Atmospheric Pressure Ionization (API)

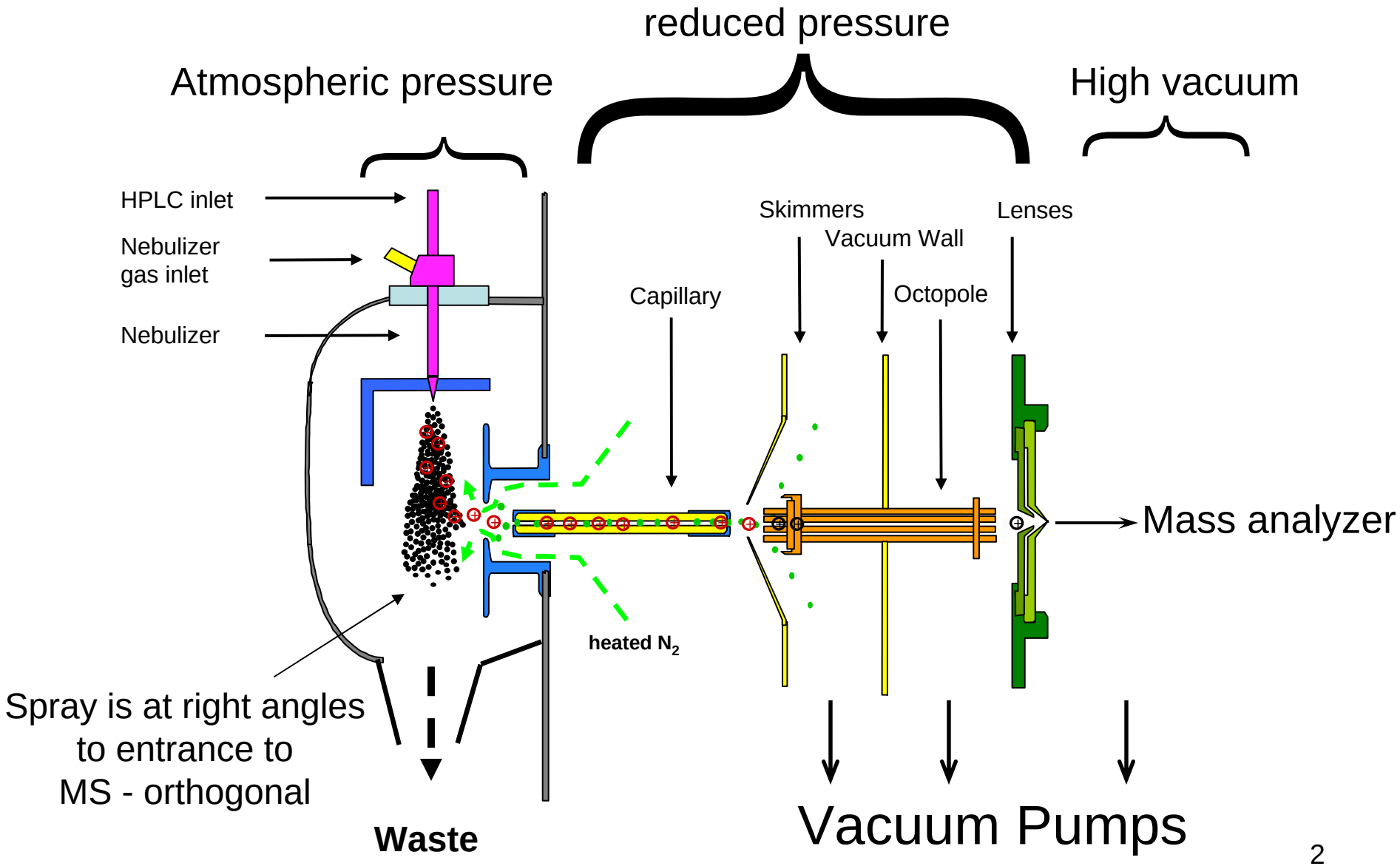
- conventional ionization methods employ sources that are at high vacuum (EI, CI, FI/FD, FAB/LSIMS, MALDI) and/or temperature (EI, CI, FI/FD)

- the introduction of API sources employing a number of different types of ionization has allowed very robust instruments to be developed for LC/MS

- These “new” ionization techniques have greatly extended the range of analytes that can be studied by MS to compounds that are high molecular weight, thermally labile and polar.

- While the sources are designed to operate at atmospheric pressure we must still maintain a high vacuum in the rest of the instrument if we want to perform mass spectrometry!!

API Source



courtesy of Agilent

API Source

- High vacuum must be maintained in the mass analyzer and detector region even though the source is at atmospheric pressure
- The region after the source is heavily pumped with rotary vacuum and turbomolecular pumps (usually)
- Also, a series of skimmers and flow restrictors are placed between the source and the mass analyzer region
- These skimmers allow ions to be efficiently transmitted to the high vacuum region while at the same time allow air, solvent vapours and other neutral volatile species to be pumped away
- The exact design will depend on the specific instrument type and manufacturer

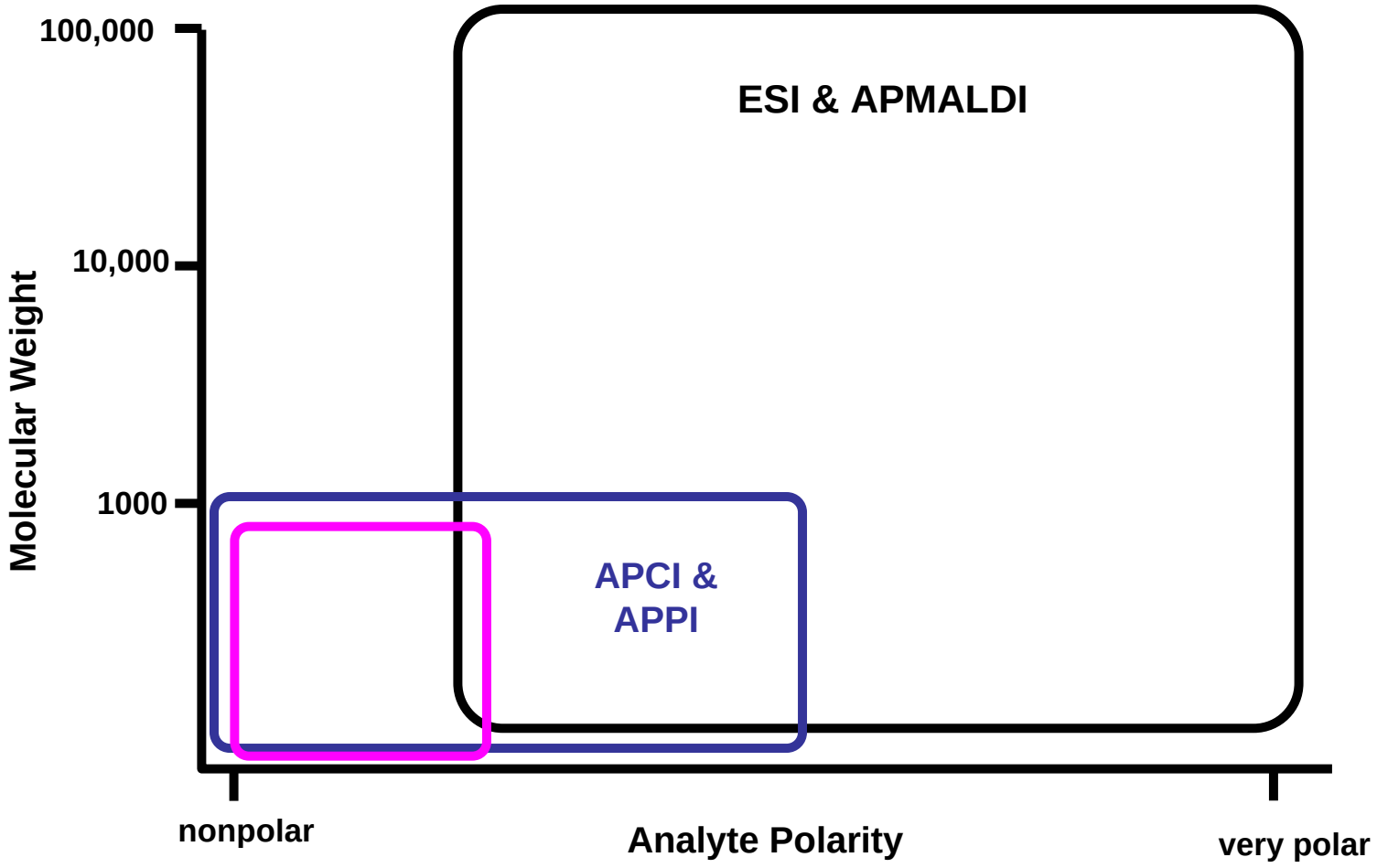
API Sources

- Electrospray (ESI)
  - high flow rate (100μL/min – 1mL/min)
  - capillary flow rate (2μL/min - 100μL/min)

  } pneumatically assisted ESI

  - low flow rate (<2μL/min)
    - nanospray (200-500nL/min) – ESI is most sensitive at these low flow rates
- Atmospheric Pressure Chemical Ionization (APCI)
- Atmospheric Pressure PhotoIonization (APPI)
- Atmospheric Pressure MALDI

Relative Applicability of API Techniques



ESI: **Electrospray Ionization & APMALDI**
APCI: **Atmospheric Pressure Chemical Ionization**
APPI: **Atmospheric Pressure Photo Ionization**
EI, CI, GC-MS

Electrospray (ESI)

- Based upon the electrostatic spraying of liquids where a solution is passed through a needle held at high voltage (kV) relative to a counter electrode (the entrance to the MS)

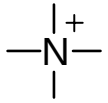
- When the solution contains an electrolyte and the needle forms part of the API source then the fine mist of droplets that emerge from the needle tip possesses a net +ve or –ve charge determined by the polarity of the needle and the solution chemistry of the bulk liquid

- These preformed and then sprayed ions, which are characteristic of the dissolved analytes, are attracted to the entrance of the MS by applying appropriate voltages

Electrospray (ESI)

- The formation of the spray must be aided by nebulization (pneumatically assisted) at liquid flow rates higher than a few μL/min

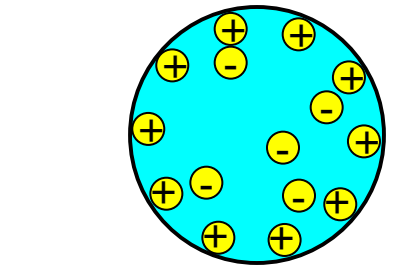
- ions exist in solution, if not, electrospray doesn't work, it is not an ion formation technique rather than a technique for extracting ions from the solution-phase into the gas-phase free of solvent for mass spectral analysis

- The analyte must be an ion in solution either as a preformed ion such as  or through modifying the solution chemistry to induce a charge

- This can be accomplished by changing solution pH or adding cations eg Li^+, NH_4^+ etc or anions to form adducts eg Cl^-, OAc^- etc

Electrospray Ionization

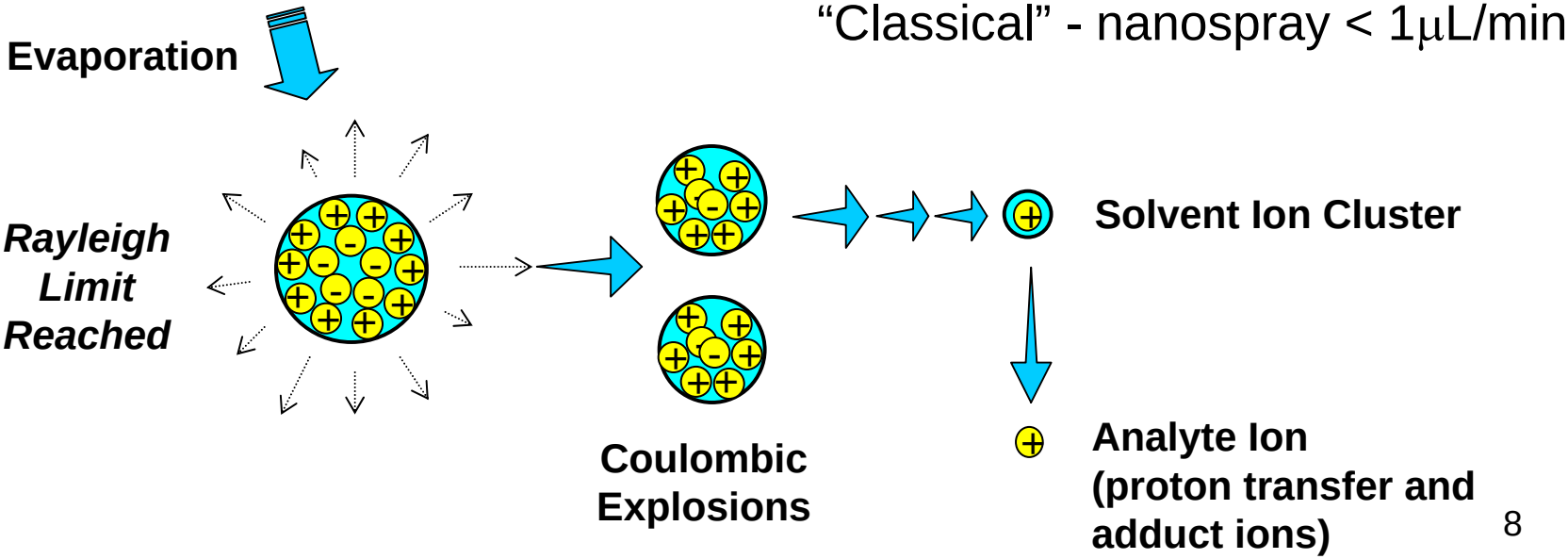
Charged Droplets containing ions in solution ⟶ **Analyte Ions in the gas phase - both +ve and -ve**



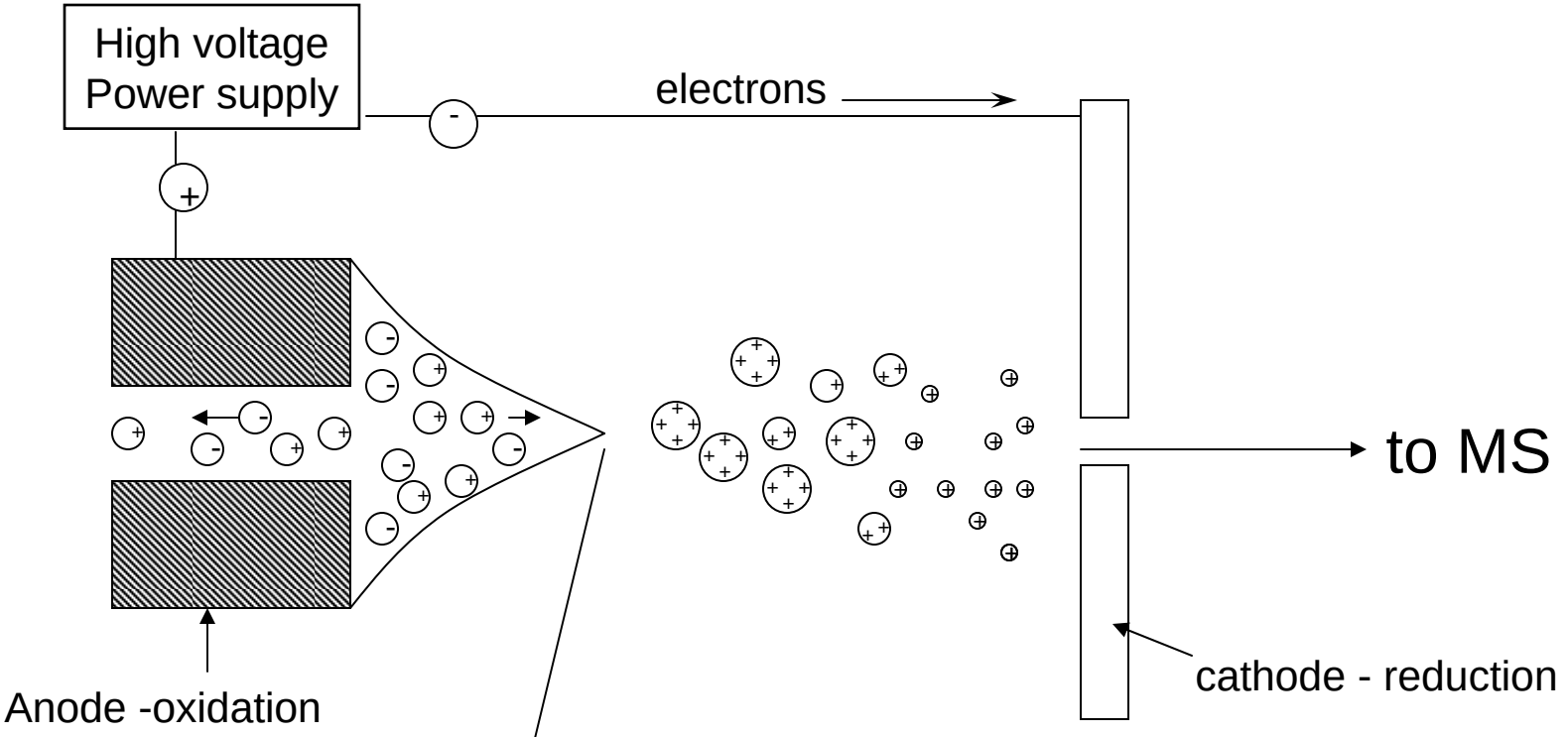
Nebulizer assisted >1μL/min
- capillary 2-100μL/min
- normal 0.1-1mL/min

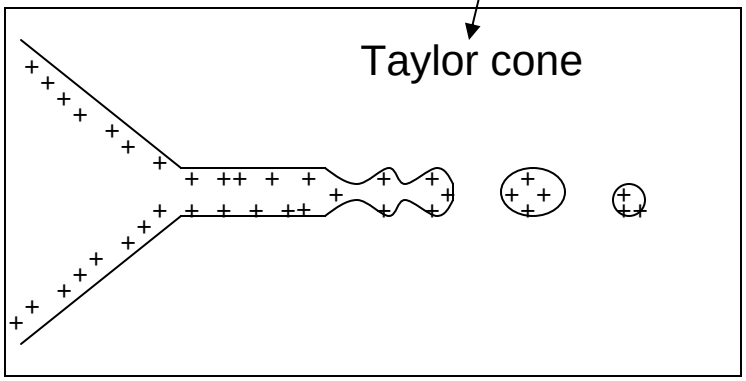
"Classical" - nanospray < 1μL/min

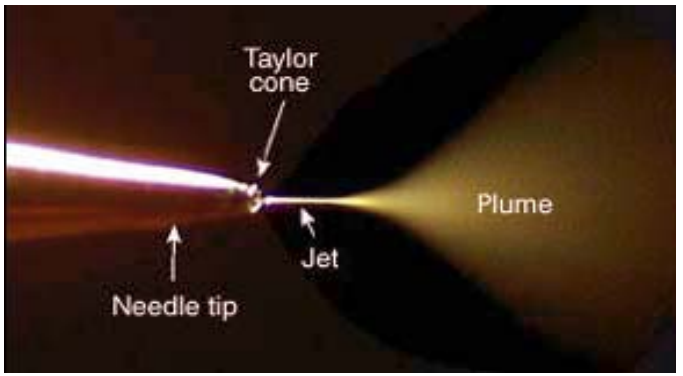
Evaporation



The “Source”


High voltage
Power supply
electrons
to MS
Anode -oxidation
cathode - reduction


Taylor cone


Taylor cone
Plume
Jet
Needle tip

Proposed Mechanisms:

1. **Charge Residue Model:** where the droplet is completely evaporated leaving “bare’ analyte ions

2. **Ion Evaporation Model:** field assisted ion desorption
    - Requires ~ $10^{7}Vcm^{-1}$ and a final droplet diameter of 10nm
    - Fits well with the observed data

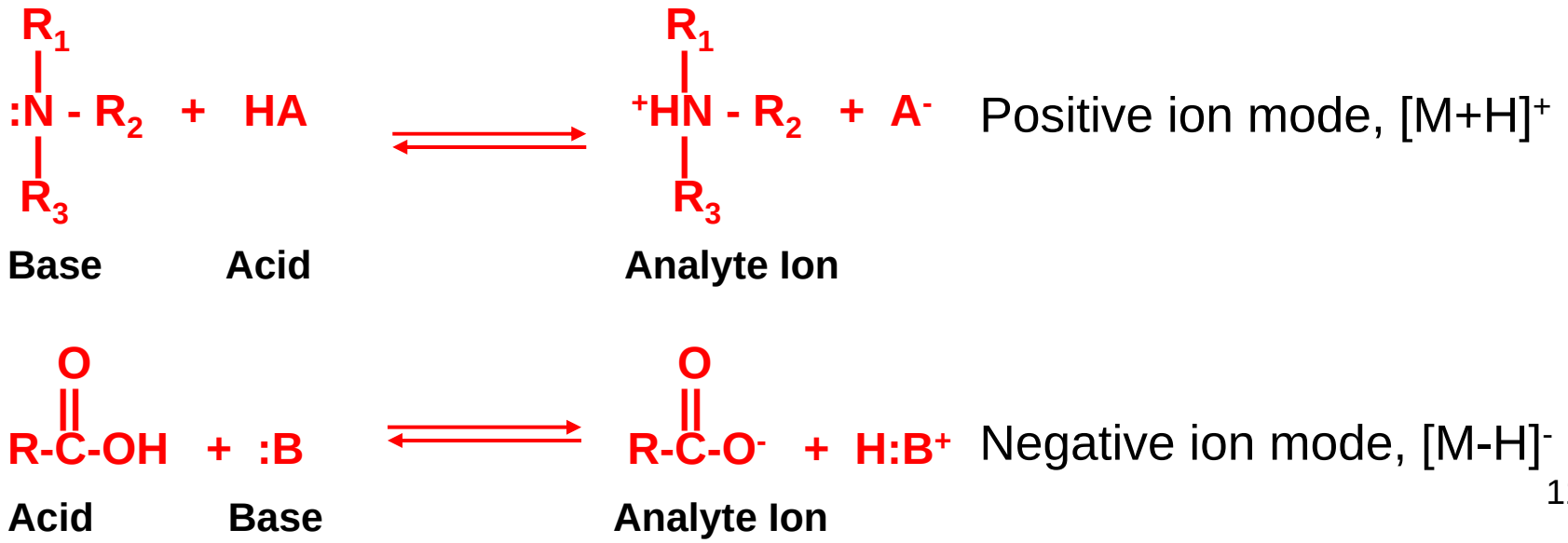
- In either case it is required that the analyte be an ion in solution (+ve or –ve) or made to be charged by modifying the solution to cause the analyte to be ionized

- This can be accomplished by changing pH, adding modifiers (Na^{+}, Li^{+})

Electrospray Solution Chemistry

- Mobile phase pH has a major effect for analytes that are ions in solution:
  - Basic pH for negative ions
  - Acidic pH for positive ions

- Changing pH can enhance performance for analytes that are not normally ionized in solution



Electrospray Solution Chemistry

- In the case of acid/base chemistry, ideally we want to be 2 pH units either side of pK in order to cause complete protonation (+ESI) or deprotonation (-ESI) to give maximum sensitivity

- In the case of batch introduction (infusion) of sample this is easily accomplished however in the case when LC is employed it is the nature of the mobile phase that determines the ions we will observe and the sensitivity

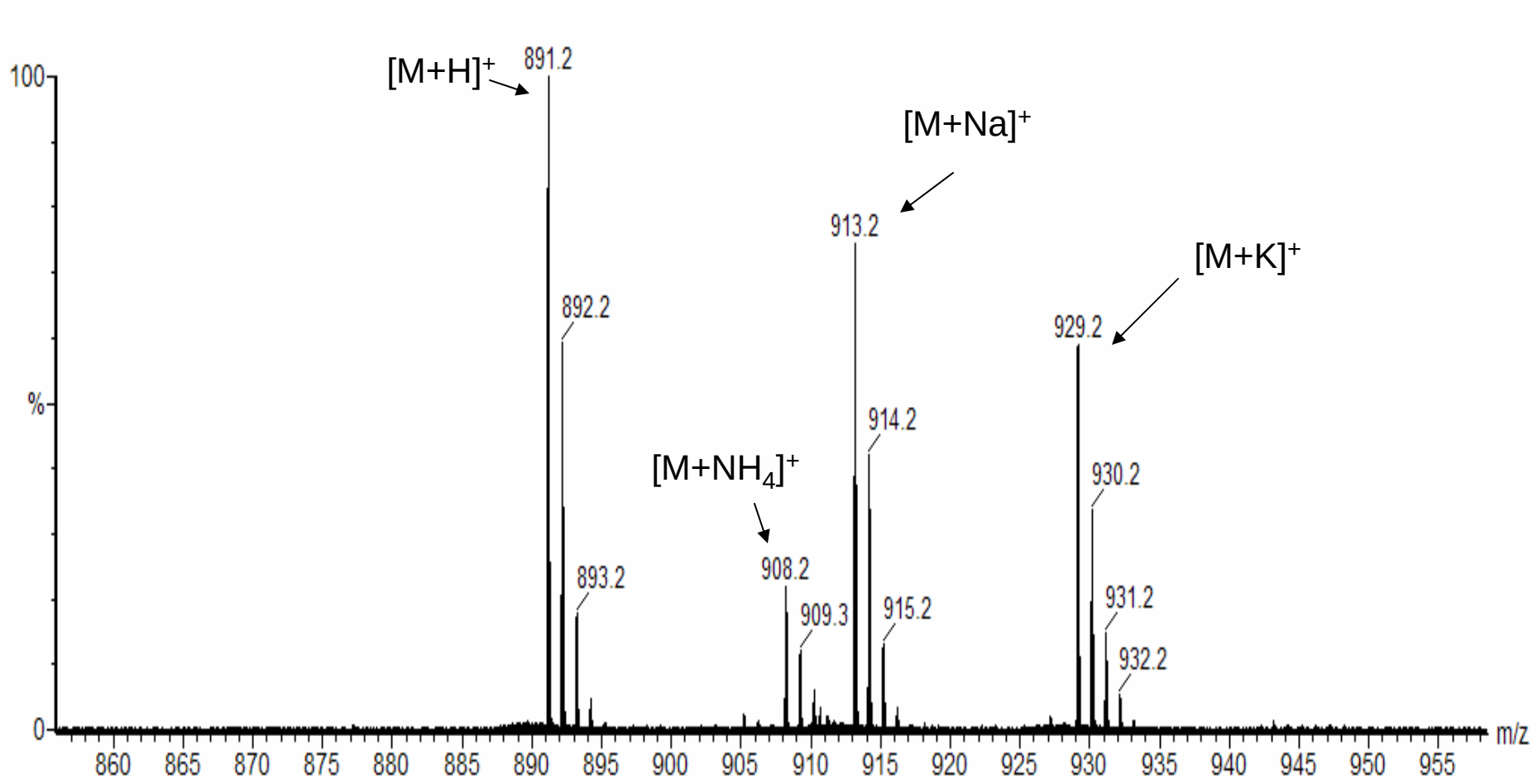
- For example, in a reversed phase (C18) separation of analytes, in order to achieve a good separation it is necessary for the analytes to be neutral in solution so that they may interact with the stationary phase and achieve a good separation. These neutral species will not yield the best sensitivity when ESI is used.

Electrospray Solution Chemistry

- Don’t forget, the ESI process is a competition for charge!
- A neutral in solution will pick up charge in a variety of ways and while we can influence which process is favoured we can not eliminate all competing ion formation mechanisms
- Not only do proton transfer reactions occur but adduct ion formation is commonly observed
- Species such as $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ in positive ion and $[M+OAc]^-$ and $[M+Cl]^-$ in negative ion are often observed even though these modifiers may not have been deliberately added to the solution containing the analyte

+ESI of Nucleotide Homologue (mw=890)

Sample in 1:1 CH_3CN/H_2O+0.2% formic acid



Electrospray Considerations

Samples:

- Ions in solution: catecholamines, sulfate conjugates, quaternary amines, carboxylates, phosphorylated compounds
- Compounds that can have a charge induced: carbohydrates
- Compounds containing heteroatoms: carbamates, benzodiazepines
- Multiply charged in solution: proteins, peptides, oligonucleotides
- A curious feature of ESI is the formation of multiply charged ions ie where z>>1 and sometimes as high as 100

Electrospray Considerations

Solution Chemistry Parameters:

- flow rate
- sample pK, solution pH
- solution conductivity

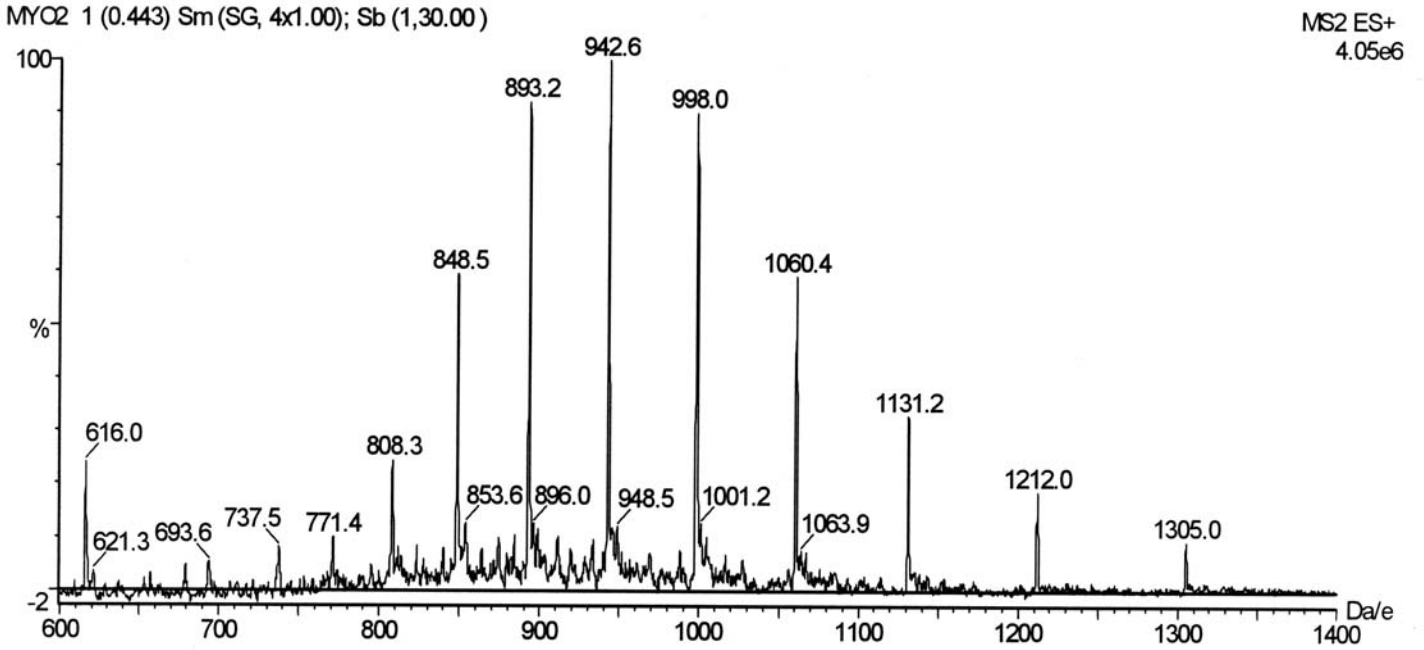
Samples to Avoid:

- extremely non-polar samples: PAHs, PCBs
- Samples containing high levels of buffers/electrolytes as this will cause ion suppression

Ion Suppression:

- Competition and interference with analyte ionization by other endogenous matrix species resulting in decreased number of ions characteristic of the analyte(s)

Protein ESI-MS



- In this mass spectrum, each peak represents the quasi molecular ion of the protein with one more charge attached, usually, but not always, a proton (H^+) eg m/z 942.6 is the $[M+18H]^{18+}$
- Consequently, each peak can be used to calculate the mwt of the protein and the resulting values averaged across all charge states.
- This results in mass accuracies for protein mwt determination of $\pm$ 0.01% or better depending on the type of mass spectrometer employed.

Protein ESI-MS

- Let the unknown mass of the protein be **M** and the # on charges be **n** corresponding to the addition of $(M+nH)^+$
- For 2 adjacent measured masses m_1 (high mass) and m_2 (low mass) we can write 2 equations:

$$m_1 = \frac{(M+n)}{n} \quad \text{(i)} \qquad \text{and} \qquad m_2 = \frac{(M+n+1)}{(n+1)} \quad \text{(ii)}$$

Solving for n:

for the ion at m/z 998.0 (m_1) $= \frac{(M+n)}{n}$ $998n = M+n$

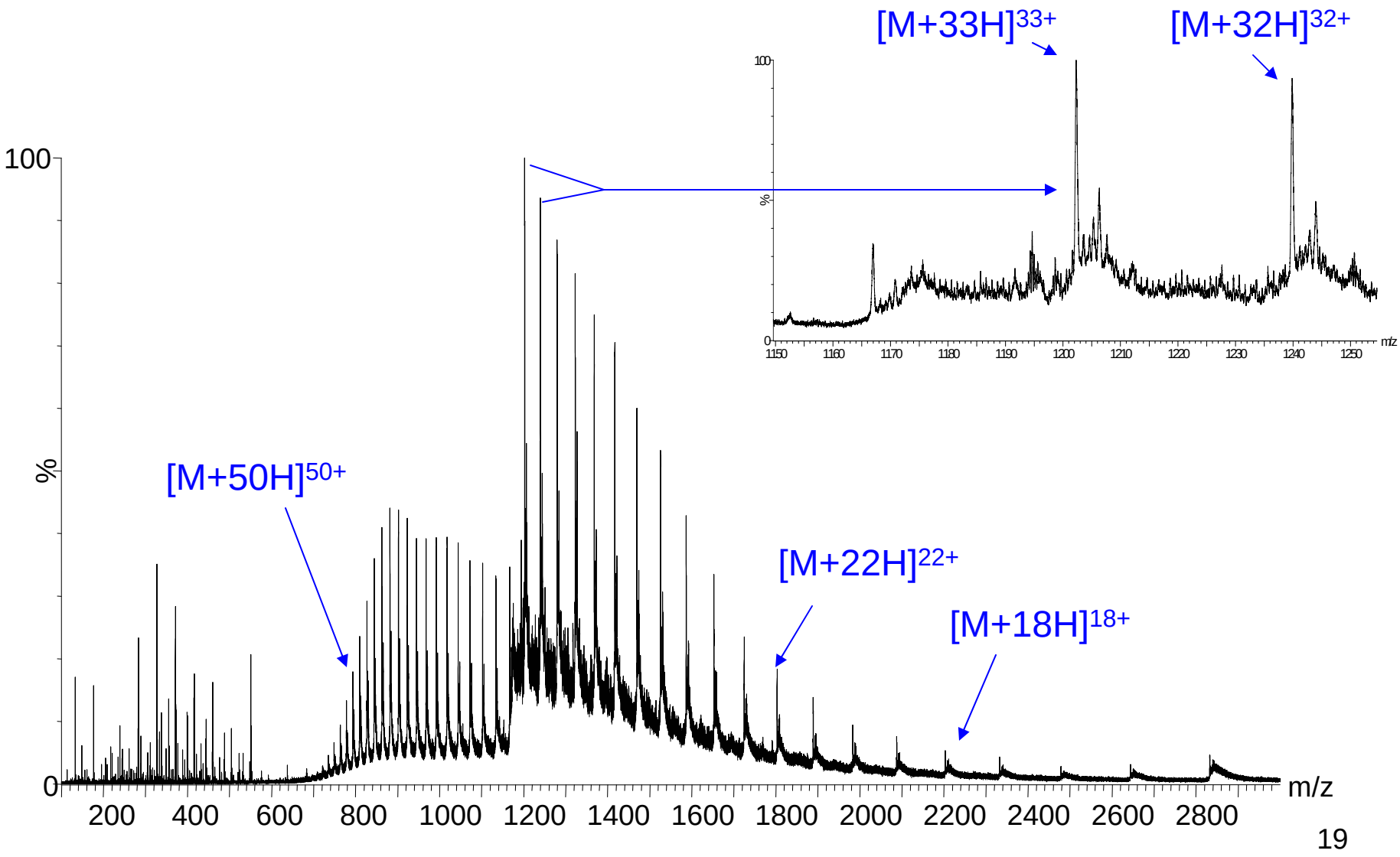
for the ion at m/z 942.6 (m_2) $= \frac{(M+n+1)}{(n+1)}$ $942.6n+941.6 = M+n$

Consequently: $998n = 942.6n+941.6$ **$n = 17$ for m_1 (m/z 998)**

Substituting n=17 in (i) gives $M = (m_1 n)-n = (998 \times 17)-17 =$ **16,949**

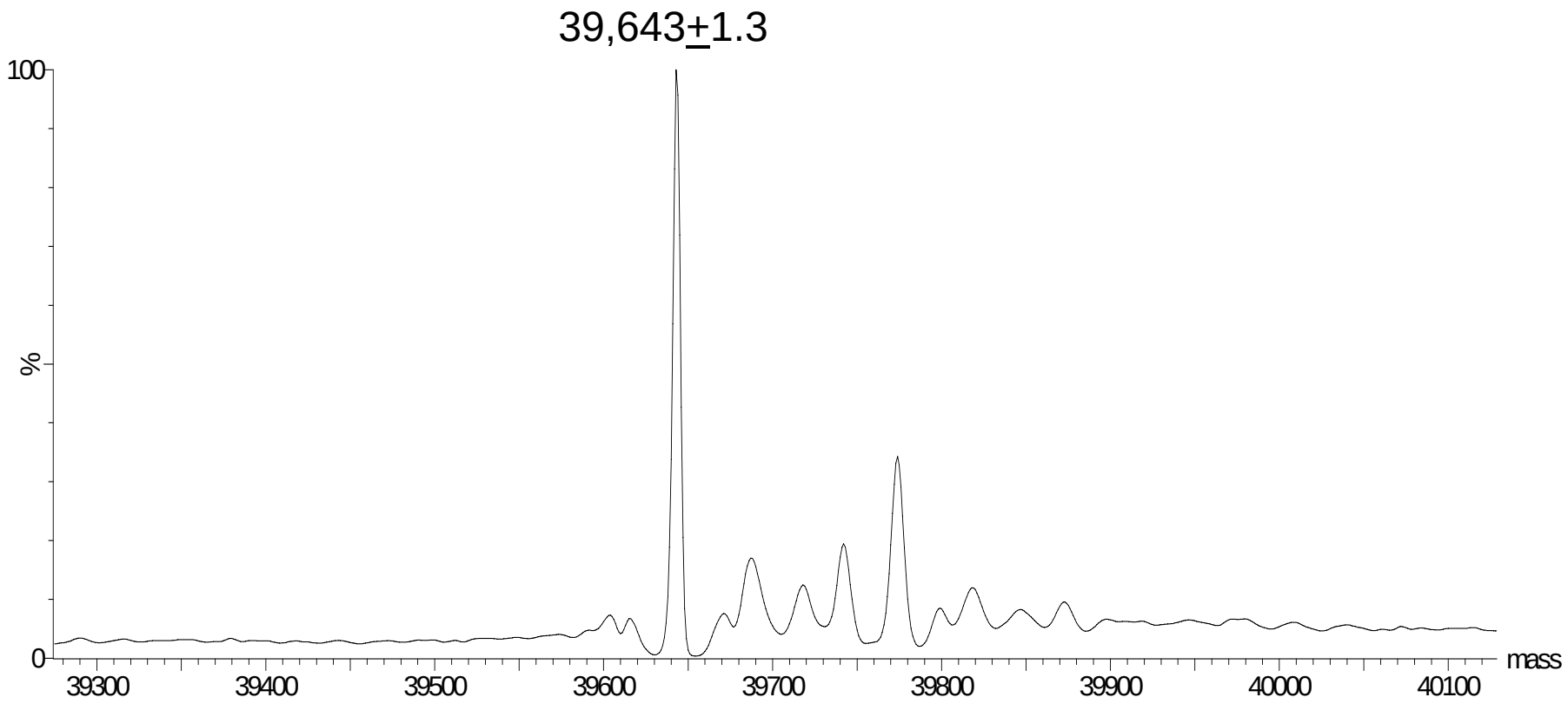
- These laborious calculations can be performed for all ion in the distribution or a software deconvolution can be performed

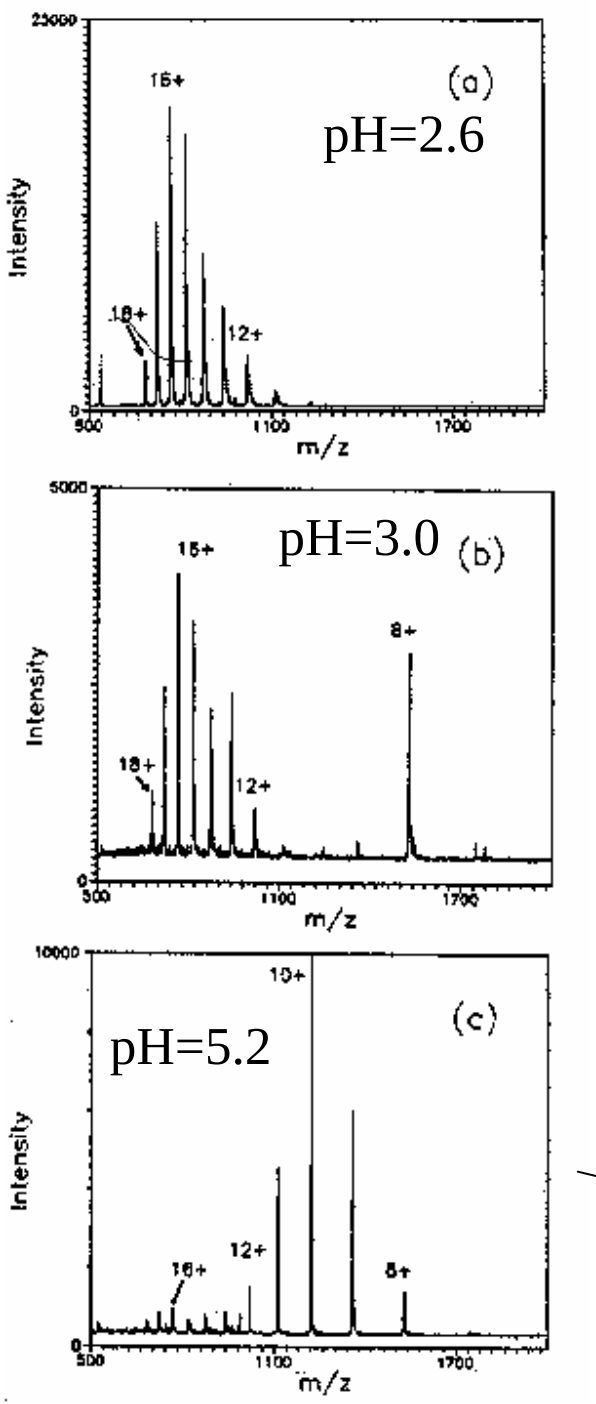
+ESI of a ~39kDa Protein - Infusion@1μL/min



Software Deconvolution

Software manipulation of the full scan +ESI data to show protein mwt





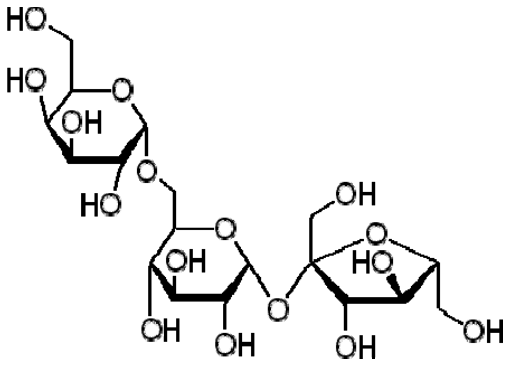
• the charge states of the gaseous ions generally represent the charge states in the condensed phase. These are sometimes modified by ion/molecule collisions. Ions such as large biomolecules are highly charged.

• the transfer of ions to the gas phase is not an energetic process. Ions are cold, in fact the desolvation process further cools ions.

• non-covalent interactions can be preserved when the species enters the gas phase. This is significant for the application of ESI to the study of biological molecules such as proteins.

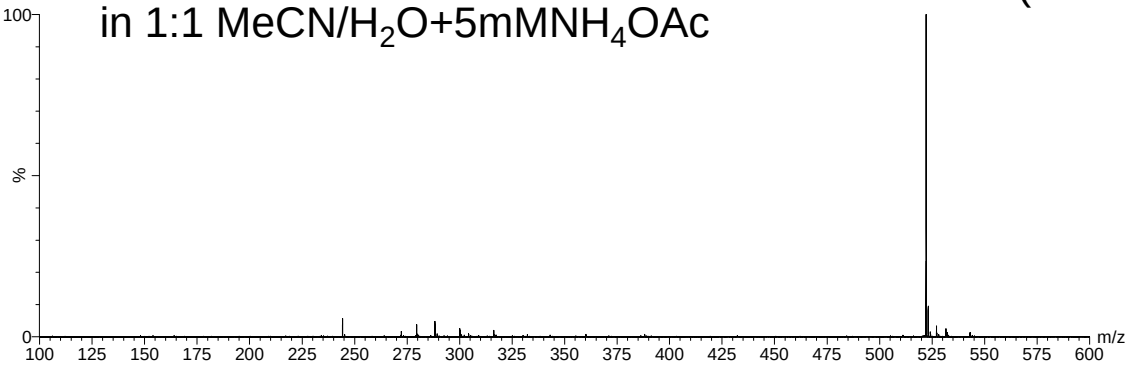
ESI mass spectra of bovine cytochrome c

Raffinose – trisaccharide, mwt=504 +ESI



in 1:1 MeCN/H_2O+5mMNH_4OAc

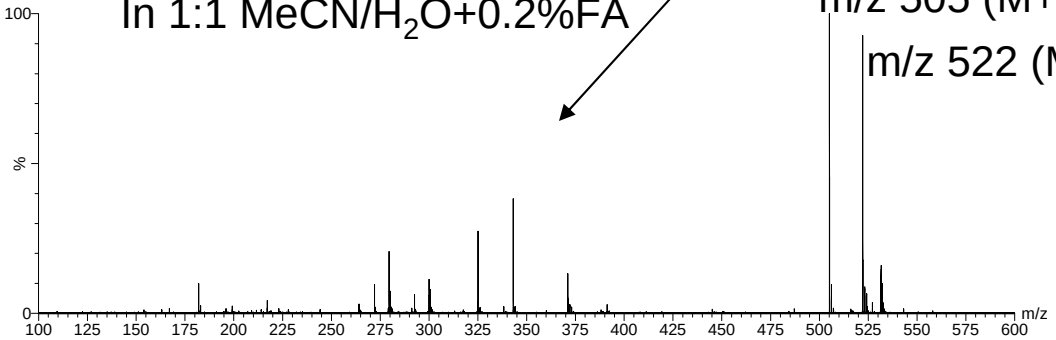
m/z 522 $(M+NH_4)^+$



In 1:1 MeCN/H_2O+0.2%FA

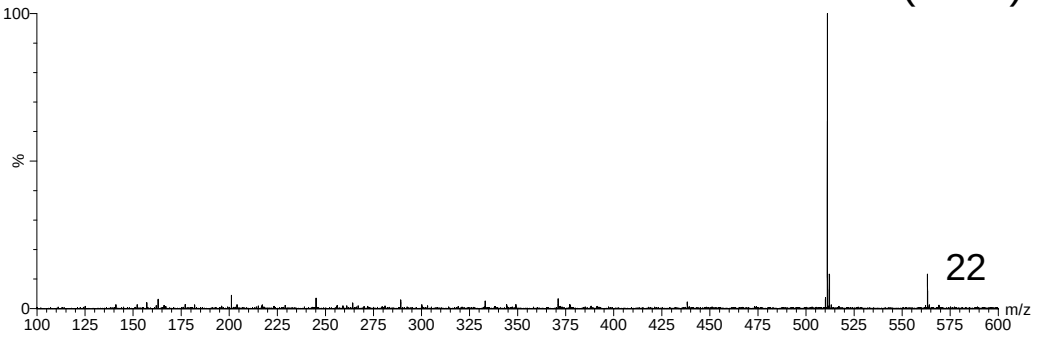
m/z 505 $(M+H)^+$

m/z 522 $(M+NH_4)^+$



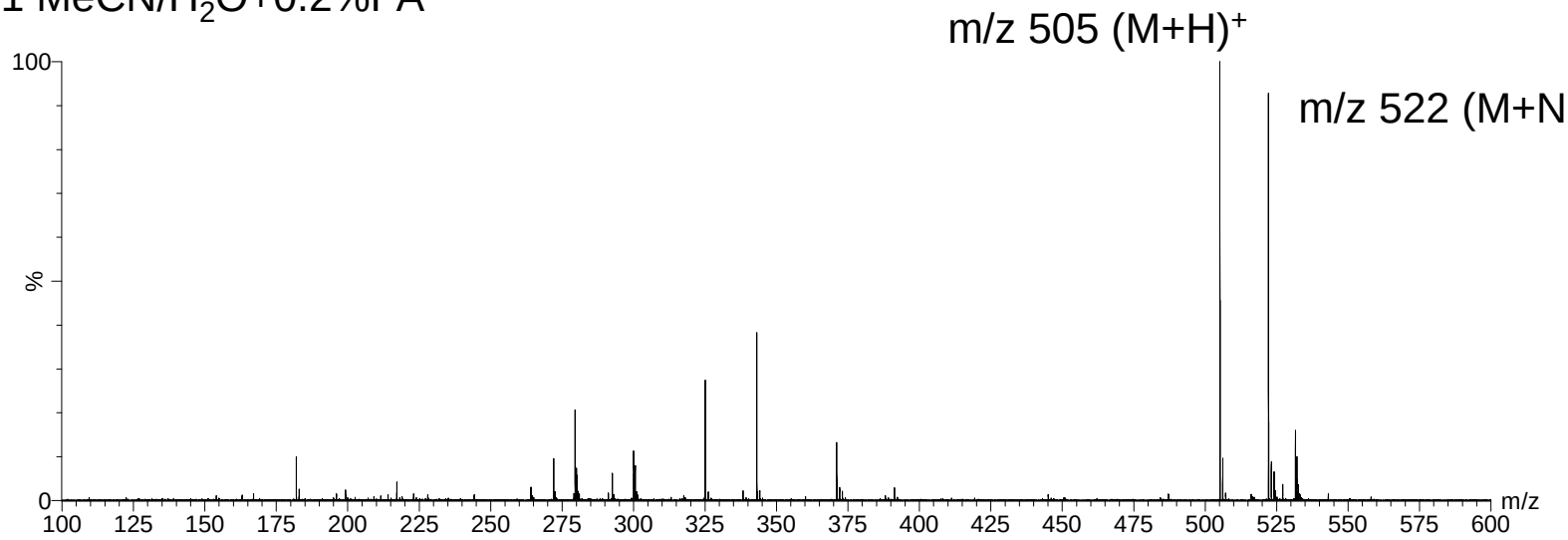
+LiOAc

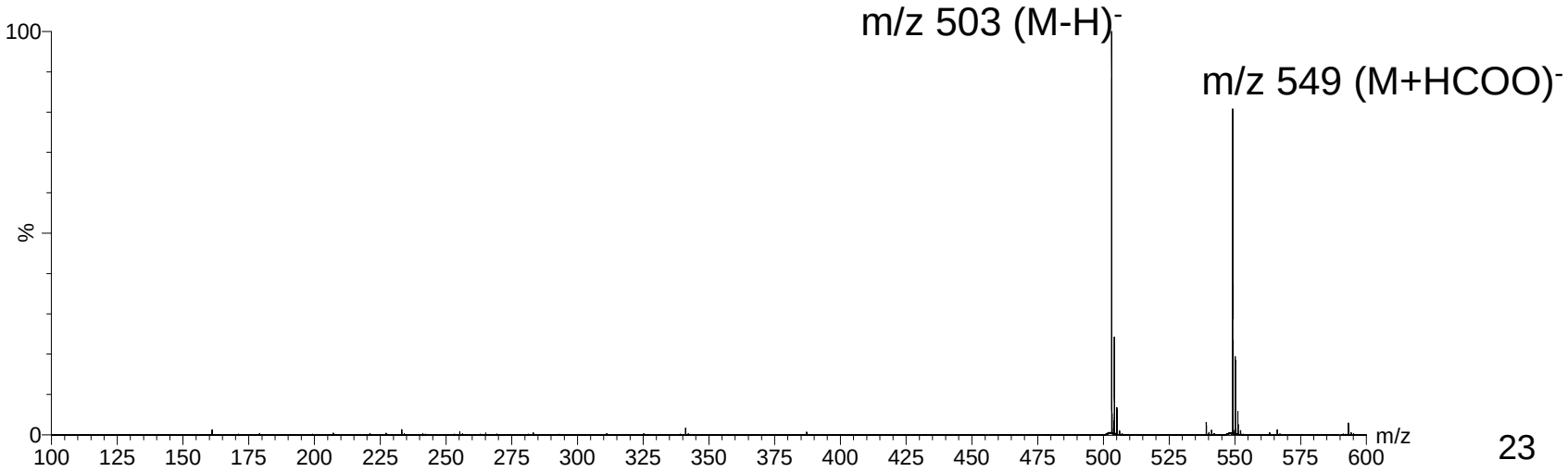
m/z 511 $(M+Li)^+$



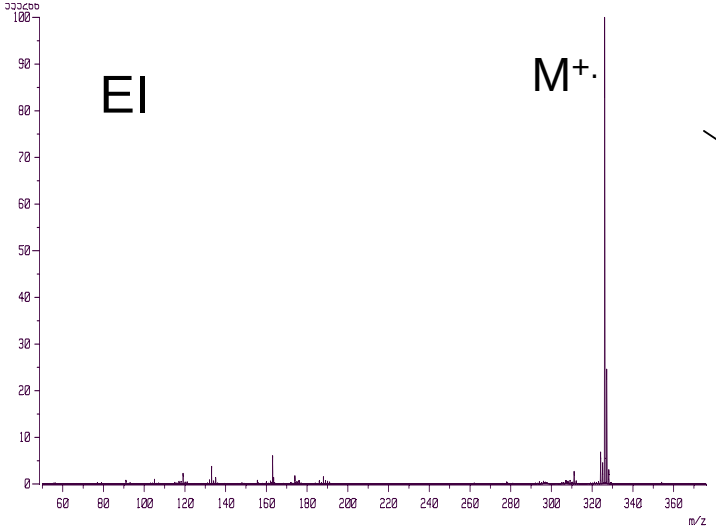
Raffinose – mwt=504 +ESI vs -ESI

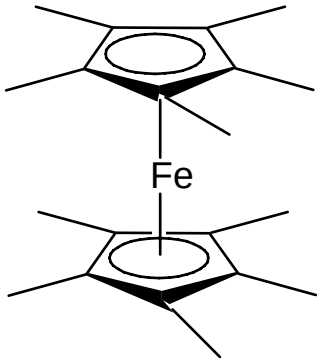
In 1:1 MeCN/H_2O+0.2%FA

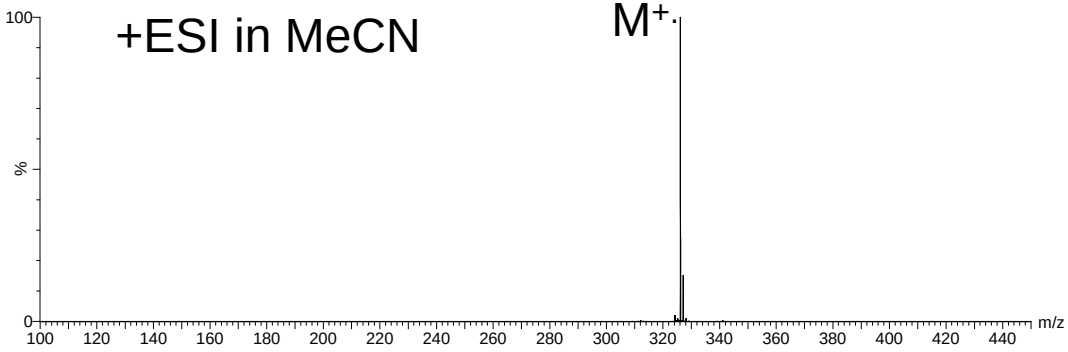




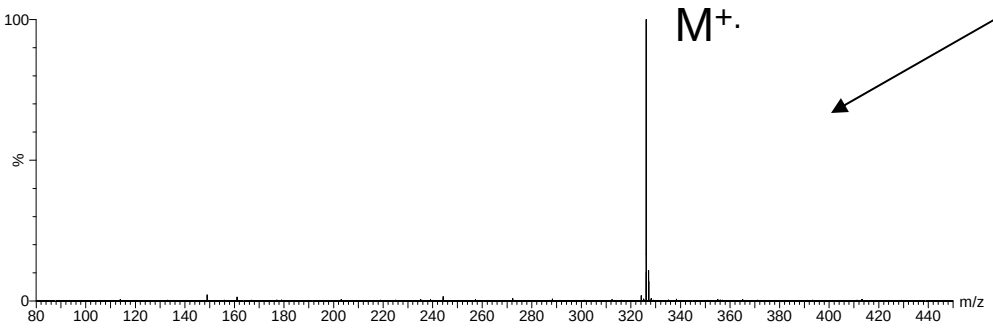
Not Always Protonated! decamethylferrocene







+ESI in 1:1 MeCN/H_2O+0.2%FA



ie no $(M+H)^+$ observed!
Electron transfer dominates
Oxidation

ESI – a MS Revolution

• Electrospray ionization (ESI) has allowed mass spectrometry to investigate a huge diversity of molecules that were very difficult or impossible to study by MS previously

- proteins, DNA, RNA, oligonucleotides
- polymers, non-volatile inorganic and organometallic molecules and salts

• As a result it has completely revolutionized mass spectrometry.

• It has also revolutionized the sales of mass spectrometers as the can be considered to be an analytical technique for biochemistry (big $$).

• Also, it has spurred the growth of more sensitive and exotic types of MS and combinations of MS analyzers.

Atmospheric Pressure Chemical Ionization (APCI)

- gas phase chemical ionization (CI) process where the vapourized LC mobile phase acts as the CI reagent gas to ionize the sample
- Mobile phase and analyte are first nebulized (N_2) and vapourised by heating to 350-550^{o}C
- The resulting vapour is ionized using a corona discharge (source of electrons)
- Subsequent ion/molecule reactions (CI) then cause ionization of the analyte
- Unlike ESI, analyte ions do not need to exist in solution
- Unlike ESI, best sensitivity is achieved at high liquid flow rates ie 200μL – 1mL/min therefore easily interfaced to conventional HPLC

- Analytes must be thermally stable and “volatile”

APCI

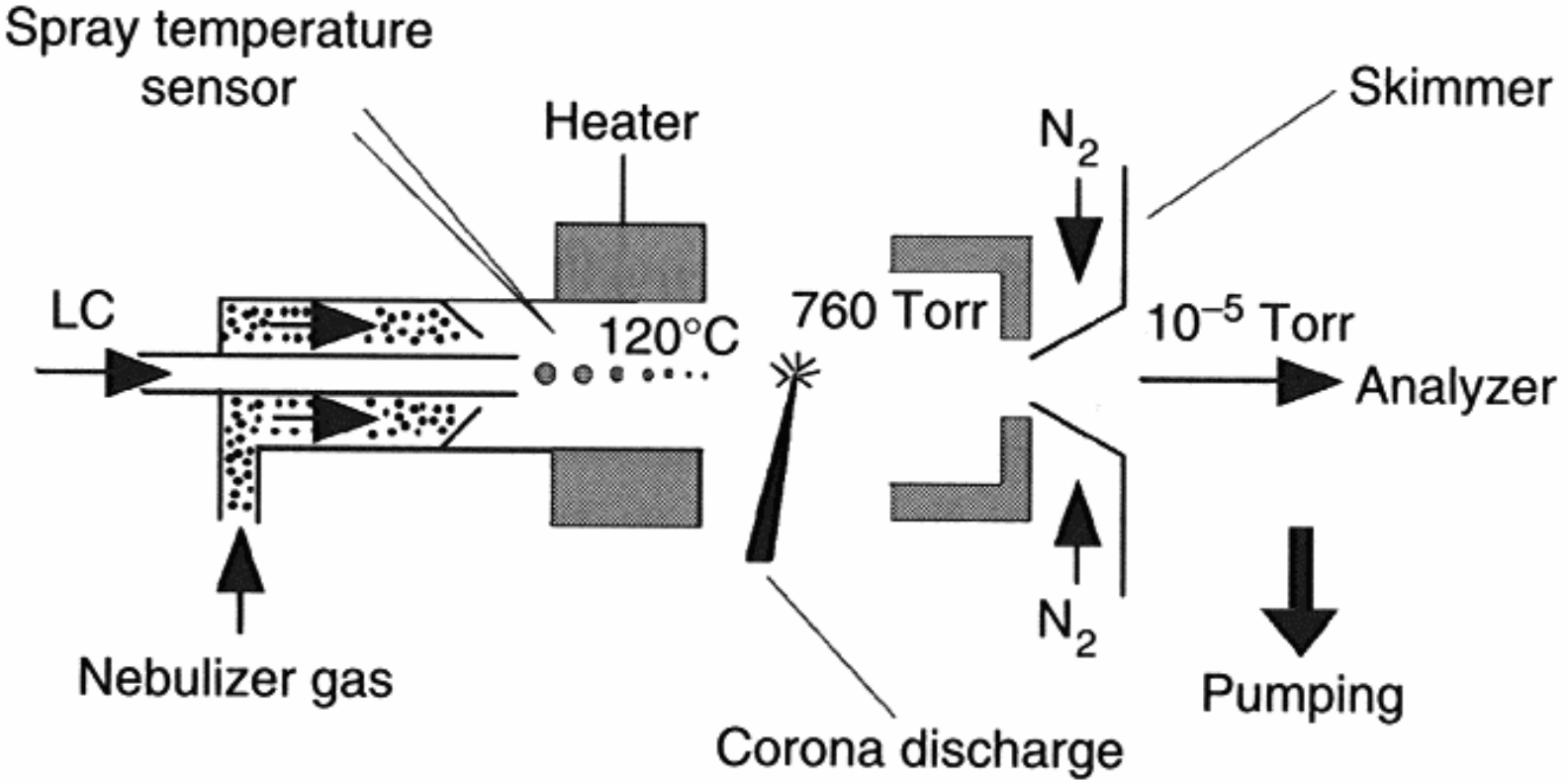
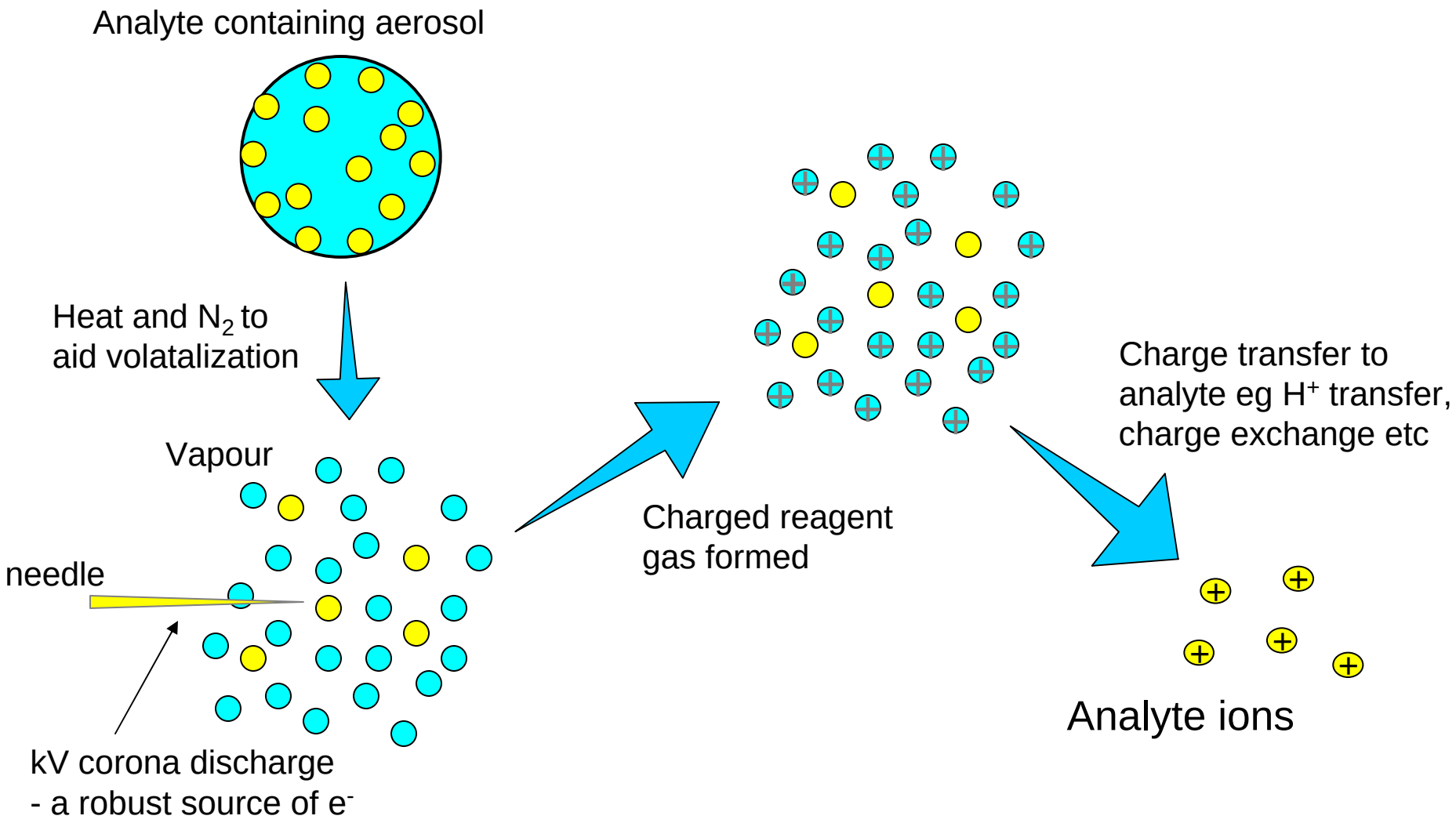


Diagram of an APCI source

APCI Process



APCI Considerations

Samples:

- Compounds of intermediate mwt and polarity: PAHs, PCBs, fatty acids, steroids, phthalates.
- Compounds that don’t contain acidic or basic sites (e.g. hydrocarbons, steroids, alcohols, aldehydes, ketones, and esters)
- samples containing heteroatoms: ureas, benzodiazepines, carbamates
- samples that exhibit a poor electrospray response, that is, APCI can be considered to be complimentary to ESI

APCI Considerations

Solution Chemistry Parameters:

- less sensitive to solution chemistry effects than ESI – ion suppression not so important
- Best sensitivity at higher flow rates than ESI
- accommodates some non-polar solvents not compatible with ESI (hexane, CH_2Cl_2 etc)

Samples to Avoid:

- thermally labile, polar and high mwt compounds due to the vaporization process

APCI Mechanism

$S + e^- \rightarrow S^{+\cdot} + 2e^-$

- Solvent molecules are ionized ($S^{+\cdot}$)
- the solvent is usually a complex mixture of H_2O, CH_3CN/CH_3OH and mobile phase modifiers

$S^{+\cdot} + S \rightarrow [S+H]^+ + S[-H]$

- $S^{+\cdot}$ abstracts a hydrogen atom ie a CI process

$[S+H]^+ + M \rightarrow [M+H]^+ + S$

- $[S+H]^+$ ionizes analyte M by proton transfer or proton abstraction

$S^{+\cdot} + M \rightarrow M^{+\cdot} + S$

- charge transfer can also occur with solvents like CH_2Cl_2

Atmospheric Pressure Photo-Ionization (APPI)

- Experimentally, you can view APPI as an APCI source where the corona discharge has been replaced with a Kr lamp

- The 1st step is complete vapourization of the mobile phase used in the LC separation employing nebulization (N_2) and heating to 350-550^{o}C

- gas phase photoionization process
  - where the vapourized mobile phase may be photoionized to form a CI plasma
  - or a modifier (dopant) is added to aid the photoionization process and formation of the CI plasma
  - or the analyte can be directly photoionized by photons from the Kr lamp

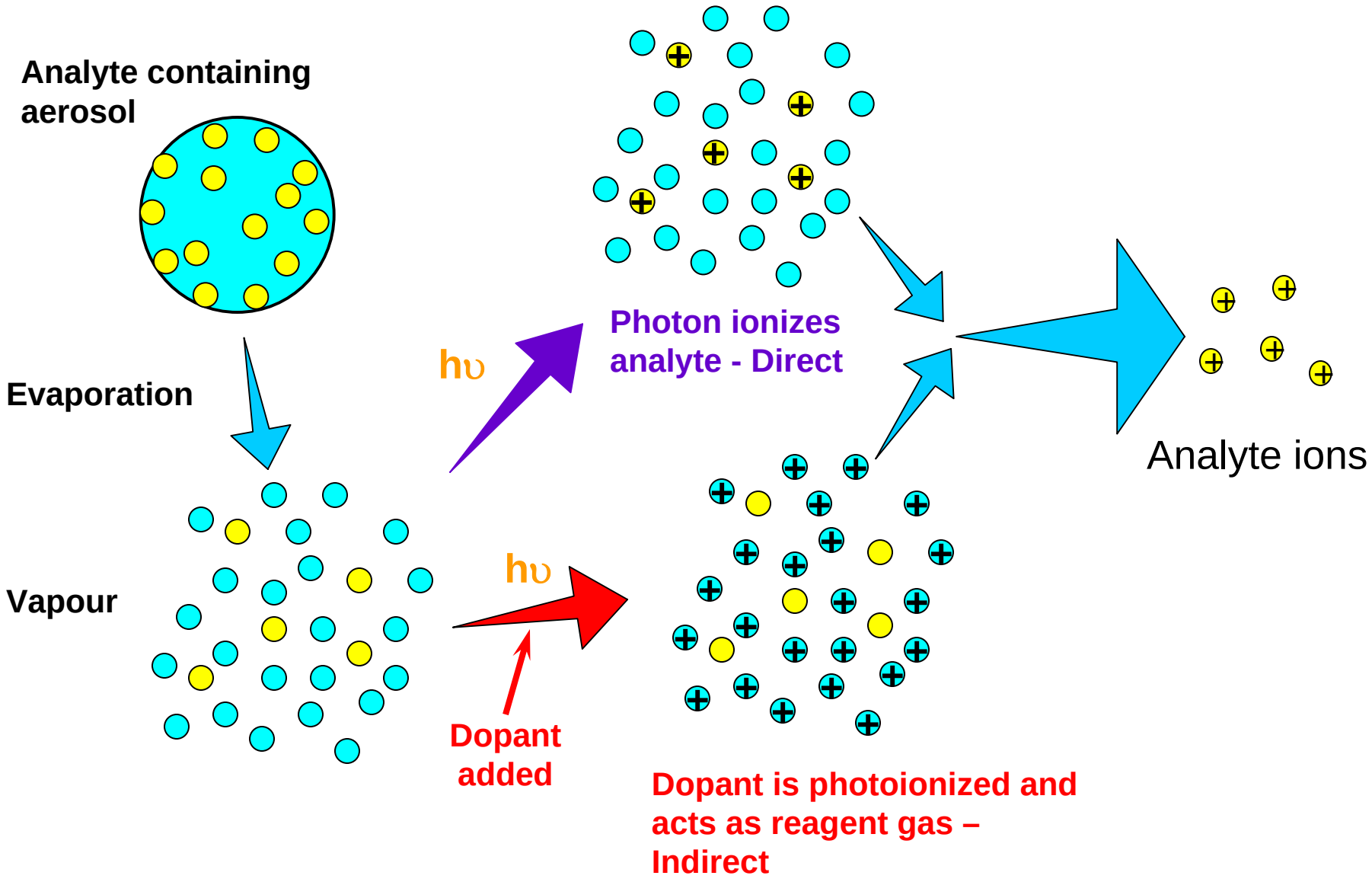
Atmospheric Pressure Photo-Ionization (APPI)

- It is ionized by high energy photons from a Kr lamp (usually) causing either direct or indirect (dopant) photoionization

- Very useful for non-polar analytes that are difficult to ionize with ESI or APCI such as PAH’s

- Unlike ESI, best sensitivity is achieved at liquid flow rates around 200mL/min therefore easily interfaced to conventional HPLC

APPI Process



APPI Mechanisms

Direct APPI:

$M + h\nu \rightarrow M^{+\cdot} + e^-$

Analyte molecule M is ionized to molecular ion $M^{+\cdot}$

- If analyte ionization potential is below Kr lamp photon energy

Subsequently:

$M^{+\cdot} + SH \rightarrow [M+H]^+ + S^\bullet$

Molecular ion $M^{+\cdot}$ may abstract a hydrogen to form $[M+H]^+$

ie a CI process

APPI Mechanisms

Dopant APPI:

$D + h\nu \rightarrow D^{+\cdot} + e^-$

- Photoionizable dopant D is in excess & yields many $D^{+\cdot}$ ions

$D^{+\cdot} + M \rightarrow \rightarrow [M+H]^+ + D$

- Analyte M ionized by proton transfer from dopant or solvent

$D^{+\cdot} + M \rightarrow M^{+\cdot} + D$

- $D^{+\cdot}$ ionizes analyte M by electron transfer ie charge transfer

Energetics for Photoionization

PhotoMate™ lamp

Krypton 10.0 eV, 10.6 eV

Ionization Potentials (IP)

Anthracene	7.4 eV
Fluoranthene	7.8 eV
Caffeine	8.0 eV
4-Nitrotoluene	9.5 eV
2,4,6-Trinitrotoluene	10.59 eV

Dopant Ionization Potentials

Toluene	8.82 eV
Acetone	9.70 eV

Solvent Ionization Potentials

Methanol	10.85 eV
Acetonitrile	12.19 eV
Water	12.61 eV

- The photons from the Kr lamp can only photoionize compounds of lower IP
- Common HPLC solvents like H_2O, CH_3OH and CH_3CN are NOT ionized and therefore cannot aid ion formation
- In this circumstance, only direct photoionization of the analyte can yield characteristic ions such as $M^{+\cdot}$ (not very efficient)
  - Subsequent ion/molecule reactions can form $[M+H]^+$
- Dopants are used that will be ionized by the Kr lamp

Atmospheric Pressure Ionization Techniques

Electrospray (ESI)

- Volatility not required
- Preferred technique for polar, high mwt, thermally labile analytes
- Ions formed in solution
- Can form multiply charged ions

APCI/APPI

- Some volatility required
- Analyte must be thermally stable
- Ions formed in gas phase
- Forms singly charged ions only

Ionization of Analytes

How do we choose which technique to use?

- is the analyte volatile?
- is the analyte thermally labile?
- Does the analyte have heteroatoms that can accept (N > O) or lose (O >> N) a proton?
- accepts a proton - use positive ion mode
- loses a proton - use negative ion mode

Ion Suppression?

- Dirty matrix would favour the use of APCI/APPI rather than ESI because they are more tolerant to matrix effects than ESI

Chromatographic Considerations

ESI:

- Concentration dependant
  - smaller i.d. column gives better sensitivity - nanospray at 200-500nL/min
- However also works well from 1µl/min to 1 ml/min
- Post-column addition can be used to adjust ionization chemistry

APCI/APPI:

- Mass flow dependant
  - column i.d. has little effect on sensitivity
- Works well from 100 µl/min to 1.5 ml/min
- Can be used with normal phase chromatography

General Mobile Phase Considerations

- Metal ion buffers interfere with ionization
- Surfactants/detergents interfere with evaporation
- Ion pairing reagents can ionize and create a high background
- Strong ion pairing with an analyte can prevent the analyte from ionizing
- Some mobile phase additives will cause persistent background problems
  - TEA interferes in positive ion mode (*m/z* 102)
  - TFA interferes in negative ion mode (*m/z* 113)

Mobile Phase Considerations

ESI:

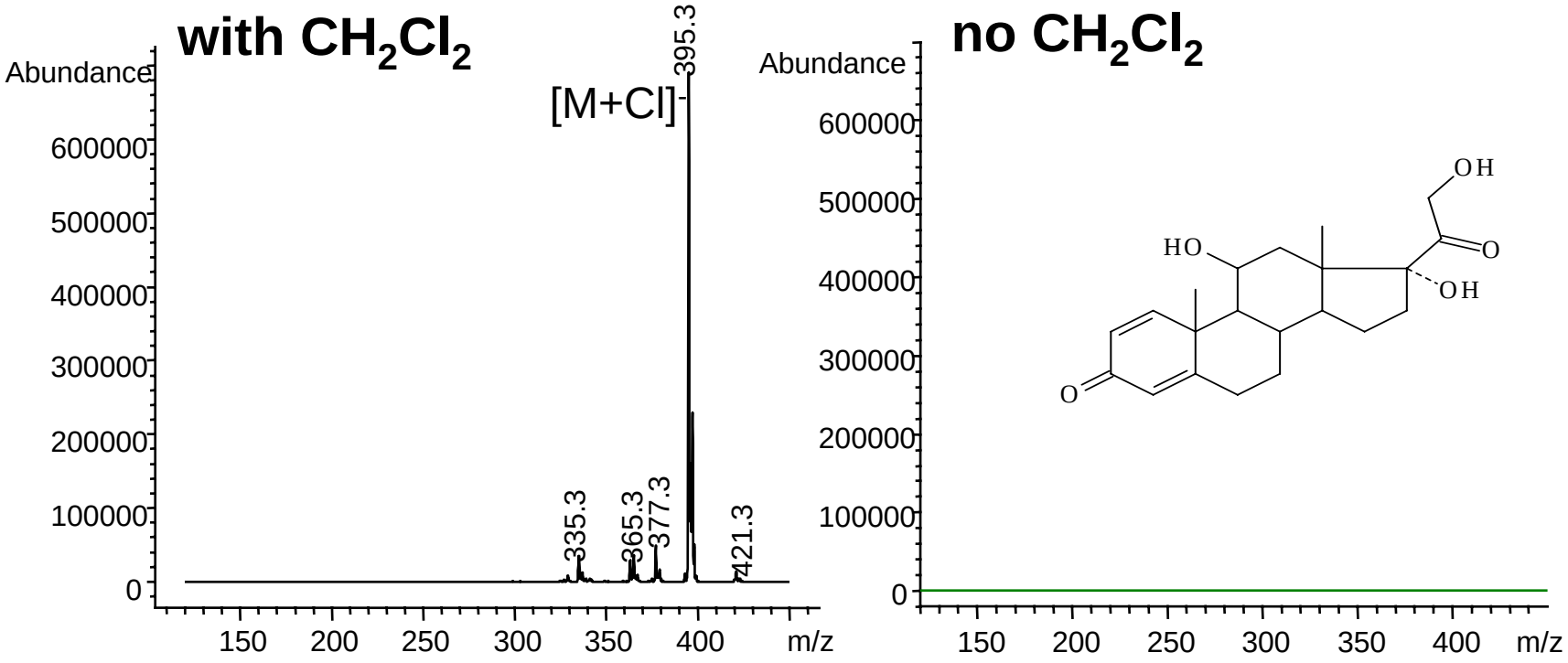
- Solution pH must be adjusted to create analyte ions
  - pH 2 units away from pK of analyte
- Organic modifier (CH_3OH/CH_3CN) has little effect on ionization
- Volatile buffer concentration should be <25mM
- Non-volatile buffers should be avoided or their concentration should be very low <<5mM
- Na^+ and K^+ adducts commonly occur

Mobile Phase Considerations

APCI/APPI:

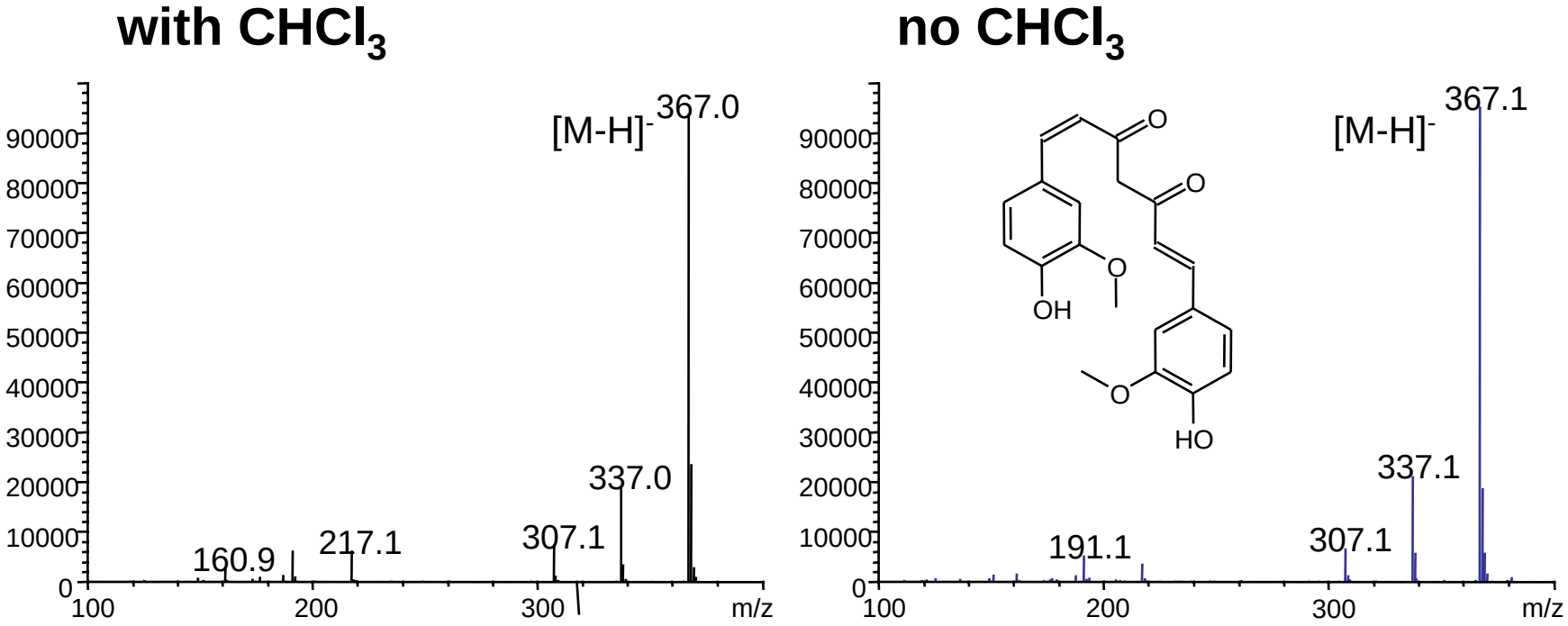
- Organic solvent should be a good charge transfer reagent
  - use methanol instead of acetonitrile
  - proton affinity of CH_3OH (182kcal/mol) vs CH_3CN (187kcal/mol)
- Chlorinated solvents can aid ionization in negative mode
- Volatile buffer concentration should be <100 mM
- Non-volatile buffer concentration should avoided or be very low <<5mM
- Ammonium adducts may occur with ammonium salt buffers
- APPI may require a dopant (eg acetone)

Mass Spectra of Prednisolone in Negative Mode APCI



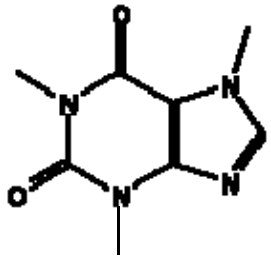
Prednisolone does not normally ionize in negative mode APCI. In the presence of CH_2Cl_2, a very intense $[M+Cl]^-$ ion is formed.

Mass Spectra of Curcumin in Negative Mode APCI

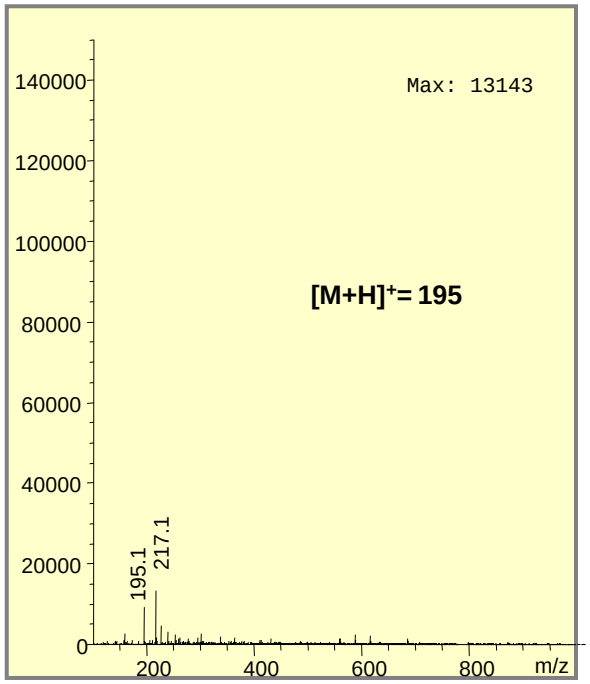


Curcumin is an example of a phenolic compound that ionizes equally well in the presence of oxygen or $CHCl_3$.

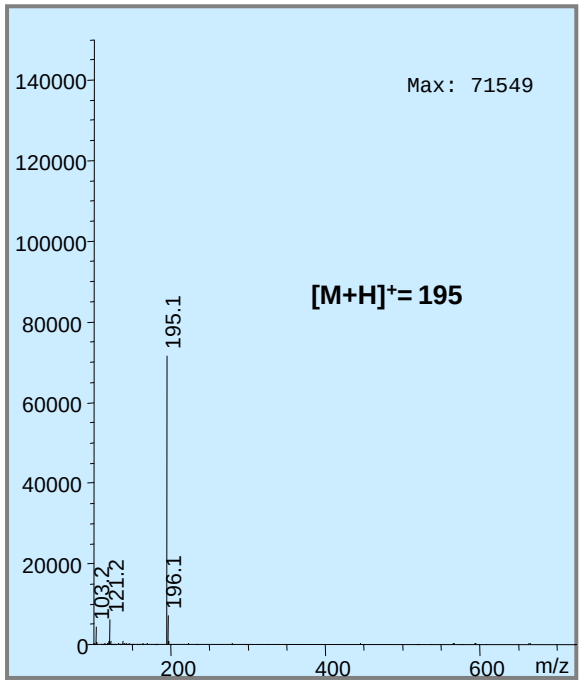
Caffeine



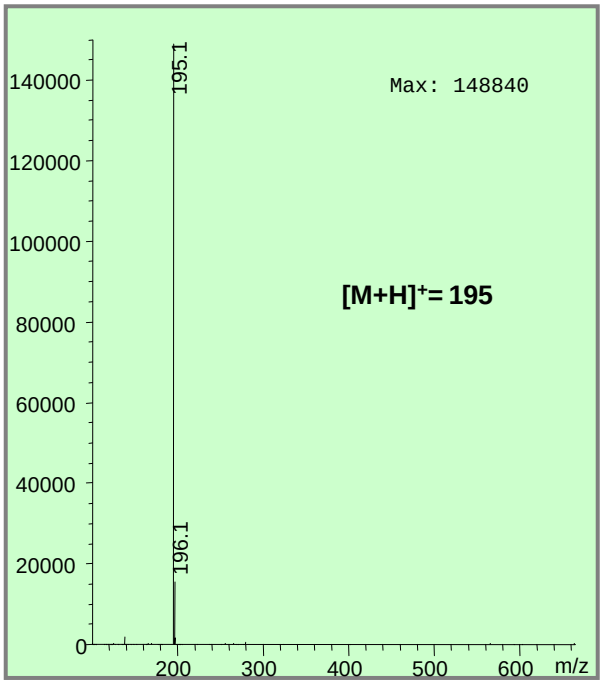
ESI



APCI

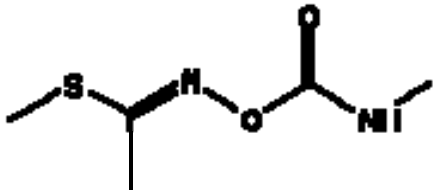


APPI

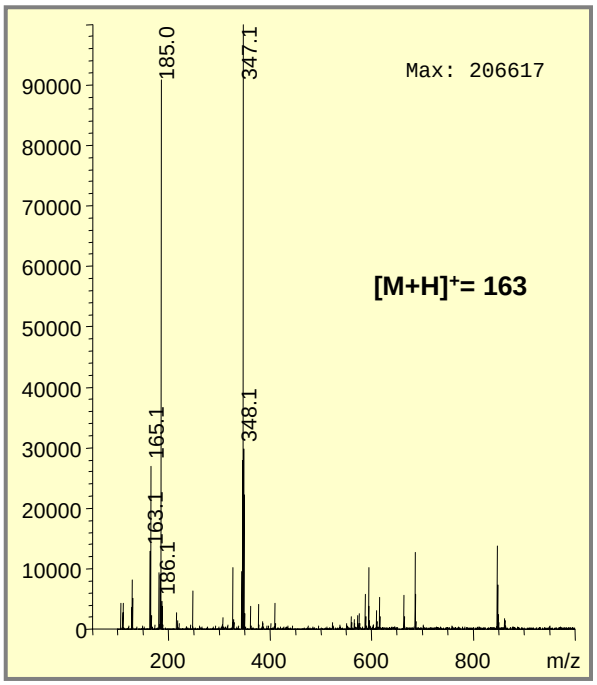


courtesy of Agilent

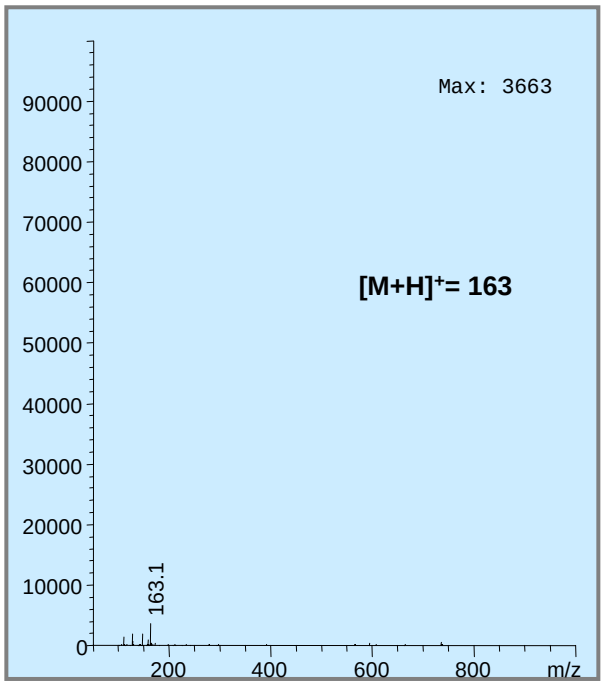
Methomyl



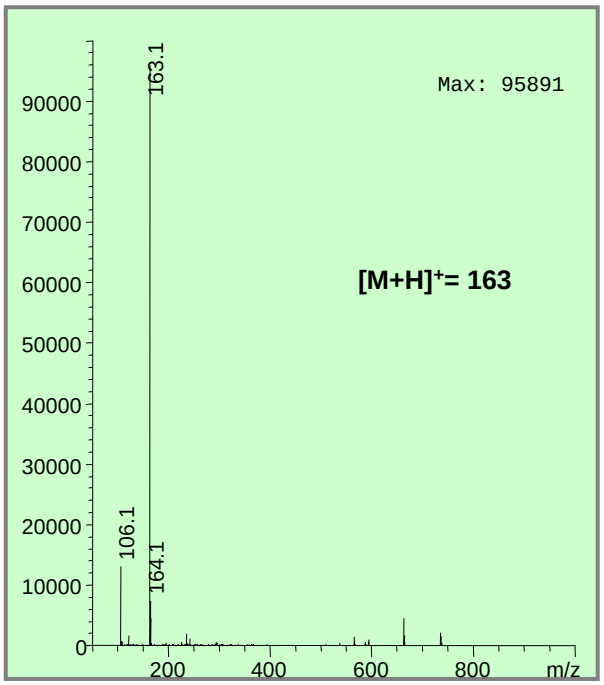
ESI



APCI

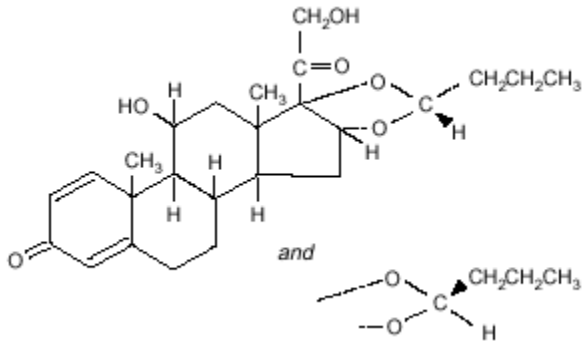


APPI

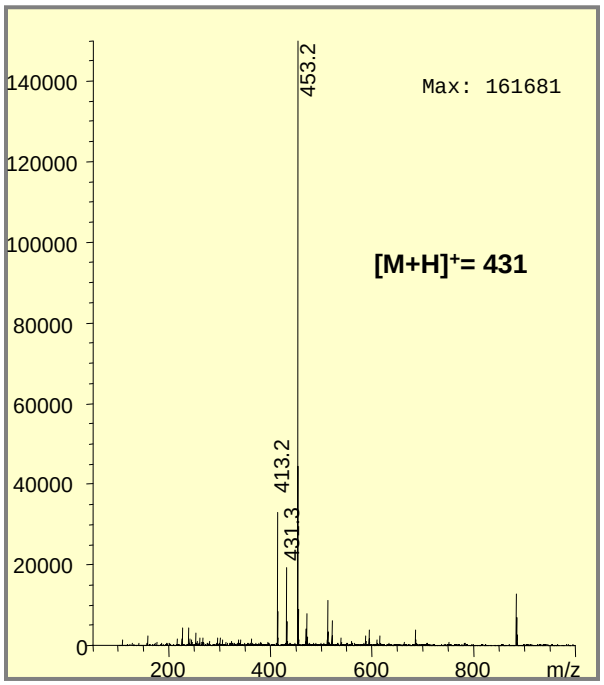


courtesy of Agilent

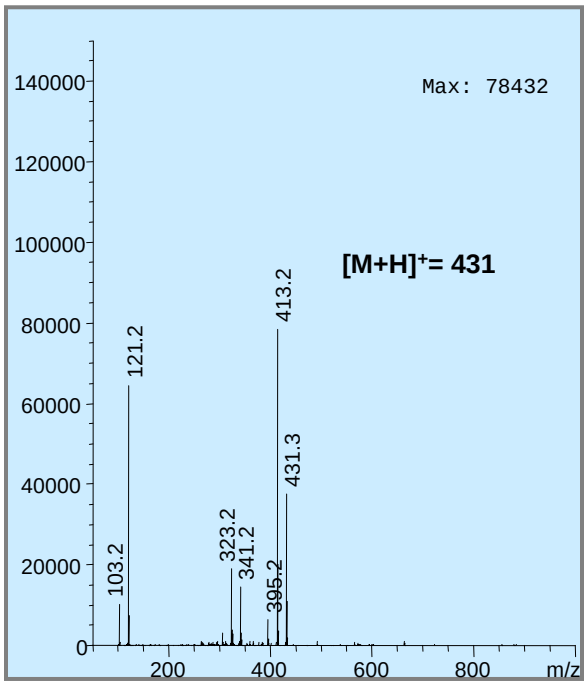
Budesonide



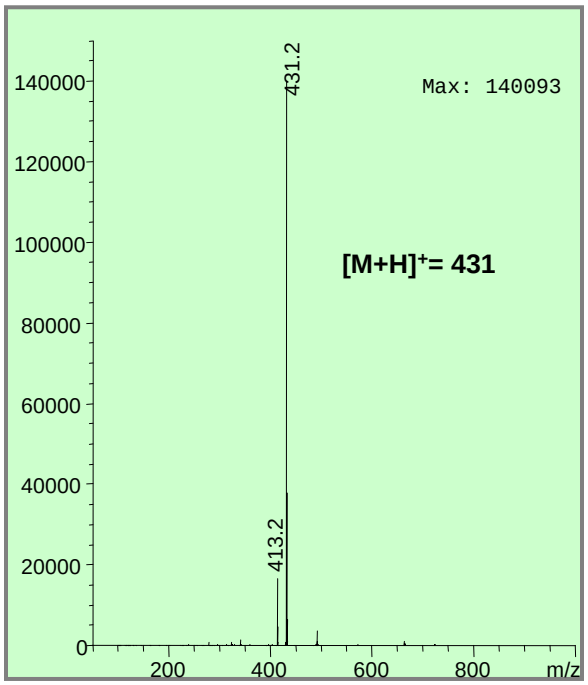
ESI



APCI



APPI



courtesy of Agilent

Sample Matrix Effects

The MS hardware is robust and tolerates non-volatile components

however…

The ionization process is effected by the concentration and type of salt/buffer and results in “Ion Suppression” and is much more prevalent in ESI

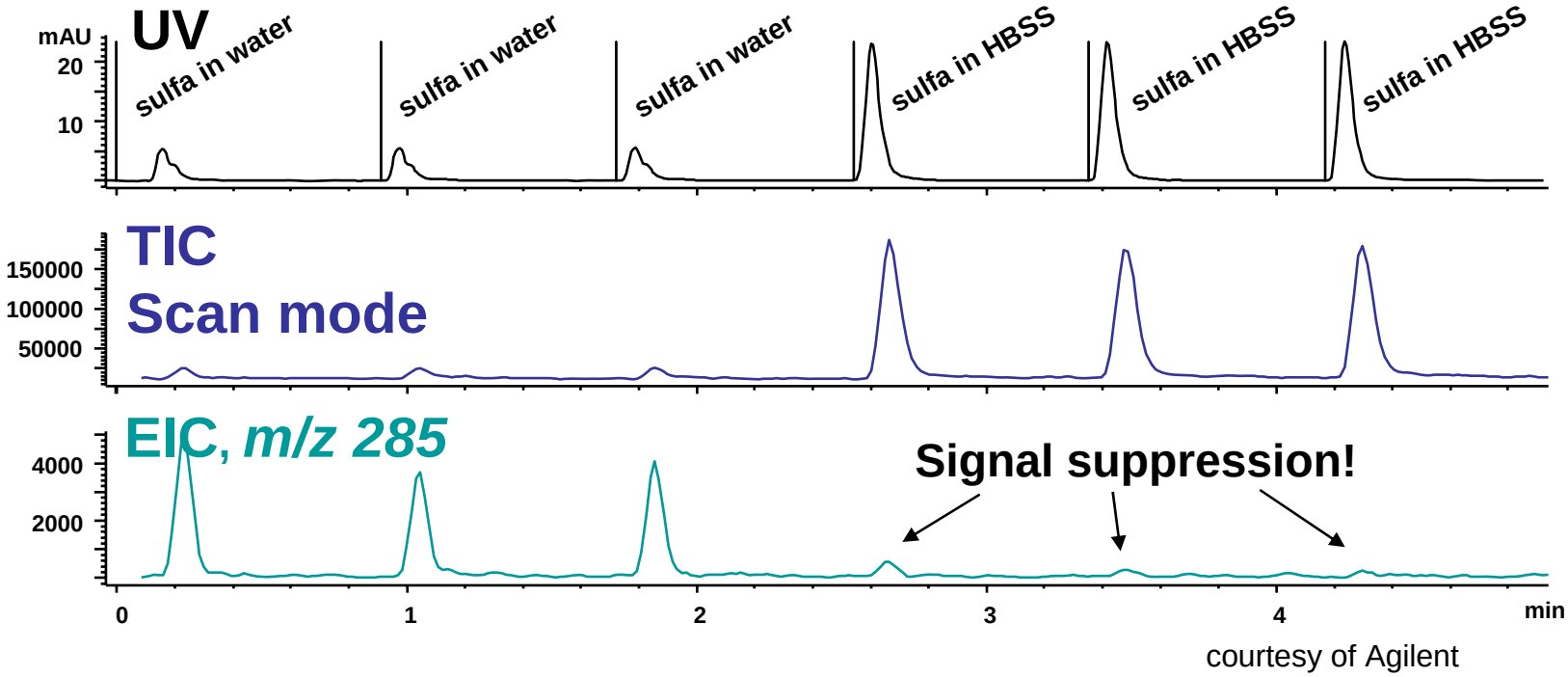
“Competition and interference with analyte ionization by other endogenous matrix species resulting in decreased number of ions characteristic of the analyte(s)”

Sample Matrix Effect in ESI

Sulfachloropyridazine (mwt=284) dissolved in water vs. Hanks Balanced Salt Solution (HBSS)

Composition of HBSS:

Component	g/L
Sodium chloride	8
Calcium chloride	0.1
Potassium chloride	0.4
Potassium phosphate monobasic	0.06
Magnesium sulfate	0.1
Sodium bicarbonate	0.35
Sodium phosphate dibasic	0.048
Glucose	1
Phenol red	0.011



courtesy of Agilent

Adapting Existing LC Methods to LC/API-MS

- Replace non-volatile buffers with volatile buffers at a concentration of <10 mM for ES or <100 mM for APCI.

- Substitute phosphates, sulfates, and borates with ammonium acetate or formate, trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), tetrabutylammonium hydroxide (TBAH)
  - If a non-volatile buffer must be used, use a buffer where only the anionic or cationic part is non-volatile, i.e. ammonium phosphate, not sodium phosphate.
  - Keep the pH the same using volatile additives:
    Formic acid, acetic acid, TFA, ammonium hydroxide

- Volatile ion pair reagents should be employed such as HFBA

Summary of Ionization Methods

Compound volatile or semivolatile:

- Electron impact (EI):
  - $M^{+\bullet}$ and perhaps substantial fragmentation
- Chemical ionization (CI):
  - Positive chemical ionization, $[M+H]^+$ (soft ionization - little fragmentation)
  - Negative chemical ionization (electron capture), $[M]^{-\cdot}$ (soft ionization - little fragmentation, can be very sensitive)
- Field Ionization (FI):
  - $M^{+\bullet}$, (soft ionization - little fragmentation)

Compounds non-volatile, methods difficult to couple to HPLC:

- Field Desorption (FD):
  - $[M+H]^+$, $[M+Na]^+$ (soft ionization - little fragmentation)
- Fast Atom Bombardment (FAB) and Liquid Secondary Ion Mass Spectrometry (LSIMS):
  - $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$ (soft ionization - quasimolecular ion and fragment ions)

Summary of Ionization Methods

Compounds non-volatile, methods difficult to couple to HPLC:

- MALDI: $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$ some multiple charging observed (both soft and hard ionization, quasi molecular ion and fragment ions, biopolymer analysis)

Compounds non-volatile, methods can readily be coupled to HPLC

- APCI: $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M-H]^-$ (soft ionization, low to medium molecular weight, medium to high polarity)
- APPI: $M^{+\bullet}$, $[M+H]^+$, $[M-H]^-$ (soft ionization, low to medium molecular weight, medium to high polarity)
- ESI: $[M+H]^+$, $[M+nH]^{n+}$, $[M+Na]^+$, $[M+NH_4]^+$, $[M-H]^-$, $[M-nH]^{n-}$ (soft ionization, low to high molecular weight, medium to high polarity, biopolymers and organic salts)