

SHIMADZU PRODUCTS

SHIMADZU LCMS Training

Practical Techniques for LC/MS Analysis

Robert Classon
rjclasson@shimadzu.com



⊕

Revised 1/12/2004

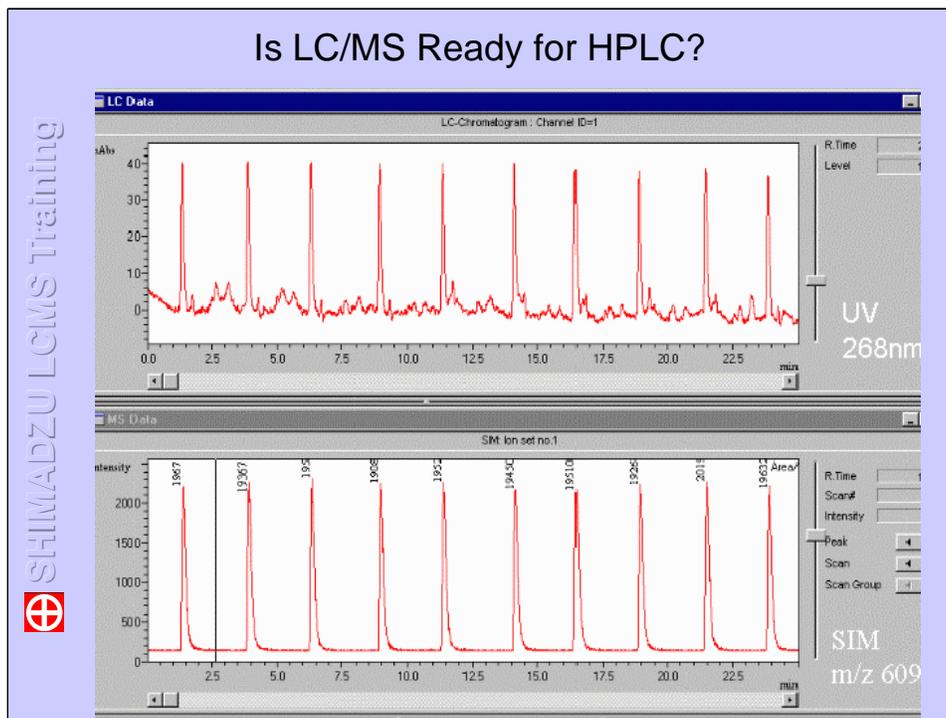
This presentation is an introduction to LCMS. It includes optimization and applications

LC/MS seminar overview

- † Getting the most out of each ionization technique
- † Practical considerations with LC/MS
- † Developing LC/MS methods



Is LC/MS Ready for HPLC?



Since this MS is intended for quantitative use as well as identification, it is important to be able to achieve good quantitative results. Here we show reproducibility on both UV and MS to show comparable results for a pharmaceutical type product (reserpine). Notice that the signal to noise from the MS is considerably better than the UV detector shows, indicating that LCMS is capable of providing better quantitative data than UV. Which signal would be easier to integrate? Which shows the best reproducibility?

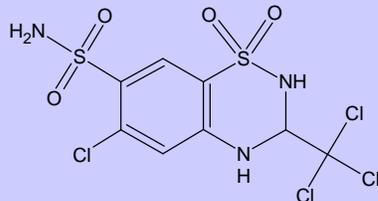


Terms and Definitions

- † API - Atmospheric Pressure Ionization
 - ⌘ Includes both APCI and ESI and all LC/MS interfaces and sources
- † APCI - Atmospheric Pressure Chemical Ionization
- † ESI - Electrospray Ionization
- † CID - Collision Induced Dissociation
 - ⌘ Same as In-Source Fragmentation



Mass versus Molecular Weight



**3-trichloromethyl
hydrochlorothiazide**

(diuretic, hypertensive)

FW = 415.10

Nominal mass = 413.00

Monoisotopic mass = 412.86

**Why the differences?
Different measurement
techniques**

Elements with multiple isotopes

Everything has mass, but there are different ways to express mass. Since mass spectrometers measure monoisotopic mass (based on the most abundant isotope present in the sample), its value will be different than average mass or molecular weight (formula weight). Mass/charge (m/z) is related to "molecular weight" (or more accurately to molecular mass), but will be slightly different in value based on the types of isotopes present. The difference will be most pronounced for compounds that contain multi-isotopic elements such as chlorine or bromine atoms in the structure, or when the molecule is especially large (such as proteins and peptides).

Ion generation

- † Atmospheric pressure ionization (API)
 - ⋈ Sample ionization takes place outside the vacuum system of the MS at atmospheric pressure.
 - ⋈ Source sample molecules are ionized to produce an ion beam of pseudo-molecular ions.

- † Ionization is carried out at atmospheric pressure for two reasons:
 - ⋈ Heat transfer is more efficient at atmospheric pressure thus enhancing solvent evaporation
 - ⋈ High electric fields do not result in strong electrical discharges that occur at reduced pressure.



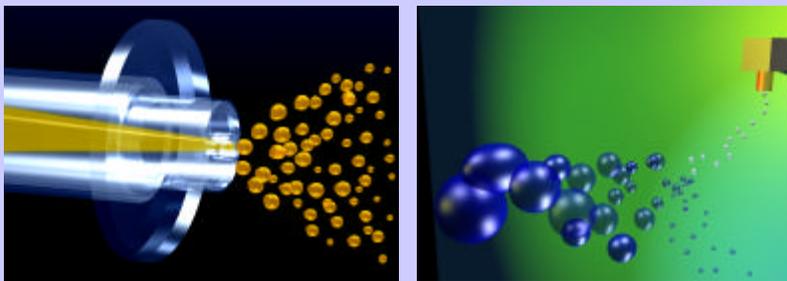
API is atmospheric pressure ionization and can refer to any source in use with LCMS today. It simply means that ionization takes place at atmospheric pressure. Although early generation LCMS systems attempted to spray into reduced pressure chambers or directly into a vacuum region, both approaches have been replaced by API techniques. The reasons: spraying directly into a vacuum does not dry as well or produce ions as well as atmospheric pressure ionization. Vacuum drying can produce 'snow' from high aqueous solvents, and will exhibit arcing that will interfere with or eliminate the ions. For these reasons, LCMS was changed over to atmospheric ionization in the early 1990's and has remained that way ever since.

Production and Loss of Ions

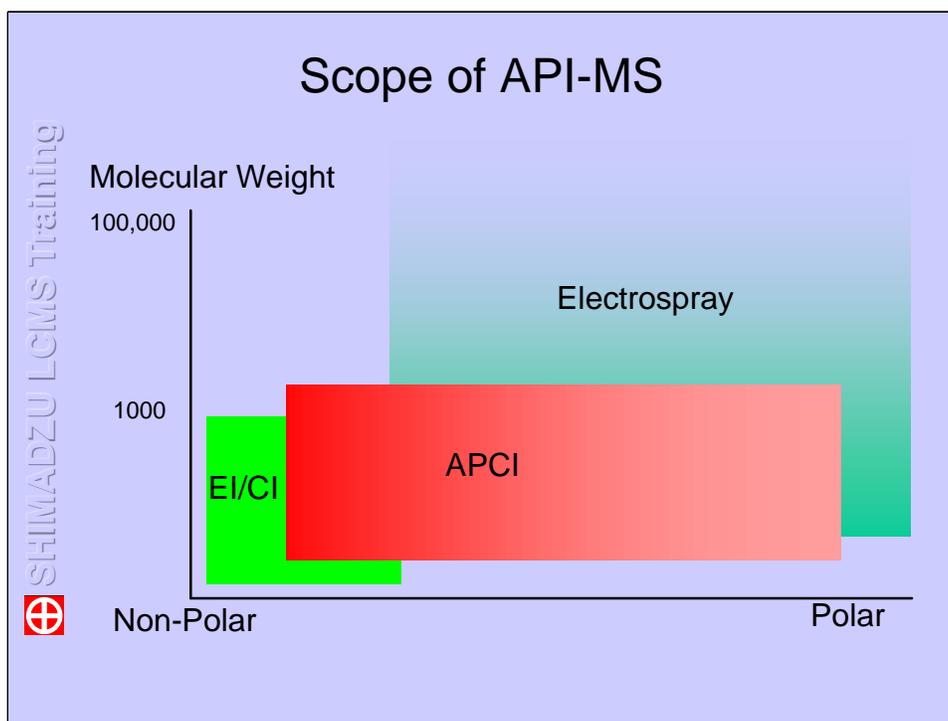
† Most Commonly Used Sources:

- ✎ Electrospray (ESI)
- ✎ Atmospheric pressure chemical ionization (APCI)

The two techniques differ in the mechanism for ion generation and consequently affect the response to different sample molecules



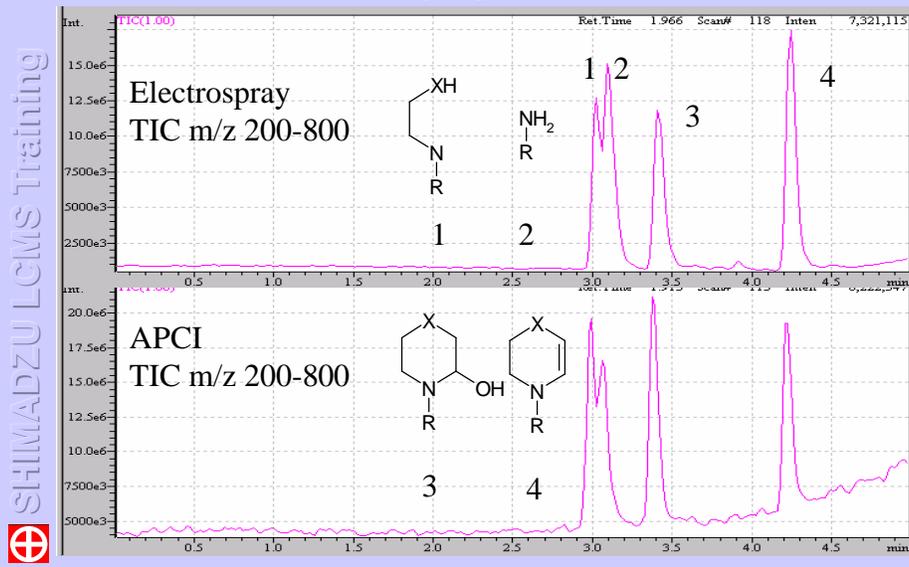
The two most common sources are electrospray and APCI. Electrospray is basically a form of creating ions in solution, evaporating that solution very quickly and generating ions from the surface of tiny droplets. As an analogy, it is similar to an electrostatic paint sprayer or an ink jet printer. The problem is that it can be very sensitive to pH and concentration of impurities. The advantage is that it is very sensitive, works with most pharmaceutical and biological molecules, and is easy to use. APCI is less well known, but generally easier to use. APCI works well for molecules under 1000 MW, and that are thermally stable. ESI is the technique of choice for proteins, peptides, pharmaceuticals, and high polarity samples such as acids and bases. APCI is the technique of choice for nonpolar molecules such as steroids, triglycerides, terpenes, etc.



Here we see a comparison of the types and ranges of compounds that can be analyzed using different ionizations techniques. (EI/CI is shown for comparison purposes only). Electrospray can cover a wide range of polarities and masses because large compounds tend to pick up multiple charges by electrospray. For example, it is easy to analyze myoglobin, a 16,951 mass protein by ESI even though the range of the LCMS instrument might only be a 2000 m/z range. This is because myoglobin typically will pick up between 10 and 20 charges.

Likewise, APCI can cover a wide range of polarities and can handle compounds that ESI would have difficulty with, such as steroids and even some aromatic hydrocarbons. This is because APCI uses a different ionization mechanism and can even produce such ions as free radicals under certain situations. APCI has difficulty with larger molecules because it needs heat as part of the ionization process. Molecules typically larger than 1000 are generally too thermally unstable to hold up to the heat of APCI. ESI is typically performed without heat other than the drying gas.

Electrospray versus APCI

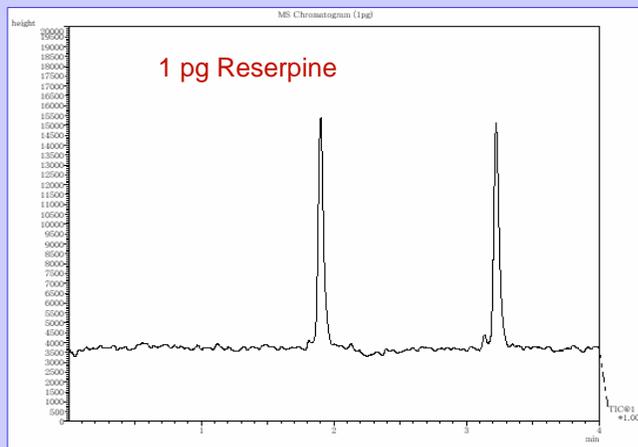


Both techniques can see a wide variety of compounds

Here is the 4 compound test mix again showing similar results for both probes. Generally APCI is used for difficult to ionize species. It is typical that it will have a higher background noise level because it can ionize compounds that ESI cannot ionize.

ESI Sensitivity

SHIMADZU LCMS Training



ESI can be very sensitive, especially to compounds that can be ionized with a pH change

Here are two injections of 1 picogram each of reserpine. This illustrates that LCMS is very sensitive for compounds like this. At this level, a UV detector would only show a flat line without any peak discernable. Reserpine is used as a sensitivity standard because of history – it was first described by Jack Henion a number of years ago and has been used by various manufacturers ever since.

What is a Picogram?



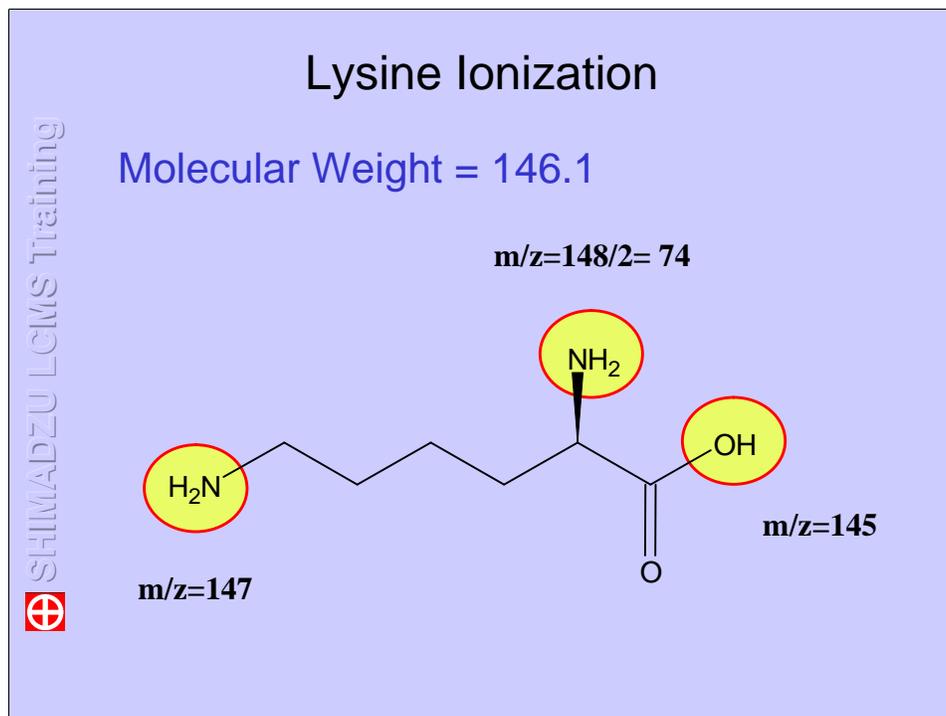
SHIMADZU LCMS Training

- † Sensitivity of quadrupole LCMS = 0.1 picograms (for ideal compounds like reserpine by electrospray)
- † Average amount of erythromycin in surface water (106 river streams according to USGS) = 1 picogram/microliter
- † Weight of about 100 *E. Coli* bacteria cells = 3 picograms
- † Total organic carbon in B&J HPLC grade water = up to 50 pg/microliter
- † EPA Arsenic in drinking water std = 55 picograms/microliter
- † EPA Cyanide in drinking water std = 200 picograms/microliter
- † One pollen grain (Alder tree) = 9,700 picograms
- † Solubility of fused quartz in high purity 18 megohm water = 11,000 picograms/microliter/4 weeks
- † **One typically messy fingerprint = 20,000,000,000 picograms**



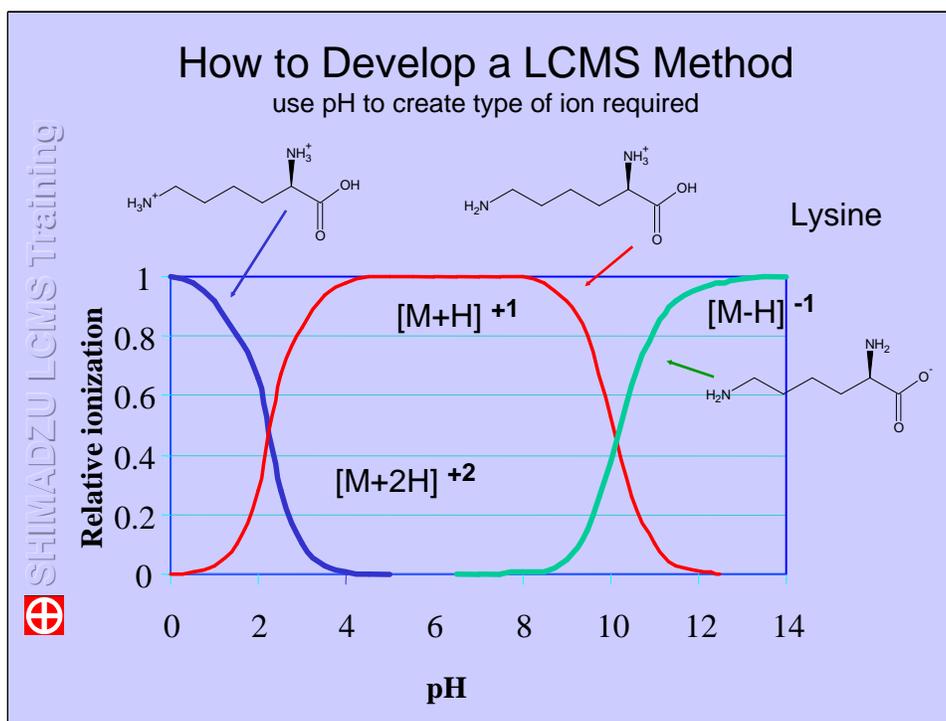
**So keep your hands off the optics and off the instrument!!!
Wear clean gloves, and use **clean** tools!**

The important thing to emphasize here is keep everything clean. Don't touch any ion optics, skimmers, or metal surfaces with fingers. Use cotton or latex gloves and rinse everything with methanol or IPA after assembly before using. Even the tiniest fingerprint contaminates the instrument over a billion times more than what many people are trying to measure! These numbers give an idea of just how much solid material can be contained in some common things. One item most people overlook is the tools (wrenches and screwdrivers) used to remove ion optics. These may have been used to fix someone car or truck and dumped into a drawer with other dirty tools. Rarely do they get more than a wipe with a paper towel (and thus have grease replaced with a detergent!). Tools and optical parts should be cleaned with solvent and air dried on a clean glass plate or in a metal tray. They should be cleaned with solvent before they are allowed to touch any critical part of a mass spectrometer. Never use paper towels to dry any critical parts!!! Keep the MS tools separate from the general lab tools!



Here we see a simple molecule called lysine. It is one of the ‘basic’ amino acids. With two amine groups and a carboxylic acid group, there are three ways it can ionize. Each will produce a different mass ion however. There are two ways to ionize it as a positive ion, and one as a negative ion. As a positive ion, the leftmost amine (primary amine) will pick up a charge first. As the acid concentration increases and the pH goes down, the amine closest to the OH group will pick up a charge (a second charge for the molecule) resulting in a doubly charged species. Since electrospray ionization can be multiply charged, the m/z will be $[146+2]/2 = 74$. It is also possible to ionize the molecule as a negative ion.

Is it possible to create a zwitterion of this molecule? Yes, however the net charge will be zero so the zwitterion species will not produce a signal by MS.



This shows the titration curves for pKa versus amount of acid or base added for lysine. Note, at certain pH values, the molecule can exist with different ionization states. For example, at pH = 2, you will have a mixture of single charge and double charge lysine present. This can complicate quantitation. Also notice that when ionization = 0.5 (midpoint crossover points for the curves), the ionization is at 50%. Going 2 pH units above or below this pH point can change ionization to 100% or 0% depending on the pH direction. Many novice users will attempt to analyze a compound at the wrong pH and may obtain either the wrong ion, or sometimes no ion. A case in point, if a simple peptide were made of two or three amino acids, you could have a distribution of charges depending on pH. Novice users have sometimes incorrectly reported a multiply charged species as an impurity in the sample.

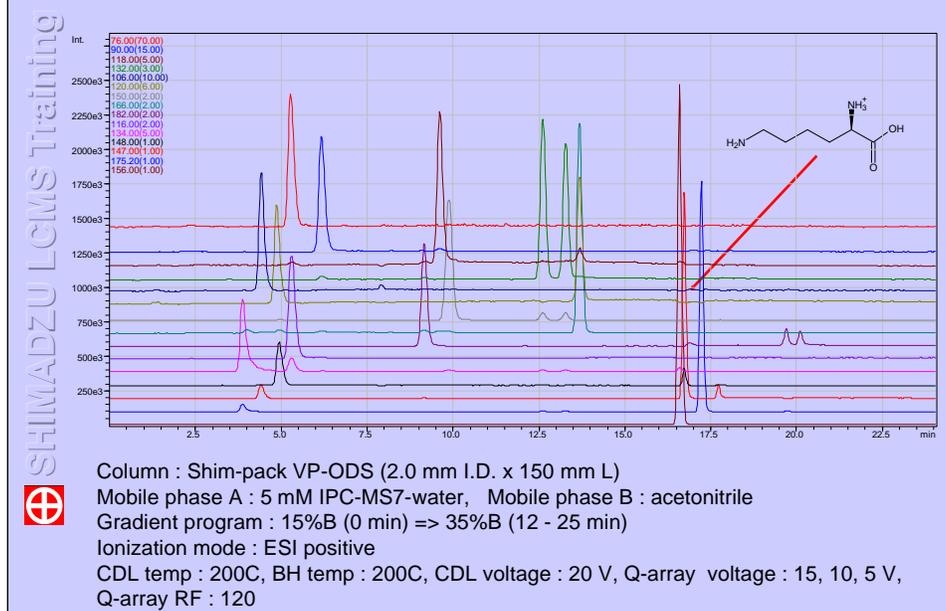
Amino Acids

- † If each amino acid has a unique pKa, how can I analyze a mixture of them without having to know or change chemistry for each one?
- † Can I avoid doing derivitization?
- † Can I separate them without having to do ion exchange separations?
- † Can they still be detected by LC/MS?
- † As positive or negative ions?



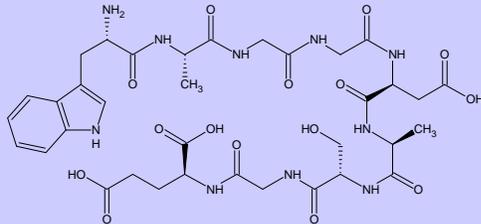
Analysis of Free Amino Acids

Use Volatile Ion Pair Reagents



The red pointer shows lysine (red peak) in a mixture of amino acids. This analysis is performed using a volatile ion pair reagent to retain the amino acids on the HPLC column. The ion pair reagent is volatile enough that it evaporates quickly in the spray chamber and leaves the amino acids to react with excess protons and produce the protonated species.

Peptides and Multiple Ions



Peptides, proteins and other large molecules can become multiply charged with ESI, so careful control of pH is essential



pKa (H1/H+L)	= 17.67 ± 0.46
pKa (H2L/H+HL)	= 17.12 ± 0.30
pKa (H3L/H+H2L)	= 16.39 ± 0.46
pKa (H4L/H+H3L)	= 16.00 ± 0.46
pKa (H5L/H+H4L)	= 15.06 ± 0.46
pKa (H6L/H+H5L)	= 15.06 ± 0.46
pKa (H7L/H+H6L)	= 14.15 ± 0.10
pKa (H8L/H+H7L)	= 13.88 ± 0.46
pKa (H9L/H+H8L)	= 13.22 ± 0.46
pKa (H10L/H+H9L)	= 13.09 ± 0.46
pKa (H11L/H+H10L)	= 8.06 ± 0.33
pKa (H12L/H+H11L)	= 4.76 ± 0.10
pKa (H13L/H+H12L)	= 4.19 ± 0.10
pKa (H14L/H+H13L)	= 3.44 ± 0.10
pKa (H15L/H+H14L)	= -0.84 ± 0.30
pKa (H16L/H+H15L)	= -1.19 ± 0.70
pKa (H17L/H+H16L)	= -1.24 ± 0.70
pKa (H18L/H+H17L)	= -1.27 ± 0.70
pKa (H19L/H+H18L)	= -1.39 ± 0.70
pKa (H20L/H+H19L)	= -1.61 ± 0.70
pKa (H21L/H+H21L)	= -1.82 ± 0.70
pKa (H22L/H+H22L)	= -1.86 ± 0.70
pKa (H23L/H+H23L)	= -1.94 ± 0.70

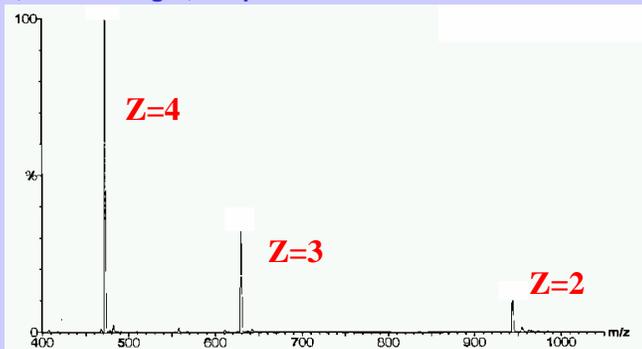
Even simple peptides can have a number of different ions with different charge states. Here we see a simple peptide can have 23 different ion charge states! Peptides usually ionize based on the number and locations of the basic amino acids, plus the terminal group amino acid.

Peptide Analysis By ESI

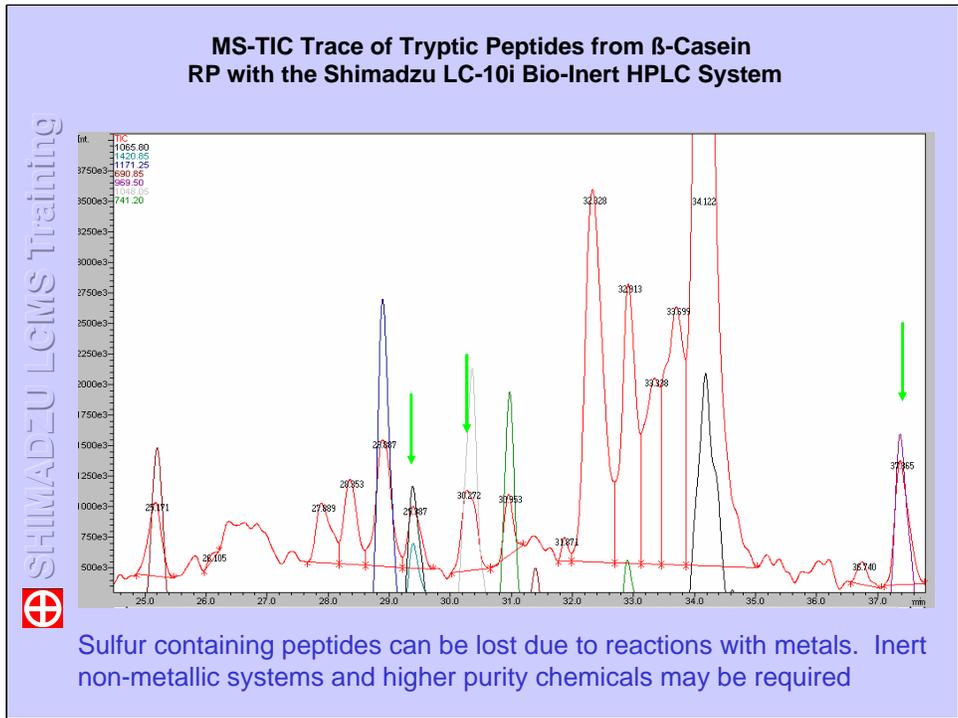
† Basic amino acids: lysine (Lys), arginine (Arg), histidine (His) can be easily protonated, i.e. ionized (in ESI, + mode). N-terminus can also be protonated

† 16-Residue Peptide: Mol mass = 1885.2

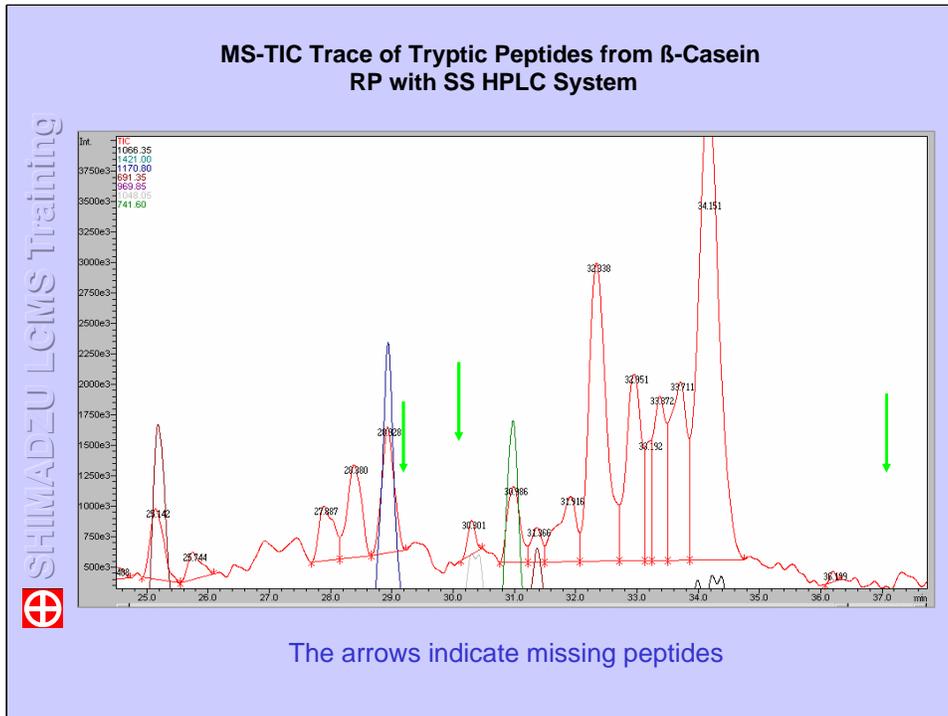
- ⚡ Tyr-Leu-Glu-Phe-Ile-Ser-Asp-Ala-Ile-Ile-His-Val-Leu-His-Ser-Lys
- ⚡ pH, ionic strength, temperature and metals can alter distribution



Most peptides will produce multiply charged ions. Generally the most basic amino acids such as lysine, arginine and histidine are the easiest to ionize and each will pick up a charge in positive ion mode. In addition, the N-terminus acid will generally also pick up a charge. Here we see a 16 amino acid peptide that has picked up 2, 3 and 4 charges. The distribution of charges is common in peptide analysis and the ratio of the different Z values can change depending on pH, ionic strength, and analyte concentration. The presence of metals can also affect the charge, especially for sulfur containing peptides.



Here we see Casein as a digest analyzed on an inert HPLC/MS system. Here are shown a number of peptides. Casein is an interesting protein because it contains some sulfur that will make certain peptides fairly reactive with metals



Here is the same Casein digest analyzed on a traditional metallic HPLC system. The arrows point out several peptides that have disappeared from view. These are the sulfur containing peptides. The difference between this and the previous slide are dramatic if you cycle back and forth between them.

Critical factors in ESI

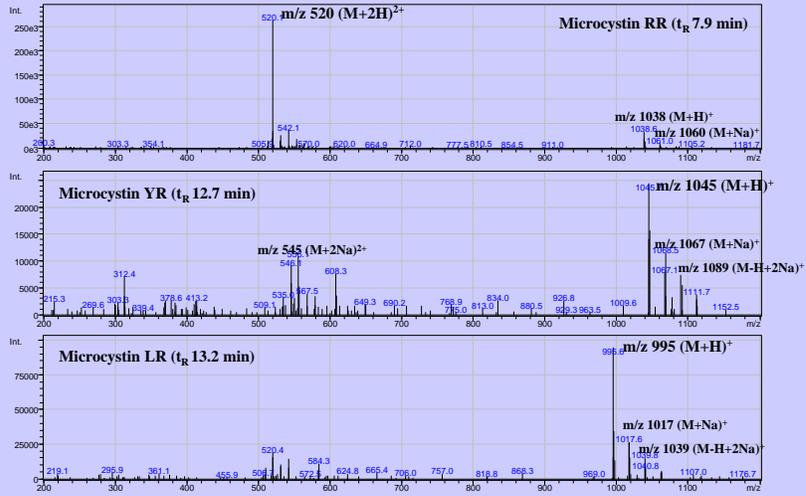
- † ESI is concentration dependent. Narrow peaks give the best results
- † Flow rate affects sensitivity
- † Solvation plays an important role on ionization efficiency.
 - ⌞ Surface tension affects sensitivity (soap, salt, organics)
- † Ion suppression must be carefully considered
 - ⌞ Trifluoroacetic acid, sample matrix, & salts can reduce signal
- † Adduct ion generation must also be accounted for in interpretation and quantitation.
- † Some compounds may ionize as multiply charged species and show up at lower masses than expected

Some comments in addition to what is shown on the slide:

1. ESI is concentration dependent – that means you want to have the smallest injection size and the sharpest peaks. From a method development standpoint, use small particle columns, narrow bore columns and connection tubing, and smaller injections. This also means that a fast gradient will produce better sensitivity results with ESI than an isocratic separation (because of peak compression with the gradient).
2. Anything that affects the surface tension will generally worsen the results. The presence of any salts in the sample, including phosphates will hurt sensitivity by suppression ion formation, restricting ion migration to the surface, and ion evaporation. So the most problems are expected to occur when no HPLC column is used (thus salts will elute with the sample rather than before the peaks of interest!).
- 3 Ion suppression – often ESI will start off looking good but peaks will set smaller over time. This is often due to a build up of more readily ionizable materials on the ion optics, skimmer or probe. For example salts can steal the charge from an ion and become ions themselves.
4. Adducts include adducts with inorganic metals (such as sodium or potassium), as well as solvent based adducts (an ion that also contains one of more solvent molecules). Adducts can distribute a common species over a number of different masses, thus reducing sensitivity.

About Adduct Ions

ESI Mass Spectra of Microcystin RR, YR, LR



These spectra illustrate the problems of adduct formation. Here we see not only simple adducts, but also multiple charged species. Many analytes will also form cation bound dimers. The key to reducing this is to eliminate cations, or to replace them with a single protonating species such as ammonium.

In general, diluting the analyte will reduce this effect as well.

Reducing Alkali Metal Adducts

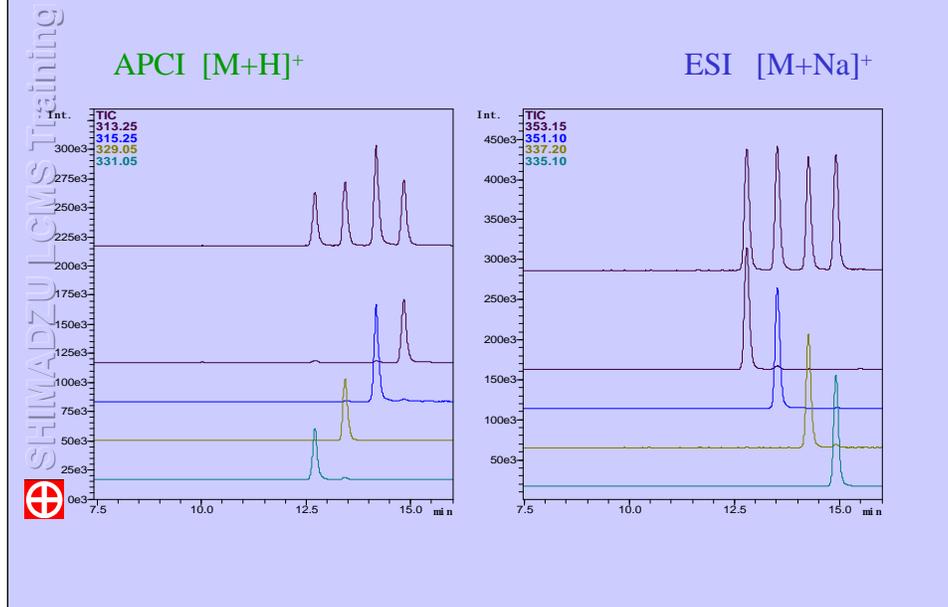
- † Add ammonium acetate at up to 20 mM
- † Dilute sample to reduce analyte concentration
- † Switch to negative ion mode ionization
- † Eliminate water from the mobile phase
- † Use plastic vials
- † Use a bypass valve to keep salts out of the source (they normally elute at void volume on C₁₈ columns)
- † Increase capillary or first skimmer voltage



Sodium and potassium are ubiquitous – they come from all living things, from glass vials, and even from high purity mobile phases. It is pretty difficult to eliminate these entirely.

Ammonium acetate will often displace sodium and potassium adducts, especially if the concentration is high enough, and ammonium adducts are easier to break up. When sodium and potassium adducts show up either as $[M + Na]^+$ or $[2M + Na]^+$, it often indicates that analyte concentration is too high. Diluting the sample can often reduce the adduct formation. Negative ion mode generally eliminates formation of sodium and potassium adducts, but may substitute acetate or chloride adducts instead, but these are easy to break up with backflow gas or adjustment of skimmer voltages. Eliminating water may seem unusual, but ESI often works well without any water present. Just substitute alcohol for water and use a more retentive HPLC column. Flint glass used in sample vials is another source of sodium, so using plastic vials may help, but some samples may be incompatible with plastic. Keeping salts out of the ionization chamber is always a good idea, so use a bypass valve. The last approach, increasing the voltage is often what many people try first, but often results in unwanted fragmentation rather than elimination of adducts. Sodium and potassium adducts are pretty stable and often are used to reduce thermal or voltage induced fragmentation.

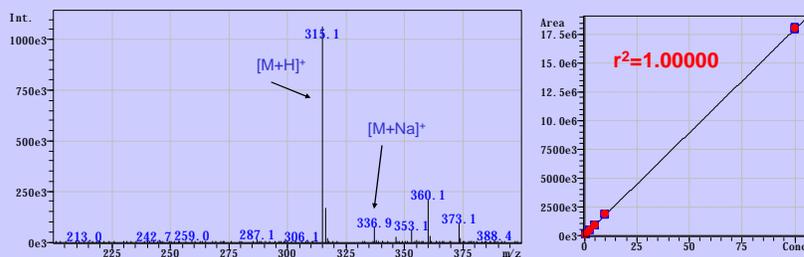
Aflatoxin Comparison



These are a series of aflatoxins. They typically form sodium adducts in ESI unless you add enough ammonium acetate to convert the ions to the ammoniated species, but will form protonated species by APCI.

Aflatoxin B₂, ESI with NH₄OAc

SHIMADZU LCMS Training



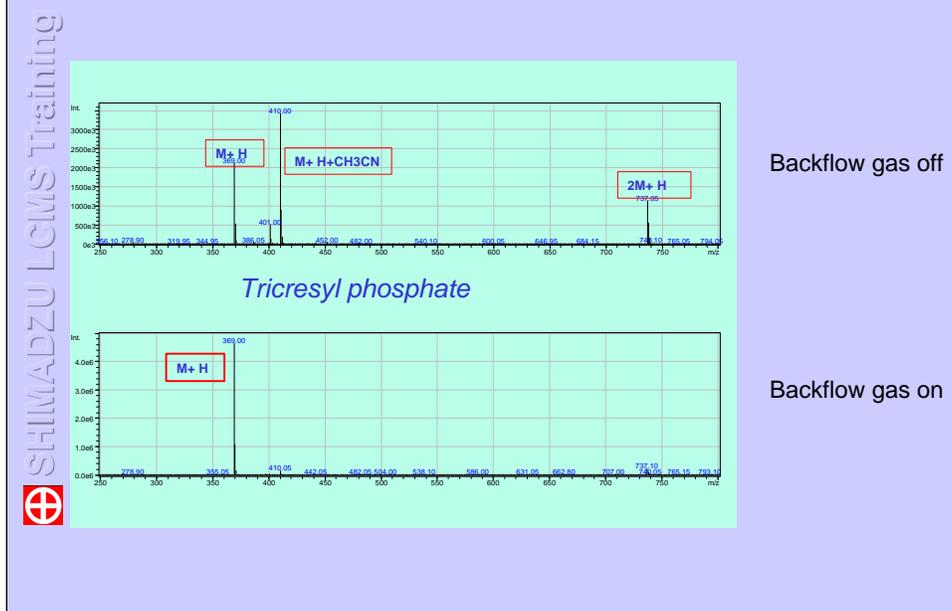
Mass spectra and calibration curves (0.5-100ppb, n=5)

Note low level of sodium adduct at 337 m/z, and no ammonium adduct at 333 m/z



Note reduction of sodium in this example. Ammonium acetate prevents formation of the sodiated species.

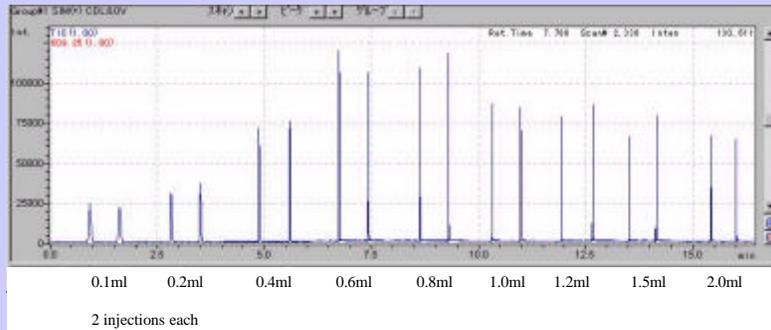
Solvent Adduct Ion Reduction



This slide shows the benefits of a backflow drying gas on solvent adduct ion production. This example is by APCI, but the benefits also apply to ESI. There is an optimum gas flow to remove adducts. Increasing flow too much can result in loss of sensitivity or an increase in noise.

ESI - Flow Performance

Find the flow rate that is optimum for your analysis!



100 % Water mobile phase, FIA mode, Reserpine 10pg/uLX5uL injected



(Results can vary depending the compound, flow rate, mobile phase composition, probe position and voltage conditions)

Most people don't try to run 100% water by ESI at 2 mL/min flow rates. Most ESI specifications are based on flow rates around 0.2 mL/minute flow, but here we see that the highest sensitivity actually could occur around 0.6 mL/min flow. The results will depend on the compound, mobile phase, probe position, and voltages used.

SHIMADZU LCMS Training

Practical Considerations - ESI Columns

Diameter	Theoretical Increase (for ESI)
† 4.6 mm	† 1
† 2.0 mm	† 5x
† 1 mm	† 21x
† 0.3 mm	† 235x
† 0.150 mm	† 2116x



The sharper the peak, the better the sensitivity b ESI. Here are some theoretical improvements possible by using smaller diameter columns (with the appropriate smaller packing material). In reality, the gains are typically not this good. For example, a 1 mm column typically only provides a 5-9x improvement because of the extra column effects (tubing, fittings, etc). To actually achieve these theoretical improvements requires that the plumbing be changed throughout the system.

Practical Considerations - ESI

† For Positive Ions

- ✗ Keep surface tension of droplet low
 - ✗ Increase organic level
 - ✗ Reduce salt levels, reduce ionic strength
- ✗ Keep additive concentration low (below 20 mM)
- ✗ Acetonitrile yields quieter baselines than MeOH but MeOH often gives better ionization for positive ions
- ✗ Keep water content low - it increases ion current & leads to baseline instability
- ✗ Keep flow rates low and use efficient columns
- ✗ Ammonium acetate tends to reduce fragmentation
- ✗ Don't switch from base to acid additives without cleaning with water in between
- ✗ Avoid TFA if possible - it suppresses ionization

Additives may be necessary to promote ionization or for chromatography. Generally additives should be as low as possible. 100mM of ammonium acetate will generally suppress ionization while 10 mM will promote it. Some additives work best at 10 mM or lower concentrations.

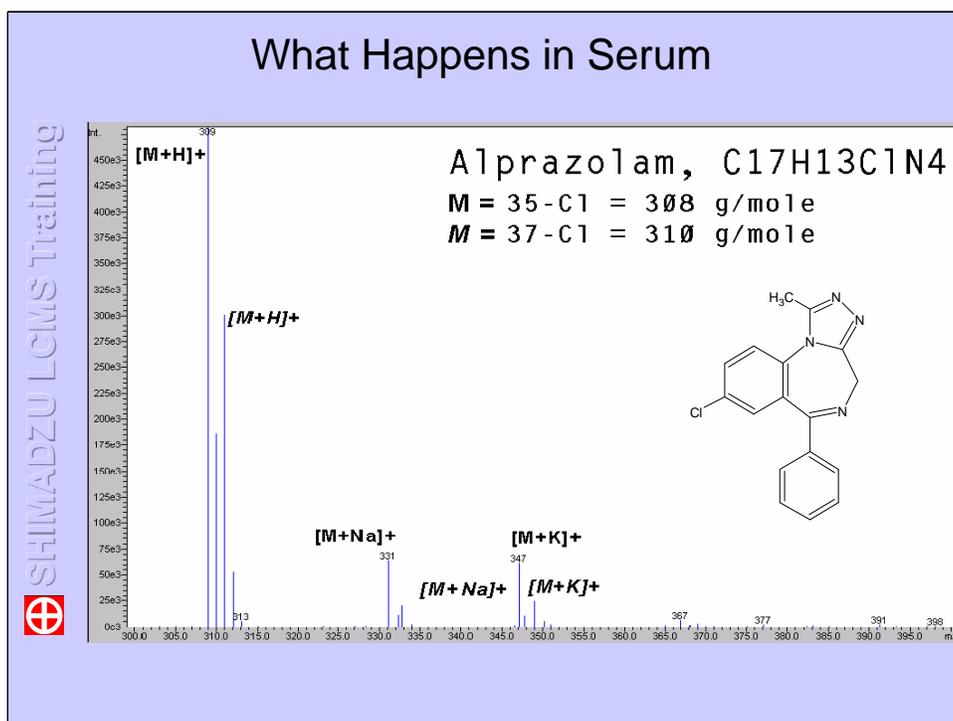
Acetonitrile is generally a cleaner solvent than methanol. It has to do with the starting materials used to prepare the solvents. Methanol is a better protic solvent however and generally produces more ions than acetonitrile. It is a good idea to run a background scan on each new bottle of solvent to see what impurities are present in the solvent before you acquire any data.

Water may be necessary for ESI ionization, but generally less is better. Too much water makes solvent evaporation more difficult, increases ion current (probe leakage current) which can result in arcing, and makes the source dirty faster (because of all the salts that dissolve in water). ESI can work in 100% organic solvents also, but is usually only done in mixtures of solvent with water.

When you switch methods from acid to base, the in between mixture sprays an acid into a chamber already contaminated with base. The result is formation of salts. Although the salts may eventually be volatile, the presence of so much salt can affect ionization. It is always a good idea to clean the source with clean water before switching from one to the other.

TFA is actually an ion pair type chemical. It can cause many compounds to produce fewer ions. For example, TFA added to nearly any peptide will produce smaller peaks than formic acid will under the same chromatographic conditions. TFA is a legacy compound left over from the days when HPLC columns didn't work well with strongly basic compounds. The TFA was

What Happens in Serum



This is a very common situation with the analysis of many compounds from biological sources. It doesn't matter whether the source was urine, plasma, wood sap or hair. All living systems contain sodium and potassium.

Since many compounds can create ions by protonation, many can just as easily replace the proton with sodium or potassium. Hydroxyl containing compounds are nearly always affected.

The presence of sodium and potassium ADDUCTS affects quantitation adversely since the ratio of the +1 ion and the +Na or +K ions will depend on the amount of sodium or potassium present as well as pH. Since these can vary, the ion abundances will also vary.

It is often possible to force the ionization in one direction by desalting the samples or by swamping the ionization by using a large excess of ammonium acetate to shift the equilibrium in one direction.

Liquid-Liquid Extraction of Bovine Serum

- ? Spike Alprazolam and Triazolam into BSC.
- ? Perform Serial Dilution of Stock Solution to create solutions with target compounds at 5, 10, 25, 50 and 100 Ng/ml in BSC
- ? Spike Internal Standard at 50 Ng/ml in each solution.
- ? Buffer BSC Samples with 1 N NaOH to pH 10.5
- ? Extract 10 ml buffered BSC with 6 ml Hexane:Isoamyl Alcohol, 97:3. Shake and Vortex for 3 minutes.
- ? Centrifuge Coagulant at r.t. then place in freezer.
- ? Decant Hexane:Isoamyl Alcohol off of frozen BSC layer.
- ? Nitrogen Blow Dry organic layer to dryness.
- ? Reconstitute in 250 μ L Methanol and Centrifuge.
- ? Transfer to conical Autosampler vials and Analyze by SIM LC/MS

Earlier we showed the recovery of reserpine from calf serum using solid phase extraction columns. For alprazolam, we will use a liquid-liquid extraction approach that reduces impurities better for trace analysis. Here we see the entire sequence for the analysis.

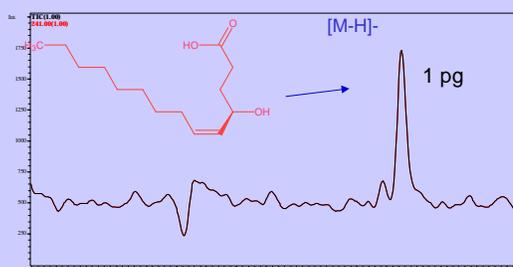
Quantitative Performance

Repetative Injection of Serum Extract				
	Extract Level	Calculated Triazolam Concentration	Calculated Alprazolam Concentration	
25 ng/mL	III (25)	26.09	24.51	
	III (25)	26.10	25.05	
	III (25)	26.06	25.56	
	III (25)	26.38	25.41	
	III (25)	26.68	25.05	
	III (25)	26.49	25.46	
	III (25)	26.72	25.56	
	III (25)	26.55	25.14	
	III (25)	26.37	25.35	
	III (25)	26.17	25.32	
N		10	10	
Mean		26.36	25.24	
Standard Deviation		0.2473	0.3187	
Percent Coefficient of Variation (%CV)		0.9383	1.2626	

These results show that even at picogram levels injected, excellent quantitative results can be obtained even from plasma. What isn't obvious here is that the HPLC method was chosen to cause overlap of the compounds with the internal standard. So even with little separation, excellent results are possible.

Practical Considerations - ESI

- † For negative ion analysis
 - ⊗ Raise pH
 - ⊗ Use methanol or IPA
 - ⊗ Consider using zero grade air as nebulizer gas
 - ⊗ Add trace (0.1% or less) chloroform, or chlorinated organic to organic portion of mobile phase
 - ⊗ Small amounts of IPA or THF promote ionization



Negative ions behave a lot differently from positive ions and you may need to change parameters to see these with adequate sensitivity. As a first step, reduce CDL voltage to 0 volts and try increasing in both directions (+ and -) from there until you have adequate sensitivity (note, this is automatic in the LCMS-2010)

Increase the DEF voltage to a value slightly higher than the tune result

Raising the pH often causes the extraction of a proton from molecules that can be ionized this way, such as organic acids, alcohols, sugars, etc.

Use acetonitrile - it works well in negative ion mode. Methanol can suppress ionization nearly completely.

Air often increases sensitivity in ESI negative ion mode. It doesn't take much oxygen to do this. The only requirement is that the air be free of organics (zero grade air with a carbon trap scavenger).

Many chlorinated compounds aid in the extraction of a proton from many molecules. It forms Cl radical ions that scavenge excess protons.

How to Obtain Good ESI Results

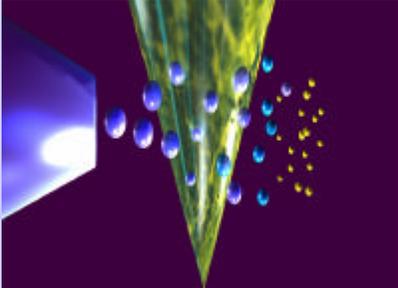
- † Eliminate sodium
 - ⌘ From glass vials, solvent containers, sample matrix
- † Use divert valve
 - ⌘ Eliminate inorganic salts at void volume of HPLC runs
 - ⌘ Eliminate poorly soluble components after peaks of interest
- † Eliminate soaps and detergents
- † Keep leakage current low
 - ⌘ Avoid TFA and strong acids
 - ⌘ Keep sample concentration low
 - ⌘ Keep acid concentration low
 - ⌘ Keep probe voltage low
 - ⌘ Increase % of organic solvents, but always have some water in the mobile phase

Sodium is everywhere. It can come in from samples, from waters, and even from the flint glass used in sample vials. Some plastic vials can be used to reduce this, but it tends to show up from sources no one expects. The best way to reduce the effects of sodium is to use HPLC =- sodium will show up at the void volume. If you use a divert valve, the sodium can be kept out of the spray chamber of the LCMS system.

SHIMADZU LCMS Training

APCI

- † **Advantages**
 - ⌘ Works with many compounds, especially non-polar
 - ⌘ Rugged
 - ⌘ Handles HPLC flow rates up to 2 mL/min
 - ⌘ Only produces singly charged ions
- † **Disadvantages**
 - ⌘ Compound must have some vapor pressure
 - ⌘ Mostly limited to around masses under 1000 AMU
 - ⌘ Thermal degradation possible
 - ⌘ Tends to form solvent adducts
 - ⌘ Can produce different ionization mechanisms for same molecule



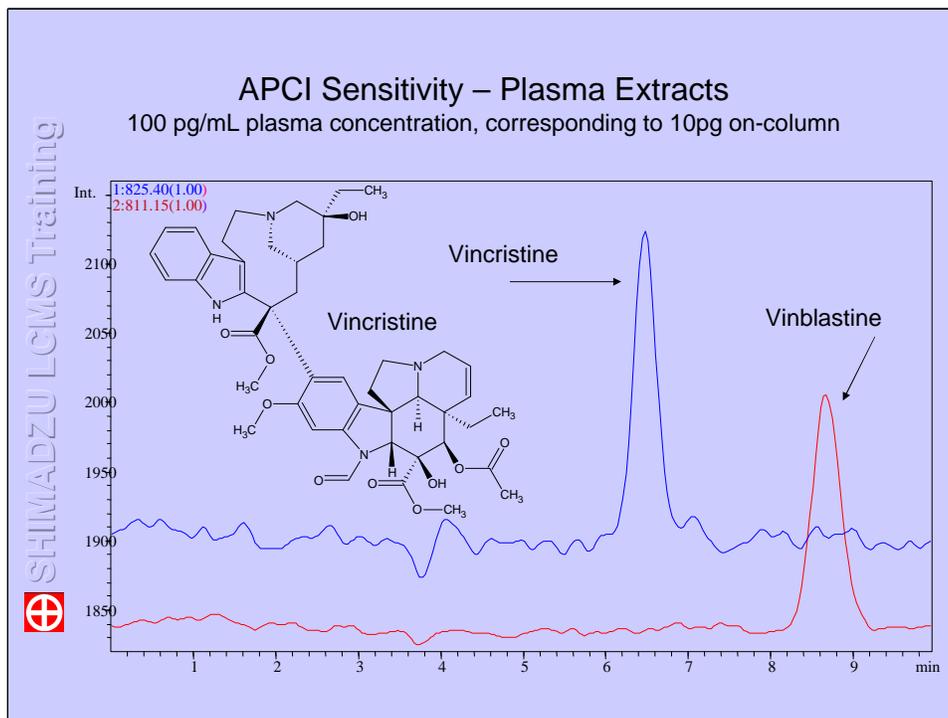

The mechanisms of ionization for APCI are different than for Electrospray. Ions are created from the nebulizer gas and mobile phase first and then these ions provide the charge for a less volatile analyte usually by charge transfer mechanisms. So you need to pay attention to the mobile phase in order to get APCI to work. Also, some compounds can ionize directly in a corona discharge without charge transfer. APCI can often produce free radical ions of conjugated aromatics, and rearrangements not seen by ESI. For example, when analyzing some long chain conjugates, it is not uncommon to find $[M+1]^+$, $[M+0]^+$ and $[M-1]^+$ all in the same run and all as positive ions. The only ions you don't see by APCI are multiply charged ions since the gas phase process only allows single charge mechanisms to take place.



APCI Practical Considerations

- † Molecule should be thermally stable
 - ≈ High vapor pressure is helpful
- † Buffers must be volatile but are usually not needed
- † Column and flow rate generally not critical
- † Detection limits are very compound dependent
- † Ideal performance depends on gas phase chemistry
 - ≈ For + ions, solute should have higher proton affinity than mobile phase
 - ≈ for - ions, solute should have higher acidity than mobile phase
- † Nebulizer gas plays a critical role in ionization chemistry
 - ≈ For + ions, nitrogen works best
 - ≈ For - ions, some oxygen is necessary

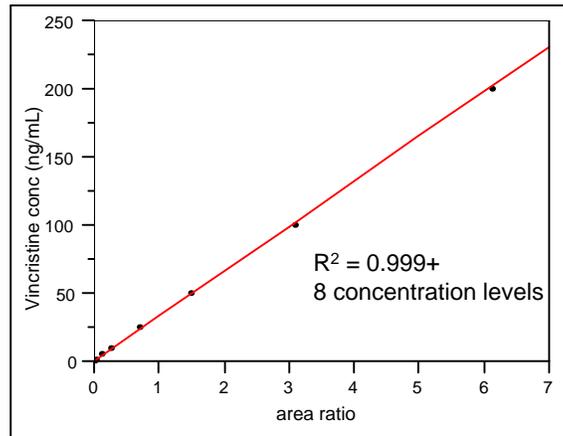
A rule of thumb: if you can smell your compound, it will probably work by APCI. Having vapor pressure indicates it should be stable enough to form a gas by APCI. Most buffers are not needed for APCI except for chromatographic purposes. APCI generates its own acids and bases in the gas phase. Regarding columns, fatter columns, and broader peaks are better for APCI because the ionization is a two step process. First you have to generate the gas phase acid from the mobile phase, then you have to do a charge transfer to your solute target. Having the solute spread out over a larger peak area allows more interactions with the gas phase ions in the APCI corona discharge area allowing more charge transfers to take place.



Here is an example of a large compound (mass = 825) being analyzed by APCI at fairly low levels. In this case the drug was spiked into human plasma, extracted using SPE, dried and reconstituted. An aliquot containing 10 pg of the drugs was injected using a HPLC column. This level is approximately 100 to 1000 times better than has been reported in the literature as of 9/2003

Vincristine Calibration – Plasma Extracts

100 pg/ml to 200 ng/ml in human plasma
(10 pg to 20 ng injected on-column)
Internal standard = vinorelbine



APCI Plasma Results

- † Both vinblastine and vincristine can be quantitated at the low picogram level from plasma (typically to 10 pg injected on-column)
- † Limit of Quantitation is 50 pg/mL in plasma
- † Limit of Detection is 1 pg/mL in plasma
- † Linearity over the range of 50 pg/mL to 200 ng/mL gives $R^2 = 0.999$ for 8 concentration levels for both vincristine and vinblastine
- † No plasma based interferences were found at the target m/z and retention times used
- † Complete analysis is possible in 10 minutes or less even at the low picogram range.



APCI Practical Considerations

† Negative mode

- ✗ Air or oxygenated solvents are needed to form ions
- ✗ Carbon builds up on APCI electrode when acetonitrile used at high concentrations

† Both polarity modes

- ✗ Impurities in solvent, and oxidized / reduced compounds in the matrix can coat the electrode.
 - ✗ Inspect often - clean or polish as needed
- ✗ Methanol and IPA tend to keep electrode cleaner



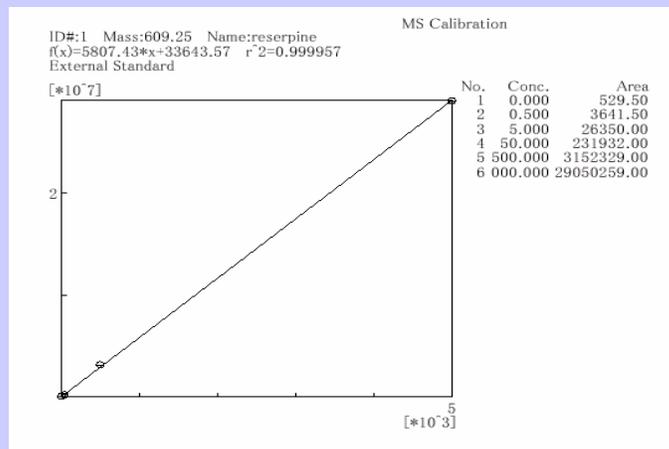
Linear Dynamic Range

- † **Related to concentration limitation aspects of ion ejection from droplets**
 - ⚡ Similar charged droplets build up charge and repel each other
 - ⚡ At high concentrations, droplets crowd out other droplets trying to 'evaporate'
- † **Affected by all other ions present in addition to solute**
- † **Linearity & Dynamic Range**
 - ⚡ Generally 2.5 to 3 orders of magnitude, occasionally 4-5 orders
 - ⚡ When doing linear fit, $1/X^2$ is typically used for weighting
 - ⚡ Best fit is usually a weighted quadratic
- † **Detectors can 'saturate' at high concentrations**

Linear Dynamic Range - ESI

4 orders magnitude

SHIMADZU LCMS Training



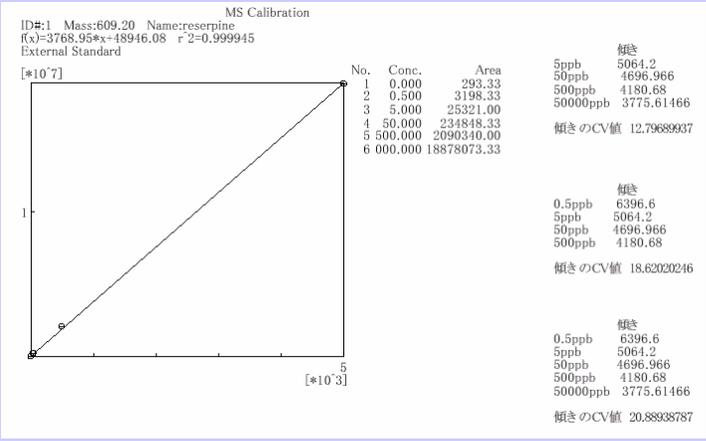
Reserpine on column:

<0.5 pg - 5000 pg, $r^2 = 0.999957$

Most LCMS systems are linear to three orders of magnitude. Quads can usually go to 4 or 5 orders of magnitude depending on the column and conditions. The limiting factors are noise at the low end, and detector dynamic range at the top end. The top end is determined by total signal – that is the signal due to the compound and the offset due to mobile phase impurities. Here we see reserpine over 4 orders of magnitude with a very linear calibration for ESI. The limitation here is how well the different concentrations can be diluted from the original stock solution.

Linear Dynamic Range - APCI

4 orders magnitude



Reserpine on column:
 <0.5 pg - 5000 pg, r² = 0.999945

Here is reserpine analyzed by APCI showing 4 orders of linearity. Some users have difficulty with APCI since it tends to be a noisier interface and will saturate more quickly, but here we see that the technique is certainly linear and has a high dynamic range.

How to Improve Linearity

- † **Keep sample concentrations low**
 - ⌘ Operate well below saturation level of detector
 - ⌘ Use smaller injections for ESI
 - ⌘ Use larger, more dilute injections for APCI
- † **Run faster acquisition speed**
 - ⌘ More data points per second
 - ⌘ SIM will have better linearity than Scan
 - ⌘ Fewest m/z channels gives best results
- † **Minimize baseline noise**
 - ⌘ Use fresh, clean mobile phases
 - ⌘ Keep additive levels low
 - ⌘ Reduce water content
 - ⌘ Keep source clean
 - ⌘ Both Probe and Inlet

These rules are fairly general and apply to both quadrupole systems and ion traps.



General Approach to LC/MS Methods

1. Pick the source for ionization (i.e., APCI or ESI) based on knowledge of the molecule and results required
2. Pick the mobile phase that gives the best sensitivity (s/n)
3. Use an additive (or buffer) only if necessary to improve the sensitivity and keep concentration as low as possible
4. Pick a column that will provide the separation, resolution and peak shape required using the mobile phase and additive conditions already selected above
5. Pick a standard based on separation from target, ability to ionize in similar fashion, and based on having a clean blank from the matrix at that mass

This may seem backwards to traditional chromatographers who pick the column first, then the mobile phase, then the detector parameters. For LCMS, it is better to pick the LCMS conditions first, then the mobile phase that provides ionization for that type of source, and lastly the column that will separate under those conditions. Traditional chromatography is done with mostly neutral species and generally produces less signal at the LCMS than this approach. For example, if you want to do ESI of a base, you would pick a mobile phase that dissolves the samples, a volatile ion pair reagent that will enable chromatography to take place, but will be volatile enough to turn ion pairs back into ions, and then select a type of column that will separate under those conditions. A traditional approach might be to do ion exchange chromatography, or to create derivatives of the compounds and then try to ionize them at the end. Both of these traditional approaches will result in more difficult methods that might not work by LCMS.



Is the molecule heat sensitive?

YES

Use ESI

- † Proteins
- † Peptides
- † Carbohydrates
- † Molecules > 1000
mw

NO

Use APCI

- † Aromatic compounds
- † Steroids
- † Nonionic Surfactants

Ionization in Positive or Negative Mode?

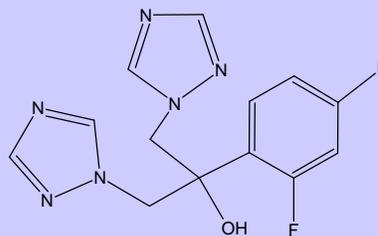
- ✍ Depends on structures and properties of compounds
 - ✍ High proton affinity: C-O, C-N double and triple bonds, basic compound tend to form positive ions
 - ✍ Low proton affinity: -COOH, -F, -Cl, -HSO₃, phenols, aniline and sugars tend to form negative ions due to stronger tendency to donate proton
 - ✍ Many compounds form both positive & negative ions due to multi function groups
- ✍ Acquire both positive & negative mass spectra in multi sequence mode and compare sensitivity and spectrum quality

5. MS method development usually starts with studying the structure and properties of target compounds.

- (1) N, O-containing compounds and basic compound generate only positive ions due to stronger proton affinity ✍ + mode only.
- (2) Acidic compounds like -COOH, F-, Cl, -HSO₃ generate only negative ions due to stronger tendency to donate proton.
- (3) Neutral compounds like benzene cannot be ionized, not suitable for LCMS analysis;
- (4) Phenols can be ionized with APCI to generate negative ions.
- (5) Aniline can be ionized with ESI to generate negative ions.
- (6) Sugars usually can be ionized to generate negative ions.

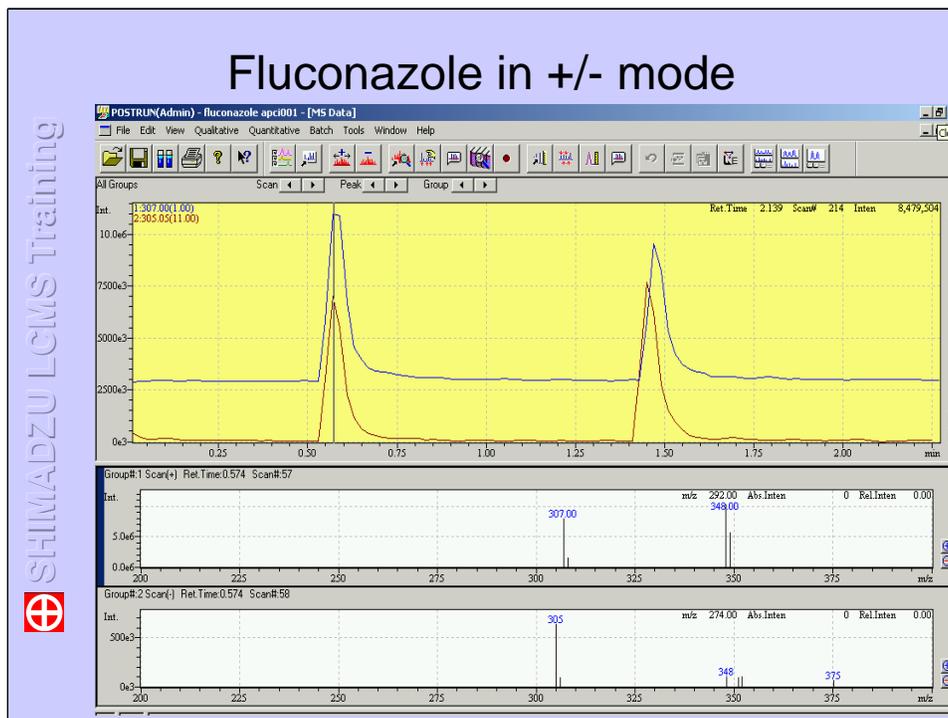
Fluconazole Analysis

- † Antifungal agent used to treat complications of AIDS
- † Relatively water insoluble drug
- † Forms adducts in ESI
- † Protonates easily at neutral pH
- † pKa = 5.28 (+), 4.47 (++)



MW=306

Looking at a compound like this most novices will expect that it will produce protonated ions easily because of all the nitrogens. Few will expect that it will also produce negative ions from the hydroxyl group. In fact it can produce both by APCI. Normally, hydroxyl containing compounds will form sodium adducts easily, and compounds like this may even produce multiply charged species by ESI at low pH. Note that the difference between singly and doubly charged is a pKa difference of less than 1 unit, so pH adjustment may be rather critical. None of these issues will be a problem if you analyze this compound by APCI



Here is an APCI analysis of fluconazole. The upper trace in blue is the protonated species (+), and the red trace is the deprotonated species (-). The spectra show that the (+) species produces a M+acetonitrile adduct, while the (-) ion as little of the adduct, and mostly the m-H species at m/z 305. APCI only produces singly charged ions, so there is no worry about a multiply charged species forming here either. One additional comment is that the signal intensity of the 307 (+) ion is about 10 times stronger than the 305 (-) ion, but notice that the chromatograms show similar signal to noise values! It means that both produce good quantitative results and that noise levels in negative ion mode are lower than in positive ion mode.

Mobile phase practical considerations

- † What pH is needed for ionization?
 - ✎ Acidic pH favors positive ion LCMS
 - ✎ Amine containing compounds, most drugs
 - ✎ Neutral pH favors negative ion LCMS
 - ✎ Many metabolites

- † What if the pH to obtain good chromatography suppresses ionization?
 - ✎ Use ammonium acetate (unbuffered)
 - ✎ Use post-column addition for pH change



HPLC Compromises for LCMS

† Change Conditions to Increase Ionization

- ✍ Change solvents
- ✍ Change buffer type
- ✍ Use additives to form adducts
- ✍ Change pH conditions post column
- ✍ Run at lower flow rate or split flow

† Control Parameters

- ✍ Control pH carefully
- ✍ Use splitter to adjust delivered flow rate

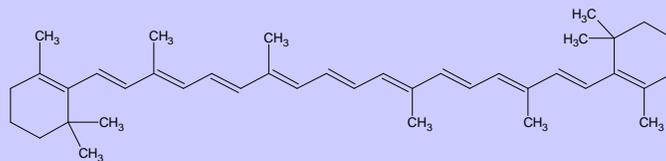
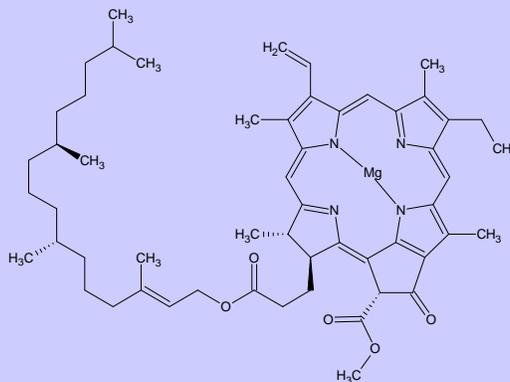


One other way to increase ionization and avoid some of the pH problems is to use a pH modifier that is more volatile than your solute. It evaporates in the desolvation step and leaves your compound as an ion. Fluorinated organic acids usually work in this situation.

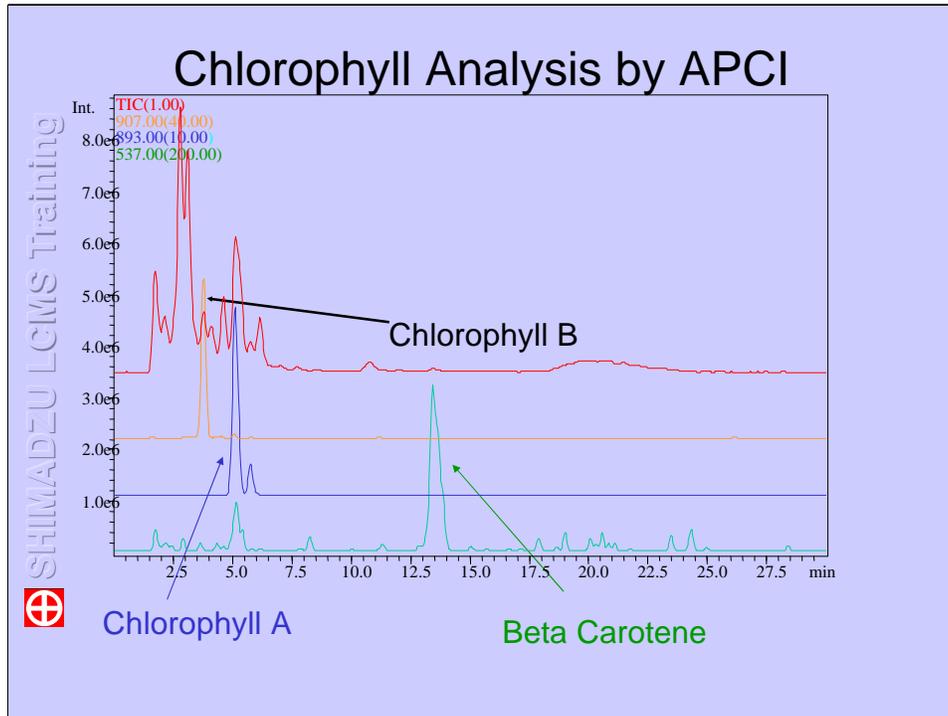
Applications - 4

† How would you analyze chlorophyll A:

in the presence of β -carotene:



Although chlorophyll b shown above contains a number of nitrogen atoms and probably can be ionized by ESI or APCI. The beta carotene contains only carbon and hydrogen and is not soluble enough for ESI. Chlorophyll is on the high side of molecular weights at m/z 906 for APCI, but is stable enough to analyze this way.



Conditions for this analysis:

Column	:	STR ODS-II (2.0 mm I.D. x 150 mm L)
Mobile phase		methanol -2-propanol (3:1)
Flow rate		0.2 mL/min
Probe voltage		+3.5 kV (APCI-positive mode)

Effects of Additives on APCI Negative Ionization

Acid	M/Z	10 mM methyl morpholine	10 mM NH ₄ Acetate	10 mM Formic
Ibuprofen	205	100	96	3
Acetylsalicylic acid	179	100	28	14
Estradiol	271	100	16	1
Benzohydroxamic acid	136	100	30	1
3-(4-methoxy 2-phenyl)-propionic acid	179	100	47	1



† source: Schaefer & Dixon, JASMS, #7, 1996, pp 1059-1069

Buffer and pH Control for LC/MS

Non-volatile buffers like phosphate are not recommended

Use volatile buffers to replace phosphate such as:

NH_4OAc /HOAc, NH_3 /TFA etc

Use a lower buffer concentration: 10 – 50 mM

pH control:

- pH 1.8 ~ 2.5 : TFA, conc. < 0.1%
- pH 2.5 ~ 4 : HCOOH, conc. ~ 0.1%
- pH 4 ~ 5 : HOAc, conc. 0.1~5%
- pH 7 : NH_4OAc
- pH > 7 : NH_3 aqueous solution



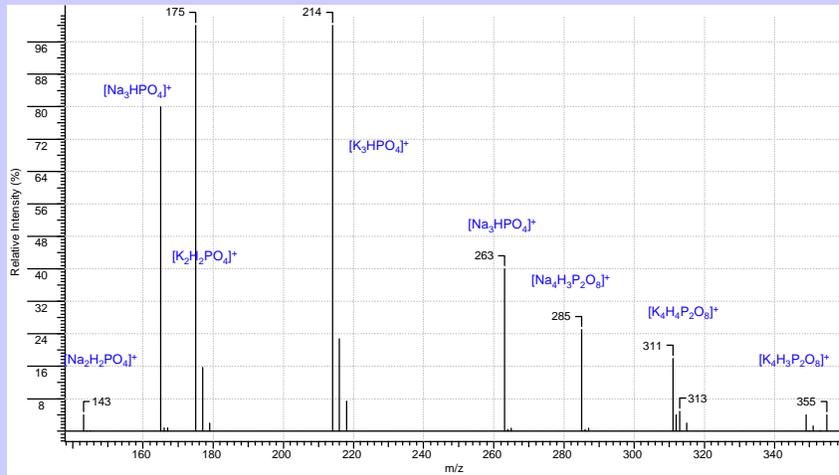
Use of Phosphate Buffer???

- † Phosphate buffer is very widely used because of the following reasons.
 - ≈ Low absorption at short wavelength
 - ≈ Wide range of pH buffer action (pH=2-4, 6-8, 11-13)
 - ≈ History of applications for UV detectors
- † One big disadvantage to use phosphate buffer with MS is crystallization that causes **clogging** in the interface of LC/MS.
- † **Volatile buffers** such as ammonium acetate or ammonium formate should be used instead of phosphate buffer.
- † Phosphates will increase interface current leading to increased oxidation or reduction.
- † If pH is adjusted using a volatile buffer, results similar to the data obtained by phosphate buffer are obtained.
- † From a sensitivity view point, volatile buffers are always preferable.



Phosphates Reduce ESI Signal Intensity

☛ Phosphates can also lead to artifact peaks:



Phosphates will reduce signal intensity for nearly everything by ESI. The reason for this is that phosphates affect the surface properties of droplets and interfere with solute ions migrating to the surface. Even a small phosphate concentration (10 mM and less) can reduce sensitivity by 50-90%.

Common LC/MS Problems

- † Dissolved gasses - Carbon Dioxide
 - ≈ CO₂ will dissolve in water lowering pH
- † Non-buffer 'buffer' - no capacity
- † Flow perturbations - increases noise
 - ≈ Baseline noise can be affected by pressure changes – consider using better pumping systems for highest sensitivity
- † Adducts with mobile phase
 - ≈ Look for M+41 (acetonitrile) adducts or M+solvent for other solvents
- † Salts (Na, K, Ca)
 - ≈ Can originate from vials, tubing, pipettes, etc
- † Purity of modifiers
 - ≈ Methanol and fluorinated acids often contain considerable impurities that give high noise levels.





Contamination Sources

- † Dirty containers (detergents and soaps)
- † Vials, pipettes, glassware (Na⁺ from soda-lime glass)
 - ⚡ Non-borosilicate vials are available
- † Bottle liners (phthalates, silicones, antioxidants)
- † Syringes with metal plungers dissolve in acids
- † TFA sticks to metal & increases background in negative ion mode (113 m/z)
- † Oils (from fingerprints)



Keeping the LCMS Clean

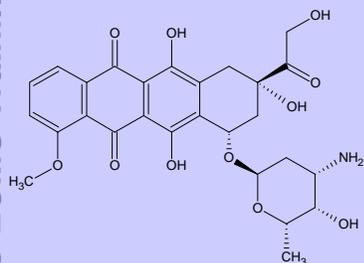
- † Use valve to eliminate salts and late eluting impurities
- † Avoid using soap to clean glassware or it will be your largest peak
- † Reduce metals in contact with sample when analyzing proteins and peptides
- † Avoid plastic containers and plastic caps
- † Watch out for glycerol in RO water
- † Keep solvent containers covered
 - ✍ Phthalates in air can dissolve in organic solvents

Typical Anticancer Drugs Analyzed by LCMS

- † Anthrocyclines
- † Taxol/Taxotere
- † Cisplatin
- † Ifosfamide
- † Vinblastine/ vincristine
- † Parthenolide
- † Combinations of various drugs



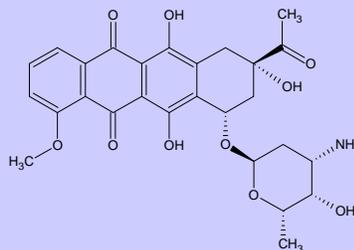
Anthrocyclines



Epirubicin

pKa = 7.12

Mass = 543.2



Daunomycin (IS)

pKa = 7.15

Mass = 527.2

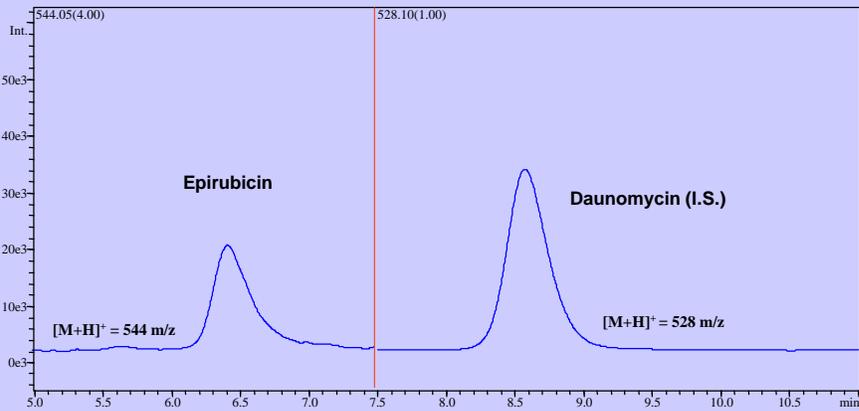
Epirubicin

- † Used as experimental antitumor agent
 - ⌘ Soft tissue sarcomas
- † Typical dosing is critical
 - ⌘ Cardiac toxicity limits upper dose
 - ⌘ Dosing often too low by a factor of 2
 - ⌘ Dose must be carefully individualized
- † Normally analyzed by fluorescence
- † H_3PO_4 sample treatment allows recovery from serum proteins



Epirubicin Chromatogram

5 ng/mL plasma



Column: Synergi Polar RP (Phenomenex)
C18 250 x 2 mm, 4 μ m
0.2 ml/min flow rate
Bypass valve 4 - 11 minutes

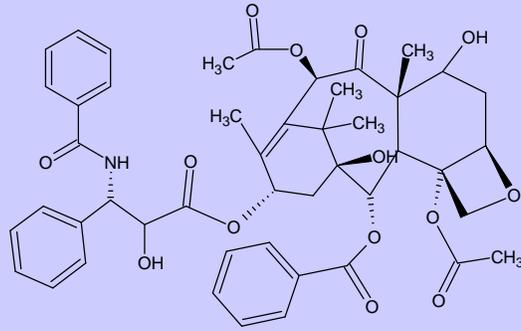
A. Aqueous phase: H₂O + 0.05% TFA
B. Organic phase: 50:50 Acetonitrile + 0.03% formic acid
Isocratic elution 60% B

Epirubicin Assay

- † 200 samples for method validation
- † 400 patient samples run
- † Sensitivity for LOQ is 0.5 ng/ml in plasma
- † Accuracy 90-105% for all samples
- † Selectivity - no interfering peaks
 - ≈ 15 different plasma lots - no interfering peaks
 - ≈ Interday and intraday CV less than 10% at all therapeutic levels
- † Stability good for up to two months



Paclitaxel

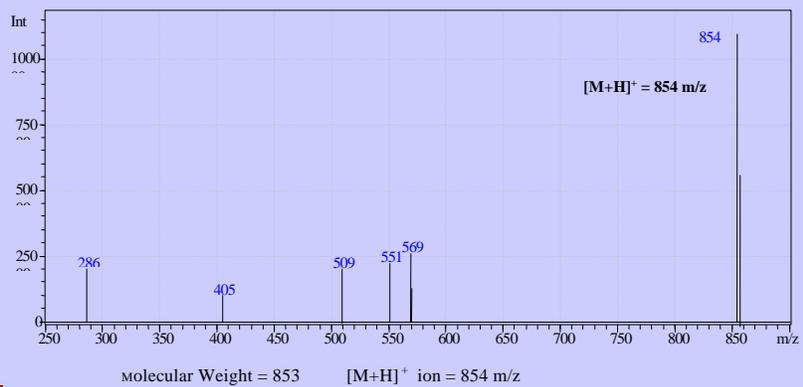


Paclitaxel by LC/MS

- † Most methods done by electrospray
 - ✍ Compound can acid hydrolyze easily
 - ✍ Forms sodium and potassium adducts easily
 - ✍ Some procedures add sodium deliberately
- † APCI
 - ✍ Ionization all done in gas phase
 - ✍ Gives simple MS spectra
 - ✍ No sodium or potassium adducts
 - ✍ Sensitivity comparable with ESI
 - ✍ Fewer adducts and decomposition problems

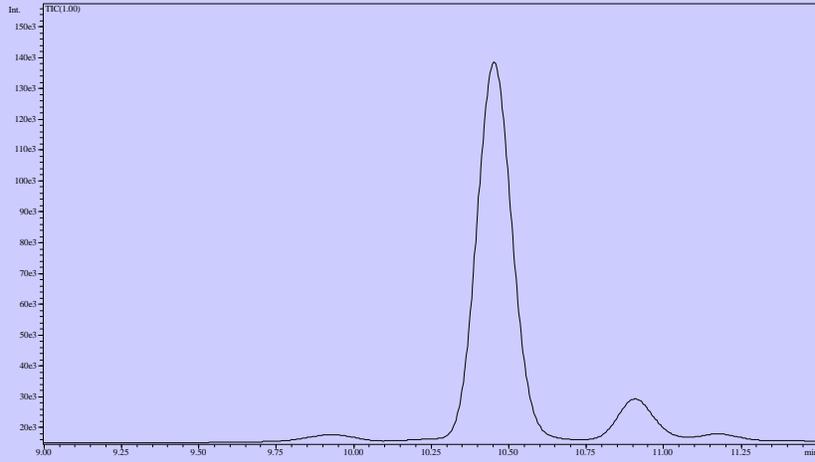
Paclitaxel Spectrum by ESI

SHIMADZU LCMS Training



Paclitaxel by APCI

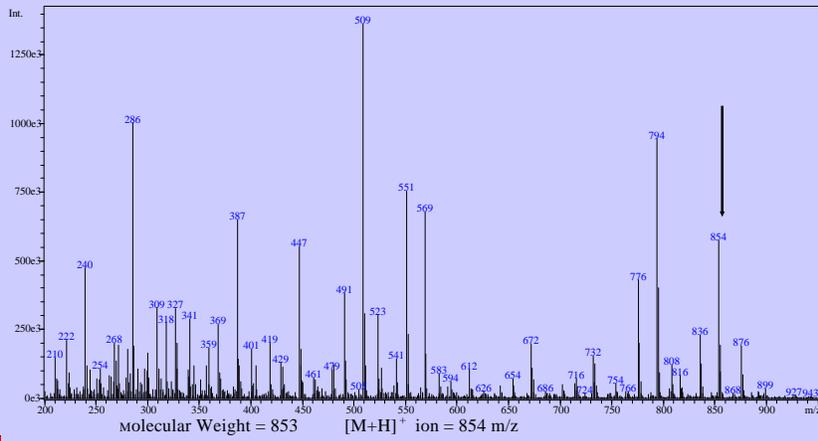
SHIMADZU LCMS Training



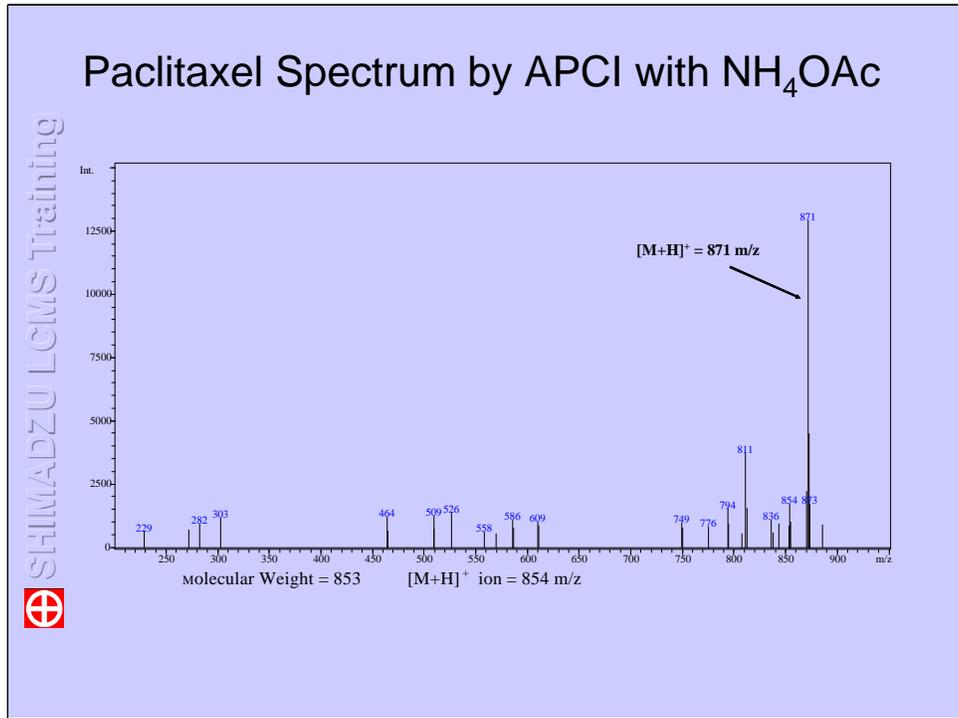
Paclitaxel Spectrum by APCI

[M+H]⁺ = 871 m/z

SHIMADZU LCMS Training



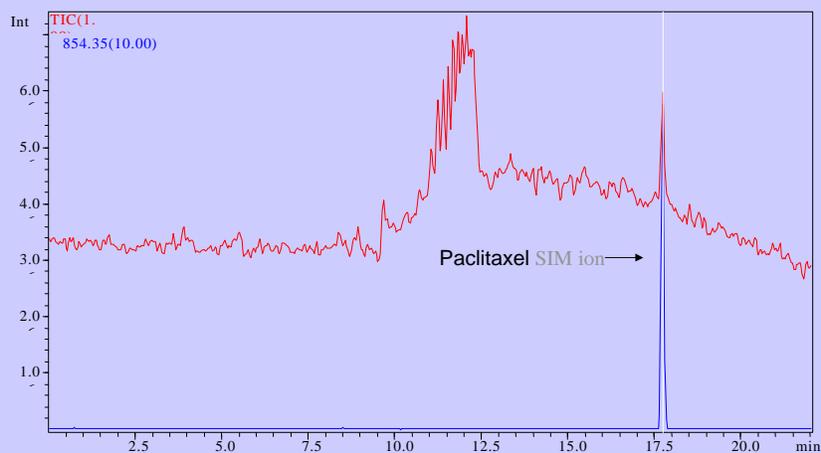
Molecular Weight = 853 [M+H]⁺ ion = 854 m/z



Here we see Taxol at 871 m/z as the ammonium adduct. 10 mM of NH₄OAc will convert nearly all of the dissociated species to the ammonium adduct and stabilize the molecule from thermal breakdown. This analysis is done with the backflow gas turned off. Turning on a counterflow gas will convert the ammoniated species back to the protonated species (next slide)

Paclitaxel Spiked Plasma by APCI

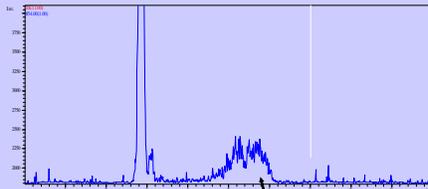
SHIMADZU LCMS Training



Use Bypass Valve to Prevent Contamination

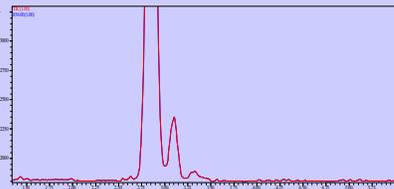
SHIMADZU LCMS Training

Taxol analysis without valve



Low solubility ethoxylated soybean oil emulsifier.
Note spikes from earlier samples.

Taxol analysis with valve



Valve used to eliminate polar impurities at V_0 , and ethoxylated soybean oil at end of chromatogram. (Note injection size has been increased to show additional peaks)

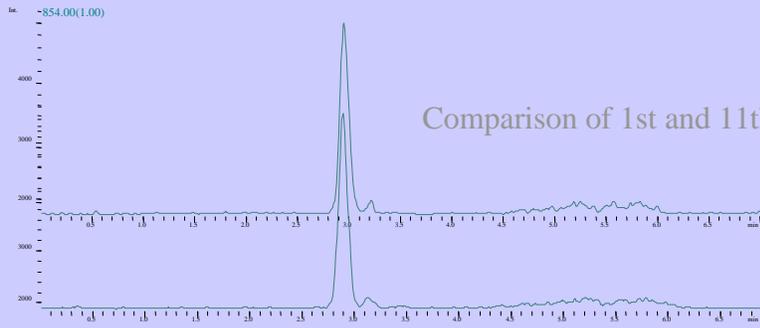


This is an analysis showing what can happen when you try to analyze a compound that has a lot of background impurities in it. Taxol is often given in a suspension in a type of emulsifier such as ethoxylated soybean oil. The oil behaves like a detergent. After a few analyses, the oil has contaminated the source of the LCMS resulting in spikes and high noise levels. This is shown in blue. The red trace is the same analysis after the same samples are analyzed using a divert valve to keep the salts and detergents out of the LCMS source. Basically the valve is closed (bypass position) during the first and last part of the chromatography. The red trace is performed with a larger injection to enable detection of several peaks of interest, but even with larger injections or greater gain settings, there is no spiking because the source is kept clean by the valve.

Reproducibility

paclitaxel + emulsifier

SHIMADZU LCMS Training



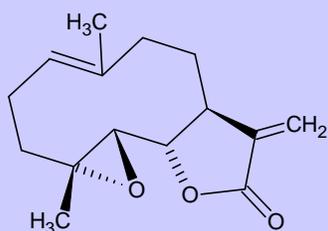
CV <10% at 25 pg/mL plasma

Paclitaxel

- † 200 samples for method validation
- † 500 patient samples run
- † Being used routinely for low dose use in a clinical trial



Parthenolide



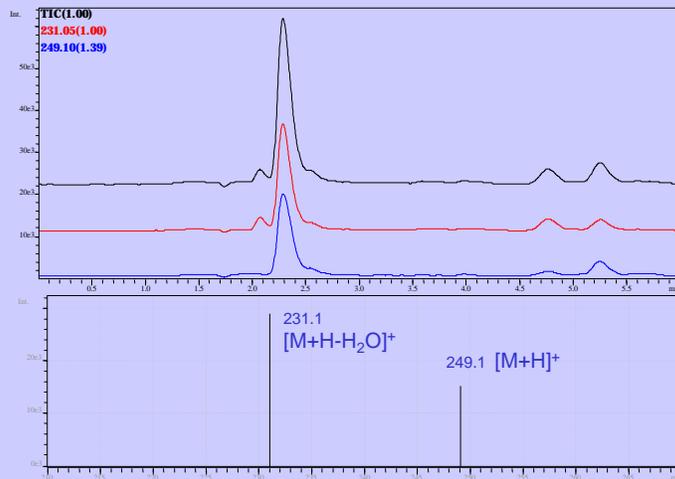
Sesquiterpene lactone
Feverfew source
In clinical trials for cancer
treatment

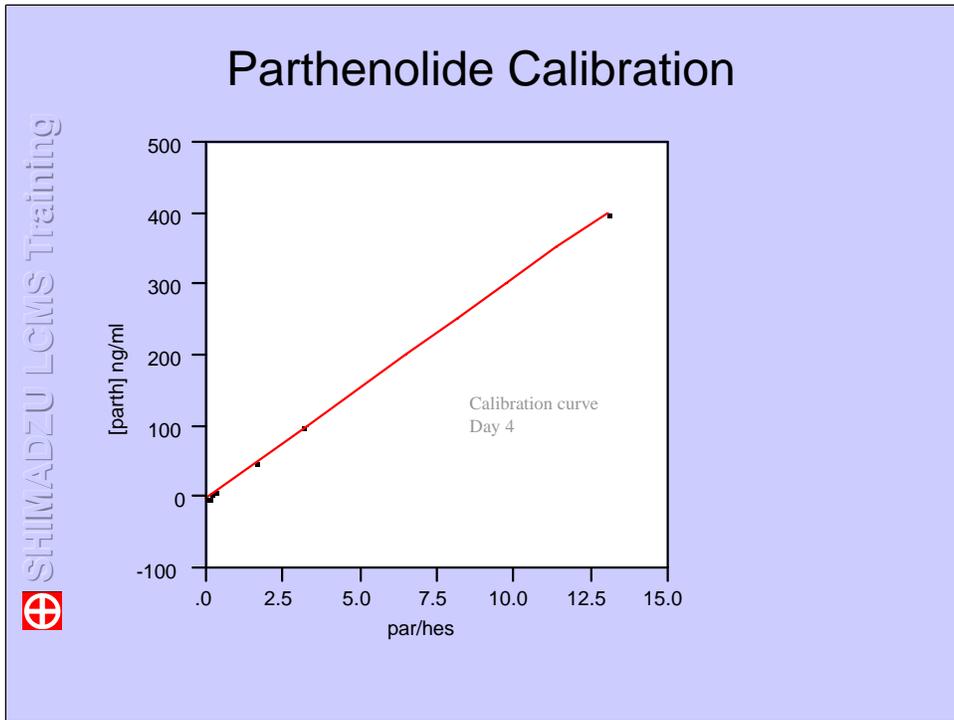
MW 248.32



Parthenolide Chromatography

SHIMADZU LCMS Training





This represents the worst data after 4 days. Standards are stable for up to 4 days use.

Parthenolide Calibration Stability

Day	Standard Curve Equation	R ²
1	PC = 0.19073 + 25.9341 (par/hes)	0.987152
2	PC = 1.09224 + 25.8044 (par/hes)	0.999844
3	PC = -0.0097 + 25.9365 (par/hes)	0.994902
4	PC = 0.1014 + 35.1139 (par/hes)	0.933759

PC = parthenolide concentration (ng/ml), par/hes = peak area for parthenolide divided by the peak area of internal standard



Parthenolide

Intra-Assay Results

Table 2: Intra-Assay Results

Target	Range	Mean \pm S.D.	CV	Recovery
2 ng/ml (n = 10)	1.73 – 2.24	1.88 \pm 0.08	4.3%	94%
25 ng/ml (n = 7)	24	24 \pm 0.01	4.5%	96%
200 ng/ml (n = 8)	214 – 219	219 \pm 0.009	0.4%	108%



Parthenolide

Inter-Assay Results

SHIMADZU LCMS Training

Target	Range	Mean \pm S.D.	CV	Recovery
2 ng/ml (n = 7)	1.7 – 2.1	1.9 + 0.16	8.5%	95%
25 ng/ml (n = 7)	23 – 25	23.6 + 0.79	3.3%	94%
200 ng/ml (n = 7)	182 – 214	197 + 11.4	5.8%	98.5%





Parthenolide Results

- † Now being used in phase 1 clinical trial
- † Calibration range: 0.5 to 400 ng/ml
- † Lower limit of quantitation
 - ⌞ 0.5 ng/ml typical levels from plasma
 - ⌞ Coefficient of variance and recovery levels were determined on three separate occasions at three concentrations (2, 25, and 200 ng/ml).
 - ⌞ Intra-Assay coefficient of variance was less than 5% in every case
 - ⌞ Inter-Assay coefficient of variance was less than 10%
 - ⌞ Recovery levels in all determinations were between 94 and 108%.

What is the Best Mobile Phase for LCMS?

- ✗ Depends on many factors including solute solubility, ionization yield, pH, polarity of ions, mass of ions, ionic strength
- ✗ Make sure mobile phase has low background signal at m/z you want to use for quantitation
 - ✗ Baseline subtraction will not correct for detector saturation!
- ✗ For ESI, you may need to use an additive to improve ion yield
 - ✗ Different modifiers can give significantly different sensitivity
- ✗ Water may be necessary for ionization, but will increase background current, increase background noise and may lead to electrochemical oxidation or reduction for some analytes.





Mobile phases for LC/MS

- † Water
- † Methanol (best for negative ion APCI)
- † Ethanol
- † Isopropanol
- † Acetonitrile
- † Hexane + IPA (often used for optical isomers)
- † Acetone
- † Ethyl acetate
- † THF (used for negative ion analysis)
- † Chloroform (sometimes used mixed with other solvents)
- † Hexafluoroisopropanol

Solvent issues



- † HPLC grade solvents vs MS grade
 - ⌞ NVR specification - should be PPB or lower
 - ⌘ Most are around 1 PPM levels or 100 to 1000 times worse than your samples (per microliter!!!! At 180 uL/min, that is equivalent to injecting 1000 samples per second every second of the run.
 - ⌘ Some are 0.02% or worse
- † Buy in small containers and use up quickly
 - ⌞ Will dissolve gases such as phthalates from air
- † Avoid plastic containers and tubing with acidic mobile phases
 - ⌞ Additives & antioxidants leach out
- † Degas mobile phase daily or continuously
- † Don't use vinyl tape, Parafilm or any plastic material to cover the container lid – only use aluminum foil.
- † Each batch may have unique low MW impurity ions
 - ⌞ Evaluate before using

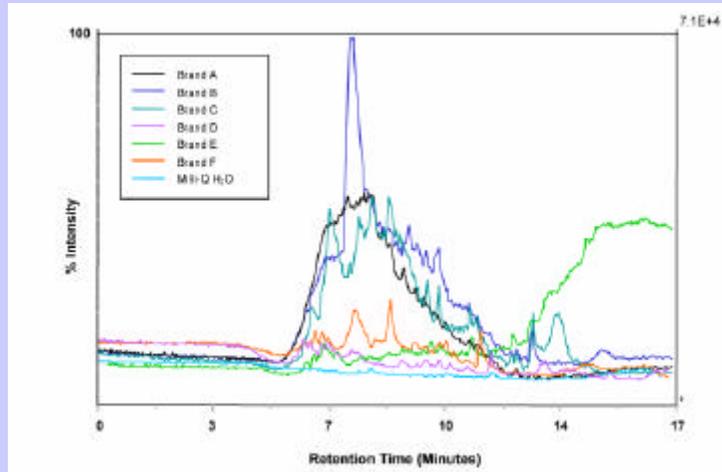
The biggest problem for most users is the purity of water. Water always contains background ion peaks and should be checked by scanning each new batch PRIOR to use. This will make sure that there are no interferences at the same mass you might be using normally. Water should be used up in a day or two and discarded. Keeping water around for a longer time will result in higher background signals, bacterial or fungal growth that will clog columns and MS capillaries, etc.

Water, water everywhere, but is any of it good enough for LCMS?

- † Distilled
 - ⚡ Can still contain volatile impurities
- † RO & Ion exchange are often acidic
 - ⚡ Ion Exchange water can have pH <5.5
 - ⚡ Propylene Glycol or Glycerol peaks from membrane, especially after installing a new membrane
 - ⚡ Be careful when using carbon based filters!
- † Bottled HPLC grades usually contain 1-2 PPM of sodium
- † TOC grade < 100 PPB organics
 - ⚡ Available in 1 quart bottles
- † Water will grow microbes upon standing
 - ⚡ These will clog tubing, pump frits, increase baseline noise
 - ⚡ Use up daily - do not keep for days or weeks
- † Degas before use and/or continuously
 - ⚡ Carbon dioxide lowers pH
- † Water can be purified using low wavelength UV with oxygen



TIC Intensity of Different Water Sources



LC-MS TIC of six HPLC-grade reagent waters and Milli-Q Gradient system water

Evaluation of HPLC reagent water purity via LC-MS, BY BYRON M. STEWART AND BRIAN L. WILLIAMSON

American Biotechnology Magazine, DECEMBER 2001, pgs. 16-18, volume 19, number 13. Copyright 2001 by International Scientific Communications, Inc. *Used by permission*

Levels of Organics in HPLC Grade Water

Table 1

LC-MS TIC and TOC results from reagent water analysis

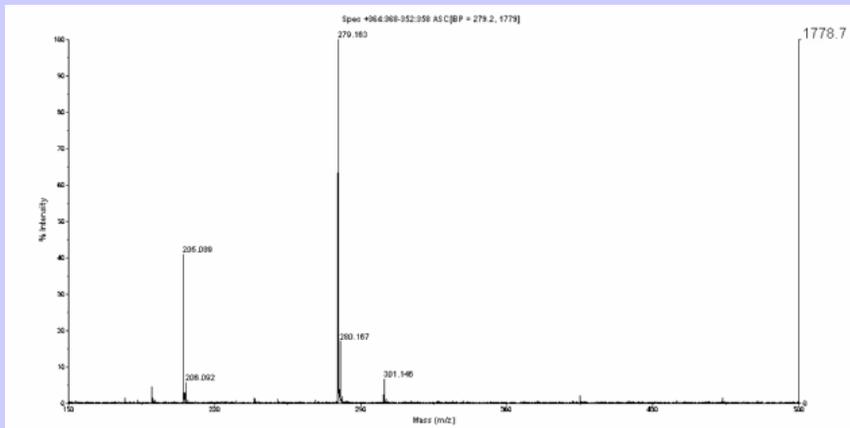
Sample	Organics (TOC in ppb)	Maximum TIC Intensity	Maximum TIC Retention Time
Brand A	100	4.3 E+4	8.1 min.
Brand B	87	7.1 E+4	7.5 min.
Brand C	777	4.2 E+4	8.2 min.
Brand D	16.5	1.5 E+4	9.9 min.
Brand E	32.4	3.8 E+4	15.1 min.
Brand F	25.5	1.7 E+4	8.2 min.
Milli-Q	7.0 (4.0*)	6.8 E+3	7.7 min.



Evaluation of HPLC reagent water purity via LC-MS . BY BYRON M.STEWART AND BRIAN L.WILLIAMSON
American Biotechnology Magazine, DECEMBER 2001, pgs. 16-18, volume 19, number 13. Copyright 2001 by International Scientific Communications, Inc. Used by permission

Milli-Q Water without UV Photooxidation

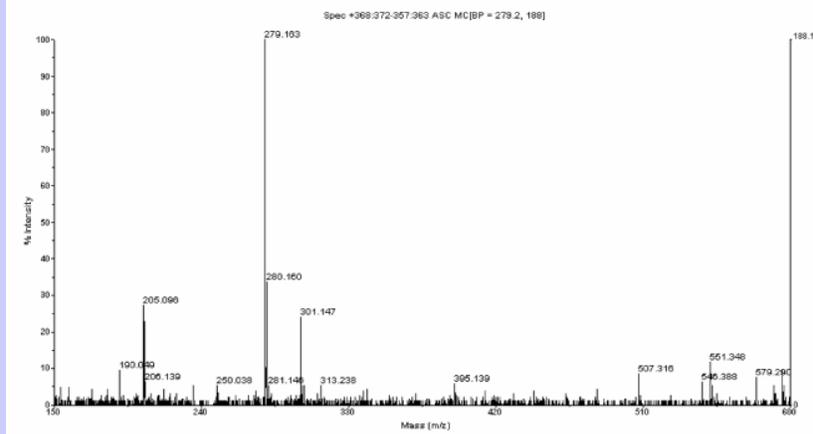
SHIMADZU LCMS Training



Evaluation of HPLC reagent water purity via LC-MS . BY BYRON M.STEWART AND BRIAN L.WILLIAMSON
American Biotechnology Magazine, DECEMBER 2001, pgs. 16-18, volume 19, number 13. Copyright 2001 by International Scientific Communications, Inc. Used by permission

Milli-Q Water with UV Photooxidation

SHIMADZU LCMS Training



Evaluation of HPLC reagent water purity via LC-MS . BY BYRON M.STEWART AND BRIAN L.WILLIAMSON
American Biotechnology Magazine, DECEMBER 2001, pgs. 16-18, volume 19, number 13. Copyright 2001 by International Scientific Communications, Inc. Used by permission

What is the Best Buffer for LCMS?

- † Utilize no buffers, if possible, when doing APCI
- † Utilize the lowest tolerable buffer concentration
 - ✍ 5 to 20 mM is typical, although 2 mM may be better for some applications
- † Ammonium acetate is the most versatile buffer for LCMS
 - ✍ Acetates absorb UV at low wavelengths!
- † Avoid TFA if possible
 - ✍ Suppresses ionization, decomposes some samples, causes corrosion to metal surfaces
 - ✍ Gives 113 background peaks
 - ✍ If must be used, keep concentration at 0.05M or lower





Practical Considerations

- † Keep salts out of mass spectrometer
 - ✎ Desalt samples
 - ✎ Liquid - liquid extraction
 - ✎ Ion exchange or suppression
 - ✎ Use water rinse when changing mobile phase from acid to basic
- † Dilute samples to reduce non-volatile load
- † Use Bypass Valve to heartcut sample
 - ✎ Salts usually elute at void volume

Practical Considerations: Avoid Detergents

- † Detergents in biological samples will suppress ionization significantly
 - ≈ 0.01% SDS can reduce protein signals by 20x in ESI
- † Even trace levels of soaps can affect quantitation of small molecules
- † Detergents may exist in samples as 'buffers and solubilizing agents' or may originate as contamination from cleaning.
- † Reference: R.R. Ogorzalek Loo, N. Dales and P.C. Andrews, *Protein Science* **3**(1994)1975-1983.



Biological samples are often prepared using detergents and solubilizers such as SDS or ethoxylated sugars. These will produce strong background signals by LCMS and will suppress ionization of target proteins and peptides. It is best to eliminate these either with sample prep or better chromatography.

Practical Considerations: Avoid Plastics!

- † All plastics contain plasticizers and oligomeric species, like PEG (polyethylene glycols) or phthalates
- † Use all-glass solvent filtering devices (ground glass connections only)
- † Store samples in glass vials with Teflon liners
- † Use glass autosampler vials with Teflon liners
- † Rinse plastic syringe filters with solvent before filtering samples



Contamination from plastics is a common problem for LCMS. Almost everyone has or will discover an ion at 391 m/z (dioctyl phthalate) that can come in from tygon tubing, or even the vapors from floor tiles. Many people find a series of ions with a 44 mass difference indicating an ethoxylated detergent is present – often from either a tune solution, or from cosmetics and hand creams. When you are looking for things in the picogram and nanogram levels, it doesn't take much of an impurity to wreck havoc with an analysis.

Summary

- † Sensitivity is not an instrument issue, but a technique issue
- † To gain the most from any instrument, look at the following:
 - ✍ What gives the best ionization
 - ✍ What solvents and additives produce the most ions
 - ✍ What HPLC conditions and column will work with the best ionization conditions, and additives for ionization

Thank You

 SHIMADZU LCMS Training



Appendix



† Additional tools and information:

- ✎ Solvents
- ✎ Additives
- ✎ Adducts and impurity peaks
- ✎ 'How To' approach to LCMS method development from Novartis



Solvents & Additives

- † ACN and MeOH - HPLC grades sometimes good, but purchase in 1 liter bottles
- † Acetic Acid, Formic Acid, TFA
 - ⌘ Purchase in small volumes & in glass vials
 - ⌘ Acetic is often dirtier than formic
 - ⌘ TFA protein sequencing grade. pKa= 0.3
- † Ammonium Acetate, Formate & Carbonate
 - ⌘ Reagent grades are good
 - ⌘ Purchase in small containers - they absorb water
 - ⌘ Ammonium acetate can be made from ammonium hydroxide titrated with acetic acid – this will usually produce a better quality product than trying to weigh out a hygroscopic solid

Additives suitable for LCMS

† Trifluoroacetic Acid (TFA)	pKa = 0.3	m = 113.99
† Heptafluorobutyric Acid	pKa = 0.2	m = 213.99
† Perfluorocaproic Acid	pKa = 0.2	m = 313.98
† Tridecafluoroheptanoic acid	pKa = 0.3	m = 363.98
† Formic Acid	pKa = 3.7	m = 46.01
† Acetic Acid	pKa = 4.7	m = 60.02
† Propionic Acid	pKa = 4.8	m = 74.04
† Butyric Acid	pKa = 4.8	m = 88.05



TFA should generally be avoided in nearly all applications. It nearly always reduces ion yields of peptides and other molecules. It is commonly used, but most people will get better results with formic acid or acetic acid instead, sometimes by 10x or more. TFA also can cause corrosion of stainless steel, especially if any chlorides are present in the samples.

Additives suitable for LCMS

† n-Methyl Morpholine	pKa = 7.4	m = 101.08
† Trimethyl Amine	pKa = 9.7	m = 59.07
† Ammonium Carbonate	pKa = 10.2	
† n-Methyl Piperidine	pKa = 10.3	m = 99.10
† Triethyl Amine	pKa = 10.7	m = 101.12
† Piperidine	pKa = 11.2	m = 85.09
† Pyrrolidine	pKa = 11.3	m = 71.07
† Ammonium Hydroxide		





Buffers for LCMS

- † Salts have no buffer capacity at $\text{pH} = \text{pKa}$!
- † Ammonium formate (pKa of formate = 3.75)
- † Ammonium acetate (pKa of acetate = 4.76)
- † Ammonium propionate (pKa of propionate = 4.86)

- † Website for buffer recipes:
 - ✉ www.bi.umist.ac.uk/users/mjfrbn/buffers/makebuf.asp



Additives to Avoid

- † Alkali metal phosphates (eg: KH_2PO_4)
- † Alkali metal salts (NaCl, NaOAc)
- † Alkali metal bases (NaOH, KOH)
- † Borates
- † Citrates
- † Sulfates
- † Sulfuric acid, Hydrochloric acid and Phosphoric acid
- † TFA

Practical Considerations

Common Mobile Phase Clusters in Positive Ion Mode

SHIMADZU LCMS Training



Water

19 $(\text{H}_2\text{O})\text{H}^+$
37 $(\text{H}_2\text{O})_2\text{H}^+$
55 $(\text{H}_2\text{O})_3\text{H}^+$
73 $(\text{H}_2\text{O})_4\text{H}^+$
91 $(\text{H}_2\text{O})_5\text{H}^+$
199 $(\text{H}_2\text{O})_{11}\text{H}^+$
379 $(\text{H}_2\text{O})_{21}\text{H}^+$
505 $(\text{H}_2\text{O})_{28}\text{H}^+$

Methanol

33 $(\text{CH}_3\text{OH})\text{H}^+$
65 $(\text{CH}_3\text{OH})_2\text{H}^+$
97 $(\text{CH}_3\text{OH})_3\text{H}^+$
129 $(\text{CH}_3\text{OH})_4\text{H}^+$
161 $(\text{CH}_3\text{OH})_5\text{H}^+$

Acetonitrile

42 $(\text{CH}_3\text{CN})\text{H}^+$
83 $(\text{CH}_3\text{CN})_2\text{H}^+$
124 $(\text{CH}_3\text{CN})_3\text{H}^+$
165 $(\text{CH}_3\text{CN})_4\text{H}^+$
206 $(\text{CH}_3\text{CN})_5\text{H}^+$
247 $(\text{CH}_3\text{CN})_6\text{H}^+$
288 $(\text{CH}_3\text{CN})_7\text{H}^+$

Common Adducts

Positive Ionization Mode

- † M + 23 (Na)
- † M + 32 (MeOH)
- † M + 39 (K)
- † M + 41 (CH₃CN)
- † 159 m/z (TFA+Na)
- † 242 m/z (Tetrabutyl ammonium)
- † 391 m/z (DOP)

Negative Ionization Mode

- † M + 45 (Formate)
- † M + 60 (Acetate)
- † M + 58 (NaCl salt)
- † M + 78 (DMSO)
- † M + 113 m/z (TFA)



Practical Considerations

Common Artifacts and Adducts in Positive Ion Mode

- † 28 Series of 300 to 600 m/z peaks separated by 28 – triglycerides from fingerprints or contamination
- † 41 Acetonitrile adduct
- † 44 Series of peaks separated by 44 = Ethylene oxide
- † 50 Series of peaks separated by 50 = CF₂ fluorinated surfactants
- † 58 Series of peaks separated by 58 = Propylene oxide,
or NaCl adduct if 58/60
- † 61.5 Series of peaks separated by 61.5 = copper adducts
- † 63 H+H₂CO₃
- † 64 ACN+Na
- † 71/72 THF
- † 74 DMF +H, or diethyl amine + H⁺
- † 79 DMSO + H⁺
- † 83 2 ACN + H⁺
- † 88 Formic acid + acetonitrile +H⁺
- † 101, 138, 183 MeOH +H₂O clusters
- † 102 Triethylamine + H⁺
- † 102 AcN + HOAc
- † 105 2x acetonitrile + Na⁺
- † 146 3x acetonitrile + Na⁺
- † 149 Fragment from dioctyl phthalate
- † 159 NaTFA + Na⁺

Practical Considerations

Common Artifacts and Adducts in Positive Ion Mode

- † 158 Amino sugar. Common from antibiotic analyses
- † 163 Nicotine
- † 169, 165, 195 (possibly 133 and 135 - dimethyl phthalate
- † 181 BHA
- † 186 Tributylamine + H⁺ (ion pair reagents)
- † 211, 227, 241, 253, 269, 281 Detergents from glassware
- † 219 Tri-tert.butylphosphine oxide from peptide synthesis
- † 221 BHT
- † 241, 253, 255, 269, 281 Fatty acids and/or soap
- † 279 Dibutyl phthalate
- † 281/282 Oleic acid soap or oleamide (Na, K or NH₄ from mold release agent used in plastic production)
- † 317, 361, 405 Triton detergent
- † 362 Dioctyl diphenyl phthalate
- † 388, 437, 444 or 463 Lubricants from HPLC components
- † 391 Dioctyl phthalate + H⁺ (common from contaminated solvents and from plastic tubing)
- † 413 Dioctyl phthalate + Na⁺
- † 427 Dioctyl sebacate
- † 447 Diisodecyl phthalate + H⁺
- † 481, 525, 569 PEG as sodium adducts
- † 503, 547, 591 PEG protonated
- † 563 Oleic acid soap



Practical Considerations

Common Artifacts in Negative Ion Mode

- † 26 CN^- from acetonitrile
- † 35 Cl^- from inorganic or organic halides
- † 59 Acetate $^1-$
- † 79 Phosphate PO_3^- (several sources - phosphoric acid, oligonucleotides)
- † 80 Sulfate SO_3^-
- † 96 SO_4 adduct (proteins & peptides)
- † 97 HSO_4 and H_2PO_4
- † 113 TFA $^1-$

