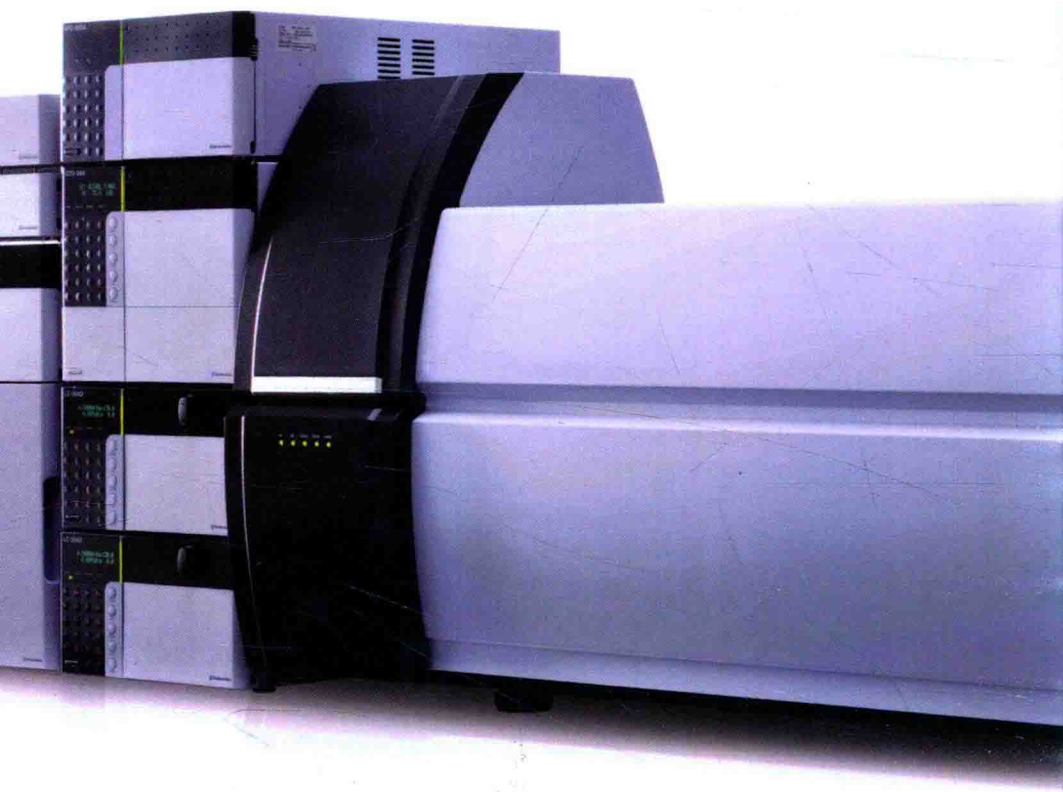
# Tandem Mass Spectrometry



Clive Flint

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Callisto Reference

New York

Published by Callisto Reference,
106 Park Avenue, Suite 200,
New York, NY 10016, USA
www.callistoreference.com

**Tandem Mass Spectrometry**
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International Standard Book Number: 978-1-63239-590-0 (Hardback)



Printed in China.

# Tandem Mass Spectrometry

# Preface

Extensive information regarding the topic of tandem mass spectrometry has been presented in this book. It provides in-depth elucidation on the theory, description as well as the instrumentation of experimental strategies and MS/MS data interpretation for the structural analysis of important molecular compounds. Analysis of carbohydrates, drugs, metabolites and protein post-translation modifications are the major topics described in this book. It aims to serve as a valuable reference for various audiences including graduate students, professionals engaged in this field as well as general readers interested in the use of modern mass spectrometry for getting answers to crucial questions of prime importance in biological and chemical sciences.

This book unites the global concepts and researches in an organized manner for a comprehensive understanding of the subject. It is a ripe text for all researchers, students, scientists or anyone else who is interested in acquiring a better knowledge of this dynamic field.

I extend my sincere thanks to the contributors for such eloquent research chapters. Finally, I thank my family for being a source of support and help.

**Editor**

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# General Aspects

Chapter 1

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

Diogo Ribeiro Demartini

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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.

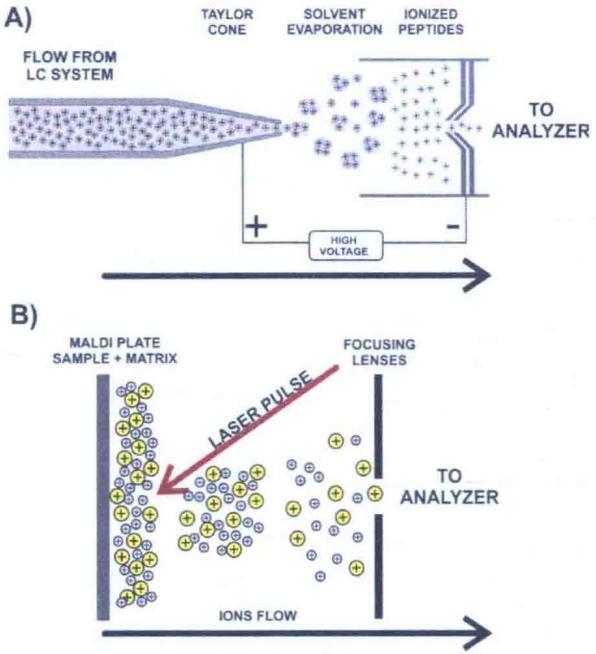


**Figure 1.** A simplified representation of electrospray ionization (ESI) is represented in **A**. The positively charged droplets and desolvated peptides are represented by the signal +. The superficial tension tends to pull back the charged droplets to the column, while the voltage applied pushes 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 explore different characteristics of the parent or fragmented ions. In case of tandem experiments, 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 proteomics 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-