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A Short Overview of the Components in Mass Spectrometry Instrumentation for Proteomics Analyses

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Additional information is available at the end of the chapter

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1. Introduction

Mass spectrometry has been widely used for analyses of biomolecules such as proteins. The soft ionization methods available nowadays, the faster and more accurate mass spectrometers, a diversity of protein databases resulting from large scale genome studies and the advances in the bioinformatics field for optimized data mining, altogether significantly contributed to high quality outputs in the proteomics area [1-3].

In order to achieve the higher number of identified proteins (and perhaps quantified), in proteomics studies, some steps are equally important and required: 1) sample preparation; 2) sample pre-fractionation; 3) peptidase digestion; 4) mass spectrometry analysis; 5) data mining. Each of these steps can be extremely challenging and the final conclusions will be based on their success [4-6].

Samples used for proteomics studies are always complex and the abundant proteins mask the low abundance proteins in several cases. Tryptic peptides originated from abundant proteins predominate, no matter what kind of analysis is performed in the mass spectrometer. Highly abundant proteins such as albumin in blood samples, cytoskeleton proteins in pelleted cells, among other examples, need to be removed to allow detection of less abundant ones, and require separate analyses [7]. The same situation happens in plant derived materials, in which the amount of RuBisCO in leaves and of storage proteins in seeds and seedlings mask the non abundant proteins in the samples. To deal with this situation, several approaches can be used. For example, these proteins can be depleted from the source sample by immunoprecipitation. Another strategy used is to create exclusion lists for the mass spectrometer during data analysis. In this case, the selected peptides will be ignored during data acquisition. Another approach widely used is the dynamic exclusion. In this case, according to user definitions, the mass spectrometer will ignore the most abundant peaks in the MS1 (for example, 5), and will analyze the

next top 5. However, if the mass spectrometer used is not fast enough during each cycle, important information could be lost [8,9].

A nice example of a proteomic study is the quantification of 1,323 proteins from *Arabidopsis thaliana* chloroplasts, using label-free spectral counting. This was achieved by coupling organelle fractionation (thylakoids and stroma) and different extractions methods, applied to enrich the abundant proteins fraction (ammonium acetate precipitation and alkaline extraction), and analysis of all fraction separately. Protein pre-fractionation using sodium dodecyl sulfate one-dimension polyacrylamide gel electrophoresis and high-resolution mass spectrometry analysis performed with two different machines, also contributed to this high number of identified and quantified proteins [10].

The challenge grows even more when the samples to be analyzed consist of membrane proteins [11]. Membrane proteins usually are highly hydrophobic and in this case chaotropic agents combined with high amounts of detergents are employed to make them soluble. However, these additives can interfere in the trypsin digestion process. The concentration of these agents must be reduced in order to maintain the trypsin activity. In case of *in solution* digestion, besides chaotropes and detergents, reducing and alkylation agents (usually dithiothreitol and iodoacetimide, respectively) must also be removed. These steps, designated as desalting process, can be performed in several ways. Most commonly it involves reverse phase chromatography in which the pH of the digested sample (peptides) is reduced using trifluoroacetic acid, formic acid, or others. The peptides are bound to a C-18 matrix and eluted with increasing concentrations of acetonitrile. After drying the samples under vacuum, they are prepared for mass spectrometry analyses [3,4,12].

The mass spectrometer itself must be correctly chosen to obtain a satisfactory balance between accuracy and speed. Fast scan rates instruments can lose accuracy in their measurements, and *vice versa.*

Tandem mass spectrometry (MS/MS) is a method where the gaseous ions are subjected to two or more sequential stages of mass analysis, which can be separated spatially or temporally, according to their mass to charge ratio, *m/z* [13,14]. The mass analyzers characterize the ions according to their *m/z*. The ion selected in the first stage of analysis is subjected to different reactions and charged products from this reaction are analyzed in a second step (a second mass analyzer or other types of analyzers) [15]. The "reaction" step is critical for data quality and performance.

A brief overview of the most used components in a mass spectrometer applied in proteomics research is discussed, specially focused on the newest researchers on the field.

2. Most used components for mass spectrometers in proteomics analyses

In this section a brief overview of the components of a mass spectrometer used in proteomics will be given.

2.1. Ionization

In order to be analyzed in a mass spectrometer, the sample must be ionized and it is imperative that the ionized molecules (proteins or peptides) turn into a gas phase to allow analysis, fragmentation and detection [14]. The extensive advance of mass spectrometry in protein analyses happened after the advance of the ESI (electrospray ionization) [16], and MALDI (matrix-assisted laser desorption/ionization) ionization methods, which allowed analysis by mass spectrometry to be extended to non-volatile and thermolabile compounds. In both cases, the production of intact molecular ions is achieved under adequate experimental conditions, with minimal fragmentation. Due to this fact, these two ionization processes are referred as soft ionization methods [13,16-18].

2.1.1. Electrospray Ionization (ESI)

Shortly, in electrospray ionization the sample (proteins or peptides) is nebulized when high voltage is applied. As previously said, this ionization process allowed extensive studies on proteomics field because almost no energy is retained by the analyte and, in general, no fragmentation happens during the ionization process [17,18]. Another important aspect is that it generates multiple charges species (specially for ionized peptides), and the m/z values are detectable in most mass analyzers [13].

The ionization process is based on a liquid dispersion [14] and the process takes place following three main steps: production of charged droplets, the fission of the charged droplets and production of desolvated ions [13,14,19,20]. The production of charged droplets takes place when high-voltage is applied at the capillary tip where the analyte solution is being injected. At this stage, the electric field causes a separation of the positive and negative charges in the solution containing the analyte. In case of operation in positive ion mode (when the capillary is set at positive potential), the positive ions move towards the counter electrode. It causes an accumulation of positive ions at the surface of the liquid in the tip. The reverse polarity will produce negatively charges. There is a deformation of the meniscus of the liquid in the tip at a critical potential forming the Taylor cone [16,17] which is a static description and does not include spraying behavior [14]. The electric potential applied to the liquid at the tip pulls it into an elliptic shape. However, there is an equilibrium between the surface tension trying to pull the liquid back and the electrostatic attraction which pushes the liquid to the counter electrode [13,14].

The capillary tip is under a constant neutral gas flow, such as nitrogen. In this case, the collision of the gas with the droplet from the tip causes the solvent evaporation, a key step in the ESI method. The second step in the electrospray process is the Rayleigh fission of the droplets. The droplets fission happens when the Coulombic repulsion between the charges is stronger than the surface tension of the liquid, due to a constant decrease of the droplet radius. The limit in which this phenomenon happens is called the Rayleigh limit: the balance between the surface tension and electrostatic attraction is lost. At this stage, the droplets decrease considerably in size and charges states [13,14].

The next step is the solvent evaporation process from the charged droplet forming a gas-phase ion-analyte [13]. Two main mechanisms explain the production of desolvated ions in the gas phase: ion evaporation mechanism and charged residue mechanism. The assumption of the ion evaporation mechanism is that, at a specific time, the electric field on the surface of the droplet is sufficiently high, which causes the emission of the solvated ion from the charged droplet [17,21]. The charged residue mechanism assumes that a series of fission events leads to a final droplet, which contains a single analyte molecule completely free of solvating solvent [17,22].

An important advantage of ESI is that it can be easily coupled with liquid chromatography systems, specially those working at nanoflow range. In a *bottom up* approach, in which the proteins are digested with a peptidase and the resulting peptides are separated in a reverse phase column, the electrospray ionization is widely used. Typical columns used to the separate the complex sample mixture of peptides are made of reverse phase materials (C-18, 3–10 μm diameter) packed into fused silica capillaries (12–100 μm diameter) with sintered silica particles or silicate-polymerized ceramics as frits [5]. The dead volume between the at the end of the column and the ionization region must be as short as possible to avoid peak broadening and mixture of the peptides which were just separated in the reverse phase column. The ESI process is the same when the sample is continuously infused without previous separation in the LC system. In these cases, the flow must be adjusted to higher values to compensate the lack of the packed resin.

A capillary column coupled to a LC system and the ESI process is represented in figure 1A.

2.1.2. Matrix-Assisted Laser Desorption/Ionization (MALDI)

In MALDI analyses, the sample must be mixed with matrix and spotted in a stainless steel plate prior the analysis in the mass spectrometer. The sample is co-crystallized with the matrix, which has an essential function in MALDI. The co-crystallized sample is ionized by short laser pulses (Figure 1B). Subsequently, the ions are accelerated and the time that they spend to flight in a vacuum tube to reach the detector is measured in a TOF (time-of-flight) analyzer [23].

The matrix has to absorb the laser energy via electronic or vibrational excitation and it must also isolate the analyte molecules by diluting during preparation/crystallization preventing their aggregation. Finally, it must be able to perform the sample ionization [24]. The ionization method by MALDI can be divided, according to Zenobi and co-workers, into two main categories. In the "primary ionization", the first ions are generated from neutral molecules, mostly matrix-derived ions. In the "secondary" ionization the ions come mostly from the analyte samples, with few contamination from the matrix [24,25]. The disintegration of the condensed phase by the laser energy has to take place without excessive destructive heating of the embedded analyte molecules. The most straightforward explanation for ions formation in MALDI, assumes that ions from primary ionization result from a laser excitation of an absorbing organic material by molecule multiphoton ionization, which leads to a matrix radical cation [25,26]. The secondary ion formation mechanism take place in the MALDI plume, which is a solid-to-gas phase transition state formed, shortly after the laser pulse

[25-28]. In case of proteins or peptides, the proton transfer mechanism is probably the most important secondary reaction. In most proteomics approaches, samples are spotted in a MALDI plate with acidic matrixes, and data collected in positive mode. Some analytes do not have a high proton affinity, then negative ions could be collected or the sample could be prepared with a more basic matrix [26]. Other important types of secondary reaction mechanisms that take place in the MALDI plume are the cation transfer, electron transfer and electron capture [26].

Ionization by MALDI can be coupled to a liquid chromatography system similar to ESI, however, since the sample must be mixed with the matrix prior analysis, a spotter must be used. MALDI works with wide dynamic ranges [29], such as 2 kDa up to 7 kDa, or more [30]. The dominance of singly charged ions, specially for proteins or peptides with molecular weighs 20 kDa is a characteristic of MALDI, in contrast with ESI which produces much more species in higher charges states [31].

An important advantage of MALDI is that this ionization process is more tolerant to salts and high concentrations of buffer. However, determination of lower masses can be sometimes difficult. This happens because the matrix is also ionized in the process and "flies" in the same range of low molecular masses molecules [32,33]. Most of the matrices used nowadays are small organic molecules, which absorb UV in the range of 266– 355 nm. Nowadays, the number of choices for matrixes is quite small for proteomics analyses, derived from benzoic or cinnamic acid. It is important to point that not all matrixes are useful only for certain all types of analytes [25], causing different ways to prepare the sample.

Different MALDI matrices that can be used according to the type of analytes [24-26,34]. In case of biological samples, some matrixes can be selected [24,35,36]:

a.   nicotinic acid: absorption at 266 nm and used for proteins and peptides;

b.   2,5-dihydroxybenzoic acid: absorption 337-353 nm, and can be used for proteins, peptides, carbohydrates and some synthetic polymers;

c.   sinapinic acid: absorption 337-353 nm and widely used for proteins and peptides;

d. α-cyano-4-hydroxycinnamic acid: absorption 337-353 nm and mostly used for peptides analyses;

e.   3-hydroxy-picolinic acid: absorption 337-353 and it is suitable for nucleic acids;

When the sample solution is mixed with the matrix, placed/spotted in a MALDI plate, and dried without vacuum, the distribution of the sample is the plate can be quite distorted. However, if vacuum is used to help drying the sample, a better chemical distribution can be achieved in each sample spot [37]. In most cases, the sample preparation is done by the "dried droplet" method, which is quite simple. The sample and the matrix are separately dissolved in a common solvent system (such as 0.1 % trifluoroacetic acid), and then mixed either before or on the MALDI plate. After that, the solvent evaporation will take place, helped or not by a gas flow. By the end, the sample will be co-crystallized with the analyte and will be ready for analyses.
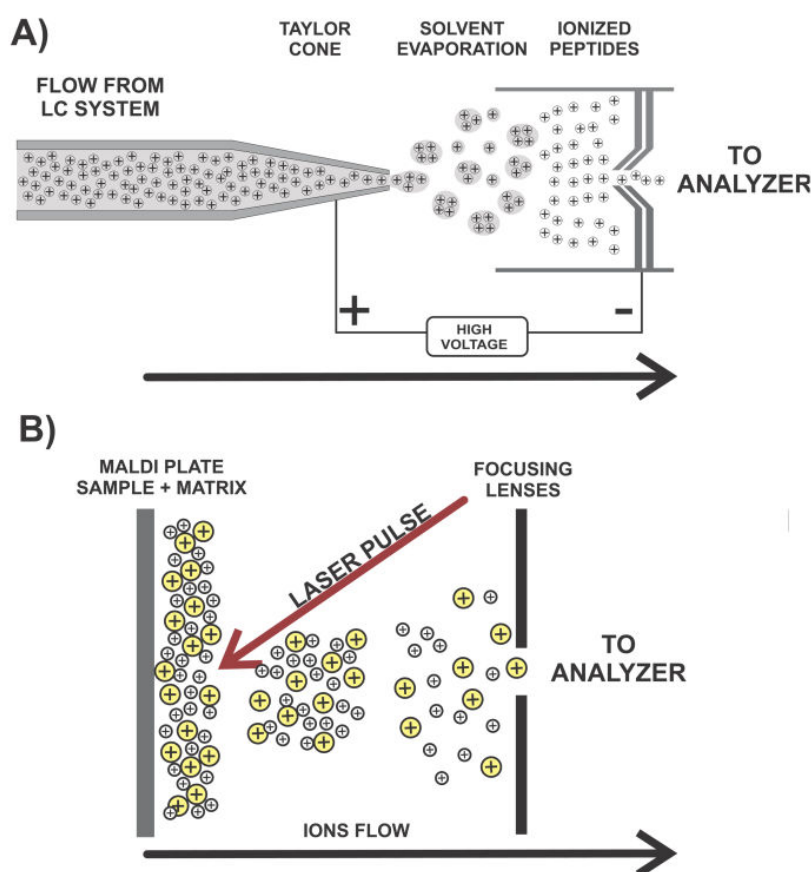
Figure 1. A simplified representation of electrospray ionization (ESI) is represented in **A**. The positively charged drop-lets and desolvated peptides are represented by the signal +. The superficial tension tends to pull back the charged droplets to the column, while the voltage applied pushes the drop away from the column. The solvent evaporates and the ionized peptides reach the skimmer and go to further analysis. In **B**, the ionization process by matrix-assisted laser desorption/ionization (MALDI) is represented. The laser pulse strikes the MALDI plate, in which the sample is (yellow circles) is co-crystallized with the matrix (grey circles). Either matrix or sample can reach the detector.

2.2. Analyzers

Once the sample is ionized, it enters the mass spectrometer itself. The mass analyzers ex-plore different characteristics of the parent or fragmented ions. In case of tandem experi-ments, the parent ion must be selected for further fragmentation, and the generated fragment ions will be detected. The combination of two or more analyzers in the same mass spectrometer yielded the high performance and resolution of the nowadays equipments. These characteristics will be briefly detailed ahead.

The main function of a mass analyzer is to separate the ions according to their *m/z* ratio [38], basically by their behavior in electric or magnetic fields [39]. Nowadays, there are few types of analyzers widely used analyzers in tandem mass spectrometry experiments, for proteo-mics analyses: quadrupole, quadrupole ion trap, time of flight and orbitrap. These analyzers vary in terms of size, price, resolution, mass range, and the ability to perform tandem mass spectrometry experiments.

In case the ions are separated "in space" (time of flight, TOF; sector; quadrupoles), the techniques are called beam techniques, because the ions "travel" across the analyzer in a "pulsed beam" mode. In this case, the MS or MS/MS analyses are performed in separated events. The ion trap analyzers, such as quadruploe ion traps and orbitraps characterize ions based on the frequency of their motion in a defined space [15]. Thus MS and MS/MS events can be performed in the same analyzer, being separated by time and not by "space" [15,40]. The analyzers use magnetic or electric fields, or even combinations of both to select ions. To avoid undesired collisions with neutral gases during analyses, the operation is performed under high vacuum [41].

2.2.1. Quadrupole mass filter

A quadrupole analyzer can work as a linear ion trap, in which ions are confined radially by a two-dimensional (2D) radio frequency field, and axially by stopping potentials applied to end electrodes.

A quadrupole mass analyzer is widely used as a "filter" prior fragmentation of the desired ions. Basically it consists of four roods assembled in two pairs, as shown in figure 2A. The first two opposite roods have the same applied voltage, which is different from that of the second two opposite roods establishing a two dimensional quadrupole field in the x-y plane (Figure 2A) [38]. The mass analysis depends on the radio frequency, and direct current voltages which are applied to the four roods [1]. Due to that reason, ions travelling in the quadrupole during analysis will be, at the same time, attracted by one set of roods and repulsed by the second set of roods. Considering the ion population being injected in a quadrupole analyzer, a selection can be made according to their m/z ratio, making some ions to have a stable trajectory in the analyzer, while a considerable number of other ions will not go all the way through (Figure 2A, arrows). The ion path occurs in the z direction, while the attraction and repulsion are occurring simultaneously in the x and y direction (Figure 2A) [38,42]. If the oscillations of an ion are stable, the ion will continue to drift down the rod assembly and reach the detector. The stable ions which "travel" all the analyzer length will go to the next steps, which can be detection, fragmentation and a second round of analysis.

The above explanation is quite simple for a complex situation. In a quadrupole mass filter ions of a single m/z maintain stable trajectories from the ion source to the detector, whereas ions with different m/z values are unable to maintain stable trajectories and do not reach the detector or collision cell [1]. The quadrupole filter is frequently used as mass filter device prior fragmentation in the collision cell, in the case of MS/MS analysis.

2.2.2. Quadrupole ion trap analyzers

Another largely employed type of analyzers is the quadrupole ion trap. The quadrupole ion trap devices are found as two-dimension (2D) also known as linear traps, or three dimension (3D) assembly. In case of 2D traps, ions are confined radially by a two-dimensional radio frequency field, and axially by stopping potentials applied to end electrodes. It traps the ions in a two dimensional field. When compared to 3D traps [43], linear traps have higher

injection efficiencies and higher ion storage capacities [38]. Besides storing ions, they can be combined with other mass analyzers in hybrid instruments and used to isolate ions of selected mass to charge ratios, to perform tandem mass spectrometry experiments [38,44].

In all cases, the ion trap is able to store either positively and negatively charged ions, or ions of one specific polarity [38]. In short words, the operation mode of an ion trap is quite similar to that of a quadrupole mass filter; the key difference is that a linear quadrupole is mainly used as a mass filter while the three-dimensional quadrupole used as an ion trap [38,39,44,45]. As the name says, these analyzers are able to trap ions for a specific period of time or to an "amount" of accumulated ions. The quadrupole ion traps analyzers are the best suitable to miniaturization among all kinds of mass analyzers, mainly because they tolerate higher pressures and can work at lower voltages. However, the extreme precision in manufacturing these devices and the lower trapping capacities, can be pointed as disadvantages [44].

When the voltage is applied to the electrodes in the trap, a "trapping potential" is formed, which keeps the ions inside the trap [38,42]. In a ion trap, the trajectories of trapped ions of consecutive specific m/z rations are affected and become unstable when the field within the trap is changed. The ions leave the trap according to their m/z ratio and reach the detector [45].

In case of 3D traps (also known as 3D Paul traps [43]), three shaped electrodes (two hyperbolic and practically identical) compose the quadrupole ion trap. A simple representation of a 3D ion-trap analyzer is shown in figure 2C. In the case of the regular 3D ion trap shown in figure 2C, the hyperbolic geometry is advantage of the ion traps is that they are used as storage chambers, mass analyzers or both [46]. Each of the end-cap electrodes has holes in the center for transmission of electrons and ions. The electrons and ions "entrance" is found in one of the endcaps, while the other one endap is the exit "electrode" through which ions will pass to a detector. The ring electrode has an internal hyperbolical surface and it is positioned symmetrically between the two end-cap electrodes [38,44,47]. These traps have mass selective detection, storage and ejection capabilities [38].

2.2.3. Time of flight analyzer

Another kind of widely used analyzer is the time-of-flight, TOF. Theoretically the mass range in a TOF analyzer is unlimited [48]. However, in practice, the range is limited by the loss of control over the kinetic energy and spatial distributions of the ions with increasing mass as they are injected into the acceleration region of the mass spectrometer. Consequently, the mass accuracy and resolution decrease as the ion mass increases [48,49]. Compared with quadrupole analyzers, the majority of the ions will reach the detector and the lost of ion will not be as expressive as in quadrupole analyzers [39,45].

In TOF analyzers, the desorbed and ionized molecules are accelerated by an electrostatic field and are then ejected through a flight tube under vacuum. In this tube, smaller ions fly faster than larger ions. The detector measures the time of flight for each particular ion. This time to reach the detector depends on the m/z of the molecule being analyzed; theoretically, all ions leave the accelerator chamber with the same kinetic energy, and the time to reach the detector will be dependent on the mass of that particular ion (Figure 2B). The ions sepa-

rated by their TOF reach the detector, and a spectrum is presented. Since MALDI produces mostly single charged species, the *m/z* values correspond to the mass of the ion [50].

The TOF analyzer can operate in the linear mode or reflectron mode (presented in Figure 2B). In case of the linear mode, the ions fly in the tube and reach the detector. In case of the reflectron mode, the ions fly towards the reflectron which focuses ions with the same *m/z* values, making these ions reach the detector at the same time. Also, there is an adjustment of the kinetic energy since the ions decelerate and accelerate again inside the reflectron. The results are considerable more accurate in the reflectron mode than in the linear mode [23,50,51].

2.2.4. Orbitrap

A couple of years ago, an orbital analyzer, named as the Orbitrap was introduced [40]. The mechanism of analysis is quite different. In an Orbitrap, quoting Michalski and co-workers [52], "the signal is recorded from the image current produced by ion packets which oscillate around and along the spindle-shaped inner electrode of the trap". An extremely simple representation of this principle is shown in figure 2D. This analyzer traps the ions radially around a central spindle electrode. The ion injection is performed perpendicularly to the longer axis of the trap (*z* axis) [40].

In an Orbitrap, the potential distribution of the field is a combination of quadrupole and logarithmic potentials. There are no magnetic or radio frequency fields, so ion stability is achieved exclusively due to ions which orbit around an axial electrode and also perform harmonic oscillations along the electrode [40,53].

The necessity of an external ion storage device prior Orbitrap analysis was pointed by Makarov when the analyzer was developed [53]. In recent years, the common feature of commercially available mass spectrometers which use an Orbitrap as analyzer is that the trap is preceded by the C-trap, which is an external injection device [54]. The process of capturing ions in the C-trap following by the injection into the Orbitrap is fast and can be easily interfaced and synchronized to any external device such as a linear ion trap mass spectrometer or directly to an ion source. The process of detection is considerably longer, since the sensitivity is proportional to the square root of number of detected oscillations [40,53,54]. Since the commercial release of the Orbitrap analyzer, there were several changes to the design of the C-trap and the higher-energy collision induced dissociation, which have improved the efficiency and speed of fragmentation [54].

The high resolution achieved by the Orbitrap helped the fast adoption of this kind of analyzer, which is considered easy to use, robust and shows excellent performance capabilities [55].

2.3. Fragmentation

2.3.1. Importance of sample preparation for tandem mass spectrometry in proteomics

There is no doubt that the fragmentation step of a precursor ion is a key point in proteomics analyses since it enables analyzes at the MS/MS or MSn levels. In tandem mass spectrometry

analyses, the first analyzer selects the ion(s) which proceeds to a subsequent section, where the excitation and dissociation steps will happen. Tandem mass spectrometry analyses are the result of two or more sequential separations of ions usually coupling two or more mass analyzers [1,39].
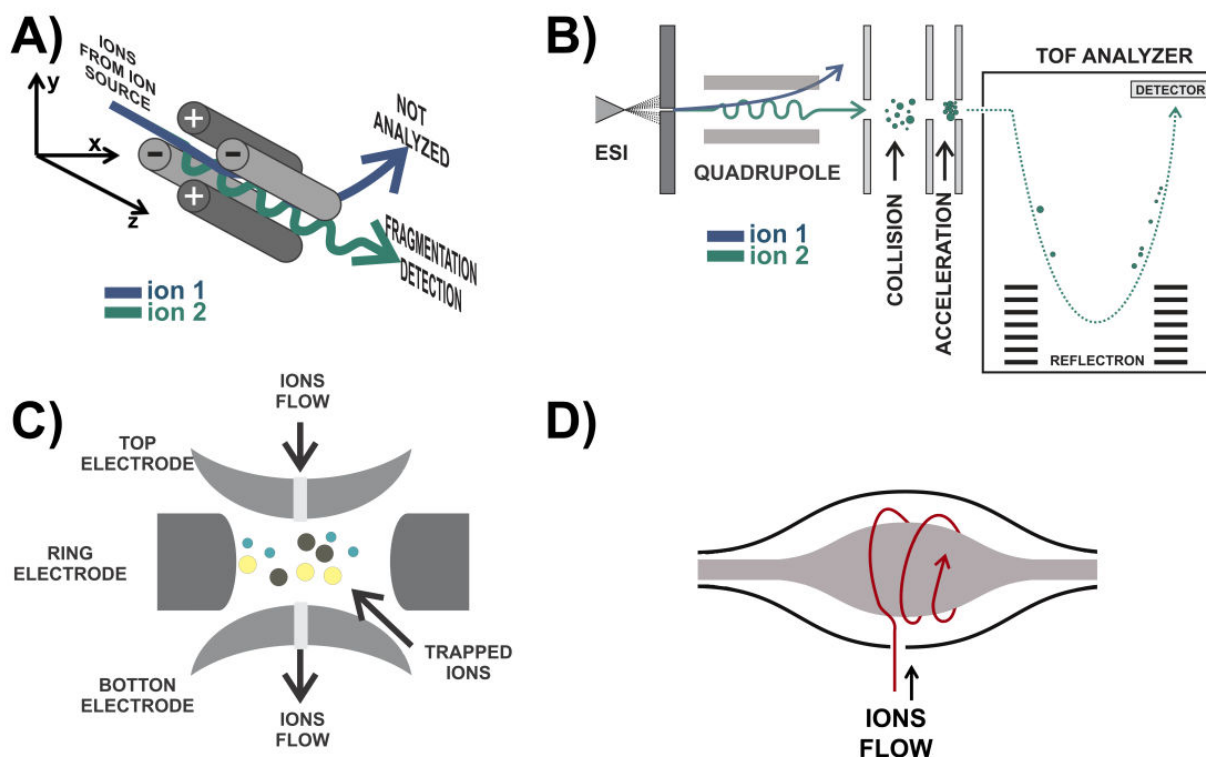
Figure 2. In **A**, there is a representation of a quadrupole mass filter. The ions generated in the ion source migrate through the quadrupole in the *z* direction and at the same time are exposed to simultaneous attraction and repulsion by opposite roods. Ions with stable trajectory are separated in the analyzer and travel all way across (ion 2, green). However, some ions with unstable trajectory are ejected before (ion 1, dark blue). In **B**, the ionization by electrospray and the quadrupole (two roods are shown) and time of flight analyzer is represented. Products from precursor ions previously analyzed and selected for fragmentation (ion 2, green) are accelerated and analyzed in the TOF analyzer. The analyzer might have the reflectron mode or not. The time that the ions spend to travel all way into the TOF is measured and spectrum is recorded. In **C**, the quadrupole ion trap is represented. Ions are trapped for a selected period of time or "amount of ions" before being released. A simple representation of the orbitrap analyzer is presented in **D** in which ions are separated based on their orbital movement around the trap.

A typical workflow for protein identification based on tandem mass spectrometry can be divided in three main steps: reduce sample complexity, perform the mass spectrometry analysis and search the data collected against a protein database. First, it is necessary to reduce complexity of a crude biological sample: several strategies can be used in this step [56]. At the protein level it may include organelle fractionation, protein enrichment by immuno-percipitation, removal of abundant proteins and fractionation by sodium dodecyl sulfate polyacrylamide electrophoresis. Multidimensional chromatography is widely used at the peptide at level [57]. The digestion of the proteins present in the sample by a peptidase can be performed in solution or in gel after SDS-PAGE fractionation. In case of liquid chroma-

tography tandem mass spectrometry approach, the peptides from the digested proteins are separated by reverse phase chromatography, ionized by ESI and analyzed by tandem mass spectrometry. Abundant proteins will produce abundant peptides, in most cases. These peptides will be selected for fragmentation regardless the approach, and will mask the low abundant proteins. Besides different strategies used to deal with this situation (exclusion lists, dynamic exclusion, affinity columns), it is still challenging and will not be focused in this chapter. For further reading, please refer to selected references cited ahead [7-10]. The last step is to combine the peptide identification results into a list of proteins that are most likely present in the sample [56,58].

The most used fragmentation methods used in proteomics analyses are briefly discussed ahead.

2.3.2. Collision Induced Dissociation - CID

In collision induced dissociation, also known by collision activated dissociation, the excitation of the precursor ion is achieved by energetic collisions with an inert gas, usually helium or argon [59]. In CID, the ions selected in the first analyzer are focused in a reaction or collision cell, which in several cases is a quadrupole [52,60], prior the reaction in gas phase. In case of peptides, precursor ions are dissociated into fragments along the backbone cleaving at the amide bonds [59,61]. Activation of peptides under low-energy collision conditions happens mainly by charge directed reactions [59].

Generally, collisions between the precursor ion and the target gas are accompanied by an increase in the internal energy, which induces decomposition with high probability of fragmentation [62]. The overall process supplies sufficient internal energy to induce covalent bond breakage, and the preferred sites of protonation are the amide bonds of the peptide backbone [62,63]. The protonated amide linkages are weakened and favored to create a series of homologous products ions upon collision-activation. Fragmentation of peptides by CID creates the complementary *b* (in case the charge is retained in the *C*-terminus) and *y* (in case the charge is retained in the *N*-terminus) ions [64].

The overall CID process can be divided in two steps: the excitation of the precursor ions and their fragmentation/dissociation. The fragmentation of a precursor ion can occur if the collision energy is sufficiently high that the ion is excited beyond its threshold for dissociation. The CID processes can be separated in low and high energy-collisions. Usually, low energy collisions (1-100 eV) is used for organic compounds of moderate masses (hundreds of daltons), while high energy collision (keV range) are produced in TOF/TOF instruments and the CID spectra resulted from low and high energy collisions are considerably different [62].

There are a number of proteins for which the digestion by trypsin will not be a good choice either because it produces too short or too long peptides. In both cases, CID fragmentation will not be effective for them. Collision-induced dissociation is very effective for short and low charged tryptic peptides, usually less than 20 residues and no more that 4 charges [65,66]. In case of posttranslational modification studies, CID helps mostly to identify the sites and types of the modification (such as phosphorylation, acetylation, etc) in a particular

protein [66,67]. Even though the sequence of the protein is known, in complexes samples they are not pure. For this reason, individual digestion of the same by two different peptidases and analyses by different fragmentation methods in tandem mass spectrometry approaches is used.

2.3.3. Electron Capture Dissociation - ECD

Electron capture dissociation is based on the dissociative recombination of multiply protonated polypeptide molecules with low-energy electrons [68]. The mechanism of ECD is not completely understood, however, in general terms, can be explained as follow. Polypeptide polycations initially capture an electron in a high orbit, followed by a charge neutralization, which leads to an excited radical species that undergoes bond cleavage [64,68]. The radical species dissociates through N–Cα bond cleavages to produce c- and z-type product ions. The mechanism (s) by which the N–Cα bond cleavages occur is believed that following electron capture, a hydrogen atom is transferred to an amide carbonyl [68-70] or a proton is transferred to an amide anion radical [68,71], both resulting in the formation of an aminoketyl radical intermediate, which dissociates through N–Cα bond cleavages.

Fragmentation in ECD happens at a very high rate, however, the time required for electron capture by precursor ions exceeds the residence time in most mass spectrometers, such as those with TOF and quadrupole analyzers. Another important point is that ECD efficiency is highest for low energy electrons [69], which is difficult to provide in ion traps, for example. For that reason, ECD is mostly used in Fourier transform ion cyclotron resonance mass spectrometry [68].

Electron capture dissociation has been shown advantageous in sequence characterization, *de novo* sequencing, disulphide bond analysis [69,71], and posttranslational modification analyses [68]. The information obtained by ECD is complementary to those obtained by CID in traditional MS/MS methods [68-70]. One important advantage of ECD is to promote a lower energy pathway than CID; even though fragmentation happens, fragile posttranslational modifications are preserved [66].

2.3.4. Electron Transfer Dissociation - ETD

The electron-transfer dissociation (ETD) is based on ion/ion chemistry [66,72,73]. It involves transferring an electron from a radical anion to a protonated peptide [72,73], resulting in cleavages at the N-Cα bond of the peptide backbone and preserving most of the posttranslational modifications [66]. Differently from CID, but similar to ECD, ETD creates the complementary c- and z-type ions. The ETD is widely used for posttranslational modification studies, such as phosphorylation specially because the predominant loss of the phosphate moiety by neutral loss generally precludes further fragmentation.

Different from ECD, in which the primary source of excess energy is the recombination energy released when the electron is captured, in EDT this recombination energy is reduced by the electron binding energy to the anion donor; ETD involves transfer of an electron to the multiply protonated precursor ion from a singly charged radical [64,71]. However, similar to ECD, ETD also induces relatively non-selective cleavage of the N–Cα bond on a peptide's

backbone and will produce the *c*- and *z*-product ions [64,66,68]. As ECD, ETD prevents fragile posttranslational modifications from fragmentation [64,66].

The ion/ion chemistry in EDT requires few milliseconds to be completed. Besides that, ETD can be performed with femtomole amount of sample and on a compatible timescale to liquid chromatography and MS analyses. Also, ETD is advantageous in the study of larger peptides, which carry three or more charges. These larger and highly charged peptides offer favorable cleavage conditions during ETD [66]. However, it is important to remember that when working with ETD, a different protease must be used, instead of trypsin, to produce larger peptides (25 aminoacids or more). Longer peptides tend to gain more charges (13 to 16) based on the increased number of basic residues [66]. Endopeptidases Lys-C and Asp-N usually yield longer peptides, and make a good choice with ETD fragmentation [66,73].

3. Concluding remarks

The main idea of this chapter was to provide an overview on the most used components/ methods in proteomics analyses employing mass spectrometer. A brief overview on the ionization methods, analyzers and fragmentation was given and can be used as a support for new researches on the field.

Acknowledgements

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