

The Role of Mass Spectrometry in the “Omics” Era

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Abstract: Mass spectrometry (MS) is one of the key analytical technology on which the emerging “-omics” approaches are based. It may provide detection and quantization of thousands of proteins and biologically active metabolites from a tissue, body fluid or cell culture working in a “global” or “targeted” manner, down to ultra-trace levels. It can be expected that the high performance of MS technology, coupled to routine data handling, will soon bring fruit in the request for a better understanding of human diseases, leading to new molecular biomarkers, hence affecting drug targets and therapies.

In this review, we focus on the main advances in the MS technologies, influencing genomics, transcriptomics, proteomics, lipidomics and metabolomics fields, up to the most recent MS applications to meta-omic studies.

Keywords: Mass spectrometry, MS, MS-based genomics, MS-based proteomics, MS-based metabolomics, MS-based lipidomics, Meta-omics

INTRODUCTION

Basic Principles of Mass Spectrometry (MS)

MS is defined as the minimum scale in the world due to the dimensions of what it weighs (Chughtai *et al.*, 2010) [1]. MS is an analytical tool to measure the mass-to-charge ratios (m/z) of ions in order to determine their molecular weight (MW). This process involves three steps: *i*) conversion of molecules into gas-phase ions by the ionization source; *ii*) ion separation by their m/z values via magnetic or electric fields through a component, namely mass analyzer; *iii*) detection of the separated ions as electric charge obtaining signals proportional to the abundance of each species. In many configurations, additional tandem MS analyses (MS/MS) are feasible. In the MS/MS mode, the instrument uses the first mass analyzer to select a single ion that is subsequently fired into a collision cell, where it collides with gas molecules such as argon (*e.g.*, collision-induced dissociation, CID) causing the ion fragmentation. The multiple fragment ions are then analyzed in the second-stage mass analyzer giving accurate information on structural features of the parent ion. In an MS spectrum, the x-axis represents m/z values, whereas the y-axis indicates total ion counts. As this extraordinary analytical technology can provide key information about analytes, including their structure, purity, and composition, it is now routinely used in either industry and research field for various purposes such as drug discovery, diagnostics and bio-analyses [2]. Due to the fact that MS analyses are infrequently performed on a single compound, the study of complex mixtures requires prior purification steps. By doing so, the mass spectrometers are coupled with dedicated separation devices such as Capillary Electrophoresis (CE), Gas Chromatograph (GC) and Liquid Chromatograph (LC) (*e.g.*, CE-MS, GC-MS and LC-MS). Fig. 1 shows different configurations of commonly used MS systems.

Soft Ionization-based Techniques

The first component of a mass spectrometer is the ion source, where charged species are produced. In the soft ionization technique, nowadays widely used, a low amount of internal energy is transmitted to the molecules during the ionization process. Electron ionization (EI) employs energetic electron beams during the ionization process and operates only under vacuum, while the analytes are already in the gas phase. A heated metallic filament produces a beam of accelerated electrons, directed to collide against a vaporized sample, causing electron expulsion and a subsequent formation of charged radical cations. These conditions are not suitable for large molecules or numerous biological materials. Chemical ionization (CI) and plasma desorption (PD) methods, introduced in 1966 and 1974 respectively [3, 4], determine the formation of protonated (or deprotonated) ions, which are more stable than the radical ions formed by EI-MS. In the CI method, energetic electrons collide with neutral molecules, producing charged ions that interact with the analytes, producing protonated species. Both EI and CI methods, are limited in terms of mass range (<1000) and are not able to ionize the most thermally instable biological compounds. The PD ionization was introduced after field ionization and field desorption methods [5, 6] and is one of the first “soft” ionization techniques able to analyze ultra-high-MW biomolecules (up to a 100 kDa). Subsequently, new “soft” ionization techniques, such as fast atom bombardment (FAB) [7, 8], liquid secondary ion mass spectrometry (LSI-MS) [9], electrospray ionization (ESI) [10] and matrix-assisted laser desorption ionization (MALDI) [11-14] were developed. The last two ionization techniques have revolutionized MS, allowing MS applications also to the study of biological macromolecules such as carbohydrates, lipids, proteins, nucleotides, organic and inorganic compounds. ESI is used to analyze labile high MW polypeptides, organometallics and polymers. The ESI source operates at atmospheric pressure and the sample is sprayed via a thin needle into a strong electric field. During sample spraying, a high electrical potential is applied to the needle (1.5-3.5 KV), resulting in the formation of highly charged droplets (*i.e.*, nebulization) electrically driven and subsequently vaporized using a tem

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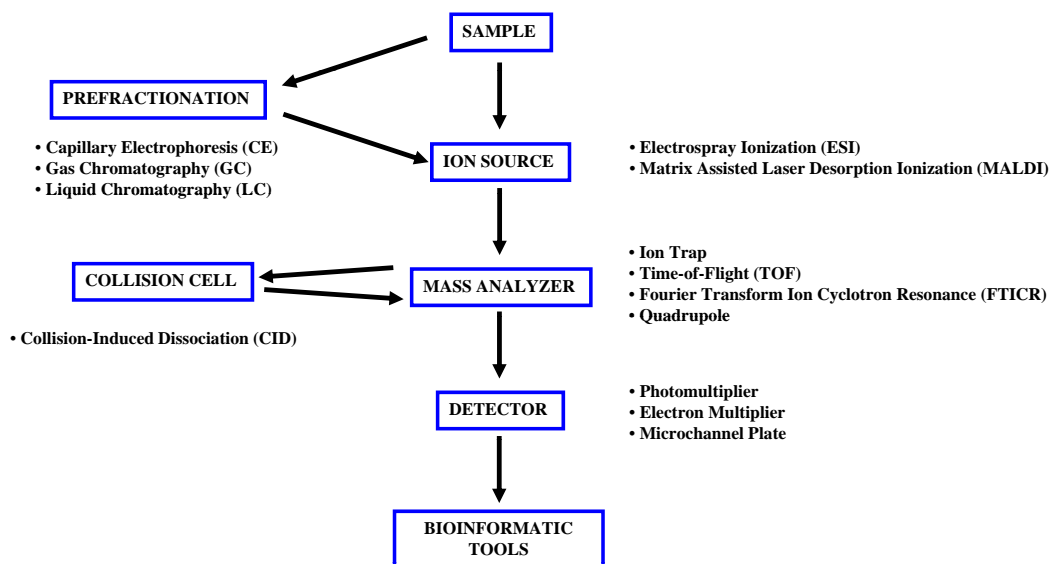


Fig. (1). Configurations of commonly used mass spectrometry systems. A pre-fractionation module usually is posed on-line with a mass spectrometer instrument. A mass spectrometer consists of three fundamental elements: *i*) an ion source, which ionizes the molecules to be analyzed; *ii*) a mass analyzer (or a combination of analyzers), which can be used as a collision cell for ion fragmentation and/or sorting by their mass- to-charge ratio; *iii*) a detector which amplifies and quantifies the resulting signals generating the final data consisting of MS and MS/MS spectra; *iv*) bioinformatic module for data processing.

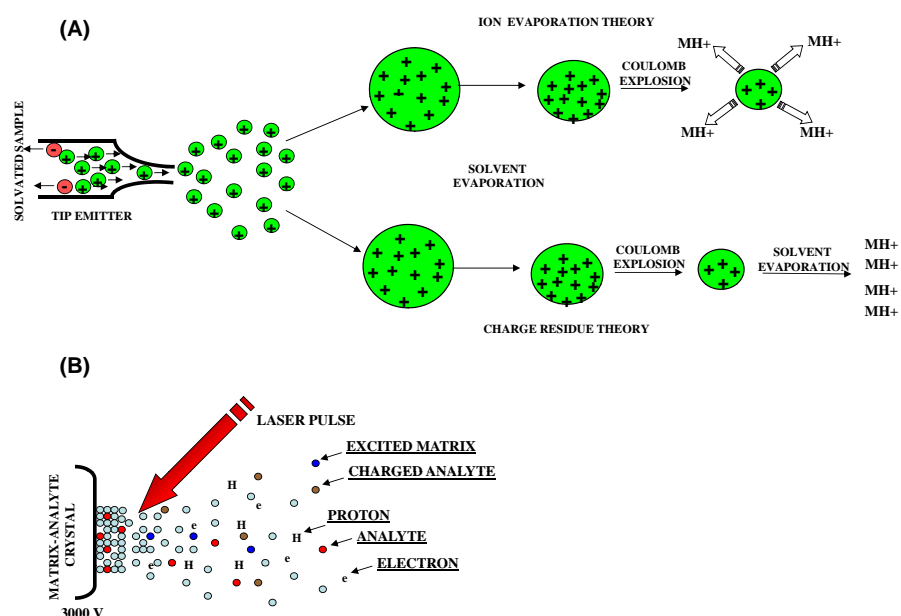


Fig. (2). Theories of ion formation in electrospray ionization technique and Matrix-Assisted Laser Desorption Ionization process. **Panel A.** As the charged droplets, created into the charged tip emitter, traverse the space between the tip emitter and the cone, the solvent evaporation occurs. Charged droplets are reduced into smaller ones and new positive ions are developed according to ion evaporation theory or charge residue theory; the same mechanism applies in the case of negative ion mode. **Panel B.** Ionization and desorption of molecules are performed by a UV laser beam (usually 337 nm), creating singly charged species.

perate neutral gas (typically nitrogen). Under these conditions, the droplets shift inside the source generating ions [15-18] (Fig. 2A). Recently, the nanospray technology, able to work in the order of nanoliters/ minute as flow rate has allowed to considerably improve ion formation mechanisms [19-21]. Similarly to ESI, the MALDI technique is able to analyze proteins [22, 23], DNA [24], lipids [25], and glycoconjugates [26] (Fig. 2B). With MALDI, ions are desorbed from a solid phase. A diluted sample is mixed with an excess of an appropriate matrix and spotted onto a MALDI plate before it dries and co-crystallizes within the matrix. The components in the mixture are brought into the gas phase by a laser beam

(typically a nitrogen laser at a wavelength of 337 nm) that hits the sample-matrix crystal causing, indirectly, the vaporization of the matrix containing analytes. The matrix absorbs the laser energy and operates as a proton donor or receptor, ionizing the analytes in both cationic or anionic species [12, 27-32]. A specific and recent application of the MALDI technique is the MALDI imaging MS (MALDI-IMS), a new and promising tool in biomarker discovery and translational medicine, because the identification of observed molecules does not require preliminary information. MALDI-IMS allows to obtain molecular images of tissues (large amounts of data with the ability to create a density map for each m/z value) and to

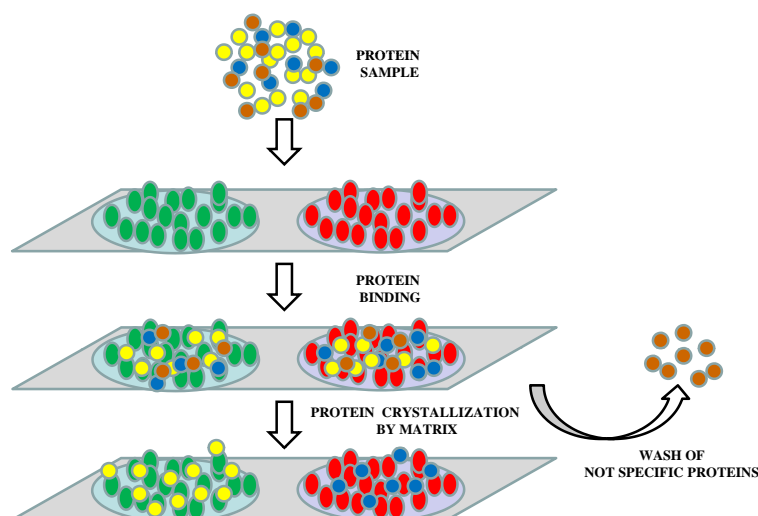


Fig. (3). Surface-Enhanced Laser Desorption Ionization process. A protein sample is applied to the spots of ProteinChip Arrays. These spots are represented by chromatographic surfaces with certain physicochemical characteristics (hydrophobic, cationic, anionic, metal ion presenting, or hydrophilic), or are pre-activated for the coupling of capture molecules (protein, DNA or RNA) prior to sample loading. After capture, nonspecifically proteins are washed away. Subsequently, an energy absorbing matrix is mixed with the proteins bound to permit the sample crystallization.

map many metabolites in tissue sections of 30-50 μm [33-41]. In the MALDI-IMS, the matrix is uniformly deposited over the tissue and the proteins are then desorbed by irradiation from several spots of the sample in an ordered array of the surface. Each spot is traced by a mass spectrum consisting of mass signals from cationic species desorbed from the tissue region. A variation of MALDI is the surface-enhanced laser desorption ionization (SELDI) method [42]. By the SELDI technique, a sample can be directly processed on protein-chip arrays (PCAs) (*e.g.*, Ciphergen, Biosystems) [43-45], which display various kinds of chemically activated surfaces able to capture molecules from biological samples through specific interactions (*e.g.*, electrostatic interaction) or affinity chromatography (*i.e.*, antibody-antigen, protein-DNA and enzyme-substrate). Proteins are then crystallized, using energy absorbing molecules, desorbed and ionized by a nitrogen laser (Fig. 3).

Mass Analyzers

A mass analyzer is the component of the mass spectrometer dedicated to ions' separation according to their m/z values. Different physical principles can be employed for the separation of ions: usually the electrically driven traditional analyzers (*i.e.*, magnetic sectors) employ a magnetic field. Currently, the ones widely used are quadrupole (Q), quadrupole ion trap (QIT), time-of-flight (ToF), and Fourier transform ion cyclotron resonance (FT-ICR) analyzers (Fig. 4). The characteristics of a mass analyzer are determined by several parameters: *i*) resolution (the ion separation efficiency, through their m/z ratio); *ii*) mass accuracy (confidence in the m/z values); *iii*) mass range; *iv*) MS/MS acquisition and precision (the ability to reproduce a mass measurement of a given compound). The Q mass analyzer was introduced in the research field in the 1950s by the Nobel Prize P. Wolfgang. It is composed of four parallel metal bars (Fig. 4), where a direct voltage is applied to two of these rods, while the other two are linked to an alternating radio-frequency potential. The applied voltages determine the flight of ions between the four rods. Specific direct and alternating current voltages, allow only ions characterized by a certain mass-to-charge ratio to pass through the analyzer. Finally, the mass spectrum is recorded by acquiring the ions passing through the quadrupole filter when the voltages are varied. Wolfgang also developed the QIT

mass analyzer [46, 47]. As shown in (Fig. 4), the ions, from the instrument source, go into the trap and are trapped inside three hyperbolic electrodes which represent the ring electrode and the entrance and exit cap electrodes. Different voltage values are applied to these electrodes, thus determining a hollow in which ions are trapped, with the ion mobility depending on the applied voltages and individual m/z ratios. The ions are then focused onto the detector by a gradual change of the potentials, producing the mass spectrum [48]. In 1929, Lawrence invented the cyclotron, an apparatus for accelerating nuclear particles to very high speeds without using high voltages. In 1974, Marshall and Comisarow fused the performance of the cyclotron system to the Fast FT, allowing the cyclotron to become a high performance mass spectrometer [49]. The ultrahigh-resolution FT-ICR consists of an ESI ion source, ion optics to transfer ions into the magnetic field (RF-Only Quadrupole ion guide) and an ICR cell or Penning trap. The ions are trapped, exposed to the magnetic field, forced into their cyclotron motion, analyzed and finally detected. The use of a Penning trap enhances the detection time and thus sensitivity and resolution. The most recent acquisition of the FT MS family is the Orbitrap analyzer. It was invented by Alexander Makarov as a modification of the QIT, where Orbitrap works with static electrostatic fields while the QIT uses a dynamic electric field typically oscillating at ~ 1 MHz. The Orbitrap was presented to the MS public at a conference of the American Society for Mass Spectrometry in 1999 and quickly made its debut in the MS mainstream in 2005 as an accurate and compact mass detector. Orbitrap mass spectrometers fundamentally differ from the most FT-ICR mass spectrometers because of their built-in excitation-by-injection mechanism [50]. Many modern mass spectrometers are composed of two or more mass analyzers to perform the *tandem-in-space* MS/MS, where different mass analyzers are involved “in different spaces”. This MS/MS technology is based on the isolation of a specific *precursor ion* (m/z), further subjected to dissociation and production of fragment or product ions. However, solving the “puzzle” created with a MS/MS spectrum, provides much valuable information about the molecular structure and the amount of analytes. As shown in Table 1, various combinations of mass analyzers can be assembled in a commercial tandem mass spectrometer, obtaining mass analyzers connected in series. In

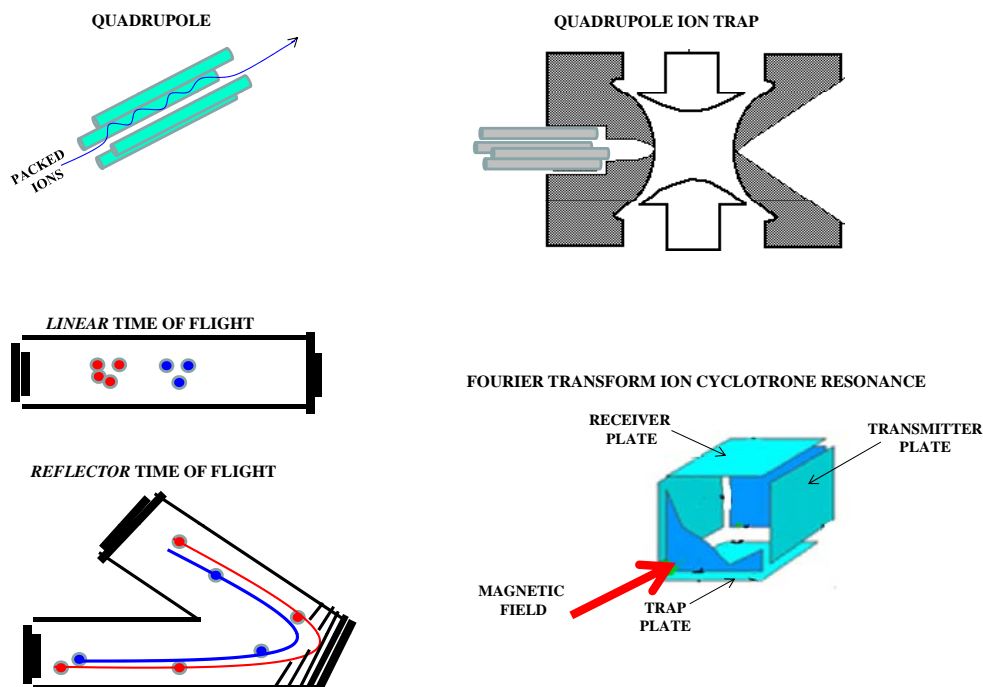


Fig. (4). Main mass analyzers currently widely used. Each one has its own special uniqueness and applications, as well as its own advantages and restrictions. The preference of mass analyzer should be based on the application, cost, and desired performance. A best mass analyzer that comprehends all for all applications does not exist.

Table 1. Common Hybrid Mass Spectrometers with their Technical Parameters

Combined Mass Analyzers	Commercial Name of the Instrument	Mass Accuracy (ppm)	Resolution ($\Delta m/z$)	Acquisition Speed (Hz)
QqQ	LCMS-8030, Shimadzu	-	0.7	15
	6490, Agilent	-	0.4	10
	Triple Quad 5500, AB SCIEX	-	1	12
	TSQ Vantage, Thermo Scientific	5	0.07	5
	XEVO TQ-S, Waters	-	1	10
Q-Linear Ion Trap	QTRAP 5500, AB SCIEX	-	0.1	20
	QTRAP 6500, AB SCIEX	-	0.05	25
Q-TOF	maXis 4G, Bruker Daltonics	< 0.6	0.02	30 (MS), 10 (MS/MS)
	micrOTOF-Q II, Bruker Daltonics	< 2	0.05	20
	XEVO G2 QToF, Waters	< 1	0.04	30
	6550 QTOF, Agilent	< 1	0.02	50
	TripleTOF 5600, AB SCIEX	0.5	0.03	50 (MS), 100 (MS/MS)
Q-IMS-TOF	Synapt G2-S HDMS, Waters	< 1	0.02	30
	MALDI Synapt G2-S HDMS, Waters	< 1	0.1	-
Q-Orbitrap	Q Exactive, Thermo Scientific	< 1	0.001	12
Q-ICR	SolariX 15T, Bruker Daltonics	< 0.25	0.0002	-
LIT-ICR	LTQ FT Ultra 7T, Thermo Scientific	< 1	0.0005	2
LIT-Orbitrap	Orbitrap Elite, Thermo Scientific	< 1	0.002	8
	MALDI LTQ Orbitrap XL, Thermo Scientific	< 2	0.004	-
TOF/TOF	TOF/TOF 5800 System, AB SCIEX	< 1	0.07	-
	UltrafleXtreme, Bruker Daltonics	< 1.5	0.08	-
	Axima Performance, Shimadzu	< 5	0.2	-
Ion Trap-TOF	LCMS-IT-TOF, Shimadzu	3	0.1	10
	Axima Resonance, Shimadzu	3	0.3	-

The list contains only main manufacturers and may not be comprehensive.

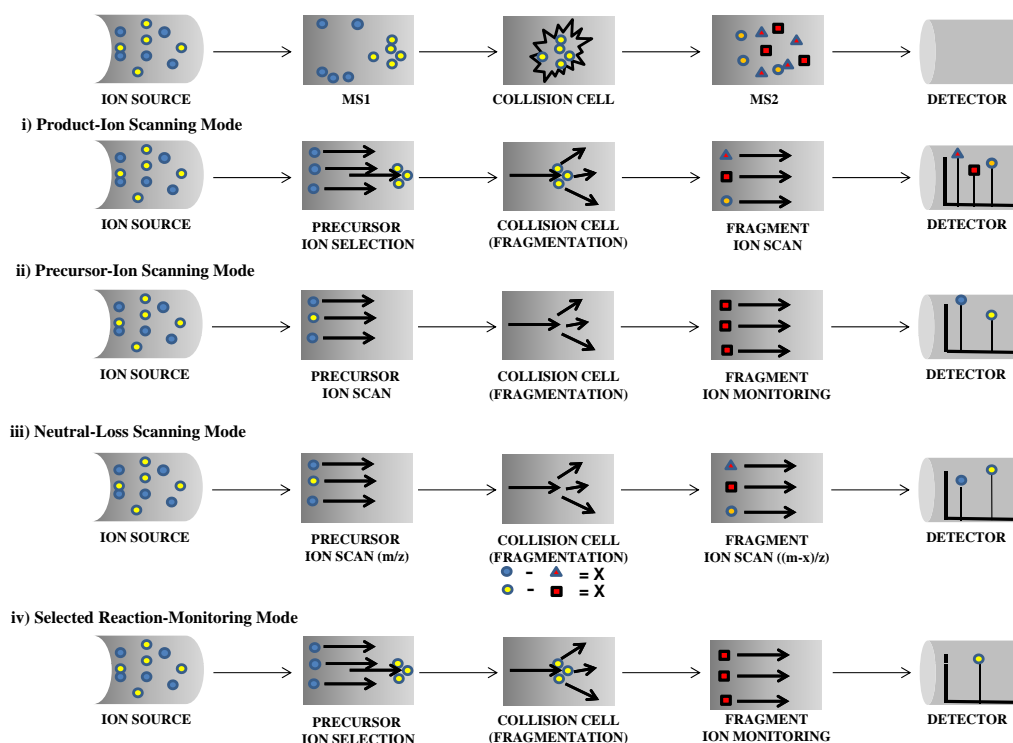


Fig. (5). Major scan modes for MS/MS tandem-in-space. The *i*) product-ion, *ii*) precursor-ion, *iii*) neutral-loss and *iv*) selected reaction-monitoring represent the four major MS/MS scan modes where the ion isolation and scan are performed by the first and third analyzers, whereas the second analyzer involves the ion collision and subsequent dissociation through atoms or molecules of a neutral gas obtaining a collision-induced dissociation (CID).

these hybrid mass spectrometers, the ion isolation and scan are performed by the first and the final analyzers, whereas the second analyzer is a collision cell that allows ion fragmentation. As shown in Fig. 5, four main MS/MS scan modes are particularly used: *i*) product-ion scanning; *ii*) precursor-ion scanning; *iii*) neutral-loss scanning; and *iv*) selected reaction-monitoring. In the (*i*), the analyzer selects a precursor ion of interest which is fragmented into the collision cell, hence producing the product ions analyzed by the second mass analyzer. During the (*ii*) process, the second analyzer focuses on a particular product ion of interest after collision, while the first mass analyzer scans the m/z ratios. By product-ion scanning mode, all precursor ions are detected. In the (*iii*), the first and second mass analyzers operate simultaneously with a constant mass offset of “ x ”. When a precursor ion is transmitted through the first mass analyzer, this ion is recorded if it yields a product ion corresponding to the loss of a neutral fragment of “ x ” from the precursor ion after collision cell. In the (*iv*) scan mode, the first and second mass analyzers are both focused on the selected ions. This modality yields high specificity and sensitivity by a high duty cycle to monitor the transitions of interest. In the case of the first or the second mass analyzer or both, multiple ions are set to monitor for multiple reactions, the term “multiple reaction monitoring (MRM)” is widely used and its technique is widely employed for quantitative analysis of individual molecular species by using high-pressure liquid chromatography (HPLC) coupled MS.

Besides the *tandem-in-space*, the *tandem-in-time* is the other available MS/MS method processing, amongst all ions, only one m/z that is subsequently fragmented “in the same space”. For example, the QIT (including FT-ICR) is a commonly used *tandem-in-time* mass spectrometer, while the triple quadrupole instrument (QqQ), composed of three connected quadrupoles, represents the widely used *tandem-in-space* mass spectrometer. Table 1 shows an overview of mass spectrometers and their technical specifications,

currently offered by the main manufacturers under LC-MS and MALDI-MS configurations. These innovative solutions offer an extraordinary advancement in mass resolution, mass accuracy and acquisition speed. The ICR resolution and mass accuracy are the highest, compared to all modern analyzers, followed by Orbitrap and TOF-based analyzers, despite the acquisition times are longer for the higher number of ion recording. However, the increase of the acquisition speed of FT mass analyzers is possible but it involves a significantly reduced resolution in comparison to the best values reported for slow scan speeds, as shown in Table 1. TOF mass analyzers have the highest scanning speed among all mass analyzers and their m/z range is theoretically unlimited in MALDI-TOF linear configuration (hundred thousands Da), despite the m/z range of TOF-based analyzers in LC-MS systems is limited to several tens of thousands. In general, the Q analyzer is the simplest and cheapest, followed by the IT and linear IT. The TOF analyzer is the cheapest high-resolution mass analyzer, with remarkable features in terms of acquisition speed, m/z range and relatively good resolution and mass accuracy. FT and ICR MS analyzers have the best operational parameters, but the instrumental complexity implies increased investment costs.

MS-coupled Pre-fractionation Techniques

Living organisms are dynamic and complex systems; the human body is composed of over a trillion cells and each cell contains over one trillion molecules. It is predicted that there are more than 100,000 different proteins, 3 billion nitrogenous base pairs and a highly complex network of metabolites. For this reason, separation methods, such as CE, GC and HPLC are necessary before analyzing complex biological human samples by MS technology. The CE methodology allows an efficient separation in a relatively short time, due to the differential mobility of charged species in an electrical field [51-58]. In a GC instrument, the liquid phase is coated

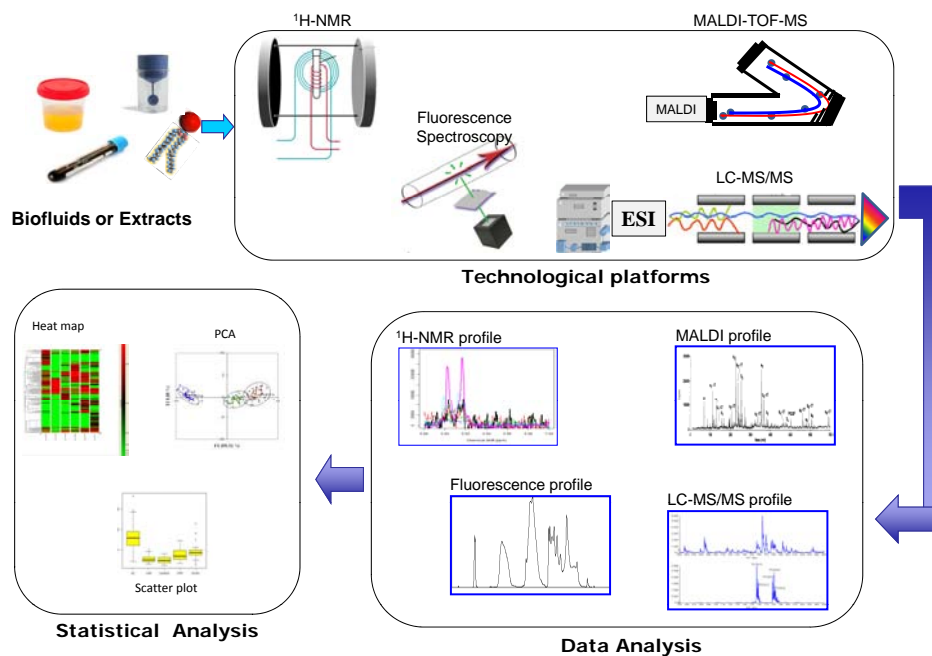


Fig. (6). Main technological approaches used in the lipidomics field. Mass spectrometry together with the modern and high performant nuclear magnetic resonance spectroscopy and fluorescence spectroscopy, are used in lipidomics field to identify and quantify the molecular lipids in complex biological systems.

onto the column inner surface [59] and the temperature directly influences the column. The carrier gas (*e.g.*, helium or hydrogen) passes through a cylinder, by a pressure or flow-rate-controlling device, to the sample injector at the column inlet. In the GC-MS system, mixture components are eluted by a column and revealed by the MS detector. The improvements of columns and instrumentation have allowed the current usage of advanced LC, including HPLC, rapid resolution (RR)-, rapid separation (RS)-, Ultra Fast (UF)- and ultra performance (UP)-LC.

“-OMICS” APPLICATIONS

MS-based Lipidomics

Lipids are constituents of cell membranes where they play a structural role as parts of organized bilayers and as precursors of various regulators of intra- and extracellular metabolism [60]. Lipidome of each cell (term first coined by Kishimoto *et al.*, 2001, [61]) refers to the entire collection of chemically different lipid species in a cell, which consists of many tens of thousands of distinct chemical moieties, which vary in content and composition during alterations in the cellular environment [62, 63] and are classified into various lipid classes and subclasses [64, 65]. Lipidomics is a research field that studies cellular lipidomes on a large scale [66-70] and involves specific identification (ID) of cellular lipid species, including the molecular structure of each lipid species, as well as their interactions with other lipids and proteins during cellular growth and development, external perturbations, and changes in nutritional status. Lipidomics is an essential field in systems biology, especially since it has provided important results in the many lipid-related diseases, including diabetes, obesity, heart disease, and neurodegenerative diseases. The first analyses of lipidomics were conducted by Gross [70] and Wood & Harlow [71], followed by Maffei Facino *et al.* [72] and Han *et al.* [73, 74]. The authors highlighted the relationship between the alterations in membrane structure, function and the biological responses to cellular adaptation in health and under disease conditions. The first application of MS-based approaches to lipidomics was performed by Han and Gross in

2003, in order to characterize specific chemical properties of lipid molecules [75]. Subsequently, many studies that used the extensive information provided by the lipidomics approaches, were published [76]. Recently, MS together with modern instrumental technologies such as nuclear magnetic resonance spectroscopy (NMR), fluorescence spectroscopy (FS), and microfluidic devices have been used in lipidomics to identify and quantify the structure and function of lipids in biological systems (Fig. 6) [76]. A high number of results in the lipidomic field was also due to the Noble prizes J.B. Fenn (2003) [77] and K. Tanaka (2003) [78], through the development of the soft ionization techniques for MS technology, such as ESI and MALDI. Recent advances in MS approaches, have significantly facilitated the accurate quantification of lipid molecules providing new insights into lipid metabolic pathways, metabolic flux, and integration systems. The direct infusion of the sample into a mass spectrometer instrument and the chromatographic separation-coupled MS represent the two main platforms now used for lipidomic studies [78, 79]. These new applications, have widely increased the amount of fragmentations' products and have allowed better IDs of lipid structures. Currently, a triple quadrupole system that operates using the four major MS/MS scan modes described above, represents the most commonly used MS/MS approach in the lipidomic field.

MS-based Metabolomics

Metabolites usually represents small molecular species subjected to temporal, spatial and diet variability [80]. Metabolites are produced during metabolism or at its end. The metabolome term (coined in the late 1990s by S.G. Oliver *et al.*, [81]) is highly inhomogeneous, because it represents a vast number of components that belong to a wide variety of compound classes, such as amino acids, lipids, organic acids, nucleotides, *etc.* in a high dynamic range of concentrations. According to Beecher [82], two thousand major metabolites seem to be a good estimate for humans and this number could greatly increase if we consider also the secondary metabolites. Global metabolic fingerprinting and quantitative metabolite

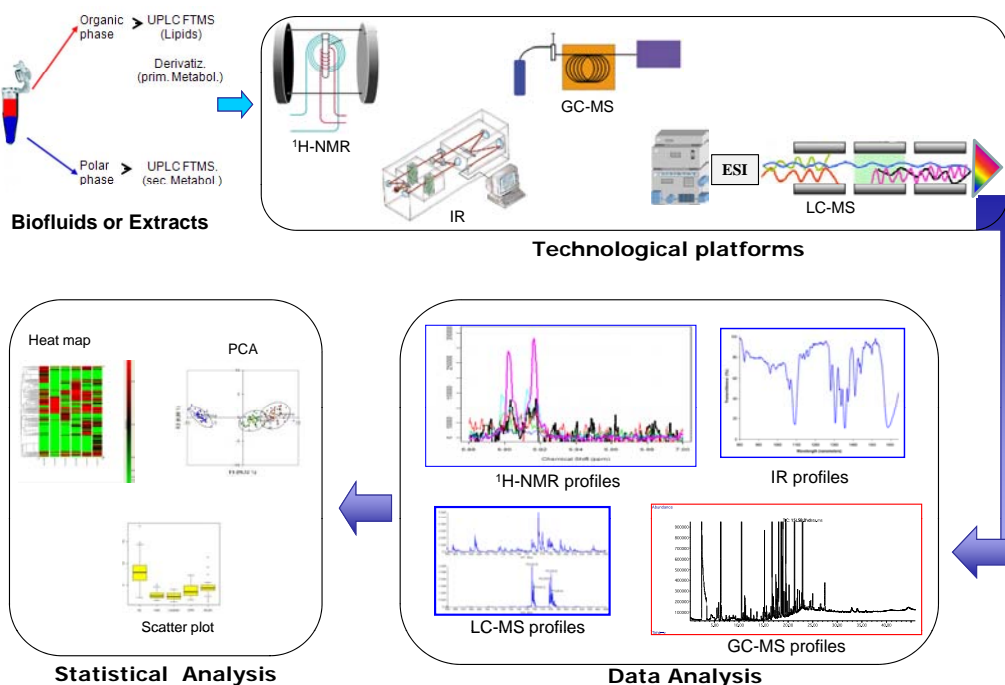


Fig. (7). Main technological approaches used in the metabolomics field. Nuclear magnetic resonance together with the infrared spectroscopy, support the mass spectrometry in the metabolomics studies to identify and quantify the molecular metabolites in complex biological systems.

profiling, represent two complementary strategies currently applied for metabolomic investigations [83]. Fingerprinting approach focuses on the changes of metabolite profiles in response to disease or environmental or genetic alterations and is principally supported by GC-MS, CE-MS, LC-MS or direct infusion into MS systems. Different technologies, such as NMR or FT infrared spectroscopy (IR), can support the MS platforms when it is necessary; however, presently, MS-based metabolomics is the main approach (Fig. 7). A typical workflow in metabolic fingerprinting approaches, previously applied to urine, plasma, saliva, cell and tissues, includes a pre-analytical stage where samples are treated to extract metabolite molecules using a liquid-liquid or solid-phase extraction with the addition of internal standards (IS). An analytical phase provides a sample derivatization and analysis through GC-MS, CE-MS and LC-MS. Subsequently to spectra acquisition, data elaborations are performed using a multivariate analysis (*e.g.* principal component analysis, PCA) to identify new potential biomarkers. A fingerprinting approach, without characterization of the metabolite species, provides only a cataloging tool and does not contribute to biochemical knowledge. The quantitative metabolite profiling approach focuses on the analysis of metabolites related to a specific metabolic pathway or group and is usually performed by using GC and LC-MS/MS systems and quantitative databases. A particular metabolite profiling is represented by targeted analysis of selected molecules, such as biomarkers of disease or products of enzymatic reactions [84]. The incredible advances of MS technology over the past ten years are allowing a constant expansion of the number of analytes that can be simultaneously quantified in a single analysis (*e.g.*, sequential window acquisition of all theoretical fragment-ion spectra, named SWATH; or global precursor ions scan mode, known as GPS) [85]. However, several quantitative approaches have been developed and are routinely used. The metabolite IDs and their quantitative modulation in specific phenotypes will provide valuable information to encode new biochemical pathways.

MS-based Genomics

Many efforts have been made in the last two decades to extract the genetic codes of distinct species, including the well-known human genome project that discovered 32,000 human genes [86]. With the recent progress of MS ionization methods, increasing attention has been dedicated to MS-based genomics in systems biology; ID of DNA or RNA is a routine job in genomic studies (Fig. 8). Schurch, Bernal-Mendez, and Leumann [87] used an ESI-Q/TOF mass spectrometer to analyze fragment ions of RNA and mixed-sequence RNA/DNA pentanucleotides. Interactions between nucleic acids and proteins are involved in various cellular pathways maintaining critical cellular functions, providing important information on the structure and dynamics of complex biological systems. To study such interactions, proteins, DNA and RNA are subjected to comprehensive examination, where MS-based methods also play an essential role. Common strategies for such investigations usually involve an enrichment or purification of target complexes followed by MS or tandem MS analysis. Hong and co-workers [88] demonstrated the use of ESI-MS/MS for the structure and distribution analysis of tandem lesions in DNA caused by the nucleobase peroxyl radical 5,6-Dihydro-2'-deoxyuridine-6-yl. Thompson and co-workers quantified oligonucleotides using EI-cleavable tandem nucleic acid mass tag-peptide nucleic acid conjugates (TNT-PNA) for the detection of specific DNA sequences by ESI-MS/MS [89]. FTICR-MS is another common choice for oligonucleotide ID due to its unique attributes allowing unambiguous mass determination. Indeed, early genomic studies using MALDI-FT-ICR-MS have demonstrated a promising performance of FT-ICR-MS for nucleotide analyses [90-93]. Hofstadler and co-workers, overviewed this topic placing a special paying on fragmentation methods [94]. Using ESI-FT-ICR-MS, ID of distinct oligonucleotides can also be achieved for genotyping purposes, such as variable number of tandem repeat (VNTR), analysis of restriction fragment length polymorphisms (RFLP), sequences and single nucleotide polymor-

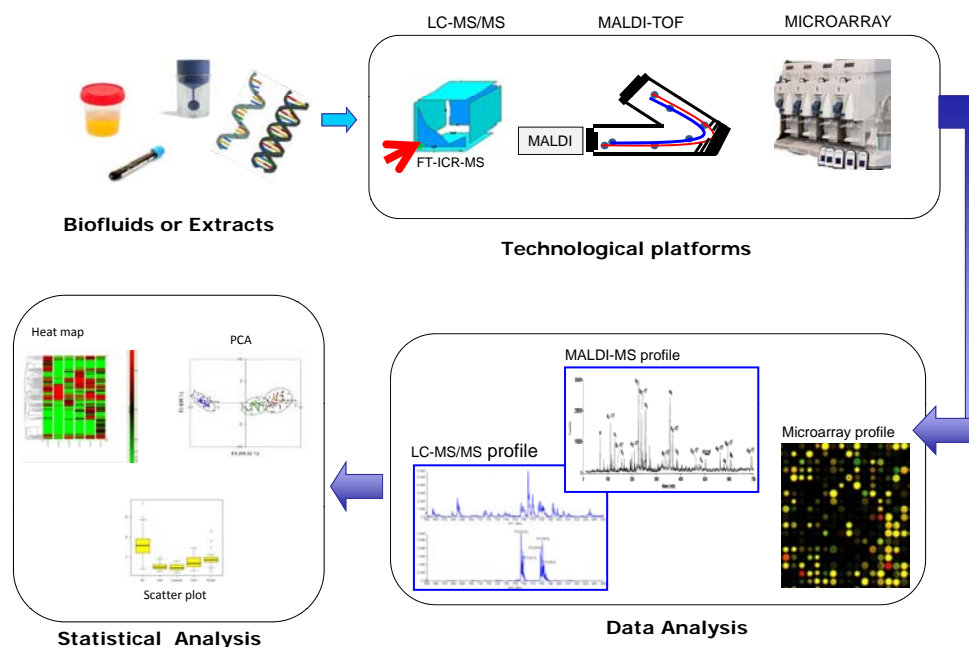


Fig. (8). Main technological approaches used in the genomics and transcriptomics fields. The mass spectrometry and the microarray technologies are applied to routinely identify and quantify DNA and RNA molecules in complex biological systems.

phisms (SNP) and short tandem repeated (STR) sequences. For instance, Null, Hannis, & Muddiman used ESI-FT-ICR-MS as a reliable approach for characterizing both simple and compound STRs by successful analysis of two tetra-nucleotide STR loci: *i*) HUMTH01, a simple STR with non-consensus alleles; and *ii*) vWA, a STR compound with non-consensus alleles [95]. Lau and co-workers [96] used MALDI-TOF for detecting SNPs in hepatitis B virus precore/basal core promoter region and Misra, Hong, and Kim (2007) [97] applied MALDI-TOF for multiplex genotyping of cytochrome P450 SNPs.

MS-based Transcriptomics

Compared to the genome, transcriptome (the full set of messenger RNA molecules produced by a given cell) is a much more dynamic system, largely varying in response to changes of external conditions. Transcriptome directly reflects the genes that are actively expressed in a given cell under a specific condition and are closely related to the changes in the proteome. In the studies on transcriptome, MS has found much fewer applications than conventional gel-based large-scale screening approaches, such as DNA microarray technology (Fig. 8) [98]. By combining DNA microarray-based transcriptomics with MS-based proteomics, enhanced understanding of cellular functions of the systems level can be achieved. For instance, Wu and co-workers conducted a global protein survey of human Jurkat T leukemic cells, one of the most important model systems for T cell signaling studies, by integrating proteomics with transcriptomics profiling [99]. In a similar way, Van Duy and co-workers in 2007 combined proteome and transcriptome analyses for the study of *Bacillus subtilis* in response to the fungal-related antimicrobial 6-brom-2-vinyl-chroman-4-on (chromanon) and 2-methylhydroquinone (2-MHQ) [100]. Additionally Schmidt and co-workers reported a comparative proteomic and transcriptomic profiling of the fission yeast *Schizosaccharomyces pombe* [101]. In the end, as a very important application of MS on genomics field, Evans and co-authors demonstrated that, for a non-model species, the sequencing of expressed mRNA can generate a protein database for MS-based ID [102].

MS-based Proteomics

The introduction of ES and MALDI (1980's), in combination with the accessibility of genome sequence information, has revolutionized MS [103, 104], thus allowing routine MS analysis of protein molecules (Fig. 9). Two main strategies for protein ID by MS are currently used in proteomics: top-down and bottom-up proteomics. In top-down proteomics, intact proteins are introduced into a mass spectrometer and then subjected to gas-phase fragmentation. However, the presence of multiply charged product ions has always been a weak point of this approach, because it may prevent the determination of product ion masses. With the introduction of the modern mass spectrometers with high mass measurement accuracy, this obstacle has been overcome (*e.g.*, modern MALDI TOF/TOF instruments). Conversely, in bottom-up proteomics, the proteins are firstly separated by gel electrophoresis or chromatography, subsequently digested by specific enzymes (*e.g.*, trypsin to cut lysine and arginine) and then introduced into the mass spectrometer. Bottom-up proteomics approach is represented by peptide mass fingerprinting (PMF) and tandem MS analysis. PMF has largely characterized the early years of the proteomic era; it relies on the acquisition of mass spectra from a tryptic digest of a protein sample and on the measure of tryptic peptide masses searched against a protein database such as UniProt, employing different database search engines (*e.g.*, SEQUEST, Mascot, X!Tandem, OMSSA, PLGS, Sorcerer, ProteinPilot) and performing, for each protein, an *in silico* tryptic digest, hence generating a theoretical spectrum. The best overlap between the experimental and theoretical mass spectra then identifies the protein. As for protein ID, it can be achieved by MALDI-TOF-based PMF and tandem MS analysis, starting from 1D- or 2D-SDS-PAGE coupled to nano-ES-MS/MS, without intermediate chromatography. Using the latter "off-line" approach, each MS/MS spectrum peptide is manually analyzed to give a partial amino acid sequence. This information, along with the peptide molecular mass is interpreted using a database search engine producing the most likely peptide match from an *in silico* tryptic digest of the entire protein database. Once the peptide sequence ID is reached, the presence of its parent protein can be therefore inferred [105]. The

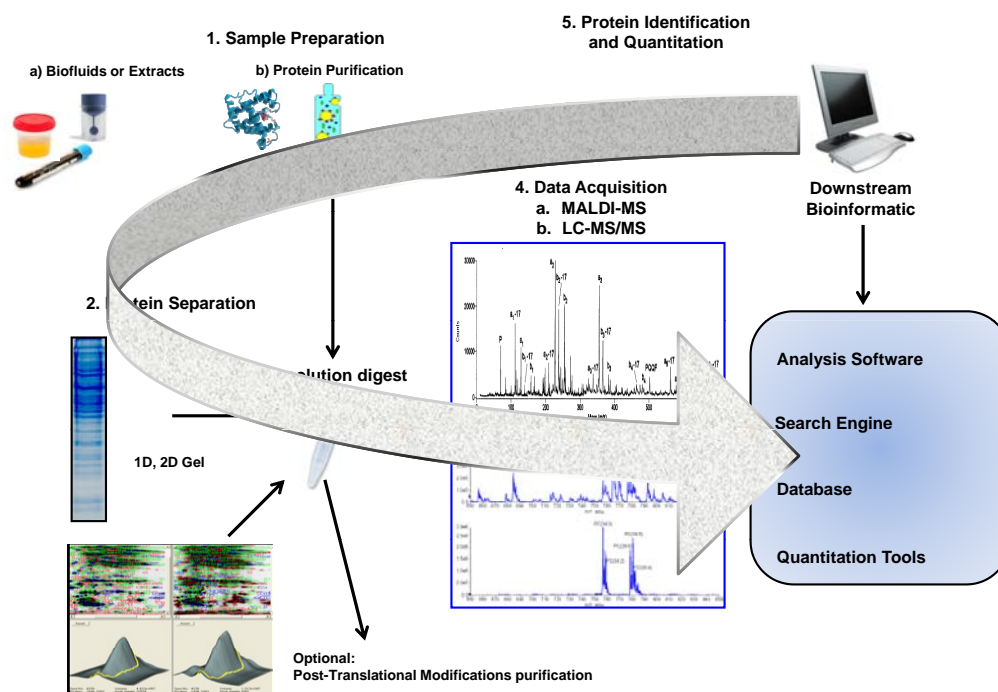


Fig. (9). Main technological approaches used in the proteomics field. Routinely analysis of protein molecules in complex biological systems are carried out by the application of pre-analytical technologies (for protein purification) and mass spectrometry approaches (for protein identification and quantification).

1D- or 2D-SDS-PAGE-LC-MS/MS based proteomics, often called shotgun proteomics, is now the main bottom-up proteomics technological approach [106]. The Nano 2D-LC-MS/MS, also called MudPIT (multidimensional protein ID technology) [107], consists of a capillary column packed with strong cation exchange (SCX) and a reverse phase (RP) resins arranged in series in a single column. In this 2D-LC system, the tryptic peptides are separated according to their acidity in the SCX column and hydrophobicity in the RP dimension and the eluted peptides are then acquired in a data dependent acquisition manner (DDA) by the MS/MS instrument. In the DDA technique, the first scan is a survey one (full MS scan), where the most abundant precursor ions are isolated and then activated. The obtained fragments are analyzed in the second MS stage avoiding a recurring acquisition of the same precursor: an exclusion list to eliminate selected ions for a given time (*e.g.*, dynamic exclusion), can be therefore generated [108]. Finally, peptides are identified by searching their MS and MS/MS spectra against a protein database using a search engine; a good ID can be evaluated by the higher score (*e.g.*, expected number of peptides with scores equal to or better than the observed score) and the lower expectation value (E value, absolute probability that the observed match is a random event). Recently, to publish MS experimental data, an estimate of the false positive discovery rate (FDR) is also required. This is obtained by matching the data against a reversed database and performing a repeated search using identical search parameters. The amount of identified false positive peptides provides a good estimate of the quantity amount of false positive IDs present in the real database searching [109]. An alternative to MudPIT is represented by 1D-SDS-PAGE-LC-MS/MS based technology. The workflow provides the protein separation by 1D-SDS-PAGE according to MW, followed by in-gel tryptic digestion, peptide analysis by nanoLC-MS/MS and protein ID by database searching, as above described [110-112]. While the application of shotgun proteomics workflows to tissues, cells, and organelles usually results appropriate, the analysis of body fluids (*e.g.*, serum,

blood, plasma, intestinal fluids, urine samples) is particularly difficult because of the complexity and of the high dynamic range of contained analytes. The human plasma is predicted to contain hundreds of thousands to millions of protein and peptide species, spanning up to 10 orders of magnitude as a concentration range. In this case, the workflows are consistently modified to deplete high abundance proteins [113] or enrich low abundant proteins [114] before MS analysis. Another MS technology, broadly used for biomarker discovery, is represented by the SELDI-TOF MS, often employing PCAs platforms consisting of chips supplied with specific chromatographic surfaces (*e.g.*, reverse phase, anionic and cationic exchange and immobilized metal affinity), purposes [115]. A few sample microliters are distributed onto the surface, under specific binding conditions that determine selective protein retention by the surface. By using different surfaces and different binding/washing solutions, a differential sample protein profile can be obtained. The proteins that bound on the array surface are then ionized and detected by the TOF MS. Remarkably, by using SELDI-TOF MS, only a limited sample preparation is needed and the system is ideally suited for low MW proteins (<20 kDa) profiling. Several studies have demonstrated that this methodology can be used to point out expression patterns for clinical diagnosis of cancers affecting ovaries [116-120], breast [121-125], prostate [126-131], liver [132-135], colon [136] and stomach [137]. However, a major disadvantage of SELDI-TOF MS technology is the lack of mass signals' ID, reason why this technology might be completely replaced in the near future with the shotgun proteomics technology, previously described. Proteomics does not only concern the ID of peptides and proteins, but also with the determination of their abundance. Quantitative proteomic studies have been mostly comparative, where one state is compared against another (*e.g.*, healthy *versus* diseased samples). The most effective quantitative proteomics technique is represented by the stable-isotope labeling (SIL), where the proteins from one state are tagged with heavy isotopes, and those from another state with light isotopes. These proteins will behave in an

identical fashion during LC separation; the mass spectrometer will distinguish between light and heavy isotope labelled forms of the same peptide and the ion-current (total ion count) of the two forms will be proportional to their relative abundance and to protein abundance. Three of the most popular methods for comparative proteomics are based on isotope coded affinity tags (ICAT) [138], isobaric tags for relative and absolute quantification (iTRAQ) [139] and SIL by amino acids in cell culture (SILAC) [140] commercial reagent kits. As an alternative to SIL protein quantification (PQ) technique, the label-free PQ technique is also available. A widely used label-free quantitative method is the redundant peptide-counting method, where the abundance of a particular protein is estimated by the number of times its peptides have been identified in a given LC-MS/MS run. Recently, an innovative label-free proteomic strategy, called selected reaction monitoring (SRM) [141], has emerged. SRM represents a very promising procedure for quantitative proteomics and has the potential to overcome the shortages of current shotgun proteomic approaches. However, the weakness of this extraordinary MS technique is limited by the overall number of proteins (~50) that can be quantified in a single analysis. On the contrary, the new SWATH MS acquisition is able to potentially quantify any protein of interest in a complex biological sample, as discussed by Gillet and co-authors [142]. For this application, data are acquired on fast, high resolution quadrupole-quadrupole TOF instruments, by repeated cycling of 32 consecutive 25-Da precursor isolation windows. This new post-acquisition ID and quantification strategy overcomes the analytical limits of current SRM methodologies, producing high quality results with a comparable consistency and accuracy. Therefore, the SWATH strategy may represent a new solution to address the challenge to decode the information residing in complex samples, which still require sample pre-treatment to achieve the appropriate sensitivity for low-abundance biomarker monitoring. The SWATH acquisition strategy is performed by the last generation nano-LC-MS/MS Triple TOF[®] 5600 (ABSCIEX, Toronto, Canada), representing the most promising MS analytical tool for both proteomics and metabolomics applications. These procedures are generally employed for a relative quantification of protein, between at least two different samples, but they are also remarkable in the field of MS absolute quantification of peptides and proteins [143-145].

Bottom-up Versus Top-down Approaches

Bottom-up proteomics is the ripest and most widely used approach for protein identification and characterization. Automated on-line nano-scale reversed-phase (RP) LC-ESI-MS/MS is universally used for bottom-up proteomics and (RP) HPLC provides high-resolution separations of peptide digests with solvents that are compatible with ESI. Commercial configurations with control software and bioinformatics tools optimized for bottom-up applications are available from several suppliers. The bottom-up strategy using MudPIT approach, has been most successful in the identification and quantification of proteins in digests derived from very complex mixtures. In the bottom-up strategy, only a fraction of the total peptide population of a given protein is identified and it represents the most significant limitation of this approach because information on only a portion of the protein sequence can be obtained. A consequence of the limited sequence coverage in bottom-up proteomics is the loss of many information about PTMs. Other limitations are encountered when bottom-up methods are used for protein identification from very complex peptide mixtures. The throughput of MudPIT approach is quite limited because it requires extended run

times of as long as 15 h or even more. Moreover, bottom-up methods include the loss of information about low-abundance peptides in mass spectra dominated by high-abundance species. Finally, narrow chromatographic peak widths can compromise the acquisition of adequate MS/MS information during elution. In top-down approach, the time-consuming protein digestion required for bottom-up methods is eliminated. The two major advantages of this strategy are the potential access to the complete protein sequence and the ability to locate and characterize PTMs. Top-down proteomics is younger than bottom-up proteomics and currently suffers from several limitations. First of all, the very complex spectra generated by multiply charged proteins limits the strategy to high purified protein mixtures. Second, the favored instrumentation (FT-ICR, hybrid ITs FT-ICR or orbitrap) are expensive to purchase and operate. Third, the top-down approach does not work well with intact proteins larger than about 50 kDa. Fourth, the molecular mechanisms of protein dissociation are poorly understood compared to those of peptide dissociation. In the top-down strategies, a greater understanding of multiply charged ions fragmentation is needed [146], including the influence of precursor ion charge state, the role of protein primary, secondary and tertiary structure, and the contribution of PTMs. Finally, bioinformatics tools for top-down proteomics are less evolved than those for bottom-up proteomics.

MS-based Metaproteomics

As described above, the proteomic analysis of complex microbial communities is a new promising research field, aiming at assigning functional activities of microbial communities. In 2004, Wilmes and Bond [147] coined the term "metaproteomics" for the large-scale ID of the entire protein content of a mixed community of prokaryotic microorganisms, given at a certain time. In this milestone study, highly expressed proteins, including an Acetyl-CoA acyltransferase, from an environmental microbiota derived from activated sludge, were characterized by 2D-SDS-PAGE and MALDI-TOF MS combined approaches. Subsequently, Ram *et al.* [148], conducted a large comprehensive metaproteomic study combining shotgun MS-based proteomics analysis with gene expression techniques in order to evaluate the *in situ* microbial activity of an acid mine drainage (AMD) natural microbial biofilm community at low complexity. In this experimental work, they characterized more than 2,000 proteins (215 as novel proteins) from the five most abundant microorganisms, including a highly expressed new protein, belonging to the iron oxidation processes. In the last few years, a rich literature is shedding light on low complexity microbial communities [148-151]. Lacerda and co-authors used 2D-SDS-PAGE and MALDI-TOF-TOF MS combined technologies to purify and characterize microbiota protein modulations over time in a bioreactor fed with cadmium [152]; Delmotte and co-authors performed a culture-independent analysis of the phyllosphere microbiota, associated with leaves of soybean, clover and *Arabidopsis thaliana* plants, employing the integration of metagenomics and metaproteomics approaches. Using 1D-SDS-PAGE LC-MS/MS technology, they identified, after tryptic digestion, 2,883 unique proteins associated with the communities of the 3 different plant species [153]. Afterwards, Park and Helm used metaproteomic analysis to track metabolic fate of extracellular proteins under both anaerobic and aerobic conditions in activated sludges, by using 1D-SDS-PAGE and LC-MS/MS combined strategies [154]. Furthermore, Wilmes and co-authors applied the shotgun proteomics technique to characterize proteins from a

complex activated sludge microbiota enriched for *Accumulibacter phosphatis* in a laboratory-scale bioreactor, by using a linear ion trap (LTQ)-Orbitrap mass spectrometer. In this experimental work, the authors identified protein modulations associated with different *A. phosphatis* subpopulations and highlighted the potential of genetic diversity in maintaining a stable process performance [155, 156]. Warnecke and co-authors applied a three-dimensional LC-MS/MS analysis by using the LTQ mass spectrometer on a gut fluid, recovered from the wood-feeding termite hindgut community, to evaluate specific enzymes associated with cellulase activity [157, 158]. The first step relied on preparative isoelectric focusing, using a free-flow electrophoresis system (FFE) and the second on a SCX step gradient chromatography. The contents of each FFE/SCX fraction were then separated according to hydrophobicity, using a microcapillary MS-coupled RP-LC system. Toyoda and co-authors studied the mechanisms of the rumen plant cell wall degradation by isolating and characterizing cellulose binding proteins from the contents of a sheep rumen, using LC-MS/MS technique combined to LCQ Deca XP mass instrument [159]. Afterwards, also Rudney and co-authors applied the three-dimensional LC-MS/MS LTQ-based approach to the first identifying of a human salivary microbiota, characterizing 139 proteins of microbial origin [160]. To confirm the functional human protein-pathogen interactions in patients with asymptomatic bacteriuria and urinary tract infection, Fouts and co-authors designed a metaproteomics approach using the LC-MS/MS LTQ-XL IT system [161]. Kan and co-authors applied the metaproteomics analysis to characterize expressed protein profiles of the Chesapeake Bay microbial communities using 2D-SDS-PAGE protein separation and MALDI-TOF, LC-MS/MS analyses [162]. On the other hand, by using LC-MS/MS technology, Sowell and co-authors in 2009 identified, proteins expressed by SAR11 (Pelagibacteraceae, a group of α -Proteobacteria very abundant throughout the oceans) in the Sargasso Sea, during the season when nutrients were highly useless [163]. Subsequently, Leiner and co-authors in 2012, discovered the metaproteome of a gutless marine worm and its symbiotic microbial community using 1D-SDS-PAGE and LC protein/peptide purifications and MS/MS with a hybrid LTQ-Orbitrap analysis [164]. To analyze the proteins isolated from dissolved organic matter, Schulze and co-authors in 2005 applied the MS-based proteomic techniques [165]. Although it is still a new science, metaproteomics has the potential to deliver extensive new functional information on high complexity ecosystems, such as the gut microbiota. In this regard, by metaproteomics approach, Klaassens and co-authors have functionally characterized the microbiota in the developing human infant GI tract in 2006, by using 2D SDS-PAGE and MALDI-TOF MS-combined analyses [166]. They discovered a temporal stability of a core proteome for an established intestinal microbiome of an adult human, as further confirmed by Kolmeder and co-authors in 2012 using 1D SDS-PAGE and LC-MS/MS LTQ Orbitrap XL mass spectrometer analysis [167]. Verberkmoes and co-authors in 2009, realized a high level of metaproteome characterization, focusing on the unaltered human adult gut microbiomes of two healthy subjects, identical human twins, highlighting the strongly integrated relationship between microbial and human proteins by the 2D nano-LC MS/MS analysis with a split phase column (RP-SCX-RP) on a LTQ Orbitrap MS system with 22 h sample runs [168]. Lastly, Erickson and co-authors, integrating metaproteomics and metagenomics, characterized the human host-microbiota signatures of human Crohn's disease using a 2D nano-LC MS/MS ap-

proach [169]. Challenges for metaproteomic investigations include an irregular species distribution, the dynamic range of protein expression levels within microorganisms, and the large genetic variety within microbial communities [170, 171]. In spite of these difficulties, metaproteomics has the great potential to link the genetic multiplicity and activities of microbial communities to their impact on the ecosystem function.

CONCLUSIONS AND FUTURE PERSPECTIVES

The tremendous developments of the MS technology and concurrent gene sequencing efforts have made the "-omic" revolution possible. The data generated by the "-omics" investigations can be integrated, hence improving the understanding of microbiota biological activities. MS already plays an essential role in the study of '-omics', because it certainly meets all the criteria to face a series of challenging tasks such as high sensitivity, selectivity, throughput, robustness, flexibility, and linear range of quantification of complex biological samples. Nowadays, genomics, transcriptomics, proteomics, metabolomics and lipidomics data from humans are copious in the literature, but their integration remains to be thoroughly addressed by the support of computational biology. The new integrated approaches will contribute to the development of personalized medicine for health monitoring and prevention.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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