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Neuroproteomics – LC-MS Quantitative Approaches

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Abstract

Neuroproteomics is a scientific field that aims to study all the proteins of the central nervous system, their expression, function, and interactions. The central nervous system is intricate and heterogeneous, and the study of its proteome is consequently complex, with many biological questions still requiring deep investigation. For this, mass spectrometry approaches, most often coupled with liquid chromatography (LC-MS), have been the number one choice in proteomics, and over the years it has added many important findings to the field. At this point it is important that proteomics turns to the quantitative expression of proteins instead of only identifying which proteins are present in a given sample, much because the most important alterations may be slight alterations in the quantity of a protein in a given situation. Therefore, many LC-MS quantitative approaches have been developed relying on the labeling of the proteins or even by using label-free techniques.

In this chapter, a brief description of the principles and procedures of several approaches used for relative and absolute, targeted and untargeted quantification of proteins is presented, complemented with a literature revision of their application in the neurosciences field.

Keywords: Neuroproteomics, LC-MS techniques, central nervous system, protein relative quantification, protein absolute quantification

1. Introduction

Neuroproteomics is a field that aims to study all the proteins of the central nervous system (CNS), as a whole or related to a specific condition (for example, disease, drug response, etc.). CNS is very complex, presenting a high degree of heterogeneity at several levels, such as distinct brain regions, cellular networks, and cell types [1], each one characterized by a different

proteome. Even slight perturbations of this structure can lead to CNS disorders, resulting in alterations in the proteome of all CNS constituents or of specific cellular networks.

Large-scale initiatives have been performed to sequence human and other organism's genomes [2], as well as the analysis of gene expression of the distinct regions and cells of the brain. However, although these studies have contributed with crucial information, the end-point of gene transcription is the synthesis of proteins, the effector molecules. This way, the complex and dynamic nature of the proteome has led to a paradigm shift in the neurosciences field, changing from the focus in genomic information to the analysis of the protein's expression levels, by resorting to several approaches [3, 4].

Proteomics methodologies aim to analyze a large number of proteins within a certain set of samples of an experiment [5], and the great development of this area may be attributed to the technological advances in mass spectrometry (MS), optimization in sample preparation, and computer sciences that are now able to deal with the large amount of information generated by the MS-based technologies [6, 7].

These approaches can deliver different types of data, such as identification of the protein in a sample at a given moment, expression levels of the proteins (quantitative proteomics), identification and quantification of post-translational modifications (PTM), and protein interactions (for example protein-protein interactions) [7].

Over the past years, MS-based proteomics approaches have been able to characterize proteins in complex mixtures; nonetheless, these approaches have largely been qualitative, successfully identifying a high amount of proteins from one sample but failing in quantifying the expression levels of these [5]. However, it has been pressing to turn the proteomics field to quantitative approaches, once most of the interesting biological alterations are slight differences in the amount of a protein present in a given situation [8].

The main goal of quantitative proteomics, or quantitative neuroproteomics in particular, is to measure the expression level of, theoretically, all the proteins in a given sample, preferably in a highly reproducible manner [9]. This quantitative information can be acquired in two distinct ways: absolute quantification, where the amount of the protein in the sample is calculated (for instance, in terms of concentration or copy number per cell); or relative quantification, where the amount of a given protein is expressed as a fold change for the same protein relative to another condition [5, 7]. The approaches to obtain relative quantification may be untargeted, where virtually all the proteins in the sample are quantified; or targeted where the quantification is obtained for a selected protein or a set of proteins. A brief summary of the most important methodologies is outlined in Figure 1.

The classical approach to obtain relative quantifications of a proteome was to perform a bi-dimensional electrophoresis (2DE-Isoelectric focusing followed by SDS-PAGE), where the identification of the proteins was obtained by a MS analysis and the relative quantification by measuring the staining density of matched gel spots [9]. Nonetheless, in this method, some types of proteins are underrepresented, and although hundreds to a few thousands of proteins may be detected, many proteins with lower abundance are very difficult to quantify. Also, the analysis of many samples by this method is laborious and time consuming. [9]

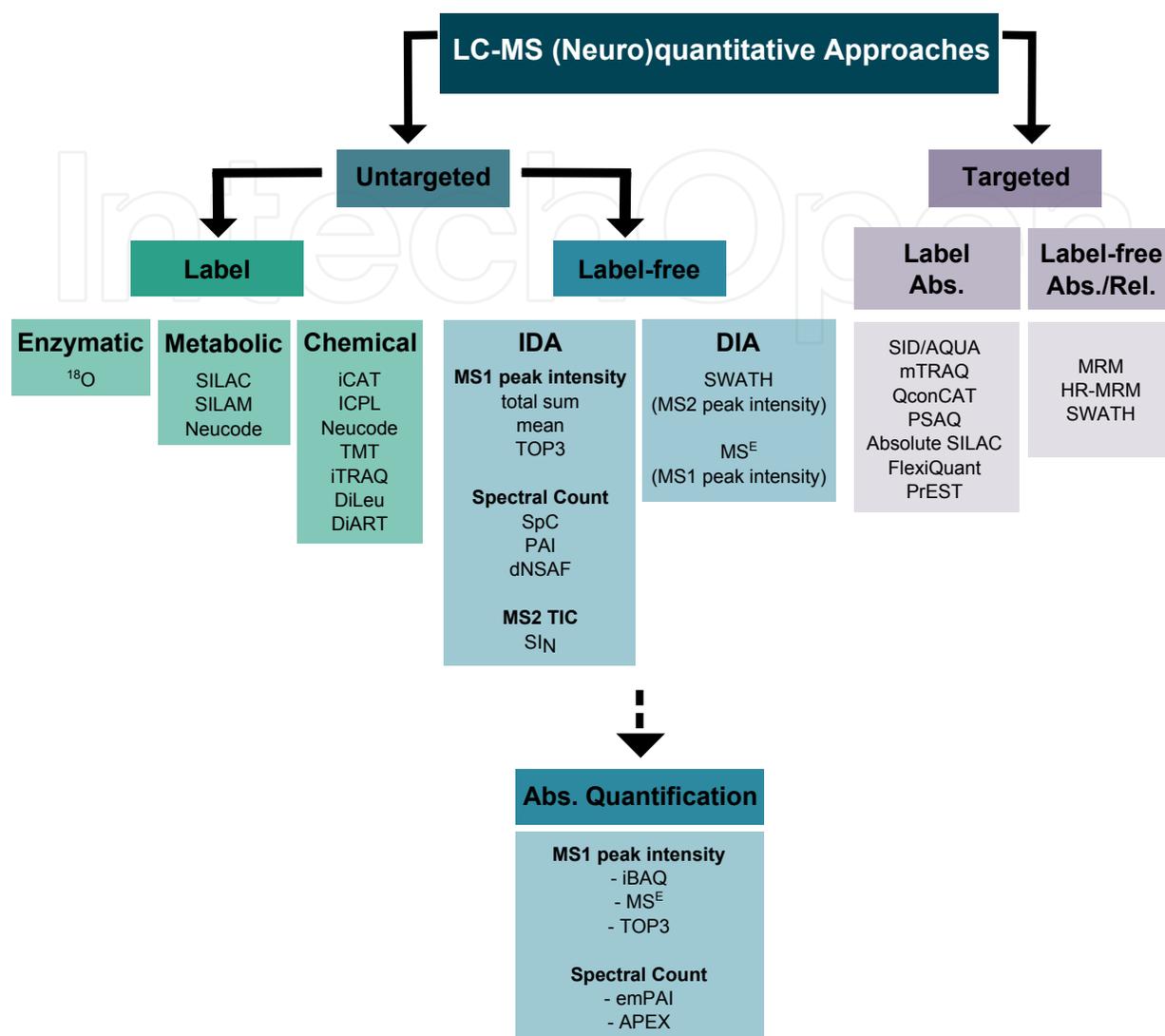


Figure 1. Diagram with brief description of the LC-MS proteomics techniques.

Therefore, over the years several methodologies were developed that support proteomic expression level quantification, and although the most popular are the so-called labeled approaches (which require the stable isotopic labeling of the samples prior to MS analysis), the label-free approaches are now gaining increasing interest mostly due to higher accuracy and sensitivity of MS instruments and improvement of the algorithms for data analysis [9].

In this chapter, a brief introduction to the different LC-MS quantitative approaches will be performed, mainly focusing on the main principle and their major achievements. Special attention will be given to the most commonly used methods in each category, and finally a revision of the literature on proteomics using those approaches will be performed, and whenever possible, examples in neuroproteomics field will be provided to elucidate the concepts.

2. Stable isotope labeling quantitative approaches

The major advantage of using MS to perform quantification instead of gel-based quantification is the possibility of slight molecular mass changes to be detectable and quantifiable by a mass spectrometer in large scale approaches and not by any other technology.

The use of stable isotopic labeling for relative protein quantification can be achieved by three different methodologies: enzymatic, as the incorporation of ^{18}O upon the protein digestion; chemical, as the incorporation of mass tags in lysines and amine-terminus of proteins or peptides; or metabolic labeling with the incorporation of heavy amino acids during protein synthesis [10].

The quantitative analysis for each approach may be performed at different levels, where some labels have mass differences that are detected (and quantified) in the precursor mass spectra (MS1), and others are based on isobaric labels that lead to peptides with the same m/z but can be distinguished (and quantified) at the fragment level (MS/MS) [9].

Each approach has its advantages and limitations, and are appropriate for different analysis depending on the biological question and on the type of sample to be used [11].

2.1. ^{18}O enzymatic labeling of peptides

The first trackable use of stable isotopes for quantification in neuronal tissue was used by Desiderio and colleagues by isotopically labeling peptide internal standards for the absolute quantification of neuropeptides [12]. To achieve this purpose, the authors used for the first time enzymatically incorporated ^{18}O (from H_2^{18}O) in the carboxylic end of the peptides [12]. Although the strategy has been used since then, it was only in 2001 that it was first reported in a study of untargeted relative quantification of the proteome of two types of adenovirus [13].

Since this first introduction, the enzymatic incorporation of ^{18}O by serine proteases has been widely used to compare the peptides produced from the protein digestion of distinct samples (usually a control sample versus a sample from the condition under study). In general, the incorporation of the heavy oxygen molecules is achieved by performing the protein digestion in H_2^{18}O using trypsin, although other enzymes such as chymotrypsin, lysine carboxylase (LysC), or GluC may also be used [13]. With this approach two oxygen atoms are introduced in the C-terminus of each generated peptide, resulting in a shift of 4Da in the mass spectra of the peptide when compared with the peptides obtained from the sample digested with regular water (Figure 2A) [14].

The advantages of ^{18}O enzymatic labeling are: the fact that virtually all the produced peptides are labeled and co-elute with the correspondent unlabeled peptide; the only reagent specifically required is H_2^{18}O ; and the procedure is easy to adapt in any proteomics lab [15, 16]. On the other hand, the procedure is labor-intensive and time-consuming; the labeling efficiency is influenced by many factors (such as pH, enzyme to be used, or the characteristics of the proteins and the peptides); and also if the ^{18}O -water to be used is less than 95% pure, some of the peptides will be labeled with only one ^{18}O , resulting in a mass spectra with both 2 Da and

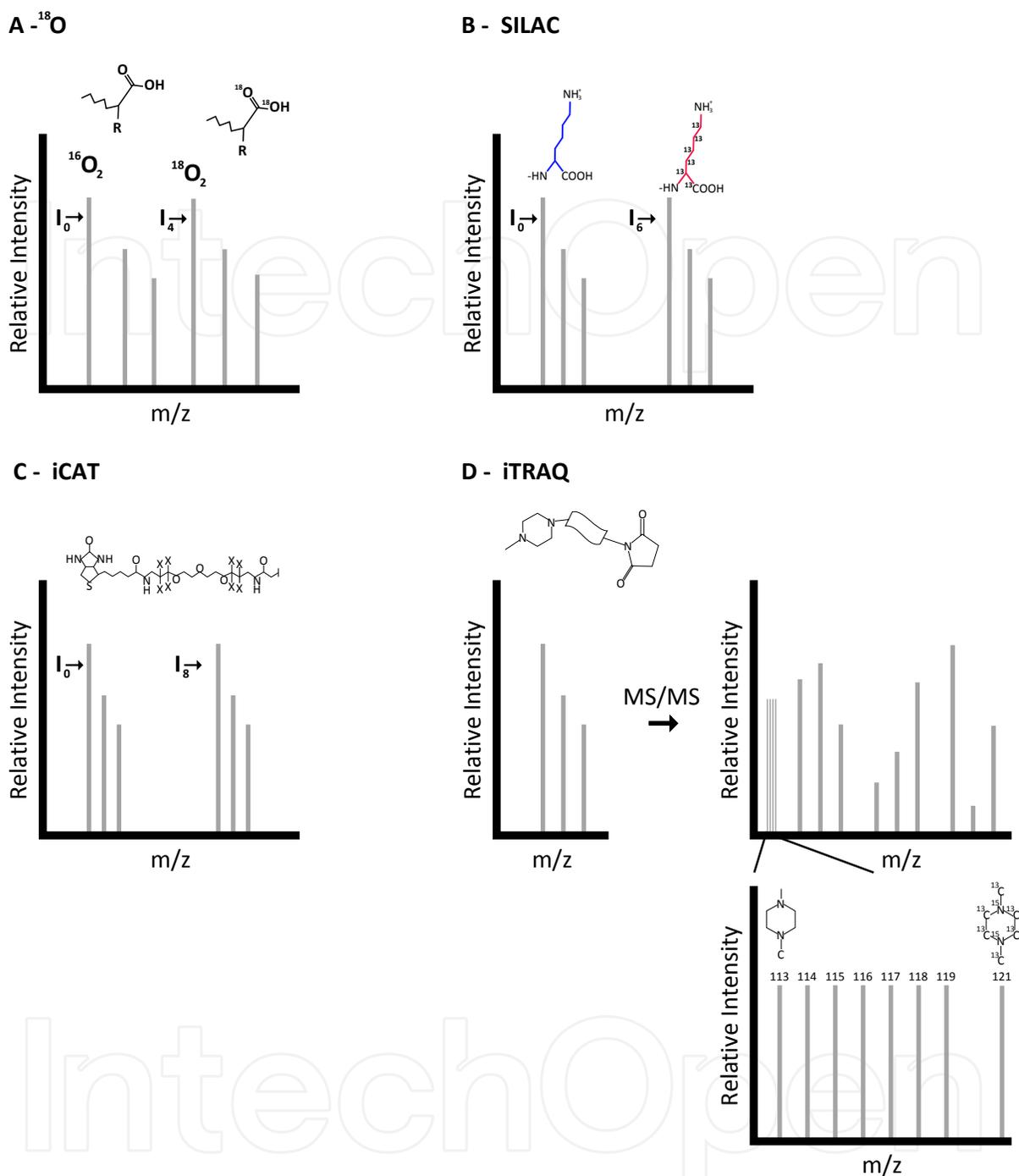


Figure 2. Representation of spectra and labelling molecules from different quantitative labeling techniques. A) Representative spectrum used for peptide identification and quantification in a ^{18}O quantitative approach with the representation of the molecules with ^{16}O and ^{18}O . B) SILAC technique with representative spectrum used for peptide identification and quantification accompanied by the representation of regular and heavy lysine. C) ICAT molecule, where the X may be hydrogen or deuterium and an example spectrum used for peptide identification and quantification. D) Representation of the iTRAQ 8-plex molecule and the spectra obtained in an iTRAQ experiment with the MS/MS spectrum that is used for identification and a zoom of the low m/z region where the reporter ions are used for quantification.

4 Da mass shifts [15]. Finally, the naturally abundant isotopes may also contribute to the peak intensities making the spectra very complex to analyze and adding the necessity for improved software for data processing [16].

In 2009, an updated ^{18}O labeling method was introduced, the acid-catalyzed labeling of the peptides, which, instead of the direct labeling of the peptides during the proteolytic digestion, it was able to separate the digestion from the labeling step, being the last performed under acidic conditions [17]. This protocol aimed to increase the distance between the unlabeled and the labeled peptide in the mass spectra (as the acidic amino acids also incorporate the heavy oxygen molecules) and also decrease the tendency of back exchange from ^{18}O to ^{16}O that was reported [17, 18]. However, the incubation in acidic conditions is prolonged, may lead to acidic hydrolysis of the peptides and deamidation of some amino acids that would increase the complexity of the spectra [18].

In order to overcome the high time consumption of the procedure, many accelerating techniques have been applied, such as heating, high pressure, or ultrasonic energy [15]. Also, other methodologies have been used, such as “inverse labeling”, which aims to decrease the influence of naturally occurring isotopes [19]. Other ^{18}O labeling approaches have been proposed throughout the years, as the incorporation of the ^{18}O molecules in cysteine (Cys) residues at the protein level by the use of ^{18}O -labeled iodoacetamide (cysteine alkylating agent) [20] or the analysis of glycoproteins after specific enrichment [21].

This ^{18}O labeling strategy has been employed to several types of samples, such as samples from the CNS, for instance, for the differential expression study of proteins in the hippocampus of rats subjected to traumatic brain injury [22] or for the quantitative profiling of CNS myelin-associated proteins in the adult mouse brain [23].

2.2. Metabolic labeling approaches

Although labeled media have been widely used in biological studies, it was only in 1999 that it was first used to evaluate protein expression by 2-DE [24] or phosphopeptides [25] in microorganisms. Nonetheless, after the introduction of SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture) in 2002, metabolic labeling approach gained higher visibility [26].

Briefly, SILAC methodology consists of growing two populations of cells, one in the presence of normal (light) medium and the other in the presence of medium that contains heavy essential amino acids [27]. The labeling of the amino acids can be achieved by substituting hydrogen for deuterium, ^{12}C for ^{13}C or ^{14}N for ^{15}N [27], and this leads to an expected mass shift in the peptides coming from the heavy medium-grown cells that is visible in the mass spectra of the peptide (Figure 2B) [28]. A shift from the first report using deuterated leucine [26] to the use of labeled lysine and arginine with ^{13}C or ^{15}N has been employed, much due to the properties of the enzyme to be used (usually trypsin or LysC). In this way, virtually all peptides in the sample will be labeled [28], and also eliminates the problem of some deuterated peptides eluting at different retention times than the unlabeled analogue [29]. In order not to introduce quantitative errors in a SILAC experiment, all the proteins must be labeled; therefore, the cells must be kept in culture with medium supplemented with dialyzed serum (to avoid unlabeled

amino acids) for at least five passages in order to have at least 97% labeling [26, 28], although a study of the labeling efficiency is advisable whenever a new cell line is used [28].

The major difference between this approach and others is that the labeling of the proteins is performed metabolically, and also the mixing of the samples to be compared is performed in the first steps of sample preparation leading to less variability in the results (Figure 3) [30]. Other advantages of the use of SILAC is its ease of use and implementation and also the possibility of multiplexing (up to 5 samples per experiment) [9, 30].

While it was proposed initially that dialyzed serum should be used to avoid the presence of non-labeled amino acids, this fact posed as a challenge for some cell culture types. In contrast, many studies have already been performed with regular serum, proving that this extra caution may not be necessary [30].

Over the years SILAC has been adapted to different cell types and with many different applications, such as the analysis of protein-protein interactions [31], identification and quantification of PTMs (for example by using methyl SILAC with labeled methionine [32]) and protein modification dynamics [33], measurement of proteome translation or turnover (by applying pulsed SILAC) [34, 35], or secretome protein quantification [36, 37].

Thus, SILAC has been applied to try to answer many neurobiological questions since it was introduced; and in the last years, many studies have been published using this technique in many different areas, such as the psychiatric field with studies of alcohol abuse [38] and schizophrenia [39]; in neurodegenerative diseases by studying the functions of Parkin [40]; or apoptosis in a neuroblastoma cell line [41].

The first rationale about SILAC was that it could only be applied to immortalized cell lines and never to cultured primary cells. However, there are now many published studies that use this technique in primary cells [9, 42], namely in primary neuronal cell lines, as in a study of neuronal phosphotyrosine proteome in response to stimulation by a neurotrophic factor [43]; in a quantitative analysis of synaptic proteins from cultured cortical neurons from a mouse model of mental retardation [44]; in the analysis of microtubule dynamics in rat hippocampal neurons [45]; or even by enabling the analysis of primary cultured astrocytes proteome and secretome [46]. Also, a strategy to diminish the number of passages necessary for the complete labeling in cultured primary neurons (60% after 6 days and 90% after 10 days) was proposed by multiplexing SILAC and using labeled amino acids for all the samples so that the protein labeling incorporation rate may be the same in both samples (because both samples will have the same heavy/light incorporation ratio) [47, 48].

In neuroproteomic studies, although neuronal-derived immortalized and primary cell lines may be considered good simplified models, the use of mammal models (such as rodents) are considered to be more complete. The general principle of SILAC was to add heavy amino acids to cells in culture, making this approach incompatible with animal models. One of the first attempts to overcome this challenge was by using the SILAC approach in cultured Neuro2A cells and then mix them with mouse brain samples to work as internal standards [49]. The first mammal to have the entire proteome labeled in vivo was a rat being fed with protein-free diet supplemented with algal cells enriched with ^{15}N [50, 51]. In 2008, the first mouse model to be

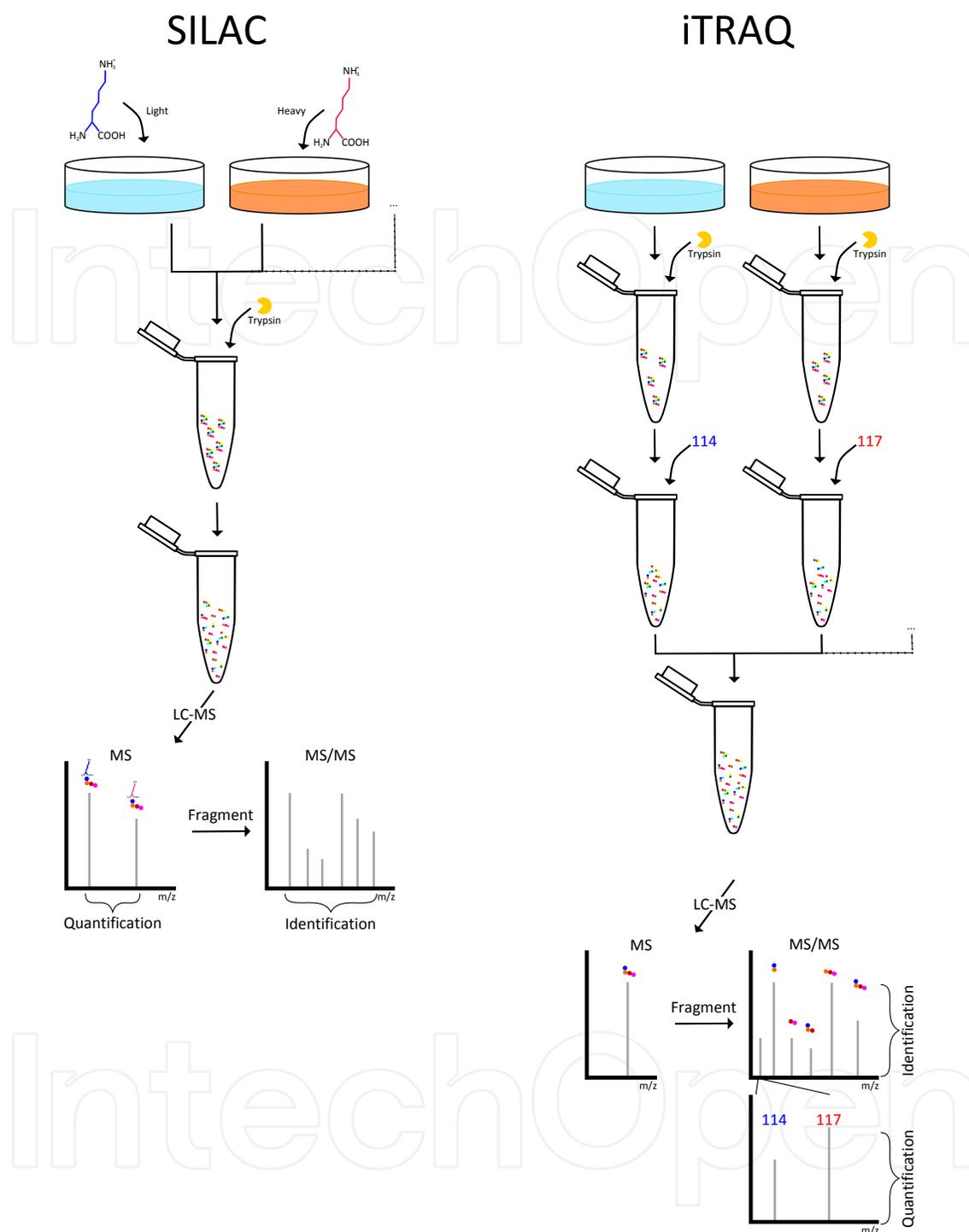


Figure 3. Comparison of the quantitative procedures of SILAC and iTRAQ.

labeled *in vivo* by using a heavy amino acid ^{13}C -Lysine [52] was introduced. This new strategy was named SILAM or Stable Isotope Labeling (by Amino Acids) in Mammals, and it has been applied in several topics of the neuroscience field as the quantification of the synaptosomal proteome of the rat cerebellum during development [53] or the proteome relative changes in barrel cortex synapses upon sensory deprivation in mice [54].

In what concerns neuroproteomics, the labeling of brain tissue *in vivo* is a great advantage, although in order to be able to completely label all proteins in the brain of rodents it is necessary to feed the animals with a special “heavy” diet at least for two generations, making this approach time-consuming and expensive [52, 55, 56]. Therefore, one of the most promising possibilities of SILAM is to use tissue from control SILAM-labeled animals as internal standards to compare between unlabeled conditions [57, 58].

Also, because of this drawback, the super-SILAC approach was introduced, where multiple cell lines are labeled with SILAC and are afterwards used as internal standards to compare with unlabeled tissue [59, 60]. This technique was firstly introduced with cancer cell lines in 2010, but it has recently been applied to the study of mitochondria from mice brain by using a super-SILAC mix of mouse brain mitochondria [61].

It was recently observed that the energy required to break down a nucleus into its component nucleons (nuclear binding energy) is different for each isotope of every element leading to a so-called “mass defect” (a mass difference of 6 mDa in the same molecule when a ^{12}C is exchanged by a ^{13}C atom and a ^{15}N for a ^{14}N) led to the hypothesis that a calculated incorporation of isotopes into proteomes would generate a MS1-centric quantification technology combining SILAC with the multiplexing capacity of isobaric tagging (see below) [62]. This new approach is named neutron encoding (NeuCode) SILAC, where peptide identifications are generated using the MS1 scans collected at 30,000 resolving power, where the same peptide with multiple labels will appear as a single peak in the spectra, whereas to obtain the quantitative information a higher resolution (480,000) MS1 scan is used, where the isotopologues can be resolved and the quantitative information extracted as for normal SILAC (with a mass shift of 36 mDa instead of 4 or 8 Da) [62]. This approach has the advantage of decreasing redundant acquisition of fragment spectra for the same precursor ion (as in classical SILAC), and because the quantitative information is acquired at the MS1 level, it is not dependent on peptides selected for MS/MS and is not subjected to dynamic range compression caused by co-isolation of precursor ions (as in isobaric labeling, see below) [62, 63].

In these first reports, the authors claim that the NeuCode approach may be used for 12-plexing by using 3-plex SILAC, each one combined with 4 isotopologues, resulting in four distinct peaks in a high-resolution spectra [62, 63], although it has already been used for 6- and 18-plex in yeast cells proteome [64]. This approach has already been used in other applications, such as C-terminal product ion annotation, based on the fact that all the y-ion in the fragment spectra will appear as doublets [65, 66], or in top-down proteomics (analysis of the intact proteins instead of peptides resulting from protein digestion) [67]. The major disadvantage of this technique is that it requires MS equipments capable of high-resolution powers ($\geq 480,000$); nonetheless, this approach is expected to be easily adapted for neuroproteomics research.

2.3. Chemical labeling approaches: Isotope techniques

The first technique using isotope labeling probes was called isotope-coded affinity tag (ICAT) and was introduced in 1999 [68]. In this approach, a specific reagent (“tag”) is added to the cysteines of proteins, once this tag has a thiol-specific reactive group, a linker with 8 deuteriums in the heavy form, and a biotin affinity tag [68]. The procedure is simple and based on some

basic steps: first the protein extracts must be isolated and the cysteines reduced, then the proteins are labeled with the heavy or light ICAT molecule and joined for protein digestion; the labeled peptides are enriched with an avidin affinity chromatography and analyzed by LC-MS, where for each precursor a pair of ions will be visible with a mass shift in MS1 mass spectra (Figure 2c) [68].

This first ICAT molecule was designed with 8 deuteriums leading most of the times to a difference in retention times of the homologue peptides, where the labeled peptide does not co-elute with its unlabeled pair, making the spectra analysis very difficult. Also, this mass difference of 8 Da may be confused with other biological modifications (such as a peptide containing 2 cysteines and an oxidation of methionine, both leading to a 16 Da mass shift).[69] On the other hand, the ICAT tag itself was quite large contributing with a mass addition sometimes bigger than advisable and leading to many fragments in the MS/MS spectra, complicating the identification of the peptides' sequence [69].

Due to these limitations of the initial approach, new strategies were introduced based on the same principles, but with a cleavable site introduced to the tag [69, 70] or also the possibility of labeling the sample in a solid-phase format [70]. This new cleavable ICAT (cICAT) has an acid-cleavable linker group connecting the biotin with the thiol-reactive isotope tag and uses 9 ^{13}C instead of the 8 deuterium, this way, after labeling and chromatographic enrichment, the biotin moiety is cleaved giving rise to a smaller modified peptide [69].

This ICAT strategy has already been applied for different approaches as the creation of aldehyde-reactive tags (hydrazide-functionalized) isotope-coded affinity tag (HICAT) for the identification and quantification of lipid-conjugated proteins [71].

This isotope-labeling technology has been applied in several neuroscience projects such as the study of the influence of aging in the proteome of CSF (cerebrospinal fluid) [72], the study of differential mitochondrial proteins analysis in the pathophysiology of Parkinson's [73] or Alzheimer's diseases [74], and also to aid the study of the expression of synaptosomal protein in cerebral ischemia [75], migraine mouse models [76], or in the study of addiction [77].

The greatest limitation of this approach is the fact that only peptides containing cysteines are labeled and enriched, making these the only candidates for protein identification and quantification, leading most of the times to poor sequence coverages. For this reason, a similar strategy, ICPL (isotope-coded protein labeling) was developed, which, instead of labeling sulfhydryl groups labels all free amine groups [78]. This strategy is very similar to ICAT, with the exception that it has specificity for primary amine groups (lysine side chains and N-termini), and has no biotin moiety so the option to enrich labeled peptides does not exist. On the other hand, it is expected that at least 70% of all peptides will have labeled lysines [78, 79], or virtually all the peptides if the labeling is performed after digestion (post-digest ICPL) [79, 80].

This post-digest ICPL can be combined with other fractionation methods such as IEF prior to LC-MS [81] or even with enrichment of peptides with specific PTM's as phosphorylation or glycosylation [82].

The original ICPL molecule could be multiplexed for three samples where the molecule had 0, 3, or 7 deuterium (d0, d3, and d7 molecules, respectively) [78], but is commercialized in a 4-plex version allowing the labeling with 0, 4, 6, and 10 Da mass shifts and may be labeled with deuterium or ^{13}C [9]. Although this approach is not widely used it has the capacity to be applied successfully to any protein samples, and it has already been used to study the proteome of postmortem prefrontal cortex from control and schizophrenic patients [81, 83] and in biopsy tissue samples from patients with glioblastoma [84].

As for SILAC, very recently, the NeuCode strategy described above has been applied to chemical labeling with the development of an amine-reactive mass tag that takes advantage of the differential neutron-binding energy between ^{13}C and ^{15}N isotopes that enables up to 12-plex MS1-based protein quantification [63]. Another NeuCode approach proposed is to use carbamylation of amine groups via urea isotopologues for protein/peptide labeling, and therefore relative quantification [85].

2.4. Chemical labeling approaches: Isobaric techniques

All the methods described above use isotopic labeling of the proteins or respective peptides, this way the calculation of the relative amounts is achieved by the analysis of the intensity of the precursor ion peaks at the MS1 spectra. In 2003, a revolutionary variation of these techniques was introduced where the mass tag that was added to the peptides is isobaric, making all the precursor ions from the samples in study appear as a single peak in MS1, but upon fragmentation it leads to the formation of reporter ions separated by 1 Da coming specifically from each of the samples [10]. The first approach applying this principle was called Tandem Mass Tag (TMT) and in this first report synthesized peptides with the tag were used [86]. A year later another approach was described, the isobaric tags for relative and absolute quantification (iTRAQ). This concept was applied for the first time to label global proteomes (yeast in this case) and even with the advantage of allowing the simultaneous analysis of 4 samples (iTRAQ 4-plex) [87].

The molecule used to tag the proteins or the respective peptides after digestion for both approaches, iTRAQ and TMT, has three main components and the principles are the same, although structurally different between the two methods (Figure 2D). The molecules are constituted by an amine-reactive group, which links the reagent to lysines and N-termini of the proteins or peptides; by a reporter group, which has differential labeling with isotopes (^{13}C , ^{15}N or ^{18}O) and is, upon fragmentation, the monitored ion for quantification in the MS/MS spectra; and also a balancer group, which aims to keep the overall mass of the reagent equal among all labels and is also differentially labeled with isotopes. [10]

A few years after this first introduction of TMT and iTRAQ, the neuroproteomics field had the highest multiplexing usage of these approaches, in this case by studying proteomic changes in CSF of patients with Alzheimer's disease undergoing intravenous immunoglobulin treatment with iTRAQ 8-plex [88] and by comparing CSF proteome in postmortem versus antemortem drawing of the samples using a 6-plex TMT approach [89]. In 2012, upon the substitution of a ^{13}C for a ^{15}N in two of the 6-plex tags it was noticed that the new tags were 6.32 mDa lighter, this way an 8-plex approach was developed even without changing the

structure of the molecule but only by changing the isotopologue used [90], and it was hypothesized that a 10-plex or even 18-plex approach was possible [90]. In fact, TMT is commercially available in four different kits (TMTzero, TMTduplex, TMTsixplex, and TMTtenplex), whereas iTRAQ is commercially available in two versions (iTRAQ 4-plex and iTRAQ 8-plex).

The TMT tags give origin to reporter ions in the 126–131 Da region of the MS/MS spectra and the molecules used in all the available kits have the same structure. On the other hand, the iTRAQ 4-plex and 8-plex molecules, which generate ions in the 114–117 Da and 113–121 Da (except the 120 because of phenylalanine immonium ion contamination), respectively, have different structures [10, 91].

Isobaric labeling of the proteins and quantification at the MS/MS level (outlined in Figure 3) has the advantage of each precursor ion appearing as a unique peak leading to an increase in sensitivity both at the MS and MS/MS level with no increase in mass spectra complexity [87, 92]. On the other hand, it is now known that the reporter ions of isobaric tags are prone to ratio compression, meaning that together with the target precursor ion some contaminating near-isobaric ions can be co-isolated and fragmented, contributing to reporter ion intensity and biasing of the quantitative information [92, 93]. This fact leads to a ratio compression around the unit, because when reporter ion intensity has interference from reporters coming from peptides derived from proteins with unchanged expression, the ratio between the two samples tend to be 1 [93]. To overcome this drawback, an MS3 strategy has been developed [93], as well as its combination with synchronous precursor selection (SPS) [93], and although with these strategies the accuracy and precision is enhanced it comes with the cost of a reduction in the number of proteins quantified [11].

Some comparative studies have been performed between the different isobaric methodologies, and when comparing 4-plex with 8-plex iTRAQ, the latter led to more consistent ratios without compromising peptide identifications [91]. On the other hand, in another report, when comparing TMT 6-plex with the two versions of iTRAQ, the 4-plex iTRAQ performed better in terms of peptide identifications and similarly in terms of precision of peptide-spectrum matches [94]. These discrepant results may be due to the use of different equipments and softwares for data analysis. [10]

The amine reactive tags were the first ones to be developed and are also more commonly used. However, new molecules have been developed to label other protein residues or PTMs. Both methods have been adapted for these applications, TMT has been adapted for iodoacetyl cysteine-reactive tandem mass tags (iodo-TMT) to identify and quantify S-nitrosylated peptides [95], carbonyl-reactive TMT (glyco-TMT), which may be used with two different chemistries, either aminoxy-TMT or hydrazide-TMT, and enable quantification both at the MS1 (coded with isotopes) and the MS/MS (coded with isotopic reporters) [96]. iTRAQ has also been adapted for the detection and quantification of carbonylation of proteins by means of functionalizing the iTRAQ molecule with hydrazine (iTRAQH) [97], for phosphoproteome identification and quantification (phospho-iTRAQ) [98], and also for identifying new N-termini generated by proteases in a strategy combining iTRAQ with terminal amine isotopic labeling of substrates (TAILS) [99, 100].

TMT and iTRAQ technologies have been extensively used by the scientific community to answer several biological questions and applied to almost all types of samples. In neuroproteomics, these approaches have been extensively used to characterize the differential proteomes of neuronal disorders, drug responses or brain regions. Many studies have been performed using these techniques in areas such as neurodegenerative disorders, as the study of the putamen proteome of an MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) monkey model of Parkinson's disease [101] or in the serum of Parkinson's disease patients [102], or even in the analysis of synaptosomes from cortical brain tissues from Alzheimer's disease patients [103]; in neuropharmacoproteomics, as in the examples of a study of protein quantitative alterations induced by antidepressants in the hippocampus of mice [104]; also in addition as in the evaluation of the effects of administration of plasminogen activator after ischemic injury in mice [105] or the alterations upon chronic exposure to cocaine [106]; in neuropsychiatric and other CNS disorders, such as schizophrenia, with the study of protein expression in the thalamus and CSF of patients [107] and a study of neurofibromin knockdown PC12 cell line as a model of neurofibromatosis [108]; or even in studies of neuronal function such as memory formation in hippocampus [109].

Once, these commercially available isobaric tags were expensive and laborious to produce, in 2010 two new isobaric approaches were proposed, *N,N*-Dimethyl Leucines (DiLeu) [110] and deuterium isobaric aminereactive tag (DiART) [111], which should serve as cost-effective alternatives to iTRAQ and TMT [10].

DiLeu was developed inspired by the chemical isotopic labeling by formaldehyde dimethylation of lysines [112], which is an inexpensive approach, and the aim is to combine it with isobaric labeling and quantitation at the MS/MS level [110]. This way a 4-plex set of dimethylated leucines for amine groups labeling was developed, and has a structure similar with the other isobaric approaches, with an amine-reactive group, a balance group, and a reporter group (115–118 Da) [110]. DiLeu has a labeling efficiency similar to iTRAQ and generates reporter ions with higher intensity; nonetheless, this approach requires an extra step of activation of the reagents prior to the labeling reaction because it uses a different chemistry [10, 110], and is also prone to the co-isolation of precursor ions (as iTRAQ and TMT). Recently, DiLeu was used to test if the implementation of ion mobility MS would mitigate this phenomena [113].

The DiLeu strategy has already been applied to study the neuropeptidome of a crustacean species [114], and for relative quantification of amine-containing metabolites [115]. A 12-plex DiLeu strategy has been introduced that takes advantage of changing isotopologues in the reporter groups, similarly to NeuCode or TMT 10-plex [116].

DiART was designed as a less expensive 6-plex isobaric labeling reagent to label amine groups of proteins and peptides and is, once more, based in a very similar structure as iTRAQ and TMT using an amine-reactive group, a balancer group, and a reporter group in the mass range of 114–119 Da. [111, 117] In a study comparing DiART and iTRAQ, the authors found that DiART leads to more intense reporter ions and consequently less ratio compression, however with the DiART approach, the common fragmentation method is not advisable due to easy

reporter ion fragmentation [118]. DiART has also proven to be compatible and valuable for PTM analysis as quantitative phosphoproteomic studies [119].

Although isotopic and isobaric techniques are based in different methods of quantification and have strengths and drawbacks, both have proven to be valuable for quantitative proteomics [11] and the combination of several methods has been applied to increase throughput of the analysis. This combination is called hyperplexing, because it enables the simultaneous analysis of a higher number of samples, such as with the combination of metabolic 3-plex labeling with isobaric 6-plex TMT that enables the analysis of 18 samples [120], also it is expected that by combining different strategies an even higher throughput and more reproducible results will be achieved [10]

3. Label-free approaches

As an alternative to the labeled methods, several label-free approaches (Figure 4) have emerged, some of them with comparable accuracy to the labeled methods and all of them with similar or higher proteome coverage and dynamic range [121, 122]. These methods gained popularity mainly due to their low cost, their simple sample preparation, the unlimited number of samples that can be compared, and their multiple applications [121]. These attributes turn label-free methods into a powerful technique for clinical applications and large screenings. However, as samples are analyzed separately, these types of methods are highly dependent on run-to-run reproducibility, therefore sample preparation and analyzes should be well implemented and standardized. Furthermore, the methods rely also on the software capacity for both data extraction and capacity to accommodate errors [123, 124].

In general, label-free approaches can be divided into two distinct groups according to the method used for data extraction. On one hand, the quantification can be inferred by counting the number of peptides or spectra assigned to a given protein, and therefore are generically called spectral counting methods. On the other hand, when liquid chromatography is coupled with mass spectrometry, quantitative values can be measured through the extraction of the area of the precursor ions' chromatographic peaks - area under the curve (AUC) or MS1 signal intensity methods. [121-123]

Traditionally, label-free methods were associated with the commonly used shotgun approaches, where mass spectrometry instruments operate in a data-dependent acquisition mode (DDA, also called information-dependent acquisition or IDA) (Figure 4A). Therefore, these methods have also the advantage of being used in data previously acquired for protein identification [125, 126].

In this type of experiments, the instruments are set to scan the precursor ions followed by the selection of a limited set to be fragmented, usually the most intense ones. The fragmentation spectra (MS/MS spectra) obtained will then be used for peptide identification. Independently of the method used to extract quantitative information, the mass spectrometers working on IDA mode must be fine-tuned in order to acquire enough data to perform both the identifica-

tion and the quantitative analysis [127]. This is particularly important for MS1 quantification methods, where enough points per chromatographic peak to perform an accurate extraction should be acquired, without misplacing the acquisition of good fragmentation spectra that allows peptide's identification. Although this balance is not so crucial for the spectral count methods, it is also important to have a good balance between survey and fragmentation scan in order to be able to achieve a higher proteome coverage. Therefore, the development of mass spectrometers with faster scans combined with higher resolution power has been fundamental for the increase in the use of label-free approaches [122, 125].

Label-free methods still rely on peptide identification, the IDA experiments tend to be biased to the most abundant proteins and are highly affected by sample complexity/dynamic range. Therefore, the use of data-independent acquisition (DIA) methods, where fragmentation spectra is acquired for the entire sample without any pre-selection of precursor ions, soon started to be used for label-free quantitative approaches as an alternative to the limitations of IDA experiments [122, 126].

Finally, although label-free approaches are mainly a method for relative quantification (Figure 4B), several groups have also taken efforts to evaluate the relationships between label-free measurements and absolute quantification (Figure 4C) of proteins in complex samples. And in fact, several adaptations came out as good correlations between label-free measurements with protein concentration, allowing the use of label-free methods for the determination of the absolute abundance of a protein [122, 128].

3.1. Spectral counting-based label-free methods

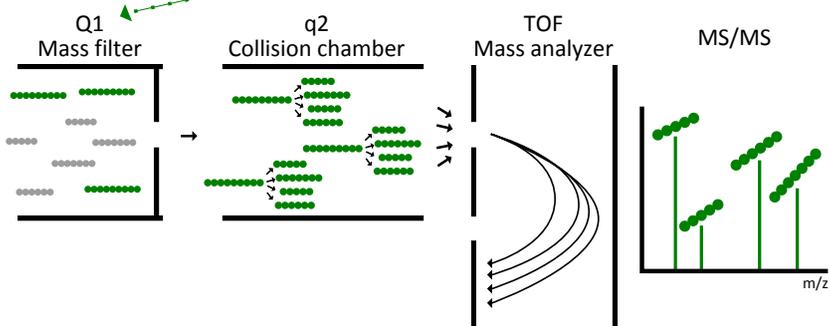
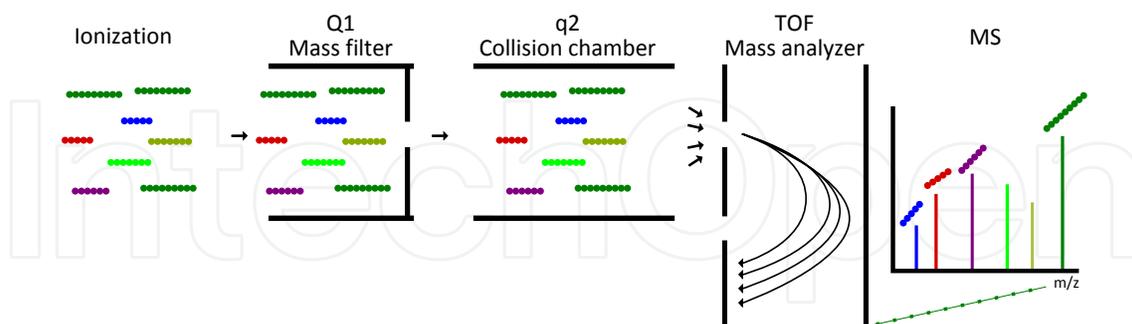
Spectral counting methods consist of simply counting of the number of peptides and/or fragmentation spectra of a particular protein, and comparing the value between conditions. Within this group of label-free methods, it is possible to distinguish some different types: 1) those that are based on unique peptide counting; 2) those based on MS/MS counting (SpC); and finally, 3) an adaptation of spectral counting, spectral TIC counting (MS2 TIC) [132].

3.1.1. Peptide counting and Spectral Counting (SpC)

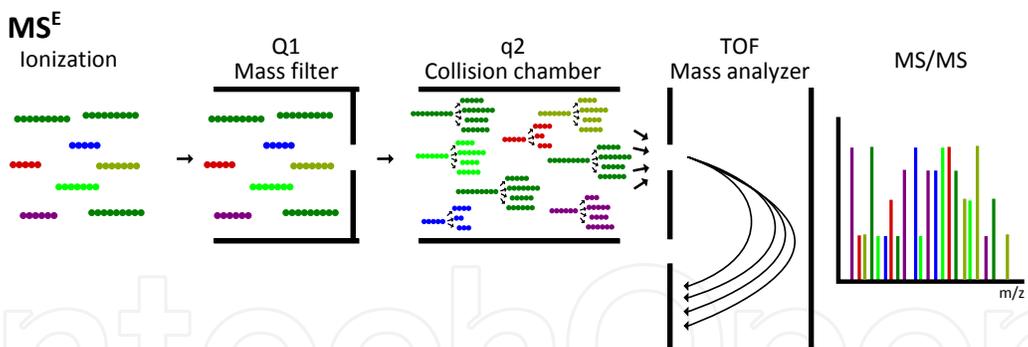
The correlation between the number of peptides acquired in an IDA experiment with the protein abundance was firstly reported in 2001 by Washburn and colleagues [133]. In this work, the authors used the codon adaptation index (CAI) as a measurement of the protein abundances, and correlated CAI ranges with the number of proteins identified and the number of peptides identified per protein. CAI relies on the evidence that mRNAs of highly expressed proteins preferably use some codons (those of which the tRNAs are present in the greatest amounts) rather than others specifying the same amino acid [134], and at that time it was already proved to correlate well with protein levels [135]. With this assessment, Washburn and colleagues were able to note that the most abundant proteins were identified with multiple peptides, while for the low abundant proteins the identification was achieved based on one or two peptides. Although no special focus at the quantitative level was performed, this evidence would be the basal principle of the spectral counting approaches [133].

A-Acquisition modes

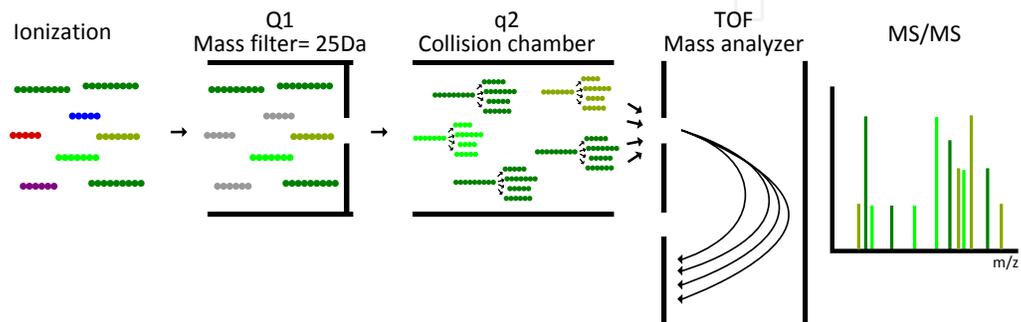
IDA



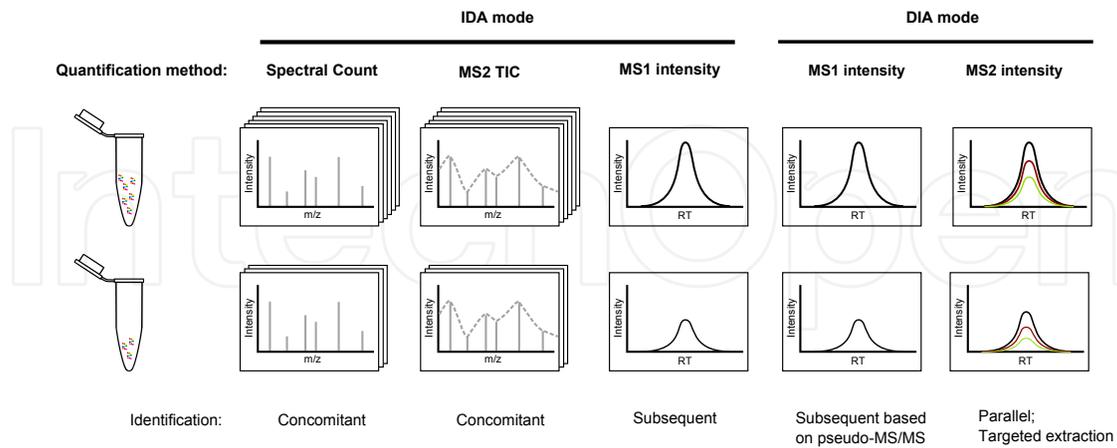
DIA



SWATH-MS



B-Relative Quantification



C-Absolute Quantification

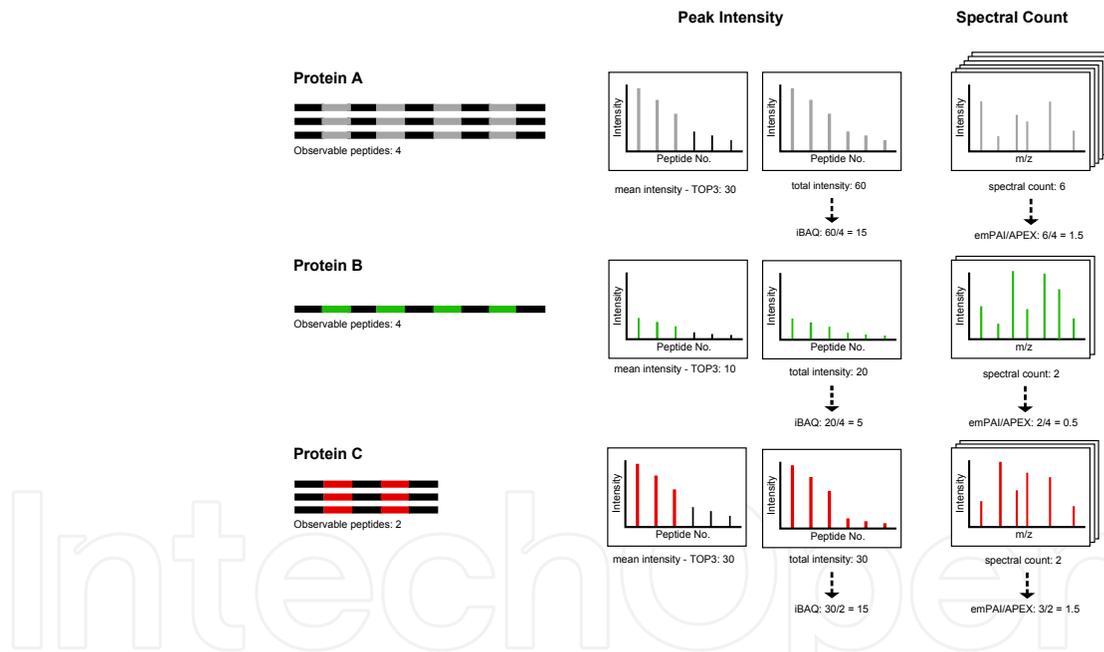


Figure 4. Overview of the label-free MS-based quantitative methods, instrumental principles and data analysis. (A) Comparison of the MS instrumental principles of the acquisition modes most commonly used in label-free approaches: 1) Information Dependent Acquisition (IDA) where fragmentation spectra are only acquired for a group of selected precursor ions based on their intensities; versus 2) Data Independent Acquisition (DIA) where fragmentation spectra is acquired for all the precursor ions independent of its intensity. Fragmentation spectra can be acquired for the entire mass range simultaneously (MS^E) or by covering the mass range in sequential smaller windows of defined size (SWATH-MS). (B) Schematic representation of the different label-free approaches for relative quantification. In the spectral-counting (SpC) approach, peptide/protein abundances can be estimated based on the number of identified MS/MS spectrum. In the MS2 TIC approach, peptide/protein abundances can be estimated based on the mean of the TIC (sum of all the fragments in a given MS/MS spectra) off all the identified MS/MS spectrum. In the precursor ion-intensity-based approach (for both IDA and MS^E method), the changes of peptide/protein abundances are determined by measuring and comparing the chromatographic peak areas of the corresponding peptides. The changing peptides

are subsequently identified based on the respective MS/MS spectra (IDA) or a recomputed pseudo-MS/MS spectra (DIA). In the SWATH-MS approach, changes in confident peptides/proteins are determined based on the fragment ion intensities (MS2 intensity), designed as peak groups of each precisely identified peptide. In this example, results would indicate a higher peptide abundance in State A. (C) Representative examples of label-free methods for absolute quantitative proteomics. In the case of the strategies based on MS1 intensities, the average of the three most intense ions (TOP3) and the iBAQ index are used to generate reliable absolute quantitative data. In the strategy based on spectral count, both emPAI and APEX strategies used the number of identified peptides normalized for the expected number of peptides (to reduce the impact of protein size) as an indicator of the protein abundance. As an example, proteins A and C, present at the same abundance, have different spectral counts but they present the same normalized spectral count. Adapted from [129–131].

At the end of the same year, the first quantitative report based on the spectral counting principle was published by Pang et al. [136]. In this work, the authors introduced the concept of peptide “hit” (now known as peptide hits technology or PHT [137]) as a measure to estimate the relative changes in protein abundance. In this method, each hit corresponds to one identified peptide and the protein abundance is calculated by summing all the hits. The method assumes the principle that the coverage of the protein increases in proportion to the protein abundance, which is reflected in the number of peptide hits of a given protein. In the same report, the authors applied this quantitative method to the identification of biomarkers for inflammation in urine samples of healthy vs. disease conditions, and performed a comparison between the proposed approach and the usual quantitative 2D-gel approach. Similar quantitative results were obtained between the methods studied, with a significant increase in the number of the proteins analyzed in the gel-free approaches combined with a significant reduction in the required amount of sample and sample processing [136].

In 2003, Gao et al. [138] applied for the first time a statistical method (Student’s t-test), already widely used for gene array experiments, in peptide hits quantitative data in order to quickly assess with statistical significance the abundance changes^o between treatments/conditions. The use of such method into quantitative proteomics was evaluated in a widely used biological system by performing a comparison with the results obtained in previous reports, revealing a high degree of concordance. Therefore, the use of such statistical evaluation can quickly highlight the proteins that are in fact altered from the entire data set of proteins analyzed in larger screenings, turning the data analysis into a more automated and reliable method [138].

After the initial report using peptide hits as a quantitative measurement of protein levels [136] and following the same principle stated in that work, some adaptations to that quantitative method started to appear in order to take into account the protein characteristics that could influence the results. Matthias Mann’s group was a pioneer in the development of such adaptations, with the first adaptation appearing in 2002 by Rappsilber and collaborators [139]. In this work, the authors characterized the human spliceosome by an exhaustive identification of the constituents of that multiprotein complex, and by obtaining the relative abundance of the different classes of proteins involved. In order to do so, the authors presented a new method to quantify protein levels, the protein abundance index (PAI), which consists of the number of MS/MS spectra identified divided by the number of theoretically observable peptides, i.e., the theoretical peptides that will feat in the mass range of MS [139]. By considering the theoretical number of peptides that can be formed from a given protein, the authors compensated the impact of the protein size, since larger proteins can give rise to more peptides within

the MS mass range. However, once the authors considered all MS/MS spectra that originated positive identifications from peptides acquired with different charge states to modified peptides, the measured values also reflect the response of a given protein to the measurement procedure and not only its abundance.

Soon, label-free approaches being performed in comparative screenings and some alternative methods based on the principle stated above started to emerge. At the same time, two independent studies focused on the proteome changes observed in the development stages of the human malaria parasite *Plasmodium falciparum* were published presenting two alternative methods to evaluate these proteomics changes. While Florens and collaborators [140] compared the protein sequence coverage between the development stages to estimate protein relative abundance, Lasonder and colleagues [141] used the total number of unique peptides identified and introduced the use of the extracted ion chromatograms (XIC) of individual peptides as a method to confirm the absence or presence of a particular protein. With the use of the MS-XIC evaluation, the authors overcame one of the limitations of IDA experiments where it is possible that a peptide is not selected for fragmentation in a particular sample due to changes in sample complexity [141].

Another disadvantage of spectral counting is that the length of the protein influences the number of theoretical peptides that can be produced from tryptic digestions [142, 143]. Therefore, in order to overcome this limitation, several modifications were proposed to take into account the protein size [121]. The most widely used is the normalized spectral abundance factor (NSAF), proposed by Zybaylov in 2006 [144], which consists of the normalization of the SpC of a given protein by the protein length (L). These values are further normalized by the sum of the SpC/L for all the proteins analyzed, thus taking into account the experimental variation. Furthermore, this method presents a high dynamic range (~4 orders of magnitude) and is able to measure smaller variations (lower than 50% variation) [144]. This method was revised by the same group, presenting an improved NSAF approach that is able to deal with peptides shared between proteins and the distributed normalized spectral abundance factor (dNSAF) [145].

The use of shared peptides for quantification has been a critical issue since the abundance of a peptide that is shared across proteins depends on the contributions of the multiple proteins to which it belongs [127, 146]. Therefore, it is incorrect to overestimate the protein abundance by counting the shared peptides multiple times, typically these peptides are simply ignored in protein-level quantification analysis [147]. However, this may significantly decrease the number of proteins for which it is possible to estimate its abundance (as much as 50%) [146]. Thus other approaches have been used to include these peptides. Some approaches try to assign the shared peptides for a particular protein (the most abundant of the group) by taking into account parameters such as the number of unique peptides to calculate the relative abundance of each protein [148, 149]. dNSAF is perhaps the most known example of such type of adaptation [145]. Finally, some authors also proposed to analyze the proteins that have shared peptides as a protein group and not individually. However, these proteins can present different regulatory mechanisms, therefore their combination fails to estimate the real variation [150].

3.1.2. Spectral TIC (MS2 TIC)

In 2008, Asara and colleagues [142] presented a new method for relative protein quantification that could be considered an extension of the spectral counting technique. In this approach, the average of the TIC for all of the MS/MS spectra that identified a protein was used as a quantitative measure. Each spectral count gets a unique abundance value, which consist of the sum of all the fragments in a given MS/MS spectra, instead of being just counted as one event. In this study, the authors proved that this “spectral TIC” method was effective and expanded the dynamic range of quantitative ratios allowing for larger protein abundance [142]. This would allow to overcome one of the limitations of the spectral counting, its intrinsic tendency to easily reach the saturation for the most abundant peptides, not being able to quantify properly large protein ratio differences, and limiting the dynamic range of the method [122]. In this approach, the authors counted all the MS/MS spectra that resulted in positive identification and the average was used, instead of the sum of the TIC, in order to overcome the sampling bias caused by different protein molecular weights (larger proteins generate more tryptic peptides than smaller proteins). The proposed method was tested by evaluating its capacity to reach the theoretical ratio of a known digestion mixture, and comparing it with other quantitative methods already well established. With this comparison, the authors showed that the spectral TIC has a similar accuracy to the AUC methods and is able to correctly calculate large variations [142] and detect relative changes in low abundance proteins [151].

This method had some improvements; it was combined with data from the SpC method in order to obtain a better characterization of the samples [152], also Griffin and collaborators proposed a new normalized label-free method that combines the three MS abundance features, namely the peptide and spectral counting with the TIC intensity [153]. This method, termed normalized spectral index (SI_N) combines the reproducibility already presented by spectral counting methods with an increase in the accuracy of the determination of protein abundance observed in TIC intensity methods. Furthermore, by correcting it for protein length, it also reduced the samples bias to large proteins [153].

3.2. MS1 signaling intensity or Area Under the Curve (AUC)

Bondarenko and Chelius [154, 155], in 2002, were the pioneers of the use of MS1 signal intensity as a measurement of protein levels. Bondarenko, in his technical work, tested the hypothesis that peak area of the peptides should reflect its concentration and therefore those peak areas should correlate with protein concentration. To test that, different amounts of a pure protein were analyzed, alone or spiked in a complex mixture, and the extracted peptides' areas were compared with peptides' concentration, revealing a high degree of correlation even in samples with high complexity. Furthermore, the authors also proposed the use of a correction factor designed as experiment-dependent correction factor that aimed to reduce the impact of some experimental parameters, such as differences in sample preparation, that could lead to some bias of the results. The use of such correction factor, which is determined from the mean tendency of the non-variable proteins, proves to improve the accuracy of the quantification [155]. Therefore, the use of normalization methods became a key feature in label-free quantification, and several alternatives have been proposed. Those alternatives can be divided into

two groups based on their basic principles. On one hand, some normalization methods are based on the principle that a large portion of the proteome does not change, therefore the mean tendency between experiments can be used to accommodate some experimental deviations. On the other hand, the normalization for housekeeping proteins, a protein or set of proteins known to be constant, or for an internal standard added to the samples before sample processing can be used since both will reflect the effect of sample processing [155, 156].

The MS1 intensity label-free methods are highly accurate since they require the use of high-resolution mass spectrometer in order to be able to distinguish the co-eluting species [121, 152]. Protein quantification based on AUC requires the comparative measurement of precursor ions intensity at a particular retention time, therefore this type of quantitative methods is also dependent on the power of data extraction algorithms, and several different methods are already available [121, 125, 130]. Independently of the software used, the data analysis of MS1 intensity peaks generically comprises a set of defined steps: feature detection, alignment of retention times, peak picking, noise reduction, and normalization of MS intensities [121, 130]. The detected and normalized peaks are then compared between the samples and their MS/MS spectra are used for protein identification [121]. The estimation of the protein abundance can be obtained mainly by three different strategies: by summing all the peptides considered in the analysis; by performing the mean of all the peptides; or considering only the 3 most intense peptides (usually using the mean value), the so-called TOP3 method [157, 158].

Since quantification is done at the MS1 level, the estimation of protein abundance is not dependent on the acquisition of a particular MS/MS spectra in all the experimental conditions. In fact, a given peptide can be identified in a single sample and quantified across all the remaining samples [141]. Thus, these methods are not so prone to the variability associated with variation of sample to sample complexity. This characteristic, associated with the unlimited number of samples to be compared, enables MS1 intensity methods as suitable methods for clinical biomarker discovery, which normally requires high sample throughput [125].

Due to the large number of modified methods and the generalized use of the terms spectral counting and peak intensity to include all the modifications, it is not always clear which particular method was used in a given experiment. Therefore, to simplify the categorization, the reports are commonly grouped in these two generic categories, taking into account only their basic principles.

The use of spectral counting and/or MS1 intensity methods in neuroproteomics is vast and usually alternates between the use of one type or another [9, 159, 160]. However, some reports combine the two methods to improve the results obtained, such as in the case of the interactomics study of the AMPA receptor performed in collaboration with our group [161] where both spectral counting and MS1 intensity were used to identify the truly positive interactors from the negative control. Within the areas where label-free methods are being used, it is possible to identify some general studies focused on understanding proteomics changes in brain regions, such as the evaluation of frontal cortex changes caused by frontotemporal lobar degeneration (FTLD) [162]; cell- and tumor-specific alterations, such as the comparison of astrocytes and astrocytoma proving evidences for the existence of important membrane

biomarkers capable to define the cell lineage of the tumor [163]; and characterization of protein architecture of secretory vesicles, key components that mediate intercellular signaling [164]. Although, regarding label-free approaches, the neuropsychiatric field is dominated by the use of MS^E, in the study of neurodegenerative diseases (such as Parkinson's, Alzheimer's, and Huntington's diseases) there is an evident tendency of spectral counting/peak intensity methods [9] in both the analysis of cell and animal models (both obtained by the use of chemical and genetic alterations) [165–167] and also CSF [168] and postmortem tissues (mainly in the case of Alzheimer's disease) [169, 170]. Those proteomics screenings led to the identification of several deregulated proteins, contributing to an increase in the understanding of the pathways that are altered in those disorders.

There is an inherent tendency for a larger number of spectral counting reports [162–165, 167, 169, 170] when compared with the peak intensity reports [166, 168], as observed from the examples stated above. This underestimation of peak intensity reports from IDA experiments is associated with the preference from the alternative peak intensity methods based on DIA acquisition (such as MS^E). Although in reduced numbers, there are also some reports on the use of MS2 TIC methods, more specifically SI_N, in the neuroproteomics field. As an example of its applicability, there are two studies involving brain tumors. In one study, the authors performed a characterization of the differentiation states of glioblastome stem cells (cells responsible for tumor formation and growth [171]), in the other study the authors were focused in the analysis of the secretome of glioma cells in order to identify the proteins that could be involved in tumor cells migration [172].

Further quantitative neuroproteomics studies were already summarized in several reviews [9, 159, 160].

3.3. Data-independent acquisition methods

As stated above, in order to overcome the limitation of the use of IDA modes, alternatives with DIA are starting to be used. The major advantage of this acquisition mode relies on its ability to record fragmentation spectra from the entire set of precursors of a given sample, without any selection that can bias the acquired data. However, in these experiments, the data analysis is very challenging, since the link between the precursor and its fragments is lost [173], these methods are highly dependent on the development of algorithms capable of extracting valuable information from the data acquired [174].

These methods operate in a cyclic mode, throughout the entire liquid chromatography (LC) time range, by alternating between survey and fragment ion spectra. Generically, these methods can be divided into two distinct groups, those that acquire the fragmentation spectra of the entire mass range simultaneously, and those that scan the *m/z* range in sequential isolation windows of different widths. The use of sequential isolation windows is a way to reduce some of this complexity, by decreasing the number of concurrent ions being fragmented at a given moment [173, 174].

Usually in DIA experiments, the quantitative information is still obtained from the precursor ion signal, while the fragmentation spectra are mainly used for peptide identification by both

the use of common tools developed for DDA, or by searching pseudo MS/MS spectra reconstituted based on co-elution profiles of precursors and their potential fragments [174].

Several DIA acquisition methods were developed based on the use of different mass spectrometers and/or different dissociation methods (see Table 1) [173, 174], however, within this chapter only the most used method, LC-MS^E, and the SWATH-MS method will be presented.

Method	Instrument	m/z selection window (width)	Dissociation method	Ref
Shotgun CID	Q-ToF	Full m/z range	CID in-source	[175]
Original DIA	Ion Trap	10 m/z	CID in-collision cell	[176]
MS ^E	QqTOF	Full m/z range	CID in-collision cell	[177]
p ² CID	Q-ToF	Full m/z range	CID in-source & collision cell	[178]
PACIFIC (Precursor Acquisition Independent From Ion Count)	Ion Trap	2.5 m/z	CID in-collision cell	[179]
All Ions Fragmentation (AIF)	Orbi-Trap	Full m/z range	CID in HDC collision cell	[180]
XDIA or DIA-ETD-CAD	IonTrap-ETD-CAD	20 m/z	ETD in cell	[181]
SWATH	QqTOF	25 m/z	CID in-collision cell	[173]
Fourier transform-all reaction monitoring (FT-ARM)	LTQ-FT or LTQ-Orbitrap	12 m/z or 100 m/z	CID in-collision cell	[182]

Table 1. List of DIA methods (adapted from [173, 174]).

3.3.1. Liquid Chromatography-Mass Spectrometry Elevated energy (LC-MS^E)

LC-MS^E was the first label-free method from DIA used in proteomics quantitative screening. This method is based in the neutral loss acquisition mode and was first reported in large datasets by Wrona and collaborators in 2005 [183] as a “all-in-one” analysis for metabolite identification. This method was further transposed to proteomics studies, mainly supported by QqTOF instruments [177, 184]. MS^E consist of the acquisition of samples in two alternate modes, first samples are acquired in a low energy mode to collect precursor ions masses (MS precursor scan) and then in a high-energy mode to induce the fragmentation of the entire samples and acquisition of all the product ions (MS/MS scan) [184]. Over the years the coupling with the continuous development of MS and LC systems (more specifically, the use of UPLC-MS^E), more reproducible and accurate quantification has been achieved. However, as an inherent issue of DIA experiments, a large amount of data acquired remains unused, therefore a considerable effort has been done in order to obtain algorithms capable to extract more information from the acquired data than that already available [174].

3.3.2. Sequential Window Acquisition of all Theoretical Fragment-Ion spectra (SWATH-MS)

In 2012, Gillet and collaborators [173] presented the SWATH-MS method, although at that time other DIA methods were already widely used. It was a method that was particularly innovator due to its proposed data extraction methodology. Here, the authors proposed a targeted data extraction by combining parallel analysis of samples with an optimized IDA method for peptide identification followed by a DIA acquisition to be used to extract quantitative information. From the IDA method, a list (called "library") containing all the information regarding a given identified peptide (such as RT, precursor m/z , and MS/MS spectra) was obtained and it was further used to extract the XICs of the specific fragment ions (called peak groups) from all the high confidence peptides identified. Thus, instead of using the precursor intensity as performed by the other methods, in SWATH-MS the use of MS2 signaling intensity-based method was introduced, which is similar to the quantification already performed in MRM and PRM experiments, for the untargeted analysis of large fractions of the proteome. Furthermore, the authors also showed that with SWATH-MS, it was possible to achieve similar reproducibility and accuracy as for the targeted methods for protein quantification [173].

For the acquisition of the fragmentation spectra of virtually all the precursor ions present in a sample, the mass spectrometer, a high-resolution Triple-TOF instrument, operates in the sequential isolation window acquisition principle introduced by previous DIA studies [174, 176]. By fractionating the sample in SWATH acquisition windows, this method leads to a reduction of the concurrently fragmented precursors and consequent reduction of the acquired MS2 spectra complexity.

As data extraction is performed by targeting the peptides already identified, the loss of precursor-fragments linkage is overcome, and a large percentage of data is effectively used. Furthermore, this targeted data extraction also allows that additional criteria, such as the transition intensity ratio, m/z error, and similarity to the identified MS/MS spectra, can be used in combination with the usual chromatographic criteria to evaluate the confidence of the peak group formed. Therefore, protein quantification is obtained from a more reliable extracted data [185].

The SWATH-MS method seems to be able to overcome the majority of the limitations of label-free methods, it is unbiased, presents a broad range of precursor ion fragmentation (covering almost the entire mass range usually analyzed), and it relies on targeted data extraction [173], thus making this method a promising strategy to be applied in large screenings, such as the discovery of biomarkers [9, 129, 186–188]. Although, being a very recent methodology, the great expectation regarding its application into the biomedical field is reflected in the several improvements already achieved into the different domains associated with this method. There are already improvements in the DIA acquisition mode with the introduction of the variable windows mode where windows with different widths are adjusted to the number of precursor ions per m/z range, thus the number of concurrent ions are reduced in the most populated regions. Moreover, several different groups, have been working on the improvement of sample preparation and library creation to increase the number of proteins quantified per sample, as well as to obtain more reprodu-

cible data [189–193]. Finally, different algorithms were also developed to address SWATH data, both in the targeted mode [194] and untargeted mode, which is mainly focused on performing protein identification directly from the SWATH data [195].

The introduction of the concept of a protein library that can be used to interrogate multiple samples has opened the door to the idea of having cell-, tissue-, and species-specific libraries containing exhaustive lists of identified proteins capable of covering the entire proteome. Those libraries can then be used in both research and clinical fields to extract larger quantitative information from the analyzed samples. Within this scope, Aebersold and co-workers have already published the first repository with 10,000 human proteins that claims to successfully detect and quantify 50.9% of all human proteins [196]. Furthermore, as the SWATH file of a given sample corresponds to the MS/MS spectra signature of that sample, that file can be interrogated any time it is required without the need to re-analyze the sample. Therefore, with these SWATH files it is possible to create a repository of samples that can be used in longitudinal studies [129].

As stated above, the use of DIA in proteomics is recent and is not a common option, therefore, this overview in the neuroproteomics field will be done for MS^E, which is the most used method, and also for SWATH-MS due to the exponential increase in the interest and development associated with this approach.

MS^E is perhaps the most used large-screen, label-free method, particularly in the neuroproteomics field, and the only DIA method that has gained enough visibility so far [9, 197]. Although MS^E was also used in different neuroproteomics areas, such as in the studies of frontotemporal lobar degeneration [198] and the profiling of phosphorylation events in different rat tissues, including the brain [199], its use was particularly potentiated by the Sabine Bahn group for the study of neuropsychiatric diseases, such as schizophrenia, major depression, and bipolar disease [160]. In general, their published works were mainly focused on differential analysis of human samples, both serum [200, 201] and postmortem tissue [202–204], from patients versus healthy controls, or including different disease groups or groups with different levels of antipsychotic medication. Those works aimed to identify differentially altered proteins that could distinguish between the disease groups, but could also contribute to a better understanding of the diseases. In fact, the authors were able to identify several different proteins that are altered between schizophrenia patients versus controls, including proteins altered in first-onset paranoid patients [201], and observed also some proteomics alterations that were dependent on the dose of antipsychotic medication [204]. Finally, to distinguish the effect of the medication from the disease alterations, Sabine Bahn's group also studied the modifications caused by some of the antipsychotic drugs in rat frontal cortex, being able to identify proteins altered by the medication, some of them altered in both types of medication used [205]. More recently, MS^E was also used to perform proteomic profiles of the first episode of major depressive disorder patients and sex-specific alterations of adults diagnosed with Asperger syndrome [206].

Being a very recent method, SWATH-MS reports are mainly associated with technical improvements, and in demonstrating its capacity to obtain large proteomics profiles with its potential use to clinical studies and biomarkers discovery, such as the study of plasma PTMs

as phosphoproteins [187] and glycoproteins [207], large screening of twins [208], and human library creation [196], and also its applicability in biopsies specimens [188]. In the neuroproteomics area, it is already possible to find some reports, such as the work published by our group [209], where we presented a pipeline for reproducible quantitative screenings using a membrane-enriched sample from rat cortex, indicating that our approach is suitable for evaluation of membrane proteins, key players in the majority of neuronal dysfunctions. There are also two other works from Fox's group regarding mitochondrial alterations: one of them corresponding to an exhaustive characterization of mitochondrial proteome from embryonic and postnatal rat brain revealing a rearrangement of proteins from glycolysis and mitochondrial trafficking/dynamics, which may suggest a development change to accommodate the required energy demands in different developmental stages [210]. Another study focused on mitochondrial functional alterations associated with deregulation of PTEN-induced kinase 1 (PINK1), a Parkinson's disease-associated protein [211].

3.4. Absolute quantification based on label-free approaches

Although the majority of the screenings are based on relative quantification, some authors started to focus on the possibility to also extend these methods to absolute quantifications [128], since the calculation of the protein abundances in a sample is essential to increase the understanding towards the biological systems and its variations [177, 212]. Overcoming the elevated cost and demanding sample preparation of an isotopic dilution-based method to perform absolute quantification, the use of label-free techniques reveals a reliable alternative (although less accurate than the referred methods). The available methods can be divided into two generic classes based on the quantification algorithms used: 1) those based on tandem MS data, e.g., protein sequence coverage or spectral counting including emPAI [213] and APEX [214]; and 2) those based on the measurement of precursor ion intensity such as MS^E [177], T3PQ [157], and iBAQ [215].

In general, all these techniques were described as having good correlation with protein amounts in both simple mixtures of proteins with known amounts (alone or spiked in complex samples) and for unknown proteins in complex samples. When complex samples were used, the accuracy of the results were confirmed by comparing the values achieved within several different techniques, such as other mass spectrometry-based quantitative methods (including isotopic labeled methods), transcriptomics analysis, and ELISA [157, 177, 212–215]. In all cases, a proper estimation of the protein abundance was achieved with or without standards.

The more cost-efficient and easier option is to exclude the standard proteins and calculate protein abundances from the fraction of each protein in the total protein pool assuming that most of the proteins that contribute to the total protein pool are identified and quantified. As examples of this quantification without standards, it is possible to find the determination of the copy number (using total protein approach (TPA) [216]) and the definition of the stoichiometry of protein complexes [217]. However, the quantification accuracy can be increased by using a standard curve from a mixture of proteins with known amounts that have different sizes and concentration [212].

Additionally, the majority of these methods are already implemented in several tools available for proteomics analysis, therefore, it is possible to combine both relative and absolute quantifications in a simple way, as in the case of the emPAI that is implemented in the MASCOT server, one of the most used servers in proteomics [214].

A brief presentation of the most used methods for label-free absolute quantification focused on the major differences between them, and some reports using these methods into the neuroproteomics field are presented below.

3.4.1. Spectral counting-based methods

Exponentially Modified Protein Abundance Index (emPAI): Mathias Mann's group presented what would be the first method for absolute quantification by proving that a transformation of the PAI values (described above) could be in fact associated with the absolute amount of a given protein [213]. In this study, the authors showed that the PAI values have a linear relation with the logarithm of protein concentration, therefore the absolute quantification of a given protein can be obtained by the exponentially modified PAI (emPAI), which is equal to the following equation $10^{\text{PAI}-1}$.

Absolute Protein Expression (APEX): In theory, the APEX method is similar to the previously proposed emPAI method since it is based on the number of peptides identified normalized for the theoretical number. However, instead of considering the redundant peptides, it relies only in the unique peptides. And furthermore, which is also its major strength, this method uses machine learning to calculate the number of theoretical peptides than can be identified in the particular experiment. To achieve the probable number of peptides, the theoretical number of peptides is normalized for a correction factor specific for the experimental settings [212, 214].

3.4.2. Intensity based methods

One of the disadvantages of spectral counting based methods already observed for relative quantification is the fact that in these methods the saturation is easily reached, therefore, failing in accurately quantifying proteins present at higher levels. On the other hand, as those methods rely on the MS/MS spectra identification, they are also biased to the most intense proteins, therefore, spectral counting-based methods are only accurate within a reduced dynamic range. Furthermore, they also present a large variability between replicates. Similar to what is observed for relative quantification with the use of MS1 intensity-based methods these limitations are overcome.

Peak intensity-based absolute quantification method (iBAQ): In this method, the amount of a given protein is calculated by the sum of the peak intensities of all peptides matching to it, divided by the number of theoretically observable peptides [215].

LC-MS^E: Silva and colleagues reported in 2006 [177] for the first time the relationship between MS signal response and protein concentration. In this work, the authors discovered that the average of the three most intense peptides is highly correlated to the effective amount of a protein in a sample. In this study the authors spiked the samples with a known amount of a

mixture of proteins (internal standards). The internal standards were then used to calculate a universal signal response factor (which was shown to be the same for all the tested proteins) that correlates the intensity calculated with the amount of the proteins, and is then used to obtain the quantification for the unknown proteins.

Three most intense peptides peak area (T3PQ): The T3PQ method is an adaptation of the method previously used in LC-MS^E [177] for IDA methods [157]. The principle of this approach relies on the evidence that for each protein (independent on its size) identified by a set of peptides, the average of the three most efficiently ionized peptides (those with the highest MS signals) directly correlated with the amount of the corresponding protein. This method proved to be more accurate and reproducible than the methods already used (in particular, when compared with the spectral counting methods) [157].

Absolute quantification methods have been used mainly in studies focusing on the understanding of complexes stoichiometry, and not in large screenings that are the most frequent assays in the neuroproteomics field. Therefore, there are only few reports using label-free-based absolute quantification, particularly in neuroproteomics, and those are mainly associated with the iBAQ method. One of the more interesting reports where iBAQ was used is perhaps the work regarding the characterization of the isolated synaptic boutons that culminate with the establishment of the amounts of the proteins that compose those vesicles [218]. iBAQ was also used to obtain a comprehensive characterization of the protein abundance in several organs, such as the brain [158], and in some experiments that focus on the determination of the amount of enriched proteins in tissue-specific (hair bundles) [219] and condition-specific (BACE1 knockouts) [220] proteomes.

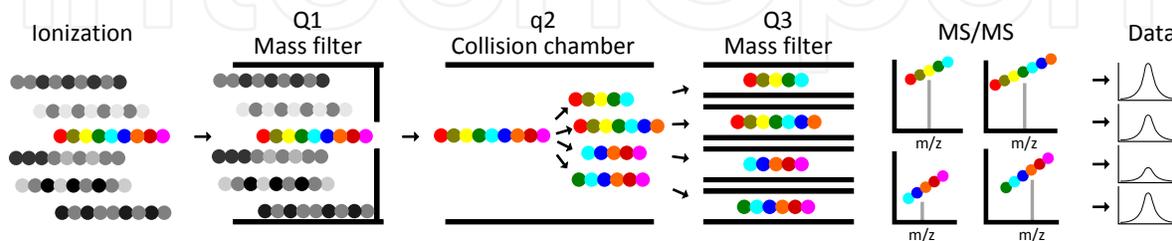
4. Multiple reaction monitoring

Multiple reaction monitoring (MRM) is a highly selective scan mode in MS that has been extensively used for the last 30 years for absolute quantification of small molecules [221]. Similarly, the knowledge acquired in small molecules targeted quantification has been transposed for targeted quantification of peptides and proteins where several reviews can be found in the literature [222–224]. Shotgun proteomics MS-based studies retrieve the identification of thousands of proteins in a single analysis, plus the relative quantification by label-free [225, 226] or isotopic labeled strategies [227, 228]. However, in these global profiling methods, low-abundance peptides may be difficult to be detected, generating “missing data” and low precision problems that can impair statistical analyses [229, 230]. Consequently, the untargeted approach has been widely used, for instance, in clinical studies of biomarker discovery to find new candidates and, the MRM targeted MS-based approach has been used in the verification/validation phase, overcoming many of the difficulties associated with antibody-based protein quantification [231, 232].

Developing and validating MRM-MS assays is a laborious process, but once constructed, it can be used for accurate and precise quantification of one or several proteins on a large scale and across laboratories [233]. The high selectivity of MRM scan mode is achieved using, most

predominantly, triple-quadrupole mass spectrometers. Quadrupoles are known as “mass filters” where in a first stage (Q1), the mass/charge ratio (m/z) of the intact peptide (precursor ion) is selected, fragmented in the collision cell (q2), and in a second stage (Q3) a specific fragment of the precursor is selected, generating the selected reaction monitoring experiment (SRM) with one transition (precursor/ fragment), or if several fragments are being monitored, an MRM experiment with several transitions (Figure 5A) [234].

A-True MRM



B-High Resolution MRM

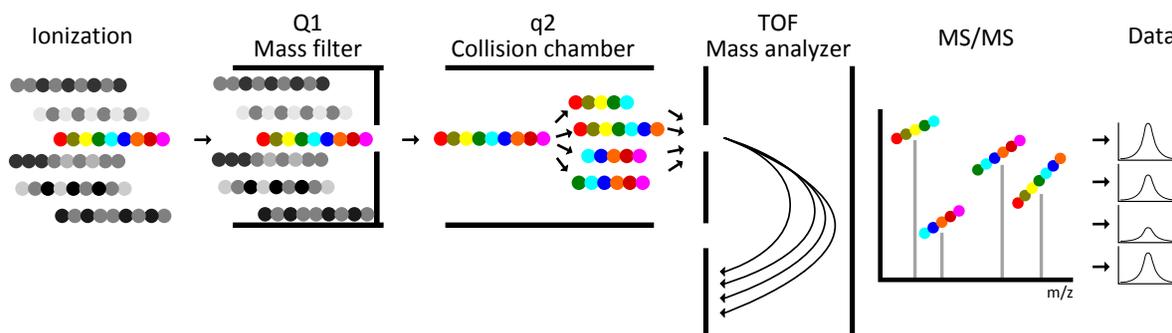


Figure 5. Schematic representation of the (A) MRM scan mode performed in a triple-quadrupole instrument and the (B) high-resolution multiple reaction monitoring (HR-MRM) scan mode performed in a QqTOF system. In classical MRM scan mode, the first quadrupole (Q1) selects the m/z of the precursor that will be fragmented in the collision cell (q2) and one of the resulted fragments is then selected by the third quadrupole (Q3) towards the detector. The two stages of mass filters (Q1 and Q3) represent a transition and more than one can be monitored in a single run. HR-MRM works similarly at the first stage (Q1) but after fragmentation, all the fragments are scanned by the TOF mass analyzer instead of selecting only one each time that the precursor is fragmented. This will generate a high-resolution mass fragmentation spectrum where extracted ion chromatograms for each fragment can be obtained by the use of specific softwares.

The peptide sequences to be monitored must be carefully selected as they have to be unique for a given protein, where peptides with less than 8 residues and those susceptible to undergo modifications during sample processing (methionine oxidation, cysteine alkylation) must be avoided. Additionally, for protein isoforms or PTM's quantification, specific peptides should be selected for accurate measurements. [229, 235, 236]. The combination of LC separation followed by the MRM acquisition (2 m/z filters) results in high precision, sensitivity, and high selective measurements for the selected peptides and, consequently, for the protein [229]. The best candidate peptide(s) to be monitored for the quantification can be selected based on

prediction tools (in silico) or on experimental evidences [236]. Selection based on empirical data involves previous LC-MS/MS experiments from the biological sample to obtain preliminary information on the peptide characteristics such as ionization and fragmentation. After the selection of the peptides (precursors) and their specific fragments, MRM transitions are evaluated by re-analyzing the sample to help in the selection of the most selective and sensitive for each peptide of interest [237]. In order to avoid long optimization and multiple rounds of analyses, there are online repositories such as PeptideAtlas, the Global Proteome Machine Database, and Pride, which contain peptide sequences and empirical MS spectra to support MRM designing without the need of preliminary sample processing and analysis [229]. Even for proteins not found in the database, there are several in silico bioinformatic tools that select high-responding peptides from candidate proteins, such as ESP predictor [238], PeptideSieve [239], PepFly [240], MIDAS [241], among others. TIQAM is another interesting software tool that selects the proteotypic peptides based on the in silico prediction and integrates that information with the PeptideAtlas repository or other sources to generate the list of transitions based on the validated fragmentation spectrum [242].

The number of proteins monitored by an MRM experiment is usually low and the duty cycle (the time for the instrument to cycle through separation and detection of each transition) will depend on the number of peptides per protein and the number of transitions per peptide. To overcome the limited number of proteins monitored in an MRM experiment, a timed acquisition mode, termed scheduled MRM (sMRM) analysis was developed where transitions are acquired only during a defined elution time window [235]. Consequently, thousands of transitions can be monitored, allowing the quantification of hundreds of proteins in a single run. Colangelo and collaborators developed a pipeline for large scale (>1000 transitions/run), label-free LC-MRM assays for the quantification of 112 rat brain synaptic proteins [243]. The workflow began with data-dependent acquisition using 5600 Triple TOF to identify the sequences of the peptides present in the biological sample of interest. The peptide library information was then converted into thousands of MRM transitions that were easily transposed to the 5500 QTRAP (demonstrating the consistency of the fragmentation patterns between the instruments) to be acquired using the sMRM methods. To address the very short dwell times due to the high number of transitions, they presented an improvement in the sMRM methods' sensitivity and robustness using an intelligence-based MRM acquisition (termed extended or xMRM). Firstly, variable acquisition windows throughout the run can be used and secondly, a "triggered xMRM", where the secondary MRM transition for each peptide was only monitored if the primary MRM exceeded a given threshold. The xMRM enabled the reduction of the number of transitions to be monitored at a given time leading to an increase of 63–68% in the dwell times for peptides and, consequently, an increase in the sensitivity for the limiting peptide concentrations.

Although MRM is considered to be a very high selective scan mode, the possibility to have non-desired peptides with isobaric or very similar m/z values can increase with sample complexity [244]. The consequence of a non-selective method is the overestimation in concentration determination of the targeted peptide. Therefore, the use of HR-MRM can increase the method selectivity and consequently improve the accuracy of the quantification. The scan

mode works as described in the first stage (Q1) and in fragmentation (Q2) for triple quadrupoles with the difference in the last stage, where rather than focusing on a single ion fragment in Q3, fragment ions of all masses are scanned by a TOF analyzer generating high-resolution MS/MS spectra (Figure 5B). Thus, fragment ions can be extracted from the high-resolution MS/MS spectra of the targeted peptides to generate extracted ion chromatograms (XICs) of high resolution [245]. Tong and collaborators performed a targeted HR-MRM analysis for the quantification of 47 tear proteins using the Triple-TOF mass spectrometer (QqTOF) with good reproducibility (CV<5%) [245]. In addition to the improvement in selectivity, the multiple steps for the selection of the best transitions are not required as in triple-quadrupole instruments.

4.1. Absolute quantification of proteins by MRM

Beyond protein identification and relative quantification by MS, absolute quantification of proteins in biological samples has also been performed using synthetic unlabeled and/or labeled peptides [12]. Absolute protein quantification has been generally performed based on the principle of the stable-isotope dilution (SID) where stable isotope-labeled synthetic analogues are spiked into the samples to extrapolate protein amounts present in a sample. Gerber and collaborators termed this approach as the AQUA methodology, where the best candidate peptides for the quantification of a given protein are synthesized with at least one residue replaced by stable isotopes, resulting in a very similar endogenous peptide (called AQUA peptides) but with a sufficient m/z difference so that they can be distinguished by MRM [246]. Protein quantification is performed by spiking the sample with a known amount of the AQUA peptide and the peak areas ratio of the unlabeled/labeled peptides are used to determine the expression levels of the protein of interest. On the other hand, Barnidge and collaborators performed a study to compare protein quantification using two different methods, one based on the AQUA approach and the other on an external calibration curve created from successive dilutions of the synthetic unlabeled peptide [247]. Quantification based on the external calibration curve resulted in better precision and accuracy values than quantification based on the sample spiking of the analogous labeled synthetic peptide. In this study, the external calibration curve was able to accurately determine the peptide concentration however, for more complex samples, the matrix effect should be evaluated so that method accuracy is not compromised. The ideal approach for accurate and precise peptide quantification would be the use of external calibration curves prepared in the representative matrix by spiking the unlabeled synthetic peptide at various concentrations and a constant amount of the analogous stable isotope synthetic (SIS) peptide as internal standard. However, proteins or peptides of interest are usually present in the representative matrix that impairs the accuracy and precision of the quantification method if the calibration curves are performed by spiking the synthetic unlabeled peptide into the matrix. For that reason, Campbell and collaborators proposed an alternative approach called the reverse curve method where varying amounts of the labeled peptide are spiked in the representative matrix to create the calibration curve [248]. In this work, seven apolipoproteins were quantified in human plasma using the three approaches: a) spiking the sample with a known amount of the analogous synthetic-labeled peptide (AQUA approach); b) spiking the representative matrix with different amounts of the unlabeled synthetic peptide and a constant amount of the labeled peptide to create the “classical”

calibration curve; and c) spiking the representative matrix with varying amounts of the labeled synthetic peptide to create the curve and a constant amount of the unlabeled peptide to work as internal standard (reverse calibration curve). For both cases using external curves, some corrections are required due to the endogenous peptide already present in the sample. The correction for the classical calibration curve is performed by subtracting the y-interception of the curve to the determined concentration of the endogenous peptide in the sample. For the reverse curve the correction factor corresponds to an increase of each curve point by an amount proportional to the ratio of the amounts of endogenous unlabeled peptide to the spiked synthetic unlabeled peptide [29]. The AQUA approach revealed to be inaccurate for endogenous peptide quantification below and above the concentration of the IS spiked into the sample. As expected, this result demonstrates that accurate peptide quantification in samples can only be achieved if the spiked IS amount into the sample is close to the concentration of the endogenous unlabeled peptide. The reverse curve has the advantage of allowing the determination of the limits of detection and quantification (LOD and LOQ) once the representative matrix does not contain the synthetic-labeled peptide. In addition, the quantification using the reverse curve revealed to be the most accurate and precise between the three methods, thus this approach can be used with confidence to quantify endogenous peptides/proteins already present in the surrogate matrix.

Absolute quantification by MRM applied to neuroproteomics was first described by Desiderio and collaborators to quantify picomole amounts of the endogenous methionine-enkephalin (ME) in the human pituitary by comparing the response of the endogenous ME to one of the deuterated ME internal standard (d5-ME) [249]. More recently, Kheterpal and collaborators determined the concentration of MIF-1 (neuropeptide) in different regions of mouse brain by using a calibration curve prepared by successive dilutions of the unlabeled synthetic peptide in the absence of matrix [250].

There are several studies in Alzheimer's disease that involve protein/peptide quantification by MRM [251–253]. Lame and collaborators developed a UPLC-MRM method to accurately quantify $A\beta_{1-38}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ in human cerebrospinal fluid that can play a crucial role in understanding disease progression and intervention [254]. The quantification was performed using calibration curves prepared with various concentrations of the synthetic peptides spiked with constant amounts of analogous ^{15}N -labeled internal standards in an artificial CSF matrix. Also, Wildsmith and collaborators described the development of an MRM assay for the absolute quantification of 39 peptides corresponding to 30 proteins to confirm previous findings for a subset of markers for Alzheimer's disease [255].

Another approach that, in combination with MRM, allows absolute quantification based on the isotope-dilution mass spectrometry or AQUA methodology is peptide labeling with non-isobaric tags reagents, the mTRAQ reagents. Originally, mTRAQ labels appeared in two versions, the lighter version (lower in mass than the iTRAQ labels by 4 Da) and the heavy version that is identical to the iTRAQ 117 label resulting in a light version ($\Delta 0$) with a monoisotopic mass of 141 Da and a heavy version ($\Delta 4$) of 145 Da. Furthermore, a new label version ($\Delta 8$) is now available called triplex mTRAQ reagents. These have been used mostly for relative quantification but DeSouza and collaborators described a method for absolute quantification

of proteins using the duplex version of mTRAQ reagent. The procedure consisted of labeling known amounts of the synthetic peptide used for protein quantification with one of the two versions while, the opposite version was used to tag the endogenous peptides that needed to be quantified [256]. At the end, these two fractions were mixed at a known amount and the resulting mixture was analyzed by unique MRM transitions for each version of the labeled peptide resultant from the different masses of the tags. The areas that resulted from each MRM transition were then used to determine the unknown concentration of the peptide in the digested sample and, consequently, the concentration of the protein of interest.

Apart from the AQUA quantification strategy, standard peptides are usually spiked at late stages of sample processing; they are poorly compatible with sample pre-fractionation; and the digestion efficiency cannot be fully determined leading to an inaccurate quantification [257]. To address these issues, other types of standards were developed known as artificial concatamers of proteotypic peptides (called QconCAT) and are generally added into the sample just before protein digestion [258]. Concatamers are artificial protein constructs that include multiple trypsin-cleavable proteotypic peptides isotopically labeled. The isotope-labeled peptides are released during protein digestion and will be used as standards for the absolute target protein quantification. The QconCAT methodology possesses the main advantage of facilitating multiplex protein quantification where typically 10–30 target analyte proteins are encoded in each QconCAT at a level of two quantotypic peptides per protein [259]. Chen and collaborators stated that a reliable quantitative approach of clusterin in brain was needed to clarify its role in Alzheimer's disease. Consequently, they developed a stable isotope-labeled concatenated peptide (QconCAT) for the quantification of clusterin in human postmortem frontal and temporal cortex [260]. Later, they applied this approach for other protein quantifications also related to AD. At this time, a multiplexed QconCAT was designed for quantification of various isoforms of amyloid precursor protein (APP). Since common tryptic peptides between all isoforms of APP were concatenated with unique tryptic peptides for specific APP isoforms, this QconCAT-MRM method allowed the clear quantification of the total APP and each protein isoform [261].

Even with the advantage of multiplexing absolute quantification, the assessment of the digestion efficiency remains undetermined once QconCAT are usually digested at high rates, not giving the true tryptic digestion efficiencies for each protein [258]. By using the isotope-labeled equivalent of the full-length target protein, the "ideal" internal standards can be added at the very beginning of sample processing, allowing the determination of the recoveries after pre-fractionation steps and the assessment of the digestion efficiencies, which is called the "Protein Standard Absolute Quantification" (PSAQ). A comparative study between AQUA, QconCAT, and PSAQ was performed for the quantification of *Staphylococcus aureus* superantigenic toxins in water and urine samples where the PSAQ strategy revealed to be more accurate than the two other methods [262]. PSAQ also revealed to be advantageous for the absolute quantification of membrane proteins that are more prone to errors on concentration determination due to protein enrichment steps usually required and incomplete digestion [263]. In this study, accurate quantification of 7 membrane proteins was achieved using as internal standards the analogous ¹⁵N-labeled full-length proteins added at an initial stage of

sample processing. There are other quantification approaches based on the addition of full-length labeled proteins internal standards for protein quantification such as FlexiQuant [264], PrEST [265], and Absolute SILAC [266].

Although, some of the approaches presented have few publications for absolute protein quantification by MRM they can be of interest for the neuroproteomics field to confirm previous findings or to find new targets with more accurate data.

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