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# Isotope labelling-based quantitative proteomics: A comparison of labelling methods

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## Abstract

In the pursuit of identifying and quantifying biomarkers, quantitative proteomics has become a very important tool. These biomarkers are differentially expressed or modified in the onset or progression of a disease and provide important information for the diagnosis, prognosis and potential therapeutic targets for treatment. Relative quantitative proteomics aims to reveal which proteins are differentially expressed between samples. Bottom-up proteomics is the identification of proteins by analysing peptides generated from the proteolysis of proteins whereas top-down proteomics analyse intact proteins directly. Bottom-up proteomics is more widely used due to peptides being more easily ionised and fragmented. This review focused on comparing the strengths and limits of the following isotopic and isobaric labelling methods: Stable Isotope Labelling by amino acids in cell Culture (SILAC), dimethyl labelling, Cleavable Isotope Coded Affinity Tag (cICAT), Isotope-Coded Labelling (ICPL), $^{16}O/^{18}O$ labelling, Isobaric tag for relative and absolute quantitation (iTRAQ) and Tandem Mass Tags (TMT). Moreover, the discussion was devoted to discussing which methods are most suited for the different kind of samples. This literature study concludes that all the different labelling methods are suited for different kinds of samples due to their strengths and drawbacks and that there is no one best method for all samples.

# Introduction

Quantitative proteomics has become a very important tool in the discovery of biomarkers in complex biological samples. Important information for the diagnosis, prognosis and potential therapeutic targets for treatment can be provided by identifying and quantifying biomarkers. These biomarkers are differentially expressed or modified in the onset or progression of a disease.[1] These proteins can be quantified either as an absolute value or relatively with regard to the amount of the same protein in a control sample. Relative quantitative proteomics, therefore, aims to reveal which proteins are differentially expressed between samples. In some cases, proteins can be absent or present in either the healthy states or sick states.[5]

Bottom-up proteomics is the identification of the proteins by analysing peptides generated from the proteolysis of the proteins.[10] In a bottom-up experiment, the peptide mixture is fractionated and then analysed with Liquid Chromatography (LC)-Mass Spectrometry (MS)/MS. The resulting mass spectra are then compared to mass spectra in a database to identify to which proteins the peptides belonged to. Top-down proteomics is another strategy which analyse intact proteins directly. The top-down approach is fundamentally different compared to bottom-up proteomics since intact proteins have distinctive characteristics in regard to fractionation, ionisation, and fragmentation in the gas phase.[10] Bottom-up proteomics is more widely used due to the peptides being more easily ionised and fragmented.[10] A general workflow is shown in **Figure 1**.

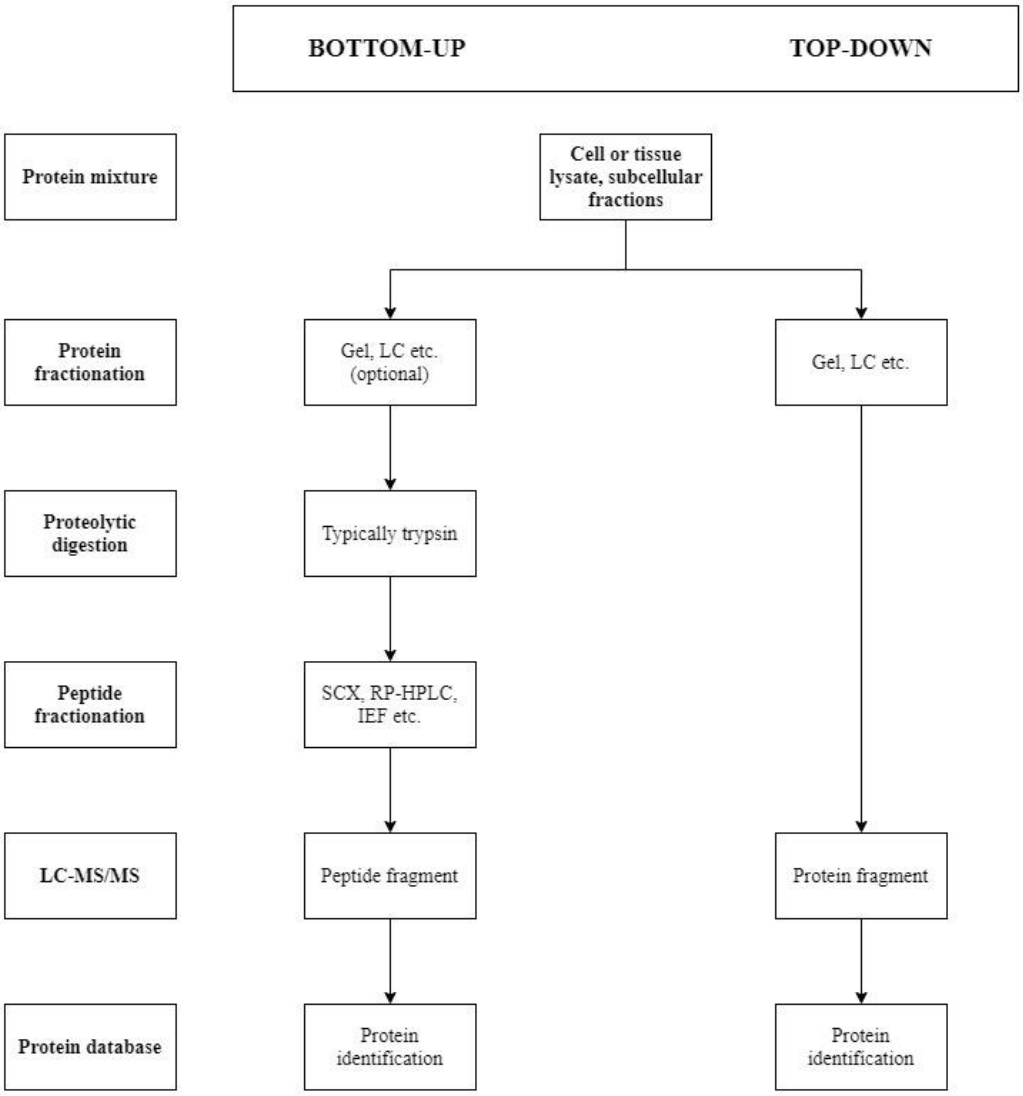



**Figure 1. The general workflow of bottom-up vs. top-down proteomics. (SCX; Strong Cation Exchange, RP-HPLC; Reversed Phase High Performance Liquid Chromatography, IEF; IsoElectric Focussing)**

MS is an analytical technique that measures the mass-to-charge (*m/z*) ratio of ions. Mass spectrometers consist of three main parts: an ion source, a mass analyser and a detector. The ions are generated from molecules in the solid or liquid phase which are transferred to the gas phase. The ionisation is commonly done with electrospray ionisation (ESI). After ionisation, the ions travel through the analyser to the detector where the *m/z* values and intensities are recorded for each ion species. These ions can either be introduced in a LC-MS ($MS^1$) setup or a LC-MS/MS ($MS^2$) setup. Peptide signals are then recorded in an intact form ($MS^1$) and a fragmented form ($MS^2$). A schematic overview of a $MS^2$ setup in the Triple Quadrupole mass spectrometer is shown in **Figure 2**. The typical mass spectrometers used in proteomics are:

orbitraps, ion-trap, quadrupole and fourier transform ion cyclotron resonance (FTICR).[12],[14],[16]

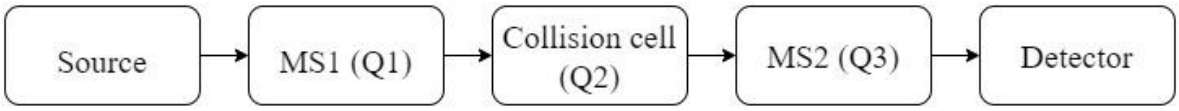



**Figure 2. A schematic overview of a $MS^2$ setup. After ionisation in the source, the ions travel to Q1 where precursor ions with a specific *m/z* value are selected, the precursor ions then enter the collision cell where they are fragmented (Q2) , the product ions enter the last mass analyser (Q3) and then pass to the detector.**

Collision-induced dissociation (CID) is a technique used to induce fragmentation of the peptide backbone. Bond breakage mainly occurs through cleavage of the amide bonds.[15] This will lead to b-ions (charge retained at the N-terminus) and y-ions (charge retained at the C-terminus). This is shown in **Figure 3**.

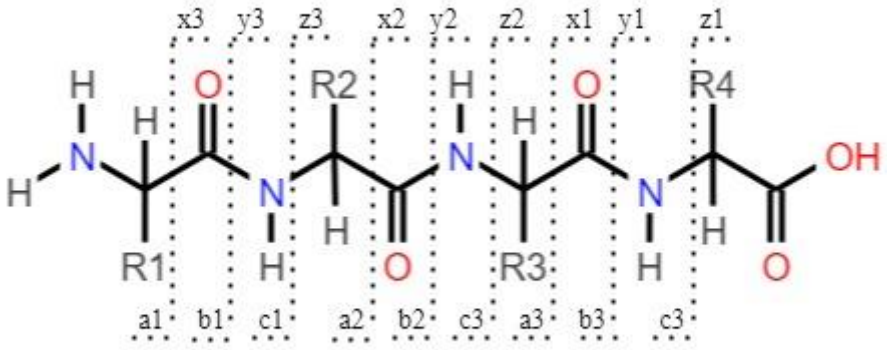



**Figure 3. Structure of a peptide with the assigned b- and y-ions.**

It is important that samples are labelled for the quantification of proteins or peptides. Stable isotope quantitative proteomics is an approach which involves different mass additions to the peptide/protein and quantifying them by comparing distinct isotope peaks. These samples are tagged with a heavy mass tag (heavy isotopes e.g. $^{2}H$, $^{13}C$, $^{15}N$, $^{18}O$) and a light mass tag (naturally abundant isotopes e.g. $^{1}H$, $^{12}C$, $^{14}N$, $^{16}O$).[2] The samples are mixed and are then analysed with MS. The specific mass shift is observed with the mass spectrometer. The mass spectrum then shows the ratio of the different mass tags and the relative abundancies of the tagged samples can then be determined. This allows to determine whether proteins are up- or

downregulated in different states (e.g. healthy vs. disease). Some well-known stable isotopes quantitative strategies are isotope-coded affinity tag (ICAT), dimethyl labelling, stable isotope labelling with amino acids in cell culture (SILAC) and $^{16}O/^{18}O$ labelling.[13]

The isotopic quantitative proteomics is limited to 2-plex, 3-plex or 4-plex set of tags.[5] Therefore, the comparison of more samples in one experiment is not possible. To overcome this limitation, isobaric tags were developed. The utilisation of isobaric tags is a major tool in quantitative proteomics and it allows the quantification of more samples in a single experiment. The isobaric tags consist of a reactive group that ligates tags to peptides, a reporter region (the signature region) and a balancer region that keep the overall mass of tags the same. After fragmentation, the relative abundancies of the tagged peptides will be revealed due to the released reporter ions having different masses. The most commonly used isobaric tags are isobaric tagging for relative and absolute quantification (iTRAQ) and tandem mass tagging (TMT).[1],[11]

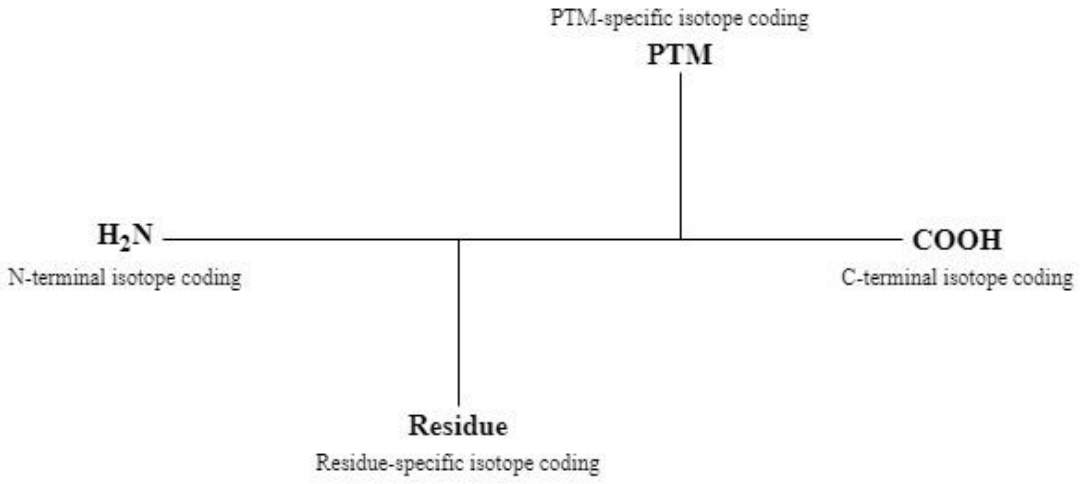



**Figure 4. Possible targets for chemical tagging**

Peptides or proteins can be tagged at specific sites or common functional groups. To ensure the highest possible coverage, common functional groups like the amino group at the N-terminus or the carboxyl group at the C-terminus of a peptide or protein are tagged, as almost every peptide will then carry the tag[2]. Specific approaches target rare amino acid residues or ones that are prone to chemical modification. Common PTMs like phosphorylation and glycosylation can also be targeted with chemical tagging[2].

There are many methods available to do the relative quantification of proteins, each with their own strengths and limits. It is vital to find accurate, sensitive and specific analytical methods

to quantify the change in expression of proteins in different states. This review will focus on comparing and summarising the strength and weaknesses of stable isotope labelling-based quantitative proteomics.

# Labelling methods

## Isotopic labelling

### Stable isotope labelling by amino acids in cell culture (SILAC)

SILAC is a metabolic labelling method where an isotopic label is added on a protein while the protein is still metabolically active. SILAC is based on cell cultures in isotopically distinct media, such as one with light amino acids (naturally abundant isotopes) and another with heavy isotopically labelled amino acids as shown in **Figure 4.** Labelling the amino acids with heavy isotopes is usually achieved with $^{2}H$, $^{13}C$ or $^{15}N$.[1] Essential amino acids for a cell are frequently targeted for isotopic labelling as each newly synthesized protein contains the isotopically labelled amino acid. This is different from other labelling methods where only specific functional groups are tagged. Leucine, lysine and methionine are used in SILAC as these are essential amino acids.[1],[6] However, arginine is often isotopically labelled in combination with lysine. The advantage of using an arginine/lysine mixture is that, during digestion with trypsin, a single isotopic label is left on each peptide.[1],[6] This makes it easier for identification and quantification. Moreover, it allows for multiplexing (up to 6-plex) due to the combination of different isotopic forms of arginine and lysine.[6],[18],[19] Choi, et al. (2020) are looking into expanding their platform to 9-plex SILAC. Due to labelling amino acids in an organism, the incorporation of a stable isotope sequence is predictable and dependent.[5] The number of cell divisions depends on the rate of protein synthesis, degradation and turnover.[5] Complete labelling of the proteins in the heavy medium is required prior to quantification. Therefore, the complete labelling needs to be tested. After complete labelling, the populations are mixed and digested, such as with trypsin, into peptides. The peptides are then analysed with LC-MS. A mass shift will be observed and the relative abundancies can then be determined.

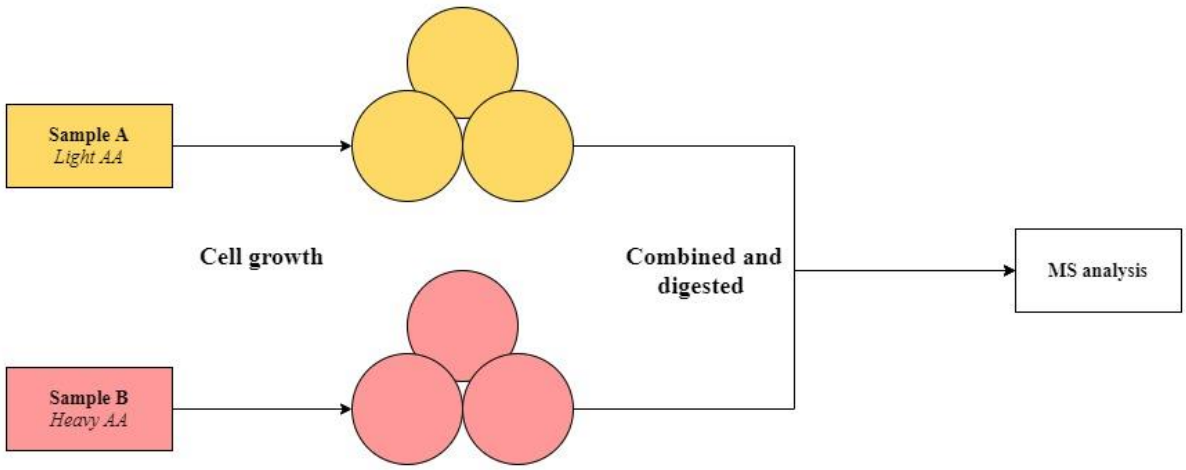



**Figure 4. Overview of SILAC. Isotopically different amino acids are incorporated in the growth media. Thereafter, the samples are combined, digested and analysed using MS.**

The major advantage of using SILAC over other methods is that, due to the incorporation of the stable isotopes at an early stage of the experiment, the variation due to sample preparation and purification is largely reduced.[1],[5] Moreover, no chemical reactions are necessary. Therefore, SILAC is very practical. PTMs have shown to play an important role in the development of a disease. Phosphorylation is one of the most important PTMs.[17],[20] It regulates enzyme activation by inducing conformational changes in protein structure. This process is reversible.[17] Important information on signal transduction mechanisms can be achieved from characterising PTM dynamics. Site specific phosphorylation can be quantified with SILAC. Sarhan et al. (2017) used SILAC to identify 116 upregulated phospho-sites and 45 down-regulated phospho-sites in response to platelet derived growth factor (PDGF) stimulation in mouse embryonic fibroblasts (MEFs). The authors studied the mechanisms by which the PDGF family of ligands induce cell proliferation and migration during development, tissue homeostasis and interactions between tumours and stroma. Hu et al. (2021) used SILAC to analyse histone post-translational modifications and protein differential expressions in MCF-7 cells under oestrogen exposure. 49 histone variants were identified and 42 were quantified. The results showed that 2 differentially expressed proteins were associated with breast cancers.

SILAC does come with its limitations. Non-dividing cells are difficult to quantify as complete labelling is necessary and the incorporation of the isotopically labelled amino acids happen during cell divisions.[1] The conversion of arginine to proline has an impact on the accuracy of the overall quantification.[1],[6] Arginine is taken up by the cells in the medium and converted by arginase to proline. This can result in proteins with proline residues that are labelled with

$^{13}C$ and/or $^{15}N$ from isotopic arginine.[23] This will cause quantification errors. However, several approaches to solve this problem have already been presented. Lößner et al. (2011) showed that the addition of unlabelled proline to the SILAC medium solves this problem. However, this approach may not be applicable to all cell lines as some cell lines or organisms have a high rate of arginine-to-proline conversion.[23] Park et al. (2012) suggested a SILAC label-swap replication approach. This approach uses geometric averaging to compensate for the errors in each replicate. Moreover, they demonstrated that this method can be extended to more complex SILAC experiments. The limitations of SILAC have been mostly addressed but to this day are still being improved. However, SILAC is less widely used due to its cost.[1] Especially in cell lines where the incorporation level is low. That said, SILAC is an excellent labelling strategy for quantitative proteomics with major advantages over other methods.

### Dimethyl labelling

Dimethyl labelling is a label-based quantification approach at the $MS^1$-level.[9] The mechanism of the dimethylation of primary amines is depicted in **Figure 5**. The reaction of formaldehyde with amine groups of peptide N-termini and lysine residues results in the formation of a Schiff base. This base is then reduced by cyanoborohydride and reacts with another formaldehyde forming the dimethylamino group. All primary amino groups are dimethylated except for the N-terminal proline which is monomethylated.[3],[8] Different isotopic forms of the reagents formaldehyde and cyanoborohydride are used for the comparative quantification of two samples. However, this can also be extended to multiplex experiments (up to 5-plex). Wu et al. (2014) designed a five-plex experiment by combining $H_2CO/NaBH_3CN$, $H_2CO/NaBD_3CN$, $D_2CO/NaBH_3CN$, $D_2CO/NaBD_3CN$ and $D_2{}^{13}CO/NaBD_3CN$. To reduce overlapping of isotope clusters, Lys-C was used for the protein digestion. At least two labelling sites were generated for each peptide, resulting in a mass difference, between the nearest labelled forms, of 4 Da.

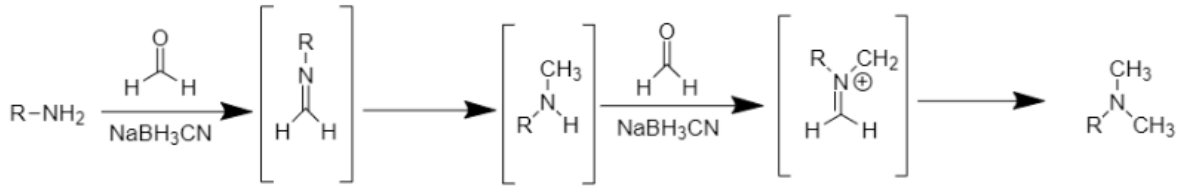



**Figure 5. Mechanism of the dimethylation of the primary amines**

SILAC can only be applied to samples derived from a cell culture or samples where labelled tissues are commercially available.[26] In contrast, dimethyl labelling can be used on any sample. Moreover, dimethyl labelling is a fast, specific and cost-efficient chemical labelling approach for quantitative proteomics.[4],[26] Lau et al. (2014) compared a dimethyl labelling approach and a SILAC approach on the HeLa cell line. They demonstrated that SILAC and dimethyl labelling have comparable quantitative performances. However, more proteins were identified with SILAC. Moreover, they reported that the repeatability with SILAC is four times better under the experimental conditions. This is due to the major advantage of SILAC having over chemical labelling approaches, being the possibility of the incorporation of labelled amino acids in an early stage, thus eliminating the variation in sample preparation. Whereas with chemical labelling approaches like dimethyl labelling, the labelling is performed at the peptide level. This requires parallel processing of samples to be compared through lysis and peptide digestion.[26] This introduces the risk for quantitative inaccuracies deriving from loss of analyte and differential processing efficiency. Nevertheless, dimethyl labelling is a solid approach and a practical substitute as SILAC cannot be freely applied in tissue or clinical samples.

### Cleavable isotope coded affinity tag (cICAT)

ICATs are the first commercially available tags on the market and are used for the relative quantitation of proteins in two different states.[5] It is a residue-specific method and can only be applied to cysteine-containing peptides. ICATs consist of three parts: biotin (affinity tag), linker and an iodoacetamide group (reactive group). The biotin moiety is included for affinity purification. Cysteine-containing peptides are isolated from a complex sample with a biotin-streptavidin affinity capture. Therefore, the number of other peptides introduced into the mass spectrometer is reduced. The iodoacetamide group reacts with the thiol group on the cysteine residue. The linker region contains the heavy isotopes ($^{13}C$ or $^{2}H$) or light isotopes ($^{12}C$ or $^{1}H$). After the peptides are labelled with ICATs, the mixtures are combined and then proteolysed. The relative abundancies of the peptides in two different states can then be determined.

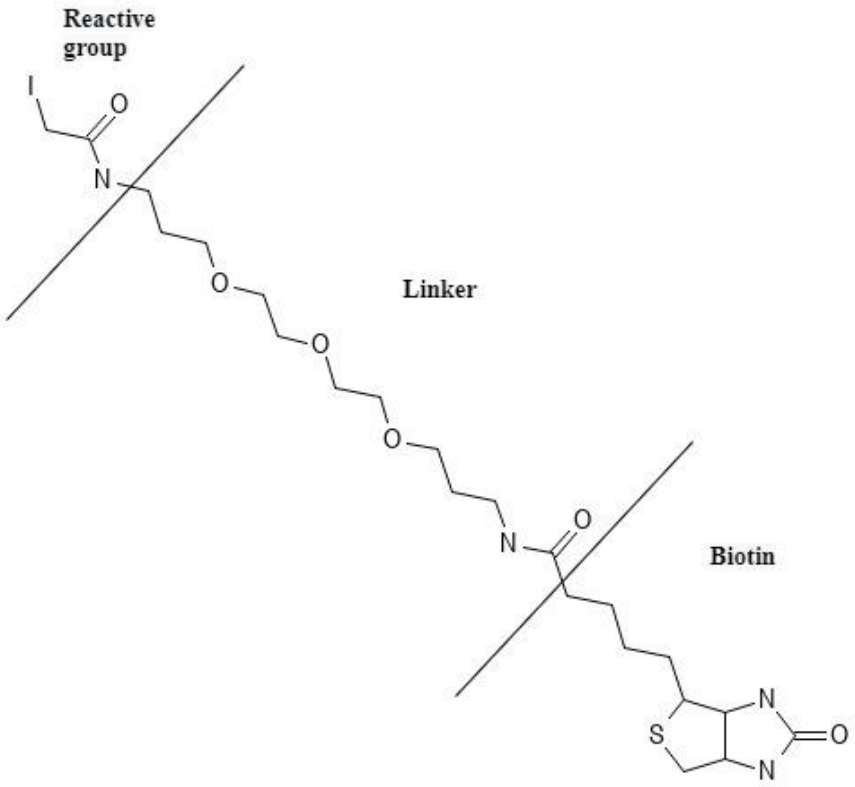



**Figure 6. Structure of an ICAT reagent**

There were, however, some drawbacks when using this ICAT reagent. Due to the use of deuterium, problems for quantification arose as there was a chromatographic shift between light and heavy peptides. The label is relatively large and did affect the peptide fragmentation which complicated the MS/MS spectra. The streptavidin-biotin affinity capture had problems such as significant unspecific and/or irreversible bindings.[2] Due to these problems, an improved reagent was developed.

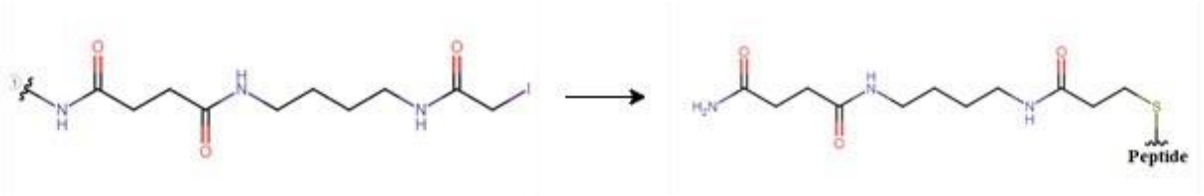



**Figure 7. (left) Structure of the cICAT tag with a cleavable biotin tag (1). (right) Labelling of a cysteine-containing peptide.**

cICAT uses $^{13}C$ labelling for the heavy tag. This generates a mass shift of 9 Da which allows the co-elution of light and heavy tagged peptides. The size of this reagent is reduced prior to LC-MS analysis due to the cleavable biotin tag. This method can be used to quantify certain

PTMs. Wu et al. (2019) demonstrated an experiment where they identified and quantified protein persulfidation. The persulfidated proteins are labelled with cICAT reagents. Streptavidin beads were used to selectively enrich the peptides after digestion. Thereafter, these peptides were fractionated by strong cation exchange chromatography (SCX), and then introduced in a LC-MS/MS setup for the identification. 74.3% of the 226 identified endogenous persulfidation sites were newly discovered. 21 persulfidation sites, out of the 44 which passed the $p < 0.05$ threshold, were differentially expressed. 19 were up-regulated and 2 were down-regulated.

Capturing only cysteine-containing peptides reduces the sample complexity. It may be the case that biologically relevant proteins do not contain a sufficient amount of cysteine residues thus making the identification more difficult. This can give ICAT an advantage over amine-labelling methods but generally this is a disadvantage. However, these methods can be combined therefore improving the ability of identification and quantification. It is currently not possible to analyse multiple samples in a single experiment using ICAT. There is less confidence and the protein identities and quantitation are based on fewer peptides.[39] There is a bias for proteins containing a high abundance of cysteine residues.

### Isotope-coded protein labelling (ICPL)

ICPL is a variation of ICAT. It is a labelling method that targets free amino groups of intact proteins instead of the thiol groups on cysteine-containing peptides. Lysine side chains and N-termini are labelled due to N-hydroxysuccinimide (NHS) being able to fully derivatise primary amino groups.[5] Similar to ICAT, reagents are either labelled with heavy atoms ($^{13}C$ and/or $^{2}H$) and light atoms ($^{12}C$ and/or $^{1}H$). Different variants can be used therefore allowing multiplex analysis (up to 4-plex).[48] Trypsin is not able to cleave peptide bonds involving ICPL-modified lysine. However, a combination of trypsin and Glu-C will significantly increase the number of labelled peptides which can be identified by MS analysis.[35]

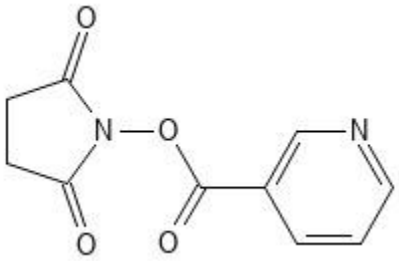



**Figure 8. The structure of an ICPL reagent**

Proteins are labelled prior to digestion. Therefore, the samples are combined at an early stage of the preparation which is an advantage. The physicochemical properties of the proteins are preserved after labelling. Different fractionation steps, such as chromatographic separation, reduce the complexity on a protein level without decreasing quantitation accuracy. Another advantage is that this method allows the separation of different species (protein isoforms and PTMs) of labelled proteins before MS analysis.[35]

Only lysine containing peptides can be quantified when using ICPL reagents. Consequently, only 60%-70% of the peptides can be quantified.[5],[35],[36],[37] However, a post-digest ICPL approach has been shown to increase the number of identified and quantified proteins. Fleron et al. (2010) demonstrated a post-digest ICPL strategy for the quantification of phosphorylated and glycosylated proteins. The authors showed that 600 more individual proteins were identified and more than 95% were quantified compared to the ICPL method. This is a significant improvement. Leroy et al. (2010) used a bacteria sample to compare the classical ICPL approach and the post-digest ICPL approach. The classical ICPL approach identified and quantified 608 and 440 proteins, respectively. Whereas the post-digest ICPL approach identified and quantified 674 and 640 proteins, respectively. However, the post-digest ICPL approach does not allow the separation of different species of labelled proteins, which is possible with classical ICPL.

### $^{16}O/^{18}O$ labelling

$^{16}O/^{18}O$ is a simple enzyme-facilitated labelling technique where the protein digestion and stable isotope labelling are performed simultaneously. Trypsin, chymotrypsin, and Glu-C[1] are typically used to catalyse the exchange of two $^{16}O$ atoms for two $^{18}O$ atoms at the C-terminal carboxyl group of peptides. This results in a mass shift of 4 Da. This method allows two samples to be quantitatively compared by digesting one protein sample in light water ($H_2{}^{16}O$) and one in heavy water ($H_2{}^{18}O$). The two samples are then pooled together and quantified using MS.

This method can be applied to any sample for bottom-up proteomics. This method is unique in that specific amino acids are not required as two $^{16}O$ are substituted by $^{18}O$ in water which reduces the complexity. No biologically important modifications are required in $^{16}O/^{18}O$ labelling. This technique is applicable to clinical samples as the required sample volume is relatively small, such as in cerebral spinal fluid.[32] Moreover, this method can be combined with other stable isotope labelling methods due to the utilisation of different proteases. Smith

et al. (2007) demonstrated that this method can be used for the relative quantification of post-translational modifications. Phosphatases were used to dephosphorylate the $^{16}O$-tagged samples and the differentially labelled samples are combined in the LC-MS. The extent of phosphorylation before the sample is treated with the phosphatase is determined with the intensity of the peaks.

A drawback of $^{16}O/^{18}O$ labelling is incomplete incorporation or exchange of the isotope which results in a biased quantification. However, this effect can be compensated for as demonstrated by Halligan et al. (2005) who developed ZoomQuant. This computational tool uses high resolution zoom spectra to quantitate the ionic species. Another drawback is that this method cannot be used for experiments where multiple samples have to be analysed in one experiment. This method can be used for size-limited samples, however $^{16}O/^{18}O$ is not used for very complex protein samples.

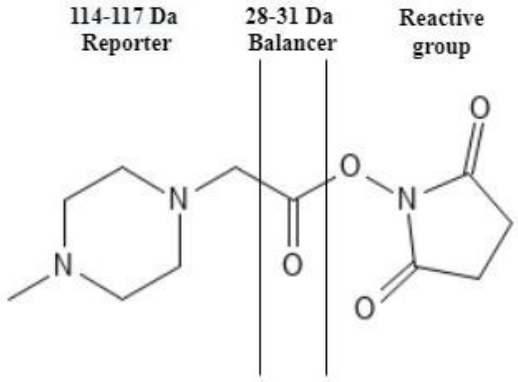
## Isobaric tagging

### Isobaric tag for relative and absolute quantitation (iTRAQ) and Tandem Mass Tags (TMT)

iTRAQ is an isobaric amine-specific chemical tagging method. Peptides are covalently modified with iTRAQ tags. The reagent reacts with all the primary amines of peptides therefore almost all peptides present in the sample are labelled. This also provides information on any modifications on the peptides. The heavy isotope distribution in the tag is used to encode the different conditions. Unlike isotopic labelling methods, each variation of an isobaric tag set has an identical overall mass. However, the heavy isotopes are distributed among the tag and after fragmentation ($MS^2$) the reporter ions yield different masses depending on which sample the peptide originated from. The total mass is kept constant by adjusting the isotope distribution between balancer and reporter-ion. Different variations of $^{13}C$ and $^{15}N$ isotopes are used. The same peptides from different samples that are identical and eluted at the same time. This results in a single peak in the $MS^1$ spectra. This avoids the increasement of complexity at $MS^1$ level when the number of samples is increased. The signals produced by the reporter ions of different masses after fragmentation, can then be used for the relative quantification of the peptides.

TMT is another isobaric tag often used in quantitative proteomics. Just as the iTRAQ reagent, the TMT reagent consists of a reporter region, a balancer region and a reactive group. The heavy isotopes are distributed between reporter-ion and balancer and after CID the reporter ions yield different masses. The mass of the reporter region is balanced with the balancer region to have a constant overall mass. A single peak is observed in the $MS^1$ spectra. After

fragmentation, the peptides can be quantified relatively and the series of y- and b-ions can be used for sequencing or identification.[5]



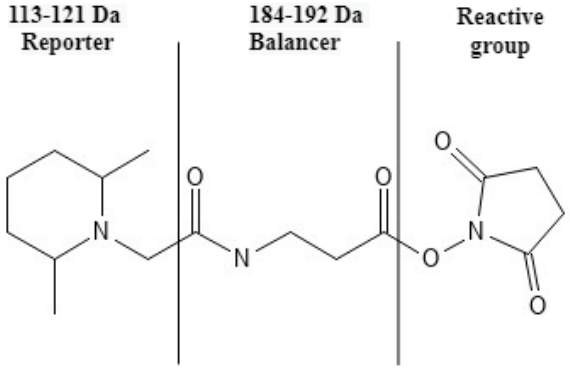




**Figure 9. iTRAQ reagent structure** **Figure 10. TMT reagent structure**

Due to the limited possibility of stable isotope labelling methods to analyse many samples in one experiment, isobaric tags were developed. The possibility to perform experiments where up to 16 samples can be analysed in one experiment with isobaric tags is a major advantage over isotopic labelling methods. Up to eight samples (8-plex) can be analysed in a single run by using iTRAQ reagents. The utilisation of TMT reagents allows up to 16-plex labelling. This limits costs and reduces time spent as multiple samples are analysed simultaneously. Liu et al. (2020) used an 8-plex approach to quantify multi-kinase activity in cell lysates after insulin stimulation or inhibition. This study is the first to report a combination of an 8-plex iTRAQ approach with MALDI-TOF/TOF MS analysis. Lastly, lower sample concentrations can be used due to multiple samples contributing to the same signal.

Although the utilisation of isobaric reagents is very attractive, there are limitations. A well-known problem is ratio distortion. This is due to background interference and isotopic contamination.[5] When two peptides have very similar *m/z* values, mass spectrometer instruments might not be able to resolve these during precursor ion selection. The resulting daughter ion spectrum will contain ion fragments and reporter ions from both peptides.[5] This can distort the reporter-ion ratios and therefore the protein and peptide abundance cannot be accurately quantified. This is very challenging to minimise, however studies have proposed approaches to reduce ratio distortion. Wenger et al. (2011) demonstrated a gas-phase purification method known as QuantMode. The charge of all peptides is reduced by one and after isolation of the new *m/z* window, interfering peptides can be removed. They showed that this method resulted in a more accurate quantification as the interfering peptides were significantly reduced. However, peptides with the same charge as the target peptide are still

co-isolated. Moreover, the process of reducing the charge is slow, therefore reducing the collected spectra. Ting et al. (2011) demonstrated that introducing another fragmentation and isolation step ($MS^3$) counteracts ratio distortion. This additional step removes interfering peptides resulting in a more accurate quantification. Initially, this method reduced the sensitivity of the quantification due to the isolation of only a single isobaric-tag labelled fragment ion from each $MS^2$ spectrum.[11] McAlister et al. (2014) introduced a MultiNotch MS3 method to overcome this. Multiple fragment ions are isolated from each $MS^2$ spectrum resulting in improved sensitivity. However, the $MS^3$ approach has disadvantages, one of them being the need of additional MS scans. This slows the process and results in a loss of ions. Moreover, it is more complex and instrumentation is expensive.

Although there are drawbacks towards using isobaric tags, the ability to analyse many samples simultaneously is a major advantage over other methods. Isobaric labelling remains one of the most commonly used methods in quantitative proteomics.

### iTRAQ and TMT multiplex labelling

Both iTRAQ and TMT are isobaric labelling methods which allow up to 8-plex or 16-plex measurements, respectively. The labels are conceptually similar but differ in structure and mass. The different iTRAQ and TMT reagents differ with respect to the depth and reproducibility of proteome coverage and differential protein expression. However, data regarding the reproducibility and comparability is lacking in the literature. Therefore, the depth and reproducibility of iTRAQ 4-plex, 8-plex and TMT 6-plex reagents were studied by Casey et al. (2017). The study found that iTRAQ 8-plex and TMT 6-plex reagents provide a lower depth of total proteome coverage and differentially expressed proteome coverage. 39% fewer proteins were identified using these two reagents. A study done by Pichler et al. (2010) found that with iTRAQ 4-plex more proteins and peptides were identified, which is in line with the study performed by the previous authors. However, these findings are in contrast with a study performed by Pottiez et al. (2012). These authors concluded that 4-plex tagging provide less consistent ratios than 8-plex tagging. However, there are multiple factors such as sample complexity, workflow, instruments, and search algorithms that could explain the discrepancies in results.

A study done by Wang et al. (2020) combined the multiplex capacities of 11-plex TMT and 16-plex TMT, to analyse 27 samples in a single experiment. Human brain tissue of Alzheimer's disease cases were analysed and then the outcome of the different reagents were compared.

TMT 16-plex performed similar to the 11-plex reagents. Over 270 000 peptide-spectrum matches (PSMs) and over 110 000 unique peptides were identified using 16-plex and 11-plex reagents. TMT 11-plex identified 15% more peptides than TMT 16-plex reagents. This finding is in line with a study performed by Thompson et al. (2019) which reported a lower performance of TMT 16-plex compared to TMT 11-plex. Over 410 000 PSMs were identified with the TMT 27-plex method (over 210 000 TMT 11-plex labelled PSMs and over 200 000 TMT 16-plex labelled PSMs). This is approximately a 50% change of identified PSMs of the individual TMT 11-plex and 16-plex analysis. The correlation of the protein fold change in the TMT 27-plex experiment is significant with the individual TMT 11-plex and TMT 16-plex analysis.

The studies indicate that analysing more samples in a single experiment result in the identification of fewer peptides and proteins. However, the obvious advantage is that more samples can be analysed in a single experiment. For example, 27 samples can be measured in one experiment with TMT 27-plex but would require multiple TMT 11-plex experiments. This would result in more instrumentation time and introduce a source of variability.

## Discussion

Every method has its own strengths and limits. There is no best method to use for every sample. This part of the review will be devoted to discussing which methods would be most suitable for the different kind of samples.

SILAC requires that freshly biosynthesised proteins in cells must be completely labelled, therefore making it difficult to use postmitotic cells as these do not divide in culture. This method has been successfully applied to compare protein expression changes during cell differentiation. The SILAC approach is mostly used on eukaryotic cells and cell cultures. A study done by Itzhak et al. (2019) used the HeLa cell line to quantify the proteome wide expression changes following proteostatic stress. The authors identified 38 proteins which were not previously linked to the unfolded protein response. The expression of these 38 proteins increased, 15 of which remediate endoplasmic reticulum stress, and the remainder may contribute to pathological outcomes. A study by Hu et al. (2021) used whole MCF-7 cells to analyse histone PTMs and protein differential expressions in MCF-7 cells under oestrogen exposure. Another study done by Sarhan et al. (2017) used MEFs to study up- and down regulated phospho-sites in response to PDGF stimulation. SILAC has an excellent quantification accuracy and a good quantitative proteome coverage and would be best suited for cell cultures and eukaryotic cells. It is not suited for samples containing undividable cells and clinical samples.

Dimethyl labelling is a fast, specific and cost-efficient chemical labelling approach for quantitative proteomics at the $MS^1$ level. It can be used on samples from any source, which is an advantage over SILAC. This method allows for up to 5-plex analysis. This was demonstrated by Wu et al. (2014) which performed a 5-plex experiment using dimethyl labelling. Dimethyl labelling yields nearly complete labelling with largely scalable peptide amounts. However, cyanoborohydride and formaldehyde are toxic and thus the handling of these chemicals requires appropriate laboratory practices and infrastructure. It is used for expression profiling and can be used for quantitative analysis of PTMs. Sato et al. (2015) used triplex stable isotope dimethyl labelling to determine the TGF-β related protein expression at the metastatic site. This allows for potentially new biomarkers. 6694 proteins were identified. Moreover, the authors found upregulated proteins in control cases compared with those in tissues exhibiting lung metastases. These proteins are part of the eukaryotic initiation factor (eIF) family. According to the data, eIF4A1 and eEF2, show a highly significant correlation

with the metastatic phenotype of advanced breast cancer. This method is suitable for any sample, but does not allow for the analysis of more than 5 samples in one experiment. Lau et al. (2014) demonstrated that this method achieves a similar quantitative performance as SILAC. However, under the experimental conditions, dimethyl labelling did identify less proteins. For the analysis of the whole proteome of a sample, this method would not be suitable as identification and quantification of a whole proteome would be better with $MS^2$ methods like iTRAQ and TMT. $MS^2$ methods have a better ratio of signal to noise, better reproducibility and allow for the analysis of more samples, than dimethyl labelling, in one experiment.[46] Dimethyl labelling would be best suited for less complex samples.

(c)ICAT is a residue-specific method and can only be applied to cysteine-containing peptides. It is a chemical labelling approach at the $MS^1$ level. This method can be applied to any sample (cells, human tissue or animal) but it should be taken into consideration that acidic proteins and those lacking cysteine-residues are not possible to be quantified using cICAT reagents. Bias for proteins containing a high abundance of cysteine residues is a drawback as well as this results in many protein identifications and measurements being based upon a single peptide.[39] Wu et al. (2019) demonstrated that this method can be used to identify and quantify PTMs like protein persulfidation. ICAT has also been used in the discovery of cancer biomarkers. A study done by Kang et al. (2010) used ICAT reagents to find potential novel biomarkers for the detection of breast cancer. Plasma proteomes were obtained from both healthy women and breast cancer patients. The ICAT approach allowed for the identification and quantification of 155 proteins. A 1.5-fold change of 33 proteins was observed between the two cases. The data indicated that biotinidase is significantly down-regulated in breast cancer plasma. This was confirmed when another set of plasmas was obtained from other breast cancer patients and healthy women. Further research is necessary to confirm whether biotinidase could be a biomarker for the detection of breast cancer. (c)ICAT does not allow for the analysis of multiple samples in one experiment. This method would be best suited for samples containing an adequate amount of peptides with cysteine residues and can be used for any complex sample. Too little cysteine-containing peptides and too many acidic proteins would not yield good results.

ICPL is a variation of ICAT but targets free amino groups of intact proteins instead of thiol groups on cysteine-containing peptides. This overcomes the problem of the low number of potential labelable residues seen in ICAT. Frequent free amino groups of isolated intact proteins, allow for this method to be applicable to any sample. Moreover, this method is

compatible with any separation method. The classical ICPL method allows the quantification of only lysine-containing peptides. However, the post-digest ICPL method approach has been shown to increase the number of identified and quantified proteins. Both Fleron et al. (2010) and Leroy et al. (2010) demonstrated that the amount of identified and quantified proteins were significantly increased with the post-digest ICPL method compared to the classical ICPL method. This method allows the analysis of 4 samples in one experiment.[48] With methods like iTRAQ and TMT, more samples can be analysed in one experiment. ICPL can be applied to any complex sample but methods like iTRAQ and TMT would be used in most cases especially when more than 4 samples need to be analysed in one experiment. However, when only an ion trap is available for use in the laboratory, then ICPL would be a good alternative for the experiment as iTRAQ and TMT reagents are possible to be analysed in an ion trap-based system but it is more complex.

$^{16}O/^{18}O$ labelling is an enzymatic labelling approach where the introduction of an isotopic tag is catalysed by proteases such as trypsin. In addition to its cost-effectiveness and the simplicity of the method, this approach is applicable to clinical samples. Moreover, it can be used for the quantification of PTMs, as has been shown by Smith et al. (2007). Another advantage is that as little as several micrograms is well suited to be analysed with this approach. There is no chemical reaction that may result in by-products during labelling of the clinical samples. The applicability of this method to limiting human clinical samples and the simplicity makes it very appealing to use. $^{16}O/^{18}O$ labelling cannot be used in experiments where more than 2 samples have to be analysed in one experiment and is not suited for complex samples. Portelius et al. (2017) sought to determine the relative amount of peptides formed by endogenous proteolytic activity in cerebral spinal fluid (CSF) using $^{18}O$-labelling. Amyloid β (Aβ) plays an important role in Alzheimer's disease. The main pathway via which Aβ is degraded by enzymes *in vivo* remains unknown. An increased leukocyte cell count was observed in patients with bacterial meningitis. Using $^{18}O$-labelling, an insulin-degrading enzyme was identified. For the detection of proteolytic activity in human CSF, $^{16}O/^{18}O$-labelling shows to be a suitable method. This method would be best suited in experiments when there is a very limited amount of clinical sample available. It is a simple, cost-effective method and there is no chemical reaction that may result in by-products during labelling.

TMT and iTRAQ are isobaric labelling methods and remain one of the most commonly used methods in quantitative proteomics. These methods allow up to 16-plex and 8-plex experiments, respectively. However, the multiplex capabilities can be extended due to higher

order multiplexing, which combines $MS^1$-techniques and $MS^2$-techniques. A study done by Wang et al. (2020) combined TMT 11-plex and TMT 16-plex to analyse 27 samples in one experiment. The sensitivity is increased in MS and in MS/MS when combining isobaric tags.[39] Isobaric labelling methods can be applied on lower sample concentrations due to multiple samples contributing to the same signal. Ion trap-based systems were not suitable for quantitation when using isobaric tags due to the low mass cut-off. This would mean that the reporter ions for most peptides cannot be observed. However, Pulsed Q Dissociation (PQD) is an alternative technique to CID which allows the analysis of isobaric labelled samples with ion trap-based systems. Studies have demonstrated that linear trap quadrupole (LTQ)-PQD detect twice as many proteins compared to Q-TOF while no loss in quantitative precision was observed.[49],[50] However, the fragmentation efficiency of PQD is less than CID, even under optimised conditions.[49] A study has demonstrated that combining PQD and CID improved the identification and quantification capabilities in iTRAQ experiments and the data suggested that this hybrid mode is especially useful for quantification of low abundance proteins in the sample.[51] Generally, the amount of information that is obtainable is limited due to the fact that only the most abundant ions in a spectrum are typically selected for fragmentation. The utilisation of isobaric tags remains one of the most used tags in quantitative proteomics (1620 hits in the last 10 years, PubMed). Issues like ratio distortion and not being able to use isobaric tags in LTQ have been addressed. Moreover, the possibility of analysing up to 16-samples in a single experiment is a major advantage as it reduces instrumentation time and no extra source of variability is introduced. Isobaric tags can be applied on any sample and is often used in complex samples. There are, relatively, a large number of steps in the workflow of isobaric tagging which leads to a greater variability. Instruments with a relatively high resolution (such as orbitraps) and tandem MS instrumentation should be available to use iTRAQ or TMT reagents. In studies where many samples (such as 16 samples) have to be analysed in one experiment, isobaric tagging would be the superior choice. Doing multiple experiments increases instrumentation time and introduces a source of variability. However, when less samples are needed, considerations such as the instrumentation available in the lab, costs and complexity of the samples and/or experiment should be made as to whether isobaric tagging would be the best method for the experiment.

**Table 1. Quantification methods and their characteristics**

| Quantification methods | Introduce label | Labelling level | Labelling site | Sample type | Multiplex capabilities | MS or MS/MS quantification |
|---|---|---|---|---|---|---|
| SILAC | Ex vivo, in vivo | Protein | Lys and Arg | Cells | Up to 6-plex | MS |
| Stable isotope dimethyl labelling | In vitro | Peptide | N-termini and Lys | Any sample (cells, human tissue or animal) | Up to 5-plex | MS |
| cICAT | In vitro | Peptide | Cys | Any sample (cells, human tissue or animal) | n.a. | MS |
| ICPL | In vitro | Protein/Peptide | N-termini and Lys | Any sample (cells, human tissue or animal) | Up to 4-plex | MS |
| $^{16}O/^{18}O$ labelling | In vitro | Peptide | C-termini | Any sample (cells, human tissue or animal) | n.a. | MS |
| iTRAQ | In vitro | Peptide | N-termini and Lys | Any sample (cells, human tissue or animal) | Up to 8-plex | MS/MS |
| TMT | In vitro | Peptide | N-termini and Lys | Any sample (cells, human tissue or animal) | Up to 16-plex | MS/MS |

n.a. – not applicable

## Conclusion

Quantitative proteomics has become a very important tool in the discovery of biomarkers in complex biological samples. Relative quantitative proteomics aims to reveal which proteins are differentially expressed between samples. There are many methods available for the relative quantification of proteins, each with their own strengths and limits. This review focused on comparing and summarising the strength and weaknesses of stable isotope labelling-based quantitative proteomics. Isotopic labelling methods such as SILAC, dimethyl labelling, cICAT, ICPL and $^{16}O/^{18}O$ labelling involve different mass additions to the peptide/protein and quantifying them by comparing distinct isotope peaks. Isobaric tags, such as iTRAQ and TMT, were developed where the relative abundancies of the tagged peptides will be revealed due to the released reporter ions having different masses. Each of these methods have their own strengths and limitations and are suited to different samples. SILAC is not suited for samples containing undividable cells and clinical samples. It would be best suited for cell cultures and eukaryotic cells. Dimethyl labelling is fast, specific and cost-efficient. Dimethyl labelling would be best suited for less complex samples. (c)ICAT is a residue-specific method and can only be applied to cysteine-containing peptides. Therefore, this method would be best suited for samples containing an adequate amount of peptides with cysteine residues. ICPL is a variation of ICAT but targets free amino groups of intact proteins. ICPL is less complex then isobaric tags and especially when only an ion trap-based system is present in the lab, then ICPL would be a good alternative to isobaric tags. $^{16}O/^{18}O$ labelling is simple, cost-effective and there is no chemical reaction that may result in by-products during labelling. $^{16}O/^{18}O$ labelling would be best suited for experiments where only a very limited amount of clinical sample is available. Isobaric tags remain one of the most used tags in quantitative proteomics. When many samples (8-16 samples) have to be analysed in one experiment, isobaric tags would be the superior choice. These methods are to this day being improved and issues are being addressed. Combinations of $MS^1$ and $MS^2$ methods are being looked into and even new tags are being developed. It is vital to have an accurate, sensitive and specific method to quantify the change in expression of proteins in different states. There is not one method best for all sample. Careful considerations should be made regarding the instrumentation available in the lab, costs, complexity of the samples and/or experiments and time.

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