Excerpt from

Peptide and Protein Characterization with Mass Spectrometry

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This excerpt demonstrates the general format used for manuals given to participants in all regularly scheduled and customized courses available through STM. A figure is presented on the left-hand page with explanatory text on the right-hand side. The figure is projected on screen during the lecture. The figure may be developed with on-screen animation but every figure presented on screen is included in its entirety in the manual. Ample white-space is provided for taking notes. This format makes the course manual easy to use and follow in class and also provides a valuable reference book which can be used again and again long after the class is over.

Our manuals are updated at least once per year to include the latest theoretical developments and an updated set of applications. Extensive references and a bibliography are included with all manuals.

Our attendees have told us many times that these are the best manuals of any they have seen in professional continuing education courses.

NOTE: Select “Continuous-Facing” view in Adobe® Acrobat® to see the manual exactly as it appears when open on a desk.

HPLC BUFFERS AND SALTS

- **Phosphates and other nonvolatile salts precipitate**
- **Useful for LC/MS pH control:**

  Ammonium Acetate/Formate/Alkylsulfonates
  Acetic/Formic/Propionic/Trifluoroacetic Acid
  Ammonium Hydroxide

- **Detergents/Surfactants in protein analysis:**

  Ion-suppression effects with infusion
  Surfactant adduct formation
  Clusters interfere in the mass spectrum

- **Na/K salts:**
  Adduct formation
  Ion suppression

Figure 6-6. Buffers and salts for use in LC/MS.

Comments on Figure 6-6

Buffers are commonly used in reversed-phase HPLC to suppress ionization, thereby improving retention and peak shape. (Ion suppression works at cross purposes to the mass spectrometer interface.) Salts also are employed to maintain a given ionic strength in solution. Both still can be used in LC/MS, but nonvolatile salts such as phosphates, borates, and sulfates must be avoided. They will precipitate in the interface and can block the small-diameter capillaries and inlets.

A variety of volatile reagents are available for adjustment of pH or ionic strength. If ionic strength is the only requirement, any of the salts shown will perform well but ion suppression effects must be considered.

As discussed earlier, pH has a significant effect on electrospray ionization efficiency. For pH control, organic acids and bases may be used to reproduce the pH achieved by the original buffer; however, the resulting solution will not be buffered. The change in reagent may require some "tweeking" of the LC method, but it generally is not a problem. Organic acid clusters may form and interfere with the mass spectrum. Cluster formation is enhanced from acetate → formate → TFA. Later in this section, we will discuss alternatives to the use of TFA in peptide chromatography.

Phosphate buffers have become popular for HPLC pH control because of their UV transparency. It is unusual that the buffering effect is needed. However, if a buffer is desired, ammonium acetate (2–10 mM) may be substituted for phosphate buffers in most applications within the acetate buffering range of 3.8–5.8. Concentrations up to 50 mM may be used. It should be noted that ammonium adducts frequently are seen with these buffers in positive-ion operation and acetate adducts in negative-ion mode. Examples of the importance of pH control in protein analysis are shown in the next few pages.

Before using any ammonium salts, investigate their proton affinity relative to your analyte molecule(s) as discussed in the previous slide.

Detergents are commonly employed in protein analysis to aid solubilization or to break up noncovalent clusters. In ES/MS, detergents have been shown to form high molecular weight clusters and protein adducts that interfere with the mass spectrum.[1] These effects seem to be reduced if injection, rather than infusion, sample introduction is used. More importantly, detergents are well known to cause ion suppression in electrospray. The effects of specific detergents will be shown later in this chapter.

[1] Ogorzalek Loo, RR; Dales, N; Andrews, PC "The Effects of Detergents on Protein Analyzed by Electrospray Ionization" in Chapman, JR, Ed., *Protein and Peptide Analysis by Mass Spectrometry*; Humana Press: Totowa, NJ, 1996.

Acetic Acid: pH 3–4
Formic Acid: pH 2–3
TFA: pH 1–2

Ammonium Hydroxide: pH 10–14

Figure 6-7A. Selection of ion-detection mode and required solution pH adjustment for electrospray.

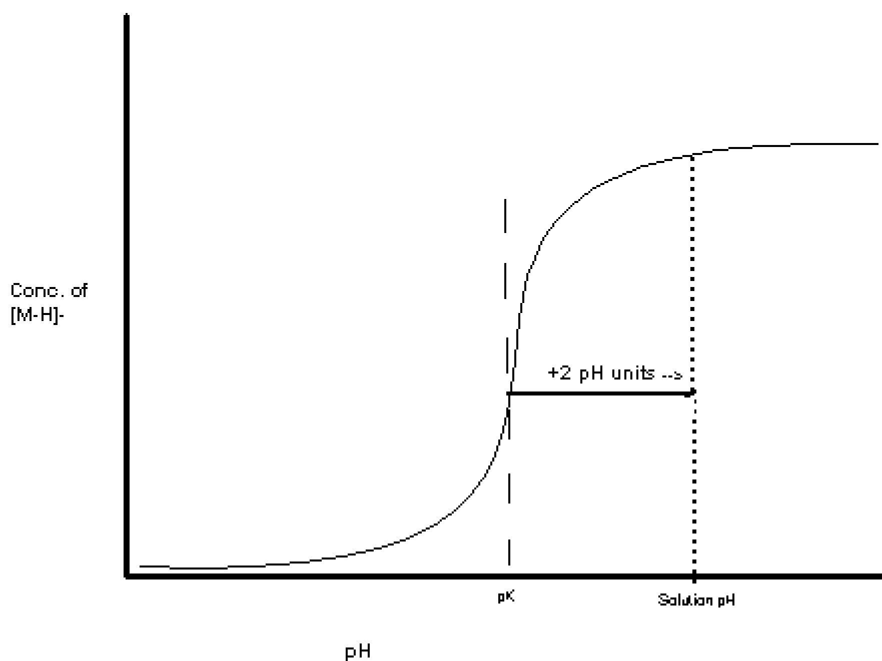
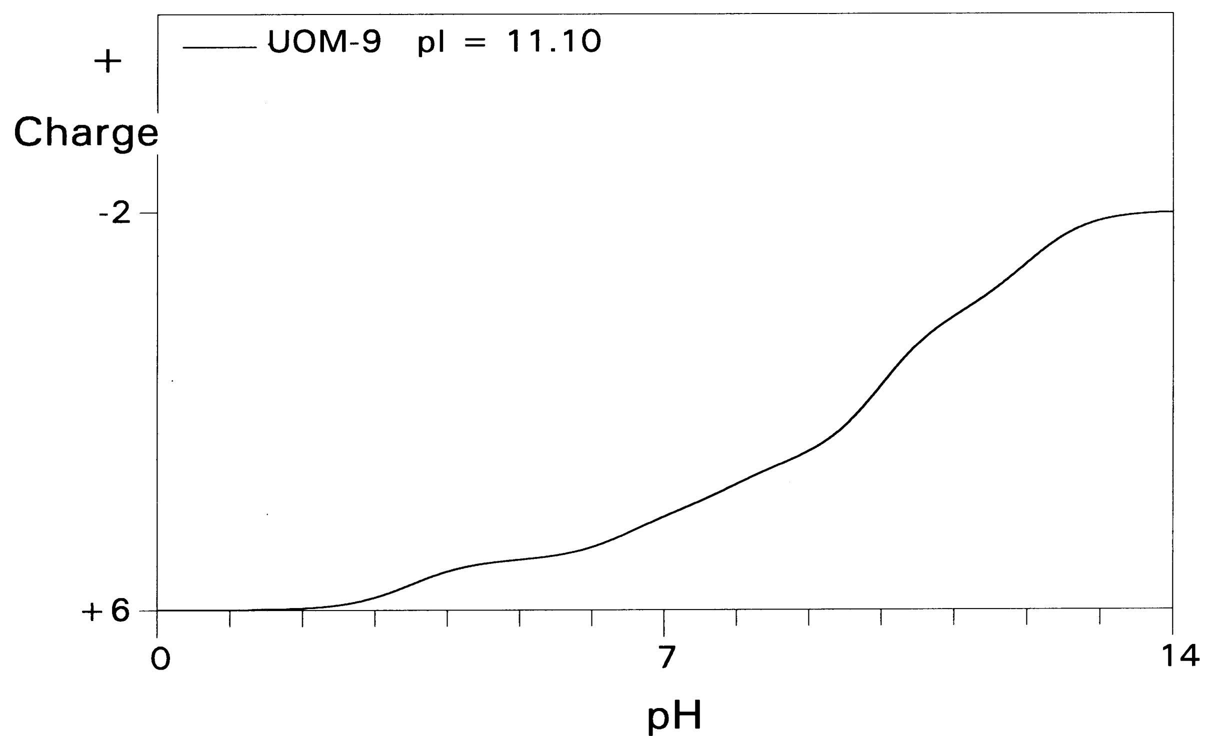


Figure 6-7B. Titration curve for an acidic compound showing selection of solution pH for maximum ion concentration.

Comments on Figure 6-7

Solution pH controls the ionization state of strongly basic or acidic compounds. Basic samples are protonated at low pH (i.e., they form positive ions). Protonation can be achieved pre- or post-column addition to the solution of acids of the types shown in **Figure A**. Acidic samples present the opposite situation. As the pH is raised, for example with ammonium hydroxide, acidic compounds lose a proton forming a negative ion.

Figure B is an example titration curve for an acidic compound. To ensure complete ionization, the pH should be adjusted to *at least* 2 pH units above the pK_a of the sample compounds. In the case of basic compounds, pH in the acidic range is adjusted to at least 2 units below the pK_a of the analytes.



UOM-9 pI = 11.10

Amount	Name	pK
1	C-term	3.5
1	Tyr-OH	10.0
1	N-term	8.0
2	Arg	12.0
2	Lys	10.0
1	His	6.5

Figure 6-8. Titration curve for a simple peptide.

Comments on Figure 6-8

As with any ionizable compound, peptides may be titrated and a titration curve generated. With multiple ionizable sites available to form both [+] and [-] ions, these curves can become quite complex.

This titration curve is for an eleven-residue peptide **KRPSQRHGSKY**. From the sequence alone, it can be concluded that this peptide would exist at low pH as an $[M+6H]^{+6}$ ion since, in addition to the N-terminus, there are five basic residue side chains: two lysines, two arginines, and one histidine. There are also two possible negative charge sites: the C-terminus and the tyrosine side chain.

The titration results show the possible ionization states for the molecule at various pH values. The pI is at a high value of 11.1 due to the preponderance of [+] charge sites on the molecule. When performing ES LC/MS analyses, titration curves provide a valuable clue to the expected results. This peptide has a molecular weight of 1423 daltons. However, if the LC mobile phase is at an acidic pH, several peaks will be seen in the mass spectrum corresponding to multiple-charge ions of the peptide. For example, the M+6H ion will appear in the spectrum at m/z = 1423/6 = 237 u.

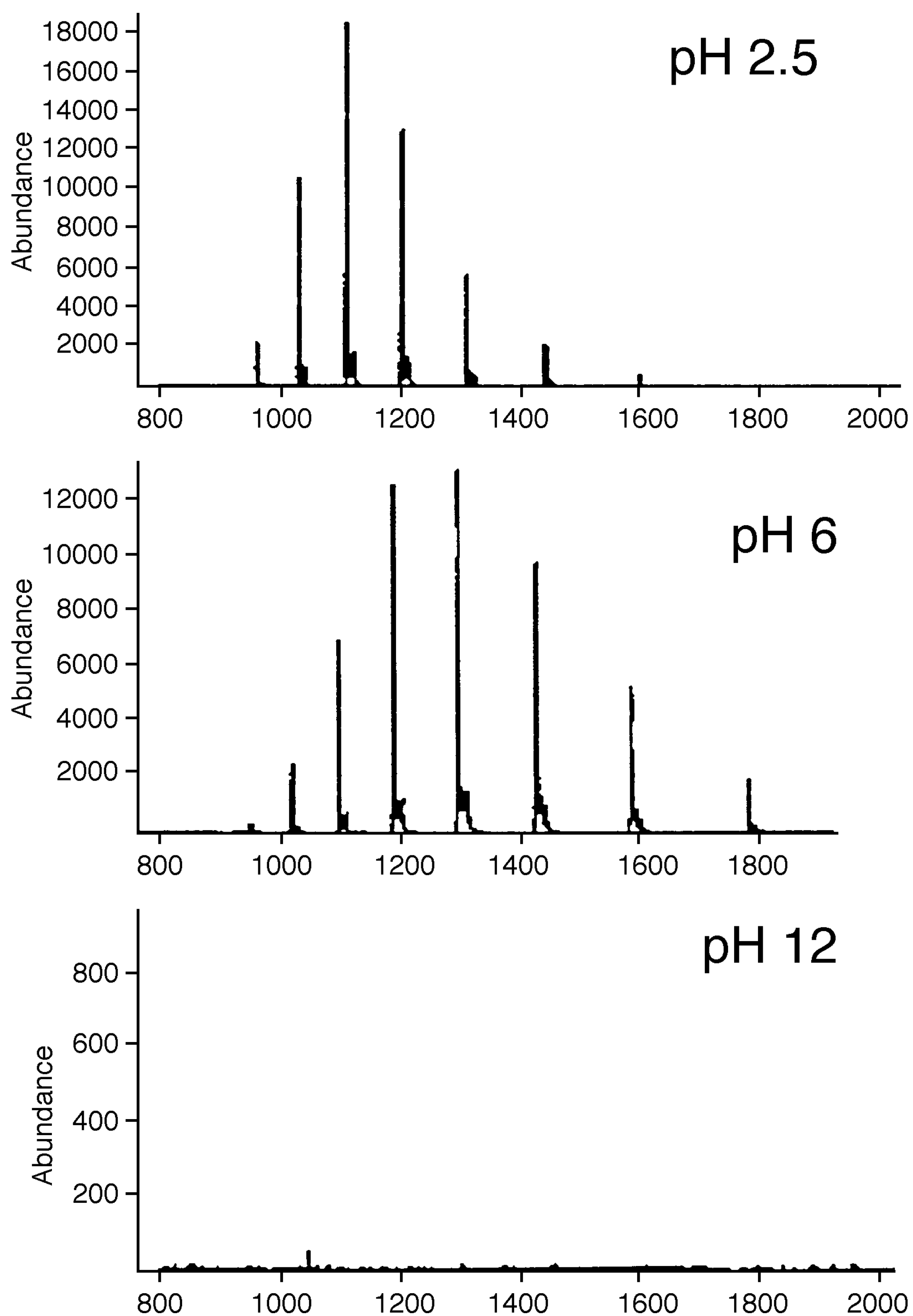


Figure 6-9. pH effects on LC/MS analysis of lysozyme using positive-ion detection.

Comments on Figure 6-9

The molecular weight of lysozyme is 14,306 daltons. At low pH, the basic sites (arginine, lysine, and the N-terminus) on the molecule are protonated to produce positive ions. In the first spectrum, the analysis is run at acidic pH of 2.5. The most abundant ion is at *m/z* 1100, which represents 13 sites with positive charges. In other words, this is the [M + 13] ion. The abundance of this ion is over 180,000.

At a moderate pH of 6, two effects are observed. First, the abundance of the most abundant ion has dropped to about 130,000. This is due to lower ionization percentage of the analyte as it enters the electrospray interface. Second, lower ionization efficiency also is seen in the number of ionized sites per molecule. The most abundant ion is now the [M + 11] ion at *m/z* 1300.

Finally, we move out to basic pH of 12 where the acidic sites of lysozyme are negatively charged, and the basic sites are neutral. As expected, no peaks are observed using positive-ion detection.

In addition to increasing the abundance of available protons, decreasing pH is also thought to eliminate tertiary structure and therefore expose more potential protonation sites to solution.