its structure, which renders the method to be more sensitive to differences, such as oxidation in surface amino acids. While this method can be used to assess purity, it is generally less sensitive to purity than RP-HPLC, because proteins can remain associated during the separation.

The SEC-HPLC is different from RP-HPLC and IEX in two major ways. The first difference is that separation is based on size only, with a small impact on the shape of the molecule. The second difference is that during SEC, the protein does not adsorb or bind to the separation media. In some cases, a protein will nonspecifically bind to the column. In these instances, it is important to use a different column matrix or to change the composition of the running buffer. The MW of a protein is determined by the comparison of its elution time with the elution time of the standard proteins of known MW. Because there is no binding of the protein with the column matrix, the protein separation is sensitive to the sample's volume. After running a set of known proteins through an SEC column, the protein of interest is loaded onto the column in a small volume and eluted under the same conditions. A standard curve is constructed, based on the MW of the standard proteins and elution time. This curve is used to determine the MW of the eluted sample. Size-exclusion chromatograph columns are available that can separate proteins with MWs as high as 1,000,000, allowing SEC to be used to identify and quantify the size and amount of aggregates in a protein preparation. Unlike SDS-PAGE, the protein is not denatured before separation, so that non–cross-linked aggregates are not disrupted and can be identified. Purity (or percent aggregation) determined by these two methods (SDS-PAGE and SEC) often differs considerably, owing to the detection of the additional aggregate forms in SEC.

9.9.3.4 Mass Spectroscopy

Mass spectroscopy is a method used increasingly to characterize proteins, in the early stages as well as through commercial manufacture. The popularity of this very sensitive technique has increased, as it has become more available in analytical laboratories, and the methods to use it have become more robust. This technique separates proteins based on their mass-to-charge ratio. To separate by MS, a protein is ionized in one of the several ways; then, it is accelerated by the electric or magnetic field. In some cases, the charged protein will break apart to produce ions. The pattern of ions produced is dependent on the structure of the protein, so that they may be used to determine the primary structure of the protein. Most MS instruments in use today ionize proteins in ways that minimize protein fragmentation to allow a true mass determination.

The information lost by reducing fragmentation in standard MS can be determined using MS/MS. In MS/MS, specific ions are subjected to an additional energy by collision, and the resulting daughter ions allow even more structural information to be determined, even to the level of the amino acid sequence. This technique is especially useful for determining posttranslational changes to the protein. The MS/MS can also be used to sequence the structure of carbohydrate side chains on glycosylated proteins and to identify the microheterogeneity that they introduce.

With large proteins, the determination of the primary sequence and PTMs is most efficiently done after digestion with trypsin or another protease to generate smaller peptides. In this case, the peptides are first separated by HPLC, most commonly RP-HPLC, and the column eluant is directed into the MS. In this hyphenated