A Review: Protein Identification by LC-MS: Principles, Instrumentation, and Applications

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Abstract:
This review will focus on protein and peptide separation studies of the period 1995 to 2010. Peptide and protein analysis have developed dramatically after applying mass spectrometry (MS) technology and other related techniques, such as two-dimensional liquid chromatography and two-dimensional gel electrophoresis. Mass spectrometry involves measurements of mass-to-charge ratios of the ionized sample. High-performance liquid chromatography (HPLC) is an important technique that is usually applied before MS is conducted due to its efficient separation. Characterization of proteins provides a foundation for the fundamental understanding of biology aspects. In this review, instrumentation, principle, applications, developments, and accuracy of the measurements of mass spectrometry will be reviewed and discussed. In addition, the principles of HPLC technology will be explained, which is necessary before applying MS.

Keywords: Mass spectrometry, Protein, HPLC, Two-dimensional gel electrophoresis.

Introduction
Proteins are bio-functional molecules that are present in living organisms. Quantification and identification of proteins are applied in several fields, such as monitoring cells functions and medicine.

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Proteins have a nutritional value as it is the main source of essential amino acids for humans [2]. They are also involved in enzymology field, which control most cell functions in living organisms [3]. Several methods are used to determine protein concentration, however choosing the suitable method depends on several factors, such as protein concentration in the sample, chemical interferences, protein availability for detection, etc. [4]. Each of these methods has advantages and limitations in terms of measurement accuracy, cost, reagent stability, etc. [5].

Mass spectrometry (MS) technology and other techniques such as two-dimensional liquid chromatography (2-D LC) and two-dimensional gel electrophoresis (2-D electrophoresis) improved peptide and protein analysis dramatically [6]. Some techniques are necessary before applying MS. For example, the HPLC technique is an accurate separation method due to the sequential basis of the one-sample a time, and therefore it is mostly applied before MS technique. In addition, different properties of proteins could be considered via HPLC separation method, such as protein charge, size, hydrophobicity, and biorecognition (Affinity). All these important features of separation make HPLC an important technique before applying MS [7]. The term proteomics was created in 1990s from merging protein and genomics. Proteomics is a branch of biochemistry related with the analysis of a large-scale of proteins and identifying their structures [8]. Application of mass spectrometry in proteomics facilitated the understanding of the dynamics and interaction of peptides/proteins as well as the investigation of microorganisms, bioremediation, and human health [9]. Moreover, mass spectrometer improved the accuracy and sensitivity of protein and peptides quantification and identification. Mass spectrometry is developed according to three scientific bases, which are mass analyzer technique, ion optics, and fragmentation [10]. Therefore, protein and peptide analytical techniques are under development towards understanding protein function and structure.

Practically, determining biomarker proteins has been well established to improve patient care along with the diagnostic and prognostic procedures of diseases [11]. Identifying and quantifying peptides/proteins by MS is also used to predict responses to therapy and help in selecting the suitable treatment [12]. From the agricultural standpoint, biomarker proteins also provide an evidence of food contamination and reveal food adulteration [13], in addition to determining allergic proteins in foods which improves food safety [14]. In this review, mass spectrometry technology in protein identification will be reviewed and discussed in term of its instrumentation, principle, applications, developments, and accuracy of measurements. Also, some other techniques, which are important and necessary before performing MS, will be explained, such as, high performance liquid chromatography (HPLC)

**Sample Preparation**

Proteins usually exist in a complex network with other biomolecule compounds that adjust a cell function. Extraction and isolation of proteins have become a critical step in protein analysis in the biological context because protein identification efficiency is affected by sample purity (interferences between a protein of interest and other compounds in the sample) [15]. Isolation, solubilization, and proteolytic digestion of proteins have proven to be fundamental steps before proteomic analysis (bottom-up) [10]. Proteins can be extracted from a liquid medium by salting out, and the precipitated proteins can be obtained by centrifuging the protein-salt mixture [16]. For example, Link et al. [17] added urea and NaCl to a yeast lysate, and the produced mixture was centrifuged to precipitate peptides. Resolubilized proteins are usually made by adding a suitable buffer to the protein pellet produced after the centrifugation step. Later, peptides can be separated by 2-D LC before being subjected to the MS [18]. All the HPLC techniques will be mentioned later in this review, which can be used as a part of preparation steps. Protein preparation steps used in Chelius and Bondarenko [19] study were almost similar in terms of the proteolysis of the protein of interest and subjecting it to the LC-MS. Ammonium bicarbonate buffer was used to reconstitute human serum with horse myoglobin, and the protein mixture was alkylated by iodoacetic acid before digesting with trypsin. Manza et al. [20] used commercially available micro-centrifuge filtration devices (spin filters with specific molecular weight cut-off, MWCO) to remove contaminants from the sample, and the protein was resuspend in buffers; the resulting protein was alkylated and digested on the filters, and the resulting peptides were isolated by the spin filter. The method was considered fast and simple, with reduced sample loss. Different preparation steps can be conducted by using two-dimensional gels and the protein/s of interest are broken down by proteases into peptides before being subjected to mass spectrometry [21]. For intact protein proteomics analysis (top-down), the proteolysis step should be

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Avoided. Wang et al. [22] used different molecular weight cutoff spin filters to separate spiked proteins, and the concentrated protein was diluted (solubilized) before it was subjected to affinity separation. The interested eluted fractions from the affinity step were subjected to LC-MS analysis. Preparation steps depend on the sample properties, such as protein concentration, types of protein in the sample, solubility, etc. as well as the purpose of the analysis. Moreover, purity level and retaining of the protein of interest are still considered the main objectives.

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is a separation analytical or preparative method that has been developed several decades ago, and it is widely used in the analytical field, protein preparation, quality control, and process monitoring. HPLC is an important technique due to the sequential basis of the one sample separation at a run (more accurate separation method) compared with other techniques that can separate several samples at a run, such as electrophoresis and planer chromatography [7]. The reasons behind the higher speed and resolving power of liquid chromatography are the improvement of column packing, column design, high pressure, and pulseless pumps [23]. Peak capacity is the best way to measure the performance of a gradient separation. A good definition of it is mentioned in Neue [24] study, which is “the upper limit of resolvable components for a given technique under prescribed conditions”. Several factors affect peak capacity, such as plate number, linear velocity and temperature. Table 1 shows different properties of proteins with different separation techniques that are used to give a successful separation.

| Protein property          | Technique                              |
|---------------------------|----------------------------------------|
| Charge                    | Ion exchange                           |
| Size                      | Gel filtration                         |
| Hydrophobicity            | Hydrophobic interaction, Revised phase |
| Biorecognition (specific ligand) | Affinity chromatography               |

**Table 1** - Different properties of protein used during purification (Hydrophobic interaction 2006)

**Pumps**

High-performance liquid chromatography has been proven to be a successful separation technique because of the excellent performance and good reproducibility. To achieve this performance, high pressures are required to rapidly transport the mobile phase through a chromatography column [25]. A good pump is necessary to achieve high pressures and subsequently a good separation [26]. Examples of pumps used usually with HPLC are:

The short-stroke piston pump is the usually used type of HPLC pump. The mobile phase is driven by a piston through a valve that opens or closes the path of the mobile phase towards the suitable direction. The pumping action is controlled by a stepper motor that drives a rotating disk [27].

![Diagram of short-stroke piston pump](image-url)
The electroosmotic flow pump (EOF) is a low voltage cascade pump that consists of a narrow channel section that involves 10 parallel channels followed by a wide channel and so on (Figure-2). Several EOF pumps work together to accumulate pressure. The narrow channel acts as a high-pressure pump with the forward electric field, while the electric field is reversed in the wide channel. However, the backpressure produced by the wide channel is small compared to the pressure produced by a narrow channel [28].

![Diagram of electroosmotic flow pump](image)

**Figure 2**-Diagram of electroosmotic flow pump [28].

**Stationary Phase and Suitable Mobile Phase**

Ion Exchange Liquid Chromatography (IEX-LC) separation method is based on the interaction between a protein with a net negative, or positive, charge and an oppositely charged medium. After a protein binds onto a medium inside a column, a mobile phase (usually using a buffer with increases in salt concentration) will compete with protein for binding [29]. Changes in pH or salt concentration are either made by stepwise or by a continuous gradient. Less polar (charged) proteins elute first with small salt concentration, while more polar proteins need more salt concentration in the mobile phase to get eluted. The net charge of a protein depends on the surrounding pH; if the pH medium is below the isoelectric point (pI) of the protein of interest, a cation exchanger is typically used. If the pH of the medium is above the pI of the interested protein, an anion exchanger is typically used. In addition to the cation and anion exchange types, there are strong and weak IEX types [30]. Strong IEX is often recommended to enhance purification because it works over a wide range of pH and the ion exchange medium will not change charge state with changing pH. Strong anion exchange (quaternary ammonium, Q column) and strong cation exchange (sulfonate (S) and sulfopropyl (SP)) columns are examples of strong IEX [31]. Diethylaminoethyl (DEAE) and carboxymethyl (CM) are examples of weak anion and cation exchangers, respectively [32]. In all chromatography techniques, the media is packed into a column to form a packed bed, and then the bed is equilibrated with eluent. Using prepacked columns is more recommended in terms of accuracy, especially with small particle size (below 15 um) [33].

Hydrophobic interaction chromatography (HIC) is used as a purification strategy in pharmaceutical industry and in research laboratories. The separation is based on the differences in the hydrophobicity of protein/peptides after interacting with a hydrophobic surface (medium), and the reaction is enhanced when the sample is in the high ionic strength buffer [34]. Usually the sample is placed in high-concentration ammonium sulphate (1.5 M) to enhance the binding, and the mobile phase gradually decreases the concentration of ammonium sulphate. The protein with weak binding to the medium will elute first, and the protein with strong binding will elute last [35]. The very hydrophobic proteins bind strongly with very hydrophobic ligands, which may require extreme elution conditions. In order to avoid this problem, it is recommended to search many hydrophobic media before selecting...
the ligand. The strength of media to binding proteins increases in the order: ether, isopropyl, butyl, octyl, and phenyl [30].

Reversed phase liquid chromatography (RPLC) differs from the classic chromatography. The latter model consists of a polar stationary phase and a non-polar mobile phase, while RPLC consists of a non-polar stationary phase and a polar mobile phase. RPLC has gained extensive interest for the separation of peptides and proteins. This is because of high resolution, high mass recovery, high capacity, and maintenance of biological activity [36]. The separation in RPLC depends on the differences between the hydrophobicity of molecules (such as proteins/peptides), so in theory, it seems very close to the HIC technique. However, RPLC and HIC are different in terms of the hydrophobicity of the used medium (column packing) in both techniques [37]. RPLC medium is more hydrophobic, which leads to stronger interaction between protein/peptide and the column medium, and as a result, it needs a more hydrophobic mobile phase in order to elute proteins/peptides. The mobile phase in RPLC must be a non-polar (organic) solvent, such as methanol or acetonitrile [38]. Proteins with less hydrophobic groups elute first with lower concentrations of organic solvent of the mobile phase, while more hydrophobic proteins need high concentrations of organic solvent to get eluted. The stationary phase that is used in the RPLC is either a hydrophilic matrix covered with hydrophobic phase of carbon chain (such as n-alkyl or aromatic hydrocarbons) or naked hydrophobic polymer matrix. Highly porous matrix gives large surface area for high binding capacity [33, 30]. Because many proteins can be denatured by high organic solvent content, one must be cautious if biological activity needs to be preserved when applying RPLC.

Affinity chromatography (AC) is a separation method that provides high selectivity, high resolution and high separation capacity of the protein of interest. The basis of protein separation depends on a reversible reaction between a protein and a specific ligand attached to a matrix media of the chromatography column in a complementary binding manner [39]. Unbound compounds are eluted directly, while the protein of interest elutes by using a competitive ligand or by changing the elution conditions such as pH, ionic strength or polarity [40]. For example, serum glycoproteins have been enriched by using lectins affinity column, which can bind to specific carbohydrate residues. Unbound compounds were eluted by using an equilibration buffer, while glycoproteins were eluted by using a stronger ionic buffer with 0.8 M galactose [41].

Gel filtration (GF) is a separation method which depends on the differences in molecular size. When solutes pass through a chromatographic column packed with porous gel particles, the molecules appear in the effluent in the order of decreasing size, because larger proteins, excluded from the gel, have a shorter path length in the column. Smaller proteins are able to enter the porous media and have a longer path, which is why they elute later [42]. Gel filtration is more ideal for final purification steps when the sample volume is reduced, because sample volume significantly affects the resolution and speed of gel filtration. The mobile phase is a specific buffer that varies depending on the sample type or next step requirements [43].

Detectors

HPLC detectors are designed to measure specific physical or chemical attributes of the solute or mobile phase of the chromatography process by one of four ways. First, bulk property detector is the most universal detectors that measure the difference in the mobile phase before and after adding the sample, such as the refractive index detector. Specific property detectors measure specific characteristics of an analyte, and UV detector is the most common example of this type of detectors. Mobile phase modification detectors measure the change in an analyte after contacting with a mobile phase, such as creating particles suspension in a gas phase. Lastly, hyphenated techniques mean adding further analytical techniques to analyze compounds eluted from HPLC, such as mass spectrometry, nuclear magnetic resonance, and infrared spectrometry [44]. HPLC detectors can be classified depending on the type of interactions they have with the analyte. They include elemental detectors, optical detectors, luminescent detectors, electrochemical detectors, mass spectrometric detectors, and other detector types such as nuclear magnetic resonance and radioactivity detectors [45, 46]. In this review, MS is the main subject, while HPLC method is the method used to enhance MS analysis.

Mass Spectrometer

Mass Spectrometry is a microanalytical technique that can be used selectively to detect and determine the amount of an analyte, elemental composition, and some aspects of the molecular
structure. These tasks can be achieved by measuring mass of gas-phase ions produced from molecules of an analyte [47]. A mass spectrometer consists of an ion source, mass analyzer, and detector [48]. A mass analyzer measures the mass-to-charge ratio (m/z) of the ionized analytes, and from this ratio and standard ratio, the compounds can be identified. Proteins and other biomolecules can be identified, characterized and quantified by using MS technique. Mass spectrometry technique is under development by increasing its sensitivity and using more complex samples. Mass spectrometry is used either to measure the molecular mass of a polypeptide or to identify structural features of a polypeptide, such as amino acid sequencing, the attachment site of a protein molecular, and the site’s modification type. In the beginning, single stage MS is used in order to determine molecular mass. Then, after initial determination of molecular weight, selected ions are subjected to fragmentation via collision, which is called tandem mass spectrometry (MS/MS). From the analysis of resulting fragments, structural features of a peptide can be inferred [49].

**Principles and Instrumentation**

**Ionization Process**

Ionization is used to volatize and ionize the peptides and proteins for mass spectrometric analysis. Below are the two most commonly used techniques:

**Electrospray ionization (ESI)** ionizes molecules out of a solution, hence ESI is well suited to be coupled to liquid-based separation methods such as liquid chromatography and, in principle, electrophoresis. However, the high ionic strength required for electrophoresis tends to interfere with ESI. Electrospray ionization is used with more complex samples [48, 50]. The analyte in a liquid phase is pumped at a low flow rate (0.5 to several μL/min) through a narrow orifice to generate micrometer-sized droplets, which evaporate rapidly, imparting charge onto the analyte. The ionization process is gentle, and it is generally seeking to avoid fragmentation of the analyte molecules. The ionized molecules (charged molecules) are transferred to the mass spectrometer with high efficiency for analysis. Electrospray ionization can be used with a wide range of compounds because the only required property of a compound is being sufficiently polar to attach a charge on it. Therefore, proteins can be analyzed by this type of ionization [51, 52].

**Matrix-assisted laser desorption ionization (MALDI)** is an ionization technique that uses laser to ionize high molecular size proteins/peptides [53]. MALDI is a soft ionization method that consists of three steps. The first step is mixing a submicroliter sample with a suitable matrix of material and applying it to a metal surface in order to produce a dry (solid) sample. The matrix is a small organic molecule that absorbs the wavelength of the used laser that excites the sample molecule [54]. Two main matrixes are used, which are α-cyano-4-hydroxycinnamic acid or dihydrobenzoic acid (DHB). The matrix type determines the amount of ions created onto a molecule depending on their absorption ability. Second, the solid sample is irradiated by nanosecond laser pulses with 337 nm wavelength, which the matrix absorbs. The laser source is used in that study was a small nitrogen laser [55]. Finally, as a result of the laser irradiation, the sample molecules are protonated (ionized). The degree of ionization depends on the incorporation of the molecules (such as peptides) into crystals [56]. A peptide molecular weight of less than 500 Da cannot be well ionized by MALDI because it is often overlapped by the matrix ions, and the proteins are fragmented to some extent during the MALDI process, resulting in broad peaks and insensitive analysis. Therefore, MALDI is used more often with peptides [51, 57].

**Mass Analyzer**

A mass analyzer is at the center of MS techniques because it identifies the mass of an ion. There are four types of mass analyzers:

**Ion trap (IT):** the ions created from ionization steps are trapped (captured) first for a specific time and then subjected to MS analysis [48]. The ions can be trapped by three-dimensional electric fields that can capture a beam of ions up to their charge limit without distorting the applied field. Then an additional electric field is applied to the trapped ions to eject one ion after another to be detected to produce a mass spectrum [58]. The positive attribute of IT is that it allows for high-throughput analysis, but it has a low ion-trapping capacity, limited resolution, and space charging effect, leading to deficient accuracy of mass measurements [49]. In theory, IT technology works perfectly, yet in practice, the resolution and accuracy do not reach the quadrupole-time-of-flight technique level.
Fourier transform ion cyclotron (FT-MS) is also a trapping technique that captures ions under a vacuum in a combination of an electric and a very high magnetic field. The generated ions are accelerated in a cyclotron (ions are held to a spiral trajectory by static magnetic field and accelerated by an electric field) in order to eject ions depending on their charges and masses. Although FT-MS technique provides sensitivity, mass accuracy, and resolution, the high expense and complexity of the instrument makes it uncommonly used [60, 48].
Time-of-flight (TOF): mass to charge ratio of a compound can be determined depending on a required time of an ionized molecule to reach the MS detector. Several ions are moving toward the same direction (detector), and the velocity is inversely proportional to the square root of the m/z ratio [62]. Time-of-flight consists of two parts: acceleration region, which contains an ion optic assembly, and an ion-free flight region that consists of an ion drift tube. The ion optic assembly has two parts, which are a repeller lens and ground aperture [63]. The potential (V) of the repeller can be raised to a specific level while the ground aperture is kept at ground potential. An electric field is made between the repeller and ground aperture to accelerate the formed ions along a distance through the ground aperture and through the drift region x toward the detector. If two different ions are formed at the same time and location, but they have different charges (z) and different masses M1 and M2 (assume M2 is heavier than M1), then M1 arrives to the MS detector first (Figure 5). If M1 and M2 are cations, the repeller potential is raised to a positive potential, and if M1 and M2 are anions, the repeller potential is set to a negative potential [64].

![Figure 5-TOF-MS. Two ions, M1 is lighter than M2. The two ions were accelerated to a constant energy. TOF is dependent on ion mass to charge ratio, acceleration potential (v), acceleration ion distance (s), and free flight distance (x) [64].](image)

Quadrupole is particularly simple and efficient in ion fragmentation. A quadrupole is a mass filter that consists of four or six cylinders (rods), which are electrodes. An oscillating electric field and a direct current in a specific ratio are applied to these rods [65]. Figure 6 shows the quadrupole components. Ions generated previously go through the empty distance between these four rods, and ions that have stable trajectories will be detected. The other ions, which are lighter or heavier than the required mass/charge ratio, do not reach the detector as they crash the rods. The direct current works to keep ions straight towards a detector while the oscillating field moves the ions up and down depending on the m/z ratio of ions and the strength of the electric field (ratio between oscillating and direct field) [51]. Therefore, ions with higher m/z ratio or lower m/z ratio than the ions that can oscillate without crashing the rods will be detected. For this reason, quadrupole is considered a double filter technique [66]. Recently, triple quadrupole and TOF have been combined to replace the third quadrupole with a TOF analyzer to have higher accuracy for mass and higher resolution of modern TOF instruments [67].
Figure 6- Conceptual diagram of the triple quadrupole mass spectrometer showing each component and its function [66].

Usually, MALDI and TOF are coupled together to measure the mass of an intact peptide. Whereas ESI is coupled with ion trap or triple quadrupole to generate fragment ions [48]. Amino acids sequencing of a peptide can be identified by using Tandem MS (MS/MS). From the first MS analysis, one peptide will be selected to be subject to the second MS analysis after dissociation by collision with an inert gas, such as nitrogen or argon [68, 69]. Nowadays, multiple ions collisions with different energies hit the selected peptide until it fragments. A peptide can be broken by the collision with the two types of ions, which are b and y ions. The b ions involve amide breakage from the N-terminus, while y ions involve breakage from C-terminus [70]. Whether individual MS analysis or tandem MS analysis is performed, some techniques are required to prepare protein/peptide samples before samples are subjected to MS; Enzyme digestion to generate smaller manageable fragments, liquid chromatography to remove interferences or isolate individual peptides from a proteolytic digest, and/electrophoretic gels are the most used techniques before applying MS [71, 72].

Protein can be analyzed by MS in three different approaches called: bottom-up (shotgun), top-down, and middle-down. Figure-7 briefly shows these approaches. Bottom-up refers to the characterization of proteins via their peptides released from proteolysis. Bottom-up is called shotgun when a mixture of protein is analyzed [73]. Top-down is used to analyze intact proteins of up to 200 kDa and identify proteins at a large-scale study (more than 1000 proteins). The potential advantage of top-down analysis is its usage for post-translational modification (PTM) and protein isomorph studies [10, 74]. In the top-down approach, using ESI is preferred over MALDI, because mono-charged protein ions (produced by MALDI) cannot be detected with high resolution. In addition, the protein separation level by chromatography is still less accurate than that of peptide separation; less protein purity negatively affects the MS analysis [75]. Middle-down is a hybrid of bottom-up and top-down; peptide fragments larger than those analyzed by bottom-up are used, which minimizes the peptide redundancy of an the protein of interest. At the same time, large peptide fragments confer similar advantages to those of top-down technique in terms of gaining further insight into PTM, without the need to deal with the challenges of analyzing an intact protein [10].
Figure 7-Proteomic strategies: bottom-up vs top-down vs middle down. The bottom-up approach analyzes proteolytic peptides. The top-down method measures the intact proteins. The middle-down strategy analyzes larger peptides resulting from limited digestion or more selective proteases. One or more protein or peptide fractionation techniques can be applied prior to MS analysis and database searching [10].

Matrix Effects

One important factor that affects the quantitative ability of a mass detector is ion suppression, which is caused by sample matrix, co-eluting compounds, and cross-talk [76]. Matrix effect occurs when molecules have an impact on the compound of interest that change the ionization efficiency of the electrospray interface [77]. King et al. [78] indicated that loss of net charge of an analyte during the gas phase reaction is not the only factor causing ionization suppression. The change of droplet solution by nonvolatile solute is the main reason behind ionization suppression in electrospray ionization. In order to evaluate matrix effect in MS analysis, three steps are required that involve internal standards. An internal standard is either a compound that differs on the sample ingredients [79] or an interesting compound and, in this case, it should be an isotope-labeled compound [80]. First, a calibrator of an internal standard is directly injected in the mobile phase. Then, the same amount of the internal standard is added to the sample after being extracted. Lastly, the same amount of the internal standard is added to the sample before extraction. The recovered amount of the extracted sample and the sample before extraction are compared with the internal standard (considered 100%) that was added directly to the mobile phase to determine the recovery percentage and subsequently the matrix effect of the sample [81]. The matrix effect is considered to be more present (less single responses of electrospray) when other organic compounds exist with the organic analyte of interest.
[82]. Therefore, removal or minimization of the matrix effect happens when two approaches are followed, which are improving sample extraction and improving separation method (LC) [77].

The recovery percentage, matrix effect percentage (% matrix effect = (peak area of analyte extracted from plasma/peak area of analyte spiked in blank plasma extract) ×100), and extraction yield percentage (% = (peak area of analyte extracted from plasma/peak area of analyte spiked in blank plasma extract) ×100) of ten different types of antiretroviral in plasma were determined. The amount of analyte that was inserted directly in the mobile phase, which is considered as 100%, the analyte added to the extraction process, and the analyte added to the sample matrix were determined by using HPLC-MS/MS. The results showed that the minimum recovery percentage was 91% for etravirine antiretroviral type, while the maximum was with Atazanavir antiretroviral type (114%). The maximum matrix effect percentage was with etravirine antiretroviral type (89 ± 11%), and the highest false positive effect was with maraviroc antiretroviral type (114 ± 9.5%); some antiretroviral types showed 100% effect with a standard deviation ≈ 10. The closest results to 100 were with extraction yield percentages, which were between 95% and 104%, which refers to a minor effect of the extraction step on the antiretroviral analysis [83].

**Pure Protein/Peptide Analysis by MS**

Protein identification by MS is based on the matching of observed peptide masses with previous peptide masses calculated from a sequence database. When there is an exact matching, the analysis is reliable. However, some of the analyzed peptides are not identical, but homologous, so they are ignored [84]. For this reason, many of studies are performed on the tryptic peptides to enhance MS analysis. Clauser et al. [85] used MALDI-TOF-MS with delayed extraction operated in the reflector mode to improve MS resolution. Delayed extraction or time-lag focusing is used to improve MS resolution by applying high voltage (25 kV) accompanied with extraction delay (100 ns) to enhance mass accuracy to about 10 ppm by alleviating the ion energy spread. This will enhance MALDI, which is the plauging of this problem. The results showed that mass accuracy was achieved to ± 0.5-5 ppm, which is necessary to distinguish the elemental composition of a peptide. It is possible to match homologous proteins that are more than 70% identical to the protein being analyzed that is found among species in the same phylogenetic tree (for example, mammals). Michalski et al. [86] described a novel version of Orbitrap analyzer combined with a linear ion trap mass analyzer (two mass analyzers) to characterize proteins and proteomes. An Orbitrap analyzer consists of an outer barrel-like electrode and a central spindle-like electrode. Originally, Orbitrap was developed in three ways; first, the line of sight of the ion bath was blocked in order to achieve a robust ion transfer operation. Second, the tandem MS acquisition speed of the dual cell linear ion trap was increased to more than 12 Hz. And, most importantly, the resolving power of the Orbitrap analyzer was increased two folds for the same transient length by using a compact and high-field analysis, which could double the observed frequencies. The original ratio between the two Orbitrap electrodes is 2.5, and it is reduced to 2 to allow an increase in the frequency to 1.8 folds in Michalski et al. [86] study. Tryptic peptides were analyzed by LC-MS/MS using this combination. The results showed a fourfold improving of the resolving power, which provides a ready isotope resolution in the bovine serum albumin mass range. Isotope-coded affinity tags (ICAT) is an approach to quantify proteins (in the microsomal fraction of HL-60 cells). The quantification of the protein is based on three functional elements, which are a specific chemical reactivity, an isotopically coded linker, and an affinity tag [14]. Gygi et al. [87] and Smolka et al. [88] used ICAT to enhance both the accuracy of quantification and sequence identification of protein, in the first study, and to enhance the quantitative analysis in the second study. In Gygi et al. [87] study, six different commercially purchased proteins were used in two concentrations and tagged to light and heavy isotopes. A synthesized biotinamide isotope was used and tagged to different proteins after disulfide bonds of each protein were reduced. The independently produced cysteiny1 group was biotinylated with a fivefold molar excess of ICTA reagent. The excess of ICTA was removed by gel filtration, and the proteins were digested with trypsin overnight at 37 °C. The tagged cysteine-containing peptides were isolated by avidin affinity chromatography and analyzed by μLC-MS/MS. The peptides tagged with light and heavy isotopes differ in mass, which can determine the relative quantities as well as the sequence identities in a single automated operation. Peptides tagged with heavy and light isotope pairs work as the ideal internal standard for accurate quantification because isotopes are chemically identical. In addition, sequencing of 5-25 amino acids of a protein is sufficient to identify the protein. In Smolka et al. [88] study, producing proteins tagged
with ICTA yielded almost the same results as those by Gygi et al. [87] study that depended on cysteine groups. The authors considered each cysteine group tagged with ICTA, so they computed labeled peptides by adding 442.2 Da (isotope weight) to each cysteine present in the sequence. The study was considered as a useful technique in the quantitative application of proteome analysis.

Mass spectrometry can be modified by improving the programs used to analyze the MS data. Two problems are frequently facing researchers that work with data analysis of tandem MS during high-throughput protein. First, it is difficult to quickly shift when large amount of data is presented to be identified because of the poor signal or contaminants, which can be ignored. Second, it is important to figure out incorrect data matches (false positives). Taylor and Johnson [84] tried to overcome these difficulties by using automatic de novo sequencing by a computer program called Lutefisk, and the sequence candidates obtained were searched in a database program called CIDentify to identify variants of known proteins. The first step in the de novo peptide sequencing algorithm imported is either raw profile data, which facilitates the analysis or makes a centroid list of ion masses and intensities, which extract the average weight that is determined by MS resolution. For triple quadrupole data, whose fragment ion resolution usually varies, the program determines the resolution by individual spectra. While, ion trap and qTOF spectra have constant fragment ion resolutions. A small set of ions (30-60 ions, depending on peptide size) is selected for sequencing analysis. The next step is to convert ions into corresponding b-type ion masses as a graph (sequencing graph). The accuracy of this program is increased to either 0.1, 0.01, or 0.001 on each node position compared with the previous work, where the accuracy was 1. Many tryptic peptides were used to detect the validity of the program, and the results showed that the ranks of the correct sequence to the total number of the sequence candidates were different. Some of the ranks were 1:1 and others were 2:10, 1:5, 1:2 etc. The authors tried to identify peptides resulting from non-consensus protease, and they could not identify them unless they used a partially correct sequence that most match with casein. Strohalm et al. [89] highlighted that most of the software of the MS was provided by vendors, and that the programs were often instrument-dependent. The authors did not create a new algorithm, instead, they made a unique combination of different utilities. The mMass program was used for fast computing of mathematical tasks and to speed up the drawing of mass spectra data. The core library (named mspy) consists of many modules that cover a wide range of data processing and provides import functions of data formats, baseline correction, charge determination, deisotoping, and smoothing. By redesigning and improving many programs, they could cover a wide range of processing tasks, such as isotopic pattern modeling to allow accurate data validation. They did not apply their programs on a real tryptic peptide analysis.

Another approach that can improve MS analysis is enhancing peptide separation before being subjected to MS. Two-dimensional liquid chromatography is utilized from two different stationary phase bases in order to enhance protein separation before it is subjected to the MS. Multidimensional chromatography coupled with tandem MS represents a promising method for proteome analysis. The sensitivity of LC-MS/MS is quite dependent on the purity of a peptide eluted from a column, which is affected by the amount of sample loaded and the slope of linear length [90]. Optiek et al. [91] used two dimensional liquid chromatography techniques, which are size-exclusion chromatography (SEC) and RPLC to separate protein mixture resulting from the lysis of E. coli cell and to separate the protein produced by this bacteria. The length of SEC was 2.4 m, that was divided into 8 SEC columns to enhance the separation, followed by two RPLC columns. The 2D liquid chromatography was quite similar to the 2D gels in terms of the analyzed quantity. After LC steps, the fractions were analyzed by 96 well microtiter plates, and the interesting fraction was further analyzed by MALDI-TOF-MS or ESI-MS depending on sample concentration. The efficiency of the system was determined by using BSA as a standard protein, with 80% recovery. The BSA loss could be absorbed to the walls of the fractions collector or binding to the column. There was no BSA detected on the subsequent blank injection. Also, tyrosine kinase and β-lactamase inserted into E. coli can be identified by the system, in addition to a number of the original E. coli proteins. Peng et al. [90] also used 2D-LC with tandem MS for large scale protein analysis that used 1 g of the expressed protein of a yeast (S. cerevisiae). A large number of yeast peptides (26815) was identified and matched the typical yeast peptides, while 7537 peptides were found to be unique by using trypsin digestion, strong cation exchange (SCX), reversed phase (RP) chromatography, and tandem MS with more than 162000 MS/MS run in total. Figure-
explains the experiment design of the identification method. The large dataset was presented, which allowed the determination of false-positive rate, which was reduced to less than 1%.

**Proteomic technology** is used to identify and report protein biomarkers that are potentially useful for diagnosis and prognosis of several diseases [92]. The concentration of biomarker proteins also gives insights on a specific biological process or disease [93]. Su et al. [92] identified urinary proteins of patients with sepsis (a fatal disease, if not controlled, causing lasting and excessive inflammation that can stimulate moderate, severe, and even multiple organ dysfunction) to pinpoint biomarker proteins that enable the discovery of the disease during early stages.

**Figure 8**-Yeast proteome analysis by using a combination of two-dimensional chromatography and MS/MS. One gm of whole yeast protein was used [90].

Isobaric tag (pairs of masses and balances that give the same weight eventually; a heavy mass with a light balance) for relative and absolute quantitation (iTRAQ) coupled with LC-MS/MS were used to identify the proteins of 30 patients. Another 54 hospitalized patients with sepsis were included in the study, and differentially expressed proteins were verified by Western Blot. Proteins (232) were identified, seven of them were associated with the biological process of lipid homeostasis. They concluded their work providing insights to the mechanisms of sepsis disease by following protein types in the patient’s urine. Hem et al. [94] also tried to understand a parasite’s (Leishmania donovani; human pathogenic protozoa) pathways via determining their proteins by using LC-ESI-MS/MS analysis. The labeling technique iTRAQ was also used in addition to treatments with phosphates. An affinity chromatography was used to clean-up and enrich phosphorylated peptides in order to enhance the protein detection by using a more purified form of protein of interest. Phosphopeptides (157) were identified covering 181 unique phosphorylation sites. These identified peptides included six functional categories corresponding to the biologically functional proteins. Adkins et al. [93] identified 490 proteins from blood serum of a healthy female donor in order to recognize serum proteins to facilitate the identification of protein biomarkers of diseases. Immunoglobulins were removed by using affinity adsorption chromatography in order to clean up the sample and, as a result, to enhance the protein identification by reducing interferences. The remaining proteins were then digested with trypsin before using SCX-RP-LC-ion trap-MS. The identified proteins were 3-5 times more than those in a previous blood-derived study, where 58 proteins were recognized, among which 51 were re-identified in Adkins et al. [93] study. Siqueira et al. [95] investigated pellicle proteins from acquired enamel pellicle (AEP), which is a thin protein film formed on the teeth because of the effect of saliva. The main challenge of identifying pellicle protein is the small amount of the protein (0.5-1 µg) on the tooth surface. AEP was collected from three human donors for three days; the samples were collected using...
dry collection strips (0.5 cm * 1.0 cm electrode wick filter paper). The proteins were transferred from strips into SDS gel by an electric field force in order to facilitate protein digestion. After trypsinization, peptides were subjected to nanoscale LC-ESI-MS/MS. Pellicle proteins (130) were identified, while 81, 89, and 84% of the proteins overlapped between subjects 1, 2, and 3, respectively. This study provided insights into pellicle protein composition, which shows a high level of consistency among subjects.

Allergenic proteins are usually detected by enzyme-linked immunosorbent assay (ELISA) and other immunological methods. Ovalbumin is an allergic protein responsible for Immunoglobulin E (IgE) -mediated egg allergy. Detection of ovalbumin in food is critical to set a food safety screen. MS techniques were used to determine ovalbumin in different solutions and foods [14]. Ovalbumin levels in a buffer solution (4% w/v), egg white, whole egg, and spiked pasta with egg white, before and after a heat treatment, were identified in order to determine protein allergy. Ovalbumin was determined by using two methods, which are LC-ESI-MS and ELISA kits for comparing results. The MS results showed that the maximum coverage (50%) was achieved with the non-heated ovalbumin solution, while the minimum coverage of the non-heated sample was 20% for the raw pasta. The coverage of ELISA was different depending on the used kit; some coverage values were higher than 100% (false positive) and others were 24%. The level of ovalbumin was higher in the non-heated samples (p < 0.05) compared with the heated ones. Ovalbumin detection in pasta was affected by the matrix and the heating process for both used methods. Prandi et al. [13] identified the marker peptides so as to detect the contamination (adulteration) of durum wheat and common wheat. Flour and ground wheat samples were used to identify the biomarker peptides following digestion with trypsin and chymotrypsin. The peptide matrix was analyzed using LC-ESI-MS and LC-ESI-MS/MS. The authors identified one peptide marker, that was coded in the DD gene, only in the common wheat. A second peptide marker was found in both types of wheat. Lagrain et al. [75] attempted to isolate and identify high molecular weight glutenin subunits (HMW-GS) from flour in order to reduce the high complexity of a reduced glutenin mixture. Glutenin is an important protein that influences wheat quality and the composition of HMW-GS may vary to up to 60% in different qualities of bread flour. To identify HMW-GS, RP-HPLC-ESI-qTOF-MS was used after some preparation steps. The preparation steps included extraction of wheat protein and evaluation by SDS-PAGE, followed by RP-HPLC and ultraviolet detection before applying MS. The method provided direct determination of HMW-GS composition and allowed further top-down proteomics of HMW-GS along with the entire wheat glutenin fraction.

Future Trends

Although different methods have emerged to analyze and quantify peptide/protein and complex proteome samples, significant improvements are required, particularly for the quantitative analysis. Bantscheff et al. [96] stated that the quantification of proteomics using MS is still not competent enough to be used in biological applications. With regard to the software programs, several computer programs are used to analyze the MS data, and these programs utilized early interpretation data. However, with high-throughput protein analysis, two problems emerged that need to be solved. The quick shift of identifying those huge spectra is difficult due to poor signal or contaminants. Therefore, the unrecognized data types are ignored. Second, it is important to figure out incorrect data matches (false positives) [84]. For the matrix effect, although effects of suppression or enhancement of matrix were determined, the reasons behind these different single responses were not determined yet. In general, a combination of different effects (analyte concentration, molecular weight, structure, and co-elute analyte) was considered to be the reason behind the suppression or enhancement of the single responses [97]. Therefore, knowing the actual reason will provide insights to enhance the quantification and identification of proteins.

Conclusions

Mass spectrometry is an essential technique in the proteomics field, so far being considered as the most commonly employed because of its accuracy in the quantification and identification of peptides/proteins compared with all other proteomics techniques. However, MS needs more modifications to identify and quantify peptides/proteins accurately. Sample preparation and isolation, instrumentation, software programs, isotope attached compounds, and other factors are required in order to progress and develop proteins/peptides analysis by MS. More accurate protein analysis will make a revolution in the biological sciences and a significant enhancement in human health care.
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