physical mass of the biopharmaceutical's API. In terms of function or biological activity, a range of pertinent *in vitro* biological or binding assays or *in vivo* testing via toxicological or clinical activity such as PK, PD, or other relevant clinical indicator will need to be assessed (see Chapters 4 and 7). However, the aspect of physical mass falls under the jurisdiction of physicochemical analysis and will be the topic of this section.

In virtually all cases, the physical mass of the API present in a biopharmaceutical's commercial product is based on the total amount of the unmodified polypeptide mass of the biopharmaceutical, which is usually expressed on the basis of the molar theoretical amino acid sequence MW of the biopharmaceutical. This simple approach provides for a reasonably more accurate assessment of the biopharmaceutical's physical mass without the need to deal with the impact of mass differences due to the complexity of PTMs and their variability.

A common approach used to assess the physical unmodified polypeptide mass in a biopharmaceutical sample involves taking a UV measurement at a wavelength that is usually at or very near 280 nm of a sample and coupling it with information about the biopharmaceutical's theoretically estimated extinction coefficient at that wavelength based on its amino acid sequence. Since the only amino acids that absorb light at this wavelength are tyrosine (Tyr), tryptophan (Trp), and cysteine (Cys), knowing the amino acid sequence of the biopharmaceutical allows one to calculate the number of Tyr, Trp, and Cys moles present per mole of unmodified biopharmaceutical (note: this approach assumes that any PTM present does not contribute to the absorptivity at the wavelength employed, which is usually the case). Combining this information with the known molar extinction coefficient (at 280 nm) for Tyr, Trp, and Cys enables one to calculate a theoretical (molar or specific) extinction coefficient for a particular biopharmaceutical to provide a moderately accurate assessment of the unmodified polypeptide mass of the biopharmaceutical that is in a given solution by simply measuring its UV absorptivity at 280 nm (Gill and von Hippel, 1989; Mach et al., 1992; Pace et al., 1995) (note: in using this approach one assumes the extinction coefficient for Tyr, Trp, or Cys by themselves and in the biopharmaceutical is the same). However, in the case of a biosimilarity assessment, the FDA has stated that the extinction coefficient used to calculate the concentration of a biosimilar should be determined experimentally to be the same as the RP (FDA, 2015c).

To experimentally assess the biosimilarity of the extinction coefficient of a biosimilar to its RP, a relative experimental approach that compares the extinction coefficient of the polypeptide mass of a biosimilar to that of the RP should be just as valid an approach as their independent absolute extinction coefficient assessment and comparison. In fact, the relative approach is most likely to be more accurate. Although several simple relative approaches can be employed, probably the simplest and most accurate involves the use of a multidetector SEC system consisting of UV, light scattering (LS), and differential refractive index (DRI) detectors (Wen et al., 1996). In this case, the simple injection of the highly purified biosimilar and RP into this SEC system, integrating the total well-resolved monomer peak area resulting from the chromatogram output from each of the three detectors, and combining this data with the rearranged form of Equation 9 from the published work of Wen et al. (1996) will yield a parameter that is experimentally directly proportional to the biopharmaceutical's (unmodified) protein extinction coefficient of the injected sample