What is Mass Spectrometry?

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

Niessen, W. M. A.; Van der Greef, J., *Liquid Chromatography–Mass Spectrometry: Principles and Applications*, 1992, Marcel Dekker, Inc., New York, p. 29.

A textbook definition of mass spectrometry. What is important to understand is that the resulting mass spectrum is based on m/z, not mass. However, when ions are singly charged, the two terms can be used interchangeably.

Steps of Mass Spectrometry

Make → Solution Phase / Probe

Move → Ion Optics

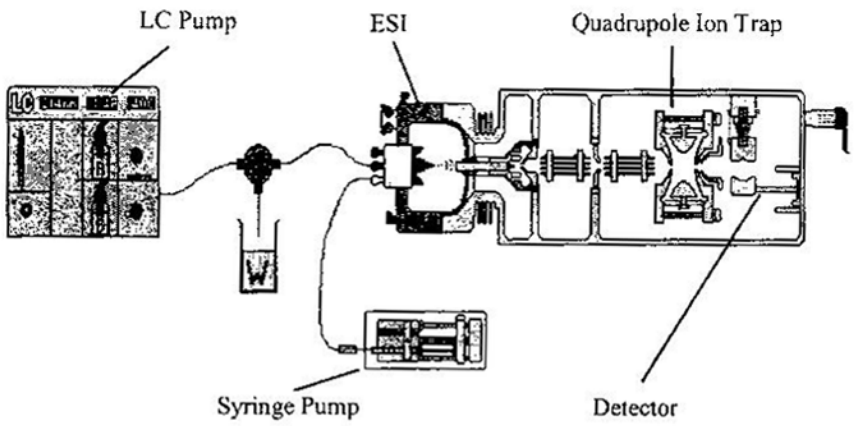
Select → Ion Trap

Detect → Conversion Dynode / Electron Multiplier

There are four steps to mass spectrometry using the LCQ. We will be using the pneumonic "MMSD" - Make, Move, Select, Detect. We first **Make** the ions in either the solution phase (when using ESI) or at the probe (when using APCI). The difference between the two will be discussed in later slides when specifically introducing the API types. The charged analytes must be **Moved** from the source to the Ion Trap without coming into contact with any of the solid internal parts of the mass spectrometer (contact would neutralize the ion, losing it in mass spectral analysis). This is accomplished by a series of ion optics that use a combination of DC voltage, RF voltage, and a vacuum gradient. The **Selection** of ions, as well as the scan event dynamics are completed within the Ion Trap itself. Only the selected ions leave the trap to be **Detected**. These may be either a selected group of m/z ions or a single m/z ion in their original form or fragments thereof, depending on the selected scan events. Detection of the ions is then completed by a combination of the conversion dynode and photomultiplier.

Next, we will be visiting each of the sections that are responsible for this MMSD process.

LCQ — MS^n Quadrupole Ion Trap



This is a schematic of the LCQ components, each of which will be discussed in more detail next.

Make

Since a mass spectrometer can only detect charged components, we must first MAKE ions. This is completed by either solution phase chemistry or gas phase chemistry depending upon the type of source probe being used. In the next few slides, we will discuss the different ways ions can be made.

API:
Atmospheric Pressure Interface (Ionization)

- Two source types:
  1)Electrospray Ionization (ESI) – Solution-phase ionization before the source
  2)Atmospheric Pressure Chemical Interface (APCI) – Gas-phase ionization in the source
- Serves four main purposes:
  1)Desolvate the sample flow for introduction into the mass spectrometer.
  2)Baffle the first vacuum region of the mass spectrometer from atmospheric pressure in the source.
  3)Ionize the analyte (APCI) or assist in ion charge distribution (ESI)
  4)Pump away neutrals and opposite charged ions which would otherwise interfere with the analysis of the desired polarity ions

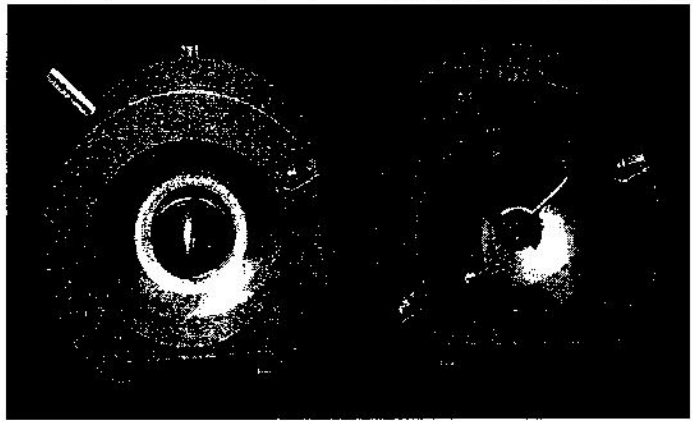
Atmospheric Pressure Ionization (API) refers to the technique by which the LC flow is interfaced to the mass spectrometer. There are three major specific purposes for the API – 1) to desolvate the sample flow for introduction into the mass spectrometer; 2) to act as the first baffle separating atmospheric pressure outside the instrument from the eventual very high vacuum inside the instrument; 3) to ionize the analyte (in the case of the APCI) or assist in preformed ion charge distribution (in the case of the ESI); and 4) to pump away neutrals, decreasing noise.

In the LCQ, the API represents a combination of the source probe and the API stack. In the "Make" section, we will be discussing the two types of source probes, ElectroSpray Interface (ESI) and Atmospheric Pressure Chemical Ionization (APCI). Following their passage through the source, the ions will pass through the API stack, which is the first section of ion optics in the LCQ and will, therefore, be discussed in the "Move" section.

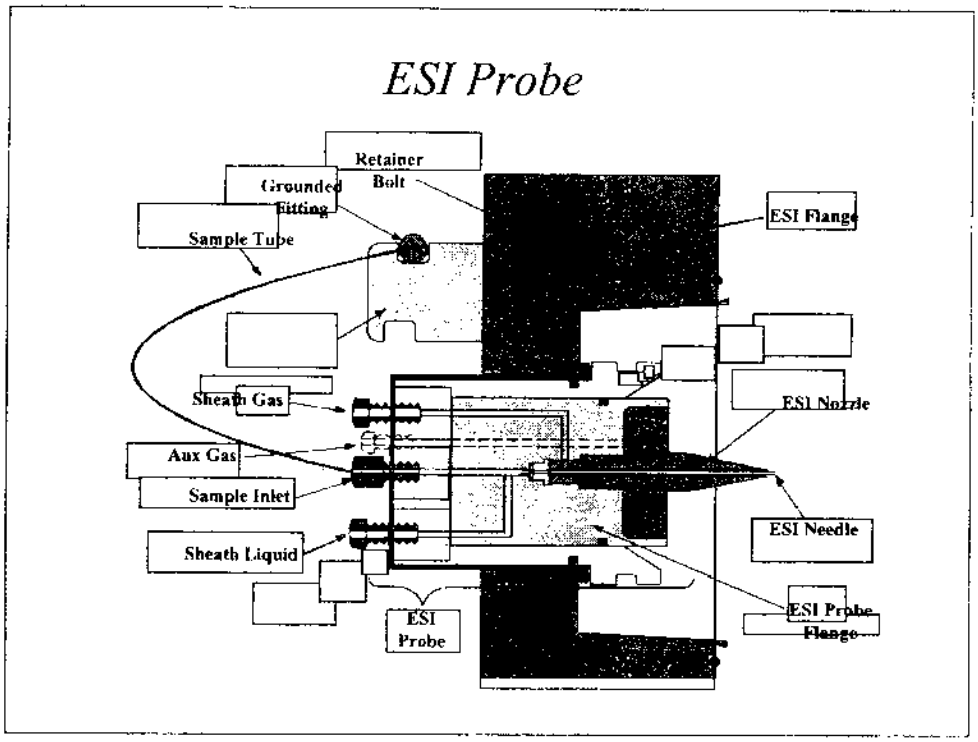
When using the ESI, the ions would be pre-formed by solution phase chemistry before the analyte ever reaches the source probe. This most commonly accomplished by adding a proton donor, such as acetic or formic acid, or a proton acceptor, such as ammonium hydroxide, to the mobile phase. On the other hand, when samples are acquired using the APCI source probe, they may reach the probe in the neutral state, where they will be protonated or deprotonated by gas phase processes occurring across the corona discharge needle.

LCQ API Probes

Electrospray Ionization (ESI) **Atmospheric Pressure Chemical Interface (APCI)**



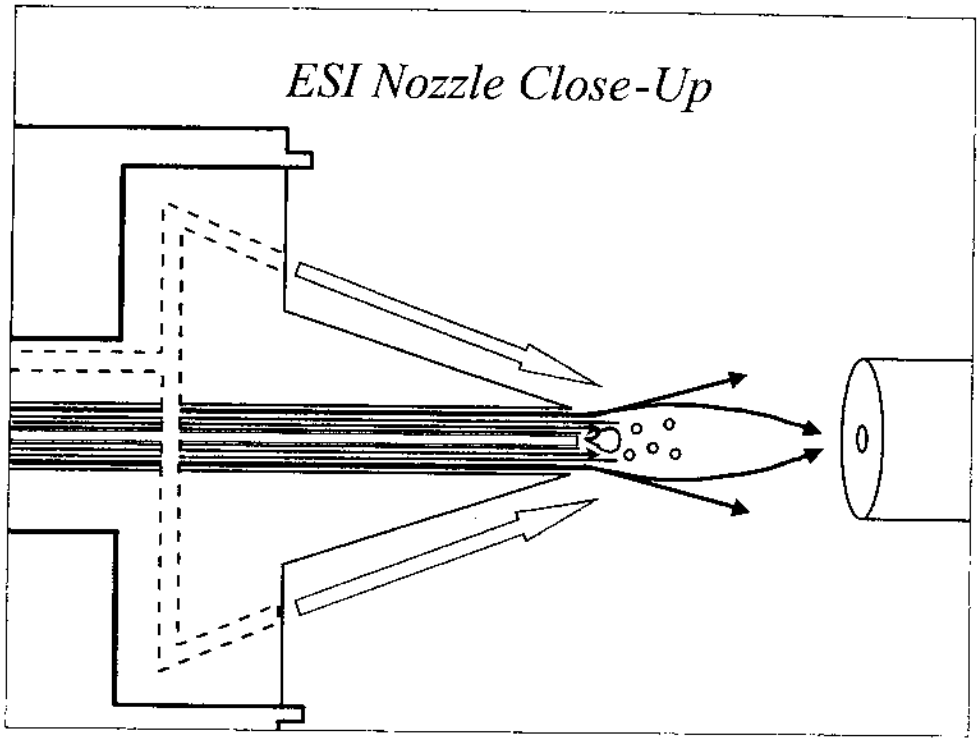
This is a picture of the API source probes for the LCQ Classic as they are viewed from the instrument side. On the Duo and Deca, these are replaced by newer API probes, on which several refinements have been made. For example, on the ESI probe here, the functional metal probe (center) is surrounded by a Teflon insulator, which is, in turn, surrounded by a metal sleeve. The Teflon/metal components have been replaced by a single uninterrupted PEEK insulator in the newer ESI probes, which increases signal stability, alleviates probe position sticking, and prevents probe shorting. Differences in the APCI probes are small.



This cross section of the original ESI probe allows the identification of the various parts that make the probe function. The sample flow enters via a metal union, which is grounded to keep a voltage from traveling through the electrolytic mobile phase. For best results, the tubing preceding this fitting should all be PEEK material. However, after this fitting, you must use fused silica tubing (0.10mm ID, 0.19 OD), unless you have purchased the ESI metal needle kit. The sample inlet is a nut/ferrule pair that is situated to hold the fused silica into place. The user should take care not to over-tighten this nut, as it is not a true union and can lead to constriction or crushing of the fused silica. The tubing then extends through the middle of the ESI needle which is, in turn, inside the ESI nozzle. The end of the fused silica should be 0.5 to 1.5 mm inside the ESI needle. Signal instability is most commonly caused by incorrect positioning of the tubing inside the ESI needle or not having a straight cut on the fused silica.

There are two gas inputs to the ESI probe, sheath gas and auxiliary (or aux) gas. The sheath gas flows through the center of the ESI nozzle but outside the ESI needle. Thus, the sample comes in contact with this gas at the end of the metal needle. The aux gas flows through an outer ring in the ESI nozzle and out holes that are angled parallel to the tapered nozzle face.

In addition to the gasses, the ESI probe has an input for sheath liquid. This liquid would be pumped into center of the ESI needle, but still outside of the fused silica. This input is not often utilized, but may be of some use in breaking down the surface tension of a very aqueous mobile phase flow by pumping in an organic, or may be used to assist in sample protonation by pumping in an acid. However, since all sheath liquids flow down the entire length of the fused silica, care should be taken as to what solvents are used as some may react with the polyamide coating on the fused silica.



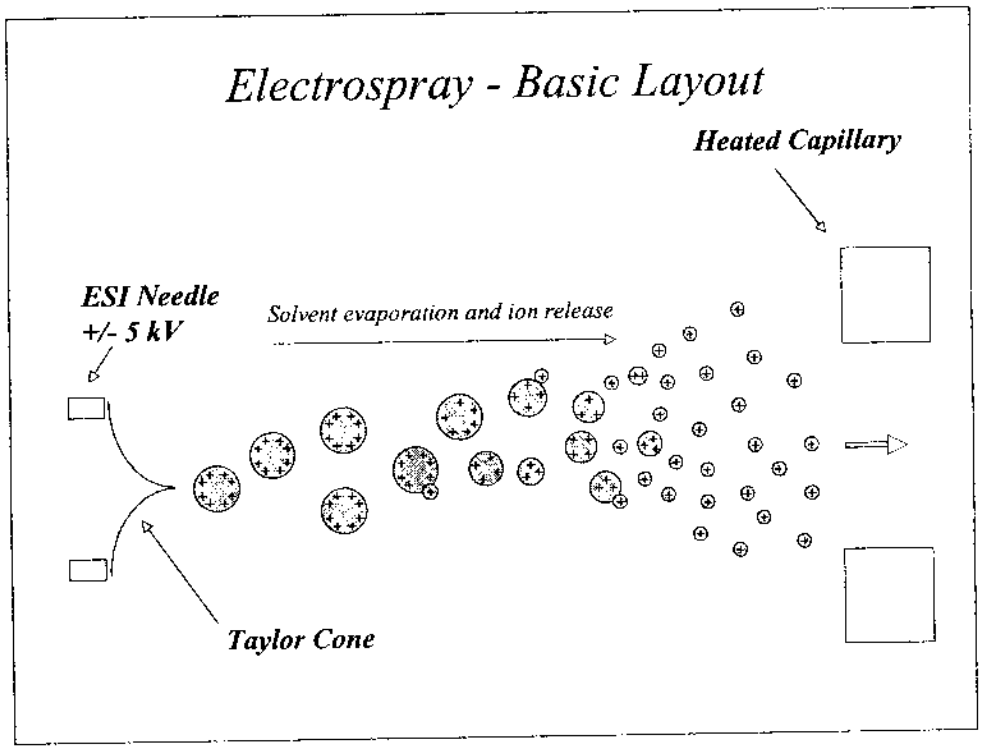
This close-up view of the ESI nozzle demonstrates the functioning of the sheath liquid and the sheath and aux gasses. The sample flow is carried to the end of the fused silica just inside of the metal tubing. If sheath liquid is flowing, it is mixed with the sample just inside the metal needle. The combination of the same polarity voltage applied to the ESI needle and the sheath gas flowing past the ESI needle ejects the sample in what is referred to as the "Taylor Cone", breaking the large droplet into many tiny droplets. At higher LC flow rates, the droplet spray expands away from the entrance to the heated capillary. Thus, some aux gas may be required to alter the flow back towards the center. The sheath gas and aux gas also serve the purpose of sample desolvation, which is completed within the heated capillary.

Common problems with the ESI probe are as follows:

1) Signal instability - Variation in spray current should be <20%. If variation is worse, you should trim the sample tube and readjust its location inside of the ESI needle (0.5 mm to 1.5 mm inside).

2) High Voltage short – If the source voltage is too high, the probe is too close to the heated capillary, or the mobile phase is highly electrolytic, the ESI nozzle (4-5 kV) may arch across to the heated capillary. Initially, this will cause surges in signal and may lead to the eventual failure of the high voltage power source. The probe position should therefore be chosen with respect to flow rate and the source voltage should never be set above 4.5 kV)

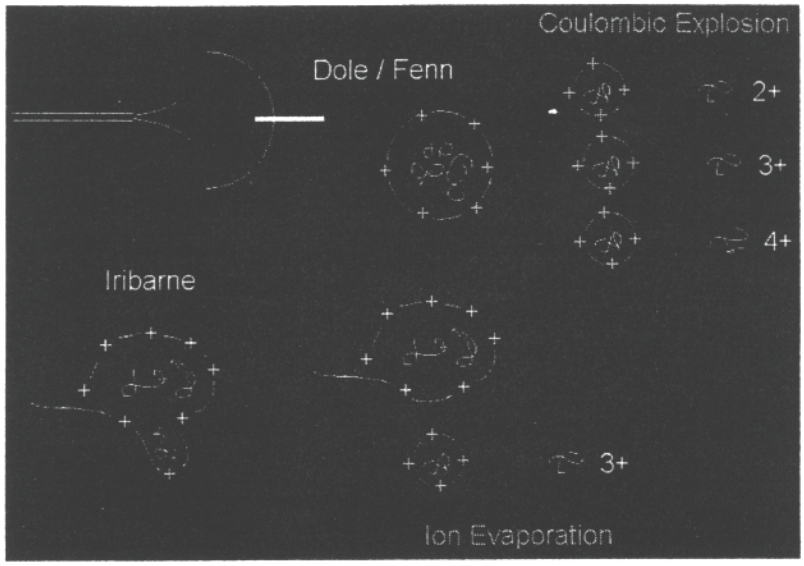
3) High HPLC pressure – If the HPLC climbs to pressure greater than those generally noted, the sample tube may be clogged, constricted, or crushed. Check all fitting and change tubing if needed.



The first step of ion formation in electrospray is droplet fragmentation at the needle tip. The interaction of the high voltage and sheath gas causes the solution flow to form a "Taylor Cone." This emits very small charged droplets. The droplets are enriched with analyte ions of the same polarity as that applied to the ESI nozzle, since those charges were repelled from the needle and opposite charges were grounded by the needle.

The second step of ion formation in ESI is desolvation and represents a process which remains under some scientific contention. There are two main theories of what happens in the process that are represented in the following slide. By whatever method, the analyte is desolvated by the combination of dry nitrogen gas and the heated capillary. Thus, dry, charged ions enter the ion optics at the end of the heated capillary to be isolated from neutrals and steered into the trap.

Two Theories of Electrospray

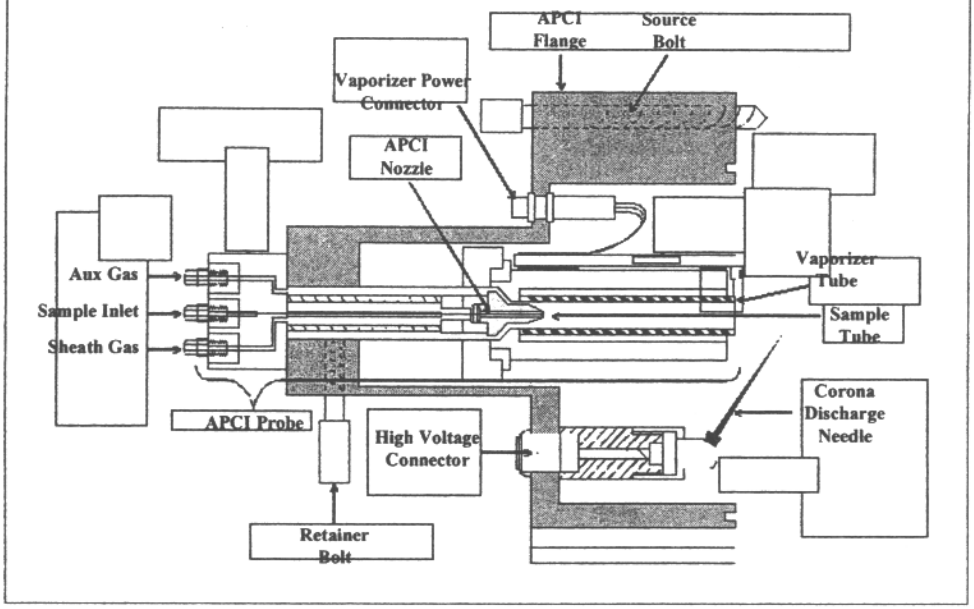


There are two competing theories as to what is occurring during the desolvation of solvent flow between the probe and heated capillary. Both agree that very small droplets are broken off of the main flow at the Taylor Cone. Then, the process by which desolvation occurs differ for each theory.

Dole and Fenn originally introduced the Coulombic Explosion theory. This postulated that solvent is being evaporated out of the small droplets by their interaction with the dry nitrogen sheath and aux gasses. However, only the neutral solvent in the droplet is evaporated. As the droplet gets smaller, it becomes more and more enriched with the charged ions. This continues until the droplet reaches the Rayleigh Limit, a physical limit in which too many charges are contained within too small an area. At this point the droplet explodes, yielding many small droplets which further desolvate and explode themselves. This eventually results in single ions with a small amount of intrinsically bound solvent. The remaining solvent is then evaporated off by the heated capillary.

Iribarne, on the other hand, postulates that it is a spatial physical processes that is responsible for the droplet fragmentation rather than an electrical physical process. He states that the droplets are not perfectly round as pictured in the case of the Dole/Fenn theory, but rather they are twisted and pocked by the turbulent sheath and aux gas flow so that tiny droplets constantly break off the main drop like tiny amoebas. This eventually leads to the same ions with intrinsically bound solvent which is completed dried by the heated capillary.

APCI Probe Assembly

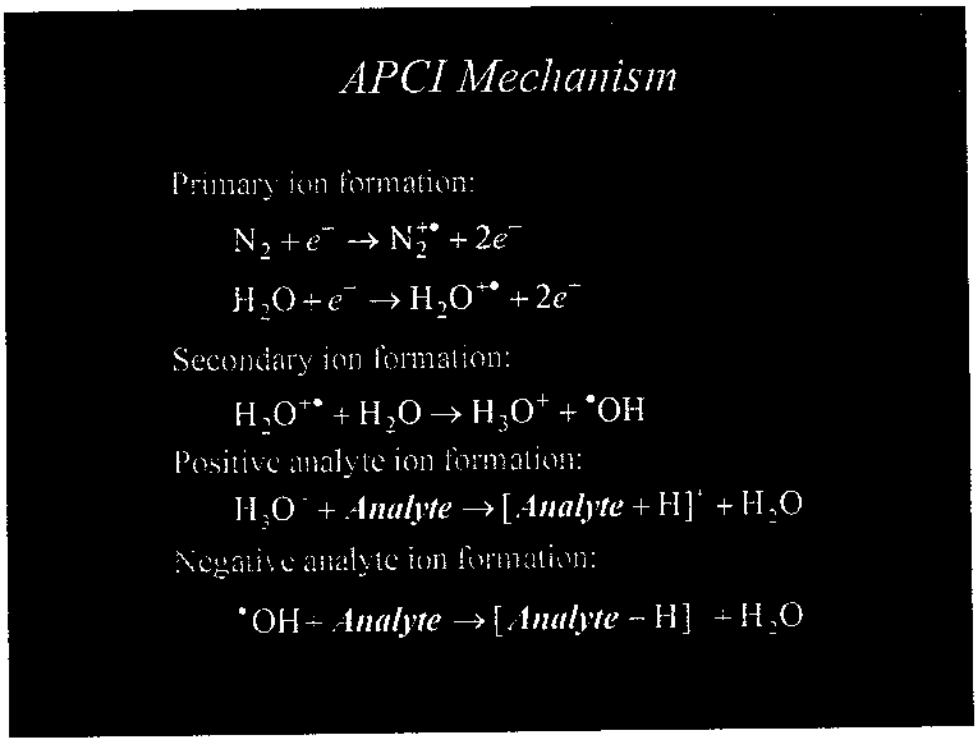


In the APCI probe, the sample flow is ejected into the sample tube of the vaporizer, where it is flash evaporated at temperatures between about 200 and 600 °C. In actuality, the ions have been shown to experience temperatures approximately 150 °C less than the temperature of the vaporizer heater. In this case the gasses don't steer the sample flow towards the heated capillary. Since the solvent is flash evaporated and each ml of liquid will yield liters of gas, the solvent itself carries the sample to the heated capillary. However the gasses are necessary to lend a drying effect tot the otherwise humid solvent gas so that the ions can be dried in the heated capillary

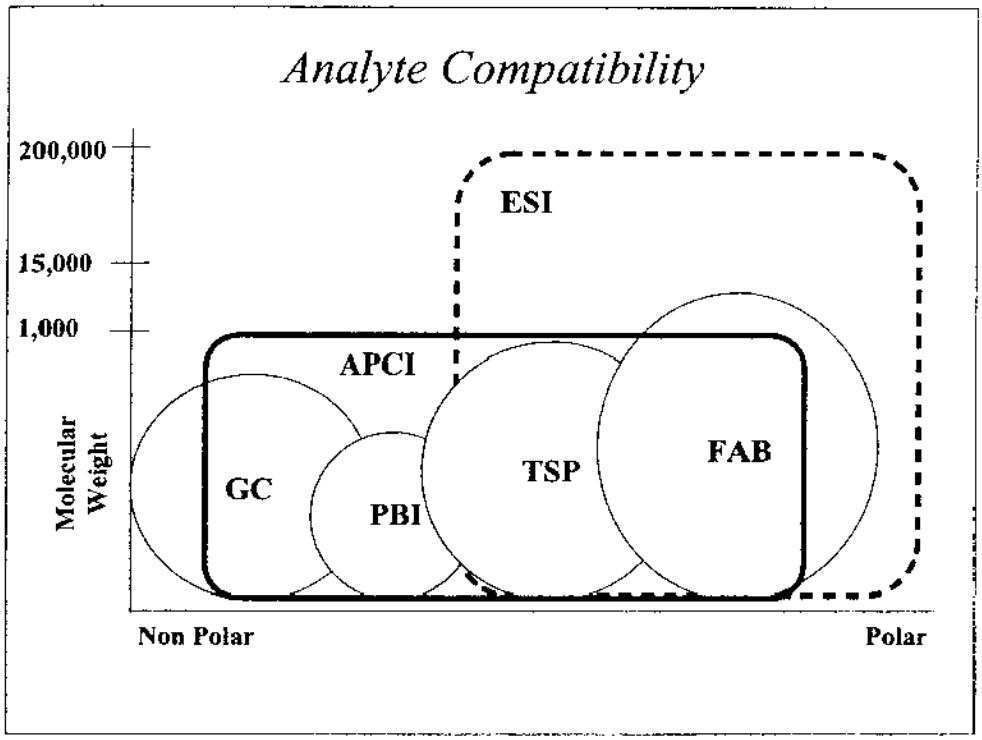
When the desolvated analyte reaches the end of the vaporizer tube, it passes through a region of high voltage plasma created by the corona discharge needle where it is ionized. The best understood chemistry for the protonation/deprotonation processes are represented in the next slide.

WHILE THE APCI PROBE IS AN EXCELLENT PROBE FOR MANY SMALL MOLECULES, IT IS NOT APPROPRIATE FOR MOST PEPTIDE / PROTEIN APPLICATIONS. The high

Heat used in the vaporization process will degrade the peptides and proteins.



APCI gas phase chemistry is best understood to occur by these pathways. The hydronium ion (H_3O^+) and the hydroxyl free radical ($^\bullet OH$) are always present in the region surrounding the tip of the corona discharge needle. When the analyte passes through this "plasma" it is protonated, $(M+H)^+$, and deprotonated, $(M-H)^-$. The desired polarity ion is chosen in the ion optics, while the opposite polarity is pumped away.



The two major factors determining source selection are the polarity and the molecular weight of the analyte. This is meant to serve as a general example only, as sample specifics may differ and there is a large region of overlap between the two. Generally speaking, however, one should choose ESI to run high molecular weight analytes (i.e. proteins/peptides) or those that are very polar. This is because large and polar molecules tend to be thermally labile or non-volatile. If run by high temperature APCI, these ions would either break down or stick to the vaporizer tube. On the other hand, small non-polar samples would generally best be run by APCI, since ESI depends largely on pre-formed ions.

Chemistry Considerations

- ESI:
  - Ions formed by solution chemistry
  - Thermally labile
  - Large Molecules (Proteins / Peptides)
- APCI:
  - Ions formed by gas phase chemistry
  - Volatile / Thermally Stable
  - Non-polar / Semi-polar
  - Small Molecules (Steroids)

This slide gives a summary of the points made on the last slide regarding the selection of ESI or APCI.

LC Flow Rates

- ESI:
  - 3 µL/min - 1mL/minute
  - Optimal Flow Rate: 200 µL/min
  - Generally, higher flow rates require higher heated capillary temperatures and higher gas flow rates.
- APCI:
  - 200 µL/min - 2mL/minute.
  - Optimal Flow Rate: 500 µL/min
  - Generally, higher flow rates require more sheath and auxiliary gas, but do not require higher heated capillary temperatures.

In addition to sample attributes, one may also want to consider flow rates in the selection of a source type. The ESI probe can analyze flow rates down to a 3 µl/min infusion and up to a 1 ml/min LC experiment. The APCI on the other hand can only be used for LC experiments down to 200 ml/min reproducibly, due to the extreme environment at the source, but can run up to 2 ml/min.

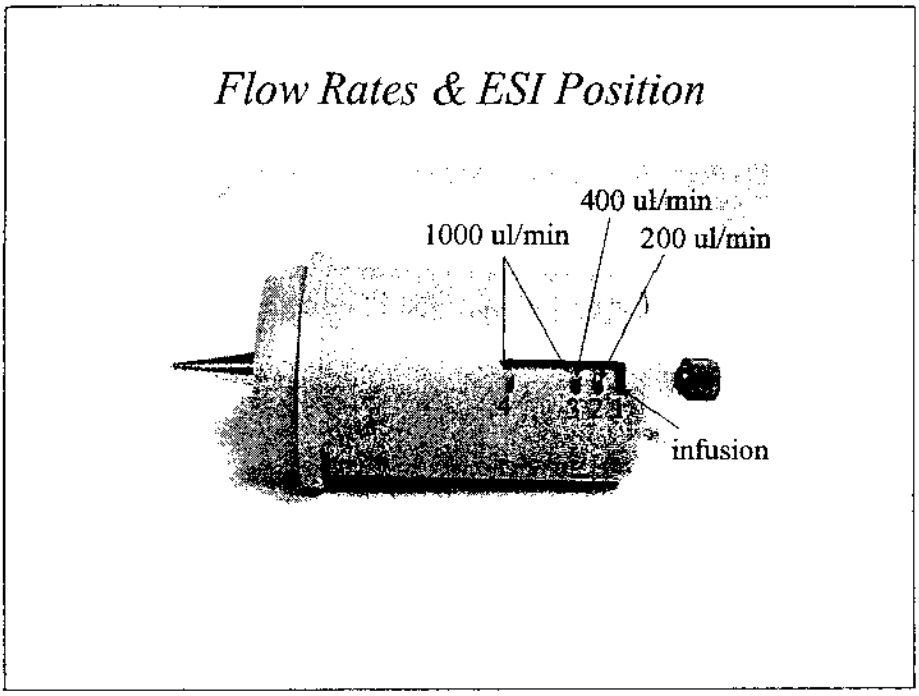
For quantitation experiments on components that can be equally run by ESI or APCI, one finds that there is an optimal trade off for the chosen flow rate. Lower flow rates result in improved sensitivity as a greater percentage of the total ions can enter the heated capillary. However, higher flow rates result in better reproducibility due to the improved LCQ flow stability. Therefore, the trade off is somewhere in the middle. In an experiment using several steroids, these optimal flow rates were determined to be 200 µl/min for ESI and 500 µl/min for APCI.

For both sources, as the flow rate is increased or has a higher aqueous composition, the sheath and aux gasses will optimize at higher flow rates. In the case of the ESI, a higher heated capillary temperature may also be necessary. In the APCI, heated capillary temperature will not alter the signal by much since the sample has already traversed through the very hot vaporizer tube.

Flow Rates & LC Columns

Flow Rate		i.d.
• 1	ml/min	4.6 mm
• 0.5	ml/min	3.0 mm
• 0.2	ml/min	2.1 mm
• 50	µl/min	1.0 mm
• < 10	µl/min	Capillary

LC columns come in a variety of dimensions, the most common ones being shown here. When interfacing LC with Mass Detection it is vital that the interface chosen can cope with the wide range of LC flows in use with the different columns.



The Duo and Deca instruments with the newer ESI source have several refinements that increase the stability of the PEEK signal at the source. One of these refinements is the switch from the Teflon/stainless steel probe present on the original probe, to the all PEEK probe shown here. The material allows the easy adjustment of the probe position within the flange relative to the heated capillary. Generally, the higher the flow rate, the further the ESI nozzle should be away from the heated capillary. This is for two reasons. First, if the ESI nozzle is too close to the heated capillary (which is commonly at 4-5 kV), the electrolytic mobile phase will cause the voltage to arch, leading innitially to signal instability, and eventually to the possible failure of a power source. Second, as higher flow rates are used, the sample spray disperses faster. Thus, more space is needed for the aux gas to node the flow back into the heated capillary.

However, in addition to the flow rate, probe positions are affected by mobile phase and additives. Thus, for optimal sensitivity, the probe position SHOULD BE EMPIRICALLY for the flow rate and mobile phase being used.

To change probe positions, loosen the retainer nut in the ESI flange to lift the pin off the probe. The pin should remain in the groves displayed here to guide the probe into the proper position. If the pin is loosened too much, the probe as the free range of motion and could be pushed too close or pulled too far.

LC Additives

- Acids
  - Do not use inorganic acids (may cause source corrosion)
  - Formic and acetic acid are recommended
- Bases
  - Do not use alkali metal bases (may cause source corrosion)
  - Ammonium hydroxide and ammonia solutions are recommended
- Surfactants (surface active agents)
  - Detergents and other surface active agents may suppress ionization
- Trifluoroacetic Acid (TFA)
  - May enhance chromatographic resolution, but causes ion suppression in both negative and positive ion mode
- Triethylamine/Trimethylamine (TEA/TMA)
  - May enhance deprotonation for Negative Ion Formation

This slide lists several recommendations for LC additives. One should avoid the use of inorganic acids and alkali metal bases as both can lead to the damage of source components. Formic and acetic acids are recommended as proton donors for positive ion mode and ammonium hydroxide and ammonia solutions are recommended as proton acceptors for negative ion mode.

One should avoid the use of surfactants such as Triton-X 100 for use with mass spectrometry as these detergents lead to ion suppression and coating of the ion optics. Both result in an overall loss in sensitivity.

TFA is commonly used in HPLC with UV detection because of its enhancement of chromatographic resolution (ion pairing). Unfortunately, this additive has an adverse effect on negative and positive ion formation. Simply speaking, negative ions cannot be formed in a low pH environment. So TFA would suppress negative ion formation. Also, since TFA contains so many electrophillic fluorine groups, it is also a proton acceptor so will lead to ion suppression of positive ions as well.

In negative ion mode, one may want to add TEA or TMA with the proton acceptor additive, which will enhance the negative ion formation.

Buffers (pH)

- Avoid using non-volatile HPLC additives such as:
  - Alkali Metal Phosphates
  - Borates
  - Citrates
- Keep Buffer concentrations below 20 mM using volatile salts such as ammonium acetate.
- When using buffers, more frequent cleaning of the heated capillary and API stack will be necessary

If the HPLC requires a buffer, one should keep these recommendations in mind.

LC/MS Additives and Buffers

Acetic Acid Formic Acid	Proton Donors
Ammonium Hydroxide Ammonia Solutions	Proton Acceptors
Trichloroacetic Acid (< 0.1% v/v) Trifluoroacetic Acid (< 0.1% v/v)	Ion Pairing Agents
Triethylamine (< 0.1% v/v) Trimethlyamine (< 0.1% v/v)	Negative ion formation
Ammonium Acetate Ammonium Formate	Buffers

Summary of the LC/MS additives and buffers that have been previously discussed.

Common LC/MS Solvents

Methanol
Acetonitrile
Water
Isopropanol
Dichloromethane
Chloroform
Hexane
THF

Common and not so common mobile phases used in reverse and normal phase chromatography. Please note that THF should not be used with a Duo or Deca instrument since it is one of the few solvents that will react with PEEK. The use of THF would, therefore, lead to irreparable damage to the ESI probe.

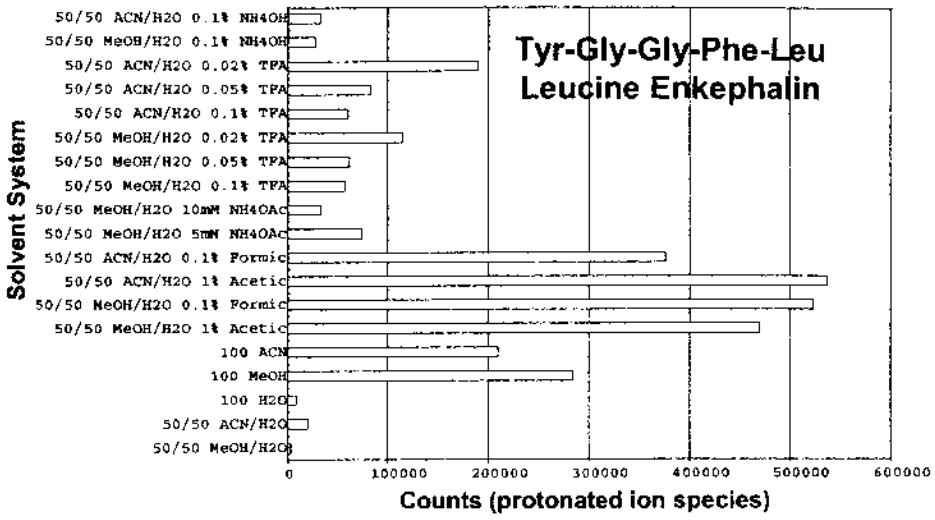
IN BIOLOGICAL SEPARATIONS the most common solvent gradient system is:

95% Water / 5% Acetonitrile with .02% TFA and 1% Acetic Acid

changing to

5% Water / 95% Acetonitrile with .02% TFA and 1% Acetic Acid

Effects of Solvents and Additives on ESI



This plot shows the results of experiments done on leucine enkephalin using several different mobile phase conditions. One may reach many general conclusions with this data. For example, if TFA is used as a proton donor, it will give a signal. However, as more of the acid is added, that signal will be decreased due to ion suppression. In this experiment, the best additive to use for positive ion formation were acetic and formic acid. Interestingly, acetic acid yielded a better signal when using 50/50 ACN/H_2O. However, when using MeOH/H_2O, formic acid gave better results. It is unknown whether this trend will persist to the analysis of other components.

For reverse phase chromatography, if it is unknown which solvents and additives should be used, one may want to begin by using a C-18 column with an organic gradient from H_2O to CAN. 1% acetic acid and 0.02% TFA should be added to both solvents so that the concentrations of these additives remain constant. The acetic acid will serve as the proton donor. Although the TFA will slightly suppress the total signal, it will increase the chromatographic resolution such that the overall signal-to-noise would be increased.