

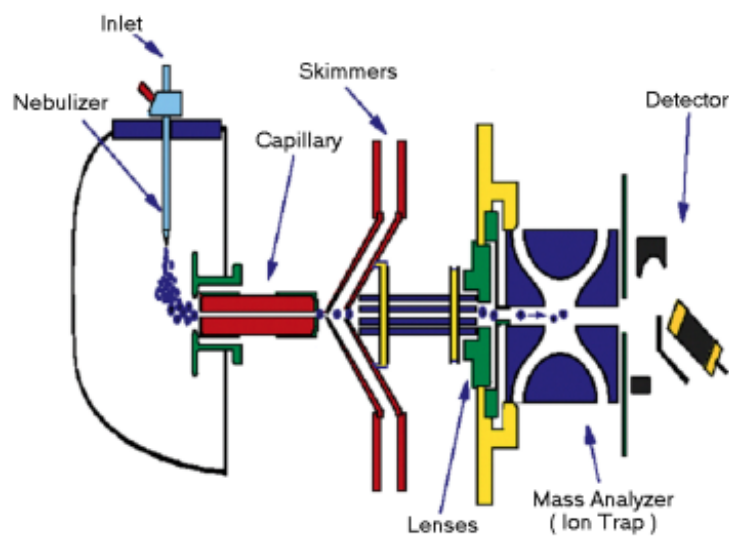
Electrospray Mass Spectrometry of Fmoc-Dipeptides

Purpose:

Verify the synthesis of the resin-bound dipeptide.

Theory

Mass spectrometry, MS, is the ideal tool for characterization of the results of synthesis experiments. However, mass spectrometers operate under high vacuum. A special source is needed to convert solid or solution samples into an ion beam under this high vacuum. Until the advent of electrospray MS, the mass spectroscopy of large molecular weight or very polar organics has required very expensive ion sources, operating with only solid samples¹. Electrospray MS allows the rapid characterization of components in solution.



An electrospray source produces a fine mist of a solution by spraying the solution from a small tip in an electric field. A nebulizer, which uses a rapid flow of nitrogen gas over the tip can also be used to aid the formation of the mist. The source uses a stream of hot, dry nitrogen to dry the mist droplets. As the droplet size decreases, the ions begin to repel each other until a "Coulomb" explosion fragments the droplets and produces gas phase ions. The electric field of the source then pulls the ions into a small capillary.

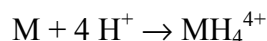
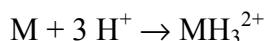
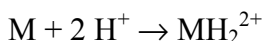
The capillary and two skimmers restrict the flow of air into the low vacuum region of the MS. Ion optics carry the ions to the mass analyzer where the m/z ratio is determined. Our MS uses an ion trap for the mass analyzer.

Electrospray ion sources are soft ionization sources, that is they produce mostly protonated molecular ions, MH^+ . The proton transfer can occur in your solution or in the droplets produced by the electrospray source.



The mass of the MH^+ ion is one greater than the molecular weight, $M+1$, because of the extra hydrogen ion that gives the ion its charge. Little fragmentation of the MH^+ ion occurs, giving very simple mass spectra. This "soft" ionization should be contrasted with electron impact ionization that is most often used with GC/MS. Electron impact ionization is violent and produces many fragment ions for each compound. Therefore electron impact mass spectra contain many peaks from many fragment ions. The simplicity of electrospray ionization mass spectra is very useful when analyzing complex mixtures, without separation.

For many compounds, ions are formed by acid-base reaction with the H^+ ion, as shown in Eq. 1. All mass spectrometers determine the m/z ratio. Electrospray ionization often produces multiply charged species (especially with proteins and nucleic acids). For example:



The m/z ratio for these multiply charged ions would be at (M+2)/2, (M+3)/3, and (M+4)/4. For example, a protein with a M of 10,000, would show MH^+ at 10001, MH_2^{2+} at 5001, and MH_3^{3+} at 3334 m/z.

Electrospray ionization is particularly well suited to biological samples for analysis of proteins and nucleic acids. One of the newest areas of biology, proteomics, is based in large part on electrospray MS. MS has become a routine tool in all the molecular life sciences.

Mass Spectrometry Procedure

1. Filter your sample through a 0.2- μ m regenerated cellulose membrane filter. Fill a 1-mL plastic syringe with filtered sample. Hold the syringe vertically and expel the bubble.
2. The `iCH242dip.m` method should already be started when you get to the MS. Select the `i Tune` tab, Figure 2, and the `i Expert` mode. Verify that the capillary voltage is 4000V. Switch to the `i Smart` mode and verify the source settings as in Figure 3. Check the MS scan range and Rolling Averaging as shown in Figure 4. The 2 rolling averages will give good signal-to-noise plots without averaging for too long a time.

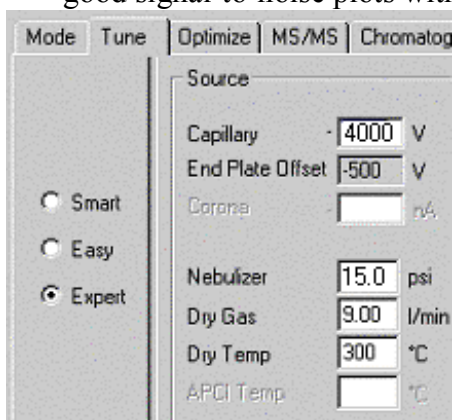


Figure 2. The capillary voltage.

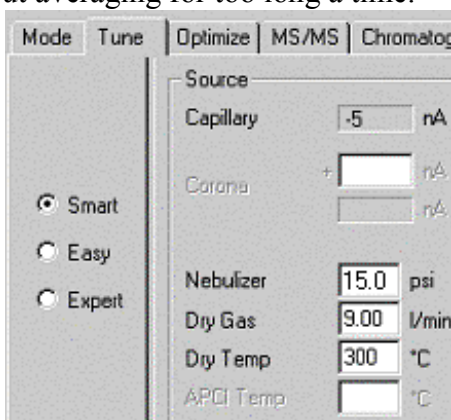


Figure 3. Source settings

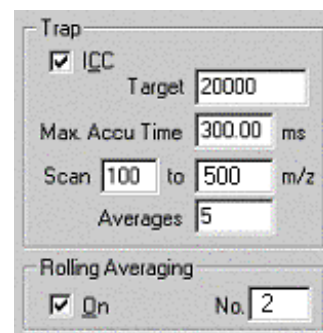


Figure 4. Rolling Averages

3. The sensitivity of the instrument can be optimized for different mass ranges and different types of samples. The settings in Figure 5 have been chosen for good sensitivity for the expected mass range of this experiment.

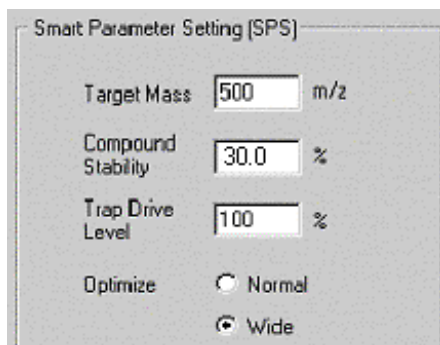


Figure 5. Ion optics settings.

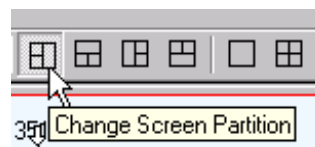


Figure 6. Normal screen arrangement with the `i stick` or `i histogram` style spectrum in the upper left.

4. Click on "Operate" if the system is in "Standby" mode. Click on the window arrangement icon shown in Figure 6, if not already chosen. The spectrum on the left is a "histogram" style spectrum, similar to the display on the GC/MS. The spectrum on the right is a "profile" spectrum that shows the full resolution of the mass spectrometer.
5. Attach your syringe to the plastic connector on the blunt-tipped syringe needle. Insert the needle in the stainless steel injection port. Place the syringe in the v-groove of the syringe pump. Lift the syringe hold-fast over the syringe. Depress the brass button on the pusher plate with your thumb and move the pusher plate against the syringe barrel. Inject sample by pushing on the pusher plate until you can see the spray in the spray chamber window.
6. Press the Run button on the syringe pump. The arrow on the LCD screen should be blinking.
7. You should see continuous mass spectra being plotted. Pull down the File menu and choose Print.
8. Expand the profile spectrum to show the isotope peaks around your dipeptide peak. To expand the plot move the mouse cursor until the cursor changes to a "↔", see Figure 7. Then use the right mouse button to control the scale expansion and the left mouse button to control the scale offset. When you have expanded the scale sufficiently, you will see the isotope peaks clearly.
9. Print the expanded spectrum.
10. Remove the syringe from the injection port.
11. Place the wash syringe containing methanol into the injection port. By hand inject about 2 mL of pure methanol to wash out the source for the next sample (in other words, you will need to refill the syringe at least 2 times). As you inject methanol by hand observe the spectrum. Note the intensity of your major peak. Repeatedly inject methanol until this peak is much smaller than the other noise peaks in the spectrum.
12. If the next student isn't waiting, place the MS in standby mode.
- 13.

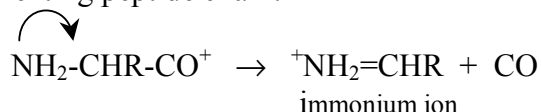


Figure 7. Mouse based scaling: use the right mouse button.

Discussion

The Agilent LC/MS is very sensitive, especially to plasticizers. Plasticizers are small to medium sized organic compounds that are used to change the properties of plastics. Plasticizers are usually added to make the plastic more pliable and can leach out of plastic lab-ware. You may see peaks at m/z 391, 279, 149, and 113. These peaks are from dinonyl phthalate, a very common plasticizer. So don't bother to try to interpret these peaks.

A small amount of fragmentation does take place in the source, which can lead to loss of protecting groups like Fmoc, fragmentation of the peptide chain, and loss of water or ammonia. Although these peaks are usually quite small, you may see some of them. Some amino acids show very typical ions, called immonium ions. Immonium ions are created from the terminus of a fragmenting peptide chain:



A table of immonium ions is given below and can be used to verify the presence of a specific amino acid in your peptide.

Table of immonium ions

Amino Acid	m/z
Arginine	70, 87, 100, 112
Proline	70 (87, 100 112 absent)
Valine	72
Lysine	84 (101 absent)
Leucine/Isoleucine	86
Glutamine	101 (84 absent)
Glutamic	102
Methionine	104
Histidine	110
Phenylalanine	120
Tyrosine	136
Tryptophan	159
Phosphotyrosine	216

Several Web applets are available to help you calculate the masses of different peptide sequences. Colby's at <http://www.colby.edu/chemistry/NMR/scripts/peptide.html> allows you to include protecting groups Fmoc and *t*-BOC, so you may find it very helpful.

If the beads aren't well washed, some of the reagents and their by-products may also appear in your mass spectrum. For example, N,N-diisopropylethylamine has a mass of 129.15 g/mol, unprotonated. The cationic part of TBTU has a mass of 321.1 g/mol. The hydroxybenzotriazole and tetramethylurea by-products of TBTU have unprotonated masses of 135.04 g/mol and 116.1 g/mol, respectively. The by-product from the deprotection step of the Fmoc-group is dibenzylfulvene, at 178.1 g/mol, unprotonated. Piperidine has a molar mass of 84.1 g/mol.

1. Assign the peaks in your spectrum. Your masses should include one or both of your amino acids. You should also find peaks with and without the Fmoc-protecting group. Remember to look for the masses of your reagents and their by-products.
2. On the basis of your peak assignments discuss the purity of your sample. Include a copy of your mass spectrum in your report.