# Common Proteomic Technologies, Applications, and their Limitations

**Anna Meyfour[1], Mostafa Rezaie Tavirani[1], Mohammad Reza Sadeghi[2]**

[1]Proteomics Research Center, Faculty of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[2]Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran.

*Corresponding Author: email address: meyfour_a@yahoo.com (A. Meyfour)

## ABSTRACT

Proteomics refers to the analysis of expression, localization, functions, posttranslational modifications, and interactions of proteins expressed by a genome at a specific condition and at a specific time. Current proteomic tools allow large-scale, high-throughput analyses for the detection, identification, and functional investigation of proteome. In this review, we have focused on the proteomics methods: gel-based and gel-free techniques and discussed their applications and challenges in the field of proteomics.

**Keywords:** proteomics; proteome; Technology; Application; Limitation

## INTRODUCTION

Proteomics refers to the analysis of expression, localization, functions, posttranslational modifications, and interactions of proteins expressed by a genome at a specific condition and at a specific time. The human genome contains 26000–31000 protein encoding genes [1]; whereas the total number of human protein products, including splice variants and essential posttranslational modifications (PTMs), has been estimated to be close to one million[2, 3]. Although genes get a lot of attention, it's the proteins that perform most life functions and even make up the majority of cellular structures [4]. Moreover, there are a wide dynamic range of proteins concentration in proteomes of mammalian cells, tissues, and body fluids [5]. In spite of new technologies, analysis of complex biological mixtures, ability to quantify separated protein species, sufficient sensitivity for proteins of low abundance, quantification over a wide dynamic range, ability to analyze protein complexes, and high throughput applications is not yet fulfilled [6]. Due to the complexity of proteomes, a major goal of proteomics is developing methods for improving sample fractionation, separation, concentration of large numbers of proteins special those proteins in low abundance. Gel-based proteomic approaches include one-dimensional and two-dimensional polyacrylamide gel electrophoresis [7, 8]. 2D gel electrophoresis (2DE) coupled to MS is a mature and well-established technique and reported by many reports. This technology is useful and current technique to monitor the expressional changes among complex protein mixtures. But 2DE has some drawbacks such as less reproducibility, difficulty in detection of scarce proteins, and incompatibility for a hydrophobic, high molecular weight, or high pI protein analysis. Gel-free high throughput screening technologies (Mass spectrometry (MS)-based approaches) such as multidimensional protein identification technology [15], isotope-coded affinity tag ICAT [9]; SILAC [10]; isobaric tagging for relative and absolute quantitation (iTRAQ) [18] apply in quantitative, comparative investigations of proteomes and play an important role in systems biology, improving our understanding of fundamental biological processes or facilitating the identification of specific protein biomarkers [1, 11, 12]. Many different bioinformatics tools have been developed to aid research in this field such as optimizing the storage and accessibility of proteomic data or statistically ascertaining the significance of protein identifications made from a single peptide match [13, 14]. In this review, we will discuss the major technical developments, applications, challenges in the field of proteomics.

***Two-Dimensional Electrophoresis***

Two-dimensional electrophoresis (2DE) was developed two decades before the term proteomics was created [15, 16]. Two-dimensional polyacrylamide gel electrophoresis (2-DE) was initially described by O'Farrell in 1975 and has evolved markedly as one of the core technologies for the analysis of complex protein mixtures extracted from biologic samples. the mixtures of proteins are separated by two properties in two dimensions on 2D gels [16]. In the first dimension, proteins are resolved in according to their isoelectric points (pIs) using immobilized pH gradient electrophoresis (IPGE), isoelectric focusing (IEF), or non-equilibrium pH gradient electrophoresis (NEPHGE). Under standard conditions of temperature and urea concentration, the observed focusing spots of the great majority of proteins using IPGE (and to a lesser extent IEF) closely approximate the predicted isoelectric points calculated from the proteins' amino acid compositions. In the second dimension, proteins are separated according to their near molecular weight using sodium dodecyl sulfate poly-acrylamide-electrophoresis (SDS-PAGE). This technique can give molecular weight estimation (+/- 10%) for most proteins. 2DE analysis presents several types of information about the hundreds of proteins investigated simultaneously, including molecular weight, p*I* and quantity, as well as possible posttranslational modifications. 2DE is commonly used but it has some limits. For example this method falls short in its reproducibility, inability to detect low abundant and hydrophobic proteins, low sensitivity in identifying proteins with pH values too low (pH < 3) or too high (pH < 10) and molecular masses too small (Mr < 10 kD) or too large (Mr > 150 kD) [2–5]. The basic proteins separated poorly due to "streaking" of spots. Although different technologies that have known and been used in some experiments, 2-dimensional (2-D) electrophoresis is currently the only technique that can be widely applied for parallel quantitative expression profiling of large sets of complex protein mixtures [17]. In addition, it shows expressional differences among proteins, changes of isoforms and post-translational modifications [18]. Good protein extraction and solubilization are critical steps for proteomic analysis using 2D electrophoresis [19, 20]. Because of precipitating highly hydrophobic proteins during isoelectro focusing (IEF), deletion of low copy number proteins during sample preparation and the insolubility of transmembrane proteins, quantitative analysis of these peptides and polypeptides are very challenging [21]. In order to improve protein extraction and solubilization, different treatments and conditions should apply to efficiently solubilise different types of protein extracts [21, 22]. combinations of zwitterionic detergents, appropriately optimised, can provide improved solubilisation of proteins for 2DE [23].

Visualization methods for protein detection following 1-DE or 2-DE are an important step in quantitative proteome analysis. There are different methods vary in limit of detection, dynamic range, and compatibility with analysis by MS. Nowadays several fluorescent staining methods are used for the visualization of 2DE patterns, including sypro staining and Cy-dyes [24]. They have gained increased popularity and offer a wide linear dynamic range, detection of nanogram amounts of protein. Although sypro ruby [25] and silver staining [26, 27] have a similar sensitivity, sypro ruby staining allows much higher reproducibility, a significantly wider dynamic range and less false-positive staining. In addition, sypro ruby detect lipoproteins, glycoproteins, metalloproteins, calcium-binding proteins, fibrillar proteins, and low molecular weight proteins that are poorly using other methods. It is interesting to know that many protein spots on contain several proteins with a similar p*I*. For solving this problem, a pH gradient with a narrow range can be used and different proteins with the same molecular weight will be separated. Increased separation distance 40 × 40 cm gels using CA-IEF [28] could increase the proteome coverage up to 5000 proteins. Finally fundamental problems of this technology have remained with some classes of proteins including low abundance and hydrophobic proteins.

***Fluorescence 2D Difference Gel Electrophoresis (2D-DIGE)***

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) is a form of gel electrophoresis that Proteins are labeled with

fluorescent dyes prior 2-D electrophoresis [29]. CyDyes are cyanine dyes (Cy2, Cy3 and Cy5) containing an N-hydroxysuccinimidyl ester reactive group that covalently binds to the amino residues of lysine in proteins. In DIGE technique [8], proteins in three different protein samples can be labeled with one of these fluorescent dyes. After that the three samples can be mixed and loaded together on the same gel. This co-electrophoresis allows the quantitative comparative analysis of three samples within one single gel. The gel is scanned with excitation wavelength of each dye one by one by a fluorescent imager (such as TyphoonTM, EttanTMDIGE Imager). Finally the images were analyzed by special led software for 2D-DIGE such as De-Cyder [30, 31].

It overcomes limitations of conventional 2D electrophoresis. It improved quantification accuracy, statistical confidence [32] and reduced bias from experimental variation. The major advantages of 2D-DIGE are the high sensitivity and linearity of its dyes. Sensitivity of the minimal dyes is similar to most sensitive silver staining but it does not have postelectrophoretic processing steps such as fixing and destaining [8, 33]. In addition, all of the sample as an internal standard can be pooled and loaded with a control and experiment samples. With this method the abundance of a protein in each sample relative to the internal standard is measured and inter gel variation is reduced. On the other hand, this technique has some limitations; proteins without lysine cannot be labeled, and they require special equipment for visualization, and fluorophores are very expensive [30, 34].

***Isotope-Coded Affinity Tag (ICAT)***

Today, several high-throughput methods are available that provide quantitative information. The most commonly used technology for monitoring changes in the expression of complex protein mixtures is still two-dimensional gel electrophoresis (2-DE) [17]. Although 2-DE is still the method of choice for proteomics, there are a lot of limitations such as reproducibility, difficulty in detection of low abundance proteins, and incompatibility with separation of hydrophobic, high molecular weight, or high pI proteins.

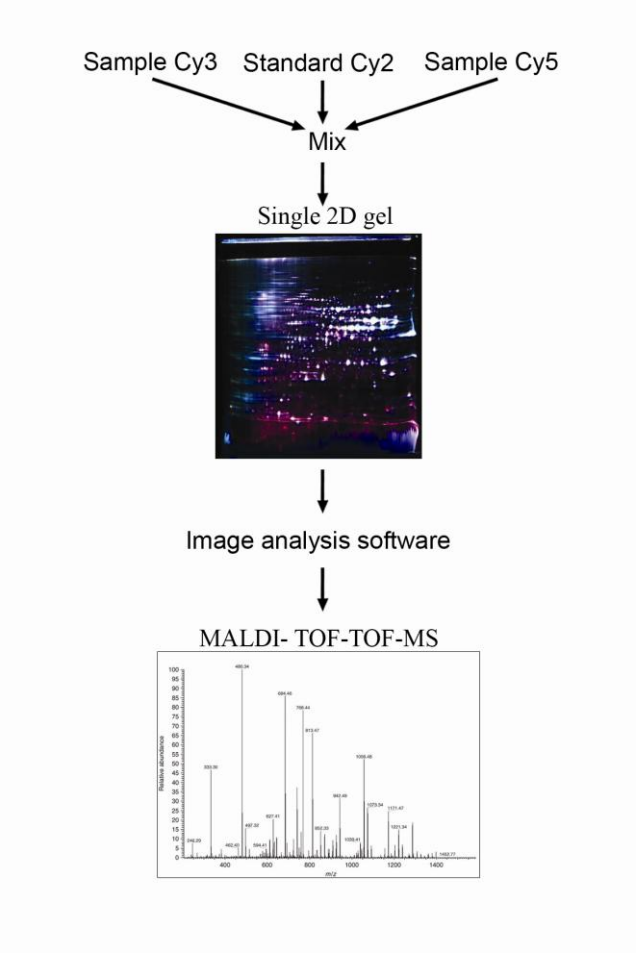


**Figure 1.** Design of a tipical 2D DIGE experiment.

Mass spectrometry (MS)-based proteomic methods have emerged as a key technology for unbiased systematic and high-throughput identification and quantification of complex protein mixtures. These methods have the potential to reveal unknown and novel changes in protein interactions and assemblies that regulate cellular and physiological processes.

ICAT is one of the most employed chemical isotope labeling for evaluating the protein content of two cell population and the first quantitative proteomic method to be based only using MS [9, 35]. Each ICAT reagent consists of three essential groups: a thiol-reactive group, an isotope-coded light or heavy linker, and a biotin segment to help the peptide enrichment process. In an ICAT experiment, protein samples are first labeled with either light or heavy ICAT reagents on cysteine thiols. Once the ICAT reagents have bound to the proteins, it is time to mix the two samples. The

protease such as trypsin is added to cut the proteins into peptide fragment. The ICAT-tagged proteins will bind to the magnet-like molecule called avidin separated through a multistep chromatographic separation procedure. Peptides are identified with tandem MS [36]. The ratios of signal intensities of differentially mass-tagged peptide pairs are quantified to determine the relative levels of proteins in the two samples [37-39]. ICAT labeled MS data are analyzed by different software programs such as proICAT, spectrum Mill and Sashimi [40]. ICAT enables identification of less abundant proteins from the large number of protein mixtures [41, 42]. However, ICAT has some limitations such as selective detection of proteins with high cysteine content and difficulties in the detection of acidic proteins [43, 44]. Also this method is so costly [45].

***Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)***

SILAC (stable isotope labeling by/with amino acids in cell culture) is a technique based on mass spectrometry that is used for comparative, quantitative proteome analysis in mammalian cultured cells [10] and detects differences in protein abundance among samples using non-radioactive isotopic labeling [46-48]. It is a popular method for quantitative proteomics [49]. It bases on the metabolic incorporation into proteins of different stable isotope labeled essential amino acids (AA) (Fig 2). Such AAs have different molecular masses due to the presence of isotopes of carbon (13C) and nitrogen (15N) heavier than the "normal" ones (12C, 14N). This results in the production of "heavy" proteins in a labeled culture, which are then mixed 1:1 with "light" proteins from an unlabelled culture before analysis. Processing and measuring samples together ensures a maximum of reproducibility and accuracy of quantitation [50 , 51] .

The use of stable isotopes to label proteins in mammalian cells has several advantages. First, SILAC requires no peptide labeling steps after harvesting proteins. Second, because the extent of incorporation is near 100%, there are no differences in labeling efficiency between one sample and the other [52]. Third, because the proteins are uniformly labeled, several peptides from the same protein can be compared to ensure that the extent of change is the same. Fourth, as the quantitative tag arises from the stable isotope containing amino acid rather than isotopic nuclei differential between two states can be specified more directly. Fifth, Compared with the ICAT the amount of labeled proteins requires for analysis using SILAC technique is far less than that with ICAT.this method can quantitate changes in small proteins, as well as those that may not contain any cysteine residues at all.

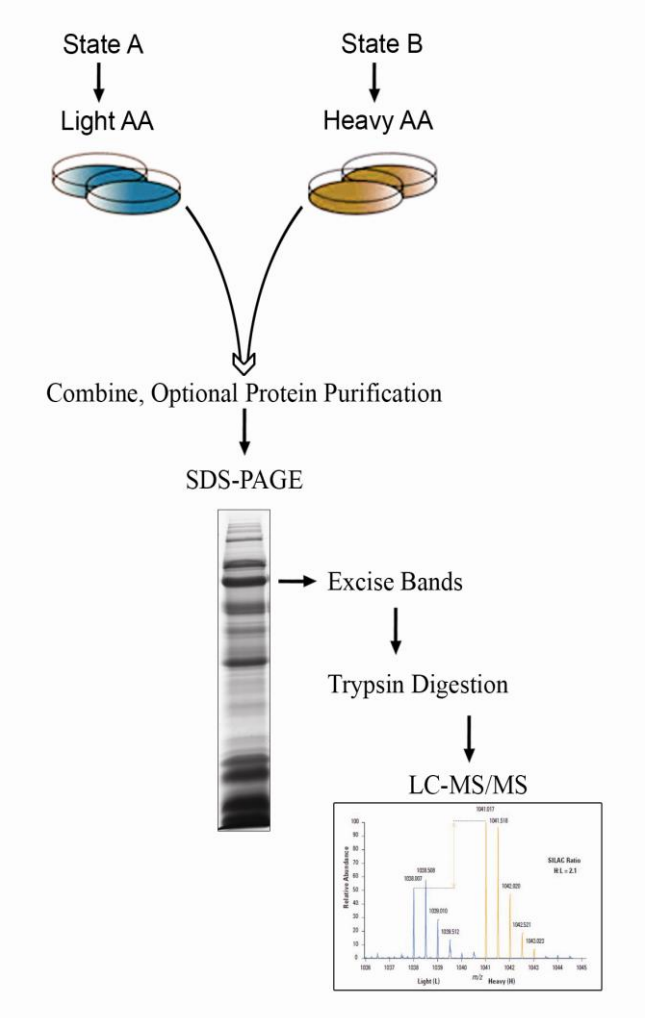


**Figure 2**. SILAC workflow. A549 cells were grown using SILAC DMEM containing 0.1 mg/ml heavy $^{13}C_6$ L-lysine-2HCl or light L-lysine-HCl supplemented. Cells from each sample (light and heavy) were lysed and protein concentration was determined. Each sample was equally mixed and SDS-PAGE was performed on 4-20%. proteins were digested and alkylated The combined peptide mixture is analyzed using an LTQ Orbitrap Hybrid Mass Spectrometer [10].

Therefore, Stable Isotope Labeling by Amino acids in Cell culture (SILAC) is an easy and reliable method for unbiased comparative proteomic experiments, which has been employed to study post-translational modifications such as protein phosphorylation and methylation, to characterize signaling pathways and to determine specific protein interactions [53-56].

Although SILAC has many rewards, its major problem is that it cannot be applied to tissue protein analysis directly [57]. To overcome this drawback, SILAC has been successfully applied to tissue proteome based on $_{15}$N isotope labeling [106]. Microorganisms such as malaria parasite can be labeled with isoleucine [58]. Recently the culture-derived isotope tags (CDITs) method was developed as an alternative quantitative approach for studying the proteome of mammalian tissues based on the application of SILAC [59].

***18O Stable Isotope Labeling***

Differential 16O/18O coding relies on the 18O exchange that takes place at the C-terminal carboxyl group of proteolytic fragments, where two 16O atoms are typically replaced by two 18O atoms by enzyme-catalyzed oxygen exchange in the presence of H218O [60]. Two atoms of 18O are introduced into the carboxylic acid group of every proteolytic peptide in a protein pool that has been catalyzed by members of the serine protease family, which includes trypsin, Glu-C protease, Lys-C protease and chymotrypsin. In the binding site of each protease, the residue of choice is covalently bound in a tetrahedral intermediate, which is then disrupted by nucleophilic attack by a water molecule, cleaving the protein. The C-terminal residue in each peptide product is re-bound by the protease, e.g., Arginine and Lysine in the case of trypsin, and released by hydrolysis. If the peptide products are incubated with the catalytic enzyme in H2 O18, the level of 18O in the peptides will eventually equilibrate with the level of 18O in the solvent, preferably > 95%. Peptide binding by the protease offers the advantage that cleavage of the protein can be optimized and carried out separately from labeling the peptide [61].

Each heavy peptide weighs 4 Da more than its 16O2 light analog. After labeling, the mixtures of heavy and light peptides are mixed, and isotope ratios of peptide pairs are determined by LC-MS. The resulting mass shift between differentially labeled peptide ions permits identification, characterization, and quantitation of proteins from which the peptides are proteolytically generated. Although the $^{16}O/^{18}O$ labeling is not the most commonly used isotope-tagging technique, its simplicity and instantaneous applicability to clinically relevant and amount-limited samples make this technique easily applicable for protein biomarker discovery that relies on MS-based profiling of human specimens. In contrast to ICAT, $^{18}O$ labeling does not favor peptides containing certain amino acids (e.g. cysteine), nor does it require an additional affinity step to enrich for these peptides. Unlike iTRAQ, $^{16}O/^{18}O$ labeling does not require a specific MS platform nor does it depend on fragmentation spectra ($MS^2$) for quantitative peptide measurements. Importantly, $^{18}O$ labeling is far less expensive than all of the stable labeling techniques mentioned earlier, making it useful in the area of biomarker discovery, where numerous samples are expected to be analyzed concurrently. 18O labeling suffers from inability to compare simultaneously multiple samples within a single experiment [62].

***Isobaric Tag for Relative and Absolute Quantitation (iTRAQ)***

iTRAQ is a non-gel based multiplexed protein quantitation technique that is well known for relative and absolute quantitation of proteins from different samples/treatments. iTRAQ is well matched for comparing normal, diseased, and drug-treated samples, time course studies, biological replicates and relative quantitation. It has simplified analysis and increased analytical precision and accuracy [63, 64].The method is based on the covalent labeling of the N-terminus and sidechain amines of peptides from protein digestions with tags of varying mass. There are currently two mainly used reagents: 4-plex and 8-plex (4 or 8 samples), which can be used to label all peptides from different samples/treatments [65]. The introduction of stable isotopes using iTRAQ reagents occurs on the level of proteolytic peptides. This technology uses an NHS ester derivative to modify primary amino groups by linking a mass balance group (carbonyl group)

and a reporter group (based on N-methylpiperazine) to proteolytic peptides via the formation of an amide bond [66]. These samples are then pooled and usually fractionated by nano liquid chromatography and analyzed by tandem mass spectrometry (MS/MS). In this techniqueDue to the isobaric mass design of the iTRAQ reagents, differentially labelled peptides appear as a single peak in MS scans, reducing the probability of peak overlapping. In MS/MS analysis, the signal intensity ratios of the reporter groups indicate the ratios of the peptide quantities and can be used to determine the relative quantities of the peptides. The MS/MS spectra of the individual peptides show signals reflecting amino acid sequences and also show reporter ions reflecting the protein contents of the samples. A database search is then performed using fragmentation data to identify the labeled peptides and hence the corresponding proteins whilst the iTRAQ mass reporter ion is used to relatively quantify the peptides. Quantitation of protein from multiple samples can be achieved in the same run. The data of the MS/MS spectra can be analyzed using software such as i-Tracker and jTraqX that is freely available. An inherent drawback of the reported iTRAQ technology is due to the enzymatic digestion of proteins prior to labelling, which artificially increases sample complexity and this approach needs a powerful multidimensional fractionation method of peptides before MS identification [64].

***Liquid Phase IEF Fractionation Methods***

Isoeletric focusing (IEF) as a electrokinetic methodologies is a popular technique for free solution prefractionation of proteins. Many commercial devices are now available for Liquid Phase IEF Fractionation. Fractionation steps reduce the sample complexicity and concentrate low abundance proteins, resulting in more confident protein identifications and quantification by 2D gels, mass spectrometry, and protein arrays. One application of liquid-phase isoelectric focusing (IEF) is prefractionation of proteins before the first dimension of 2D gel electrophoresis [67, 68]. For more consistent p*I* separation, the Zoom IEF fractionator [68, 69] and multicompartment electrolyser (MCE) [70] are being used to prefractionate the proteins. In order to perform 2DE, the fractionated samples can be loaded on standard narrow range IPG strips. With this method, 10000 to 15000 proteins can be separated. Also Liquid Phase IEF Fractionation has been used in shotgun proteomic experiments [71]. IEF runs in a buffer-free solution containing carrier ampholytes or in immobilized pH gradient (IPG) gels. The use of IPG-IEF for the separation of complex peptide mixtures has been applied to the analysis of plasma and amniotic fluid [72, 73] as well as to bacterial material [74]. The IPG gel strip is divided into small sections for extraction and cleaning up of the peptides. This technique recovers the sample from the liquid phase and was demonstrated to be of great interest in shotgun proteomics [75]. In addition to gain high resolution peptide separation, IEF can provide additional physicochemical information like their isoelectric point [76, 77] that is useful information to confirm peptide sequence identification during database search for MS/MS [78]. The recent introduction of commercially available OFFGEL fractionator system by Agilent Technologies provides an efficient and reproducible separation technique [79]. This separation is based on immobilized pH gradient (IPG) strips and permits to separate peptides and proteins according to their isoelectric point (p*I*) but is realized in solution [80].

***Large-Scale Western Blotting Proteome Analysis***

In this procedure, a large well is used to separate the sample by PAGE and lanes are created on the membrane containing immobilized protein with the use of a manifold [81]. Compatible combinations of primary antibodies are predetermined, with the criterion of being able to identify proteins that do not comigrate. Different combinations of primary antibodies are added to each well, with appropriate dilutions of each primary antibody so that expressed proteins are detected in a single condition. The scalability of the system depends on defining suitable combinations of primary antibodies, with up to 1000 antibodies in 200 lanes being used in the largest screens. Detection software is used to identify proteins based on their expected and observed gel mobility. It greatly facilitates the verification and functional analyses of detected

proteins. Furthermore, this approach provides important basic information on expressed proteins, their isoforms, post-translational modifications, protein function, such as cell signaling molecules [82]. This method have some disadvantages: first, it only identifies proteins for which antibodies are already available and in compare with 2D PAGE and HPLC-MS/MS, this method is not proper selection for identifying uncharacterized proteins. Second because of high antibody utilization, it is expensive experiment.

***Multidimensional Protein Identification Technology (MudPIT)***

Multidimensional protein identification technology (MudPIT) (1) developed is a method to analyze the highly complex samples necessary for large-scale proteome analysis [83]. In the MudPIT approach, enzymatic digestion of protein samples usually is carried out using trypsin and endoproteinase lysC. Peptide mixtures are separated by strong cation exchange (SCX) and reversed phase (RP) high performance liquid chromatography (HPLC) [84, 85].

Peptide fractions from the RP column are identified by electrospray ionization, tandem mass spectrometry (MS/MS), and database searching [85]. The success of MudPIT for proteomics is a result of the two-dimensional resolution of peptides and the ability of database searching programs to identify proteins based on a search with one or more peptides. By using peptides for identification, unbiased identification of proteins can be made; even proteins of relatively low abundance, extreme hydrophobicity or pI, and large molecular weight can be identified [66, 86]. A combination of HPLC, liquid phase isoelectric focusing, and capillary electrophoresis provides other multimodular options for the separation of complex protein mixtures [87].

***Concluding remarks***

Proteomics refers to the analysis of expression, localization, functions, posttranslational modifications, and interactions of proteins expressed by a genome at a specific condition and at a specific time. The most commonly used technology for monitoring changes in the expression of complex protein mixtures is still two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry. But a lot of unsettled issues such as reproducibility, difficulty in detection of scarce proteins, and incompatibility for a hydrophobic, high molecular weight, or high pI protein analysis still remain beyond two-dimensional gel electrophoresis abilities. Mass spectrometry (MS)-based proteomic methods have emerged as a key technology for unbiased systematic and high-throughput identification and quantification of complex protein mixtures [88-90]. These methods have the potential to reveal unknown and novel changes in protein interactions and assemblies that regulate cellular and physiological processes. Finally both gel-based (one-dimensional [1D] gel electrophoresis, two-dimensional [2D] polyacrylamide gel electrophoresis, 2D difference in-gel electrophoresis [DIGE]) and gel-free (liquid chromatography [LC], capillary electrophoresis) approaches have been developed and utilized in a variety of combinations to separate proteins prior to mass spectrometric analysis. Advances come from the development of new and improved separation methods and strategies, mass spectrometers, and computer software. This creates a highly dynamic technological environment in the field of proteomics, permitting new applications and driving new discoveries [91].

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