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The changing impact of the collision-induced decomposition of ions on mass spectrometry

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Abstract

The first observations of the products of the collision-induced decomposition (CID) of ions were made in the early days of the development of mass spectrometry and for many years, they were regarded as little more than a nuisance. Early in the 1960s, systematic work on CID began, and, although it proved to be very useful in the investigation of the structures of ions, in general, the mass spectrometry community regarded CID as a subject for fundamental studies but of little value in analytical work. In essence, CID was a technique looking for a problem. With the advent of soft ionisation methods, first fast atom bombardment and later electrospray ionisation and matrix-assisted laser desorption ionisation, all of which gave molecular weight information but no structural information, the situation changed, and CID became an integral part of analytical mass spectrometry. High-performance, compact tandem mass spectrometers based on the quadrupole, time-of-flight and ion trap mass analysers play an increasingly important role in biological mass spectrometry and Fourier transform ion cyclotron resonance instruments provide very-high-resolution CID capabilities. Tandem mass spectrometry is already the method of choice for the sequencing of proteins and will undoubtedly be important in helping us understand protein function. (Int J Mass Spectrom 200 (2000) 479–493) © 2000 Elsevier Science B.V.

Keywords: Collision-induced decomposition; Ions

1. Introduction

Ions that decompose in the flight tube of a mass spectrometer have been known since the early days of mass spectrometry and were originally known as *Aston Bands* [1]. In a single-focusing magnetic sector instrument, if an ion m_1^+ dissociates in the field-free region before the magnet to give a product ion m_2^+, a diffuse peak is observed at an apparent mass $m^* = m_2^2/m_1$, as first shown by Hipple, Fox and Condon [2]. Initially, the peaks were assumed to arise from unimolecular decomposition of the precursor ion, m_1^+, the collection of the resulting m_2^+ ions giving rise to what became known as a *metastable peak*. Some peaks, however, were undoubtedly due to fragmentation arising from the collision of ions, after acceleration, with molecules of residual background gas and hence were products of collision-induced decomposition (CID). When trying to apply the Quasi-Equilibrium Theory (QET) [3] to the fragmentation of ions, it was important to be able to distinguish between unimolecular and CID processes. For the most part, however, such peaks were regarded as a nuisance or, at best, a curiosity, and instruments were fitted with metastable suppressors, which reduced the intensities of these peaks relative to those of normal peaks. It is

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interesting to observe that many spectra are now obtained in which the normal peaks are suppressed and the products of CID are used to identify a compound or to obtain structural information about it.

For various reasons, metastable transitions proved to be of limited use for identifying samples and probing their structures, and, at best, they were seen only as a method of supporting inferences made from other more direct observations. The internal energy content of the ions is lower on average than that of ions that fragment in the source, and the time at which fragmentation occurs is considerably later. As a result, the relative abundance of the two sets of product ions are very different and cannot readily be related. Furthermore, the time interval between ion formation and fragmentation is sufficient to allow ions in some cases to isomerise before they fragment, thereby reducing the value of the resulting spectrum.

The QET states that the fragmentation products on an ion depend only on the internal energy of the ion but not on the method by which the ion acquires the internal energy. It therefore seemed possible that the products of collision-induced decompositions could provide useful additional information, in that the resulting spectrum should more closely resemble that of ions formed in the source. The average internal energy could be increased by converting a small fraction of the translational energy to internal energy by colliding the ions with residual gas such as helium or air in the flight tube. These products can be observed using techniques very similar to those used for observing products of metastable transitions, and the problem of isomerisation is reduced to some extent because the higher average internal energy leads to faster fragmentation of ions in a CID experiment.

2. Tandem mass spectrometry in space

In all the early work on the CID of ions, the selection of the primary ions is carried out in the first part of the flight path of the ions as they pass through the instrument. After passage through the collision region, the observation of the product ions is carried out in a second mass analyser in a later part of the flight path of the ions. The selection of the precursor ion and observation of product ions are therefore carried out in parts of the instrument that are spatially separated.

2.1. The age of magnetic sector instruments

The availability of commercial double-focussing magnetic sector mass spectrometers from the early 1960s made the observation of the products of metastable transitions available for the first time to a wide variety of mass spectroscopists. In a forward geometry instrument, such as the MS9 and MS50 (AEI Ltd.) and the 21-110 (CEC Inc.), decompositions that occurred between the electric and magnetic sectors gave rise to peaks as described above, and early work made use of this. Reverse-geometry instruments, such as the ZAB (Vacuum Generators Ltd.) gave spectra free from such peaks, because the electric sector did not transmit the lower energy fragment ions formed by decomposition after acceleration. In some of the earliest experiments on the CID of simple aromatic molecular ions produced by electron ionisation at a nominal 12 eV, the pressure in the analyser tube of an MS9 instrument was increased first by loosening a flange and later by baking the electrostatic analyser [4]. The spectrum of product ions observed at m_2^2/m_1 given by CID was shown to be similar to the normal 70-eV electron impact spectrum after allowing for variation in detector response. A section of the spectrum given by the CID of the toluene molecular ion is illustrated in Fig. 1. This was in accordance with the predictions of the QET, and the CID of molecular ions could therefore, in principle, be used to obtain structural information. This method is of very little general use, because, at higher electron energies, many fragment ions are produced and transmitted by the electric sector. Consequently, the diffuse peaks observed at m_2^2/m_1 are often difficult to interpret since the masses of neither m_1 nor m_2 can be identified with certainty. Clearly, if one of them could be identified, the position of the peak would allow one to identify the other ion and so characterise the fragmentation yielding the product ions.

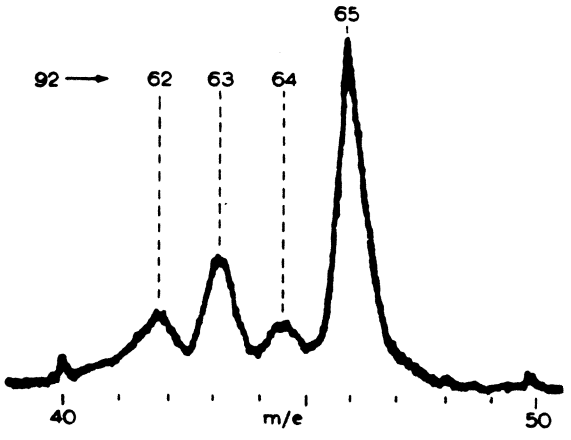

Fig. 1. Part of the CID spectrum of the toluene molecular ion observed in an MS9 double-focusing magnetic sector instrument. (Reproduced from Ref. 4.)

For both forward and reverse geometry instruments, a simple modification allows one to observe the products of metastable transitions in the absence of peaks of the normal mass spectrum by decoupling the accelerating voltage V from the electric sector voltage E. In forward geometry instruments, products of decompositions occurring in the field-free region between the source and the electric sector can be observed by tuning the instrument to collect normal m_2^+ ions and then increasing the accelerating voltage from its normal value of V_0. If $m_1^+ \rightarrow m_2^+$ in this region, the m_2^+ product ions can be observed if V is increased to V_1 such that $V_1/V_0 = m_1/m_2$. This was first accomplished by using the ΔV control of the MS9 instrument to demonstrate the loss of H or H_2 by an ion [5] and later extended to allow the observation of the loss of much larger neutral species by modifying the MS9 source supply electronics [6]. A scan of V indicates all m_1^+ precursors of the chosen m_2^+ product ion, because whenever $V_1/V_0 = m_1/m_2$, m_2^+ ions formed from m_1^+ possess the correct energy to be transmitted by the electric sector and the correct momentum to be transmitted by the magnetic sector. They are therefore collected at the normal position on the mass scale. A spectrum showing the precursors of the product ion C_3H^+ in the mass spectrum of toluene is illustrated in Fig. 2. A reverse geometry instrument is tuned to collect m_1^+ ions formed in the source, and these are transmitted normally by the magnetic sector. All products formed by the decomposition of these ions can be collected if the electric sector voltage is scanned to lower values than its normal value of E_0 [7]. If $m_1^+ \rightarrow m_2^+$ in the field-free region between the

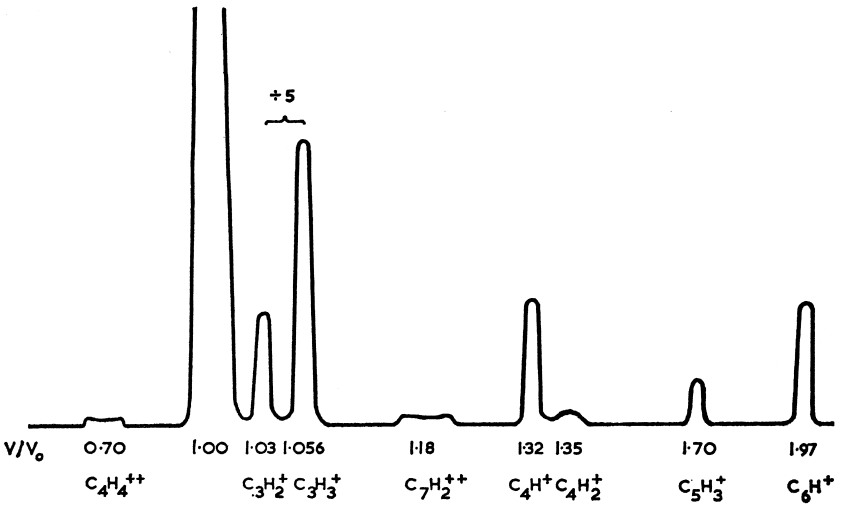

Fig. 2. Precursor ions of the C_3H^+ product ion in the mass spectrum of toluene obtained on an MS9 instrument by scanning the accelerating voltage, V, at constant E and B. (Reproduced with permission from K.R. Jennings, Some Aspects of Metastable Transitions, in G.W.A Milne (Ed.), Techniques and Applications, John Wiley and Sons, Inc., New York, 1971, p. 419.)

magnetic and electric sectors, m_2^+ ions are collected when $E_0/E_2 = m_1/m_2$ when the electric sector passes all m_2^+ ions of the appropriate energy. These two scans are known respectively as the V-scan and MIKES-scan (mass-analysed ion kinetic energy spectroscopy) and were the main methods of investigating metastable transitions from the mid-1960s and throughout the 1970s.

The instrumentation was improved by effecting the CID in a cell at a focal point in the analyser tube, controlling both the flow rate and nature of the collision gas and automating the scanning. This allowed one to produce complete fragmentation schemes for the molecular ions of small sample molecules and to determine the major fragmentation routes of larger molecular ions [8]. Helium was usually chosen as the collision gas, because, for low mass ions, the conversion of translational energy into internal energy during collision was sufficient to lead to fragmentation, its low mass minimised loss of ions by scattering and its high ionisation energy minimised loss of ions by charge exchange. In fact, it is probable that much of the CID was a result of residual air in the tubes connecting the helium and the mass spectrometer because absolutely pure helium is relatively inefficient in effecting the CID of ions [9].

For the most part, however, the mass spectrometry community did not find CID of ions of great interest. Rather than seek a method for increasing the fragmentation of ions, the main interest at the time was in developing a softer method of ionisation, such as chemical ionisation, which would give molecular weight information and structural information from controlled fragmentation of the MH^+ ion. Fundamental work on collision processes was carried out by a number of groups, such as Futrell and Tiernan [10], who were concerned primarily with ion–molecule reactions. Others, most notably McLafferty and coworkers, used CID to great effect to investigate the structures of ions that could exist in two or more isomeric forms [11]. The structural information can be inferred from the fragment ions produced in much the same way that it is deduced from electron ionisation or chemical ionisation spectra but the main use of CID was to investigate structures of fragment ions of identical elemental composition formed from different neutral species. A fundamental assumption made in the use of CID spectra is that differences in spectra arise from structural differences in the ions undergoing CID and that internal energy effects are negligible. Although this assumption is usually valid at high collision energies, it was later found to be more difficult to justify at low collision energies when small differences in collision energy can lead to substantial differences in the CID spectrum. Isomerisation to a common structure or mixture of structures prior to fragmentation occurs in certain cases so that structurally different isomeric compounds, such as isomeric alkenes, give nearly identical CID spectra. In such circumstances, little can be deduced from such spectra. On the other hand, if the CID spectra of two ions having the same molecular formula are different, it is almost certain that the ions have different structures.

In general, CID was seen by many as a technique waiting for a problem, because electron and chemical ionisation spectra were adequate for most analytical problems in chemical mass spectrometry at the time, especially when used in combination with gas chromatography, and CID seemed to offer little useful additional information. Towards the end of the 1970s, various linked scans were developed for use with double-focussing magnetic sector instruments [12–15]. The most widely used for analytical work was that in which the ratio of the magnetic field strength, *B*, and the electric sector field strength, *E*, remained constant (sometimes known as the *B/E* linked scan) when both were scanned [13]. The instrument is tuned to collect normal m_1^+ ions, which determines the initial value of *B*, and *B* and *E* are then scanned such that *B/E* remains constant throughout the scan. For any m_2^+ ion formed from the chosen m_1^+ ion, both the energy and momentum of the m_2^+ ions is m_2/m_1 times that of the m_1^+ ions so that the ions are passed by each sector if the ratio *B/E* remains constant. This allows the collection of all product ions formed by the CID of the chosen m_1^+ precursor ions. This scan largely replaced the scans of *V* and *E* described above, since it has the advantage for analytical work of providing higher resolution than these scans because it discriminates against the collection of ions formed in pro-

cesses in which translational energy is released. The technique continued to be of interest primarily to those working on fundamental aspects of gas-phase ion chemistry but its use for demonstrating the presence of a specific substance in a complex mixture was, however, beginning to be recognised [16].

2.2. The advent of the triple quadrupole instrument

Two developments at the end of the decade stimulated interest in CID. Following construction of a triple quadrupole for fundamental work [17], the analytical triple quadrupole instrument by was introduced by Yost and Enke [18], and this was quickly followed by commercially available models. The quadrupole instrument was widely used for GC/MS work and the addition of a radiofrequency-only quadrupole collision cell and a second quadrupole mass analyser produced a compact, easy-to-operate tandem mass spectrometer, often referred to as a QqQ instrument. One can select a precursor ion in the first mass analyser, allow it to undergo CID in the rf-only quadrupole cell at collision energies up to 200 eV and observe products by scanning the second analyser. Conversely, the product ion can be selected in the second analyser and all precursor ions observed by scanning the first analyser. Unlike the two-sector magnetic deflection instruments, the triple quadrupole is a true tandem mass spectrometer, and the ease with which different types of scan could be carried out and with which it can be interfaced to GC and LC equipment meant that it was quickly adopted and widely used [19–21].

2.3. The beginning of biological mass spectrometry

The second development was the description of fast atom bombardment (FAB) ionisation by Barber and co-workers in 1981 [22]. This very simple ionisation technique enabled one to ionise compounds having a molecular weight of several thousand and was free from the practical difficulties that were associated with plasma desorption, described a few years earlier [23]. Because FAB is a relatively soft ionisation technique, the major peak in the spectrum is frequently that due to MH^+ ions so that although the molecular weight can be determined, the spectrum gives little or no structural information. Finally, here was a problem that required the use of CID to produce structurally significant fragment ions from the MH^+ ions given, for example, by many peptides and fragments of proteins. CID was, for the first time, an integral part of the protocol required for obtaining structural information for biological molecules.

For much of the 1980s, a debate raged over the best way to carry out the CID experiments. The triple quadrupole approach was seemingly very simple and straightforward in that separate analysers of unit mass resolution were used in the CID experiment. A major uncertainty was the variable transmission of the quadrupole mass analysers, especially at higher mass-to-charge ratios (*m/z*), together with the use of a low collision energy (typically 30–60 eV) [24]. Small variations in the collision energy led to rather worrying changes in the CID spectrum observed, so that use of libraries of CID spectra was difficult. The transmission of early QqQ instruments for ions of *m/z* above about 1000 was frequently quite low, and, although they were capable of giving very useful results, the resolving power had to be reduced to maintain sensitivity [25]. The use of the linked scan at constant *B/E* on a two-sector magnetic deflection instrument had the advantage of producing CID spectra at high collision energies that were relatively reproducible [14]. The major limitation of the technique is that although the resolving power for product ions is typically about 1000, for large commercial instruments such as the MS50 instrument (Kratos), the resolving power with which the precursor ion can be selected is typically only 300–400. Hence, for samples of relative molecular mass above about 400, it is impossible to select a monoisotopic ion from the isotopic envelope in the region of the molecular ion.

2.4. The introduction of hybrid tandem mass spectrometers

It seemed that what was wanted was high collision energy coupled with high resolving power with which to select the precursor ion. Consequently, during the

1980s, this led to the development of large tandem mass spectrometers incorporating a two-sector magnetic deflection instrument as the first mass analyser for precursor ion selection. Because such instruments typically have a resolving power in excess of 100,000, one can select monoisotopic ions for CID experiments at high resolving power and with reasonable sensitivity up to relatively high masses. The upper limit of about *m/z* 3000 was determined primarily by the efficiency of the ionisation and the CID processes. In general, lower resolving power suffices for the second mass analyser, which is scanned to identify product ions. In the so-called hybrid instruments of BEqQ geometry, a quadrupole mass analyser was used for the second stage, based on work originally carried out by Cooks and his co-workers [26,27]. In such instruments, CID could be carried out either at high collision energy followed by a deceleration stage or at low collision energy in the rf-only quadrupole after deceleration of the precursor ion beam. In either case, however, the need to convert a high-energy ribbon-like beam of ions into a low-energy beam of approximately circular cross section led to considerable transmission problems [28]. Because the quadrupole mass analyser must be scanned to record the product ion spectrum, the sensitivity is further reduced, as all ions not being collected are lost. In addition, the low-energy CID of large molecules, such as those of interest to biochemists, is often inefficient, and relatively few fragment ions are therefore produced.

2.5. Four-sector tandem mass spectrometers

To combat this, commercial four-sector magnetic deflection instruments were introduced at the end of the 1980s. Much earlier, Futrell and Miller had described the first four-sector instrument but this was designed for the study of ion–molecule reactions at near thermal energies rather than for CID studies [29]. In the commercial instruments, CID occurs at a collision energy of several keV in a cell placed between the two mass analysers [30]. In principle, both precursor and product ions could be selected at very high resolving power, but this was rarely possible because of sensitivity problems. Usually, the first mass analyser was operated at a resolving power just sufficient to select m_1^+ ions unequivocally, and the second mass analyser was operated at a resolving power sufficient to give unit mass resolution for the highest mass fragment ions. Floating of the collision cell to allow postacceleration detection reduced scattering effects and allowed the use of heavier collision gases that improved the sensitivity significantly, particularly for low-mass fragments, so that the overall sensitivity was significantly greater than that of the BeqQ hybrid instruments discussed above [31,32]. Product ions were observed by use of a modification of the linked scan in which *B/E* remains constant. The low sensitivity of magnetic sector mass analysers arising from their being scanning instruments was to some extent overcome by placing an array detector along the focal plane of the second mass spectrometer [33]. This allowed one to collect ions simultaneously and to integrate the signal over a small *m/z* range, typically 4–5%, the complete spectrum being obtained by stepping the *m/z* range covered. This type of detector was not widely adopted primarily because of the high cost.

Throughout the 1980s, the main ionisation technique used for high-molecular-weight compounds was fast atom bombardment (FAB). This produced singly charged ions and hence a requirement for mass analysers that could resolve ions of high *m/z*. The upper practical mass range for the production of ions was raised from ~1500 Da to ~5000 Da, but the upper mass range for obtaining informative CID spectra was somewhat lower. For example, using a four-sector instrument with a floating collision cell and argon as collision gas, we were able to sequence a peptide of mass 2465 Da from its CID spectrum [34], as shown in Fig. 3. At the time, this was one of the largest peptides to be sequenced in this way. Large magnetic sector instruments had a *m/z* range of up to 15,000, but their size, cost and complexity made it highly unlikely that they would become the standard instruments for biological mass spectrometry. Furthermore, the limited upper mass range of FAB ionisation, the need for picomole quantities of sample for structure determination and the severe discrimination effects that were frequently observed when it was

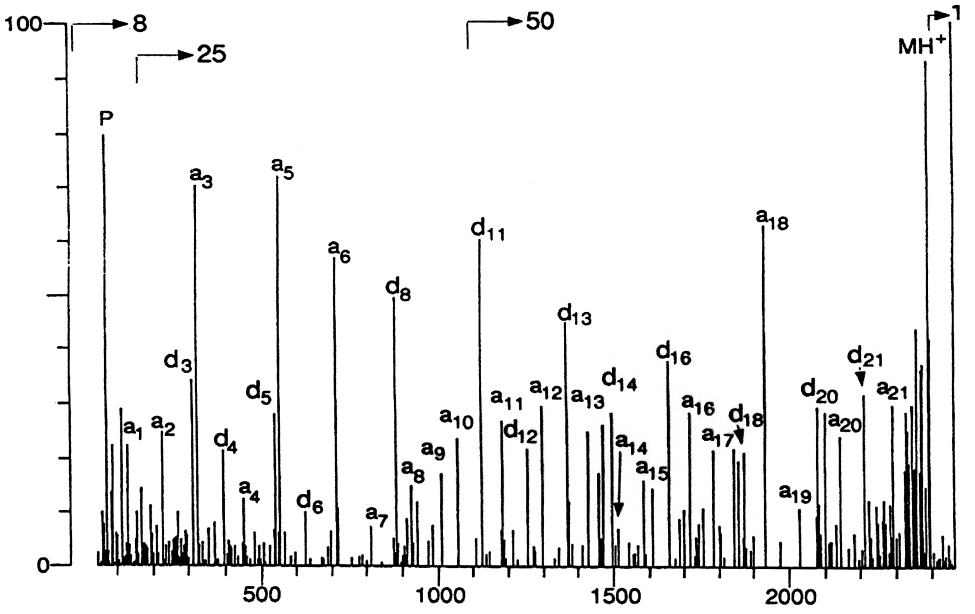

Fig. 3. A CID spectrum obtained by use of a four-sector instrument to sequence the peptide CLIP, *m/z* 2465, with argon as collision gas, showing a complete sequence of **a** ions yielding the sequence RPVKVYPNGAEDESAEAFPLEF.

employed to ionise mixtures were further disadvantages. Some new methods of ionisation and mass analysis were clearly needed.

2.6. ESI and the CID of multiply charged ions

Of the various ionisation methods based on sprays, it was electrospray ionisation (ESI) that offered the first breakthrough for extending the mass range of samples that could be ionised [35]. The very useful feature of this type of ionisation is that for many samples of relative molecular mass above about 1000, two or more protons are attached to the sample molecule producing a range of ions of the type $[M+nH]^{n+}$. Because the ions carry multiple charges, almost all samples produce ions of *m/z* 500–2000. Furthermore, as the mass of the samples increases, it becomes increasingly impossible to resolve monoisotopic peaks and in many cases, the observation of a single, unresolved peak giving the average or chemical molecular mass is adequate to identify a sample. Consequently, the need for mass analysers capable of focussing ions of very high *m/z* at very high resolving power diminished considerably. In addition, difficulties associated with interfacing the atmospheric pressure ESI source at 8–10 kV with a magnetic sector instrument led to a marked drop in the use of such instruments. The ESI/QqQ instrument became increasingly common and this added a new dimension to CID experiments. For the first time, it was usual for the precursor ion to carry more than one charge so that if a small, singly charged ion was lost, the remaining larger product ion would have a higher *m/z* ratio than the precursor ion. This has the added advantage that as the size of the molecule increases, so that more energy is required to promote CID, the increasing charge leads to a higher collision energy so that CID can be observed for ions of much higher mass than is possible for singly charged ions. Furthermore, in many cases, structural information can be obtained on 10–100 fmoles of sample.

2.7. MALDI and the resurgence of the time-of-flight instrument

Towards the end of the 1980s, matrix-assisted laser desorption ionisation (MALDI) was introduced [36].

In this technique, the sample is dissolved in a matrix, usually a substituted aromatic unsaturated acid, and ionisation is effected typically by the use of a pulsed nitrogen laser giving radiation at 337 nm; ions are extracted by the application of 25–30 kV. This pulsed source is ideal for use with time-of-flight (TOF) mass analysers, especially when time-lag focussing is used to reduce the effects of ion energy spread [37], but when a linear mass analyser is used, no tandem mass spectrometry can normally be carried out. Frequently, a reflectron is fitted [38], and this is normally tuned to reflect all ions with the full translational energy of ions leaving the source and improves resolving power by compensating to some extent for the energy spread of ions leaving the source. For CID studies, product ions of lower energy, formed in decompositions after acceleration, can be focussed over a limited mass (i.e., energy) range if the reflectron is tuned to focus ions of the appropriate energy range [39]. By tuning the reflectron in a series of steps, different groups of product ions of decreasing energy can be brought to focus and a complete spectrum obtained by stitching together the different partial spectra. This is best accomplished by means of a two-stage reflectron, which can be adjusted to focus a greater mass range of product ions than is possible when a single-stage reflectron is used. This is particularly useful when only a few of the major products of CID are of interest for use in database searches. Alternatively, a nonlinear reflectron may be used to acquire spectra of a wide mass range of product ions but at some cost in sensitivity and resolving power [40]. This technique has been called post-source decay (PSD) spectrometry to cover all forms of ion decomposition that occur in the flight tube [39]. Some ions will be formed with sufficient energy to fragment in the region of the sample surface; others undergo multiple low-energy collisions before experiencing the main accelerating voltage after which high energy CID may occur in the flight tube. Precursor ion selection is achieved by ion gating in which all ions except a narrow mass window are deflected close to the source before most decompositions have occurred.

The duty cycle of a modern orthogonal TOF mass analyser is high, and a relatively high fraction of the ions leaving the source are, in principle, detected [41]. As a result, its sensitivity is one to two orders of magnitude greater that that of a typical scanning instrument in which all ions not currently being detected are lost. Furthermore, its upper mass range and resolving power are considerably greater than that of a typical quadrupole instrument. It has therefore replaced the second quadrupole in the QhQ tandem mass spectrometer to produce a QhTOF configuration, such as in the Micromass Q-TOf instrument [42] as illustrated in Fig. 4. A recent example illustrating its higher resolution is in distinguishing mutant β-chains of haemoglobin differing in mass by only 1 Da by observing the CID of a resolved doubly charged ion at *m/z* 1030 rather than working with the isotopic cluster of ions [43]. Both positive and negative ion CID spectra have recently been used to demonstrate its use in determining the structures of a number of model gangliosides [44].

In each of the above methods of studying the products of CID, the precursor ion is selected after the ions formed in the source have been subjected to an accelerating voltage of up to 30,000 V in a TOF instrument down to about 30 V in a quadrupole instrument. A second mass analyser is used to observe product ions. If one wishes to look at a consecutive fragmentation process, such as $m_1^+ \rightarrow m_2^+ \rightarrow m_3^+$, one requires a third mass analyser and there have been a few instruments of the type QqQqQ [45] built. The overall sensitivity of such instruments is often low because of transmission problems, and it became clear that, for studies of this type, an alternative method was required.

3. Tandem mass spectrometry in time

An alternative to the use of separate mass analysers to select each ion in the decomposition sequence is to make repeated use of a single mass analyser so that the observations are separated in time rather than in space. This has the advantages of reducing the size and cost of the instrument and of allowing one to investigate consecutive fragmentations of the type m_1^+

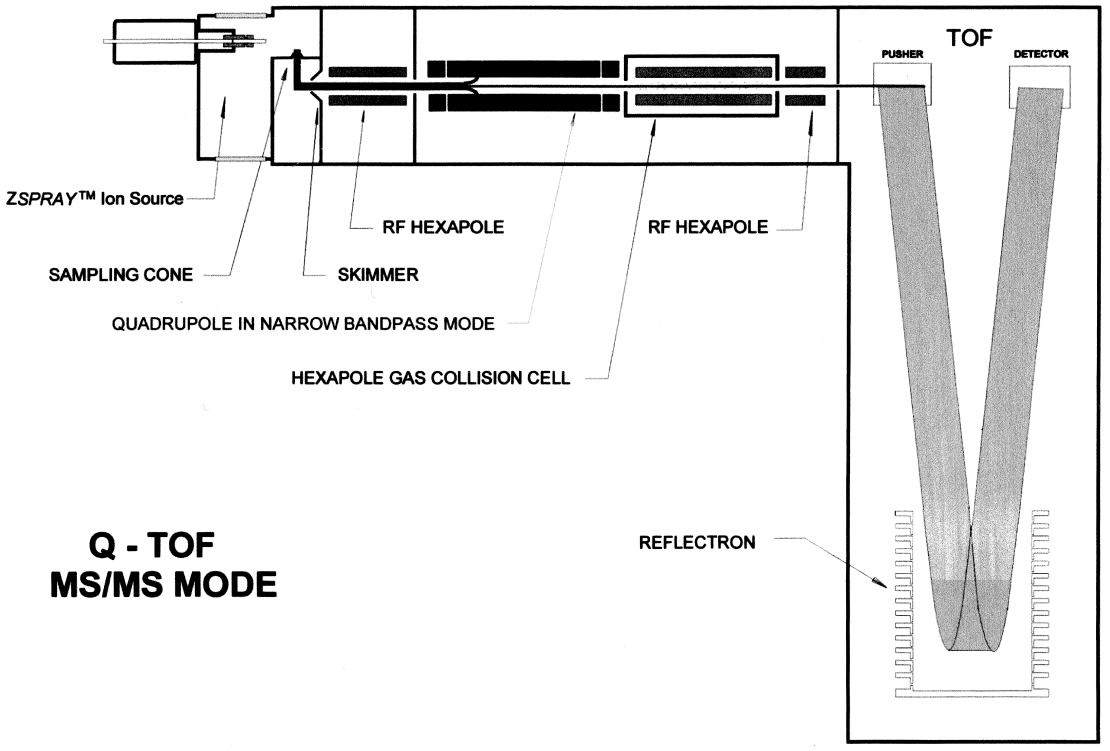

Fig. 4. Schematic of the Micromass Q-TOF tandem mass spectrometer. (Courtesy of Micromass UK Ltd.)

$\rightarrow m_2^+ \rightarrow \cdots \rightarrow m_n^+$, often referred to as MS^n, where n can be typically up to 5 or 6.

3.1 The ion trap mass analyser and $(MS)^n$

The first description of the ion trap was by Paul in 1956 [46] and the first mass analyser based on this device was described by Stafford et al. in 1984 and formed the basis of the first commercially available instrument of this type [47]. The ion trap consists of a doughnut-shaped ring electrode and two circular end-cap electrodes, so that it is axially symmetrical and, ideally, has hyperbolic surfaces for maximum performance. It is able to confine ions for long periods in a small volume close to the centre of the trap where ions may undergo reactions prior to mass analysis. As its name suggests, ions are stored within the trap and detection is accomplished by ejecting ions of a given mass-to-charge ratio to strike a conversion dynode before amplification of the signal by an electron multiplier. A mass spectrum is obtained by ramping a rf voltage, but ions that are not being ejected are stored rather than being lost, as in the scanning of a magnetic sector or quadrupole instrument. A general introduction to the properties of ion traps has recently been given [48] and recent instrumental developments have been described elsewhere [49].

Since the ion trap is operated in a pulsed mode, it allows one to store mass-selected ions between pulses, and these can be caused to collide repeatedly with helium buffer gas to effect CID of a chosen ion. Because this is a very mild form of excitation, one can selectively promote fragmentation of almost all the precursor ions by the lowest energy process to give a single product ion in high yield [50]. One method of carrying out CID experiments is illustrated in Fig. 5, for investigating the CID of the *m/z* 320 and 322 molecular ions of dioxin in which the low mass cutoff

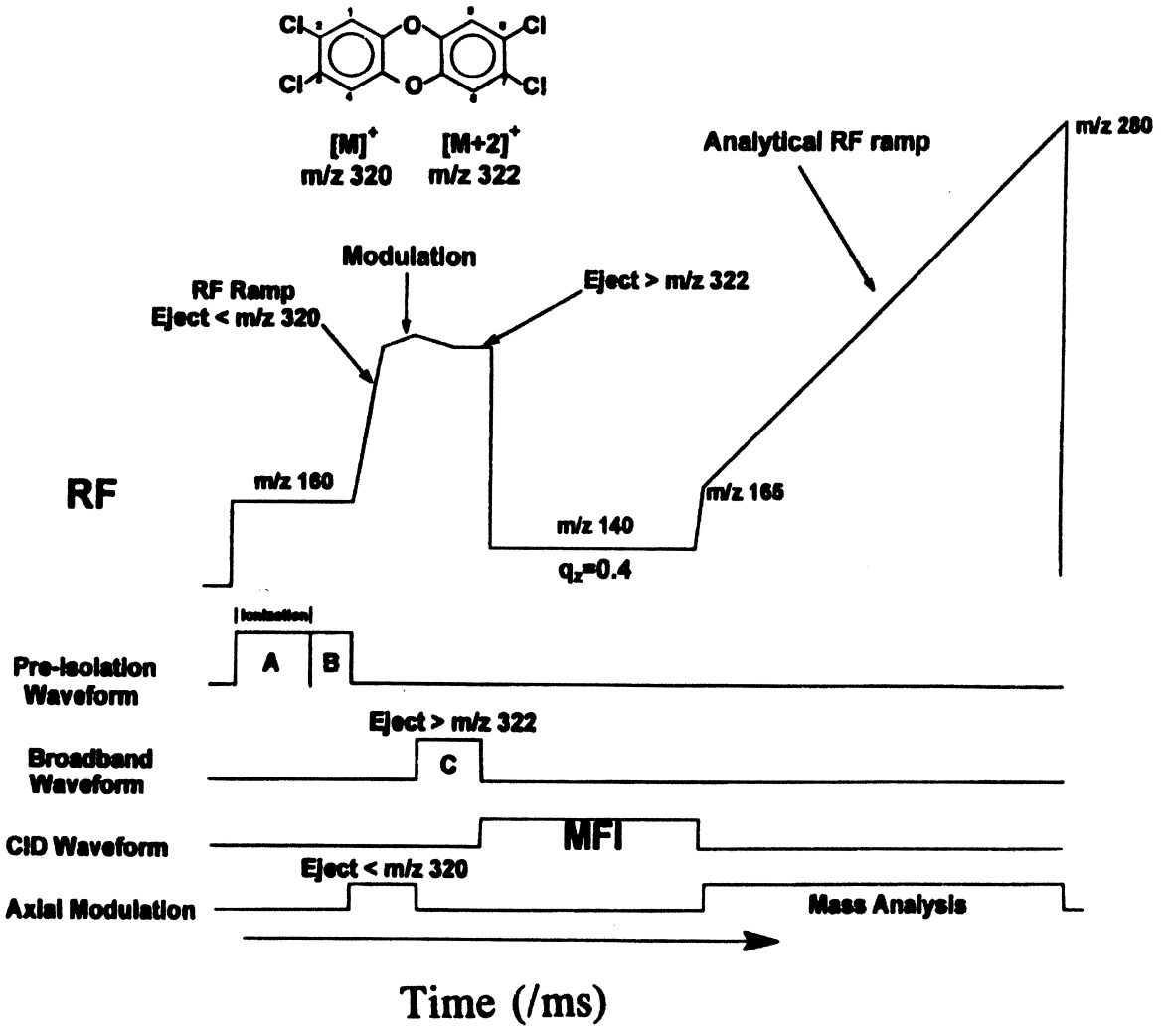

Fig. 5. Scan function for the CID of the *m/z* 320 and 322 molecular ions of dioxin in an ion trap. (Reproduced with permission from Ref. 48.)

(LMCO) was set at *m/z* 160 during ionisation. A preisolation radiofrequency voltage is applied to the end-cap electrodes, during (Fig. 5, A) and immediately after ionisation (Fig. 5, B) during which all ions of *m/z* <320 are ejected. This consists of a multiple frequency waveform with a 1-kHz notch so that all ions except those of the chosen *m/z* ratio are ejected. Fine isolation was achieved by use of a rf ramp until the LMCO was just below *m/z* 320 followed by a broadband voltage applied to the endcaps (Fig. 5, C) to remove all ions of *m/z* >322. A low-amplitude narrow bandwith mutifrequency rf voltage (MFI) is then applied to the end-cap electrodes for a number of milliseconds to effect CID, after which product ions may be selectively ejected and collected externally by gradually increasing the amplitude of the analytical rf ramp voltage [51]. Fig. 6 illustrates the resulting spectrum of product ions formed from the two isotopic molecular ions previously isolated.

Alternatively, the methods described above may be used again to isolate one of the product ions of the CID process, after which it may be subjected to further collisions to give new product ions in a process described as $(MS)^3$. This may be repeated

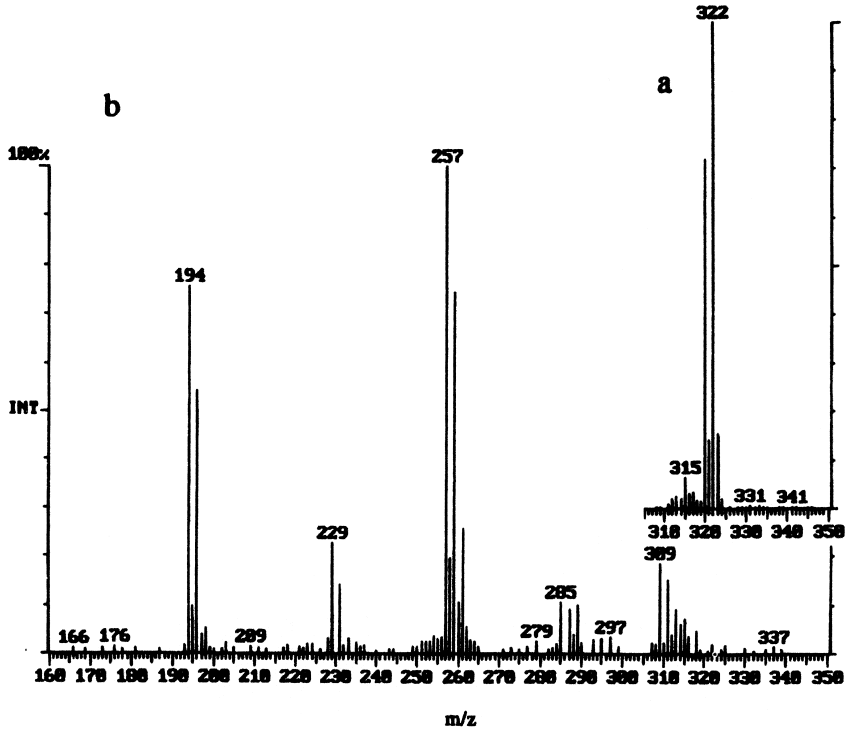

Fig. 6. Ion trap spectra (a) showing the isolation of the *m/z* 320 and 322 molecular ions of dioxin and (b) showing the CID spectrum of these ions. (Reproduced with permission from Ref. 48.)

several times so that a complete decomposition sequence may be observed in $(MS)^n$ experiments, the limit being imposed by the diminishing ion abundances available for the later stages of CID. An example of $(MS)^6$ of oleanolic glycoconjugate is shown in Fig. 7, in which one or two neutral species are lost in each CID experiment [52]. Although such experiments are of interest from a fundamental viewpoint, the ion trap is most commonly used for $(MS)^2$ or $(MS)^3$ experiments because of the falling sensitivity and increasing number of scans required as the number of stages of CID is increased. Nevertheless, the compact nature of the ion trap, its ease of coupling to an electrospray source and its intrinsically high sensitivity make it a very useful instrument for CID studies of peptides and proteins. A typical commercial instrument has a range of up to about *m/z* 2000 with unit resolving power, but these may be extended to much higher values by increasing the rf voltage and reducing its frequency and the rate of scanning [53].

3.2. CID in a Fourier transform ion cyclotron resonance spectrometer

Fourier transform ion cyclotron resonance (FTICR) spectrometry was first described by Marshall and Comisarow in 1974 [54], but, although it offered very high resolving power, the control of ions within the ICR cell became increasingly difficult as their *m/z* increased. It was therefore seen by many as a very interesting technique, useful for fundamental studies of, e.g., ion-molecule reactions, but an upper *m/z* limit of under 3000 meant that its use as an analytical instrument for high mass samples such as peptides and proteins was not widespread. Since the introduction of ion cyclotron resonance mass spectrometry in the 1960s, the technique known as double resonance had been used to increase the translational energy of a chosen ion by a few electron volts [55]. Its use was primarily to study the change in ion–molecule reaction rates as a function of the translational energy of

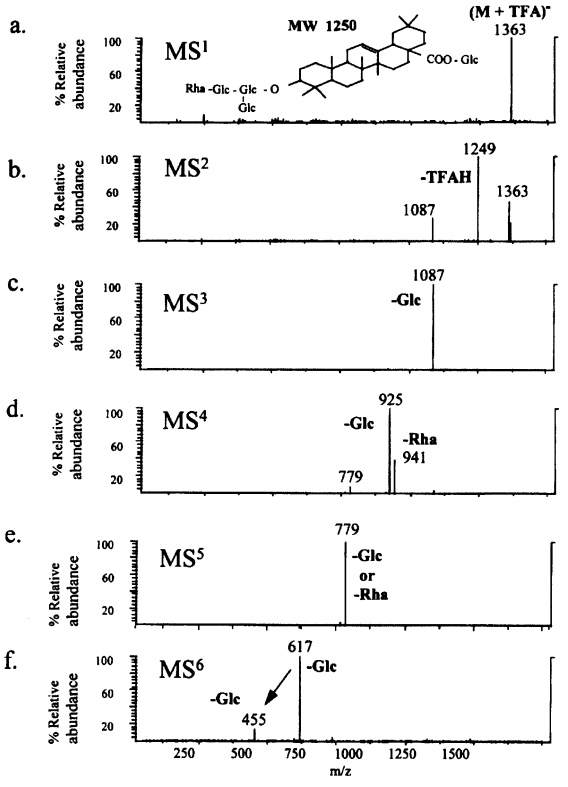

Fig. 7. The $(MS)^6$ CID spectrum of oleanolic acid glycoconjugate (molecular weight 1250) obtained by means of an ion trap. (Reproduced with permission from Ref. 52.)

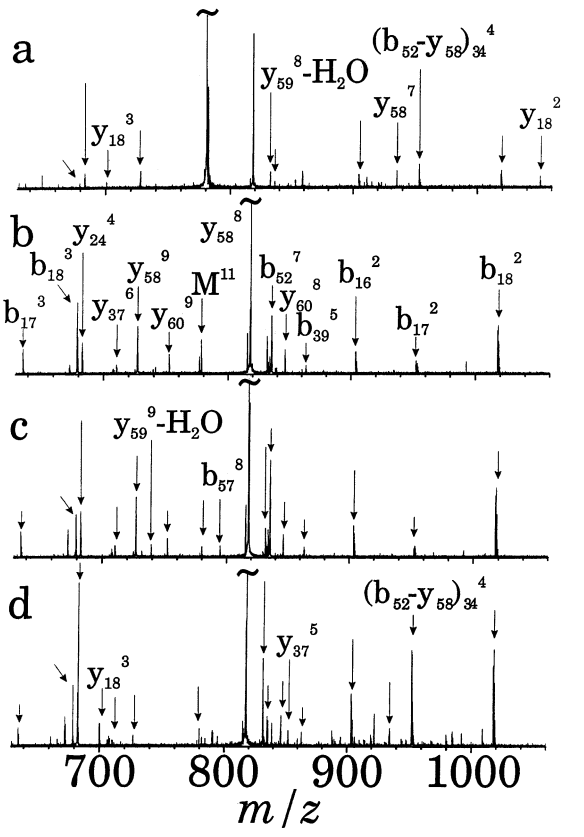

Fig. 8. The SORI/ESI/FTICR/CID spectra of bovine ubiquitin for (a) MH_9^{9+}, (b) MH_{10}^{10+}, (c) MH_{11}^{11+} and (d) MH_{12}^{12+} ions. [Reproduced with permission from M.W. Senko, J.P. Spier, F.W. McLafferty, Anal. Chem. 66 (1994) 2801.]

the reactant ion. In principle, it can be used to effect CID of a chosen precursor ion by first removing all ions of lower and higher masses by applying pulses of appropriate frequencies and amplitude prior to increasing the radius of the orbit of the chosen ion, thereby increasing its translational energy. A problem is that this process causes the ions to follow paths that are increasingly further from the centre of the cell, where the control of their motion becomes more difficult due to fringe field effects [56]. A particular strength of the technique, however, is that ions are detected by the absorption of rf power and are not destroyed, as is the case when an electron multiplier is used. This allows one to carry out $(MS)^n$ experiments with suitable pulse sequences without requiring modifications to the hardware.

The introduction of ESI changed this picture completely. High-mass samples, such as proteins produced multiply charged ions, typically of *m/z* between 500 and 2500, ideal for the FTICR instrument. By working at a sufficiently high resolving power to allow one to measure the *m/z* separation of isotope peaks, the problem of determining the number of charges on a product ion was solved. Early work on the CID of multiply charged ions made use of nozzle-skimmer or cone dissociation in the ESI source [57,58], a technique first introduced by Smith and co-workers for use with a triple quadrupole instrument [59]. McLafferty's group demonstrated that it was possible to dissociate over 80% of carbonic anhydrase ions to give a large number of product ions the masses of which could be determined with sufficient accuracy to determine variations in the amino-

acid sequences of fragment ions [58]. The lack of precursor ion selection is a particular problem with the use of ESI, and, during the past decade, a number of techniques have been described in which precursor ion selection is possible prior to the use of CID.

Two such techniques are infrared multiphoton dissociation (IRMPD), pioneered by Baykut et al. [60] and surface-induced decomposition (SID) developed by Cooks et al. [61], each of which were shown to yield product ions quite efficiently in an FTICR instrument [62,63]. They have the advantage of not requiring the introduction of a pulse of collision gas so that the operating pressure can remain low. The most efficient technique developed, however, is based on sustained off-resonance irradiation (SORI) of the precursor ion, a technique that was originally introduced to dissociate ions of low mass [64]. In this technique, a low amplitude rf pulse of frequency close to the resonant frequency of the precursor ion is applied, which causes the ion's motion to be alternately in phase and out of phase with the applied field, causing the radius of its orbit to increase and decrease repeatedly. The low amplitude ensures that the ion remains close to the centre of the cell and a pulse of gas leads to the ion undergoing many low-energy collisions yielding product ions close to the centre of the cell. This allows them to be detected most efficiently at high resolving power once low-pressure conditions have been restored. In principle, this process can be repeated a number of times so that $(MS)^n$ experiments are possible. This technique is particularly suitable for the investigation of structures of large biomolecules [65], experiments that would have been quite impossible only 10 years ago (Fig. 8).

4. The present and the future

Over the past 40 years, the CID of ions has been transformed from a nuisance, through being a curiosity to being a major technique used in determining structures of compounds of all types and in detecting traces of specific components in complex mixtures without the use of chromatography. The Human Genome Project has created a considerable interest in proteomics, the study of the full complement of proteins expressed by a cell under specified conditions, and CID plays a major role in sequencing tryptic peptides in the identification of unknown proteins. It is widely used in the study of molecular diseases, such as those arising from aberrant haemoglobin and it is of considerable importance in providing valuable information on the linkage, branching and structures of oligosaccharides and glycopeptides.

Most biological applications of mass spectrometry now use ESI or MALDI to produce ions that require the use of CID to provide structural information. They have the advantage of being very sensitive, having a high mass range and being relatively free from discrimination effects. The large and expensive magnetic sector instruments, that for so long dominated mass spectrometry, are not readily interfaced with these ionisation techniques and the relatively low sensitivity of multisector magnetic deflection instruments has contributed to the decline in their use. For biological applications, tandem mass spectrometers are now based entirely on quadrupole, ion trap and time-of-flight mass analysers and the large increases in mass range, sensitivity and resolving power that have become available in recent years have contributed to their greatly increased use. The FTICR instrument has become the instrument of choice for work requiring very high resolving power. These instruments are operated under computer control and in the case of the ion trap and FTICR, additional stages of CID simply require additional pulse sequences controlled by software.

Although CID is likely to be an important technique for the foreseeable future, more and more proteins can now be identified without recourse to extensive CID experiments. An increasing number of proteins that have already been sequenced are now contained in protein databases, and these can in many cases be identified quickly from the masses of tryptic digest peptides obtained by means of MALDI/TOF measurements. CID is used, if at all, merely to sequence tag the peptides by determining the masses of only a very limited number of fragment ions. If the protein is unknown, a more

complete CID spectrum must be obtained, but as more genomes are identified, the need for this will presumably diminish. Nevertheless, sequence information derived from CID experiments is much more specific than the peptide masses and allows one to search a wider range of databases [66]. Mass spectrometry is one of the few techniques suitable for direct analysis of peptides containing posttranslational modifications, such as phosphopeptides. A MALDI peptide spectrum indicates the presence of a single phosphorylation by an increase of 80 Da in the molecular weight and nano-ESI CID locates the position of the phosphorylated residue [67].

As the use of mass spectrometry in medical research gathers momentum, CID will play an ever-increasing part in providing structural information. As the sophistication of databases grows and the need for increasingly rapid identification of proteins rises, it may be that for known proteins, nano-ESI CID experiments will be used only to provide confirmatory sequence data. The ability of mass spectrometry to access posttranslational modifications and noncovalent interactions will be of increasing importance as interest switches from protein sequencing to protein function. For the present, I believe that we can rely on biological complexity to ensure that CID will not become part of history for some time to come.

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