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Review

Progress and pitfalls of using isobaric mass tags for proteome profiling

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Abstract

Introduction: Quantitative proteomics using mass spectrometry is performed *via* label-free or label-based approaches. Labelling strategies rely on the incorporation of stable heavy isotopes by metabolic, enzymatic or chemical routes. Isobaric labelling uses chemical labels of identical masses but of different fragmentation behaviours to allow the relative quantitative comparison of peptide/protein abundances between biological samples.

Areas covered: We have carried out a systematic review on the use of isobaric mass tags in proteomic research since their inception in 2003. We focused on their quantitative performances, their multiplexing evolution, as well as their broad use for relative quantification of proteins in pre-clinical models and clinical studies. Current limitations, primary linked to the quantitative ratio distortion, as well as state-of-the-art and emerging solutions to improve their quantitative readouts are discussed.

Expert opinion: The isobaric mass tag technology offers a unique opportunity to compare multiple protein samples simultaneously, allowing higher sample throughput and internal relative quantification for improved trueness and precision. Large studies can be performed when shared reference samples are introduced in multiple experiments. The technology is well suited for proteome profiling in the context of proteomic discovery studies.

Keywords: isotopes, iTRAQ, labelling, liquid chromatography, mass spectrometry, proteomics, quantification, reagents, tagging, TMT

Article highlights

- Protein quantification using mass spectrometry (MS) can be achieved *via* label-free or label-based approaches.
- Label-based approaches rely on the incorporation of heavy stable isotopes. Labelling with isobaric chemical mass tags is one option for label-based relative quantification of proteins.
- Multiplexed comparison of two to sixteen samples is currently achievable with isobaric labelling in routine use providing adequate precision and trueness. Higher order multiplexing options have been proposed.
- Increasing multiplexing capabilities allow a number of new applications in pre-clinical and clinical studies. Isobaric mass tags have been successfully employed in large scale studies dealing with several hundred (up to a thousand) of samples.
- While quantitative performances of isobaric mass tags suffer from the well-known co-fragmentation issue (*i.e.*, peptide precursor of interest isolated together with other peptides) that interferes with accurate quantification, solutions such as an additional round of fragmentation (*i.e.*, MS/MS/MS or MS^3) or further peptide separation/isolation (*e.g.*, with ion mobility) can significantly improve performance.
- We consider that quantitative precision is a must while the trueness might be less of an issue in proteomic discovery applications.
- Data completeness is achieved within samples from a single experiment using isobaric mass tags. Missing values arise in multiple experiment comparisons due to the stochastic nature of the data-dependent mode of MS used for data acquisition. This limitation can be partly alleviated by replication of the liquid chromatography-MS analysis, sample fractionation, or refined data processing.

1. Introduction

Proteomics has rapidly turned from qualitative to quantitative [1] when faced with biological questions related to protein changes in expression and abundance. Current mass spectrometry (MS)-based proteomic methods offer mainly relative quantification, allowing the comparison of proteomes in pre-clinical models and clinical studies in a wide area of applications, such as biomarker discovery and exploration of biological mechanisms. In essence, liquid chromatography (LC)-MS enables the unbiased identification of proteins in samples and their relative quantification between samples. Possible approaches rely on comparison between LC-MS analyses (*i.e.*, label-free) or within LC-MS analyses (*i.e.*, label-based).

The principle of isobaric labelling for MS-based proteomics was introduced in 2003 with a first generation of tandem mass tag (TMT) reagents [2]. Rapidly after, Applied Biosystems launched a commercial version of isobaric mass tags so-called isobaric tag for relative and absolute quantitation (iTRAQ) following the works by Pappin and co-workers [3]. ExactTag reagents and newly designed TMTs [4] became soon after available to scientists. As a matter of fact, the use of isobaric mass tags has gradually increased over the years (**Figure 1a**). While the iTRAQ reagents have for a long time dominated the field, TMTs are gaining increasing popularity (**Figure 1b**), most likely due to their improved multiplexing capabilities. Isobaric mass tags are chemical reagents, which react with specific moieties (*i.e.*, primary amines; other reactive groups are available but less used (see **Section 3**)) in proteolytic peptides; this makes the quantitative approach global as every peptide is labelled as least on its N-terminus. Besides the reactive group, tags are composed of a mass reporter and a mass balancer group embedding sets of stable heavy isotope atoms. The combination of these two groups confers the primary characteristics of a set of isobaric mass tags, *i.e.*, identical mass for each individual tag. This is basically achieved by counterbalancing the incorporation of stable heavy isotopes either in the mass reporter or mass balancer groups as depicted in **Figure 2**. For further illustration, tag chemical structures are given in **Figure 3** for some of TMT reagents currently commercially available.

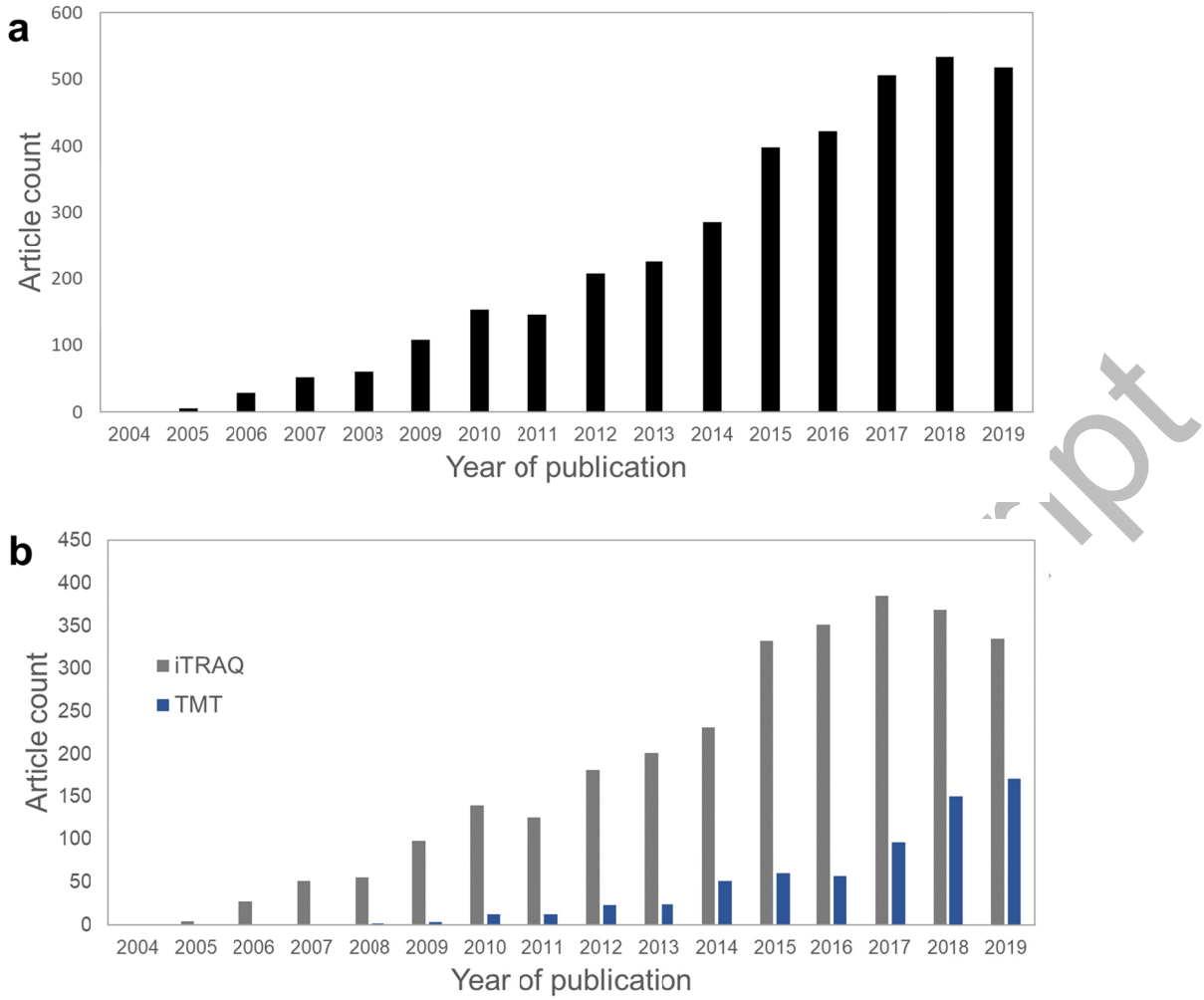

Figure 1. PubMed searches (**a**) for ((((((((((((((("isobaric labelling") OR "isobaric labeling") OR "isobaric tagging") OR "isobaric label") OR "isobaric labels") OR "isobaric tag") OR "isobaric tags") OR "TMT") OR "TMTs") OR "tandem mass tag") OR "tandem mass tags") OR "iTRAQ") OR ("isobaric tag for relative and absolute quantitation")) OR ("isobaric tags for relative and absolute quantitation")) OR ("isobaric tag for relative and absolute quantification")) OR ("isobaric tags for relative and absolute quantification")) AND proteomics) and (**b**) for ((((("TMT") OR "TMTs") OR "tandem mass tag") OR "tandem mass tags") AND proteomics (in blue) or (((((("iTRAQ") OR ("isobaric tag for relative and absolute quantitation")) OR ("isobaric tags for relative and absolute quantitation")) OR ("isobaric tag for relative and absolute quantification")) OR ("isobaric tags for relative and absolute quantification")) AND proteomics (in grey). Searches were performed on 3rd October 2019.

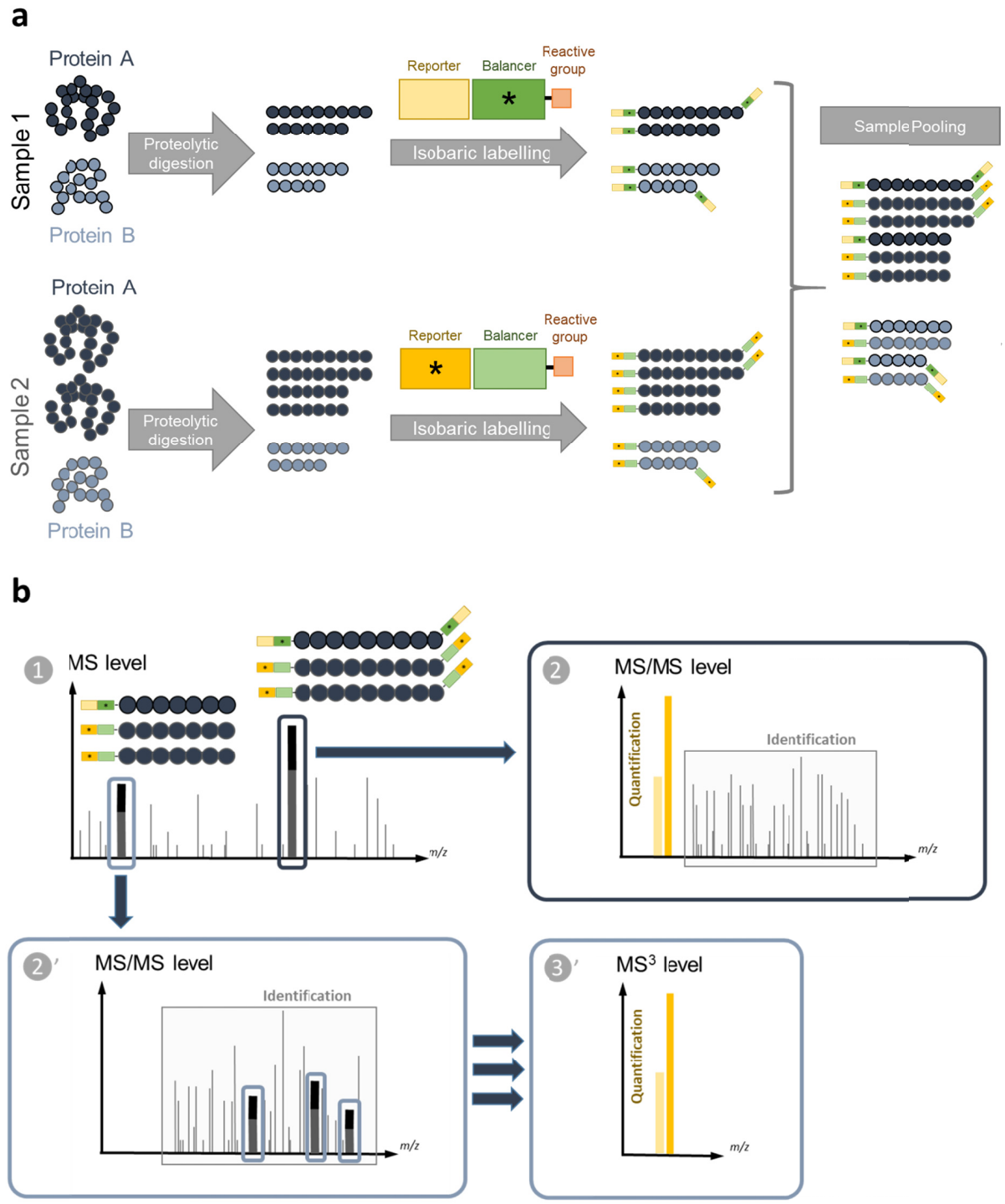

Figure 2. (**a**) Overall scheme of the isobaric labelling workflow. Proteins (exemplified by proteins A and B) in samples to be compared (here samples 1 and 2) are digested by a proteolytic enzyme, usually trypsin as illustrated herein; obtained peptides are differentially labelled (on their N-terminus and lysine residues) in samples using different forms of the isobaric mass tags (here, one stable heavy isotope atom (noted *) was incorporated either in the balancer/linker or the reporter group for comparison of two samples); after labelling is completed, samples are pooled; the pooled sample is then analyzed with LC-MS and MS/MS. (**b**) While MS (❶) cannot help differentiating peptides of identical amino-acid sequence between the samples (because of their identical masses), MS/MS (❷) directly provides relative quantification of those peptides in the samples (by comparison of the reporter ion signal intensities), on top of allowing their sequence matching from the tandem mass spectra. An additional level of fragmentation (❷' and ❸') can provide a more accurate quantification (see **Section 4**).

A common proteomic workflow used with isobaric mass tags is the shotgun approach (**Figure 2a**) where proteins are digested with a proteolytic enzyme (trypsin being the classical one) after reduction of disulfide bridges and alkylation of the thiol groups. The obtained peptides are then directly labelled with isobaric mass tags using one different form of the tags for each of the samples to be compared. After completion of the labelling reaction, the differently-labelled peptides and therefore the samples, are mixed and subjected to LC-MS and tandem MS (MS/MS) analysis (**Figure 2b**). The full survey MS scan (*i.e.*, MS^1) detects peptide ions (one signal being constituted of the addition of the peptides of the same sequence originating from each of the mixed samples). MS^1 cannot distinguish the origin of the labelled peptides but, when exceeding a defined threshold, triggers their selection, isolation and fragmentation in an MS/MS scan (MS^2). MS/MS fragments the peptides, revealing the peptide backbone fragment ions, and cleaves the attached isobaric mass tags, releasing the mass reporter ions from the different tags. Fragment ions are used for peptide identification while mass reporter ions in the low-mass range of tandem mass spectra enable relative quantification of the corresponding peptides between the different samples. Information is then inferred at protein level and compiled for all peptides of the same protein.

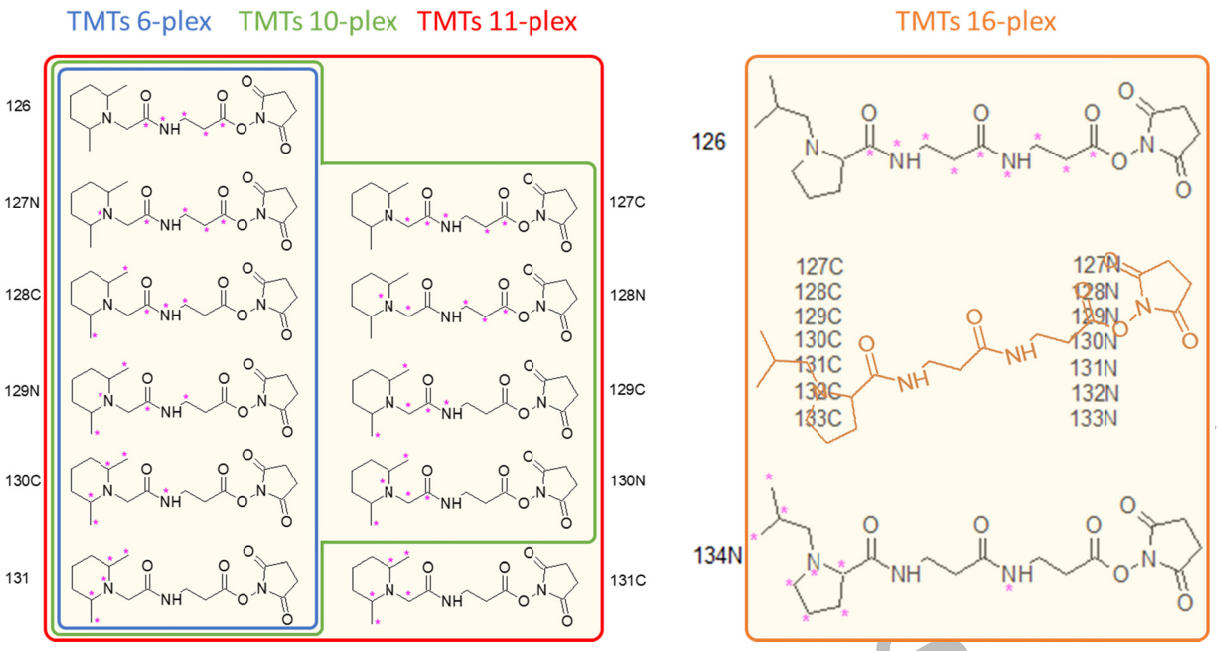

Figure 3. Structures of TMTs in different multiplexing sets (heavy isotopes are marked with an asterisk). For 6-plex TMTs, five heavy isotopes are incorporated in the mass reporter or mass balancer groups; playing with the position of these isotopes allows building unique tags (mass reporter groups are of different masses incremented by 1 Da) with the same global mass. For 10-plex and 11-plex TMTs, difference also arises from the differential isotope incorporation on carbon or nitrogen atoms and their discrete mass defects (difference of 6.32 mDa); still five heavy isotopes are distributed in each of the reagents. The newly released 16-plex TMTs, so-called TMTpro, are displayed on the right panel; they are designed with the incorporation of nine heavy isotopes and also exploit the mass defect properties of carbon and nitrogen.

In this review, we focus our discussion on: i) the multiplexing capabilities of isobaric mass tags, ii) their quantitative performances in particular with regard to other MS-based relative quantification techniques used in proteomics, iii) their major reported limitation, referred as quantities' ratio compression or distortion, iv) the processing and analysis of isobaric labelling-type of data, and v) some relevant applications in pre-clinical models and clinical studies. Isobaric labelling has been reviewed previously [5-7] but we believe that the present report will give pertinent additional perspectives to the reader and to the field.

2. Multiplexing of samples and experimental conditions

The main advantage of the isobaric mass tag technology is its sample multiplexing capability [8], for the relative quantification of multiple proteome samples at a time. While the very first tag set was a 2-plex version [2], enabling comparison of two samples, versions of 4-plex [3], 6-plex [4], and 8-plex [9] were subsequently introduced and commercialized as iTRAQ or TMT reagents (**Figure 4**). Exploiting the mass defect of $^{12}C/^{13}C$ and $^{14}N/^{15}N$ isotopes, that arises from differences in nuclear binding energy for each isotope [10], and the high resolving power of recent MS instrumentation (for instance the Orbitrap analyser [11]) that can baseline-resolve this small differences, 8- and 10-plex version of TMTs were recently described and commercialized with mass reporter groups containing one extra neutron incorporated into either carbon or nitrogen [12,13]. A 16-plex version of the TMTs was very recently released (**Figure 3**) [14].

Larger labels (*e.g.*, 4-plex *versus* 8-plex iTRAQ) were shown to induce lower protein and peptide identification rates [15]. This is caused by additional internal fragmentation of the larger tags or increased charging during electrospray ionization [16], a fact however that was not confirmed using matrix-assisted laser desorption/ionization-tandem time-of-flight (MALDI-TOF/TOF) instrumentation [17]. Therefore, replacing carbon (^{12}C by ^{13}C) and nitrogen (^{14}N by ^{15}N) atoms has been pursued as the preferred option to increase the multiplexing capabilities of isobaric mass tags while limiting their size increase.

Researchers have developed alternative isobaric mass tag reagents primarily aiming at reducing the cost and dependence on commercial solutions described previously. A cost-effective 12-plex *N,N*-dimethyl leucine (DiLeu) isobaric mass tag for high-throughput quantitative proteomics was presented [18], based on the original development of a 4-plex version of the DiLeu isobaric mass tags by the same authors [19]. Deuterium isobaric amine reactive tags (DiART) were also described [20,21]. In contrast to TMT and iTRAQ reagents, built with stable isotopes of carbon, nitrogen and oxygen, DiART reagents were synthesised using less-costly incorporation of deuterium. The negative effect observed with deuterium-based mass tagging, namely retention time shifts in RP-LC, has been shown to be negligible when the deuterium is placed closed to a hydrophilic group [22]. More recently, the same researchers introduced the 10-plex isobaric tags (IBT) finally relying on the inversion of

$^{12}C/^{13}C$ and $^{14}N/^{15}N$ isotopes [23]. In addition, novel designs of isobaric mass tags also go beyond multiplexing and cost considerations and include such aspects as enhanced ionization properties of the labelled peptides in MS and increased selectivity and yield of the tagging reaction [24].

By exploring hybrid labelling strategies that combine pair mass labelling with multiplexed isobaric mass tagging, scientists demonstrated the possibility to perform higher order sample multiplexing, or hyperplexing. For instance, simultaneous comparison of 18 yeast samples was achieved by combining 3-plex metabolic labeling with 6-plex isobaric mass tags [25]. More recently, Frost *et al.* reported the hyphenation of compatible 2-plex stable isotope dimethyl chemical labelling with 12-plex DiLeu isobaric labelling [26], in a so-called combined precursor isotopic labelling and isobaric labelling (cPILOT) approach [27].

Overall, sample multiplexing displays very interesting advantages. The ability to compare multiple sample simultaneously offers a significant gain in time for LC-MS and MS/MS analysis (*i.e.*, all labelled samples are pooled and only the mixture is analyzed) and thus an increase in throughput which is a key enabler to consider for large scale studies.

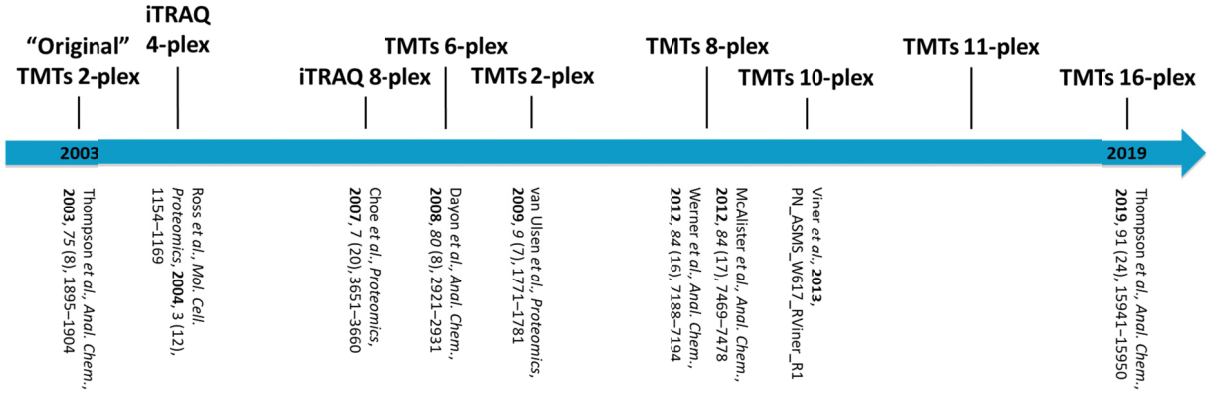

Figure 4. Evolution of the multiplexing capabilities of iTRAQ and TMT reagents. From the classical incorporation of heavy isotopes in the reporter and balancer groups, small mass differences induced by the mass defects of carbon and nitrogen isotopes have allowed increased multiplexing possibilities of these reagents.

3. Quantitative performances and benchmarking against other relative quantification approaches

3.1. Comparison to label-free methods

Beside of the sample multiplexing, isobaric mass tags provide some compelling quantification advantages with respect to label-free methods.

Quantification that uses isobaric mass tags is basically independent of LC or MS instrument variations due to the fact that the quantification vector is incorporated by sample labelling and pooling prior to the instrumental analysis. This is a common feature of label-based

methods offering in general better precision and trueness of the quantitative measurements than label-free approaches.

However, an important limitation linked to isobaric mass tags with increased multiplexing capabilities is the dependence on high resolution mass analysers which are able to resolve the small mass defects. Higher mass resolution often comes with longer analysis time, as it is the case with the Orbitrap analyser. Because increasing mass resolution is detrimental to the instrument duty cycle, reduction of proteome coverages is usually observed. Yet, the proteome coverages can be rescued or even increased by pre-fractionation of the pooled sample that is straightforward and compatible with label-based approaches. Comparing a 10-plex TMT workflow (that includes high pH reversed-phase pre-fractionation) with a label-free single shot data-independent acquisition (DIA) workflow for the analysis of ten samples, higher proteome coverage was achieved at fixed MS instrument time for the TMT-based method [28], confirmed also for an iTRAQ-based approach in another study [29]. In the same report by Muntel *et al.*, the 10-plex TMT workflow offered slightly better precision while quantitative trueness was higher for the label-free DIA method [28]. In another experiment, a TMT-based workflow was compared to label-free proteomics using data-dependent acquisition (DDA); comparable levels of trueness of median quantitative ratios but superior sensitivity and precision were obtained with the use of the labels [30]. In large-scale phosphoproteomics, better precision was confirmed using isobaric mass tags but better trueness was obtained with label-free quantification [31]. Finally, label-free methods present in general lower performance in terms of precise and reproducible quantification with respect to the approaches based on isobaric mass tags [28,31,32].

One further advantage of isobaric mass tags often described is the completeness of the generated data matrix despite the use of DDA for the LC-MS analysis. While this is true for a single multiplex experiment where samples are directly compared, this argument falls short when several multiplexed experiments are necessary to accommodate larger numbers of samples and conditions. Experiments are put in relation using a shared/common sample (usually a reference sample obtained from the initial pooling of all samples). However, the stochasticity of the DDA process cannot guarantee the necessary primary identification of the same peptides and proteins in all isobaric mass tagging experiments [30]. In a recent review, Wühr and co-workers suggested the use of DIA, which mostly overcomes the missing value problem, to analyse the specific complement ion clusters (see **Section 4** for technical details) in a systematic way, and cope with such an issue [6].

Importantly, isobaric mass tag reagents can be easily obtained from several commercial sources and are thus directly available to scientists. However, the relatively high cost of these commercial reagents constitutes a severe constraint for core facilities and research laboratories. Less costly alternatives exist as mentioned before (*e.g.*, DiLeu tags) but their use it still not widespread for other reasons (*e.g.*, patent infringement). A clear advantage of

label-free approaches is the absence of these reagent costs, but then the LC-MS analysis often requires additional instrument time for the sequential processing of all samples. This time factor needs to be considered in the overall cost estimation.

3.2. Comparison to other label-based methods

Among label-based methods, isobaric labelling displays quite some interesting advantages. The isobaric mass tagging approach does not increase complexity of the survey MS signal as opposed to mass-differential isotopic labelling methods such as the stable isotope labeling with amino acids in cell culture (SILAC) [33,34], where doublets or triplets of MS signals are created. An increase in the number of precursor ions to be selected induces a reduction of the data acquisition speed and sensitivity. A mass defect-based labelling strategy for MS-centric quantification was more recently developed using metabolic or chemical routes to circumvent such limitation related to the classical mass-differential isotopic labelling [35,36]. However, metabolic labelling with its early isotope incorporation presents clear benefits in terms of quantification accuracy as sample handling and manipulation before mixing is reduced to its minimum. Incorporation of isobaric mass tags, on the other hand, occurs later in the process after protein digestion. Therefore, initial sample preparation steps prior to digestion, efficiency of the digestion as well as the labelling reaction needs to be carefully controlled in order to generate reproducible and accurate results [37].

It is worthwhile to note that non-isobaric versions of iTRAQ or TMT reagents can also be used for mass-differential labelling and therefore quantification based on precursor ions. Non-isobaric mTRAQ [38] and more recently mTMT [39] technologies are available for such purposes, enriching the pool of chemical labelling methods [40].

Fundamentally, chemical labelling presents an important advantage over metabolic labelling (*e.g.*, SILAC) that is the accessibility to a wide range of samples. As such, isobaric labelling is applicable to cultured cells, tissues and body fluids among others and, most importantly as an *in vitro* procedure, is fully compatible with human samples. In that regard, it has been extensively applied and explored in clinical research studies (see **Section 6**).

3.3. And other advantages…

Isobaric mass tags have been modified for the more specific analysis of post-translational modifications (PTMs). For example, some particular chemistries have been developed to directly target protein carbonylation, glycan modifications or cysteine residues. iTRAQ hydrazide (iTRAQH) was used to probe carbonyl groups [41], aminoxyTMT was described for the quantification of carbonyl-containing compounds [42], and iodoTMT labelling was employed for studying cysteine-containing peptides [43,44]. Indirect methods employing a combination of specific peptide enrichment techniques before or after isobaric labelling have been used to study phosphoproteomes [16,31,45,46] and acetylomes [47,48]. An approach

called TAILS for terminal amine isotope labeling of substrates [49], uses isobaric mass tags to distinguish the N-termini of proteins from the N-termini of their protease cleavage products [50,51].

Advantageously, with isobaric mass tags, signals of precursor ions result from the sum of the individual but identical peptides originating from the compared samples. The concept of enhancing MS signal by using one sample of different origin and/or in higher quantity within an isobaric labelling experiment has been put forward [52-54]. The boosting to amplify signal with isobaric labelling (BASIL) was recently reported for phosphoproteomic analysis to increase by a factor of four the number of quantifiable phosphorylated peptides in a limited amount of human pancreatic islet cells [55]. Similarly, this approach was also exploited towards single-cell proteomic analysis [56,57].

Finally, while isobaric mass tags are predominantly used for screening/discovery and relative quantification applications, the technology was nevertheless also evaluated for more targeted and absolute quantification purposes [53,58]. For instance, Erickson *et al.* proposed a combination of chemical mass labelling using a light non-isobaric variant of the isobaric mass tags (*e.g.*, so-called mTRAQ or TMTzero initially used for selected-reaction monitoring (SRM) MS applications [59,60]), and sample multiplexing using classical versions of isobaric mass tags, which they called triggered by offset, multiplexed, accurate-mass, high-resolution, and absolute quantification (TOMAHAQ) [61]. Similarly, Zhong *et al.* described a combined approach called hybrid offset-triggered multiplex absolute quantification (HOTMAQ) that allowed high throughput application of isobaric mass tags for absolute quantification in targeted proteomic experiments [62]. Even higher throughput was reported in targeted proteomics with a 54-plex quantification in a single LC-MS analysis [63].

4. Addressing the co-fragmentation and ratio distortion

The main limitation currently recognized in the quantification performance of isobaric mass tags results from the co-fragmentation of more than one peptide species (**Figure 5**). This induces inaccurate measurements and leads to quantities' ratio compression (or distortion) [64,65]. Ratio compression refers to the underestimation of true quantitative changes obtained with isobaric mass tags. In complex mixtures, a majority of proteins are used to display a 1:1 ratio between comparative experimental conditions. This is a logical consequence of the generally stable concentrations of the majority of the proteins within the investigated proteomes. These proteins, constant in abundance between conditions, and by consequence their proteolytic peptides, represent a stable background. When those "constant" peptides are isolated for fragmentation together with peptides regulated in abundance (*i.e.*, co-isolation and co-fragmentation), their reporter ions released from the isobaric mass tags contaminate those of the latter, more or less reducing the quantitative changes of truly differentially regulated peptides. As a matter of fact, if such co-fragmentation

of peptides also happens between differentially regulated peptides, measurement accuracy *via* the reporter ions is randomly and possibly more strongly impacted. One can expect that low-abundance peptides are more prone to suffer from this phenomenon.

Several research groups have proposed solutions to address this impairment in quantitative accuracy. For instance, Savitski *et al.* used delayed fragmentation to trigger MS/MS at the apex of LC peak and optimized isolation width to reduce the occurrence of interfering species [66]. Logically, sample pre-fractionation and state-of-the-art LC separation are other options that were implemented [53,67,68]. Yet, those approaches not always yielded sufficient improvement. Mathematical methods have also been developed [69-74] but generating best quality data nonetheless remains essential.

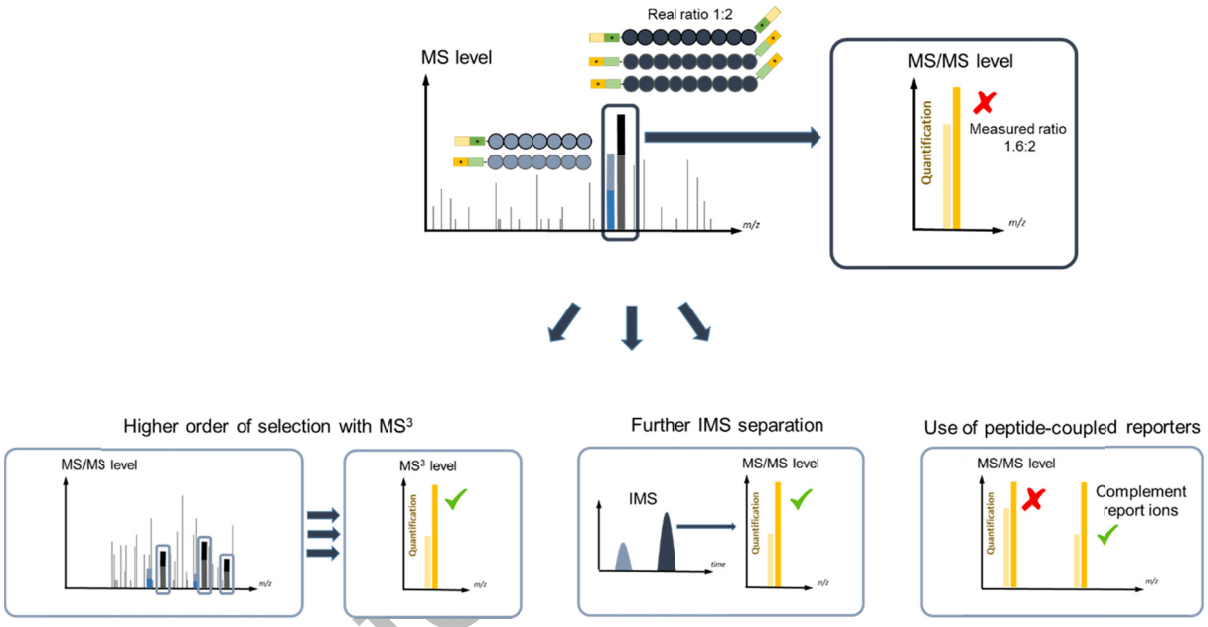

Figure 5. Ratio distortion encountered when performing quantification with isobaric mass tags results from the MS/MS co-fragmentation of the precursor ion of interest (grey/black peak) together with an interfering species (blue/grey peak). Proposed solutions to cope with such an issue encompass additional level of fragmentation (*i.e.*, MS^3), improved precursor ions separation with IMS, or the use of higher-mass peptide-coupled reporter fragments (also called complement reporter ions).

Coon and colleagues proposed gas-phase purification by proton-transfer ion-ion reactions to reduce the charge state of precursor ions, expand the *m/z* space they populate, and therefore decrease the probability of concurrent isolation of multiple peptide ions [75]. Gygi and co-workers pioneered MS/MS/MS, or MS^3, acquisition in order to eliminate ratio distortion observed when using isobaric mass tags [76]. In this approach, first-generation fragments obtained from collision-induced dissociation (CID) are further fragmented to reveal the reporter ions (**Figure 5**). Because of the additional isolation and MS^3 fragmentation, the interference effect is reduced due to the lower chance of isobaric peptides to generate also isobaric fragments. Today, this is probably the most commonly used approach to mitigate co-isolation and co-fragmentation of interfering species. Several further improvements of the

methodology were more recently implemented. One key improvement was the introduction of the so-termed MultiNotch (or synchronous precursor selection (SPS)) MS^3 to systematically co-isolate and co-fragment several MS/MS fragment ions using hybrid Orbitrap mass spectrometers [77]. MultiNotch MS^3 has to a large extent addressed the initial sensitivity issue resulting from the additional MS^3 round of fragmentation and selection of a single MS/MS fragment ion for MS^3 [76,78]. Moreover, the limiting but necessary use of Lys-C enzyme to generate proteolytic peptides tagged at both N- and C-termini, ensuring release of reporter ions from both b- and y-type ions during MS^3, could then be replaced by the use of classical trypsin [78]. A more recent development, now available on the last generation of tribrid Orbitrap mass spectrometers, is the real-time database searching (called real-time search (RTS)) to trigger subsequent MS^3 scans in an adaptive manner only when confident peptide identification occurs [79,80]. These methods clearly help to alleviate the issue with the longer and inefficient duty cycles of the systematic MS^3 acquisition mode.

Another interesting approach to deal with the co-isolation and co-fragmentation of isobarically mass tagged peptides is the use of ion mobility spectrometry (IMS) (**Figure 5**). Lilley and co-workers were indeed able to reduce precursor contamination using travelling wave ion mobility separation (TWIMS) applied after quadrupole (Q) mass filtering on Q-TOF mass spectrometers [81]. High-field asymmetric waveform ion mobility MS (FAIMS) was also shown by the Thibault's group to reduce the occurrence of chimeric tandem mass spectra and provide more accurate quantification using isobaric mass tags [82]. The latest generation of FAIMS devices confirmed improvement in quantitative figure-of-merits, leading to a 2.5 fold increase in the number of quantified peptides compared to that obtained with MultiNotch MS^3 [83]. Schweppe *et al.* further showed the benefit of FAIMS [84] on the quantification of a TMT-based interference standard [85]. FAIMS was efficiently combined with MultiNotch MS^3 and standard high-resolution MS/MS, improving in both approaches measurement accuracy by reduction of the occurrence of interfering species [84].

Another proposed strategy was to base the MS/MS quantification on complement reporter ion clusters at higher *m/z* originating from the partial loss of TMT tags (*i.e.*, intact peptides remaining fused to most of the mass balancer groups) that is precursor-specific (**Figure 5**) [86]. Due to several constraints (*e.g.*, improper design of TMTs to favour the formation of complement reporter ion clusters and lowered multiplexing due to limitation in current resolving power of mass analysers to distinguish the clusters for 10-, 11- and 16-plex TMT technologies), this approach has still not been broadly adopted [87]. The so-called easily abstractable sulfoxide-based isobaric tag (EASI-tag) was recently introduced and similarly uses peptide-coupled reporter fragments for interference-free MS/MS-based quantification [88]. The 6-plex EASI-tag labels dissociate at low collision energy, produce a neutral loss and thus retain the charge on the peptide-coupled reporters, optimizing the quantitative signal in such a strategy. Absence of ratio compression was demonstrated as well as the

accurate measurements of large ratio fold changes exemplified. The SO-tag is another recent reagent materializing these alternative solutions [89].

Many efforts have been made to improve the quantitative accuracy of isobaric mass tags, coping with issues of interference and quantitative ratio distortion. The MS^3 acquisition mode has been demonstrated efficient in numerous works. Alternative methods based on IMS become nonetheless more attractive as they can be implemented on different MS platforms, are sensitive and fast, and provide easy to process tandem spectra containing both the identification and quantification information.

4. Facing computational challenges

4.1. Processing of tandem mass spectra

Inherently to the use of isobaric mass tags, several challenges also arise for the processing of the complex data generated, as well as its analysis.

Recording of the mass and tandem mass spectra can be performed with different flavours of analysers when using isobaric mass tags. Similarly, tandem mass spectra can be obtained with different fragmentation techniques (*e.g.*, CID or higher-energy collisional dissociation (HCD) available on hybrid Orbitrap mass spectrometers), depending for instance if they are used for identification and/or quantification purposes [90,91], as well as different levels of fragmentation (*i.e.*, MS/MS or MS^3). Spectral processing tools are available to handle such level of diversity and complexity. They are either embedded in MS vendors' software (*e.g.*, Proteome Discoverer from Thermo Fisher Scientific), part of commercial data analysis tools (e.g., PEAKS from Bioinformatics Solutions), or supported by researchers' custom solutions [92,93]. The open-source software MaxQuant is an alternative tool to process and analyse both MS/MS and MS^3-based isobaric mass tagging data [93].

4.2. Calculation of relative quantification values

After the initial step of peptide and protein identification, the second step consists in retrieving the quantitative values. This involves a first correction to adjust area under the curves (for profile mode data), or peak intensities (for centroid mode data) of the reporter ion signals [92,94] because isotopic impurities are contained in isobaric mass tag reagents. Optionally, filtering based on minimum threshold of reporter ion intensity and/or level of occurrence of co-fragmentation can be implemented as well. Relative quantification values for all peptides are then calculated in different forms such as ratio fold changes or relative abundances (*e.g.*, Libra methodology implemented in the Trans-Proteomic Pipeline [95]) between the compared conditions. Finally, quantification values for proteins are computed from the different peptides for inference at the protein level using different approaches (*e.g.*, average, media, and weighted average).

4.3. Data normalization

Data normalization is a key step to remove systematic error due to, for instance, sample preparation or instrument variability when multiple experiments are performed. Quantile normalization has been often used for data obtained with isobaric mass tags [96,97] but other methods have been proposed more recently such as constrained standardization [98], median sweeping [99] and analysis of variance (ANOVA)-based normalization [100]. These methods allow reducing the systematic and technical errors and could be valuable when applied to larger sample sizes (*i.e.*, when multiple isobaric labelling experiments are performed).

4.4. Missing data imputation

While missing data are almost absent from a single isobaric mass tagging experiment, their number increases when multiple experiments are performed [101,102]. In such cases, data imputation can be performed for instance with k-nearest neighbours or singular value decomposition [103] in order to avoid restricting the data analysis to the proteins solely measured in every sample and condition, reducing the power of the statistical analysis, or introducing unwanted bias. An alternative way of spectral data processing that looks for similar tandem mass spectra between experiments (based on a roughly similar concept than the match between runs algorithm in MaxQuant [93], but applied specifically at MS/MS level for isobaric labelling applications) was proposed [102]. In this report, the peptide match rescue module saved quantitative information from unmatched/unexploited tandem mass spectra and significantly reduced the initial occurrence of missing values in the raw data.
In summary, data processing and analysis solutions are multiple [96,97,104,105] but the reproducibility of these pipelines has been challenged [106]. In that regard, analysing data from isobaric mass tags should be performed with caution. Approaches that focus on peptide quantification prior to peptide/protein identification may also be attractive to process and analyse isobaric labelling-type of data [102,107].

5. Applications in pre-clinical and clinical studies

Isobaric mass tags have been applied over the last years to a wide variety of proteomic studies resulting in an ever increasing number of publications, as depicted in **Figure 1**. In this section, we do not intend to provide a comprehensive review of these applications. More specifically, we focus on a few of those that smartly exploit the multiplexing capabilities of this technology and elegantly demonstrate the wide applicability of chemical isobaric labelling.

Compartmentalization and exchanges of proteins between organelles within cells are pivotal to cellular processes, and their alterations are associated to various diseases [108]. The localisation of organelle proteins using isotope tagging (LOPIT) was introduced by Lilley's

and co-workers [109,110]. This approach, combining cellular fractionation and proteomic profiling, takes full advantage of isobaric mass tags to decipher spatial subcellular maps using density gradient-based hyperplexed LOPIT (hyperLOPIT) [111,112] or LOPIT after differential ultracentrifugation (LOPIT-DC) [113]. High spatial resolution is achieved to map cells using MS-based proteomics, providing, among others, information on localization of protein isoforms and complexes and PTM dynamics.

The study of PTMs is also efficiently performed with isobaric mass tags. One main advantage is the possibility to directly compare a reasonable number of samples while avoiding important occurrence of missing values. This is particularly important with PTMs because of their low stoichiometry. PTMs can be detected in one sample and missed in another using label-free approaches, for example. Combination of all samples after labelling also helps to increase detection of modified peptides. Phosphopeptides can be probed after specific enrichment, with titanium dioxide for instance, that has been shown to be compatible with isobaric mass tags [114]. This approach was efficiently applied in the study of glucose-dependent phosphorylation in insulin secreting cells [115]. Likewise, acetylated peptides are identified and quantified using a combination of isobaric labelling and immuno-based enrichment of the acetylated peptides before LC-MS and MS/MS analysis [47,116,117].

The thermal proteome profiling (TPP) developed by Savitski and co-workers [118] is used to study protein aggregation and disaggregation, to phenotype cells and to detect protein interactions with drugs, metabolites, and other compounds [119-122]. TPP is facilitated by the use of unbiased multiplexed quantitative MS, and therefore isobaric mass tags, to explore the thermal stability of thousands of proteins *via* construction of melting curves [123,124]. Digging into the structural proteomes and establishing links to protein functions and pathological states using such strategies [125] represent a key task ahead in the proteomic field and accurate quantitative techniques based on isobaric mass tagging are critical tools to achieve this extraordinary challenge [126].

Last but not least, human clinical research studies represent a strategic application field for isobaric mass tag technologies. Sample multiplexing enables increased throughput, a fundamental element to perform large sample-size proteomic studies [37,127]. Due to important biological variability in humans, maintaining a proper study design with a sufficient number of individuals and data points are nowadays becoming a priority in research studies [128-131]. In this regard, isobaric labelling was used to study the plasma and cerebrospinal fluid proteomes in increasingly large cohorts of individuals, from hundreds to thousands of body fluid samples [132-135]. Similarly, iTRAQ and TMT have been used to probe a wide range of diseases and identify putative biomarkers in human plasma [136].

6. Conclusion

Isobaric labelling technologies are amongst the most popular techniques for the relative quantification of proteins with MS. They provide adequate relative protein quantification performance for discovery exercises. Isobaric mass tags can be applied to almost all sample types, and importantly offer multiplexing capabilities for concomitant comparison of two to sixteen samples in a single experiment (multiple experiments can be linked with the use of a common reference sample). They come with certain drawbacks similar to other proteomic techniques currently used, the primary one being the co-fragmentation limitation that results in quantitative ratio distortion. This issue has been a topic of intensive research over the last ten years. End-users of proteomic technologies generally look for lean workflows and efficient processes but still need to deal with trade-offs when performing differential screening of proteomes in large sample-size studies. All those factors require detailed considerations when opting for isobaric mass tagging. Selecting the appropriate workflow is key. It should integrate optimal sample preparation, efficient analytical separation, MS detection and acquisition, all combined with intelligent data processing, analysis and interpretation.

7. Expert opinion

In fifteen years, isobaric mass tagging has significantly evolved into a mature proteomic technology. Since the initial concept introduced in 2003, multiplexing capabilities and the possibility to compare an increasing number of samples have greatly improved, *e.g.*, from two samples at a time to sixteen with the last commercial set of tags available. Moreover, quantitative performances, mainly relying on improved sample preparation and adapted MS data acquisition methods, have transformed the tool into a robust quantitative technique widely used in proteomic research laboratories, core facilities and contract research organizations.

The deployment of state-of-the-art workflows, however, still remain cumbersome and to some extent limited to experienced proteomic specialists. Efficient management, coordination and execution of sample preparation, MS data acquisition, and processing/analysis of the data is key for a successful application of isobaric labelling techniques. The dependence to selected mass spectrometer manufacturers or to particular data processing and bioinformatic solutions adds another level of complexity, especially when they evolve over time. Significant associated costs (mainly the reagent cost [137] and that of high resolution mass spectrometers) can also act as a potential barrier for the full adoption of isobaric mass tags.

In our laboratory, we have aimed at implementing easily manageable workflows with automated steps such as sample preparation and data processing. Up to now, we have privileged the simplicity of MS/MS scanning for data acquisition. While scouting for

alternative MS acquisition modes and their implementation (*e.g.*, SPS-MS^3 with enhanced trueness but reduced sensitivity), MS/MS has yet offered sufficient precision and very good repeatability of our quantitative measurements as also recently described by others [31]. While such data is inherently suffering from quantitative ratio distortion, they have allowed to reproducibly decipher relative protein changes in human plasma samples from large independent cohorts [132]. The results have been furthermore confirmed using label-free DIA MS [138] that is free of such quantitative distortion. Sample complexity and protein abundances have certainly an influence on the quality of the readouts and careful evaluation of the quantitative needs is essential to correctly address scientific questions. Interestingly, the emergence of alternative solutions to cope with the quantitative ratio distortion such as the use of IMS tends to offer an excellent compromise between implementation effort and quantitative performances. Importantly, isobaric labelling, considered mainly as a relative quantification technique, is not intended to substitute quantitative targeted MS-based assays. While important developments have demonstrated the use of the technology in multiplexed targeted assays for hypothesis-driven research or confirmatory studies, we currently see the primary application field in exploratory and discovery studies and generation of novel biological hypotheses.

In the future, we expect further democratisation of the isobaric mass tagging technology that requires strict consolidation of MS data acquisition and processing tools. A universal approach with precise implementation is critically needed to reduce the risk of irreproducible and divergent results within and between laboratories. Efforts of proteomic method standardization are pursued by several scientific initiatives and the isobaric labelling technology should be an integral part of it.

An increase of sample multiplexing still remains highly desirable to explore larger cohorts and sample sizes and thus bringing proteomics closer to other omics platforms in terms of sample throughput. This will help to face the multiple challenges uncounted in proteomics, such as the broad diversity, high dynamics, and complex structural changes of the proteomes, where large-scale accurate protein quantification is essential. Positive competition and benchmarking against other label-free and label-based quantitative approaches is important and will lead at the end, hopefully, to a few optimal solutions. Surely, it can be speculated that those MS-based approaches will further evolve and enable more efficiently to translate proteomic discoveries into real world outcomes.

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* of interest

** of considerable interest

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