

A Precision change, b accuracy change, c both a and b , d no change

(d) Leaking plunger in the injector for LC/MS

Precision change, b accuracy change, c both a and b , d no change

(e) carry-over from a previous determination

Precision change, b accuracy change, c both a and b , d no change

(f) adsorption of the sample on-column

(g) Precision change, b accuracy change, c both a and b , d no change

(g) detector saturation

Precision change, b accuracy change, c both a and b , d no change

(6) Will an isotopically labeled IS aid in the precision and/or accuracy for a-g above?

(a) dilution to the wrong volume after extraction

a Yes, b no,

(b) error in making the IS stock solution for generating the calibration curve

a Yes, b no,

(c) fluxions with the pipette for diluting the sample

a Yes, b no,

(d) Leaking plunger in the injector for LC/MS

a Yes, b no,

e

(e) carry-over from a previous determination

a Yes, b no,

(f) adsorption of the sample on-column

a Yes, b no,

(g) detector saturation

a Yes, b no,

(7) Why should LOQ ≠ LOD?

A cannot meet accuracy criteria at LOD, cannot meet recovery criteria at LOD, c signal to noise too low at LOD, d all of the above

(8) Why is reconstituted extract stability at room temperature determined for up to 24 hours? A required in a validation, b last sample in a sample set that runs overnight, c both a and b, d neither a or b

(9) 3 days?

(10) A required in a validation, b last sample in a sample set that runs over the weekend, c both a and b, d neither a or b

Questions

(9) What is the purpose of GLP's

A make more work for the analyst , b guarantee data quality, c help ensure data quality. D none of the above

(10) Once written, can a GLP be changed?

A yes, b no c do not know

(11) What is the error in molecular weight if the calibration drift by 0.3 m/z for an ion containing 40 charges?

A 0 daltons, b 6 daltons, c 12 daltons, d 24 dattons, e none of the above

(12) Answer Yes or No to the following statements on GLP's/SOP:

a) SOP's repeat what is written in the instrument manual?

A yes, b no c do not know

b) Only the 5 most recent calibration records are kept on file?

A yes, b no c do not know

c) Once written, the SOP should be locked in a file, hidden from everyone's view?

A yes, b no c do not know

d) SOP's can be short, only 3-4 pages in length?

A yes, b no c do not know

e) Operator training records should be kept?

A yes, b no c do not know

f) An SOP should include what is to be done if performance criteria are not met?

A yes, b no c do not know

Questions

g) GLP requires 3 ions must be detected for quantitation of a target analyte?
A yes, b no c do not know

h) Are non-linear calibration curve acceptable for GLP work?
A yes, b no c do not know

(13) Issue in specificity for quantitation of an analyte:

a) Is a low or high m/z ion usually more specific?
A low mass, b high mass c makes no difference

b) Can a confirmation be made on less than 3 ions?
A yes, b no c do not know

C Is MS3 on the ion trap equivalent to detecting 3 ions on a triple quadrupole?
A yes, b no c do not know

What information is lost in MS3?
A parent ion m/z, b product ion mass,c ion ratios of product ions, d all of the above, e none of the above

(14) If a common ion interference from the matrix gives a response equivalent to a 100 pg/mL solvent standard of analyte, what is the estimated LOD of the analysis?
A 50 pg/ml , b 100 pg/ml, c 300 pg/ml , d 1000 pg/ml , 3000 pg/ml

LOQ?
A 50 pg/ml , b 100 pg/ml, c 300 pg/ml , d 1000 pg/ml , 3000 pg/ml

(14) What is the minimal mass increase for an isotopic labeled internal standard that is need to prevent interference with the unlabeled compound for:

(1) C20 H22 N3 O Cl2
A 3, b 6, c10, d 14, e 20, f unknown

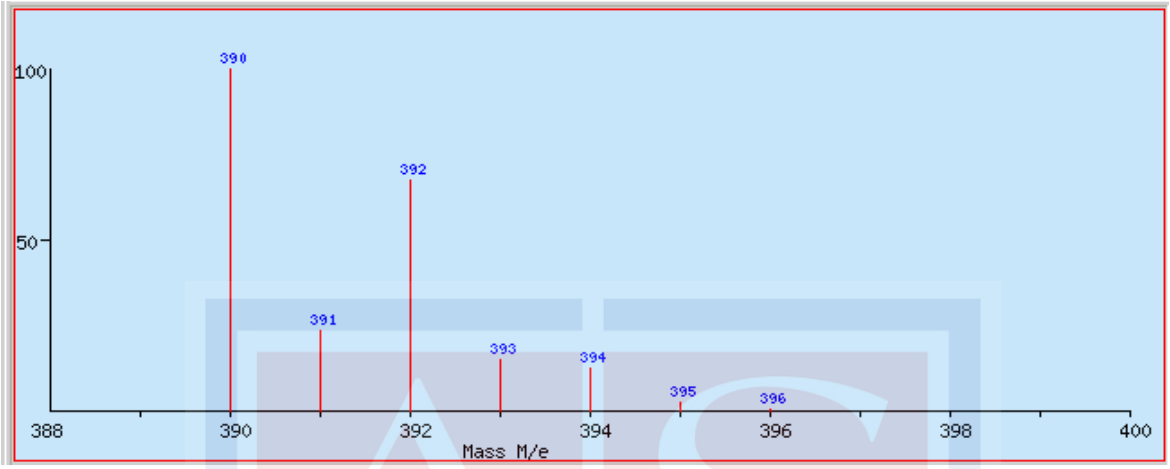
(1) C20 H22 N3 O I
A 3, b 6, c10, d 14, e 20, f unknown

(1) C20 H23 N3 O
A 3, b 6, c10, d 14, e 20, f unknown

(1) C44 H48 N5 O7 Br Cl5
A 3, b 6, c10, d 14, e 20, f unknown

Answer Question #15

1



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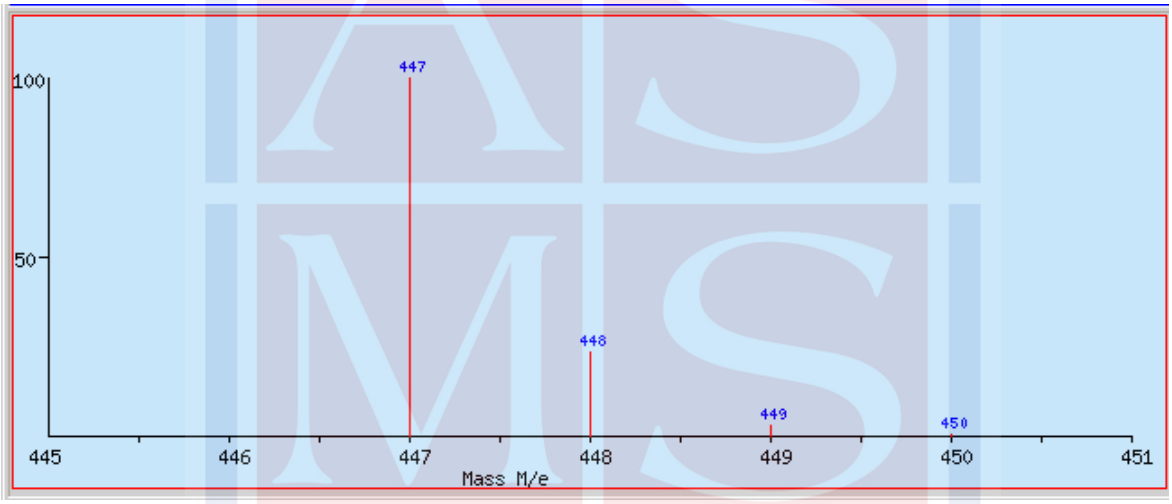

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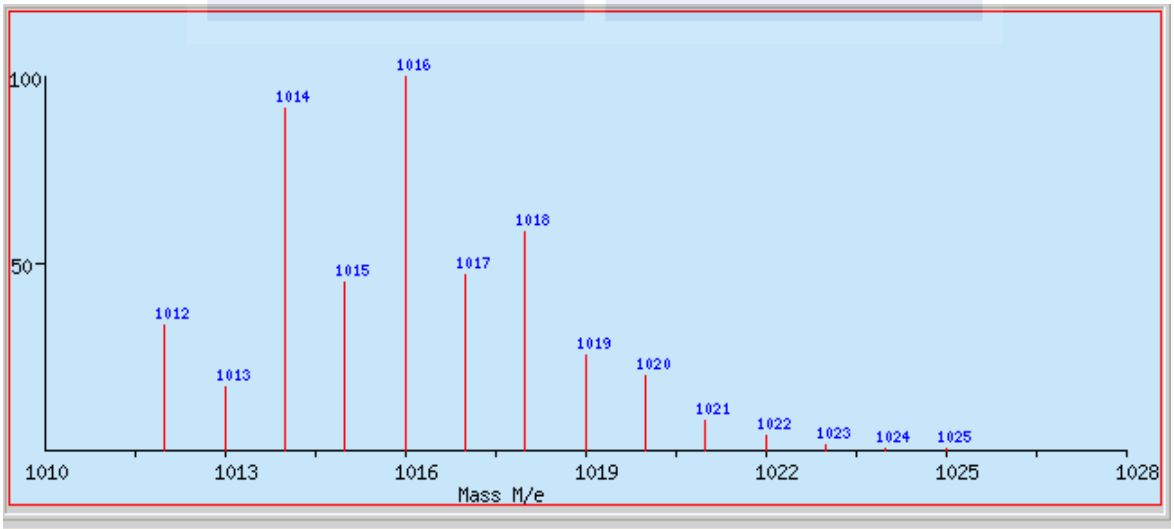


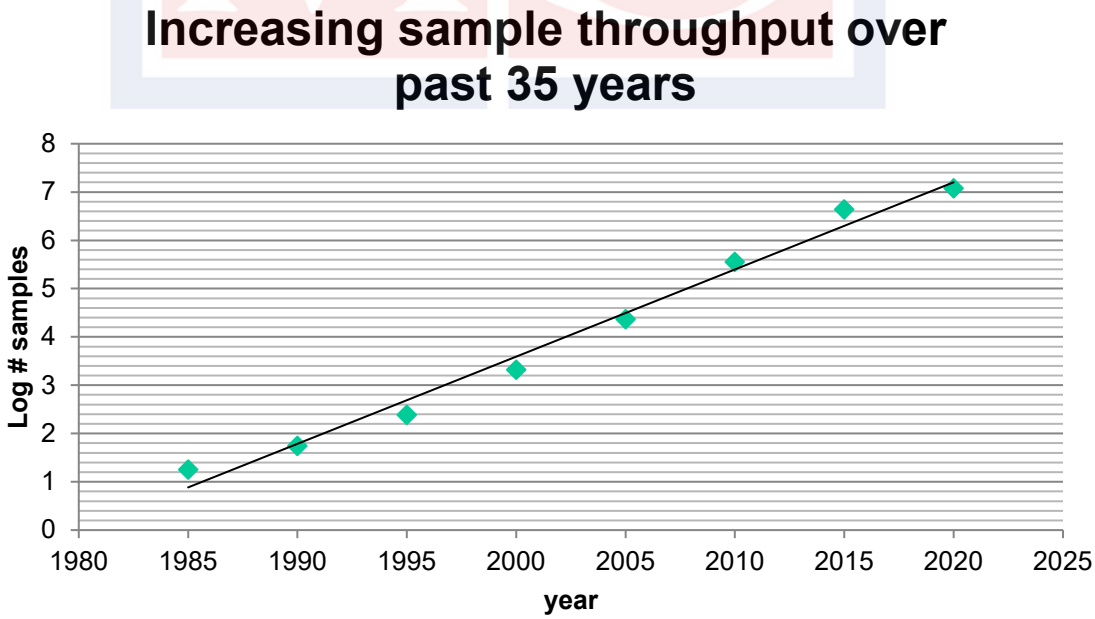
New Techniques:

Chip Based Systems

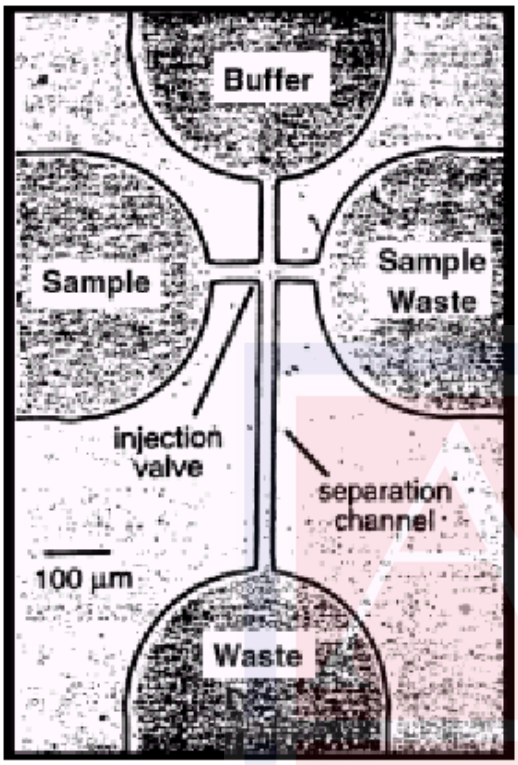
Direct Analysis Approaches

Why Microchip Devices ?

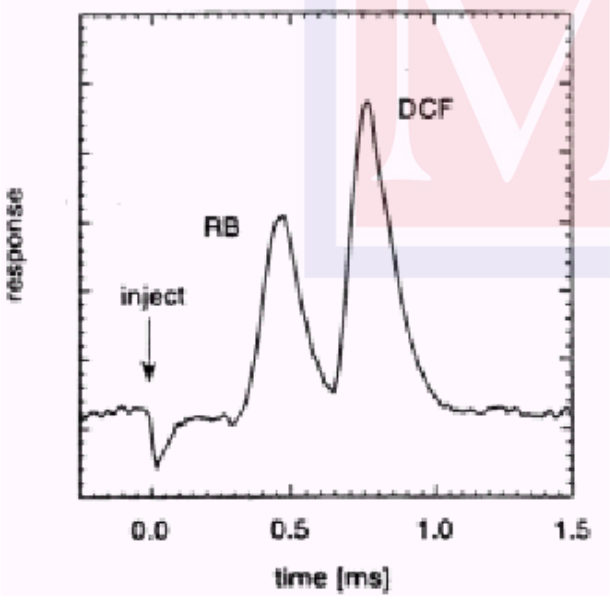
- Speed- *short separation times, parallel processing*
- Costs- *low usage of reagents, short analysis times, low unit cost*
- Automation- *computer controlled*
- Sensitivity- *limited sample handling, minimal sample losses and contamination*
- Ruggedness- *bulk production, easy and inexpensive to replace*



Microchip CE/LIFD Determination Made in Less than 1 msec



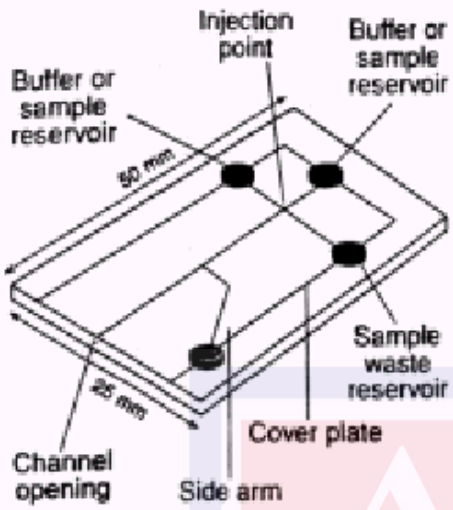
Photograph of microchip used for high-speed electrophoretic separations. The area of the photograph is 0.8 x 1.2 mm, and the injection valve and separation channel are shown. The wide channels are 440 µm wide, and the narrow channels are 25 µm wide.



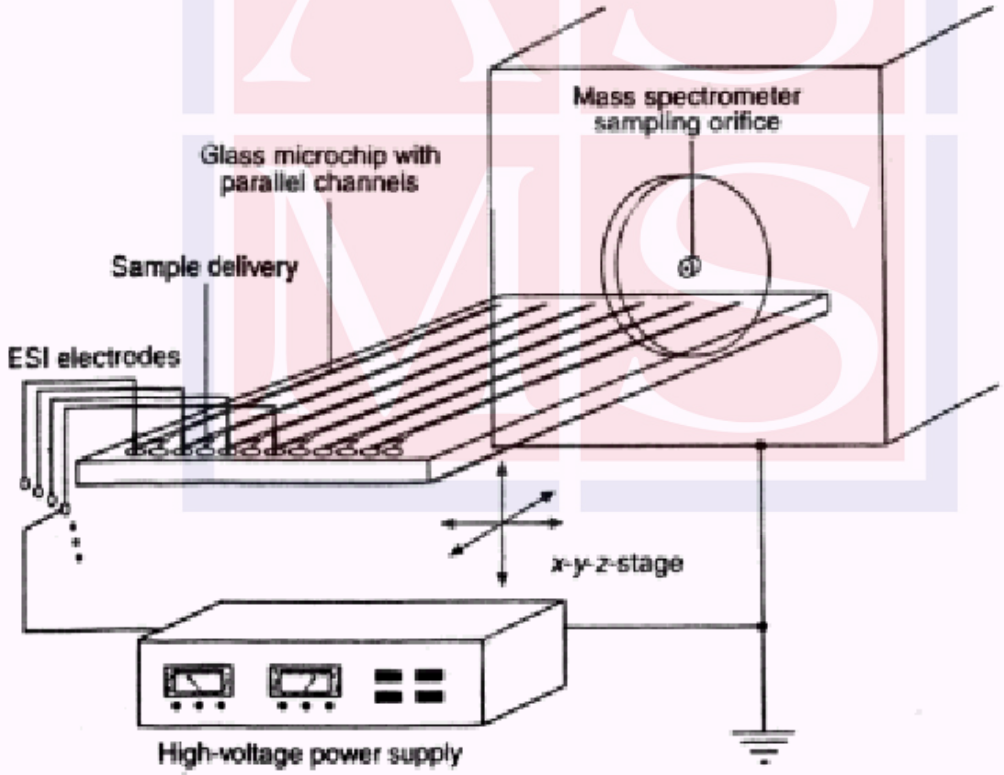
High speed electropherogram of rhodamine B and dichlorofluoroscein resolved in 0.8 ms using a separation field strength of 53 kV cm^{-1} and a separation length of 200 µm. The start time is marked with an arrow at 0 ms.

From Anal. Chem., 70 (16), 3476 (1998)

Microchip CE/MS



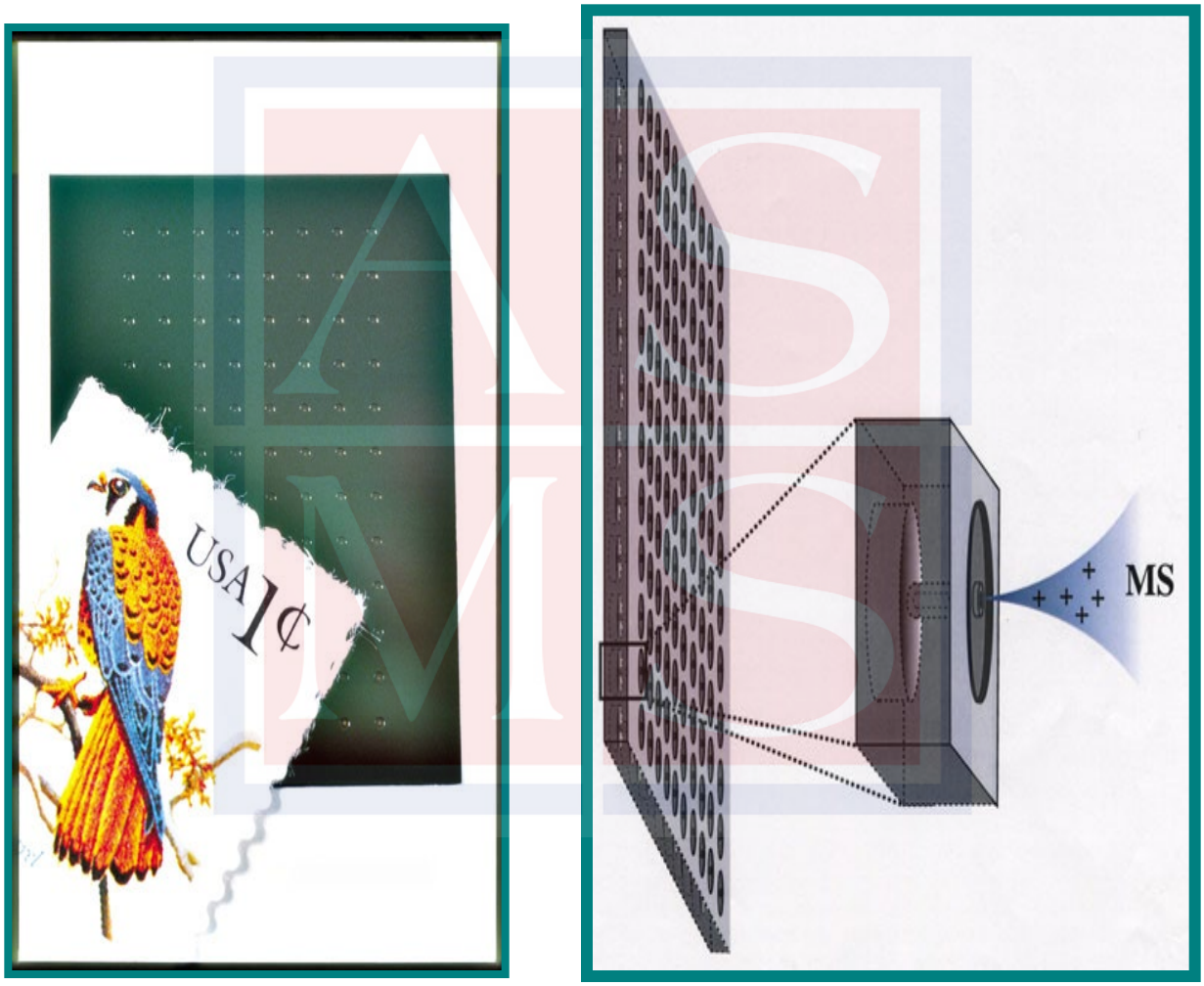
A schematic of the side-arm chip for electroosomotic pumping



A schematic of the multichannel electrospray chip interfaced to a mass spectrometer

From Anal. Chem., 69 (11), 359A (1997)

The ESI Chip



Chip dual LC (trapping and analytical Column) with nanospray tip

Multiple-Nozzle ESI

Multiple nozzle ESI devices add reliability and ease of use to low flow electrospray.

Good performance is achieved when some nozzles are not performing properly or plugged.

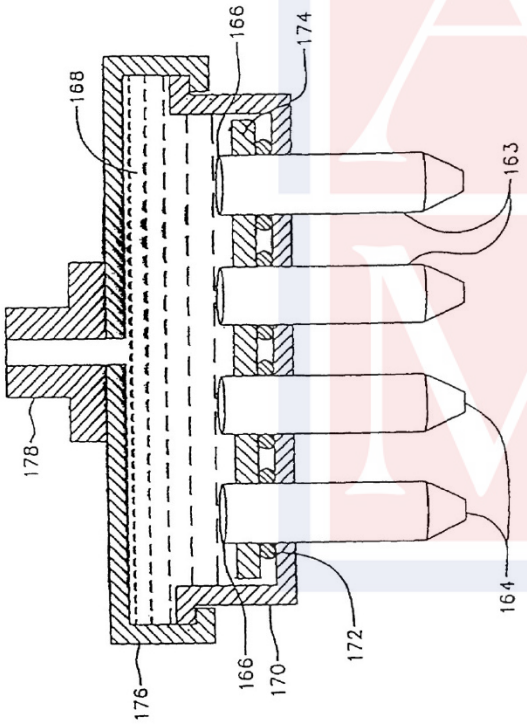
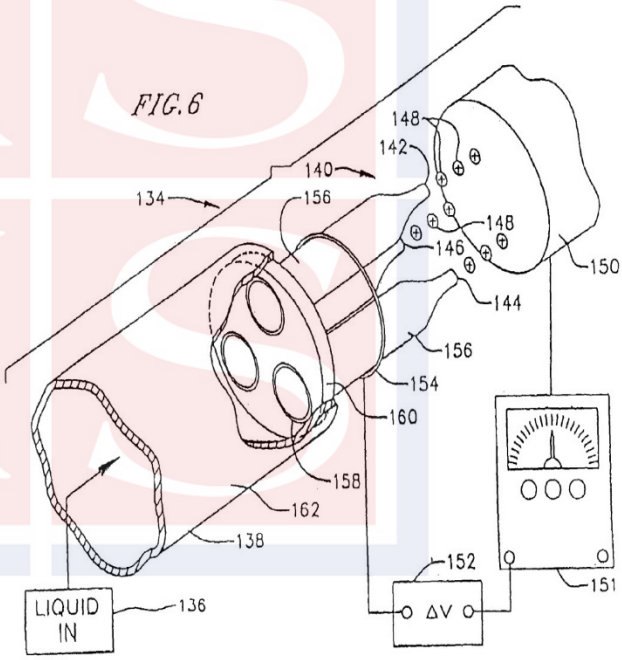


FIG. 7

FIG. 6



U.S. Patent Apr. 17, 2007 Sheet 5 of 7 US 7,205,536 B2

Micro Ion Trap Mass Analyzer

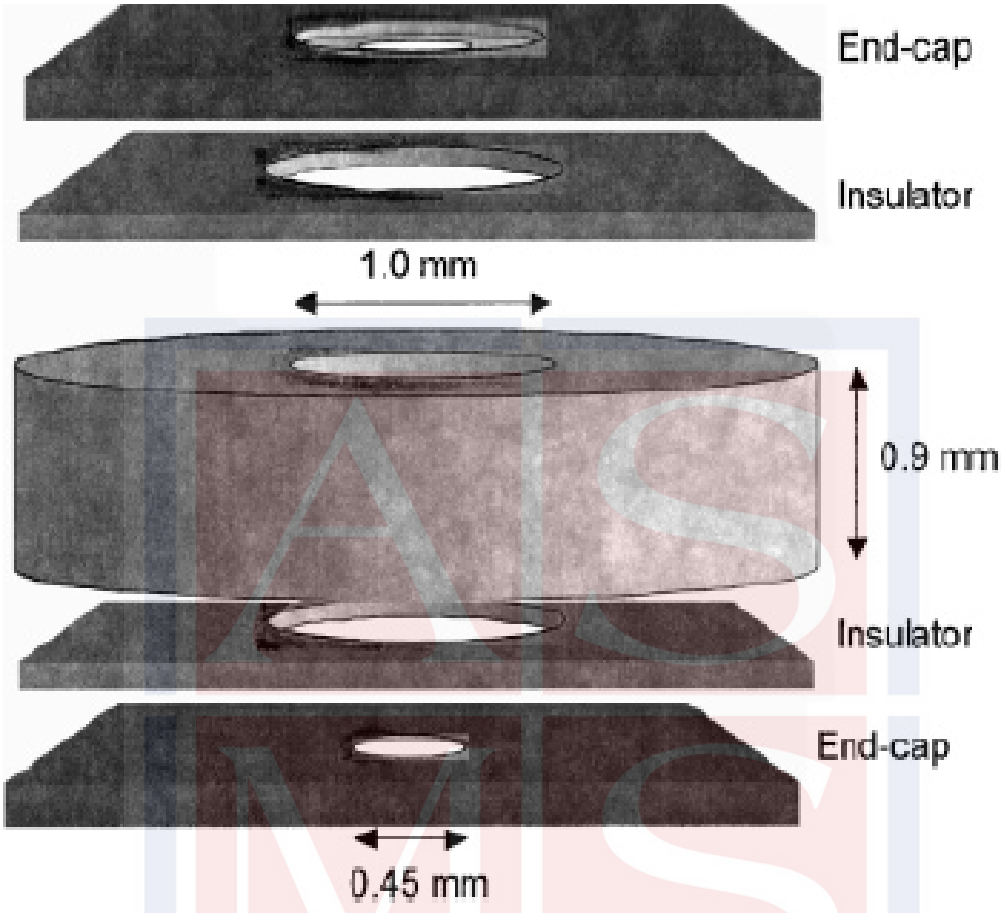
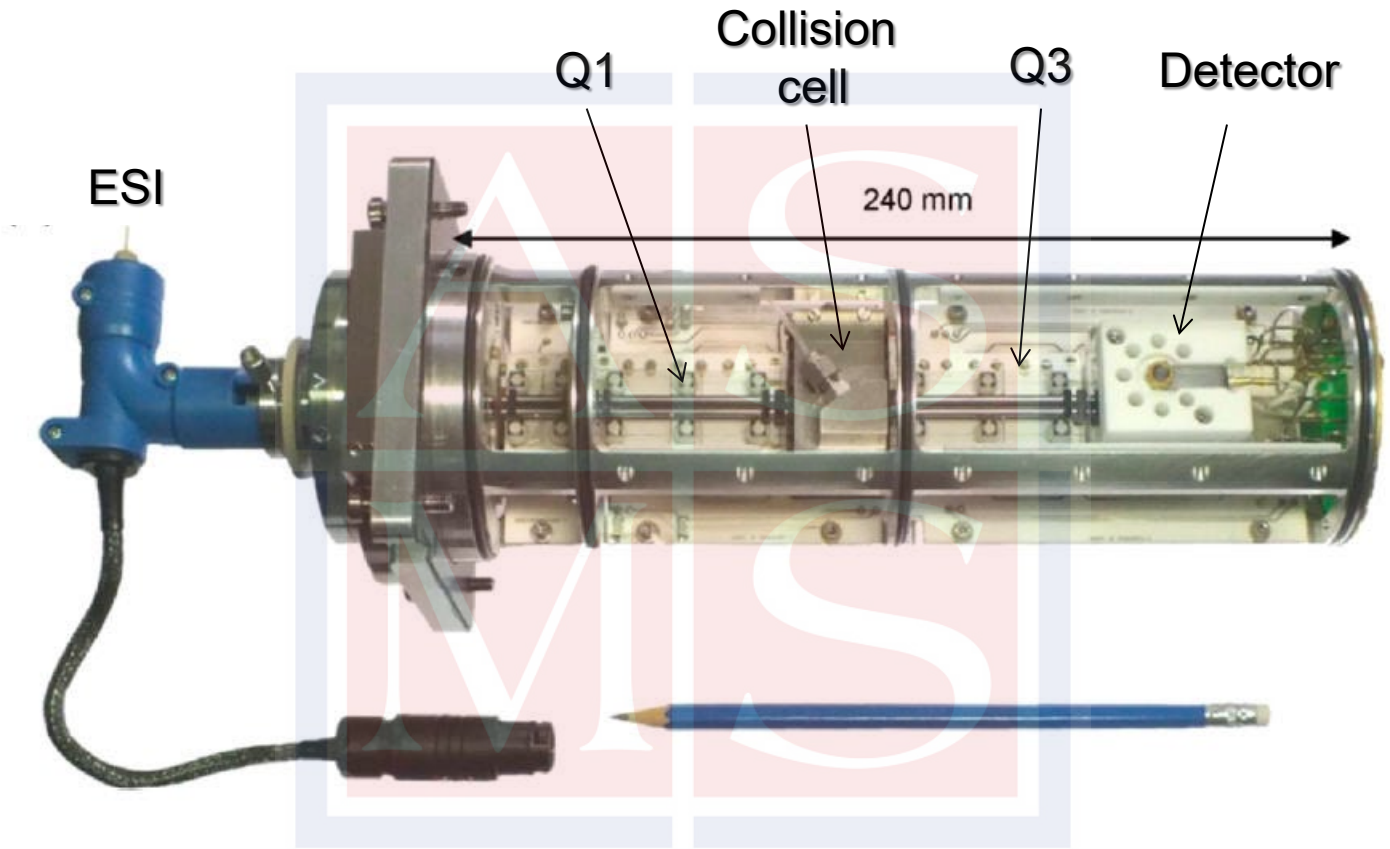


Figure 2. Micro ion trap assembly.

Rapid Commun. Mass Spectrom. 13, 50–53 (1999)

Trap I.D. = 1 mm
Ring electrode 0-200V @ 5.8 MHz

Miniature ESI triple quad MS



http://dx.doi.org/10.1021/acs.analchem.5b00311

Why use Direct Analysis Approaches?

Potential Advantages:

- No or limited sample preparation
- High throughput- a sample per second
- Simple (all work at atmospheric pressure)

Potential Disadvantages

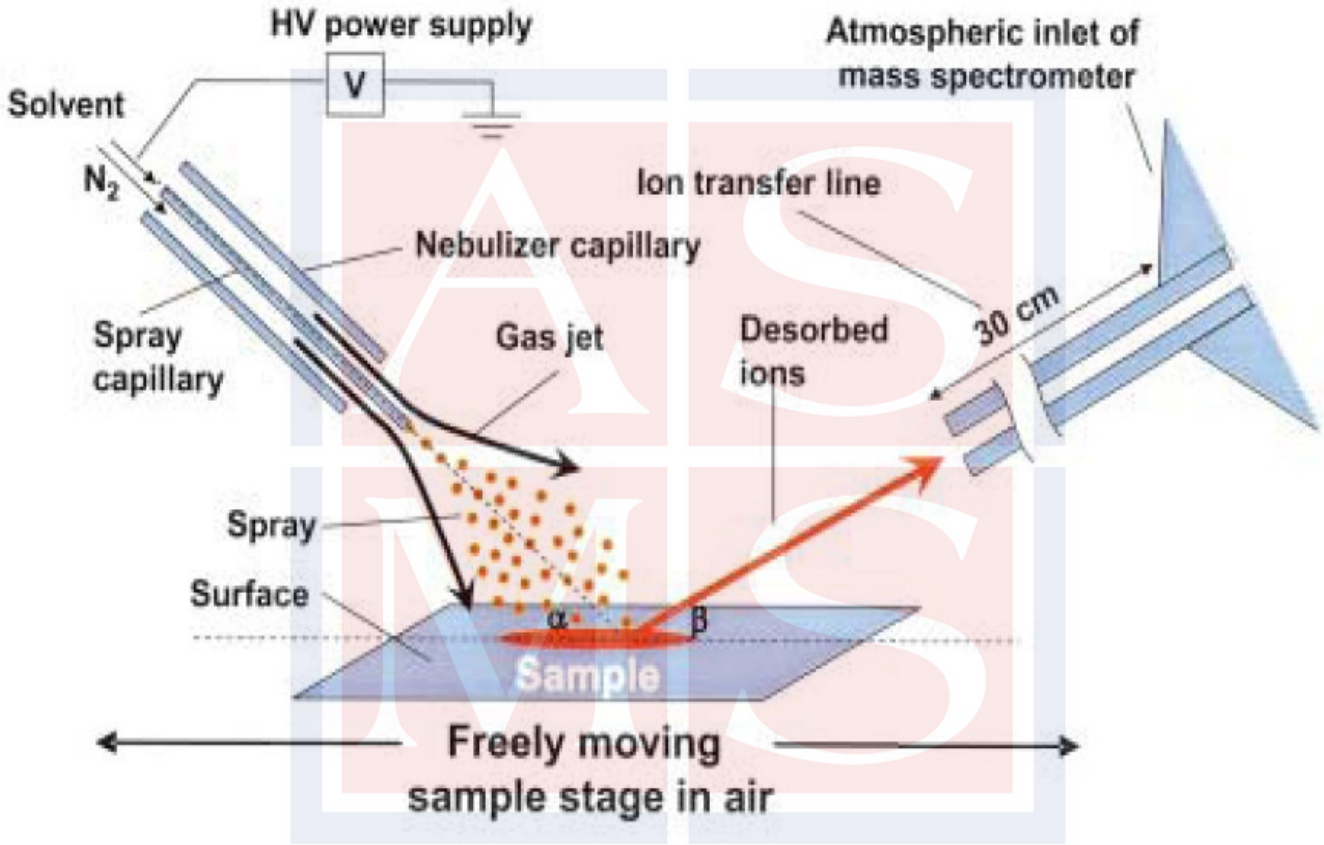
- Matrix suppression and common mass interferences for detection of a target compound.
- Requires MS/MS or high resolution MS
- Some approaches only amenable to volatile compounds
- Approaches are usually not compatible with LC

Approaches that will be discussed

- DESI-Desorption Electrospray Ionization
- DART –Direct Analysis in Real Time
- ASAP – Atmospheric-pressure Solids Analysis Probe

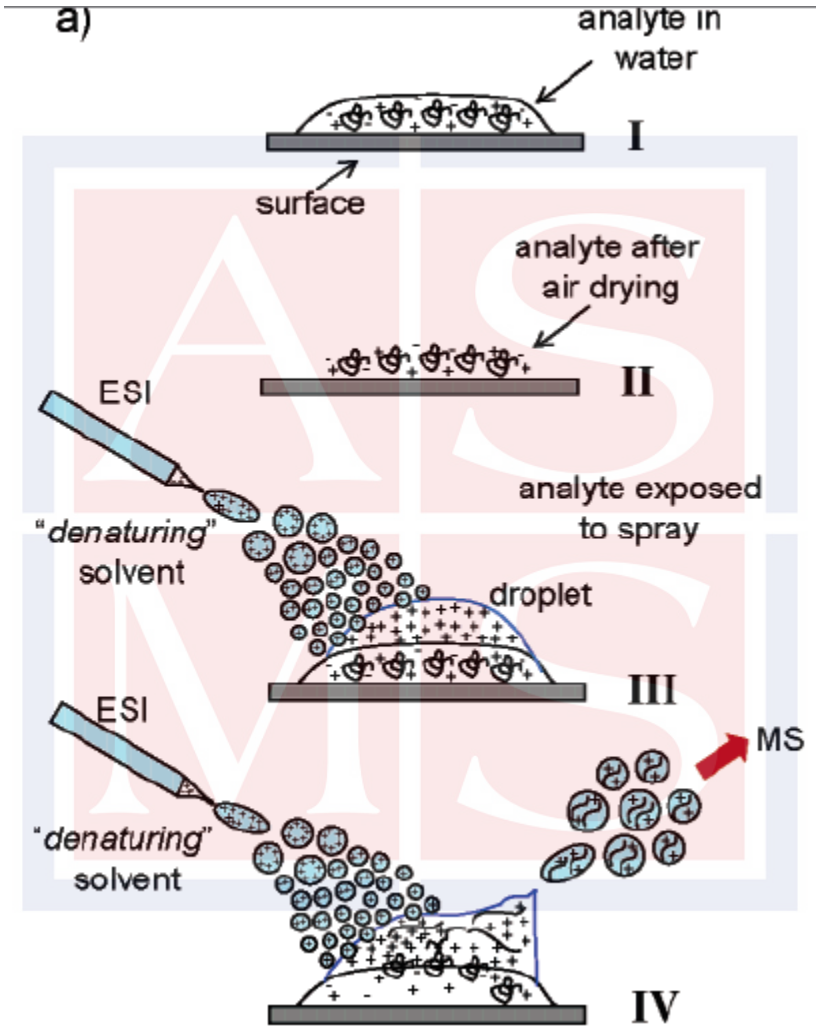
DESI

Instrument schematic



Sci 306, 471, 2004

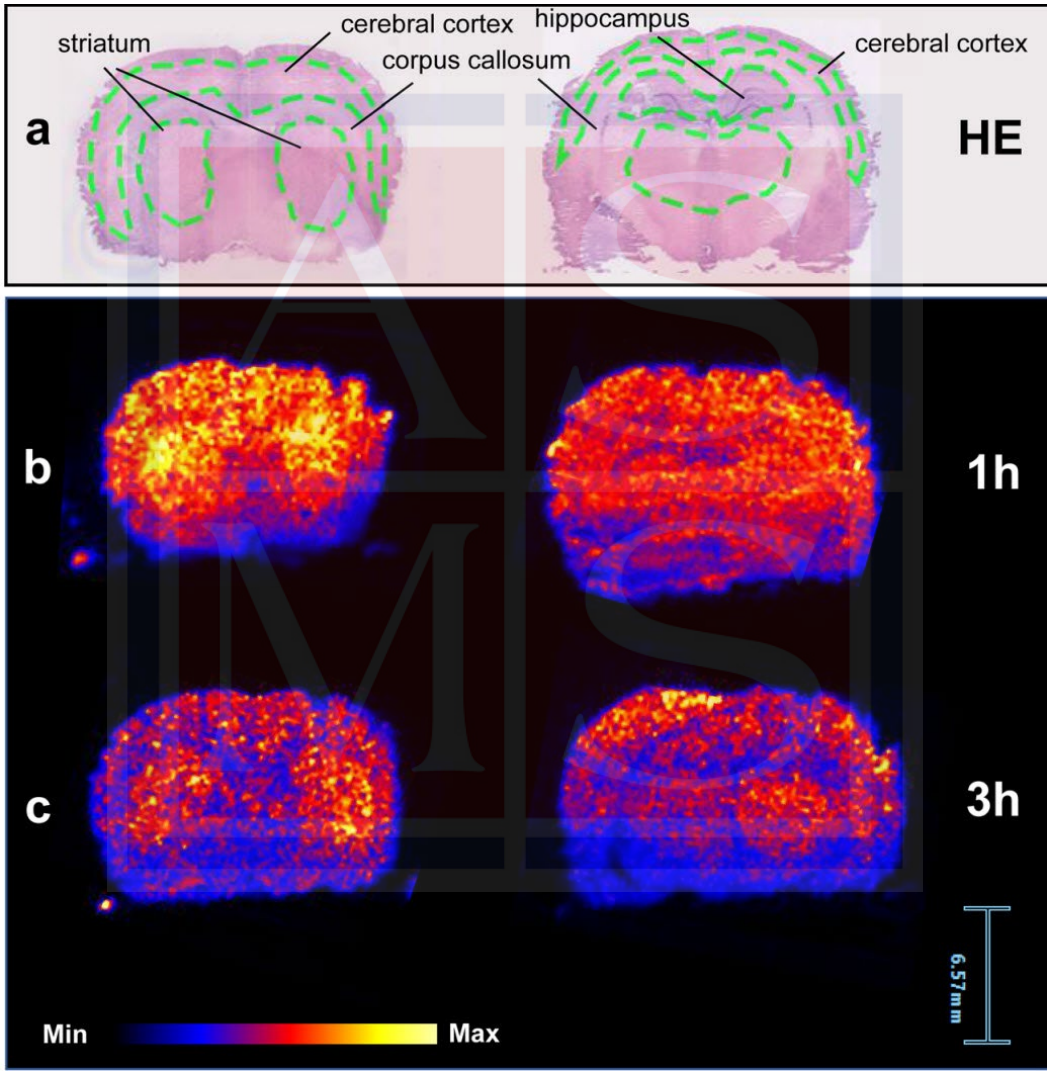
Proposed Ionization Mechanism for DESI



J Phys Chem 110, 5049, 2006

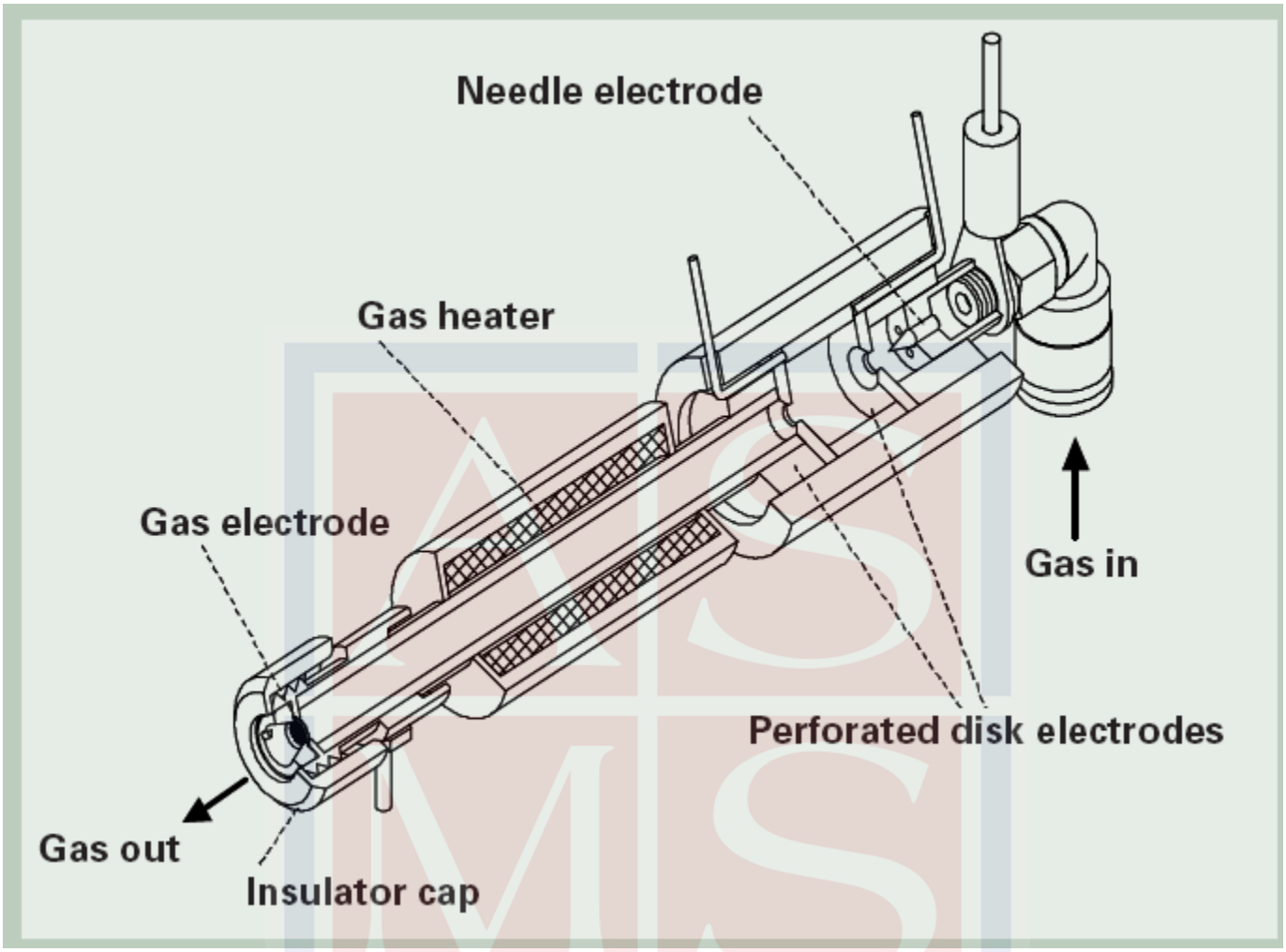
Application of DESI for chemical imaging

Analysis of brain slices for Fluoropezil, (an acetylcholinesterase inhibitor) at 1 and 3 hours after dosing monitoring m/z 416



Analytical and bioanalytical chemistry, 09/2021, Volume 413, Issue 23

DART



Gas phase ionization Mechanism

$$Gas^{*} + M \rightarrow M^{+\cdot} + Gas^{-}$$

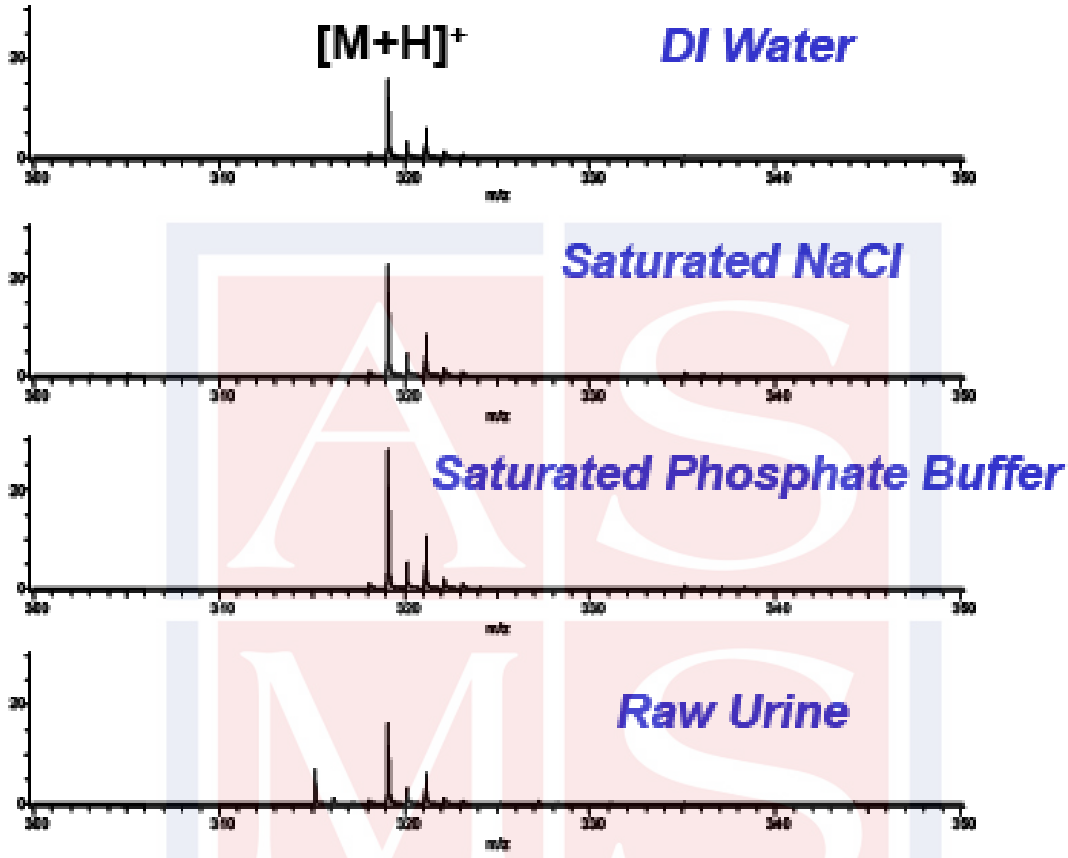
$$Gas^{*} + surface \rightarrow Gas + surface + e^-_{\text{high en}}$$

$$e^-_{\text{high en}} \rightarrow e^-_{\text{low en}} \text{ (via collisions)}$$

$$e^-_{\text{low en}} + O_2 \rightarrow O_2^-$$

$$O_2^- + M \rightarrow M^- + O_2$$

Application of DART



DART analysis of chlorpromazine in various solutions.

ASAP

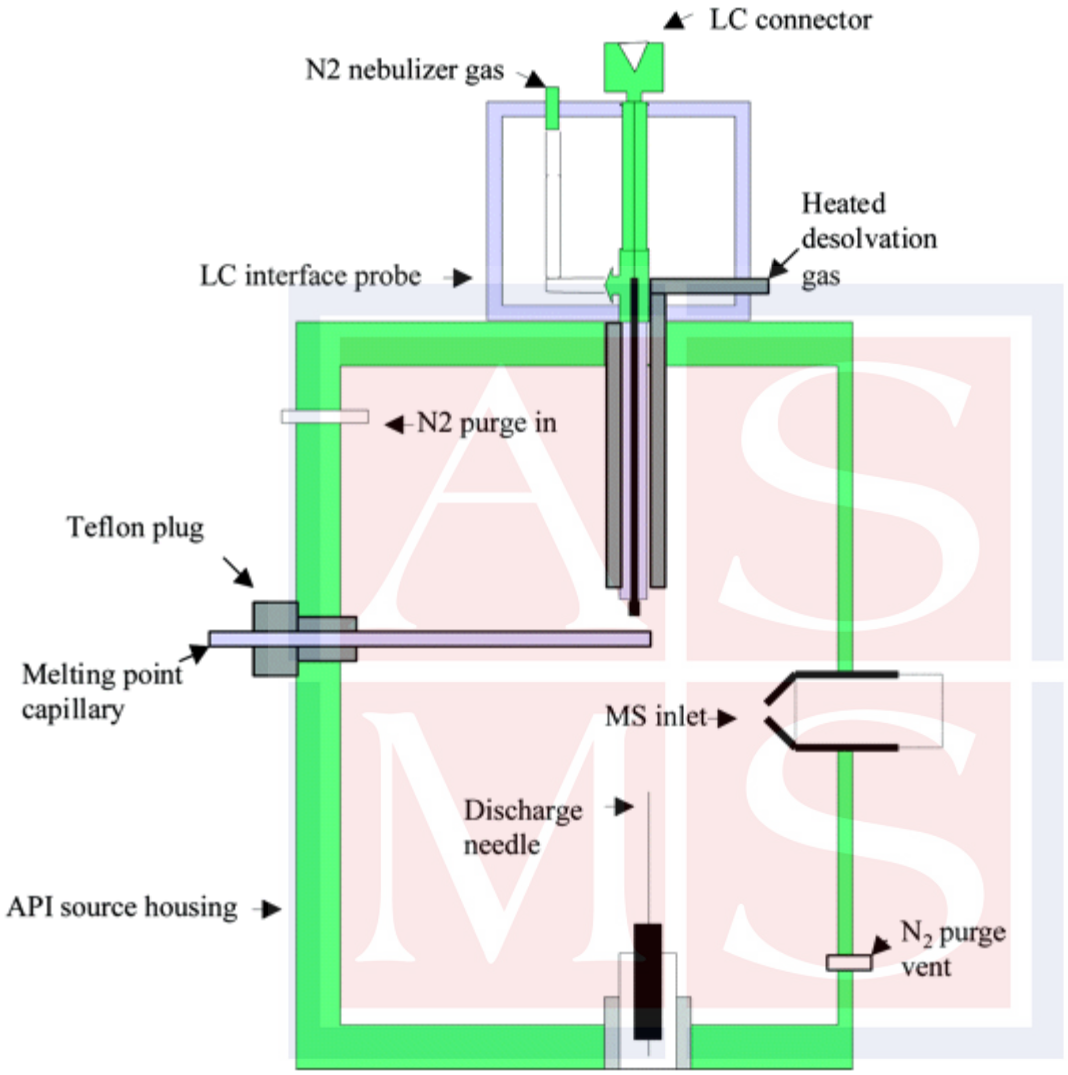


Figure 1 Cross-sectional drawing of an atmospheric pressure LC/MS ion source modified for ASAP analysis.

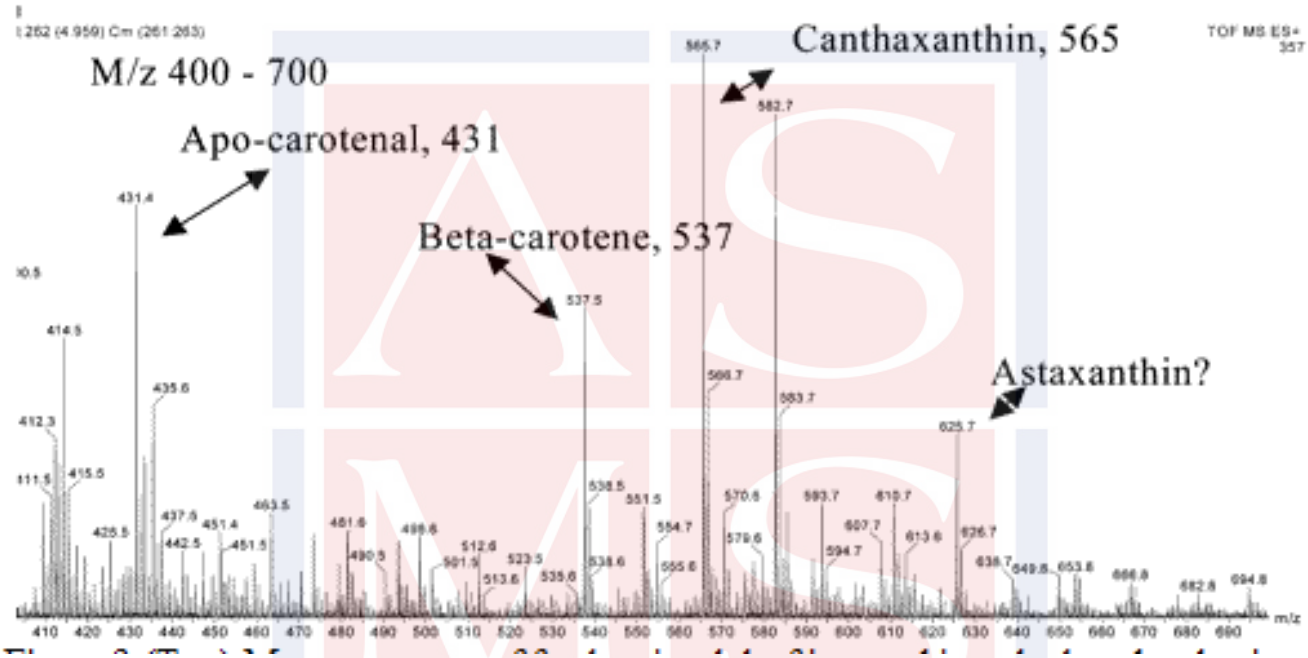
Ionization is gas phase- analogous to APCI discussed previously

ASAP source can be easily fitted onto most API instruments

Anal Chem 77, 7826, 2005

Application of ASAP

ASAP analysis if spinach leaf



Anal Chem 77, 7826, 2005

Discussion and Problems

1. Discussion time on attendee problems
2. Measuring Simvastatin in plasma
3. Quantifying 10 Benzodiazepines in plasma
4. Measurement of a peptide in plasma
5. Quantifying a protein in food

Mass Spectrometry
McCullagh, James; Oldham, Neil
Published by Oxford University Press (2019)
ISBN 10: 0198789041 ISBN 13: 9780198789048

Discussion time

Opportunity for attendees to bring up any questions involving their problems with current LC/MS/MS analysis. This can include but limited to:

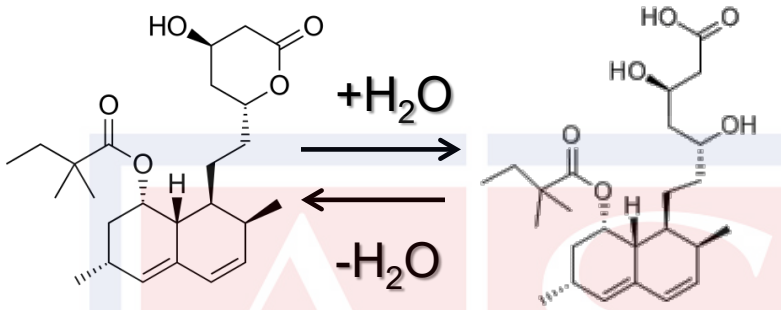
1. Method development
2. Trouble shooting method or instrument
3. Improving sensitivity and specificity
4. MS interpretation
5. Quantitative issues like matrix suppression
6. Any aspect of electrospray, APCI or APPI
7. Instrument evaluation- what is best for my needs?

For existing problems for analysis, Ideally have what has been performed already, compound structure and any data that can be shared for the discussion and what byou would like to solve.

For future analysis that have not been tried yet, ideally have a compound structure and goals for the assay.

Measuring Simvastatin in plasma

Problem 1 Discuss all aspects in developing a LC/MS/MS method for Simvastatin (sv) in plasma. The method should be able to detect 0.1 ng/ml levels in plasma sample size of 200 ul



SV SVA

- Sample prep
- Separation
- MS conditions

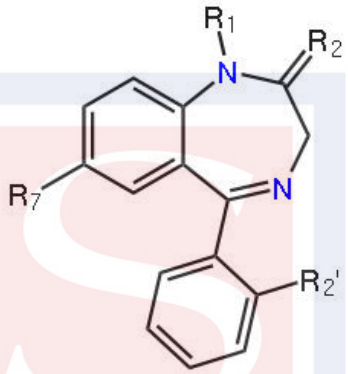
•What can be done if interferences are present

•What can be done in sensitivity is lacking

Quantifying 10 Benzodiazepines in plasma

Problem 2

Discuss all aspects in developing a LC/MS/MS method for Benzodiazepines in plasma. The method should be able to detect 0.1ng/ml in 1 ml of plasma



- Sample prep
- Separation
- MS conditions

- What can be done if interferences are present

- What can be done in sensitivity is lacking

Quantifying a peptide in plasma

Problem 3

Discuss all aspects in developing a LC/MS/MS method for peptide in plasma. The method should be able to detect 0.1ng/ml in 1 ml of plasma

VSLETNYSLLQY

MW = 1429.5993

- Sample prep
- Separation
- MS conditions

- What can be done if interferences are present

- What can be done in sensitivity is lacking

Quantifying a Peanut Allergen Protein in food

Problem 5

Discuss all aspects in developing a LC/MS/MS method for the protein (MW 58450) below in food . The method should be able to detect 1 ng/g in a maximum sample size of 1 g of food

1	ISFRQQPEEN	ACQFQRLNAQ	RPDNRIESEG	GYIETWNPNN	QEFECAGVAL
51	SRLVLRRNAL	RRPFYSNAPQ	EIFIQQGRGY	FGLIFPGCPS	TYEEPAQQGR
101	RYQSQRPPRR	LQEEDQSQQQ	QDSHQKVHRF	NEGDLIAVPT	GVAFWLYNDH
151	DTDVVAVSLT	DTNNNDNQLD	QFPRRFNLAG	NHEQEFLRYQ	QQSRQSRRRS
201	LPYSPYSPQS	QPRQEEREFS	PRGQHSRRER	AGQEEEHEGG	NIFSGFTPEF
251	LAQAFQVDDR	QIVQNLRGEN	ESEEQGAIVT	VRGGLRILSP	DRKRGADEEE
301	EYDEDEYEYD	EEDRRRGRGS	RGSGNGIEET	ICTATVKKNI	GRNRSPDIYN
351	PQAGSLKTAN	ELNLLILRWL	GLSAEYGNLY	RNALFVPHYN	TNAHSIIYAL
401	RGRAHVQVVD	SNGNRVYDEE	LQEGHVLVVP	QNFAVAGKSQ	SDNFEYVAFK
451	TDSRPSIANL	AGENSVIDNL	PEEVVANSYG	LPREQARQLK	NNNPFKFFVP
501	PSQQSPRAVA				

- Sample prep
- Separation
- MS conditions
- What can be done if interferences are present
- What can be done in sensitivity is lacking

Tryptic digest of Protein

Digest showing peptides > m/z 400 with no missed cleavages

m/z	Sequence	m/z	Sequence
532.295	(R) GRGSR(G)	1617.7715	(R) GENESEEQGAIVTVR(G)
584.2899	(R) GQHSR(R)	1659.8449	(R) NRSPDIYNPQAGSLK(T)
587.3624	(K) KNIGR(N)	1707.8582	(R) GSGNGIEETICTATVKK(N)
629.3842	(R) RNALR(R)	1730.8721	(R) RFNLAGNHEQEFLR(Y)
635.3148	(R) EFSPR(G)	1762.8871	(R) RSLPYSPYSPQSQPR(Q)
656.4566	(R) LVLRR(N)	1879.9178	(R) GSRGSGNGIEETICTATVK(K)
700.3988	(R) ILSPDR(K)	1955.869	(R) LQEEDQSQQQQDSHQK(V)
729.4114	(K) NIGRNR(S)	1980.9345	(-) ISFRQQPEENACQFQR(L)
733.3628	(K) NNNPFK(F)	2000.9996	(R) GENESEEQGAIVTVRGGLR(I)
740.391	(R) GQHSRR(E)	2004.0086	(K) NNNPFKFFVPPSQQSPR(A)
809.39	(R) YQQQSR(Q)	2022.945	(-)ISFRQQPEENACQFQR(L)
828.4938	(R) ILSPDRK(R)	2051.0457	(R) RPFYSNAPQEIFIQQGR(G)
853.489	(R)QIVQNLR(G)	2111.9701	(R) RLQEEDQSQQQQDSHQK(V)
870.5156	(R) QIVQNLR(G)	2149.0309	(R) SLPYSPYSPQSQPRQEER(E)
872.4948	(R) EQARQLK(N)	2314.2091	(R) NALFVPHYNTNAHSIIYALR(G)
1031.5381	(R) YQSQRPPR(R)	2348.0974	(R) LQEEDQSQQQQDSHQKVHR(F)
1083.5654	(R) LNAQRPDNR(I)	2365.1432	(R) FNLAGNHEQEFLRYQQQSR(Q)
1083.6269	(R) GGLRILSPDR(K)	2417.1231	(R) GYFGLIFPGCPSTYEEPAQQGR(R)
1085.5738	(R)QLKNNNPFK(F)	2452.2427	(R)QIVQNLRGENESEEQGAIVTVR(G)
1102.6004	(R) QLKNNNPFK(F)	2469.2692	(R) QIVQNLRGENESEEQGAIVTVR(G)
1160.5331	(R)QEEREFSPR(G)	2505.311	(R) NALRRPFYSNAPQEIFIQQGR(G)
1177.5596	(R) QEEREFSPR(G)	2525.1698	(R)QQPEENACQFQRLNAQRPDNR(I)
1180.5818	(R) YQQQSRQSR(R)	2527.3317	(R) NALFVPHYNTNAHSIIYALRGR(A)
1187.6392	(R) RYQSQRPPR(R)	2528.8856	(R) GADEEEEYDEDEYEYDEEDR(R)
1187.6392	(R) YQSQRPPRR(L)	2541.2984	(R) VYDEELQEGHVLVVPQNFAVAGK(S)
1200.5868	(R) EFSPRGQHSR(R)	2542.1964	(R) QQPEENACQFQRLNAQRPDNR(I)
1269.7525	(K) TANELNLLILR(W)	2573.2242	(R) GYFGLIFPGCPSTYEEPAQQGRR(Y)
1289.6637	(K) FFVPPSQQSPR(A)	2640.4355	(R) SPDIYNPQAGSLKTANELNLLILR(W)
1295.6451	(R) AHVQVVDSNGNR(V)	2684.9867	(K) RGADEEEEYDEDEYEYDEEDR(R)
1389.7009	(R) SPDIYNPQAGSLK(T)	2684.9867	(R) GADEEEEYDEDEYEYDEEDRR(R)
1434.6536	(K) SQSDNFEYVAFK(T)	2792.5094	(K) TANELNLLILRWLGLSAEYGNLYR(N)
1460.6223	(R)QQPEENACQFQR(L)	3013.3632	(R) IESEGGYIETWNPNNQEFECAGVALSR(L)
1477.6489	(R) QQPEENACQFQR(L)	3325.5032	(R) AGQEEEHEGGNIFSGFTPEFLAQAFQVDDR(Q)
1508.7677	(R) GRAHVQVVDSNGNR(V)	3494.7009	(R) IESEGGYIETWNPNNQEFECAGVALSRLVLR(R)
1530.8063	(K) FFVPPSQQSPRAVA(-)	3497.7507	(K) TDSRPSIANLAGENSVIDNLPEEVVANSYGLPR(E)
1541.7747	(R) WLGLSAEYGNLYR(N)	3610.6469	(R) ERAGQEEEHEGGNIFSGFTPEFLAQAFQVDDR(Q)
1574.771	(R) FNLAGNHEQEFLR(Y)	3817.9256	(R) AHVQVVDSNGNRVYDEELQEGHVLVVPQNFAVAGK(S)
1579.7632	(R) GSGNGIEETICTATVK(K)	3836.966	(R) WLGLSAEYGNLYRNALFVPHYNTNAHSIIYALR(G)
1606.786	(R) SLPYSPYSPQSQPR(Q)	3956.9341	(R) VYDEELQEGHVLVVPQNFAVAGKSQSDNFEYVAFK(T)
		3981.9901	(K) TDSRPSIANLAGENSVIDNLPEEVVANSYGLPREQAR(Q)