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Strategies for Protein Separation

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1. Introduction

The proteome of a cell or tissue depends on cellular and environmental conditions, showing a dynamic system subject to large variations. To study these large changes of variability and quantity, proteomics has emerged, providing techniques dedicated to global characterization of all proteins simultaneously. The expectation is that this information will produce new insights into the biological function of proteins in different physiological states of a cell or tissue.

The proteome has a dynamic and complex nature that is the result of many post-translational modifications, molecular interactions, and a variety of proteins arising from alternative mRNA splicing. With this in mind, the number of modified and unmodified proteins found in any biological system is much bigger than the number of genes (Anderson et al., 2004), which is why mRNA expression may not correlate with protein content (Rogers et. al, 2008). In addition, not all proteins are expressed in the same or similar level in the proteome. For example, the enzyme Rubisco comprises 30–50% of leaf proteome (Feller et al., 2008), which is a big issue in the proteomic assessment of low-abundance proteins. In fact, the majority of proteins are in the low-abundance level. To overcome these challenges, the proteome must be fractionated for effective detection and quantification by mass spectrometry (MS). Consequently, the analysis of proteins on the large or small scale is dependent on separation methods.

As the ultimate goal in proteomics is to resolve all individual proteins in the cell, although it is quite difficult to find a separation method that could accommodate the diversity of proteins equally, protein separation methods directly affect the achievement of reliable results. Such methods are based on the physical or chemical properties of different proteins, such as their mass or net charge.

The combination of sequential methods exploiting different properties can provide high-resolution analysis of very complex protein mixtures. Then, current analytical strategies can reach different levels of resolution depending on the platform used. Two-dimensional gel electrophoresis (2DGE) and multidimensional liquid chromatography (MDLC) are the two methods that dominate the separation steps in proteomics. The differences of each strategy are basically related to sensitivity, automation, and high-throughput possibilities. In this chapter, the limitations and principles of these techniques will be discussed.

2. 2D-PAGE: Principles, advantages and limitations

The 2D-PAGE (two dimensional polyacrylamide gel electrophoresis) was developed by Patrick H. O'Farrell who successfully combined two known electrophoresis methods, isoeletric focusing (IEF) and sodium dodecyl sulfate electrophoresis (SDS-PAGE) (O'Farrell, 1975) with the objective of resolving more complex proteomes. The author was brilliant in his idea of combining both techniques once now proteins could be separated by two non-related properties given a uniform distribution throughout the gel. Surprisingly, the paper "High Resolution Two-dimensional Electrophoresis of Proteins" was firstly rejected by the JBC (Journal of Biological Chemistry) journal because of its "speculative character", as pointed out in the commemorative issue of the JBC (2006), but the power of 2D gel electrophoresis in resolving proteomes had already spread rapidly. Although the combination made by O'Farrell had immediately caused great impact on proteins separation, its commercial application in proteomics become possible only after a technical modification that made the 2D gel electrophoresis reproducible. In the mid1980s, was introduced to the 2D-PAGE system, commercial strips with immobilized pH gradients (IPG strip) and instruments for IEF (isoelectric focusing) (Bjellqvist et al., 1982) and, since then, the 2D-PAGE assume a central role in proteomics. Together, the 2D-PAGE and mass spectrometric techniques provided the characterization of thousands of proteins in single gels.

2.1 Principles of 2D-PAGE

To perform proteins separation, the two dimensional electrophoresis uses sequentially two non-related physical proprieties. In a first dimension, proteins are separated owing their migration in an immobilized pH gradient. Then, in a second dimension, proteins that occasionally took the same migration point after the first separation could now be separate in the polyacrylamide gel, according to their molecular weight, what guarantees to this technique a greater resolution power than achieved in one dimensional electrophoresis. Protein separation can be achieved as low as 0.1 isoelectric point (pI) unit and 1 kDa in molecular weight (MW) (Figeys, 2005). The spots visualized in a second dimension gel are unique proteins or simple mixture of proteins depending on certain factors that can influence technique resolution. To improve resolution, proteins should be completely denatured, reduced, disaggregated from protein complex and solubilized to disrupt macromolecular interactions (Chevalier, 2010).

In the 2D-PAGE protocol, preparation of protein samples is a fundamental and determining stage in electrophoresis efficiency. Usually, to solubilize samples, buffers containing chaotropic agents (urea and/or thiourea), nonionic or zwitterionic (CHAPS or Triton X-100) detergents, reducing agent (DTT) and proteases and phosphatase inhibitors are used. The chaotropic agents will act in the non-covalent macromolecular interactions, interfering in hydrophobic interactions; surfactants (CHAPS and Triton X-100) will act synergistically with chaotropics preventing the adsorption or aggregation of hydrophobic proteins which after the action of thiourea will have their hydrophobic domains exposed; the reducing agent will reduce protein disulfides breaking up intra and inter molecular interactions and proteases and phosphatase inhibitors will avoid modifications in the proteome. Also to optimize proteases and phosphatase inhibition, diluted TCA or TCA-acetone can be used in the solubilization process. One important aspect in solubilization process is to avoid salts accumulation through dialysis or precipitation. Salts can migrate through the pH gradient in

the IPG strip and accumulate in the ends, inducing water accumulation and electric current reduction, what interferes in the focalization process.

The first dimension in 2D-PAGE, also called isoelectric focusing (IEF), is performed in acrylamide gel strips with immobilized pH gradient (IPG strips). The gel in the strip is formed through acrylamide polymerization with amphoteric acrylamide monomers named immobilins. Immobilins with different pKa are added to the acrylamide mixture and after gel polymerization; immobilins are immobilized in the strip generating the pH gradient, that's why strips used in IEF are called immobilized pH gradient or IPG strips. The IPG strips are commercially available in many pH ranges such as 6–9, 6–11 or 7–10. They are sold dried and should be rehydrated to be used. In this process, the rehydration solution must be composed by a commercial mixture of carrier ampholytes containing molecules corresponding to all pIs (isoelectric points) in the strip pH range and by the solubilized protein sample to be separated. Ampholytes act as good buffering agents next to their pIs, assisting proteins in the mixture to migrate in the gel.

Isoelectric focusing like the whole electrophoresis process is based on the migration of charged biomolecules under an electric field. The separation of a protein mixture in a pH gradient occurs because proteins are amphoteric molecules and thus can present negative or positive charges in their ionized groups depending on the pH medium. When an electric current is applied, proteins migrate in the gel while the balance between their charges is positive or negative until the difference between charges became equal to zero (isoelectric point – pI), in this point protein migration ceases and protein get focused. Proteins positively charged, i. e., the ones those are in the strip region where pH value is lower than their pI, keep migrating directly to the positive pole until reach their pI. In the other side, proteins negatively charged, i.e., the ones those are in the strip region where pH value is higher than their pI, keep migrating directly to the negative pole until reach their pI. Focusing process can last from 12 to 20 hours.

After IEF ends, the strip containing focused proteins must be equilibrated with the anionic detergent sodium dodecyl sulfate (SDS) solution that denatured these proteins and forms negatively charged protein/SDS complex. The amount of SDS linked into the protein should be directly proportional to its weight, thus proteins that are totally coupled to SDS will migrate in polyacrylamide gel (SDS-PAGE) only due to their weight. Other reagents in the reaction include Tris-HCl buffer, urea, glycerol, DTT, iodoacetamide and bromophenol blue.

The second dimension is performed by placing the IPG strip above and in direct contact with the gel in a system composed by two spaced glass. An electric current is applied and proteins migrate from the strip to a second dimension where they are solved due to their molecular weight. In the second dimension, gel can be heterogeneous: with a superior phase or stacking gel with acrylamide 6% and with an inferior phase or resolution gel containing 12 to 15%. In some cases, gel can be homogeneous with acrylamide 13% (Görg et al., 2000). The second dimension can be performed vertically or horizontally, but only the horizontal systems allow multiple runs simultaneously. Gels usually run with 1 or 2 W of current in the first hour, followed by 15 mA/gel overnight with temperature regulation (10°C to 18°C) (Chevalier, 2010).

To visualize the spots in the gel dyes visible to naked eye or fluorescent dyes can be used. In both cases, are necessary to fix the gel after the run, using an acid (phosphoric acid or acetic acid) or an alcoholic (ethanol or methanol) solution depending on the chosen dyeing protocol (Görg et al., 2000). Among the non-fluorescent dyes are Coomassie Brilliant Blue, Colloidal Coomassie Blue and silver nitrate which detect spots respectively with minimal protein of 50,

10 and 0.5 ng (Patton, 2002 & Smejkal, 2004). Usually, fluorescent dyes used are SYPRO dyes, Flamingo and Deep Purple. All these three dyes are sensitive enough to detect spots with up to 1 ng protein (Patton, 2002) however, because of their high costs, they are less used.
Once stained, gels are scanned and gel image can be analyzed using specific software available. It's always recommended to reproduce the proteome of the sample in at least three gels, representing identical technical repetitions. Software will search for a representative spots profile among all repetitions and, if desirable, compare this generated profile with others previously obtained. To perform the comparison, normally markers spots are designated in the gel and the position of all others spots is determinate using these spots as reference. It's also possible to estimate the volume of interested spots assigning them relative quantification values when the objective is to compare proteins differentially expressed. Through the use of these tools a proteomic map for a determined sample can be assembled and yet information on protein differential expression can be obtained. Among the software available are: Image Master, Progenesis, PDQuest, Samespots, the Melanie package from the Swiss Institute of Bioinformatics, the Phoretix 2D software from Phoretix and Gellab II from Scanalytics.

2.2 Why to use 2D-PAGE

The 2D-PAGE cannot be used alone to directly identity proteins through the visualization of resulting spots in the gel, even when proteomic maps and sequence information are available to the tested sample. That's because there's a great variation in the proteome of two identical samples, which beings in the protein extraction technique and solubilization and ends in the electrophoresis acrylamide gel. Thus, the identification of proteins in the spots depends on a sequencing stage performed through mass spectrometry (MS or MS/MS). This workflow is usually assumed in proteomics laboratories once can be easily conducted, is applied in many laboratories despite the structure and offers a resolution power enough to detect hundreds of proteins in one gel. Besides that, the 2D-PAGE system is unique about the possibility to visualize the protein profile of a studied sample, allowing immediate comparison with distinct profiles, interesting spots isolation to further studies or yet the enrichment of labeled proteins or specially stained. All these characteristics guarantee its massive application in proteomics characterization. Many other high throughput "gel free" strategies to perform protein separation are available today, but the 2D-PAGE system keep being an important toll in different workflows proposed to protein studies.
The proteomic map assembly is, until the present moment, realized merely by two-dimensional electrophoresis. Gel images are digitalized and made available in data banks what enables the *in silico* comparison between different profiles and the selection of interesting spots. The Japanese Bank containing rice proteomic maps shows for example, more than 13000 characterized spots to different tissues and development phases (http://gene64.dna.affrc.go.jp/RPD/). In humans, there are a great number of studies that report the generation of proteomic maps directed to protein identification that works as biomarkers to reproductive dysfunctions and tumor development (Guo et al., 2010 & Klein-Scory et al., 2010).
Another important contribution of 2D-PAGE system to proteomics is found in the identification and relative quantification of differentially expressed proteins between samples, i.e., differential-display proteomics. Until 1997, this assignment was not easy due to proteome variation in identical samples and gel-to-gel variation frequently observed in the repetition of runs from the same sample. In that time, it was necessary to obtain a great

number of gels to reach the required reproducibility in the "average gel" and then perform the comparison between samples. Since DIGE technique or difference gel electrophoresis (Unlu et al., 1997) was developed, the reproducibility problem of 2D-PAGE gel was bypassed. The DIGE system consists in a modification in the conventional protocol of 2D-PAGE that make possible to analyze in a unique gel three different samples giving the electrophoresis system a "multiplex" character. Samples are pre-labeled with fluorescent markers such as Cy2, Cy3 and Cy5, pooled and separate in a single run. Therefore, in addition to solve the 2D-PAGE reproducibility problem, DIGE system allow the direct quantification of spots from different samples resolved in the same gel and is much more sensitive due to fluorescent dye labeling raising the gel resolution dynamic range up to 1,000 times (Chevalier et al., 2010).

The third sample used in the DIGE system is an internal running control composed by identical aliquots from each experimental sample. The mixture: internal standard and sample 01 and 02 are labeled, pooled and resolved in the same gel what avoid diversion on sample preparation. The internal standard control is normally labeled with Cy2 dye and the other samples with Cy3 and Cy5 dyes. The quantification of each protein is obtained from the signal Cy3:Cy2 and Cy5:Cy2 ratio. The Cy3:Cy2 and Cy5:Cy2 ratios for each protein are then normalized across all the gels in a large experiment, using the Cy2 signals for separate normalization of each protein under survey (Lilley & Friedman, 2004).

A problem in DIGE lies in the hydrophobicity of the cyanine dyes, which label the protein by reacting to a large extent, with surface-exposed lysines in the protein, and lead to removal of multiple charges from the protein. Consequently, this decreases the solubility of the labeled protein, and in some cases may lead to protein precipitation prior to gel electrophoresis. To address this problem, minimal labeling is generally employed in DIGE. In this reaction only 1-5% of total lysines in a given protein are labeled avoiding protein precipitation. Alternatively to minimal labeling the saturation labeling method can be done for Cy3 and Cy5 dyes by reacting to free cysteines in a protein (Shaw et al., 2003). This strategy circumvents the sensitivity problem of minimal labeling but limits the proteome analysis to proteins that show free-cystein residues (Chevalier, 2010).

The 2D-PAGE can also be very useful to identify post-translation modifications (PTMs). The affinity chromatography systems are usually used to enrich samples containing a specific PTM, but the 2D-PAGE visual character enable the direct selection on spots differentially expressed to a specific PTM. Proteins resolved in gel can be for example, specifically labeled to detect phosphorylations or glycosylations, and after visually selected; proteins are excised and identified using mass chromatography.

2.3 Limitations of 2D-PAGE

Technical characteristics related to gel reproducibility and others that can prevent or influence protein resolution are considered the main limitation of 2D-PAGE technique. However, gel reproducibility in 2D-PAGE method is also strongly influenced by own sample biology, what cannot be considered a limitation from the system *per se*. Some limitations associated to 2D-PAGE are pointed and discussed below.

2.3.1 Reproducibility

Protocols available for protein extraction can be applied to various types of biological samples, but the efficiency varies a lot depending on the biological characteristics of the sample. It's much simpler to reproduce the proteome from samples with unique cellular

types, like a cell culture for example, than from samples containing many distinct types of cells or cells in different development phases, like for example, from an onion root. The cellular type also offers challenges to protein extraction and solubilization procedures, with a higher reproducibility to animal cell than to plant cells, which are cover by cellular walls and are rich in membranous compartments (plastids). Besides these intrinsic factors associated to sample biology, the proteome dynamism represents an important variation source in experimental repetitions from a same sample, especially when the objective is to perform a protein relative quantification. The proteome can be promptly modify by degradative pathways or by any of the hundreds post-translational modification that exists. Furthermore, small variations due to differences in the genetic backgrounds between sample repetitions can introduce relevant variations in the proteome.

Another factor that can reduce importantly gel reproducibility in 2D-PAGE is gel-to-gel variations which begin in the sample preparation and extend to focusing process and SDS-PAGE. Even in simultaneous runs that preserve exactly the same experimental conditions the gel-to-gel oscillations are present. To minimize or eliminate this effect, two alternatives are available: built an average gel from at least three replicates or use a multiplex run system (DIGE).

It's also important to emphasize that the reproducibility problem of 2D-PAGE system can restrict the applicability of proteomic maps databank that are being generated when no sequencing information to interesting spots are available.

2.3.2 Resolution

2.3.2.1 Proteins with high molecular weight

Proteins with molecular weight higher than 250kDa cannot be resolved in polyacrylamide gels. To realize this, the ideal is to use an agarose gel followed by an isoelectric focusing (Yokoyama et al., 2009).

2.3.2.2 Low abundance or rare proteins

Low abundance proteins operate in cellular activities of high interest, participating in signal reception, gene activity regulation and in signal transduction cascades. The detection of these proteins is masked in 2D gels by abundant proteins, which depending on the sample can be present in a magnitude concentration up to 12 times higher. That's the case for example, of albumin protein present in plasma samples. One possible strategy to avoid this problem is the depletion of abundant protein through methods such as affinity chromatography (Greenough et al., 2004). This is normally used to plasma sample, but is not yet possible to other many systems. In plant cells, the abundance of ribulose bisphosphate carboxylase/oxygenase (RuBisCo) enzyme mask low abundant proteins and the current used strategy to understate this effect is to reduce sample complexity through the use of IPG strips with overlapping narrow pH ranges (Görg et al., 2000).

2.3.2.3 Hydrophobic proteins and membrane proteins

In 1998, an important paper was published (Wilkins et al., 1998) in which was demonstrated that hydrophobic proteins were almost absent in 2D gels done using urea as the only chaotropic agent in the protein solubilization solution. This information was very valuable once hydrophobic proteins comprise the proteins present in cellular membranes and represent around 30% of total proteome (Molloy, 2000). After this observation, proteins solubilization began to be realized using a combination of higher concentration of urea and

a lower concentration of thiourea, a chaotropic agent much more efficient than urea. The elevated concentration of urea was necessary to solubilize thiourea, which was used in lower concentration, because if in higher concentration, thiourea can interfere in protein focusing process (Molloy, 2000). This modification in the original protocol of protein solubilization resulted in a greater efficiency to solubilize hydrophobic proteins but yet the combination of urea-thiourea cannot keep the proteins in solubilized forms in the aqueous environment necessary to IEF. Other variations in the solubilization protocols combining urea and others nonionic or zwitterionic detergents were suggested, but all resulted in a additional identification of only some membrane proteins spots (reviewed by Rabilloud et al., 2008). It was clear that the solubilization of membrane proteins, mainly those with high hydrophobicity (multiple transmembrane domain), could not be achieved under IEF compatible conditions (reviewed by Tan et al., 2008). The gel systems intent to resolve membrane proteins should use strong detergents for solubilization of this kind of proteins and agents that can add charges to the proteins preventing their aggregation. Such gel-based systems (blue native-PAGE or BN-PAGE, clear-native-PAGE or CN-PAGE, benzyldimethyl-n-hexadecylammonium chloride or BAC, and SDS/SDS or dSDS-PAGE) exclude the IEF resulting in a severely impaired gel resolution (reviewed byTan et al., 2008). The resulting spots are generally composed by a misture of proteins that carry different post-translational modification and/or by complexes of membrane and soluble associated proteins (Rabilloud et al., 2008). Other strategies to detect membrane proteins are available using gel free systems, sample pre-fractionation through subcellular fractionation or affinity purification, and the avidin–biotin technology (Elia, 2008). However, there is still a great necessity of development of protocols that allow the high resolution detection of membrane proteins and the simultaneously detection of membrane and soluble proteins. This is especially important when we are looking for desease responses or physiological phenomena because membrane proteins play key functions in normal development, participating in cellular recognition and signal transduction. Identification of altered membrane proteins could lead to the discovery of novel biomarkers in the disease diagnosis (Adam et al., 2002 & Jang & Hanash, 2003) and targets to therapeutic approaches (Bianco et al., 2006).

2.3.2.4 Basic proteins

The basic proteins represent approximately one third to half of total cellular proteome. Among them, are ribosomal proteins and nucleases which exhibit pI superior to 10 and because of this reason are poorly resolved in pH ranges available for alkaline proteins (pH ranges 6–9, 6–11 or 7–10). This 2D-PAGE limitation began to be settle with the commercialization of IPG strips comprising pH ranges of 3-12, 6-12 and 9-12 which are successfully used in the resolution of strongly alkaline protein, with pI superior to 11 (Drews et al., 2004 & Görg et al., 1997).

3. Principles of liquid chromatography in proteomics

Chromatographic separation methods have been applied in different laboratories around the world to decipher the many complex problems in industry and science, involving, for example, amino acids and proteins, nucleic acids, carbohydrates, drugs, pesticides, etc. This method separates the components of a mixture by the distribution of these components into two phases, where an immiscible stationary phase remains fixed while the other moves through it. The sample components more strongly connected to the stationary phase move

very slowly in mobile phase flow, while those linked more weakly to the stationary phase move more quickly. This process results in differential migration of these components.

The main criteria for classification of chromatographic separation are related to the separation mechanism involved and the different types of stages used. Thus, the physical form of the system classifies the general technique as planar or column chromatography. In the former, the stationary phase is prepared on a flat surface, while in the latter, the stationary phase is arranged in a cylinder. In Gas Chromatography (GC), the mobile phase is an inert gas that does not contribute to the separation process, whereas in Liquid Chromatography (LC), the mobile phase is a liquid that can interact with the solutes, so their composition is very important in the separation process. Supercritical Fluid Chromatography (SFC) utilizes a substance with temperature and pressure higher than the critical temperature (Tc) and critical pressure (Pc) proper to fluids, with the advantage of having lower viscosity than the liquid while maintaining the properties of interaction with the solutes (Skoog et al., 2006).

The LC techniques may be further divided into classic liquid chromatography (LC) and High-Performance Liquid Chromatography (HPLC). LC utilizes glass columns at atmospheric pressure, and the flow rate is due to gravitational forces. HPLC is the automation of LC under conditions that provide for enhanced separations during a shorter time. It utilizes a metal column and the mobile phase flow rate is due to a high-pressure pump, which increases the efficiency achieved in the separation of compounds, thus making HPLC one of the main techniques used in the separation of proteins and peptides from a wide variety of synthetic or biological sources.

The HPLC equipment comprises a reservoir of mobile phase, which contains the solvents used as the mobile phase to achieve selectivity in HPLC; a pumping system; sample injector; columns; and detectors (Figure 1). The pumping system is required to pump the mobile phase and overcome the pressure exerted by the particles of the column. The major requirements for an efficient pumping system include the ability to generate pressure to

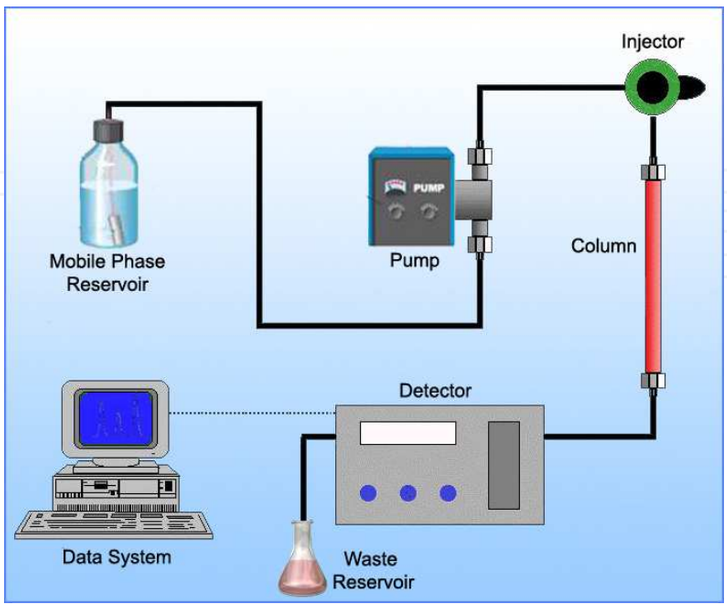

Fig. 1. Scheme of a HPLC system.

6000 psi, with no pulse output; flow rate ranging from 0.1 to 10 mL/min; constant solvent flow (with no variations greater than 0.5%); and corrosion-resistant components. There are two main pumps for HPLC: reciprocal pumps, which are employed in 90% of HPLC systems, consist of a small chamber in which the solvent is pumped by an oscillatory movement of the piston controlled by a motor. Because of this, the flow is not continuous, requiring a shock pulse. Syringe pumps consist of a large chamber equipped with a plunger that is activated by a screw mechanism. The rotation of the screw provides a continuous movement of the mobile phase that is free from pulsations from 0.1 to 5 mL/min. The most common injection system is sampling loops, which allow the introduction of samples up to 7000 psi with excellent precision. These loops can be manual or automated (Oliver, 1991; Meyer, 2010).

In a chromatograph, there are two types of columns: a guard column and separation column. The guard column has a length of 2 to 5 cm and is placed between the injector and separation column, allowing it to retain possible solids that can block the filters of the column and, in some cases, retain materials that can precipitate chemical reactions in the stationary phase. The separation columns (stationary phase) are the heart of a chromatograph, since they are responsible for the separation of the components present in the sample. They consist of a tube of inert material, usually stainless steel, and uniform internal diameter (i.d.), capable of resisting high pressures. They can be classified according to i.d (Saito et al., 2004).

Column designation	Internal Diameter [mm]
Conventional HPLC	3 – 5
Narrow-bore HPLC	2
Micro LC	0.5 – 1
Capillary LC	0.1 – 0.5
Nano LC	0.01 – 0.1
Open tubular LC	0.005 – 0.05

Table 1. Classification of liquid chromatography according to internal diameter (ID) of columns (Saito et al., 2004)

Silica is the most common stationary phase in HPLC because of advantages such as resistance to high pressures and physicochemical properties. Despite these advantages, silica has two limitations: the first restricts its use in a pH range of 2 to 8 because at pH below 2, bonds of Si-O-Si, which compose the silica and are responsible for maintaining the organic groups immobilized on the silica surface, become more susceptible to hydrolysis. On the other hand, at pH above 8, the hydroxyl groups (OH-) can easily react with the residual silanols, promoting silica dissolution that result in low efficiency and peak enlargement. The second limitation refers to the presence of residual silanol groups that can result on the asymmetry of the peak when basic samples are analyzed (Neue, 1997; Oliver, 1991; Meyer, 2010).

The particle structures are classified as porous, non-porous, and pellicular. The porous particles are most often used for HPLC, since it allows for greater surface area for interactions between the stationary phase and the analyte. Non-porous particles allow faster chromatography without losing efficiency because there is no diffusion of the analyte inside

of the particles. However, to keep the sample capacity, it is necessary to use particles with diameters of 1 to 2 μm, as the capacity is 50 times less than that of porous particles. Pellicular particles are constituted of a solid nucleus coated with a thin layer (1–3 μm) of the stationary phase, and they have a good efficiency when analyzing macromolecules due to the fast mass transfer kinetics. The particle shape may be regular (spherical), irregular, or monolithic. The columns packed with spherical particles have a higher resistance to high pressures and good efficiency. The columns packed with irregular particles can have good efficiency when compared to regular particles; however, they have no mechanic stability and can result in higher pressures in the system (Meyer, 2010). Recently, monolithic particles have been introduced in HPLC. They are single pieces of porous silica or a highly intercrossed porous polymer such as polyacrylamide. The skeletons of monolithic particles contain macropores with diameters of approximately 2 μm and mesopores with diameters of approximately 13 nm. Because of those characteristics, they can provide higher flow rates without increasing the pressure, as well as great chemical stability and high permeability (Neue, 1997; Meyer, 2010).

In HPLC, there are different ways to detect the compounds eluting from the column. The ideal detectors are linear, selective and non-destructive and have adequate sensitivity, good stability and reproducibility, and a short response time. However, there are no detectors with all the features mentioned above, so the choice of the detector should be based on objective analysis as well as the type of sample to be analyzed. Liquid chromatographic detectors are basically of two types. Bulk property detectors respond to mobile-phase properties, such as refractive index, dielectric constant, or density. In contrast, solute property detectors respond to properties of solutes, such as UV absorbance, fluorescence, or diffusion current, which are not present in the mobile phase (Skoog et al., 2006; Meyer, 2010). Table 2 shows the major detectors used in HPLC.

Type of Detector	Limit of Detection	Commercial Available
Absorbance	10 pg	Yes
Conductivity	100 pg – 1 ng	Yes
Electrochemical	100 pg	Yes
Element Selective	1 ng	No
Fluorescence	10 fg	Yes
FTIR	1 μg	Yes
Light Scattering	1 μg	Yes
Mass Spectrometers	< 1 pg	Yes
Optical Activity	1 ng	No
Photoionization	< 1 pg	No
Refractive Index	1 ng	Yes

Source: Skoog et al., 2006

Table 2. The most common detectors used in HPLC.

High-performance chromatography supplanted gas phase chromatography because it is more versatile; it is not limited to volatile and thermally stable samples, thus allowing a

wide choice of mobile and stationary phases. Because the mobile phase carries the solutes through the stationary phase, the correct choice of mobile phase is extremely important in the separation process, as it can completely change the selectivity of separations. The solvents used must be compatible with the stationary phase and detector and the high power of sample solubilization. The elution mode can be isocratic or gradient. In isocratic elution, the separation employs a single solvent or solvent mixture of constant composition, and the mobile phase remains constant with time. Gradient elution, in contrast, utilizes two or more solvent systems that differ significantly in polarity. In this case, when the elution process is begun, the ratio of the solvents varies with time, and separation efficiency is greatly enhanced by gradient elution (Skoog et all., 2006).

The major separation modes that are used to separate most compounds are normal-phase chromatography (NP), reverse-phase chromatography (RP), size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), and affinity chromatography (AC).

In **normal-phase chromatography**, the stationary phase is polar while the mobile phase is non-polar. The retention of analytes occurs by the interaction of the stationary phase's polar functional groups with the polar groups on the particles' surfaces, and they elute from the column by addition of the low polarity compound followed by other compounds of increasing polarity (Figure 2). This method is widely used to separate analytes with low to intermediate polarity (Skoog et al., 2006).

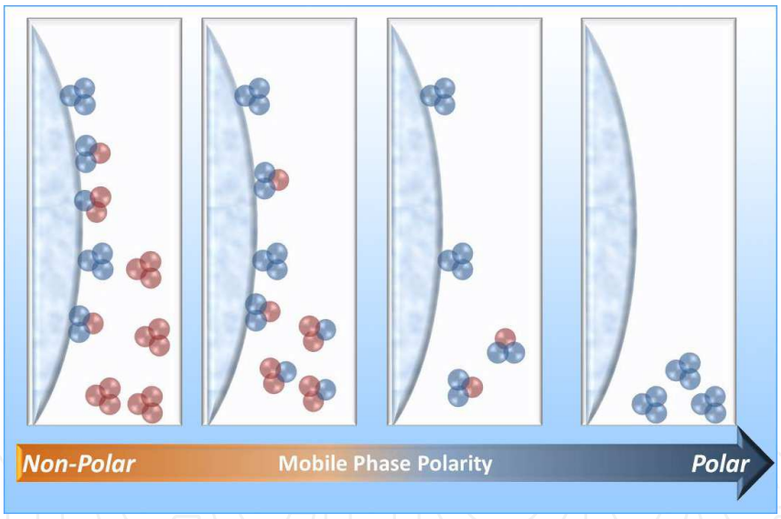

Fig. 2. Diagram of normal-phase chromatography separation. The stationary phase is polar and retains the polar molecule (blue) most strongly. The relatively non-polar molecules (red circles) are quickly eluted by the mobile phase, a non-polar solvent. An increase in mobile phase polarity will move polar molecules through the column.

Reverse-phase liquid chromatography has become a powerful tool widely used in the analysis and purification of biomolecules because of the high resolution provided by the technique. It is considered a very versatile technique because it can be used for non-polar, polar, ionizable, and ionic molecules. In RP-HPLC, the separation principle is based on the hydrophobic interaction between the analytes and non-polar groups bound on the stationary phase. Silica is the most common material used for column packing, which consists mainly of silicon dioxide (SiO_2) and has octadecyl (hydrocarbons having 18 carbon

atoms) and octyl (hydrocarbons having 8 carbon atoms) groups chemically bound to the surface. The mobile phase composition is usually water or a water-miscible organic solvent (methanol, acetonitrile). The analytes adsorbed on the hydrophobic surface remain bound until the higher concentration of the organic solvent promotes the desorption of the molecules from the hydrophobic surface (Figure 3). More hydrophobic analytes are eluted slower than are the hydrophilic analytes (Skoog et al., 2006; GE Healthcare, 2006).

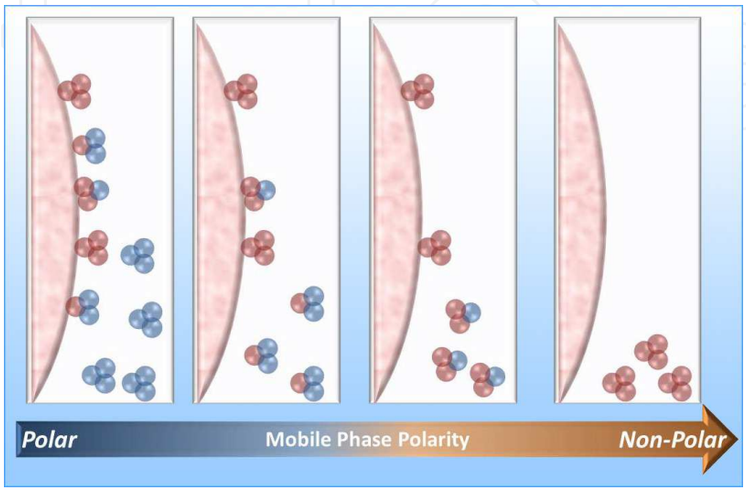

Fig. 3. Diagram of Reverse-phase chromatography separation. The stationary phase is non-polar and retains the non-polar molecule (red) most strongly. The relatively polar molecules (blue circles) are quickly eluted by the mobile phase, a polar solvent. A decrease in mobile phase polarity will move non-polar molecules through the column.

Affinity chromatography is the most specific chromatographic method. The separation is based on specific biochemical interactions such as enzyme-inhibitor, antigen-antibody or hormone-carrier. The stationary phase involves an inert matrix coupled with an *affinity ligand* specific for a binding site on the target molecule. The substance to be purified is specifically and reversibly adsorbed to a ligand, immobilized by a covalent bond to a chromatographic matrix. The samples are loaded in an affinity column containing the specific ligand, and the analyte of interest is adsorbed from the sample, while the molecules which have no affinity for the ligand pass through the column (Figure 4). Recovery of molecules of interest can be achieved by changing experimental conditions such as pH values, temperature, or ionic strength or by adding a stronger ligand to the mobile phase. For success in affinity chromatography, some important points have to be considered, such as finding a ligand specific enough and determining the ideal conditions for safe binding between analyte and ligand, as well as the ideal conditions for the retention and elution of the molecules involved (Skoog et al., 2006; GE Healthcare, 2007; Hage, 1999).

Ion-exchange chromatography (IEC) is based on the charge properties of the molecules. A stationary phase matrix constituted from a porous and inert material contains charged groups that interact with analyte ions of opposite charge. If these groups are acidic in nature, they interact with positively charged analytes and are called cation exchangers; however, if these groups are basic in nature, they interact with negatively charged molecules and are called anion exchangers. As the matrix material, they can be classified as organic (most common) and inorganic, natural or synthetic. Charged groups binding to the matrix

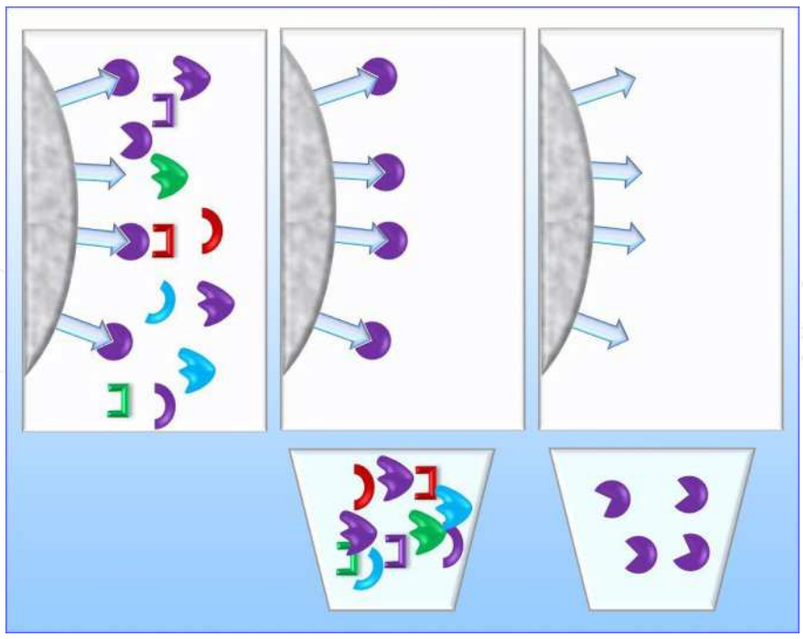

Fig. 4. Affinity chromatography column. The sample is loaded under ideal binding conditions. The target molecules bind specifically to the affinity ligands, while all other sample components, are not adsorbed.

are classified as strong ion exchangers that are completely ionized at a wide range of pH levels, while weak ion exchangers are ionized within a narrow pH range. Thus, weak exchangers offer more flexibility in selectivity than do strong ion exchangers, although the strong ion exchangers are used for initial development and optimization, because binding capacity does not change with pH. For the separation, the column is equilibrated with a start buffer, and then an analyte containing an opposite charge binds to the ionic groups of the matrix, whereas uncharged molecules, or those with the same charge as the ionic groups, are not retained. The adsorbed analyte of interest can be eluted by a gradient of ionic strength, pH values, or a combination of both in the mobile phase. The action mechanism of ion exchanger is shown in Figure 5 (Oliver, 1991; GE Healthcare, 2004; Meyer, 2010).

Size-exclusion chromatography (SEC) is a preparative and non-destructive analytical technique that, unlike other methods, is not based on interactions between molecules and the stationary phase, but on the size of molecules (Figure 6). The column is packed with inert material with pores of controlled size, within the stationary phase, such that the small molecules can enter most of the pores and therefore will be retained the longest time, while the larger molecules cannot penetrate and are kept for a shorter time period. The SEC can be classified according to the mobile phase used in *gel filtration chromatography* or *gel permeation chromatography*. Gel filtration chromatography (GFC) uses an aqueous mobile phase, which may contain organic modifiers or salts to change the ionic force or buffer solutions to change pH. Gel permeation chromatography (GPC) is a method used to separate high polymers, and it has become a prominent and widely used method for estimating molecular-weight distributions. Unlike GFC, GPC uses organic mobile phases such as tetrahydrofuran (THF), toluene, chloroform, dichloromethane, or dimethylformamide (Oliver, 1991; Meyer, 2004; GE Healthcare, 2010).

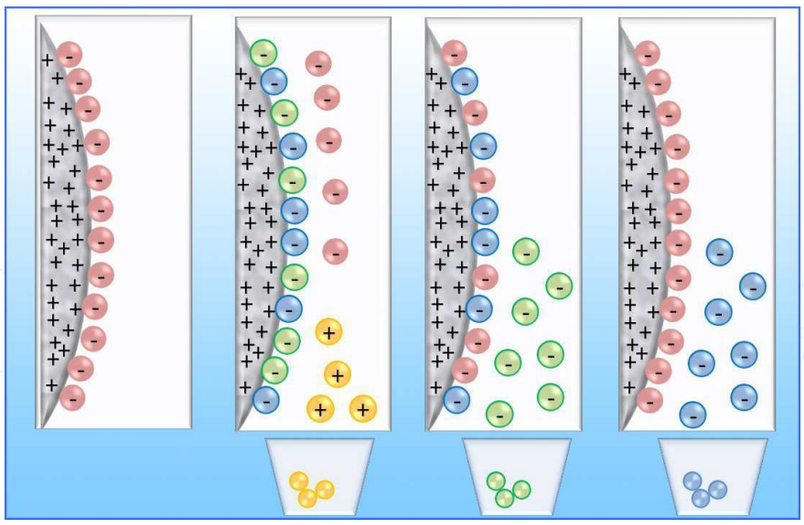

Fig. 5. The principle of IEC Separation. The mobile phase has ions negatively charged (red circles) that binding to the stationary phase (positively charged). The sample containing mixture of positively and negatively charged groups flows through the column. Those analytes containing negative charge are able to displace the mobile phase ions and bind to the stationary phase, while the positives groups (yellow) are eluted. The sample bound in the stationary phase can be eluted by increasing the concentration of a similarly charged species. The analyte that binds weakly (green) in the stationary phase will be eluted by buffer with salt ions at lower concentration. The analyte that binds strongly (blue) in the stationary phase will be eluted by buffer with salt ions at higher concentration.

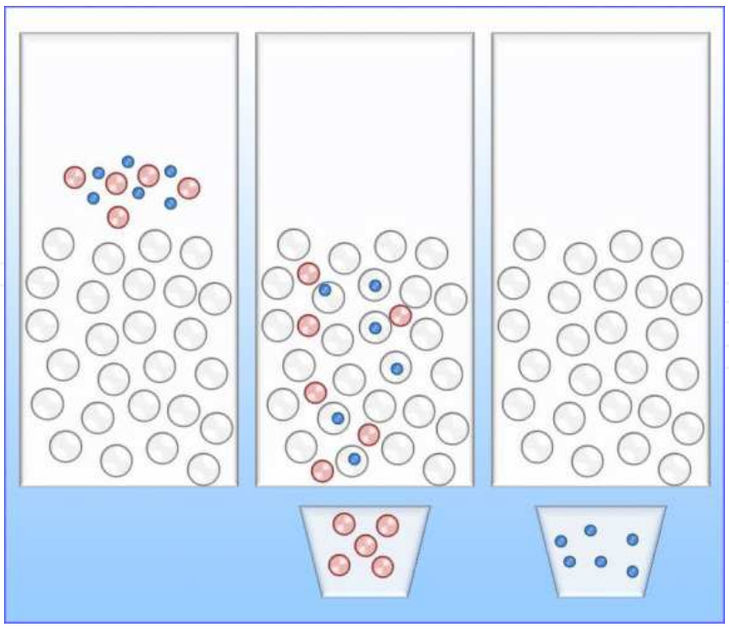

Fig. 6. Molecules smaller than pore will become trapped in the matrix. Those of larger molecular weight will not be trapped but will flow through column. Thus, larger molecules elute first, while smaller molecules are held longer inside the pores and will be eluted last.

3.1 Liquid chromatography coupled to MS

In proteomics, LC can be performed downstream of 2D gels to fractionate peptides from excised spots before MS analysis, upstream of 2D gels to prefractionate the protein sample, or instead of 2D gels as the main separation method in a multidimensional protein identification technology (MudPIT) experiment (item 4). Two strategies for protein identification and characterization by MS currently are employed in proteomics. In the bottom-up strategy, purified proteins or complex protein mixtures are subjected to enzymatic digestion, and the peptide products are analyzed by MS (Andersen et al., 1996; Pandley & Man, 2000) (Figure 7). In top-down proteomics, intact proteins or big protein fragments are subjected to fragmentation during MS analysis (Kelleher, 2004; Siuti & Kelleher, 2007). The major problem in bottom-up proteomics is that many peptides are generated for subsequent mass analysis, so it is not possible to get full protein sequence coverage and protein inference can be a problem. Different proteins can share some peptides, and consequently identification through bottom-up proteomics could be ambiguous. For these reasons, many efforts have focused on separation methods such as multidimensional chromatography to improve sensitivity and resolution. On the other hand, top-down proteomics permits high coverage sequence, thus overcoming one of the most important challenges in bottom-up strategy; however, it is a newer approach with several instrument limitations that will benefit from some hardware MS advances.

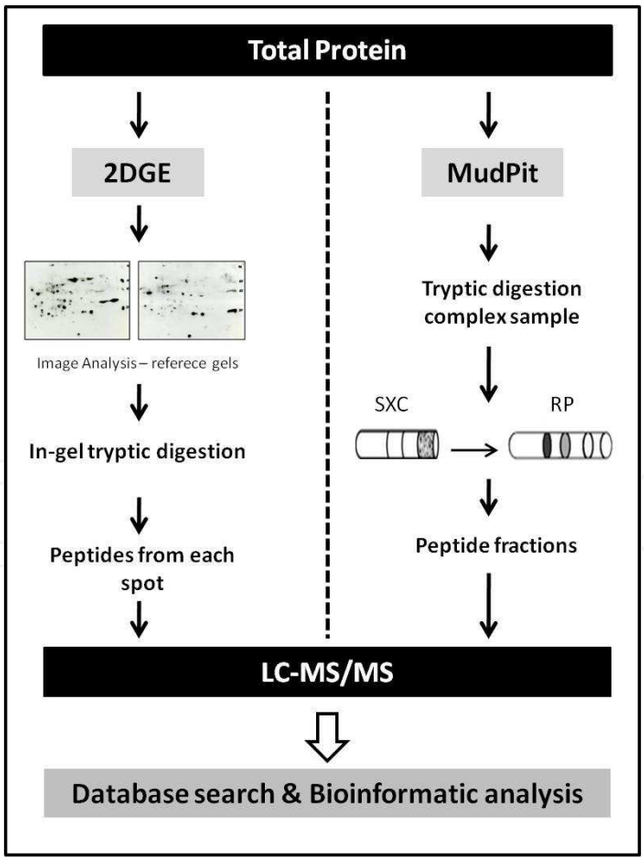

Fig. 7. Schematic representation of separation methods in bottom-up proteomics approaches (Adapted from Agrawal et al.,2008).

Also, the so-called "shotgun proteomics" is applied in a typical bottom-up approach and involves the utilization of HPLC coupled to tandem mass spectrometry (MS/MS) for identification of proteins on a large scale. This platform has been facilitated by the use of MudPIT, discussed below. As bottom-up proteomics is the most mature and most widely used approach for protein identification, in the following section we focus on chromatographic platforms for peptide separation.

4. Multidimensional liquid chromatography in proteomics

As previously mentioned, bottom-up proteomics offers some challenges to be overcome, such as sample complexity and large differences in protein concentration. As we know, these problems are far from being solved by single chromatographic or electrophoretic methods. Basically, two main approaches have been developed to face these difficulties: methods to separate abundant proteins from lower abundance proteins and multidimensional separation methods to maximize fractionation of peptides, thus increasing the proteome coverage analyzed by MS.

Abundant proteins can be a big problem when analyzing complex samples from certain tissues. Some tissues have high percentage (around 50% or more) of some classes of proteins. For example, leaves show approximately 50% of Rubisco (Feller et al., 2008) and the great majority of human blood serum proteome is comprised of albumin, fibrinogen, and immunoglobulins, transferrin, haptoglobin, and lipoproteins (Burtis & Ashwood, 2001; Turner & Hulme, 1970). If these abundant proteins are not removed from the sample, the peptides resulting from these proteins for proteomics analysis will overlap the peptides from the low-abundance proteins. To remove abundant proteins in complex samples, immunoaffinity separation or gel-based fractionation could be good options, although immunoaffinity separation can also remove low- abundance proteins that have any level of interaction with those abundant proteins (Granger et al., 2005).

Sequential chromatographic separations utilizing different chemical or physical properties have improved the assessment of classes of proteins that are difficult to handle in gel electrophoresis. MDLC can reach the same resolution of 2D gel electrophoresis with added advantages like automation, better sensitivity, and increased proteome coverage. MDLC was first described by Giddings in 1984 (Giddings, 1984) as a technique which combines two or more types of LC to increase the peak capacity and selectivity, contributing to a better fractionation of peptides that will enter the mass spectrometer.

The peak capacity is the number of peaks that can be resolved in a given time. To increase the peak capacity, the combination of two or more orthogonal separation dimensions is necessary. In other words, the properties affecting the separation in the first dimension do not affect the separation in the subsequent dimensions; thus, the process simplifies the sample complexity and improves the power of resolution, fractionating more components in a given time.

Many LC combinations have been reported utilizing chromatographic methods like strong cation exchange (SCX), strong anion exchange (SAX), size-exclusion (SEC) and reverse-phase chromatography (Zhang et al., 2007; Hynek et al., 2006; Moritz et al., 2005). Some factors are essential when considering an MDLC approach. The choice of different types of chromatographic columns to reach a satisfactory MDLC peak capacity is the most important point within this strategy. In order to separate a large variety of peptides in high-performance chromatography, the columns used in each dimension have to work with no

correlated properties. Other factors to be considered include the fact that the first column should have a larger loading capacity and have solvent compatibility with the subsequent columns. Thus, this compatibility between dimensions is essential in online systems. The last step of chromatography immediately before MS analysis should be compatible with electrospray ionization (ESI). For this purpose, the reverse-phase (RP) chromatography is frequently used, as it can desalt the samples and it is completely compatible with ESI, thus providing effective resolution and facilitating MS detection.
Several proteomic studies using different multidimensional configurations have been done along these years (reviewed by Gao et al., 2010) Here we point out some examples of the most applicable approaches.

4.1 Ion-exchange and reverse-phase chromatography (IEX-RPLC)

MUDPIT was first introduced by Yates and co-workers (Link et al., 1999; Wolters et al., 2001) using a biphasic column sequentially packed with SCX particles and then with C18 particles for peptide separation prior to MS analysis. Other works reported the use of an SCX column and an RP column connected to perform an online SCX-RP-MS/MS analysis (Tram et al., 2008; Gilar et al., 2005). Besides their orthogonality, SCX and RP columns show mobile phase compatibility between each other and with MS analysis.
In the SCX-RP configuration, peptides from a complex mixture are acidified and applied to an SCX column, in which the elution steps can be done using a salt gradient. Then, a fraction of peptides are absorbed in the RP column, and after washing away salts and buffers, peptides are eluted from the RP column into the mass spectrometer using a gradient of an organic solvent. Finally, the RP is reequilibrated and new fraction of peptides from the SCX column is eluted to be absorbed in the RP column, and the process repeats.
Several studies came out to improve the application of the MudPIT technique. One of the limitations of SCX-RPLC is related to the use of salt gradient SCX separation, resulting in limited resolution and peak capacity for peptide separation. Extensive salt usage could also cause ion suppression, thus reducing MS performance. Second, by using coupled SCX-RP columns, separation on both dimensions has to be performed at the same flow rate, which may sacrifice MS detection sensitivity for low-abundance components. As an alternative approach, offline SCX-RP separation has been used to identify more than 1200 proteins from zebrafish liver (Wang et al., 2007).However, the implementation of offline configurations is not always the best option, as extensive offline sample handling increases the overall analysis time and causes sample loss and sensitivity reduction.
Dai et al. (2005) reported an integrated column composed of SCX and RP where peptides were fractionated by a pH step gradient. The exclusion of salt removal steps could lead to fast 2D-LC separation and MS/MS detection. Some years later, the same research group developed a SCX and SAX combined pre-fraction strategy called Yin-Yang multidimensional chromatography (Dai et al., 2007). Peptides eluted from a SCX column at pH 2.5 were then separated by a SAX column using a pH gradient solution. Subsequently, SAX fractions were analyzed by RPLC-MS/MS. This approach revealed proteins from a broad range of different pH values.

4.2 Reverse-phase-reverse-phase chromatography (RP-RPLC)

The use of RP columns in both dimensions of a MudPIT analysis exhibited high-throughput, automatability, and performance comparable with that of SCX-RP. Despite all successful

studies based on SCX-RP, these approaches are frequently encountering higher complexity samples than can be accommodated by their power of resolution. Accordingly, new strategies have been developed, including the RP-RPLC. Gilar et al. (2005) investigated the use of a pH gradient in a RP-RP platform. The RP-RP approach provides higher peak capacity in both dimensions, and the pH gradient has the most significant impact on the selectivity of this platform. The pH gradient modulates the peptide hydrophobicity during the elution of fractions of peptides.

Another factor that has been increasing the sensitivity of this approach is the integration of a high-pressure system in RP-RP platforms. This was first implemented in the Agilent 1100 2-D liquid chromatographic system and more recently by the 2D NanoACQUITTY system from Waters, frequently used with a pH gradient and two columns of C18 particles. Zhou et al. (2010) revealed that RP-RP fractionation outperforms SXC-RP. It was also demonstrated that the combination of RP-RP systems with the nanoflow format had good impact in the efficiency of electrospray ionization prior to MS/MS analysis.

4.3 Affinity chromatography

Another type of chromatography largely used in proteomics is affinity-based. The main usage of this technique is to enrich post-translationally modified (PTM) peptides or proteins subject to MS analysis. Due to their low stoichiometry and dynamic modification patterns, PTM materials have to be enriched in complex mixtures prior to MS analysis. Affinity chromatographic columns can be used online or offline to RP columns and the mass spectrometer.

4.4 Enrichment of phosphopeptides or phosphoproteins

In phosphoproteomics, the interest focuses on the identification and quantification of phosphorylated proteins or peptides. With this purpose, several selective enrichment methods are applied to increase the content of phosphopeptides or phosphoproteins in complex mixtures, thus preventing the suppression of ion signals by unphosphorylated molecules.

Immobilized metal affinity chromatography (IMAC) is one of the most popular techniques employed in phosphoproteomics. IMAC contains immobilized positive ions (Fe^{3+}, Ga^{3+} and Al^{3+}) interacting with the negative ions of phosphate groups. The elution of the interacted molecules makes the enrichment possible. Tsai et al. (2011) showed a pH/acid-controlled IMAC protocol for phosphopeptide purification with high specificity and lower sample loss. They characterized over 2,360 nondegenerate phosphopeptides and 2,747 phosphorylation sites in the H1299 lung cancer cell line, showing that a low pH buffer increases the specificity of IMAC for phosphopeptides. The buffer composition was directly associated to the specificity and selectivity of the IMAC technique (Tsai et al., 2008; Jensen et al., 2007).

Another important enrichment technique widely used is the metal oxide affinity chromatography (MOAC). MOAC uses a principle similar to that of IMAC, incorporating metal oxides, such as titanium dioxide TiO_2, zirconium dioxide ZrO_2, or aluminum hydroxide $Al(OH)_3$. Metal oxides tend to have higher selectivity for phosphopeptides, making it easier to trap them in the column (Zhou et al., 2007). During the last few years, TiO_2 has emerged as the main MOAC-based phosphopeptide enrichment method (Pinkse et al., 2004). The principle is the same as that of IMAC; however, when loading peptides with DHB (2,5-dihydroxybenzoic acid) non-specific binding is reduced, thus increasing the selectivity of TiO_2 (Larsen et al., 2005).

4.5 Enrichment of glycopeptides or glycoproteins

Glycosylation is the most complex PTM presented in eucaryotes. Like other PTMs, glycopeptides are often very minor constituents compared to peptides derived from proteolytic digestion. Therefore, enrichment of glycoproteins or glycopeptides is essential in a glycoproteomics study (Ito et al., 2009). The lectin affinity approach is the most common tool for glycoprotein/glycopeptide enrichment. Lectins are sugar-binding proteins that are highly specific to sugar moieties. For example, concanavalin A (conA) is a mannose-binding lectin largely used to study N-glycosylated proteins, while lectin from *Vicia villosa* (VVL), which preferentially binds to alpha- or beta-linked terminal GalNAc, has a different preference. All lectins have sugar specificity, and therefore serial lectin affinity columns (SLAC) have been developed to reduce the complexity of proteolytic digests by more than one order of magnitude. SLAC can be used to study O-glycosylation proteins, which are difficult to access because lectins for studying them are not specific enough. Jacalin, a lectin, is relatively specific for O-glycosylation but has the problem that it also selects mannose N-glycans. This problem can be overcome by first using a ConA affinity column to first remove mannose, and then using Jacalin columns. When used in a serial configuration, O-glycosylated peptides can be accessed (Durham & Regnier, 2006).

4.6 LC-MS repeatability and reproducibility

First of all, it is important to understand the difference between repeatability and reproducibility. The first, represents the variations in the measurements on the same sample, made in the same instrument and by the same operator (Bland & Altman, 1986). The second, is the variation observed for an analytical technique when operator, instrumentation, time, or location is changed (McNaught & Wilkinson, 1997). So in proteomics, it is possible to calculate variations in results from run to run estimating the repeatability of the analytical technique or estimating the reproducibility between different laboratories in completely differently instruments. These measurements are imperative to the clinical utility of biomarker candidates and must be expected (Baggerly et al.,2005).

During proteomics analysis there are many potential contributors to variability that can compromise the approach repeatability and reproducibility. The variations can begin in sample collection, specimen processing techniques, storage, and instrument performance (Hsieh at al, 2006; Banks et al., 2005; Pilny et al., 2006). All steps in the proteome analysis can offer a source of variations. The proteomics analysis by LC-MS begins with the digestion of complex mixtures followed by peptide fractionation in LC systems. Then MS/MS scans are acquired and spectra matches are resulted from bioinformatics analysis. The complexity of these steps leads to variations in peptide and protein identification and quantification. Minor differences in LC can result in modified peptide elution times (Prakash et al., 2006) or change which peptides are selected for MS/MS fragmentation (Liu et al., 2004).

In this context, the use of MudPIT also can introduce more variation. Delmotte et al. (2009) showed that an introduction of a separation dimension decreases the repeatability by approximately 25% upon 1D or 2D chromatographic separations. Slebos et al. (2008) reported superior reproducibility in isoelectric focusing (IEF) compared to SCX separations. They found that IEF more quickly reached maximal detection within three replicate analysis. In contrast, the SCX required six replicates. In this study, approximately 90% of all peptide identifications are found in a single fraction. In contrast, SCX is characterized by spread of peptides into adjacent fractions. Peptides at lower abundance or those generating lower signal intensity are more likely to be selected for MS/MS if they appear in multiple

fractions. Thus SCX produces more chances of peptides detection which is consequence of the greater SXC sensitivity of peptide identifications. Though, greater sensitivity leads to much higher variability in peptide detection at each fractionation step from run to run, demanding a greater number of technical replicas, which compromises the overall throughput. Several groups have evaluated the number of replicates necessary to observe a particular percentage of the proteins in a sample (Liu et al., 2004; Slebos et al., 2008; Kislinger et al., 2005).

On the other hand, Tabb et al. (2009) concluded that a standardized platforms results a high degree of repeatability and reproducibility (around 70-80%) in protein identifications. This is an indication that LC-MS/MS platforms can generate consistent protein identifications. They showed that instrumentation can also increment variations. In this study, they observed that the high resolution of Orbitraps outperforms lower resolution of LTQs in repeatability and reproducibility. They also observed that reproducibility between different instruments of the same type is lower than repeatability of technical replicates on a single instrument by several percent.

Standardizations of methodologies and system configurations, as well as appropriate instrument tuning and maintenance would result in lower noise level from biological, chemical or instrumental sources between LC-MS/MS analysis. A comprehensive understanding about components that affect reproducibility and repeatability can help in the determination of the best alternative of experimental design to reach the reliability desired in proteomics studies.

5. Concluding remarks

Protein separation methods are vital for the characterization of proteomes. All these methods exploit one or more general properties of proteins and are directly linked to the effectiveness of any proteomic analysis. Traditionally, 2D gels have been the most frequently employed protein separation tool, capable of separating up to 10,000 components. However, 2D gels have some limitations, as discussed above, preventing efficient automation. In addition, LC methods coupled to MS analysis have been emerged as a promising strategy to achieve better automation and sensitivity. Combining orthogonal chromatographic columns increases peak capacity and permits a large dynamic range to be more efficiently measured in complex samples. Also, affinity chromatography allows the selection of a subgroup of proteins (proteins with certain PTMs), directing the study into specific biological pathways and increasing the generation of relevant information. Although its resolution has been improved resolution, LC fractioning has several concerns to be circumvented, such as the amount of time required, reproducibility, and protein inference from peptides. An experiment involving RP analysis can take 1.5 to 2 hours for a single analysis. Then, considering 15 fractions (taken from an IEX) from a complex mixture, there will be 22.5 hours required. Moreover, there is the analysis of biological (10 individual) and technical replicas (3 injections) that could reach 56 days of analysis, taking into account an experiment comparing control and affected. The required time increases instrument and maintenance costs and influences the experimental throughput. The second concern involves reproducibility during fractionating, which can be related to the number of dimensions employed and if they are inline coupled to the mass spectrometer or not. Another point that has received considerable attention is the development of data analysis pipelines to effectively process LC data from multiple peptide fractionation. The challenge is to reach

better accuracy in protein inference, mainly in bottom-up proteomics. In parallel, top-down proteomics is developing rapidly, with significant progress in MDLC systems for protein separation and probably warranting more attention in the near future.
Finally, the achievement of better representation, resolution, sensitivity, and automation has been developed with the application of LC methods coupled to MS, but they are far from being considered as the perfect strategy and are subject to improvements. The use of 2D gels or LC as the central separation method or both in combination is debatable, depending on the study objective. However, the constant advancement of these platforms is resulting in successful proteomics studies.

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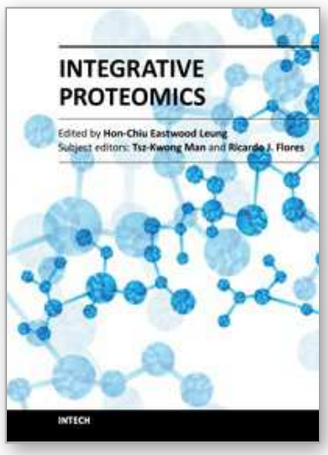

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