ITRAQ: A PROTEOMIC APPROACH FOR QUANTIFICATION OF PROTEIN AND DATA ANALYSIS

Abstract

Proteins are large, complex molecules and are considered the basis of life as they perform a wide range of functions in living organisms, including structural support, enzymatic catalysis, signalling, and regulation of gene expression. Proteomics, a branch of molecular biology, helps understand the comprehensive analysis of proteins, particularly their structures, functions, interactions, and abundances within a biological system. Over the years, several quantitative proteomic methods have been developed to measure protein abundances and compare protein expression levels between different conditions or samples. This facilitated studying dynamic changes in the proteome in response to various stimuli. Isobaric Tags for Relative and Absolute Quantification (iTRAQ) is a groundbreaking technique that has revolutionized proteomic research. iTRAQ is a bottom-up approach and shotgun-based quantitation technique that enables simultaneous identification and relative quantification of hundreds of proteins in up to eight different biological samples in a single experiment using mass spectrometry. This book chapter aims to provide a comprehensive overview of the iTRAQ technique, highlighting its principles, experimental workflow, data analysis strategies, and applications in various fields.

Keywords: Proteomics, protein quantification, mass spectrometry, iTRAQ, data analysis

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I. INTRODUCTION

Since the beginning of biological research, the dynamic role of biomolecules insustaining life has been well documented. In 1838, Berzelius proposed the title “protein” to emphasize the significance of molecules [1]. The term protein is derived from the Greek term, *proteios,* meaning “the first rank”. The proteome refers to the entire set of proteins that are produced or expressed by a cell, tissue, or organism at a specific time or under specific conditions. Proteins are large, complex molecules that perform a wide range of functions in living organisms, including structural support, enzymatic catalysis, signalling, and regulation of gene expression. In comparison to genomics, proteomics offers a greater grasp of the composition and operation of the organism. However, it is considerably more challenging than genomics due to changes in response to various factors such as developmental stages, environmental conditions, and disease states [2]. It is influenced by the genetic information encoded in the genome, but additional factors like post-translational modifications and protein interactions also contribute to the complexity of the proteome. Studying the proteome is important for understanding biological processes and functions, as proteins are the primary effectors of cellular activities [3]. The proteome of eukaryotic cells is quite striking in its complexity due to post-translational modification and dynamic range [4,5].Prokaryotic proteins have a variety of characteristics, such as variations in abundance, size, and propensity either attract or repel water, which makes it challenging to analyze them [6]. In addition to the quantity of corresponding mRNA, which is a key factor in determining protein abundance, the host organism's ability to control and regulate translation also plays a significant role in biological processes. In order to fully describe a biological system, proteomics proves to be the most important set of data [7].

In 1996, Marc Wilkins coined the term “proteomics” to refer to the complete set of proteins coded by a genome[8]. The broad study of proteins known as proteomics focuses on understanding the properties of the proteome, encompassing protein expression, roles of proteins, expression profiles, post-translational modifications, and protein-protein interactions in driving cellular processes under stress as well as control conditions [9, 10, 11]. Proteomics entails understanding the properties of the proteome, including protein expression. Proteomics plays a vital role in the prediction and detection of disease as well as the development of a drug as a targeted molecule. The proteome undergoes variation periodically, between different cells, and in reaction to external triggers. Plant responses to stress and the intricacy of biochemical processes are both studied using the proteomics method [12, 13]. Plant stress proteomics offers the capacity to locate potential candidate genes that could be utilized to genetically modify plants to withstand stressors. Under abiotic stresses, plants always experience a noticeable change in protein expression. A proteomic approach will be very helpful in elucidating the role of protein accumulation under stress conditions and its association with stress tolerance [14, 15, 16].

Among the omics approaches, genomics and transcriptomics are valuable, but have the limitation of providing indirect measurements of cellular conditions that do not accurately reflect changes in proteins. A change in posttranslational modifications (PTMs), such as phosphorylation and protein degradation, is not mentioned in the data. Hence, purely relying on genomic data does not provide a complete understanding of the disease mechanisms. Investigation of proteomic changes related to a disease frequently aids in understanding the mechanisms behind the disease, as well as pinpointing valuable biomarkers and targets for

treatment. These proteins rarely exhibit either total "presence" or total "absence". It is more likely that they will have varying degrees of abundance. To measure these changes accurately and sensitively, it is essential to adopt an appropriate methodology.

The use of shotgun proteomics methodologies allows for the detection of proteins that are either increased or decreased in levels under certain conditions, which can be investigated in various cells and tissues. The process of multiplexing iTRAQ is simple and allows for the analysis of up to eight distinct samples in a single experiment. The purpose of this chapter is to explain in considerable depth the iTRAQ methodology in the laboratory, the extent of its functionality, and the pipeline for data analysis with iTRAQ.

II. THE GOALS OF PROTEOMICS ARE DIVERSE AND INCLUDE

1. **Protein Identification and Characterization:** Proteomics aims to identify and describe proteins present in a sample, including their amino acid sequences, post-translational modifications, and three-dimensional structures. This information provides insights into protein functions and their roles in biological processes.

2. **Protein Quantification:** Proteomics techniques allow for the measurement of protein abundance levels in different samples or under different conditions. By comparing protein quantities, researchers can identify proteins that are differentially expressed and gain insights into cellular responses and disease mechanisms.

3. **Protein-Protein Interactions:** Proteomics helps in elucidating the interactions between proteins, identifying protein complexes, and understanding how proteins interact to carry out specific cellular functions. This information aids in unravelling complex biological networks and signalling pathways.

4. **Biomarker Discovery:** Proteomics plays a crucial role in the identification of protein biomarkers, which are specific proteins or protein patterns that can indicate the presence or progression of a disease. Biomarkers have potential applications in disease diagnosis, monitoring treatment responses, and developing personalized therapies.

   Overall, proteomics provides a comprehensive approach to studying proteins, enabling a deeper understanding of cellular processes, disease mechanisms, and the development of new diagnostics and therapeutics.

III. TYPES OF PROTEOMICS

Proteomics can be categorized into different types based on the specific aspects of protein analysis and the goals of the study. Here are some commonly recognized types of proteomics:

1. **Expression Proteomics:** Expression proteomics aims to analyze and compare protein expression levels between different samples or conditions. It involves the identification and quantification of proteins to understand changes in protein abundance. Techniques such as mass spectrometry-based quantitative proteomics and protein microarrays are commonly used in expression proteomics [17].

2. **Structural Proteomics:** Structural proteomics focuses on determining the three-dimensional structures of proteins. It involves techniques such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy (cryo-EM). Structural proteomics provides insights into protein folding, conformational changes, and interactions with ligands or other molecules [18].

3. **Functional Proteomics:** Functional proteomics aims to understand the functions of proteins in a biological system. It involves investigating protein-protein interactions, post-translational modifications (PTMs), enzymatic activities, and cellular localization. Techniques such as protein microarrays, yeast two-hybrid assays, and phosphoproteomics are used in functional proteomics [19].

4. **Interaction Proteomics:** Interaction proteomics focuses on studying protein-protein interactions. It involves identifying and characterizing protein interaction networks, protein complexes, and signalling pathways. Techniques used in interaction proteomics include yeast two-hybrid assays, co-immunoprecipitation (Co-IP), and affinity purification coupled with mass spectrometry [20].

IV. TECHNIQUES OF PROTEOMICS

In the field of proteomics, many approaches are being utilized and developed to enable quantitative comparison of samples between states. These can be categorized into mass spectrometry-based or gel-based approaches. According to comparative research, each approach to proteomics has advantages and disadvantages and complements one another. Isobaric Tags for Relative and Absolute Quantitation (iTRAQ), a shotgun technique, is one methodology that has attracted more attention [21, 22]. In comparison to other techniques like 2DE [23], ICAT [24], and DIGE [25], iTRAQ provides greater quantitative repeatability, and higher sensitivity and has numerous applications in proteomics research [26].

1. **Gel-Based**
   SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis)
   2-DE (Two-dimensional gel electrophoresis)

2. **Off Gel Base**
   LC (Liquid Chromatography)

3. **Quantitative Proteomics**
   iTRAQ (Isobaric tags for relative and absolute quantitation)
   ICAT (Isotope-coded affinity tag)
   SILAC (Stable Isotope Labeling by Amino acids in Cell culture)

4. **Mass Spectrometry**
   MALDI - TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry)
   LC-MS (Liquid chromatography-mass spectrometry)
   CE-MS (Capillary electrophoresis–mass spectrometry)

Isobaric tag for relative and Absolute Quantitation:

- Applied Biosystems Incorporation created the isobaric tag for relative and absolute quantitation, or iTRAQ, in 2004. iTRAQ is one of the most popular and very simple methods for proteome investigation.
- iTRAQ is a bottom-up approach and shotgun-based quantitation technique that enables simultaneous identification and relative quantification of hundreds of proteins in up to eight different biological samples in a single experiment using mass spectrometry.

V. PRINCIPLE AND WORKFLOW OF ITRAQ

The isobaric tagging reagents consist of a unique charged reporter group, a peptide reactive group, and a neutral balance group. This technique enables rapid protein identification and relative quantification at the MS/MS level by utilizing peptide fragments and low-mass reporter ions [27]. All of the peptides in a specific sample digest are labelled by the peptide reactive group, which covalently joins an iTRAQ reagent isobaric tag with each lysine side chain and N-terminus group of a peptide. The iTRAQ reporter ions are released during collision-induced dissociation (CID), and their relative intensities are used to quantify proteins.

1. Components Used in iTRAQ

- **Isobaric Tag**: The MS-based method known as iTRAQ uses four isobaric, isotope-coded tags to mark the proteins via their N-terminal and allows for relative and absolute protein quantification in up to four cell preparations. A reporter group, a balancing group, and an amine-specific reactive group make up the three main parts of the iTRAQ tag molecular backbone.
- **Protein Samples:** The samples whose proteins need to be quantified by the iTRAQ procedure.
- **Trypsin Digestion:** Trypsin is a proteolytic enzyme that breaks down proteins at the C-terminus of arginine and lysine residues, with the exception of when proline is present after those residues. As a result, huge protein samples can be divided into manageable peptide fragments.
- **Peptide Fragments:** The smaller fragments obtained upon cleavage of the protein samples after trypsin digestion.
- **iTRAQ Label:** The N-terminus of all peptides is labelled by a protein reactive group in the iTRAQ reagent, which also includes free amine groups on lysine side chains, a neutral balance component, and a reporter group.The labels are isobaric due to the differing isotope distributions between the reporter and balance groups, which allows for their detection in MS upon fragmentation and release (Fig. 1).
- **SCX Purification:** To eliminate any unbound iTRAQ reagent and to simplify the peptide mixture, tagged peptides are separated on a strong cation exchange column.
- **LC-MS/MS Analysis:** It is reverse-phase liquid chromatography is used to further purify the iTRAQ-labelled peptides that are obtained, and tandem mass spectrometry is used to analyze them. In order to determine the relative abundances of the peptides,

each tag causes the release of a unique mass reporter ion following peptide fragmentation.

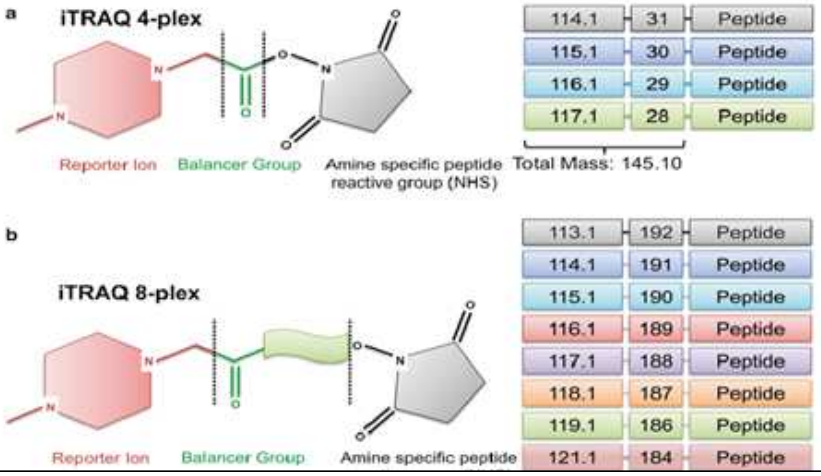

Figure 1: Chemical structures for iTRAQ (a) 4-plex and (b) 8-plex isobaric tags. Balancer + reporter ions add up to 145 Da in 4-plex and 304 Da in 8-plex experiments.
Source: Aggarwal and Yadav, 2015 [28]

2. Workflow of iTRAQ:

- **Sample Preparation:** The protein sample was extracted from the cell/tissue, and the extracted samples were digested with trypsinto break down the protein samples into smaller peptide fragments. Unless lysine and arginine residues are followed by a proline residue,trypsin cleaves proteins at their C-terminal regions. The 1-DE was used to assess the efficiency of the digestion process. The simplest method for desalting is to mix a smallamount of protein solution with centrifugal filters with C_{18} tips and various MWCB membranes.

- **iTRAQ Labelling:** The peptide generated is separated by SDS-PAGE to simplify the mixture and then tagged with the iTRAQ label [29]. The iTRAQ reagent consists of a reporter group, a balance portion, and a peptide reactive group that interacts with the N-terminus of the peptide or free amino group of lysine residues, giving it an overall mass of 145. The reporter group used to label each peptide sample is unique, with mass varying between them, thereby enabling the labelling and quantification of four samples simultaneously. This has been further improved to allow the labelling of eight samples simultaneously.

The amount of sample isobaric labelling is a key parameter that determines the amount of material that can be labelled with isobaric reagents. Labelling too many samples would waste the isobaric reagents, and labelling too few samples would result in inadequate labelling because of a lack of tags. In research involving the characterization of protein post-translational modifications (PTMs), it is imperative to label as much material as possible because the quantity of beginning materials directly correlates with the number of PTM sites found and quantified [30].

- **Concentration of Peptides and Proteins for Labelling:** Samples can be calculated either before or after digestion (at the protein or peptide level). The amino acid analysis (AAA), Lowry protein assay, bicinchoninic acid (BCA) protein assay, Bradford protein assay, Qubit fluorescence assay, or ultraviolet (UV) absorbance can all be used to determine the protein content [31, 32]. Depending on the sample composition, the best protein assay technique should be chosen because some components can impede the results of particular protein assays. Using UV absorbance at 280 or 205 nm, AAA, the BCA peptide assay, or peptide concentration, can be calculated [33].

- **Fractionation:** For quantitative proteomic analysis, fractionating isobaric-labelled peptides provides various benefits, including a reduction in sample complexity, an increase in coverage of complicated proteomes, and an improvement in the analytical dynamic range of the samples. As the interferences from precursor ions depend on the complexity of the sample and the quantity of coeluting peptides, they also partially affect the ratio compression of isobaric labelling [34]. The isobaric-labelled peptides are fractionated using chromatographic techniques such as High-pH RPC [35, 36], strong cation exchange chromatography [37], or HILIC [38] in order to expand the proteome coverage and decrease sample complexity. Despite the fact that SCX chromatography has strong orthogonality to acidic pH reversed-phase LC, it necessitates a further desalting step after the fractionation, which causes sample loss and extends the processing time for samples.

  The labelled samples are then combined, poured into a column in the direction shown, and allowed to run through the column before being collected in the empty beaker below. For the purpose of getting rid of any extra unbound iTRAQ reagent, the pooled samples are purified on a powerful cation exchange column. The IPG gel strips were rehydrated for 15 minutes using the peptide IPG strip rehydration, and the sample was poured into each well. The peptides were electrofocused, and peptide fractions were removed after focusing, and the wells were thenrinsed. The washing solutions were then combined with the matching peptide fraction.

- **Separation:** The "injector" should be inserted into the sample with the plunger lowered and injected into the column. It must be demonstrated that liquid flows via the tube that connects the "pump" and the "column". The colour of the column must change once the liquid is flowing, and it must be demonstrated that the liquid has passed through the tubing at the outlet. The SCX-purified peptides are further purified using reverse-phase liquid chromatography, in which the sample is run through a column containing a packed stationary phase matrix that only adsorbs specific analyte molecules. MS is used to further characterize the eluted fractions.

- **LC-MS /MS Analysis:** It was initially difficult to analyze samples with MS that had been isobarically labelled. However, isobaric-labeled peptide-based quantitative proteomics investigations have become commonplace and analyses the quick advancement of MS technology. It was initially only possible to analyze isobaric-labeled samples using mass analyzers that could detect low mass range ions, such as tandem time-of-flight (TOF/TOF) or quadrupole-time-of-flight (Q-TOF) [39, 40].

Isobaric labelling uses fragment ions from the low m/z range of the MS2 spectra for peptide quantification.

Following purification, the labelled peptide fragments are analyzed by MS/MS. The reporter groups' various masses enable the identification of the peptide fragments. Fragmentation results in the loss of the reporter group. iTRAQ now allows for the relative quantification of up to eight samples. Analyzing samples that have been isobarically labelled requires certain MS specifications. The best configurations for various LC-MS/MS systems should be identified for collision energies, isolation windows, instrument voltages, and ion target settings depending on the isobaric tags used, the sensitivity and speed of the MS instrument, and the chromatographic resolution [41].

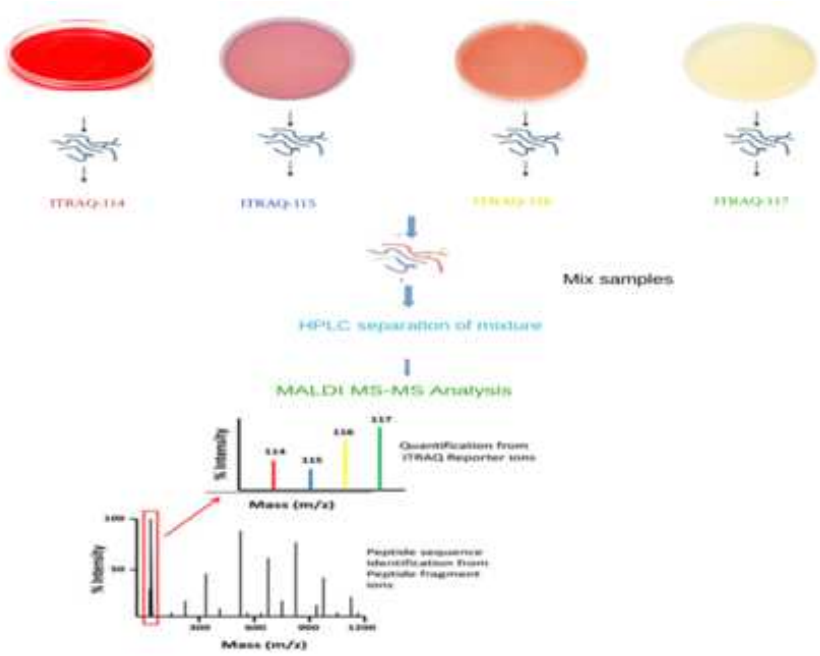

Figure 2: Workflow of iTRAQ

- **Peptide Spectra Analysis:** In addition to the field for PMF, the MS/MS data analysis software offers other inputs, including Quantitation, MS/MS tolerance, peptide charge, instrument, etc. They need the user's input on the experimental parameters employed, such as the protein name, alterations, and enzyme cleavage, as well as the desired search criteria, such as taxonomy and peptide tolerance. The NCBI, MSDB, and SwissProt protein databases are frequently used while processing MS data to retrieve sequence data. The data file produced by MS is submitted, and a search is run. The search is run after the data file produced by MS is uploaded.

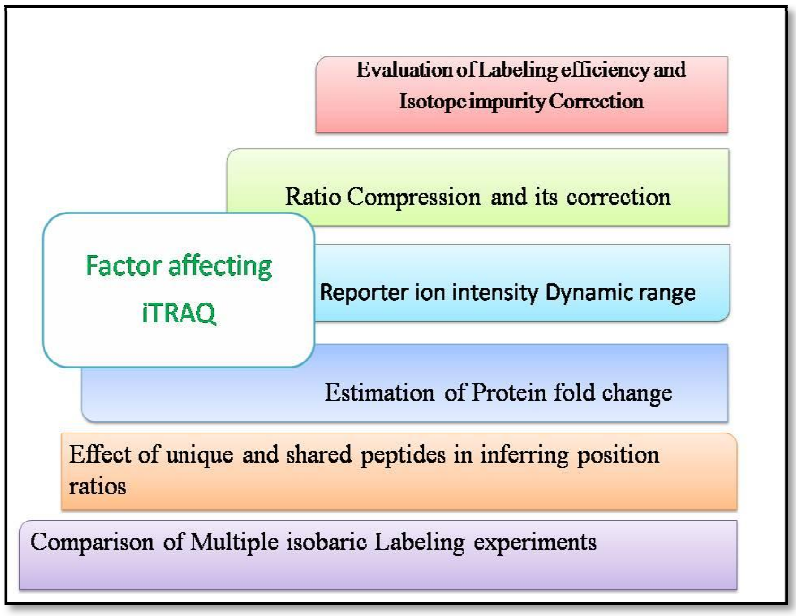

Figure 3: Factors Affecting iTRAQ

VI. DATA ANALYSIS

Protein identification and quantification, isobaric labelling data are analyzed with proteomic software after MS data capture. Data from isobaric labelling can be analyzed using a variety of proteomic programs. There are certain programmes designed expressly for the analysis of isobaric labelling data, including MSnbase [42], OCAP [43], Isoprot [44], PQPQ [45], MaxQuant [46], and LIQ-iQUANT [47].

Two procedures are employed to process the MS raw files during data analysis: one for protein identification and the other for protein quantification. After that, the results of the two procedures are combined to create a protein list with identification and quantification details. The quantitation process entails a number of phases, including pre-processing of the data, area and error calculations, isotopic correction, ratio calculations, determining protein abundances, and statistical analyses for group comparisons. It is important to remember that quantification happens at the level of the spectrum, which represents peptides rather than proteins.

1. **Pre-processing of Data and Database Search:** The removal of low-quality spectra from the data is necessary for accurate quantification. For the purpose of removing low-quality data, data preparation comprises selecting peaks and removing noise from the MS/MS spectra. Compared to quantitation, which just requires a very small amount of data filtering in order to calculate an exact area, this processing can be very different. The quantitative accuracy of isobaric labelling is greatly influenced by the co-isolation of interfering precursor ions in the MS/MS selection window. This phase just calls for baseline correction and noise removal below the signal-to-noise threshold. While clean and processed spectra are beneficial for identification, too much processing may damage quantitation. Therefore, it is advised to use profile mode data for quantitation, whereas central data should be used for identification.

For peptide and protein identification, a number of database search techniques, including X!Tandem [48], Mascot [49], MassWiz [50], OMSSA [51], and Paragon [52] can be employed.

2. **Reporter Area and Error Calculation:** The raw peak areas are integrated, and the estimation error for the area is computed. This stage necessitates the accurate identification of the reporter ion peaks in a specified low-mass m/z band (100-125 Da) within a tolerance range. After determining the peak area, the quantification error is calculated to account for any potential errors that might occur during the area calculation. Error (E) can be determined by referring to the ProteinPilot help manual.
   $E=\sqrt{I}$
   Where,
   I= total intensity computed for a single reporter ion.

   Various software applications employ distinct error models that could produce marginally varied outcomes [53, 54, 55]. In general, the software utilizes a specific error model, including its documentation, due to the absence of a general agreement. The i-Tracker reference provides a comprehensive explanation of how to calculate errors.

3. **Isotope Correction**: The intensity of each reporter ion includes overlapping isotopic contributions from nearby tags because the isobaric labelling reagents are not completely pure, and isotope correction should be applied to the reporter ion intensities using the reagent purity values [56, 57]. The apparent change in protein expression would be distorted by the uncorrected data, whereas the isotopic correction would aid in precise quantification [56]. As a result, the isotope correction table has been introduced to the data analysis pipeline by some tools, including PD, PEAKS Q, Census, and MilQuant. The real area can be determined for each batch's purity values 16 and 32 purity values for 4-plex and 8-plex, respectively which correspond to the percentages of each reporter ion with masses that varied by +2, +1, 2, and -1 Da.

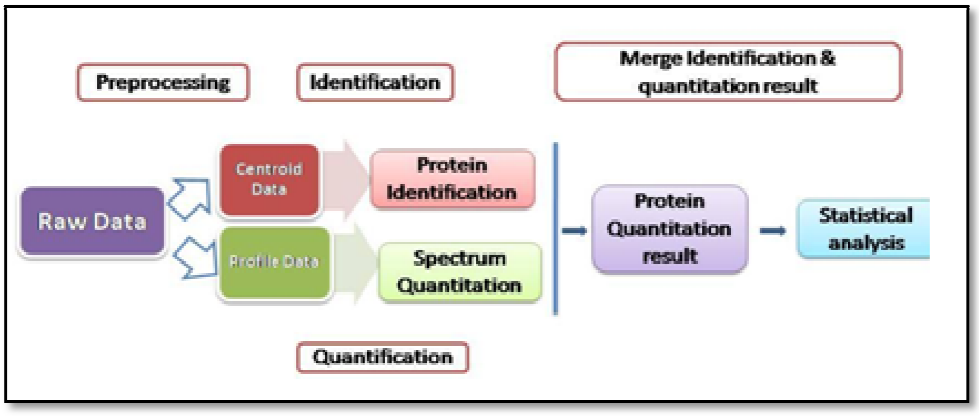

Figure 4: iTRAQ Data Analysis Workflow with Identification and Quantification

4. **Data Normalization:** It is necessary to normalize the data to reduce any variability brought on by experimental parameters, such as protein extraction, digestion efficiency,

and isobaric labelling efficiency, before using the protein quantification data for additional studies. Data with insufficient or improper normalization can lead to inaccurate results. To normalize the data, a variety of algorithms have been created. A global normalization, for example, is based on correction factors obtained from the total or median peak intensities of all reporter ions or based on computing ratios by taking the median, arithmetic averages, or intensity-weighted averages [58].

The peak normalization is applied as follows:
$T_N = T_R / T_{Total}$

Where,
T_N = Normalized peak area
T_R = true area of the reporter ion
T_{Total} = Total of all true area for all reporter ions

The data is normalized by PD v2.1 (and later versions) using the overall peptide amount and the specific protein amount. The latter adds up the abundance values of all the peptides found in each channel and then uses the abundance value from the channel with the highest overall abundance to normalize the abundance values of the other channels' peptides [59].

5. **Estimating Bias and Variance:** The root mean square error (RMSE) was used to assess the peptide quantification error. The RMSE represents a weighted average of the variations between the mass spectrometry-measured values (y2) and the ideal values (y1). The measured values were scaled to the same level in order to make the RMSE independent of the signal strength. By dividing the measured values by the slope of the regression line and comparing the measured values to the ideal values, scaling was performed. A regression technique known as scaling factor, robust linear regression, which is less susceptible to outliers, was used to determine the slope [60]. The bias was investigated by plotting the iTRAQ peptide ratios against the minimum peptide intensity.

6. **Protein Identification and Quantitation:** All MS data were gathered using MicrOTOFcontrol, and DataAnalysis (Bruker Daltonics) was used for analysis. To identify proteins, Mascot v2.3.01 (Matrix Science) was used. The database known as the Universal Protein Resource (UniProt) [61] served as a guide.

The quantification of proteins is the end result of many proteomics investigations. This task is entirely dependent on the accuracy of the processes that have already been covered, particularly the results of the peptide quantification and protein inference steps.

7. **Methods for Peptide and Protein Quantitation:** The first involves determining several ratios from the protein's peptides, then adding up these ratios to get a single fold change. Although label-free ways have been added to the usage of this technique, which is frequently used in stable isotopic labelling [62]. Its key benefit is the ability to calculate the protein ratio's standard deviation from the peptide ratios. The second method involves determining a single fold change at the protein level after estimating the protein abundance from its peptides. A statistical estimator typically converts peptide ratios into protein ratios by determining the most pertinent protein ratio from the distribution of

peptide ratios. Robust statistics is an alternative approach based on the postulate that the ratios of the spectrum are various measures of protein ratios [63]. Thus, a large number of measurements will boost the reliability of the protein ratios. There are also intensity-based approaches, although they can result in significant inaccuracies [64].

The peptide ratio and error can be obtained by combining the ratios and errors computed at the spectra level in one of three ways [65]:

- **Average-Based:** Following a database search, all of the spectra corresponding to the same peptide will be averaged to provide the peptide ratio and error.
- **Median-Based:** Peptide ratios are calculated based on the median of all the spectral ratios that belong to a certain peptide. If the spectrum counts are even, the geometric mean will be the median in this case.
- **Score-Based:** In this method, the peptide ratio and error will be determined by the ratio and error of the spectra that best define the provided peptide and the peptide ratios and errors will be combined to obtain the corresponding protein ratios and errors.

The following three techniques can be combined after identifying the peptide ratios and errors to obtain the corresponding protein ratios and errors:

- **Average:** The protein ratio and error are determined as the average of all peptide ratios and errors.
- **Median:** The protein ratio and error are used to generate the median for all peptide ratios and errors.
- **Weighted Average:** The protein ratio (PR) is computed as follows [66]:

$$PR = \frac{\sum_{j=1}^{n} PER_j \times PW_j}{\sum_{j=1}^{n} PW_j}$$

Where,
PER = Peptide ratio
PW = Peptide weight
j =Number of peptides from 1…n

8. **Ratio Compression**: There is a known tendency for ratios to be compressed towards 1 when coeluting peptide fragments concurrently. This has the consequence of bringing most reporter-ion ratios closer to 1, which can lead to big quantitative changes (fold changes) being underestimated and missed. The right mass spectrometry and careful separation can overcome these problems. The bias and unpredictability resulting from differing intensities in isobaric tags are another issue. Area estimates for low-intensity peaks have larger inaccuracies, and vice versa. In order to address this issue, a model known as variance-stabilizing normalization (VSN) has been proposed.

9. **Statistical significance analysis and data mining:** Quantitative proteomic experiments aim to compare protein expression levels between different groups. Data mining and the functional interpretation of datasets pose analytical challenges. Many software tools automatically output protein abundance ratios but lack proper algorithms for statistical analysis, data mining, and visualization. These issues are usually resolved using statistical platforms like MATLAB, R, or dedicated software packages. A statistical test is used to estimate a p-value and a specified cut-off, determining significant protein changes. The two-sample t-test is the most common statistical test used to evaluate differences between groups. If the first hypothesis is not met, the log transformation can be used to convert the observed abundance distribution into a more symmetric distribution. Nonparametric tests, such as permutation tests or the two-sample Kolmogorov-Smirnov test, are useful when the sample size is small. If the second hypothesis is not met, the one-sample t-test is used.

VII. ADVANTAGES & DISADVANTAGES OF ITRAQ

Advantages of iTRAQ:

- **Multiplexing**: iTRAQ enables multiplexing, meaning several samples can be labelled with different iTRAQ tags and combined into a single experiment. This reduces experimental variation and costs compared to performing separate runs for each sample.
- **Accurate Quantification**: iTRAQ provides accurate and reliable relative quantification of proteins between different samples. It measures peptide abundance ratios, allowing researchers to compare protein expression levels under various conditions.
- **High-Throughput**: The ability to analyze multiple samples simultaneously makes iTRAQ a high-throughput method. It can efficiently analyze large datasets, making it suitable for complex experiments.
- **Reproducibility**: When properly executed, iTRAQ experiments exhibit good reproducibility, which is essential for obtaining reliable results.
- **Quantification of Post-Translational Modifications (PTMs)**: iTRAQ can be adapted to measure PTM changes, providing insights into regulatory processes.
- **Reduce overall time and variation**: It has higher specificity and sensitivity, therefore reducing the analysis time and variations.

Disadvantages of iTRAQ:

- **Cost**: Although multiplexing reduces costs compared to individual runs, iTRAQ reagents can still be relatively expensive, especially for experiments with many samples.
- **Complexity**: iTRAQ analysis involves several steps, including protein extraction, digestion, labelling, and mass spectrometry, which can be technically demanding and may require specialized equipment and expertise.
- **Sample Complexity**: iTRAQ may face challenges when dealing with highly complex samples, such as those containing low amounts of abundant proteins or extensive protein modifications.
- **Quantification Accuracy**: While iTRAQ is generally reliable, it is sensitive to experimental variability, and inaccurate quantification can occur if not adequately controlled.

- **Isobaric Interferences**: Isobaric ions with similar mass-to-charge ratios might interfere with accurate quantification, leading to potential misidentification and quantification errors.
- **Data Analysis Complexity**: Interpreting and analyzing the large datasets generated by iTRAQ experiments can be challenging and may require bioinformatics expertise.

VIII. APPLICATION OF ITRAQ

Species	Stress	Result	Source
Plants			
Oryza sativa	High Temperature	Another response to high temperature	[69]
Brassica napus	Chlorophyll deficiency	Pathway associated with chlorophyll deficiency	[70]
Brassica napus	Methyl Jasmonate Responsive Proteins	Guard cells of stomata	[71]
Boehmeria tricuspis	—------	Differential proteomic analysis to identify proteins associated with Apomeiosis	[72]
Agrostis stolonifera	Exogenous application of (GABA) under drought stress condition	GABA-induced drought tolerance is possibly involved in the improvement of nitrogen recycling, photosystem II protection	[73]
Camellia sinensis L	Application of GABA in cold temperature tolerance	Identified protein related to amino acid transport	[74]
Adzuki bean	Drought tolerance during seed germination	Differential expression proteins related to biological process, molecular function and cellular component in condition of drought stress	[75]
Microbes			

Lactobacillus plantarum K25	Cold-stress response	DE proteins involved in carbohydrate, amino acid and fatty acid biosynthesis and metabolism recorded downregulation	[76]
Cyanobacterial Synechocystis sp. PCC 6803	Biofuel ethanol	Synechocystis cells treated with ethanol recorded up-regulation of photosynthesis related proteins	[77]
Staphylococcus epidermidis	Biofilms of dormant bacteria	Proteins related to ribosome synthesis pathway upregulated and proteins involved in metabolism of purine, argin-ine, and proline recorded downregulation	[78]
Streptococcus pneumoniae	Bactericidal mechanism of sodium new Houttuyfonate	----------	[79]
Estrogen-degradation strain Pseudomo-nas putida SJTE-1	Global response to 17β-estradiol	Identify proteins involved in energy metabolism, transportation, cell motility, chemotaxis, and carbon metabolism	[80]
Bacillus cereus	Synergistic antibacterial mechanism of phenyllactic acid and lactic acid	PLA inhibited transport of K+ and down-regulated kdpB, LA inhibited transport of P and identified periplasmic phosphorus binding protein down-regulated which disrupt ribosome function	[81]
Vibrio parahaemolyticus	Proteomic profiling under various culture	Identification of membrane protein of metabolism process	[82]
Animals			
Przewalski's gazelle	Selenium deficiency response	Identification of differentially expressed proteins related to extracellular region, nodal proteins involved in the cellu-lar and biochemical processes,	[83]

		pathwaysof signal transduction	
Humans	Protein alterations after traumatic brain injury	Identify 3937 proteins among them 146 proteins were changed after traumatic brain injury	[84]
Humans	Effects of diurnal variation on the composition of Human Parotid Saliva	Identify novel peptides	[85]
Humans	Involvement of GABA transporters in atropine-treated myopic retina	Identified a total of 3882 unique proteins among which 30 proteins recorded upregulation and 28 were downregulated. After atropine treatment ABA transporter 1 in myopic retina reduced	[86]
Sheep and goat	Fibre density	Identify differentially expressed proteins that are and are associated with variation in fiber features. Proteins related to growth of hair (keratin) and fatty acid synthesis recorded different abundance	[87]
Humans	Differential urinary proteins to diagnose coronary heart disease	A total of 100 differential proteins recorded associated with activation of the Liver X receptor pathway, atherosclerosis signaling and the production of nitric oxide	[88]

IX. CONCLUSION

In the last two decades, there has been a tremendous advancement in the technology in the field of proteomics. These technologies are rapid, sensitive, and provide greater proteome coverage. ITRAQ is one such technology. iTRAQ is a bottom-up approach and shotgun-based quantitation technique that enables simultaneous identification and relative quantification of proteins. It provides accurate and reliable relative quantification of proteins between different samples. It enables multiplexing and has higher specificity and sensitivity, therefore reducing the analysis time and variations.

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