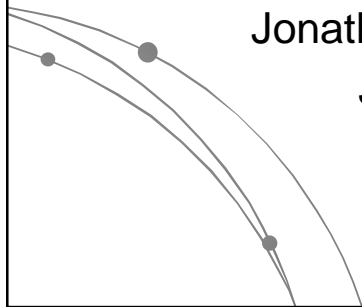


Electrospray Ionization Time-of-Flight MS for Small Molecules

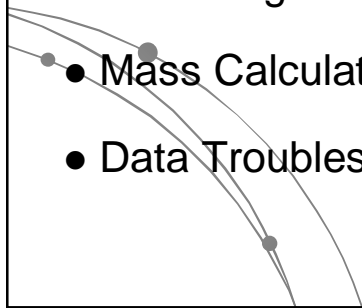
Jonathan A. Karty, Ph.D.

July 28, 2008



Topics Covered

- Sample Requirements
- Instrument Overview (technical)
- Collecting Data
- Mass Calculations
- Data Troubleshooting



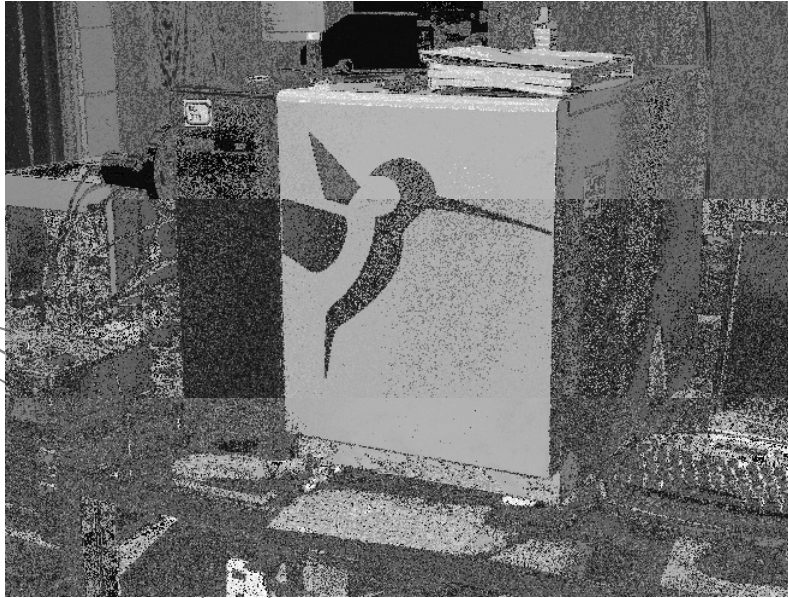
Some Important Caveats

- Switching to self-serve is an evolutionary process
 - We are adapting to issues as they come up
 - Procedures are likely to change in the first few months
- Much of this information is a generalization
 - Not all procedures will apply to every sample
 - However, we are open to try new things

LCT System Description

- Electrospray Ionization/Time-of-Flight (ESI-TOF) mass spectrometer
 - 75-4,000 m/z range
 - ~6,000 resolving power
 - 50 ppm mass accuracy
 - APCI source also available
- Inlet is a Waters CapLC System
 - Ternary gradient system (3 solvents)
 - 1-40 $\mu\text{L}/\text{min}$ flow rate (0.25-1.0 mm i.d. columns)
 - Autosampler 0.2-5 μL injection volume on KC-379
 - 0.2-10 μL injections on on KC-366
- Waters 2487 Dual λ Detector (KC-379 only)
 - 190-600 nm UV-VIS detector
 - Can alternate between 2 wavelengths during a run

LCT Picture



What Samples Can Be Analyzed by ESI-TOF-MS?

- Non-volatile organic molecules
 - Should be soluble in polar, volatile solvents
 - Molecule must be ionizable (MS detects ions)
 - R-NH₂, R-CO₂H, R-HSO₃, R-OH, R-H₂PO₃ work best
- Biopolymers
 - Peptides, proteins, DNA, RNA
 - K, R, H residues and unblocked N-termini make positive ions
 - D, E, residues, unblocked C-termini, and phosphate backbone of nucleotides make negative ions
 - *Sequence determines which ionization mode works best*
- Organometallic complexes
 - Organometallic salts work especially well
- HPLC-MS
 - With appropriate mobile phases

What Samples Are Inappropriate for ESI-TOF-MS analysis?

- Samples in non-polar or non-volatile solvent:
 - Hexane, benzene, CH_2Cl_2 , DMSO, etc.
- Buffer systems incompatible with ESI
 - 6M urea, 10% glycerol, 0.1 M NaH_2PO_4 (involatile)
 - 100 mM HCl solution (too conductive)
 - detergents
- Molecules that have no ionizable groups
- Complex mixtures yield complex spectra

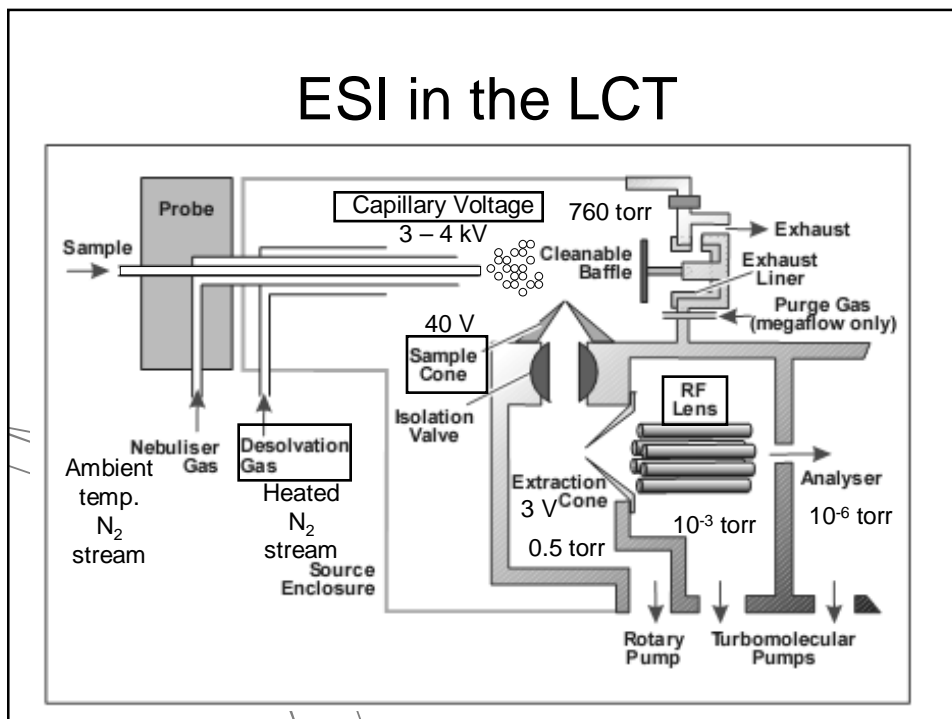
General Sample Guidelines

- **Purify analyte if possible**
 - Analyte should be 5 – 100 μM in concentration
 - Colored samples should be translucent
 - **Sample must have no particulates or precipitates**
- **Use only volatile solvents/buffers**
 - MeOH, H_2O , EtOH, acetone, CH_3CN , THF, etc.
 - HOAc, HCOOH, NH_4OAc , NH_3 , etc. (weak acids/bases)
 - **Trifluoroacetic acid suppresses ionization**
 - Ionic strength < 20 mM is best (e.g. 0.1% v/v HOAc)
 - Include 20-80% volatile, polar organic solvent if possible
 - 1 – 5 μM NaOAc for acid/base labile samples
- **Need at least 15 μL for flow injection analysis**
- **Non-aqueous samples can be analyzed by ESI**
 - THF, 1:1 CHCl_3 :MeOH, acetone, EtOH: CHCl_3

Characteristics of ESI Ions

- ESI is a thermal process (1 atm in source)
 - Little fragmentation due to ionization process (cf EI)
- Solution conditions affect ESI
 - Species often observed with same charge distribution as exists in solution (e.g. organometallic salts)
 - Sample pH drastically affects charge state distribution
 - Think about pH and pKa/pl relationship
- ESI ions are mostly generated by ion transfer
 - $(M+H)^+$, $(M+Na)^+$, or $(M-H)^-$, rarely M^+ or M^-
- ESI often generates multiply charged ions
 - $(M+2H)^{2+}$ or $(M+10H)^{10+}$
 - Most ions are 500-1,500 m/z (even for large proteins)
- ESI molecular weights listed as mass/charge (m/z or Th, not amu or Da) 1 Th = 1 Da/z

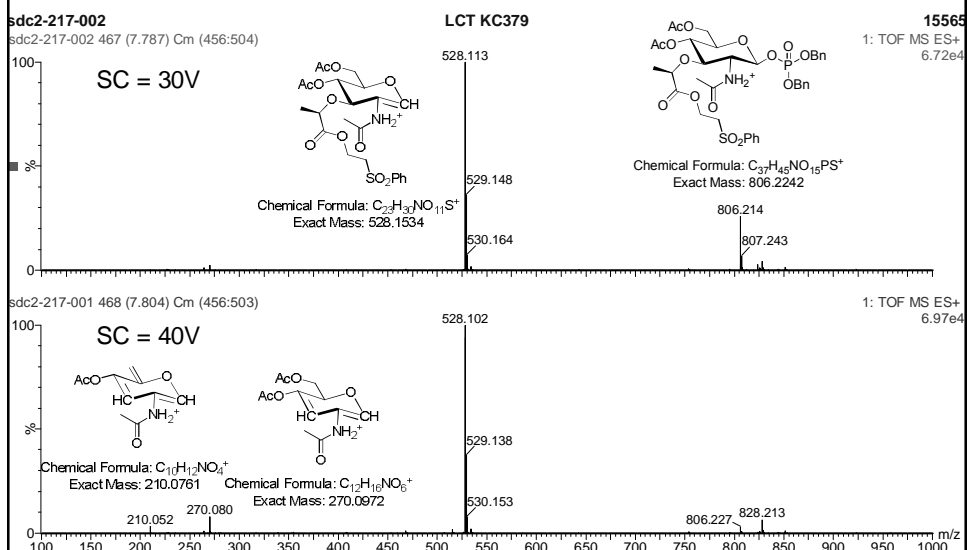
ESI in the LCT



Sample Cone Voltage

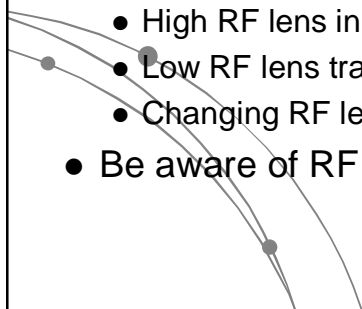
- Sample Cone (SC) region at moderate vacuum
 - Typical SC value 25-85 V (default = 40 V)
 - Typical extraction cone value 3 V
 - Collisional cooling reversed in partial vacuum
 - Ion attains significant velocities between collisions
 - Collision-induced heating can cause ion to fragment
- SC voltage difference affects observed mass spectrum
 - High SC can cause fragmentation
 - High SC limits ion/solvent clusters
 - High SC can improve sensitivity (counteracts diffusion)
 - High SC alters charge state distribution to favor lower charge states (protein/DNA)

Sample Cone Example

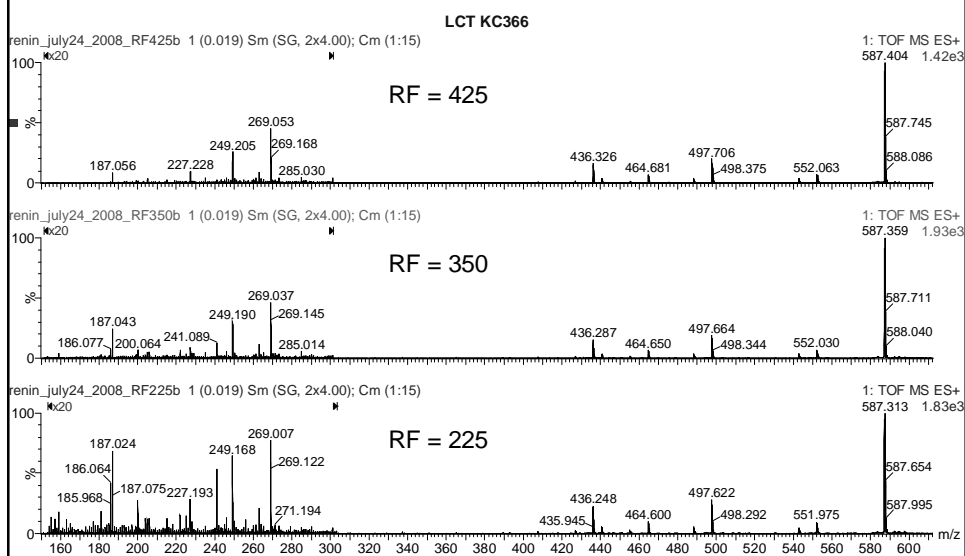


RF Lens Voltage

- Hexapole lens focuses ions into TOF
 - RF lens is amplitude of the radio frequency signal applied to the hexapole
- Although operated in RF only mode, it does induce some mass discrimination
 - High RF lens inhibits transmission of low m/z ions
 - Low RF lens transmits high m/z ions poorly
 - Changing RF lens slightly alters m/z calibration
- Be aware of RF lens voltages in your analyses



RF Lens Example



What This All Means

- Tune files are named as follows
 - Mode_polarity_CAPVOLTS_RF#_CV#_T#.ipr
 - ES_POS_3600_RF225_CV40_T125.ipr is electrospray, positive ion, 3600 V on capillary, 225 V on RF lens, 40 V on sample cone, 125 °C desolvation gas temperature
- MS experiment files are named:
 - Mode_polarity_RF#_time_lo#_hi#.exp
 - ES_POS_RF225_3min_150_1300.exp is electrospray, positive ion, 225 V on RF, recording from m/z 150-1300 for 3 minutes
- Be aware of the RF value for both tune files and MS experiment files
- See Jon or Angie to make new tune and/or calibration files

About the HPLC

- KC-379 is set up for reversed-phase HPLC-UV-MS
 - Solvent A is 95% H₂O, 5% CH₃CN, 0.1% HCOOH
 - Solvent B is 95% CH₃CN, 5% H₂O, 0.1% HCOOH
 - Solvent C is 1:1 CH₃CN:H₂O
 - Column is 0.5 mm i.d.x150 mm 5 µm Zorbax C18-SB300
 - UV-VIS detector has a 3 µL flow cell + tubing
 - At 15 µL/min, ~0.6 minute delay introduced by UV-VIS
- LC methods are named as follows
 - Time_grad_lambda_flow.clc
 - 20min_10-50-85_216_15.clc means a 20 minute gradient starting at 10% B, linear to 50%B, then linear to 85% B, monitoring 216 nm with a 15 µL/min flow rate

More HPLC Info

- KC-366 is set up for flow-injection analysis
 - no column, 3 min run time
 - Solvent C is 100% methanol, A and B are same as KC-379
- Liquid must be at least 5 mm deep
 - Just below the 0.5 mL line on a 12 mm vial
 - Low volume insert requires only 15 μ L
- See Jon or Angie if you need to make up new methods, change the eluents, or try a different column

Quick Sample Prep Instructions

- Place 750 μ L of ESI solution into an autosampler vial
 - Vials and caps are in blue container on the middle shelf by the balance
 - Solution is methanol with 0.5% v/v acetic acid and 5 μ M sodium acetate
- Add 2 μ L of your analyte solution (if NMR sample)
- Seal with a septum cap and shake well to mix
 - Label vial with sample name and/or online #
- Place vial into autosampler of LCT KC366 (closest to hood); keep track of where you put it

About the Autosampler

- Both LCTs are configured to use HPLC pump and autosampler as sample inlet
- Solvents in HPLC and autosampler can be changed if MeOH is not compatible
- Source region is purged with N₂ gas from a liquid N₂ dewar
- Talk to Jon or Angie if you need to change HPLC solvents

Sample Entry Page

- All samples are analyzed using the HPLC
- Pull up the MassLynx window
 - Note project name to find files later
- Fill out all columns
 - File name must follow Windows XP rules
 - File text can be anything including &%' etc.
 - Sample location is always column,row (1,C)
 - Injection volume should be 2 μ L
- Up to 48 samples may be analyzed at one time
- Sample ID is the online sample number
 - You can have up to 39 samples under a single number

Sample Page View

	File Name	File Text	MS File	Inlet File	Bottle	Inject.	Sample ID	MS Tune File
1	pep + cholesterol e undiluted	pep + cholest...	c18_20min_15_6..._20_min_test		1.A	5.000	15684	proten_pos
2	water blank	water blank	c18_ballistic_15_bio_C8_C18_15_ballistic		1.1.B	2.000		proten_pos
3	1-11-2 A IM	1-11-2 A IM	c18_20min_15_L..._20_min_test		1.1.C	2.000	15688	ESI_pos_lo
4	1-11-2 B IM	1-11-2 B IM	c18_20min_15_L..._20_min_test		1.1.D	2.000	15689	ESI_pos_lo
5	1-11-2 C IM	1-11-2 C IM	c18_20min_15_L..._20_min_test		1.1.E	2.000	15689	ESI_pos_lo
6	1-11-2 D IM	1-11-2 D IM	c18_20min_15_L..._20_min_test		1.1.F	2.000	15689	ESI_pos_lo
7	1-11-2 E IM	1-11-2 E IM	c18_20min_15_L..._20_min_test		1.2.A	2.000	15689	ESI_pos_lo
8	1-11-2 F IM	1-11-2 F IM	c18_20min_15_L..._20_min_test		1.2.B	2.000	15689	ESI_pos_lo
9	1-11-2 G IM	1-11-2 G IM	c18_20min_15_L..._20_min_test		1.2.C	2.000	15689	ESI_pos_lo
10	1-11-2 H IM	1-11-2 H IM	c18_20min_15_L..._20_min_test		1.2.D	2.000	15689	ESI_pos_lo
11	1-11-2 I IM	1-11-2 I IM	c18_20min_15_L..._20_min_test		1.2.E	2.000	15689	ESI_pos_lo
12	1-12-1 IM A	1-12-1 IM A	c18_20min_15_L..._20_min_test		1.2.F	2.000	15689	ESI_pos_lo
13	1-12-1 IM B	1-12-1 IM B	c18_20min_15_L..._20_min_test		1.3.A	2.000	15689	ESI_pos_lo
14	1-12-1 IM C	1-12-1 IM C	c18_20min_15_L..._20_min_test		1.3.B	2.000	15689	ESI_pos_lo
15	1-12-1 IM D	1-12-1 IM D	c18_20min_15_L..._20_min_test		1.3.C	2.000	15689	ESI_pos_lo
16	kh 1-24-1 MQ water Jon_vial	1-12-1 IM D	c18_20min_15_L..._20_min_test		1.3.E	2.000	15694	ESI_pos_lo
17	kh 1-24-1 DI water Jon_vial	1-12-1 IM D	c18_20min_15_L..._20_min_test		1.3.F	2.000	15694	ESI_pos_lo
18	kh 1-24-1 PBS Jon_vial	1-12-1 IM D	c18_20min_15_L..._20_min_test		1.4.A	2.000	15694	ESI_pos_lo
19	kh 1-24-2 LysRA of Polystyr...	kh 1-24-2 Lys...	c18_20min_15_L..._20_min_test		1.4.B	2.000	15694	ESI_pos_lo

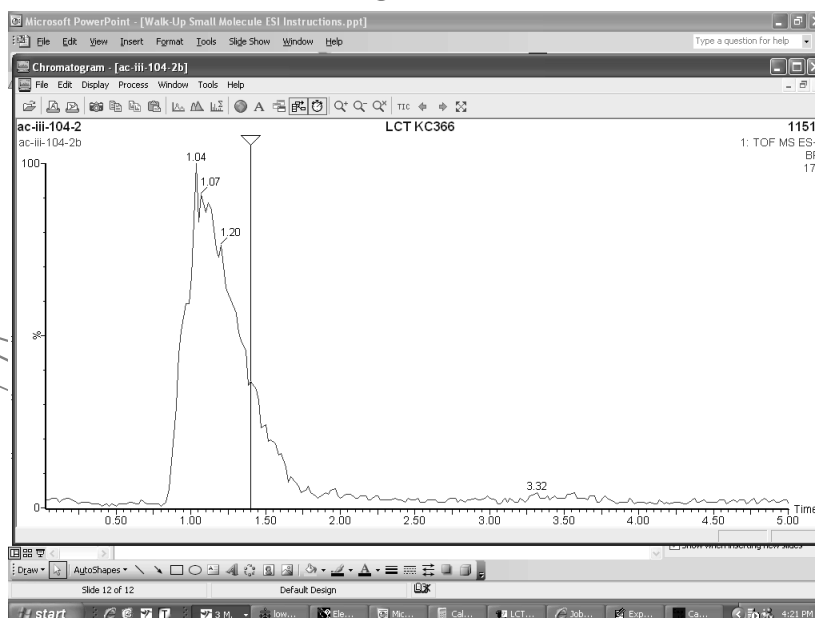
Starting the Analysis

- Save the completed sample table
- Highlight the rows you wish to run
 - Entire row should turn black when selected
- Click the “Start Analysis” icon (looks like a play button)
- Check and make sure the sample numbers in the popup are correct
 - Press OK to add runs to the queue
 - More samples can be added to the queue while instrument is in use

Analyzing Data

- Highlight the row containing the sample you wish to view
- Click on the “Chromatogram” link along the top of the sample list
 - A chromatogram will appear
 - One can open also files from the “Chromatogram” window
 - Files are in Project/Data directory
- The left mouse button is used for zooming in on data
 - Drawing a horizontal line expands the X-axis
 - Drawing a vertical line expands the Y-axis
 - Drawing a box makes whatever is in the box fill the screen
- The right mouse button is used to extract mass spectra
 - Dragging across a region sums the mass spectra
 - Double right-clicking extracts a single mass spectrum

Chromatogram Window



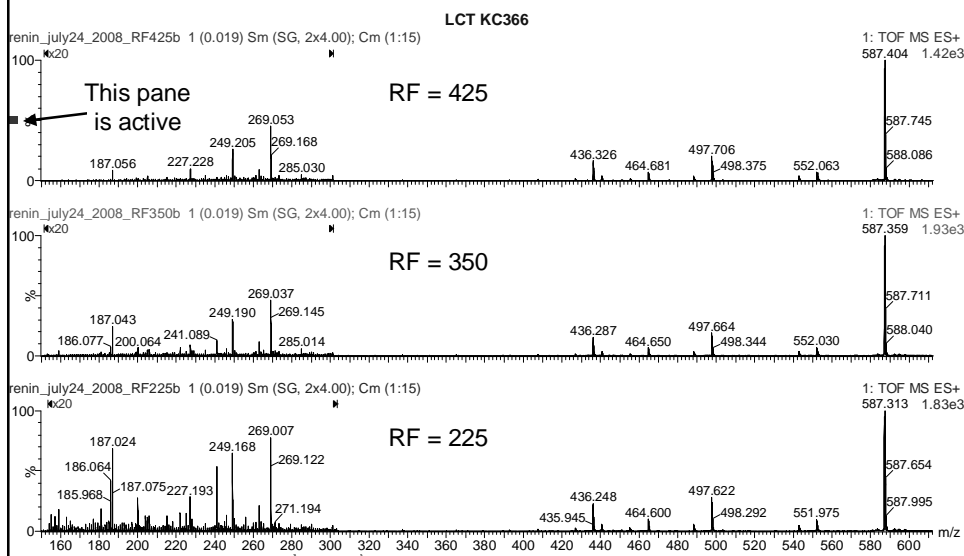
Analyzing Data 2

- Zooming in Spectrum app works just like in Chromatogram app
 - Right clicking generates a plot of the selected m/z ratio (or range) as a function of time
- Smooth the data by clicking on the smooth icon
 - 2x4 point Savitzky-Golay is adequate for most data
- Find the centers of the peaks by clicking on the centroid icon
 - 6 points wide for things <800 m/z, 10 pts for things over 800 m/z
- Mousing over any button will cause a description of its function to appear along the bottom left border of the window

Analyzing the Data 3

- Sample text appears in upper-left corner
- Colored text below sample text describes the processes performed on data
- Camera icon copies current view to clipboard for pasting into other programs
- Overwrite icon determines whether a process creates a new pane
 - Colored dot on left indicates the active pane
 - Clicking in a pane makes it active
- Intensities can be added from the Display Menu
 - Display → Peak Annotation...

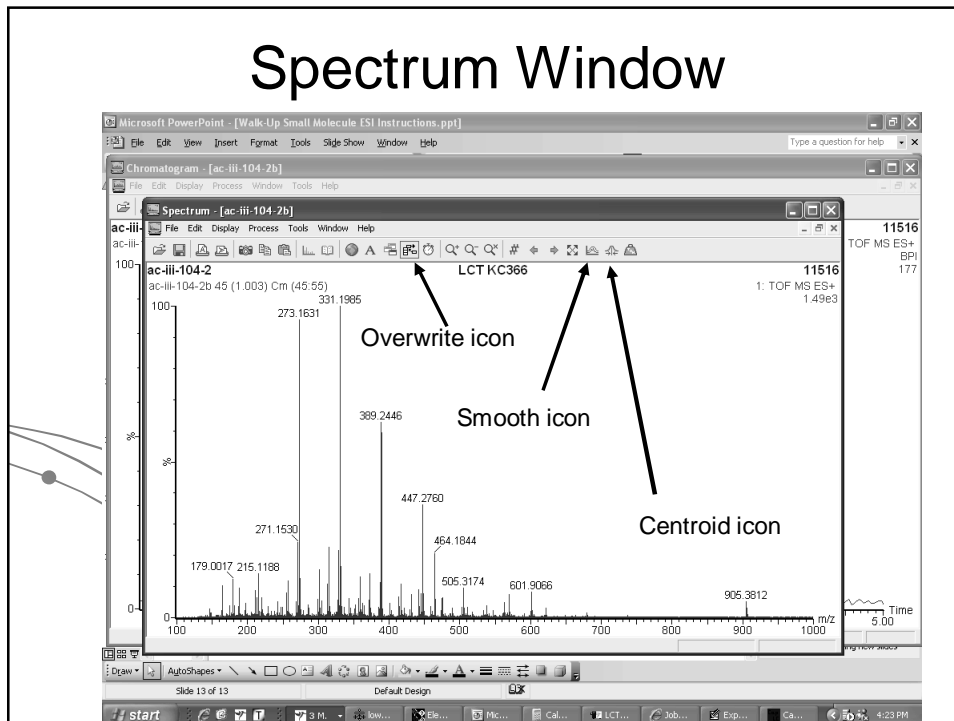
Active Pane Dot



Labels and Printing

- Clicking the “A” icon allows one to create a text box to place anywhere in a data pane
- Data can be printed or sent to a pdf
 - Select the printer using File→Printer Setup...
 - MSF-2420 is printer on MALDI instrument
 - DeskPDF creates pdfs
 - Printer icons determine portrait or landscape orientation
- Both of the LCT computers are on the IU network so you can transfer pdfs to your PC or slashtmp
 - Due to security concerns, flash drives are **NOT** permitted on MSF computers

Spectrum Window



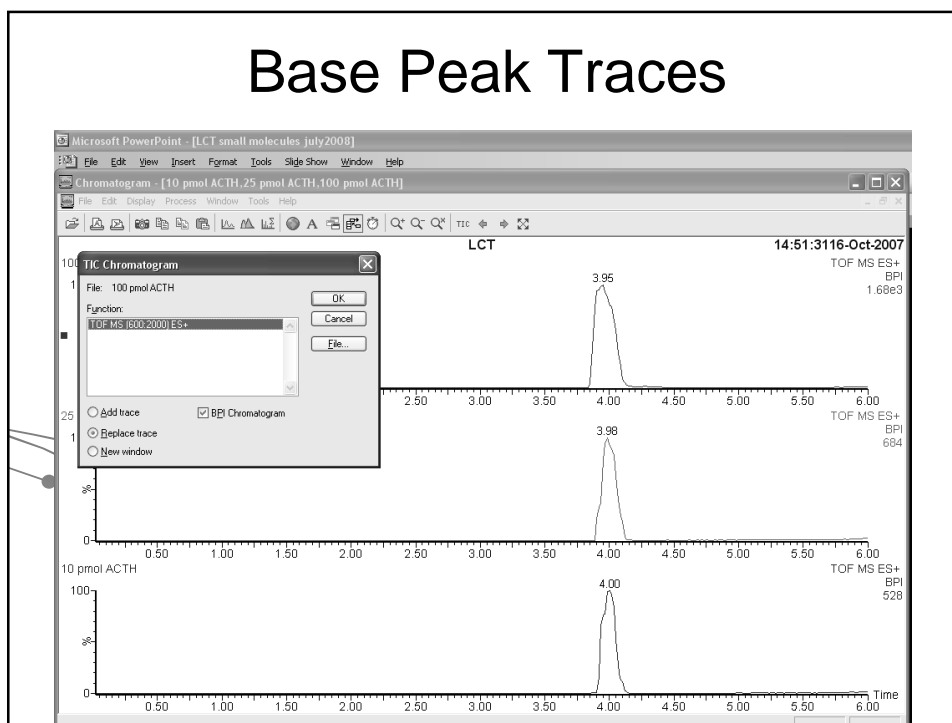
Interpreting Data Overview

- Is LCT overloaded?
- Molecular ion mass
 - $(M+H)^+$ or $(M+Na)^+$ or other
- Isotope Pattern
- Mass Defect
- Fragmentation/Multimers

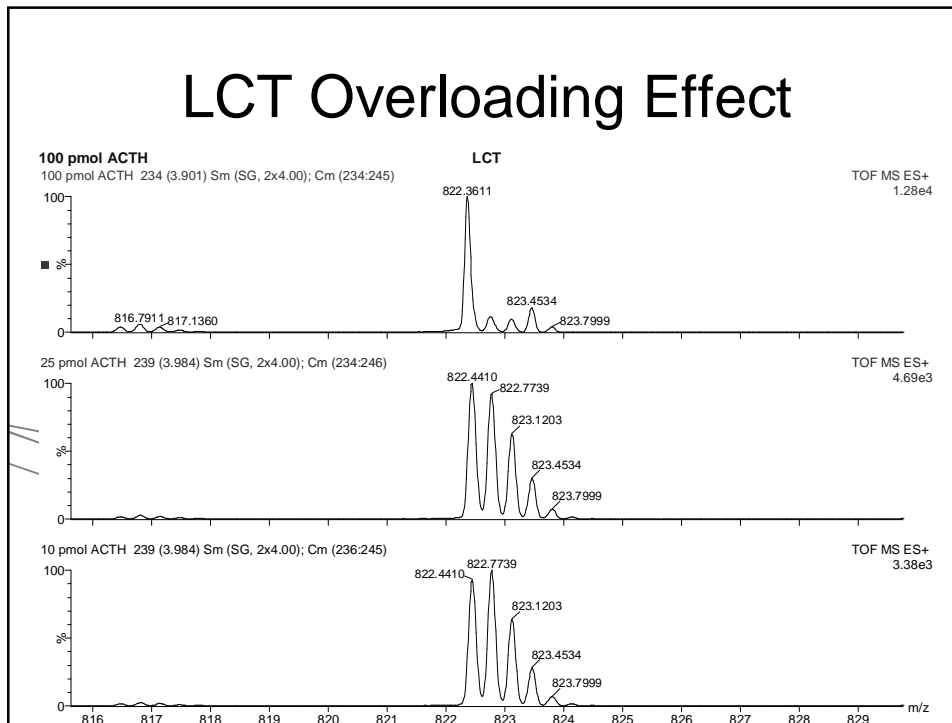
Overloading and TDC Dead Time

- Digitizer in LCT only records ion arrival times
- If 2 ions arrive simultaneously, they are counted as 1
 - Can cause peak to appear at a lower m/z
 - High ion currents also suppress sensitivity for adjacent isotope peaks
- Must have fewer than 600 counts per second in any single peak in a single scan
- Use Base Peak (BPI) trace to check for detector overloading
 - BPI tracks intensity of most intense ion in any scan as a function of time
 - Total ion chromatogram (TIC) tracks sum of all ion current in any single scan
 - Traces can be added by clicking Display \rightarrow TIC

Base Peak Traces



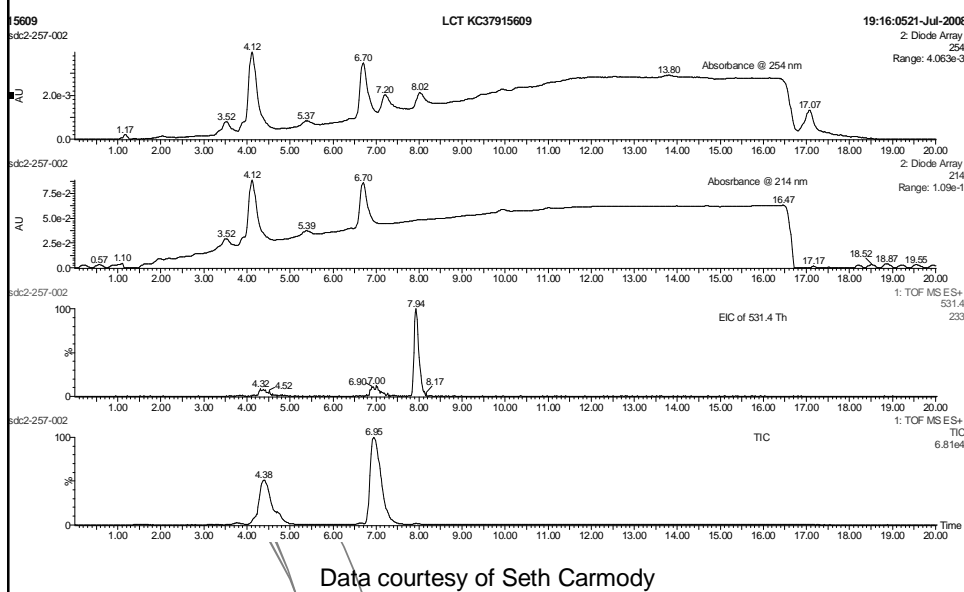
LCT Overloading Effect



Adding Other Chromatograms

- 3 other chromatogram types exist beyond TIC and BPI
- Extracted ion chromatograms (EIC)
 - EIC plots intensity of a particular m/z as a function of time
 - Create by selecting Display→Mass
 - A TIC, BPI, or EIC trace must be active to create an EIC
- Diode array trace shows sum of absorbance at both UV wavelengths
 - Create by selecting Display→TIC→Diode Array
 - Highlighting BPI plots most intense absorbance
- Wavelength trace can extract individual absorbance chromatograms
 - Create by selecting Display→Wavelength
 - A Diode array or wavelength trace must be highlighted first

Alternate Chromatogram Examples



Isotopes and Charge States

- Many elements have more than one naturally occurring isotope (Cl has 2, Se has 6, Ru has 7, Sn has 10)
- Mass spectra show distribution of all isotopes of all atoms
- Isotope pattern can be useful when interpreting data (indicate presence/absence of a particular element)
- Isotope peaks are spaced by ~ 1 Da/charge
 - Spacing can be used to discern charge state of an ion
 - e.g. @ +4, ^{13}C isotope peaks are 0.25 Th apart
- Small molecules tend not to support multiple charges
 - +2 small organometallics tend to either cluster with a counter ion and/or reduce their metal atoms to make a +1 ion
 - No solvent to stabilize higher charges in vacuum

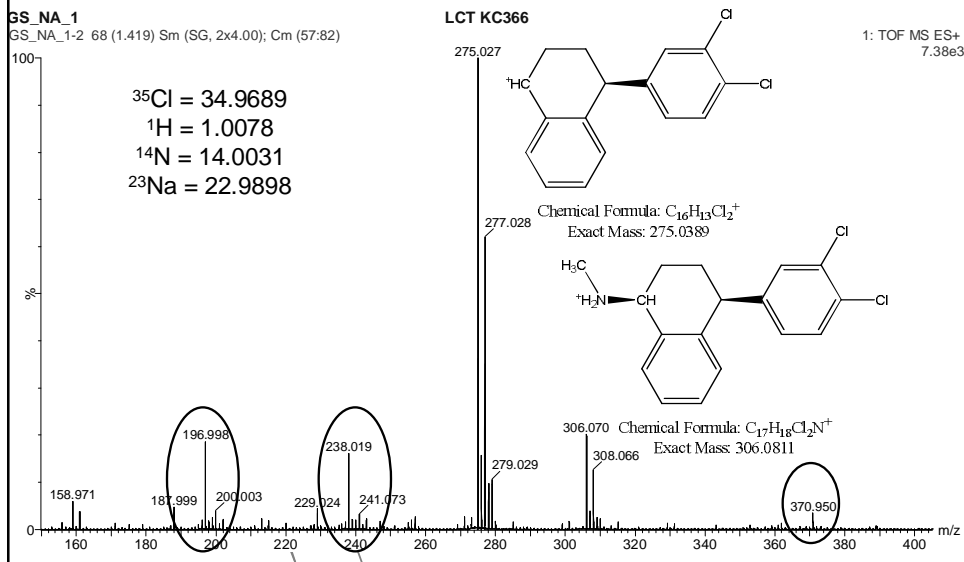
Interpreting Data: Mass Defect

- **Atomic weights are not integers (except ^{12}C)**
 - $^{14}\text{N} = 14.0031 \text{ Da}$; $^{11}\text{B} = 11.0093 \text{ Da}$; $^1\text{H} = 1.0078 \text{ Da}$
 - $^{16}\text{O} = 15.9949 \text{ Da}$; $^{19}\text{F} = 18.9984 \text{ Da}$; $^{56}\text{Fe} = 55.9349 \text{ Da}$
- Difference from integer mass is called “mass defect” or “fractional mass”
 - Related to nuclear binding energy
- **Sum of the mass defects depends on composition**
 - H, N increase mass defect
 - Hydrogen-rich molecules have high mass defects
 - Eicosane ($\text{C}_{20}\text{H}_{42}$) = 282.3286
 - O, Cl, F, Na decrease it
 - Hydrogen deficient species have low mass defects
 - Morphine, ($\text{C}_{17}\text{H}_{19}\text{NO}_3$) = 285.1365

Solvent/Salt Clusters

- The low m/z (<300) region of the mass spectrum is often dominated by solvent cluster ions
- These are distinguished by low mass defects due to Na^+ and K^+ incorporation
- To confirm presence of solvent cluster, try injecting a blank of ESI solution with your solvent system

Mass Defect Example



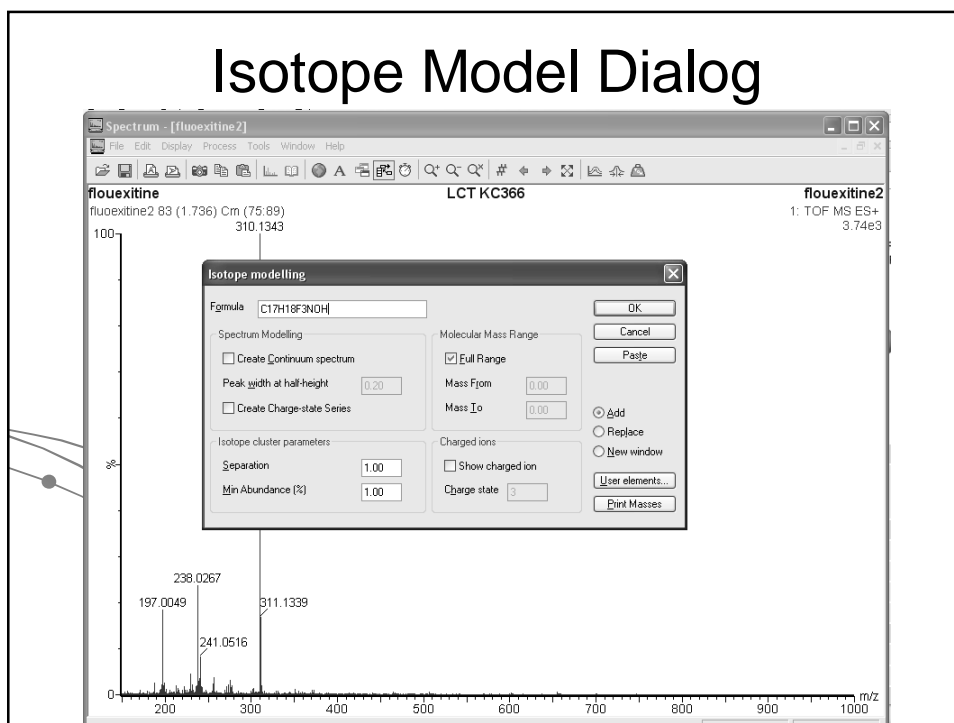
Multimers and Fragments

- As seen previously, labile ions can fragment in the sample cone region
- Conversely, strongly associating ions can make dimers and trimers
 - Multimers are usually singly charged
- Remember, if ion is $\text{M}+\text{Na}$, dimer is $(2\text{M}+\text{Na})$
 - Thus, dimer mass is $2 \cdot (m/z_{\text{mono_obs}} - 23) + 23$
- If ion is $\text{M}+\text{H}$, dimer is $2 \cdot (m/z_{\text{mon_obs}} - 1) + 1$

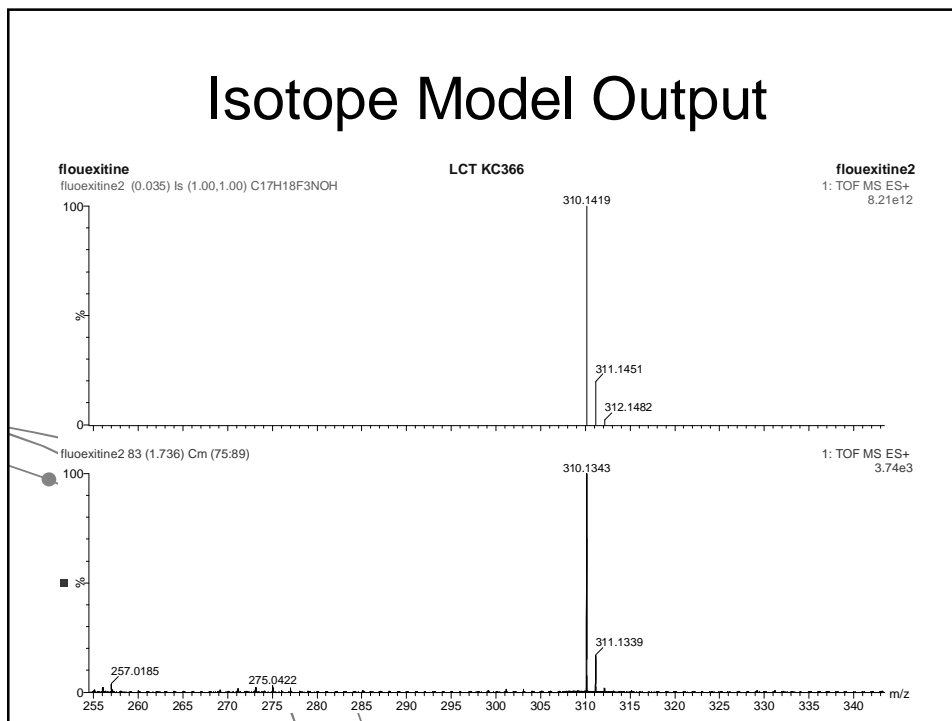
Isotope Model Module

- MassLynx has an isotopic prediction module
 - Found under Tools → Isotope Model...
- Just type in chemical formula for ion
 - For morphine (M+Na)⁺, C₁₇H₁₉NO₃Na
 - For singly charged, do not click show “Show charged ion”
 - Check status of Add or Replace buttons
- Isotope model can predict multiply charged ion isotope patterns
 - Click “Show charged ion” and enter the charge state
 - However, software adds a proton for each charge
 - For (M+2Na)²⁺ of morphine, I would type C₁₇H₁₇NO₃Na₂

Isotope Model Dialog



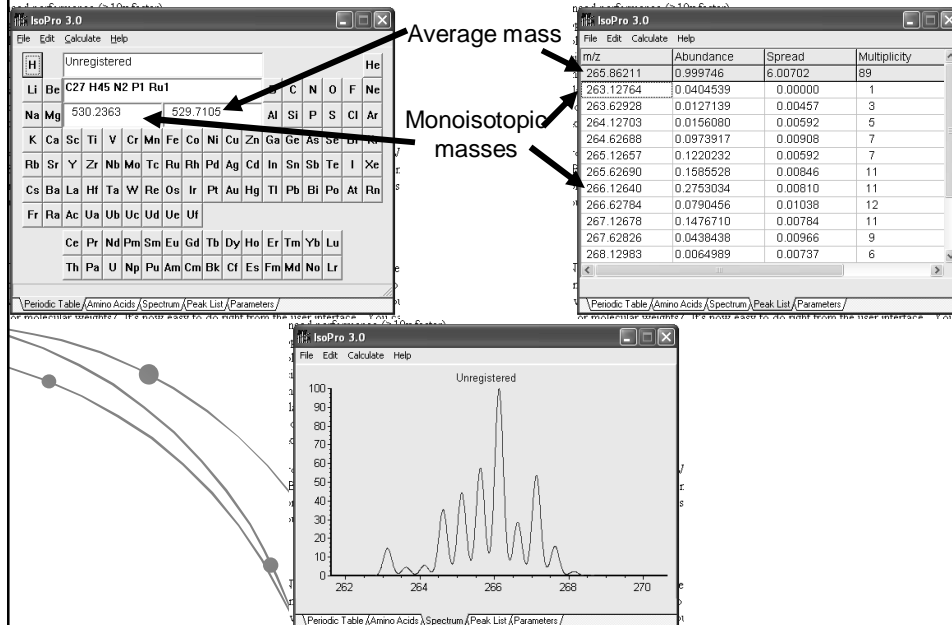
Isotope Model Output



IsoPro on Your PC

- IsoPro 3.0 can be used in the MSF or downloaded
- Type in formula by selecting Formula from the Edit menu (use proper capitalization)
- Select Distribution from Calculate (text table)
- Select Display from Calculate (picture)
- Use Parameters to alter charge state or theoretical resolution
- Recalculate distribution/display after making changes
- Protein “sequences” can be entered by entering amino acid composition on Amino Acids page
- Remember to account for ionization cations
 - Charge state function on Parameters page automatically adds 1 H per charge

IsoPro 3.0 Screens



Hands-on Training

- Groups of no more than three
- One hour or so to complete
- No charge for first session
- After training, students must demonstrate competency by running their own samples prior to being granted after-hours access