

is a major task and requires analyzing those using highly sensitive and accurate methods. While the goal is to have the lowest possible number of process-related modifications, these are often inevitable as a result of sample handling process, for example, in proteins that have been stored for some time during a stability study. They are unintentional and a source of structural heterogeneity in protein. These changes are known as PTMs and may be undesirable. Mass spectrometry can be used to detect even the smallest modifications, which are usually analyzed using a combination of electrophoretic, HPLC, and peptide mapping methods. Normally, mass spectrometry data are qualitative rather than quantitative. However, a quantitative approach can be taken to evaluate the degree of modification taking place. Several types of analyses are possible including the study of deamidation, oxidation, glycosylation, phosphorylation, N- and C-terminal truncation, acetylation, and pyroglutamate formation. All of these can be analyzed using a combination of two-dimensional (2D) gel electrophoresis, HPLC, and peptide mapping.

*Deamidation* is commonly observed as a PTM of the amino acid asparagine, which turns into a mixture of isoaspartate and aspartate via a succinimide intermediate. The rate of modification is influenced by factors such as the presence and interaction of surrounding amino acids and the pH and temperature buffers. Deamidation of glutamine also occurs, but much less frequently. It will be desirable to find out whether the protein has undergone deamidation because it leads to a change in pI and, therefore, the presence of charge heterogeneity. This can be reliably observed by one-dimensional IEF or IEX-HPLC. These variants can affect the effectiveness and even immunogenicity.

*Oxidation* of proteins is usually observed in methionine residues and to a lesser extent in tryptophan or tyrosine. As the name suggests, it is promoted by a form of oxygen, such as peroxide, but is also influenced by light, pH, temperature, buffers, and other factors. In many proteins, methionines in certain positions of an amino acid are especially prone to oxidation, presumably due to differences in solvent accessibility. The relative impact of oxidized products is less severe than that of deamidated variants.

*Proteases* are an enzyme class showing very diverse specificities and characteristics. Some have one specific substrate *in vivo*, while some are much more promiscuous. This makes it very difficult to detect unknown protease contaminations, which are a major problem in production and storage of protein- or peptide-based pharmaceuticals and diagnostic assays. Highly specific fluorescence resonance energy transfer (FRET) substrates (the sequence either directly deriving from drug substance or adapted to assay issues) can be used to detect proteolytic enzymes for the in-process control of the upstream and the downstream production of therapeutic proteins. Peptidic FRET substrates contain a donor (a fluorophore) and an acceptor (quencher) which absorbs at the emission