Competitive ionization may be avoided by varying the pH conditions or the matrix, through chemical derivatization of the peptides contained in this mixture, or through the partial fractionation of the mixture through reversed-phase liquid chromatography so that each fraction contains peptides of a similar hydrophobicity.

For both ESI and MALDI, the concentration of the sample and the complexity of the contaminants play important roles in both the sensitivity and the mass accuracy. Biological samples most often are diluted solutions of peptides or proteins containing a great number of contaminants. These two problems, dilution and contaminants, are not easy to handle, especially when the total amount of sample is low, such as picomoles.

The contaminants are of different origin and include buffers, non-volatile salts, detergents and many compounds of unknown origin. ESI can tolerate only low quantities of contaminant ions. These ions can reduce the abundance of the ions from the compound of interest and can even totally suppress them. They also very often result in the formation of adduct ions, further reducing the sensitivity by distribution of the ion current over several species. Furthermore, they may complicate the determination of the molecular mass, or reduce the accuracy of the molecular mass if some adducts are not separated from the ions of the protonated molecule.

Generally, MALDI is more tolerant than ESI to many contaminants. This can in part be due in part to some separation occurring during the crystallization of the sample with the matrix [30]. Whatever the ionization method, the quality of the mass spectrum is higher if the contamination is reduced.

The second problem is the generally low concentration of the compound of interest in the biological samples. The volumes needed for the analysis are very low, in the microlitre range for both MALDI and ESI [31], and only part of it is actually consumed during the analysis. But the concentration has a marked influence on the observed spectra.

As a rule, a separation method should be used for both purification and concentration of the sample. The classic method for peptides and proteins is a reverse-phase liquid chromatography preparation of the sample, followed by a concentration step (often lyophilization) of the fraction of interest. During those steps performed on very small quantities of sample, loss on the sample can occur if care is not taken to avoid it. Lyophilization, for instance, can lead to the loss of the sample absorbed on the walls of the vial. The use of separation methods on-line with the mass spectrometer often are preferred. Micro- or nano-HPLC [32,33] and capillary electrophoresis [34], both coupled mainly to electrospray ionization/mass spectrometry (ESI-MS), are used more and more.

8.2.2 Structure and Sequence Determination Using Fragmentation

8.2.2.1 *Fragmentation of Peptides*

In order to generate structural data by mass spectrometry, the molecule that is studied must undergo fragmentation of one or several bonds to match the *m/z* of the resulting fragments with the chemical structure. However, the various techniques we have considered so far imply the formation of stable ions that do not yield any fragment. This property is used to facilitate the determination of the molecular mass of peptides or proteins, even when they appear in a complex mixture. However, the same property leads to a lack of information concerning the structure. This drawback is overcome by transferring at least the extra energy required by fragmentation to the stable ions produced during

the ionization. Although various techniques allow an energy transfer, the most common method remains collision-induced dissociation (CID). Consequently, tandem mass spectrometry (MS/MS) has become an essential technique for structural analysis of peptides and proteins.

This technique consists of selecting the ion to be fragmented using a first mass analyser and sending it into a collision cell, where it collides with uncharged gas atoms. Thus the kinetic energy is transformed partly into vibrational energy and the resulting fragments are analysed by a second analyser, hence the name CID tandem mass spectrometry or CID MS/MS. If the instrument resolution is sufficient, the first analyser can select only the isotopic peak containing the main isotopes, such as ^{12}C and ^{16}O, which allows a fragmentation spectrum free from complex isotopic clusters (especially at high masses) to be obtained.

The tandem mass spectra may be obtained using many different instruments, such as sector, reflectron TOF, ion trap, triple quadrupole, ICR or hybrid instruments. The main difference from a practical point of view is in the kinetic energy of the ions. In magnetic and TOF instruments the precursor ion kinetic energy is several kiloelectronvolts whereas in the other types of analysers, such as the quadrupole, ion trap or ICR, the ion kinetic energy never exceeds 100 eV. This difference influences the fragmentation process [35]. As will be discussed in more detail below, the high-energy tandem mass spectra present a broader range of fragmentation pathways, some of which are not observed at low energy. A greater number of fragment ions often carry more information but also increase the complexity of the spectra, thereby rendering their interpretation more difficult.

The fragmentation of peptides can be observed also by a technique named post-source decay (PSD) when reflectron TOF instruments are used. In this technique, which is not only used for peptide analysis, the ions of the molecular species produced by MALDI indeed contain enough energy to fragment but this metastable fragmentation occurs during the flight between the source and the detector. With an linear TOF spectrometer the fragments reach the detector together with the precursor ions. In contrast, they will have different flight times after passing through the reflectron and thus their masses can thus be determined. A chosen precursor can be selected by a gating system at the origin of the flight tube. The resolution for this selection is low. This is in general sufficient to select a peptide in a mixture, but not to select one isotopic peak. MALDI-TOF/TOF instruments remove several disadvantages of MALDI-TOF PSD instruments and allow high-energy CID and high-speed analysis. Indeed, in these instruments, ions of high velocity that are produced by MALDI are selected with an ion gate and subject to CID. The resulting fragments are further accelerated and analysed in the second reflectron TOF analyser.

The fragmentation of peptides can also be obtained by FTICR instruments. Besides the most commonly used activation method, namely CID, the activation can alternatively be performed without gas by infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD). These methods fragment peptide ions in the ICR cell by emitting a laser beam or electron beam, respectively.

The MS/MS analysis of many peptides with known sequences permits identification of the various existing fragmentation processes [36]. The high- and low-energy fragmentation spectra of a peptide are presented in Figure 8.2.

From a practical point of view, the fragments may be classified in either of two categories: those derived from the cleavage of one or two bonds in the peptide chain and those that also undergo a cleavage of the amino acid lateral chain. The nomenclature suggested by

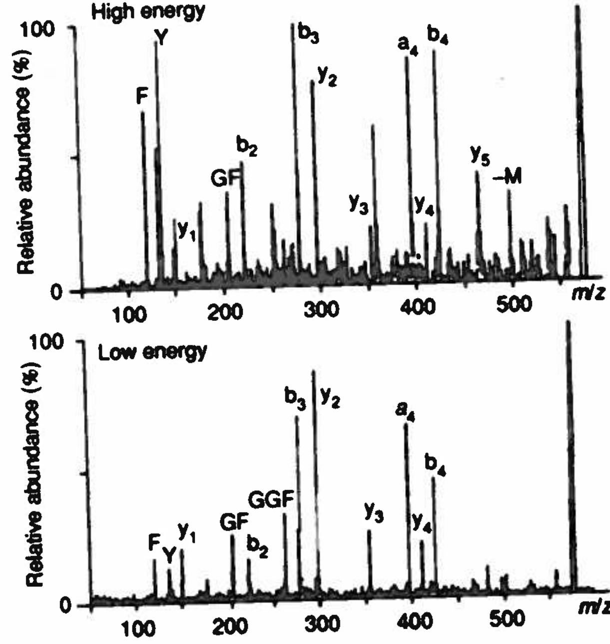

Figure 8.2
High- and low-energy fragmentation spectra of methionine–enkephaline (sequence YGGFM). The notation used is described in the text. Reproduced (modified) from Bean M.F., Carr S.A., Thorne G.C., Reilly M.H. and Gaskell S.J., Anal. Chem., 63, 1473, 1991, with permission.

Roepstorff and Fohlman [37] and later modified by Biemann [38] allows the labelling of the various fragments that are obtained.

The first fragments that were identified were produced by the cleavage of a bond in the main chain. The cleavage of a bond in that peptide chain can occur in either of three types of bonds, Cα–C, C–N or N–Cα, which yields six types of fragments that are respectively labelled a_n, b_n, c_n when the positive charge is kept by the N-terminal side and x_n, y_n, z_n when the positive charge is kept by the C-terminal side. The c_n and y_n fragments implicate the transfer of two extra hydrogen atoms, the first one responsible for the protonation and the second one originating from the other side of the peptide. The subscript n indicates the number of amino acids contained in the fragment. Figure 8.3 shows the various types of fragments produced through the cleavage of a bond in the peptide chain.

The mass difference between consecutive ions within a series allows the identity of the consecutive amino acids to be determined (see Table 8.2) and thus deduction of the peptide sequence. Indeed, the 20 common amino acid residues have distinctive elemental compositions and consequently distinctive masses. There is one exception with Leu and Ile, which are isomers. However, a low-accuracy measurement may be incapable of discriminating between Gln and Lys, which differ by 0.036 u. In addition, there are combinations of amino acid residues that yield the same nominal mass or even the same elemental composition.

Table 8.2 Mass increments of the various amino acids.

Amino acid	Code (3 letters)	Code (1 letter)	Monoisotopic mass	Chemical mass
Glycine	Gly	G	57.021 47	57.052
Alanine	Ala	A	71.037 12	71.079
Serine	Ser	S	87.032 03	87.078
Proline	Pro	P	97.052 77	97.117
Valine	Val	V	99.068 42	99.133
Threonine	Thr	T	101.047 68	101.105
Cysteine	Cys	C	103.009 19	103.144
Isoleucine	Ile	I	113.084 07	113.160
Leucine	Leu	L	113.084 07	113.160
Asparagine	Asn	N	114.042 93	114.104
Aspartate	Asp	D	115.026 95	115.089
Glutamine	Gln	Q	128.058 58	128.131
Lysine	Lys	K	128.094 97	128.174
Glutamate	Glu	E	129.042 60	129.116
Methionine	Met	M	131.040 49	131.198
Histidine	His	H	137.058 91	137.142
Phenylalanine	Phe	F	147.068 42	147.177
Arginine	Arg	R	156.101 12	156.188
Tyrosine	Tyr	Y	163.063 33	163.17
Tryptophan	Trp	W	186.079 32	186.213

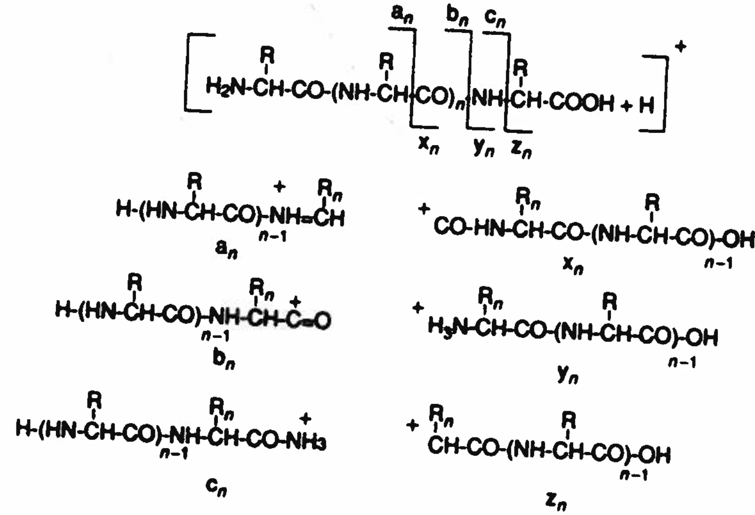

Figure 8.3
Main fragmentation paths of peptides in CID tandem mass spectrometry.

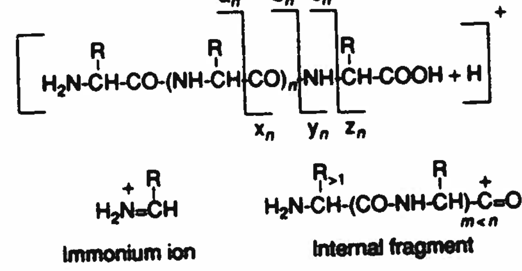

Figure 8.4
Fragments derived from a double cleavage of the peptide main chain.

Normally the spectra show several incomplete series of ions that produce redundant data and make the spectrum very complex and difficult to interpret. The peptide sequence can be deduced even if several incomplete series of ions are present, provided that there are other series of ions present and as long as there are overlaps in the deduced sequence information.

The types of fragment ions observed in an MS/MS spectrum depend on many factors including the amino acid composition, the peptide sequence, the amount of internal energy transferred, the ion activation method used, and so on. There is a marked difference between the fragmentations observed at high and low energy. At high energy, all the fragmentations described in Figure 8.3 can be generated in principle. However, all those fragments are not observed in the spectra, because the fragmentation can be influenced by the nature of the amino acids present in the sequence, as will be shown below. Unlike low-energy CID, ions do not readily lose water or ammonia. At low energy, the observed fragments are mostly the b_n and y_n ions. These fragments then lose small molecules such as water or ammonia from the functional groups on the side chains of the amino acids.

Two other types of fragments found in most spectra result from cleavage of at least two internal bonds in the peptide chain. The first type is called an internal fragment because these fragments have lost the initial N- and C-terminal sides [39]. They are represented by a series of simple letters corresponding to the fragment sequence. Fortunately, this type of ion is often only weakly abundant and, because they rarely contain more than three or four amino acid residues, they appear in the spectrum among the low masses. These peaks confirm the sequence but are often more of a nuisance than a help. Peptides containing a proline are an exception to this as the proline imino group is included in a five-atom ring and thus has a higher proton affinity than the other amide bonds in the peptide. Hence protonation and cleavage of the proline amide bond are a favoured to yield an internal fragment, extending from the proline in the direction of the C-terminal side. The various fragments requiring cleavage of two bonds are shown in Figure 8.4.

The second type of fragment that results from multiple cleavage of the peptide chain appears among the low masses in the spectrum. These are the immonium ions of the amino acids, labelled by a letter corresponding to the parent amino acid code. Even though these fragments are rarely observed for all of the peptide amino acids, those that appear yield information concerning the amino acid composition of the sample, especially when other diagnostic ions in the low-mass region are taken into account [40]. A list of immonium ions commonly found in spectra is given in Table 8.3 [41,42].

Table 8.3 Masses of the low-mass ions characteristic of natural amino acids, most often immonium ions.

Amino acid	Characteristic mass
Proline (P)	70
Valine (V)	72
Leucine (L)	86
Isoleucine (I)	86
Methionine (M)	104
Histidine (H)	110
Phenylalanine (F)	120
Tyrosine (Y)	136
Tryptophan (W)	159

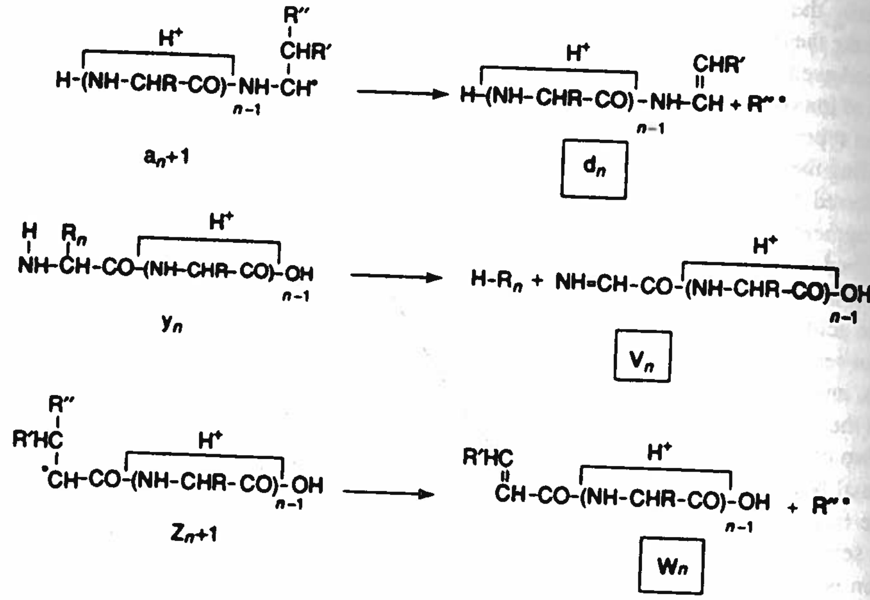

Figure 8.5
Fragmentation paths yielding the ions characteristic of amino acid lateral chains.

In addition to the ions described earlier, three new types of fragments that require cleavage of the peptide chain and the amino acid lateral chain were highlighted only in the high-energy spectra. These fragments are useful for distinguishing the isomers Leu and Ile. Figure 8.5 shows the mechanisms and structures of the corresponding fragments [43–46].

Two types of these fragments result from cleavage of the bond between the β and γ carbon atoms of the side chain of the amino acids. These fragments are symbolized as d_n or w_n respectively according to whether the positive charge is retained by the N-terminal or the C-terminal fragment. They are useful to distinguish the isomers Leu and Ile. Amino acids carrying an aromatic group attached to the β carbon atom (His, Phe, Tyr, Trp) either do not display these two fragments or have very low abundances.

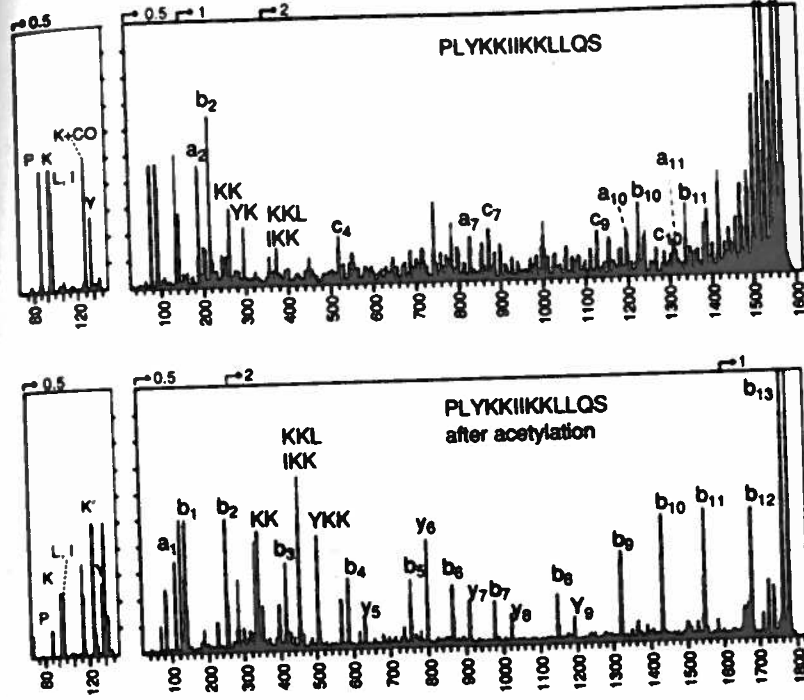

Figure 8.6
Influence of the presence and the position of the charge on the resulting fragments. Reproduced (modified) from Biemann K., Meth. Enzymol., 193, 455, 1990, with permission.

The last type of these fragments results from the complete loss of the side chain and is symbolized as v_n. This fragment containing the C-terminal moiety is intense for amino acids that do not easily yield w_n type fragments. No equivalent containing the N-terminal moiety has ever been observed.

The types of fragment ions observed in an MS/MS spectrum are influenced by charge position and charge delocalization. Protonation occurs mainly at the more basic sites. In peptides, the terminal amino group is basic. If more protons are added they will be located first at other basic amino acids, if present, and then on the amide groups. These last will be more statistically distributed along the chain.

It has been shown that charged ions from small peptides that do not contain basic amino acids display comparable abundances for all the y_n and b_n ions. When the chain becomes longer, b_n ions become favoured [47].

However, the presence and the position of a basic amino acid within the peptide influence the fragmentation process. The presence of basic amino acids such as Arg, Lys, His or Pro at the C-terminal amino acid induces mainly the formation of ions containing the C-terminal side (y_n, v_n and w_n), whereas the presence of these amino acids on the N-terminal side favours the formation of ions containing the N-terminal side (a_n, b_n and d_n). However, the absence of a basic site within the peptide is characterized by a distribution of ions containing one of the two sides (y_n, b_n), as shown in Figure 8.6 [48].

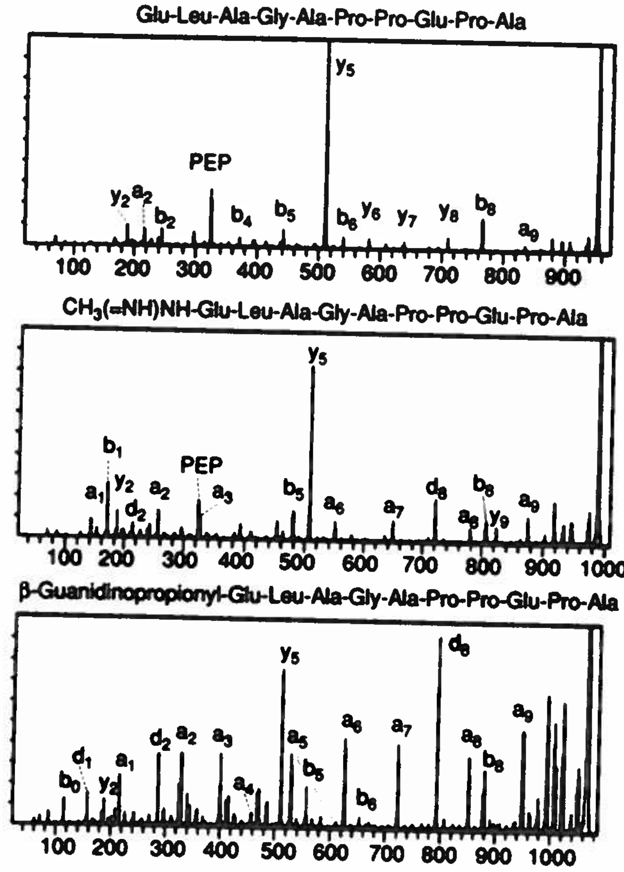

Figure 8.7
Influence of the charge delocalization on the type of fragmentation obtained. Reproduced from Martin S.A., Johnson R.S., Costello C.E. and Biemann K., in 'The Analysis of Peptides and Protiens by Mass Spectrometry' edited by McNeal C.J., Wiley & Sons, New York, 1988, pp. 135–150, with permission.

Similarly, increasing the localization of the positive charge on the peptide increases the a_n and d_n fragments that end up outnumbering the b_n and y_n fragments if the charge is carried on the N-terminal side or increases the v_n and w_n fragments if the charge is carried on the C-terminal side, as shown in Figure 8.7 [49].

The rule describing the relationship between the position and the charge localization on the one hand and the fragmentation process on the other is summarized in Figure 8.8.

The discussion up to now has only been concerned on the CID spectra of singly charged precursor ions, but the development of ESI allows the study of the dissociation of multiply charged ions. As a rule, the fragmentation spectra of multiply protonated peptides, at high as well as at low energy, display fragments analogous to the one observed for the monocharged species [50,51]. Multiply charged ions require lower acceleration voltages before CID because the kinetic energy is proportional to neV, where n is the number of charges, e is the electron charge and V is the acceleration voltage. At first glance they appear to fragment easier. It is best, when possible, to acquire spectra of different charge states because some fragments can be more abundant for some charge states.

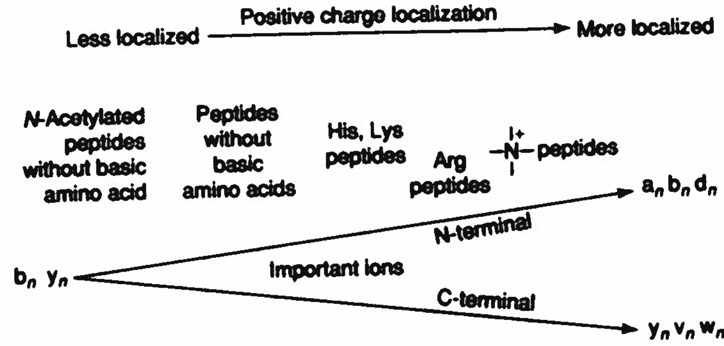

Figure 8.8
Charge and fragmentation with respect to the nature of the peptide.

The dissociation of multiply charged ions is interesting but can lead to more complicated spectra. Firstly, the presence of protons on different protonation sites induces fragmentations from a variety of starting points. Secondly, the ions obtained can have different charge states and the charge state has to be determined. Because two adjacent peaks of the isotopic cluster are separated by 1 Da, if the number of charges is n, they will appear as being separated by $1/n$ Th. If the resolution of the instrument is sufficient, the charge state can be determined by this way. Tryptic peptides have two basic sites, one at each terminal, and thus easily yield doubly protonated ions: on the basic C-terminal amino acid and on the free amino group of the N-terminal. As a consequence, they mainly yield monocharged b_n and y_n ions, as shown by the example in Figure 8.9 [52,53].

Fragmentation of peptides can be obtained in MALDI during ionization as observed by ion source decay (ISD). In this case, the most abundant fragment ions observed in ISD are c_n and y_n ions. Fragmentation of peptides can be obtained also with an reflectron TOF instrument by the PSD technique [54,55]. An example of such a spectrum is displayed in Figure 8.10. The fragment ion types observed in MALDI-TOF PSD are close to those observed at low-energy CID [56]. With MALDI-TOF/TOF instruments where collision gas is used, the spectra are similar to those observed at high-energy CID. All ion series can be accompanied by ions resulting from losses of ammonia or water.

Fragmentation of peptides can also be observed with FTICR instruments. Infrared multiple photon dissociation (IRMPD) and electron capture dissociation (ECD) have been introduced as two alternative dissociation methods to the low-energy CID method. The IRMPD method produces many fragments that make the spectrum very complex and difficult to interpret. Some of the fragment types observed with IRMPD are b_n and y_n type ions or these ions that have lost ammonia or water. However, most of them are not these types of fragment ions.

ECD has recently been introduced as an alternative activation method to obtain fragmentation of multiply protonated peptides [57]. An example of an ECD fragmentation spectrum

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