Chapter 3

Instrumentation for LC-MS/MS in Proteomics

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Abstract

Mass spectrometers now have sufficient sensitivity and acquisition rates to allow analysis of complex proteomic samples on a chromatographic timescale. In this chapter the different instrument options for protein and peptide analysis will be presented, along with their relative strengths and weaknesses for producing different types of information, such as protein identification, modification characterization, or reporting quantitative measurements.

Key words: Quadrupole, Orbitrap, Fourier transform, mass spectrometry, proteomics, metabolomics.

1. Introduction

The two major types of ionization for analysis of biomolecules are electrospray ionization (ESI) (1) and matrix-assisted laser desorption/ionization (MALDI) (2). One of the fundamental differences between these methods is that MALDI is employed on samples in a solid state, whereas ESI is employed on samples in a liquid state. Hence, interfacing liquid chromatography with ESI is relatively straightforward, whereas LC–MALDI analysis is an offline process, where fractions are collected and then analyzed by MALDI at a later stage. Partly as a result of this, ESI is the dominant ionization process for analysis of samples separated by liquid chromatography and will be the emphasis of instrumentation discussed from hereon.

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2. Liquid Chromatography Instrumentation

Although electrospray starts from the solution phase, the mass spectrometer is ultimately detecting gaseous ions. Hence, during the ionization process, all the solvent has to be removed. The process of ESI converts a solution into a mist of charged droplets. These droplets shrink as the solvent is evaporated and when the charge density in the droplet reaches a critical level, coulombic repulsion causes desorption of charged gaseous ions from the droplet (3, 4). This process continues; as solvent continues to evaporate, droplets get smaller and more ions are ejected into the gaseous phase. Unsurprisingly, this conversion of liquid sample into gaseous ions is more efficient when less solvent is present. Hence, electrospray is referred to as a concentration-sensitive process. This means that the smaller the volume of sample introduced, the better the efficiency and sensitivity of the process. Increasing sample concentration can be achieved in two ways using liquid chromatography. First, use of a narrower column and lower flow rate will cause elution in smaller volumes. Second, by improving the resolution of separation, the same amount of sample will elute in a narrower profile, giving a higher concentration at the maxima of peak elution.

Nanospray is more sensitive than ESI approaches at higher flow rates (5); so sub-microliter flow rates are typically used for proteomic analyses. Many chromatographic systems cannot natively produce reliable gradients at these low flow rates due to the presence of solvent-mixing chambers in the plumbing that have too large volumes in comparison to the solvent flow rate, leading to inconsistent solvent mixing and irreproducible gradients. Hence, pressure-based flow-splitting systems are commonly employed and built into the chromatography system. Generally, a split in the range of 1:100 to 1:1,000 is used post-pump but prior to the separation column, allowing efficient mixing of solvents prior to chromatography to produce consistent separations, albeit with large amounts of solvent waste. Recently, some systems have been developed that use air pressure as the pumping mechanism and allow splitless delivery of reproducible nanoliter per minute flow rates.

Different stationary phases in chromatography columns provide variable levels of resolution. Reverse-phase chromatography is highly compatible with subsequent mass spectrometric analysis due to the lack of salts in the buffers and provides relatively high-resolution separation, so is the dominant separation method in use for proteomic analysis. Most reverse-phase stationary phases for LC-MS analysis consist of silica beads of 3–5 μm in diameter with alkyl chains of either eight or eighteen

carbons in length (C8 or C18) attached. The resolution of separation can be increased through the use of smaller particle-size resins, e.g., 2-μm-diameter beads, which leads to higher efficiency but also higher pressure separation; as a result this is sometimes referred to as ultrahigh-pressure liquid chromatography (UPLC) and generally requires specialized chromatography instrumentation to cope with the associated higher pressures (6, 7). Another form of UPLC involves the use of long columns (up to 80-cm-long columns have been reported for ultrahigh-pressure LC by the group of Richard Smith), which also results in high-resolution and high peak capacity separations (8) .

3. Mass Spectrometry Instrumentation

For identification of peptides in complex mixtures, measurement of peptide mass alone is not sufficiently informative. While the combination of accurate mass and retention time can be employed for identification in well-defined samples (9), the most flexible and generally applicable approach involves fragmentation analysis of components. In an initial scan, the masses of intact components are measured, and then in a subsequent scan/s, individual components are isolated in the mass spectrometer and then fragmented. A selection of different instrument configurations can be employed to perform these two steps of analysis.

3.1. Ion Traps

An ion trap is the only analyzer that can be used for measurement of peptides, isolation of selected components, and subsequent fragmentation analysis in the same chamber. There are three different types of ion traps employed in mass spectrometers: the quadrupole ion trap, the Penning ion trap, and the Kingdon trap (orbitrap).

3.1.1. Quadrupole Ion Traps

A quadrupole ion trap, sometimes called a Paul trap after Wolfgang Paul, who received the Nobel Prize in 1989 for its development, consists of a ring electrode and electrostatic end caps that create a box-like chamber to trap ions. The classical version of these (sometimes referred to as a 3D trap) uses two linear rods as end caps and a single ring electrode, but more recently a linear or 2D equivalent has been developed and made commercially available, which uses a set of four linear rods and two end-cap electrodes (10, 11). The 2D and 3D refer to the number of dimensions in which the ions are being oscillated during the trapping mechanism: in a 3D quadrupole trap, ions are sequentially "squeezed" and "stretched" in all three dimensions, whereas in a linear trap, the end caps are purely performing trapping and so

their voltage is not changing and ions are oscillated only radially. The main advantages of linear ion traps over 3D traps are their more efficient ion trapping and larger ion capacity, allowing them to produce spectra with higher signal-to-noise and better dynamic range (11).

The equations describing the stability of ions inside a quadrupole ion trap are not simple. However, commercial quadrupole ion trap instruments are operated in a way that maintains stability in the axial direction; so manipulation of stability in the radial direction, by altering a parameter known as the q_z, is used for determining whether an ion is trapped or not. The parameter q_z is defined by a combination of the m/z of the ion, the ion trap radius, and the frequency and amplitude of the RF voltage applied. Scans are performed by changing the amplitude of the RF voltage to sequentially eject ions radially, normally from low to high mass (12). As ions are ejected, they are detected, and by correlating the time of detection to the RF amplitude, it is possible to determine their mass-to-charge ratio (m/z).

Ions of a given m/z in the quadrupole ion trap will be moving in a motion at a certain frequency. By applying an AC voltage on the end-cap electrodes at the same frequency as this motion, it is possible to resonantly excite ions of that m/z. These ions collide with gas molecules in the ion trap and can cause fragmentation through collision-induced dissociation (CID). Unfortunately, low-mass ions (below about one-third of the precursor m/z) formed from CID analysis will have a q_z value too high to be trapped, meaning the low-mass region of the fragmentation spectrum is missing. By resonantly exciting for a very short period of time, it is possible to still trap a few low-mass ions (a technique called pulsed Q dissociation or PQD). However, this compromises the fragmentation efficiency. By applying a higher amount of resonant excitation, it is possible to eject ions from the trap without fragmentation, and this provides an alternative method for isolating a particular ion to scanning ions out at the extremes of the q_z stability range.

Quadrupole ion traps are the most common type of ion trap in use, and almost invariably if someone states that they are using an ion trap mass spectrometer, they mean a quadrupole ion trap instrument. The instruments are known for their high sensitivity. Their resolving power (ability to distinguish between components of similar masses) is dependent on the rate at which they scan the mass range. However, as one cannot detect new ions until the previous ions have been scanned/ejected out of the trap, fast scan rates are typically employed to maximize sensitivity and speed. As a result, ion trap data are typically of low resolution and relatively poor mass accuracy.

If too many ions are isolated in any type of ion trap, the charge density causes ions to interact with each other (referred to as

space-charging effects), causing distortion of the electric field and subsequent mass spectrum. Hence, the number of ions trapped is usually regulated by a process known as automatic gain control (AGC), where the number of ions is monitored over a very short period and then the same ion flux is assumed to produce a target number of ions isolated in the trap (13).

Some quadrupole ion trap instruments now have a chemical ionization source either attached to the back end of the instrument or interfaced to a quadrupole region before the quadrupole ion trap. These allow introduction of anions that can be used for electron transfer dissociation (ETD) fragmentation (14) and can also be used to introduce chemicals for proton transfer reactions that allow manipulation of the charge state of ions (15). ETD will be discussed later in this chapter.

3.1.2. Penning Ion Traps

By placing an ion chamber of similar geometry to a Paul trap in the center of a magnetic cylinder, it is possible to trap ions using a combination of magnetic and electrostatic fields, and this type of ion trapping was first used as part of a mass spectrometer by Alan Marshall (16). Ions are trapped in the center of the magnetic field and through application of an oscillating electric field in the same direction as the magnetic field, the ions are moved into a cyclotron motion. The rate at which they cycle through this motion is dependent on the m/z ratio of the ion. Ions can be detected as an image current as they pass a pair of plates on either side of the chamber in a motion referred to as ion cyclotron resonance. The sum of all the image currents for all ions trapped can be converted into an m/z scale using Fourier transform mathematics; so this technique is generally referred to as Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry.

Fragmentation can be performed in the Penning trap using a wide variety of methods, including variants of collision-induced dissociation (17, 18), infrared multiphoton dissociation (IRMPD) (19), and radical-based fragmentation using electron capture dissociation (20). For a thorough review of FT-ICR mass spectrometry, *see* (21).

FT-ICR mass spectrometry provides the highest mass precision and resolution, and can be used for determining elemental composition of components. These facets are useful for peptide analysis but are particularly beneficial for analysis of large peptides and small proteins (22).

3.1.3. Kingdon Ion Traps/Orbitraps

An orbitrap consists of an outer electrode of a barrel shape surrounding a co-axial inner electrode (**Fig. 3.1**) (23). Through electrostatic forces the ions are attracted toward the central electrode, but these forces are balanced by centrifugal forces that cause the ions to cycle around the inner electrode. The frequency of this oscillation is related to the m/z of the ion, and the ions

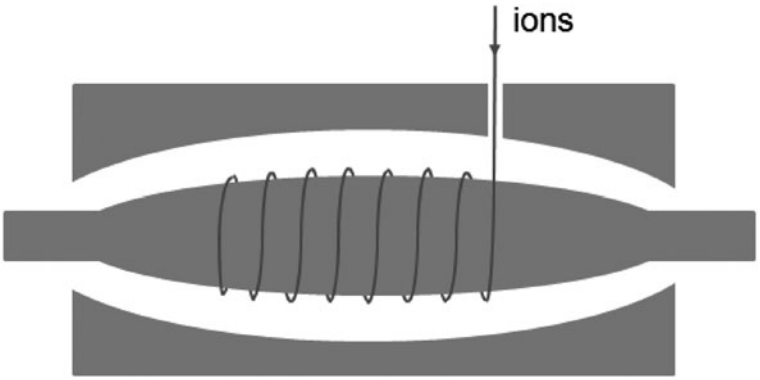

Fig. 3.1. Schematic of an orbitrap mass analyzer. Ions are injected into the orbitrap off-center and with a velocity perpendicular to the long axis of the orbitrap. The off-center injection also gives the ions potential energy parallel to the length of the orbitrap, causing them to oscillate along the plane of the orbitrap in an analogous fashion to a swinging pendulum as they cycle around the central electrode.

can be detected as an image current and converted to m/z using Fourier transformation in the same way as in FT-ICR.

An orbitrap provides high-resolution and mass accuracy detection, and has a higher dynamic range than a Penning trap has (24), meaning low-level components can still be detected in the background of more abundant co-eluting compounds.

3.2. Hyphenated Instruments

Many of the most popular mass spectrometric instruments used for proteomic analysis employ a combination of more than one analyzer type. As different analyzers are better for certain types of experiments than others, by combining analyzers together, the user can sometimes get "the best of both worlds."

3.2.1. Triple Quadrupoles

A quadrupole is a set of four linear rods, similar to a linear ion trap, but without the end caps. Variations exist with six (hexapole) or eight (octupole) rods, but they are all functionally the same. DC and RF AC currents are applied in a similar way to in a quadrupole ion trap and at given voltages, only ions of a certain m/z successfully traverse the chamber. A quadrupole can be operated in a mass selection mode, where all ions of a certain m/z can be separated from the others, or in a scanning mode, where voltages are ramped across a range of values and at a given time point, only ions of a given m/z traverse the chamber.

The triple quadrupole is a comparatively cheap mass spectrometer for fragmentation analysis. As the name suggests, it consists of three sequential quadrupoles. For measurement of intact components, the first two quadrupoles function essentially as ion transmission devices allowing all ions through and the third quadrupole is operated in a scanning mode to measure the m/z of all components (**Fig. 3.2a**). For fragmentation analysis, the first

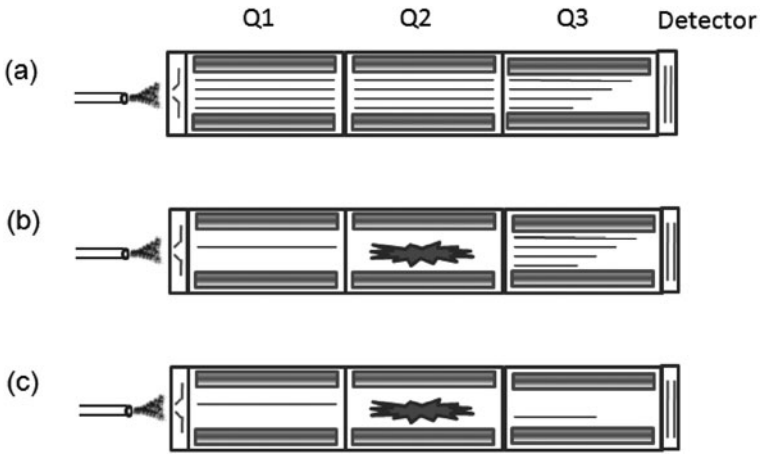

Fig. 3.2. Different operating modes of a triple quadrupole mass spectrometer. (**a**) For measurement of intact components, both quadrupoles Q1 and Q2 transmit all ions and Q3 is operated in a scanning mode where only ions of a given *m/z* pass through the chamber at a given time point during the scan. (**b**) For measurement of fragmentation spectra of a component, Q1 is operated in a mass selective mode to transmit only ions of the *m/z* of the desired precursor, Q2 is used as a collision cell, and Q3 is operated in a scanning mode to measure the fragment ion *m/z*. (**c**) For selective reaction monitoring, Q1 transmits only the *m/z* of the precursor ion, Q2 is used as a collision cell, and Q3 measures only one fragment *m/z*.

quadrupole is operated in a mass selection mode to isolate a single *m/z* component for subsequent analysis. The second quadrupole is used as a collision chamber where applied energy and introduction of low levels of a collision gas (often nitrogen, but sometimes helium or air) cause collisions and form fragments that can then be measured using the third quadrupole (**Fig. 3.2b**).

Quadrupoles are low-resolution and low mass accuracy analyzers. In a mass selection mode, they exhibit high sensitivity, but in a scanning mode, they are insensitive compared to other analyzers. Hence, triple quadrupoles are generally not the instruments of choice for discovery of peptide identifications through fragmentation analysis. However, they are powerful in studies where you already know how a component fragments and you want to either detect this component at very low levels and/or quantify its level. In experiments known as selected reaction monitoring (SRM), a single mass can be monitored in the first quadrupole, components are fragmented, and then the mass of a single fragment ion is monitored in the final quadrupole (**Fig. 3.2c**). By monitoring a single reaction (given mass precursor forms a given mass fragment), components can be detected at high sensitivity and specificity, even in the background of a very complex mixture. It is possible to cycle between a series of these SRM experiments within an LC-MS analysis (a technique often known as multiple reaction monitoring or MRM), allowing high-sensitivity

and high-specificity monitoring of a set of components (25), and this approach has great applicability for monitoring biomarkers in complex biological fluids (26).

3.2.2. Q-Traps

The Q-trap is identical to a triple quadrupole instrument except that the final quadrupole has been replaced by a quadrupole ion trap. This dramatically improves the sensitivity of the instrument for scanning mass ranges and makes the instrument more suitable for analyzing peptide fragmentation spectra. The final ion trap can also be used as a quadrupole; so SRM studies can also be performed using this instrument (26, 27).

3.2.3. QqTOF

The basis of mass analysis by time of flight (TOF) resides in the fact that when ions are accelerated with the same kinetic energy, ions of higher m/z will have a lower velocity. Hence, by measuring the time ions take to travel along a flight path to a detector, it is possible to determine their m/z. Most TOF mass spectrometers, rather than having a simple linear flight path to the detector, employ an ion mirror or a reflectron that "bounces" the ions back to a detector toward the other end of the flight tube (28). This is a mechanism that allows partial compensation for any variation in initial kinetic energy that ions may have had; if two ions of the same m/z had slightly different initial kinetic energies, then the one with higher kinetic energy will have slightly higher velocity and so will penetrate further into the ion reflectron and take more time to be reflected. By positioning the detector at the correct focus point, the two ions of slightly different initial kinetic energies but same m/z will be detected at the same time. The use of a reflectron improves the resolution and mass accuracy of TOF mass spectrometers, giving performance in between a quadrupole ion trap and an orbitrap or FT-ICR mass spectrometer. In a TOF analyzer, all accelerated ions are measured. Hence, because of their short duty cycle, TOF instruments are relatively sensitive when compared to quadrupoles.

QqTOF instruments employ a first quadrupole for precursor isolation and a second as a fragmentation chamber in the same way as triple quadrupole and Q-trap instruments. However, the final analyzer is a TOF with a flight path orthogonal to the axis of the quadrupoles (29), as shown in **Fig. 3.3**. For TOF analysis, ions need to start with no initial kinetic energy, then a packet of ions are accelerated together, and flight times are measured. Ions passing through the quadrupoles in a QqTOF have kinetic energy in the plane of the quadrupole axes but none in the orthogonal direction and so are suitable for TOF measurement perpendicular to their flight path.

QqTOF instruments are good compromise instruments between sensitivity, mass accuracy, and resolution; they are more

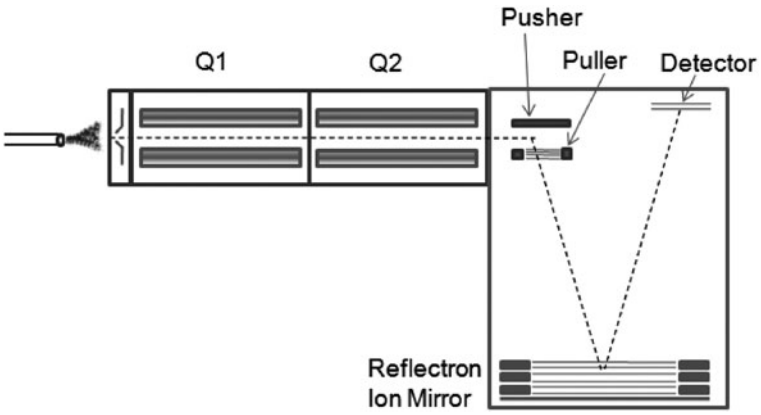

Fig. 3.3. Schematic of a QqTOF geometry instrument. Q1 can either allow all ions through or select a single *m/z* for subsequent fragmentation analysis. Q2 is used as a collision cell when CID analysis is desired. Ions entering the pusher/puller region have no velocity perpendicular to the path through the quadrupoles and so can be accelerated in pulses and their time of flight measured as they are reflected off the ion mirror and back to an ion detector.

sensitive than FT-ICR instruments but have better mass accuracy and resolution than do quadrupole ion traps.

3.2.4. Quadrupole Ion Trap–Orbitrap

As previously mentioned, quadrupole ion traps are able to measure ions, perform ion chemistry reactions to fragment components, and measure their masses, all in the same chamber. However, their routine low resolution and mass accuracy is a problem for peptide identification using database-searching strategies. Hence, there are clear advantages to an instrument where there is the alternative to measure the ions at high mass accuracy and resolution. The LTQ-Orbitrap is an instrument that combines a linear quadrupole ion trap and an orbitrap (24). All ion isolations and manipulations are performed in the quadrupole ion trap, but then the user has a choice of either measuring ions at high sensitivity but low resolution and mass accuracy in the quadrupole ion trap or transferring ions to the orbitrap (through a chamber known as the C-trap) for high-resolution and mass accuracy detection. Unfortunately there is some ion loss during transmission of ions from the linear quadrupole trap to the orbitrap; so there is a sensitivity hit in making this measurement. For identification of peptides by database-searching strategies, mass accuracy of the intact peptide is significantly more important than for fragment ions. Hence, the typical mode of operation for a LTQ-Orbitrap is to measure intact masses in the orbitrap but measure the fragments at higher sensitivity in the linear quadrupole trap.

The LTQ-Orbitrap XL (schematic shown in **Fig. 3.4**) is the same as the LTQ-Orbitrap described above, except that it has a hexapole located after the C-trap. This allows quadrupole-type

CID spectra to be acquired (although they must be measured in the orbitrap) (30). It is also used as a transmission cell for introduction of anions for electron transfer dissociation (ETD) fragmentation (31) (see below).

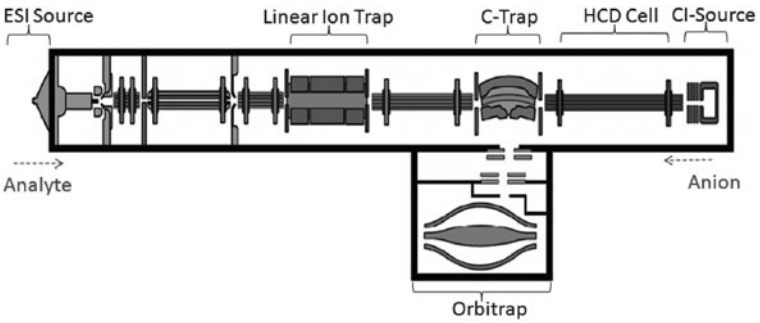

Fig. 3.4. Schematic of an LTQ-Orbitrap XL enabled with ETD. Analyte ions are introduced on the left of the instrument and pass through into the linear quadrupole ion trap. Ions can be measured in the linear trap at high sensitivity or passed out of the trap and diverted into the orbitrap through the C-trap for high mass accuracy and resolution measurement. For ETD analysis, anions are produced in the CI source at the right of the instrument and are passed through the HCD cell and C-trap to the linear ion trap, where ETD fragmentation occurs. Yet again, ions can then be measured in either the linear ion trap or orbitrap. Quadrupole (rather than quadrupole ion trap) fragmentation analysis can be performed in the HCD cell but can be measured only in the orbitrap.

3.2.5. Quadrupole Ion Trap–FT

Interfacing a quadrupole ion trap with an FT-ICR gives very similar functionality to an LTQ-Orbitrap, with the ability to measure ions either at high sensitivity or with high mass accuracy (32). FT-ICR can produce higher resolution measurement than can an orbitrap, but for proteomic applications, both have more resolution than strictly required. The advantages of the orbitrap are better dynamic range and sensitivity; the transfer distance between quadrupole trap and orbitrap is significantly shorter than into the cell inside an FT-ICR magnet; so there is less sample loss during ion transfer. However, the FT instrument does provide the opportunity to employ different fragmentation mechanisms.

3.3. Fragmentation Alternatives

CID is the major fragmentation approach employed in mass spectrometers but is not the only approach employed for proteomic studies, and particularly the use of ETD is likely to increase in the near future. Also, CID in a quadrupole ion trap produces slightly different fragmentation to CID performed in a quadrupole and the two methods are found to be complementary (see below).

3.3.1. CID in a Quadrupole Ion Trap vs in a Quadrupole

CID fragmentation in a quadrupole ion trap is produced by resonant excitation of a specific m/z. Hence, once the component has been fragmented, its products are not further excited. This means that the majority of the fragments observed are the products of

a single-bond cleavage and so mainly b and y ions are observed (33). It also means that if there is a labile bond in the peptide, for example, the O-phosphate linkage in a phosphopeptide, then the fragmentation spectrum is dominated by a single fragment ion (34), sometimes precluding the ability to identify the peptide.

In quadrupole CID, all ions, precursors and fragments included, are excited. Hence, products formed by secondary fragmentation are also formed. Two backbone cleavages can form internal ions. In addition, b and y ions can be further fragmented. The b ion structure is relatively unstable and so readily fragments further to form smaller b ions (35). However, the b2 ion is stable. Hence, in quadrupole CID spectra, a large percentage of b ions are fragmented down to a b2 ion giving relatively intense b2 and a2 ions.

It should also be pointed out that resonant fragmentation spectra in a quadrupole ion trap lose the low-mass region of the spectrum, whereas quadrupole CID spectra contain a full mass range spectrum. This has important implications for certain quantitation strategies based on MS/MS peaks, e.g., iTRAQ (36), but this will be discussed in a later chapter.

3.3.2. Electron Capture Dissociation and Electron Transfer Dissociation

Electron capture dissociation (ECD) (20) and electron transfer dissociation (ETD) (14) provide orthogonal alternatives to CID for fragmentation analysis (37). Both of these approaches form unstable radical ions that then fragment at sites that are not the weakest bonds in the molecular structure. These techniques produce fragmentation spectra that are less dependent on the peptide sequence (with the exception of the inability to cleave N-terminal to proline residues).

ECD involves firing a beam of electrons at the trapped cloud of sample ions (20). Electron capture by the analyte produces a radical ion, which is unstable and fragments to produce predominantly c and z. ions from peptides. ECD is almost exclusively performed in FT-ICR instruments.

ETD uses anions, most commonly fluoranthene ions, to transfer electrons to the analyte, forming radical ions that then fragment similarly to ECD (14). ETD can be performed in quadrupole ion traps, making the technique much more sensitive and affordable than ECD in an FT-ICR instrument.

Both of these fragmentation approaches are more effective on highly charged and charge-dense precursors. As electron capture or transfer by definition reduces the charge of the precursor, one cannot produce charged fragments from singly charged components; so ETD/ECD MS/MS analysis of singly charged species cannot be performed. Also, the efficiency of fragmentation of doubly charged species is not as high as triply charged or greater precursors (38). Hence, these approaches are not as generally applicable as CID, but as they are effective on higher charged

species that generally are less efficiently fragmented by CID, they can provide useful complementary data. Indeed, a decision tree that decides whether to perform CID or ETD based on the precursor m/z and charge has been shown to provide more comprehensive results than does either fragmentation approach alone (39). An area where these radical-based fragmentations are very powerful is in the analysis of labile post-translational modifications, where sites of modification can be determined on peptides bearing modifications that are highly labile under CID conditions (40–43). They also have the potential to be used for LC-MS analysis of large peptides and small intact proteins.

In conclusion, the correct choice of mass spectrometer to use for proteomic studies is somewhat dependent on the type of analysis to be performed. With any selection a trade-off has to be made between sensitivity versus mass accuracy and resolution, but hybrid instruments provide more flexibility in workflows to suit different types of experiments. The availability of different fragmentation mechanisms on the same instrument also allows a wider range of sample analysis strategies, and this can be particularly important for the analysis of protein and peptide modifications.

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