Published in final edited form as:
Bioanalysis. 2012 October ; 4(20): 2525–2541. doi:10.4155/bio.12.208.

Relative quantification of biomarkers using mixed-isotope labeling coupled with MS

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Abstract

The identification and quantification of important biomarkers is a critical first step in the elucidation of biological systems. Biomarkers take many forms as cellular responses to stimuli and can be manifested during transcription, translation, and/or metabolic processing. Increasingly, researchers have relied upon mixed-isotope labeling (MIL) coupled with MS to perform relative quantification of biomarkers between two or more biological samples. MIL effectively tags biomarkers of interest for ease of identification and quantification within the mass spectrometer by using isotopic labels that introduce a heavy and light form of the tag. In addition to MIL coupled with MS, a number of other approaches have been used to quantify biomarkers including protein gel staining, enzymatic labeling, metabolic labeling, and several label-free approaches that generate quantitative data from the MS signal response. This review focuses on MIL techniques coupled with MS for the quantification of protein and small-molecule biomarkers.

Mixed-isotope labeling for biomarker analysis

The dynamic nature of living organisms provides a significant challenge in the quest to develop a complete understanding of the important chemical relationships within biological systems [1]. The ability to detect and quantify changing concentrations of biomarkers provides unique insight into the cellular responses to external stimuli or disease pressures on an organism [2,3].

The great sensitivity and mass accuracy afforded by MS has made it the platform of choice for modern biomarker quantification. MS methodologies have been coupled in many varied arrays to yield complimentary approaches to biomarker analysis. The choice of which MS technique to utilize is guided by the type of biomarker to be analyzed and the choice to include or preclude the incorporation of isotopic variants of the biomarkers or a chemical-labeling step. The four general approaches to quantitative biomarker analysis are detailed in Figure 1. Within each approach, extensive bioanalytical validation has been performed ensuring capable strategies for eventual clinical biomarker analysis [4,5].

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Financial & competing interest disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Isotopic- and chemical label-free approaches allow for a broad, non-targeted approach to biomarker analysis that relies solely on chromatographic resolution and mass spectrometric signal intensity to overcome the inherit complexity of proteomic and small-molecule systems [6–8]. The use of elegant multidimensional chromatography, tightly controlled MS parameters, internal retention time and ionization standards, as well as sophisticated data analysis software, has allowed for a significant increase in the use of these isotopic- and chemical label-free approaches during the past decade. One such advance is spectral counting, wherein the number of spectra observed for a protein is used as a measure of protein abundance [9]. As an isotopic- and chemical label-free approach, spectral counting has the advantage of not requiring difficult chemical labeling steps or expensive isotopic labels, but generally performs poorly for protein quantification when individual spectral counts and signal-to-noise levels are low [10].

Isotopic labeling of biological systems can be achieved in a number of chemical labeling and chemical label-free methods. Each strategy is predicated on the observation that chemically identical but isotopically unique chemicals will exhibit identical behavior in chromatographic and mass spectrometric analyses [11–13]. The unique masses of the biomarker and its isotopically labeled equivalent can be detected by the mass spectrometer and used to perform relative or absolute quantification.

Chemical label-free isotopic incorporation can be achieved through spiked standards, enzymatic labeling, or metabolic labeling. Spiked standards or stable-isotope dilution (SID) strategies are targeted approaches wherein an isotopic standard of known concentration is added to a biological sample prior to MS analysis. Both proteomic [14–18] and metabolomic [19–22] biomarkers have been successfully analyzed using this approach. The isotopic partner allows for absolute quantification of the biomarker of interest. The scope of this technique is impressive and has been reviewed previously [23]. Two of the major limitations to the SID strategy are high costs and limited availability of the isotopic reagents. A labeled reference peptide method has provided a cost-effective alternative to SID by utilizing a single isotopically labeled peptide as a reference standard for all measured peptides. Analysis of un-normalized peak areas is the basis for quantitation and has been shown to exhibit identical performance to SID [18].

Enzymatic labeling, unlike SID, allows for a global analysis of protein concentrations through post-digestion protease catalyzed incorporation of either ^{16}O or ^{18}O atoms (via $H_2\,^{16}O$ or $H_2\,^{18}O$, respectively) at the C-terminus of all peptides in two separate samples [24,25]. The need to procure an isotopic form of a biomarker is unnecessary since the enzymatic reaction incorporates isotopic labels (sample 1: light; sample 2: heavy). Pooling of the light- and heavy-labeled peptides allows for a relative quantification of all peptides present, thereby yielding the relative native protein levels in each sample. The challenge of enzymatic labeling is in the need to control the efficiency of the enzymatic $^{16}O/^{18}O$ labeling step, as well as to resolve the small mass differences (+2/+4) between the light- and heavy-labeled peptides.

Metabolic labeling harnesses synthetic biology machinery to incorporate isotopic labels throughout living organisms, though it has seen limited organismal level use due to the prohibitive costs associated with isotopically enriched diets. Instead, metabolic labeling is generally performed with the use of isotopically enriched media – usually amino acids – in cell culture. The isotopically enriched amino acids within the media are incorporated by the organism and further manipulated through normal cellular processes. MS quantitation of isotopically enriched biomarkers can be compared with control biomarkers grown under standard media conditions yielding relative quantification values. Stable-isotope labeling

with amino acids in cell culture [26] is a classic metabolic labeling approach that has found ever increasing use in protein and peptide biomarker discovery [27–29].

Detection of biomarkers from the chemical label-free approaches, mentioned above, is a result of the intrinsic ionization potential of the native biomarkers since no chemical transformation has been performed on the biomarkers. An obvious challenge to each of these scenarios is the realization that many biomarkers, particularly small-molecule biomarkers, lack the necessary chemical functionality to strongly ionize under most MS conditions and, thus, most label-free approaches are targeted towards peptides/proteins. An evolving and creative solution to this ionization challenge is to create a chemical label that introduces both isotopic labels and an ionizable group into a biomarker structure.

Chemical labeling strategies provide targeted approaches to biomarker analysis. The chemical label will select all molecules that carry the particular functionality (e.g., amine, thiol, carboxylic acid, alcohol, ketone, aldehyde) that is necessary to form a covalent bond. This chemical labeling approach has proven to be useful for quantification in genomics (e.g., two-color fluorescent dye labeling) [30,31], proteomics [32] and metabolomics [33,34].

In these chemical labeling approaches, each sample is reacted with a reagent that differs only in its isotopic composition, thereby creating ‘heavy’ and ‘light’ versions of derivatized metabolites, which are easily distinguished by MS (Figure 2). Samples are mixed after the labeling reaction and then analyzed by LC–MS. Labeled metabolites co-elute from the chromatographic column and appear in the mass spectrum as pairs of peaks with a mass shift equal to the difference in mass of the two isotopic labels. The ratio of peak intensities for each pair yields the relative concentration of each metabolite between the two samples.

This isotopic labeling strategy has a number of advantages. First, it improves the precision of relative quantification by minimizing or negating errors associated with run-to-run irreproducibility. Such errors can arise from variations in MS detection sensitivity, such as those caused by ionization suppression in electrospray, or from retention time differences between runs [35,36]. The isotopic pair of labeled compounds in MIL co-elute within a single run and, therefore, have identical retention times and are electrosprayed from identical solution conditions. A second benefit of utilizing a derivatization reagent is that it can help identify a metabolite by indicating the presence of a certain functional group – the one targeted by the reagent. Furthermore, well-designed labeling reagents can improve the chromatographic separation, as well as enhance the detection sensitivity and provide the opportunity to do multiplexed analyses [37–39].

Protein biomarkers

A big challenge in proteomic biomarker techniques is the need to reduce sample complexity while minimizing errors during sample handling. A chromatographic separation using gelor liquid-based approaches is most often used prior to mass spectrometric analysis. In MIL approaches, samples are modified for increased detection for both quantification and identification. The scope of MIL in proteomics is continually expanding and now encompasses targeting nearly every reactive site on a protein or peptide. In addition, several novel tagging reagents have been developed that are compatible with many MS and MS/MS platforms.

Mixed-isotope tags are chemical tags that are added to protein samples that form covalent bonds at a single functional group type. There are typically two versions of the tag: light and heavy, the heavy tag being the isotopic version and the light tag being the non-isotopic incorporated form. Isotope-coded affinity tags (ICAT) were the first tags of this type used extensively in proteomics [11]. There are three major components to the ICAT tag: an

iodoacetamide group to form covalent bonds with cysteine side chains, a biotin affinity tag to reduce sample complexity to only those samples that had been labeled, and an isotopically coded linker for relative quantification by MS. The ICAT reagent introduced isotope labeling to proteomics and made possible the ability to analyze samples for low-abundance molecules. Much of the proteomic efforts since this seminal paper have focused on efforts that utilize the sensitivity and speed of MS analysis coupled with isotope labeling [40,41].

A lack of diagnosis biomarkers leaves the prognosis of hepatocellular carcinoma (HCC) poorly understood. As a leading cause of cancer death worldwide, it is clear that better methods for diagnosis are crucial. In an attempt to identify the different expressed serum proteins in HCC patients, ICAT reagents have been utilized in combination with LC–MS/MS. A study by Kang *et al.* found that the serum concentration of α-1-acid glycoprotein was significantly higher in patients with HCC, identifying a biomarker that can be used for future diagnosis [42].

More recent introductions to MIL for proteomic analysis includes isotope-coded protein labels (ICPL) [43], reductive methylation [44–46], tandem mass tags (TmT) [47–49], global internal standard technology for comparative proteomics [50], isobaric tags for relative and absolute quantification (iTRAQ®) [51] and mass differential tags for relative and absolute quantification (mTRAQ™) [52]. ICPLs were developed with the goal of increased sequence information from protein and peptide samples. The original ICAT reagent provided limited sequencing information that made protein identification challenging. ICPLs have improved sequencing by accounting for post-translational modifications (PTMs). The larger coverage from ICPL labeling was derived from the covalent bond the label makes with amine groups of proteins [52,53]. A near global representation of peptides and, thus, proteins, are possible when efficient amine labeling is chosen as a labeling strategy. An amine-targeting nicotinyl-NHS ester tag is used in ICPL labeling. Chemical labeling through ICPL has also made available multiplex proteomic analysis [54]. This multiplexed approach enables the comparison of multiple samples, three in this case, within one analytical run, with accuracy and precision comparable to that of the binary analyses. For example, ICPL has been used to compare protein expression between four regions of the glioblastoma, the most frequent primary tumor of the central nervous system. Using this method, 31 proteins were identified to be overexpressed in the tumor compared with the periphery, leading to new insights in the understanding of glioblastoma progression [55].

A similar procedure, reductive alkylation, has been used to isolate highly homologous proteins such as isoforms within samples [44,56]. Reductive methylation consists of the addition of alkyl groups to the amino residue of the proteins from an alkyl-donating compound. For example, reductive dimethylation involves the addition of formaldehyde and sodium cyanoborohydride to a protein sample. This leads to the addition of two methyl groups at each of the amino groups. Reductive methylation has many benefits such as low cost, fast reaction rate, mild reaction conditions, high specificity to amino groups, high labeling and ionization efficiency and stable post-derivative products; but, since a large number of labeled protein/peptide peaks are produced, alternate methods are needed to ensure a pure sample or to create a greater separation between peaks [44,57].

Reductive methylation was the chosen chemical derivatization procedure for the introduction of an enrichment method using a solid-phase active ester reagent to selectively capture and quantify nitropeptides by LC–MS. This method involves *N*-dimethylation of aliphatic amines followed by a reduction of nitrotyrosines to their corresponding aminotyrosines, which are then selectively captured using solid-phase active ester reagent on glass beads (Figure 3). The nitropeptides are then released by acidcatalyzed hydrolysis and are quantified using LC–MS to detect low-abundance PTMs, such as protein nitration, an

important biomarker for various diseases. In human serum, reductive methylation was shown to quantify nitroubiquitin at diminishing nitroprotein content, down to concentrations as low as 18 pmol/ml [58]. Dimethylation can completely label lysine sites due to the small size of the reagents (formaldehyde and sodium cyanoborohydride) and the reduction of steric hinderance from denaturing and alkylation of proteins prior to derivatization. In addition, dimethylation based proteomics has shown the ability to differentiate and quantify differences in protein isoforms.

Cerebrospinal fluid (CSF) surrounds the brain and spinal cord, and is commonly used an indicator of the pathological state of the central nervous system. A study by Giron *et al.* demonstrated the potential of TmT to identify differential brain-related proteins by using post-mortem and ante-mortem CSF samples [59]. Protein quantification revealed that concentrations of PRDX5 were significantly higher in the CSF of deceased patients versus control patients. PRDX5 is an important biomarker of the inflammatory response in human CNS as a result of the oxidative stress caused by many neurological disorders. Protein quantification in CSF by TmT reagents may assist in the diagnosis and prognosis of these disorders.

iTRAQ and mTRAQ are both powerful isobaric labeling approaches for proteomic analysis. The iTRAQ approach, originally developed in 2004 [51] and later commercialized [60], quantifies peptides by measuring the ratios of reporter ions, which are measured in the low *m/z* ranges and are produced by precursor ion fragmentation. mTR AQ, in comparison, measures relative quantification by relative intensities of differing mass tags [52]. mTRAQ uses multiple-reaction modeling and focuses the mass spectrometer on specified *m/z* values, which allows for more accurate quantification of low-abundance proteins. These two methods are easily compared since they both use identical tags; the tags differ only in their positioning and number of ^{13}C, ^{18}O and ^{15}N atoms.

Both approaches are available in multiplex formats, yet iTR AQ has many benefits over mTRAQ for protein analysis. iTRAQ has been shown to read twofold the amount of proteins compared with mTRAQ when used for protein identification. It is believed that iTRAQ has an additive effect on precursor intensities while mTRAQ can lead to redundant reads on the MS due to multiple readings on a peptide containing different mTRAQ labels. iTRAQ has also found more success in the analysis of low abundance kinases. iTRAQ has shown higher sensitivity, less variability and better reproducibility than mTRAQ, but has also proved to be less accurate due to peak compression [61].

In a recent study, iTR AQ reagents were used for quantitative proteomics of cyclin E1-interacting proteins in adult versus embryonic brains [62]. Previously, the function of the core cell cycle protein, cyclin E, in non-proliferating brain cells was unknown. Results from the study suggest that in terminally differentiated neurons, cyclin E is responsible for regulating synapse formation and may provide a better understanding of neurological disorders, including Alzheimer's and Parkinson's diseases. Although the iTR AQ reagents have proven utility, they have remained ineffective in consistently detecting specific proteins in complex samples, leading to the availability and use of the mTRAQ reagents. Specifically, these reagents have been used to quantify pyruvate kinases in biopsied endometrial cancer tissue. The amount of pyruvate kinases in the malignant tissue was 85 nmol/g of total proteins, approximately fourfold higher than the concentration found in the non-malignant tissue. The level of pyruvate kinase reported by the mTRAQ technique was twofold higher than those determined using the iTRAQ technique [63].

With the sustained popularity of isotopic tags, there have been numerous strategies to lower the costs associated with tagging reagents by carefully choosing the isotope source and

minimizing synthetic schemes. Deuterium is the least expensive isotope available for common use in tagging, yet it is rarely implemented due to the interference it causes in chromatographic separation [64]. Under most conditions, deuterium does not perfectly co-elute with its corresponding protium partner, making effective MS identification and quantification challenging. ^{15}N and ^{13}C do not cause chromatographic retention time differences from their lighter isotopomer, but are significantly more expensive to purchase and incorporate into a tagging reagent. One way that deuterium labeling has been used without loss of chromatographic homogeneity between two samples is by strategic placement of the deuterium label on the hydrophilic region of the tag. The hydrophilic region of the tag has little contact with the hydrophobic stationary phase, thereby eliminating retention time discrepancies. Two examples of these types of deuterium-incorporated isotopic reagents are: deuterium isobaric aminereactive tags (DiART) [22,65] and *N,N*-dimethyl leucines (DiLeu) [66]. The DiART reagent allows for six-plexed analysis, while the DiLeu reagent allows for fourplexed, by the analysis of labeled reporter ions. Both sets of reporter ions have the same chemical structure, differing only in their isotopic labeling states. The DiART reagent, which is more cost effective and easier to label with, provides greater response of reporter ions and better quantitative coverage of proteome samples than the more widely used iTRAQ proteomics approach [67]. Recently, DiART labeling was used to quantify intracellular amine metabolite changes from hyperglycemic human aortic endothelial cells to gain insight into macrovascular diabetic complication. DiART was shown to improve chromatographic resolution of derivatized amines, resulting in a 100-fold S/N enhancement in MS and demonstrated a limit of detection below 10 nM/100 amol [68]. Rather than increasing the multiplexing potential, another approach used to deal with complex protein samples has been to increase the throughput potential, which allows for the analysis of common analytes in different samples. The ultra-throughput multiple reaction monitoring method was used to accurately quantify a signature peptide in 25 different samples and is envisioned as a facile method with broad applicability [69].

The field of proteomics is an expansive and continually developing field, now capable of targeting almost every reactive site on a protein or peptide. The use of biomarkers is critical to the understanding of biological systems, owing not only to the constant effort to reduce cost, increase speed, enhance detection, and integrate profound throughput potential, but also to the creativity of its applications. Researchers will apply techniques, such as ICAT, iTRAQ, mTRAQ, ICPL, TmT and reductive alkylation, to a growing number of protein-based systems (Table 1). As mentioned previously, iTRAQ is an outstanding example of how a protein biomarker technique has enhanced the understanding of a disease state to a level that was technically unobtainable prior to this method. This development gives an excellent first step to the critical understanding of the biological systems that underlie disease states in humans.

Small-molecule biomarkers

Lipids

The study of small molecules as biomarkers has grown as interests in systems biology approaches has expanded. One of the fastest growing areas of biomarker quantification is the study of lipids and lipid oxidation compounds. The now-apparent roles of lipids in matters of health and disease make them a target for modern quantitative chemical analysis [70–72]. Lipids act as an integral part of cellular function, including membrane formation [73,74], energy storage and cell signaling [75], organ and tissue physiology [76], and lipid oxidation (and resulting biomarkers). There are also implications of lipids in metabolic diseases, specifically the disruption of lipid metabolic enzymes and pathways in disease states [77]. These discoveries have led to increased attention to lipids in the fields of biomedical and analytical chemistry. The quantification of lipids is of interest to analytical

chemists with the goal of comparing lipid concentration in various functioning and diseased organisms. Popular diseases of interest are extensive and include: cancer, diabetes, obesity, atherosclerosis, hypertension, stroke and other neurodegenerative diseases [78].

Lipidomics is defined as the global quantitative analysis of cellular lipids. Performing lipidomics studies on the organism level has been difficult in the past due to the complexity of lipids and the need for stronger analytical techniques. The wide range of compounds that fit the term 'lipid' make them hard to classify and may include between 10,000 and 100,000 chemical entities [78]. The introduction of new MIL techniques, coupled with LC and MS, have allowed for new and exciting lipidomics studies to proceed.

LC–MS

LC–MS provides an increasingly popular platform for acquiring relative quantification of lipidomic species [79–81]. Relative quantification of lipids using LC–MS has been achieved in part by the use of MIL. MIL is used for biological samples that cannot be labeled *in vivo* (urine or plasma) [79], or for samples for which other quantitative techniques, such as SID, are not feasible. Labeling of lipid compounds is achieved by chemical derivatization (with the light and heavy forms of the tags) at the carboxylic acid group. Several derivatization reagents have been adopted that are capable of targeting the carboxylic acid functional group while incorporating appropriate isotopic mass shifts, and maintaining or enhancing ionization characteristics of the labeled lipids. Many of these MILs are similar conceptually to earlier proteomics chemical labeling approaches with a generally stronger ionization component added to compensate for the weakly ionizing lipids.

Optimization of reagents that would be effective in separation of lipids on LC–MS comes in various forms, but with similarly varying results. One such derivatization reagent is an adaptation of a compound known to be effective in LC–MS analysis. 7-(*N,N*-dimethylaminosulfonyl)-4-(aminoethyl)piperazino-2,1,3-benzoxadiazole (d_6) (DBD-PZ-NH_2 [D]) is a benzo-furazan compound, which has been used previously in LC–MS with favorable results [82]. DBD-PZ-NH_2 (D) adopted for the relative quantification of lipid compounds reacts with organic acids, which allows it to target lipids in biofluids. Six ^1H atoms in the dimethylamino group are replaced with deuterium atoms giving the heavy and light forms a 6-Da differential, resulting in an appropriate separation for relative quantification in MS. Results show that this reagent, coupled with LC–MS, gave not only sufficient separation and accurate relative quantification, but also isotope internal standards that were then used in subsequent quantitative studies of the materials. The DBD-PZ-NH_2 reagent was shown to determine fatty acid concentrations in rat plasma as low as 0.1 μM, proving its utility as an accurate reagent for quantification [82].

Other lipid-labeling approaches have sought to enhance the detection of lipids through the judicious choice of an ionizing group to partner with isotope labels. One method for enhanced detection is the use of a quaternary ammonium group on the labeling reagent [83–85]. The quaternary ammonium allows the derivatized lipid to hold a permanent positive charge, thus assisting with the sensitivity of detection for the tagged compounds using LC–MS in the positive-ionization mode. With a charged group, the analyzed compound experiences better (and more efficient) chromatographic resolution since the LC component of the separation runs optimally under acidic pH conditions that do not affect an already positively charged compound. One such labeling process adds 3-carbinol-1-methyl-d_3-pyridinium iodide (CMP-d_3). The resulting heavy form has a 3-Da shift compared with the light (3-carbinol-1-methylpyridinium iodide [CMP]) forms. The results reported from the CMP-d_3 derivatization followed by LC–MS analysis resulted in a sensitivity 2500-fold higher than that of the traditional negative-mode ionization with underivatized samples [83].

We have used a similarly acting reagent, (2-aminoethyl)trimethylammonium chloride hydrochloride (cholamine), for relative quantification of lipids [84]. The three methyl groups of the quaternary nitrogen on cholamine were each labeled with three deuterium atoms, giving a 9-Da shift of the heavy form of cholamine. Similarly to the CMP label, cholamine labeling endows each labeled compound with a permanent positive quaternary ammonium ion. The cholamine tag itself is relatively small compared with the mass of the target lipids. This important feature allows the chromatographic behavior of the lipids themselves to distinguish their independent retention time, as opposed to all lipids co-eluting due to the characteristics of the labeling reagent. Very good limits of detection (15–30 fmol) and coefficients of variation (6%) were obtained when using cholamine in chicken egg fatty acid analysis. The larger shift resulting from the 9-Da heavy form allows for the possibility of other isotopic forms of cholamine to be synthesized and used in a multiplexed lipidomics analysis platform [84].

In addition to charged ammonium groups as labeling reagents, other basic functional groups have been used to enhance sensitivity of labeled lipids. The derivatization reagent, *p*-dimethylaminophenacyl (DmPA) bromide, yields a tertiary amine-labeled lipid that is detected at levels two- to four-times lower than the unlabeled lipid [86]. One of the main components of the tag that allows this optimization is its integrated ring structure. The ring on the DmPA tag is hydrophobic; adding this to the hydrophilic end of a lipid molecule increases its hydrophobicity and allows for enhanced retention for LC. This increased hydrophobicity allows the compound to remain on the surface of the droplets during ESI-MS resulting in a stronger signal. This method also gives clear peaks, allowing for a wider range of metabolite analysis and has been used recently in the quantification of acylglycines, which play a crucial role in regulating inborn errors of metabolism. Using human urine samples in which these acylglycines may be found, the DmPA tag provided a LOQ as low as 1–5 nM [87].

Although the focus of this review is analytical techniques, there have been many recent technological advances in LC–MS, including the development of multiple instruments [88] and accompanying software [89] that have led to improved metabolic profiling. These options allow for personalization of techniques for quantitative analysis, making LC–MS one of the most versatile tools for metabolomics.

GC–MS

GC coupled with MS has also grown as a technique for the quantification of lipids. Early GC lipidomics approaches were challenged by the limited volatility of lipids and their thermal stability [23]. Recent GC–MS techniques have overcome the obstacle by converting lipid compounds into volatile substances that can be detected and quantified by GC without compromising sample integrity. Silylation isotopic labeling creates volatile lipid members for efficient relative quantification by GC–MS [90]. One of the challenges of this silylation approach is the need for anhydrous conditions and heating of the lipid samples.

A more recent approach to lipid analysis using GC–MS is termed isotope-coded fatty acid transmethylation (‘iFAT’) [91]. Li *et al.* demonstrated that relative quantification of fatty acids could be performed after ultrasonic irradiation in the presence of light methanol-d_0 or heavy methanol-d_3 [91]. The iFAT approach has shown robustness in the analysis of various lipid sources as well as maintaining low percent errors in validation experiments with a detection limit at the picogram level [91–94]. In addition, iFAT has the added benefit of being a faster, simpler, and less error prone labeling technique than the common fatty acid methyl ester GC–MS approach because it does not require the saponification, methylation and extraction of fatty acid methyl esters.

With these improved techniques for relative quantification of lipids (Table 2), the fields of lipidomics and biomedical research will continue to expand. In addition, the accurate relative quantification of lipids may be enhanced by the development of stronger analytical techniques. The elimination of preparatory steps for standard techniques, such as extraction and saponification, may remove unnecessary errors as well as the potential of compromising the samples, further reducing interference on MS and improving quantification. The importance of lipids in cellular functions makes them a considerable target for quantification, and has once again led to the use of MIL. Enhancing the sensitivity of the LC–MS analysis by the addition of a quaternary ammonium group has proven to be an effective method for relative quantification of lipids.

Scientists in these fields are able to gain a better understanding of the metabolic roles of lipids, their role in signaling and regulation of gene expression and, perhaps, most importantly, tracking changes for disease biomarker discovery [86]. The advancement of lipid metabolic drugs can also benefit immensely from relative quantification studies. Lipidomics can assist in identifying where the break down in lipid metabolism is occurring in disease states. One area of particular interest is the study of neurological disorders, as the nervous system is known to have the second highest lipid concentration for organs (only behind adipose tissue) [77].

Metabolites

The field of metabolomics provides a challenge to analysis and quantification not seen in proteomics or lipidomics due to the global approach required for complete analysis of the metabolome [95]. The metabolome includes a wide array of small molecules that all contribute to metabolic function. Unlike groups such as lipids and proteins, there is no common chemical functionality present in all metabolites that can be used as a site for chemical labeling.

One solution to the challenge of labeling the wide range of chemicals that are present in the metabolome is to focus on a specific functional group within the metabolome. It has been demonstrated that one of the most effective methods is to isolate functional groups, analyze each group separately, and then combine these data for a full view of the metabolome [96–98]. These targeted metabolomics approaches can include groups such as: amines [99,100], alcohols [101], thiols [102] and carboxyl groups [103], among others.

The role of amines and their derivatives as effective biomarkers in physiological processes makes them a rich target for analysis in metabolic studies [68]. One methodology for the isotopic labeling of amine metabolites that has emerged is the use of formaldehyde and ^{13}C-formaldehyde to label all primary and secondary amines present in a sample through reductive methylation, as in the proteomic analyses mentioned previously. Primary amines experience a dimethylation, while secondary amines experience a monomethylation. The monomethylation versus dimethylation of the amine compounds provides the ability to deduce information about the number and types of amine groups present since the quantity of isotopes introduced to the analyte should match up with the number of primary and secondary amines in the sample.

Reductive alkylation can also increase the detection of the labeled amine metabolites (one-to tenfold, depending on the target compound) [104]. The methylation provides a slightly more hydrophobic molecule while also producing a tertiary amine that is more readily protonated for ESI detection. This method was used to relatively quantify the amount of primary and secondary amines, and absolutely quantify 20 amino acids and 15 amines found in a sample of human urine. Although the absolute quantification is limited by the standards available for comparison, this method demonstrates the ability of reductive methylation labeling to

work with complex biological samples, and it will likely be applied to diseased biological fluids that are known to have large quantities of amine metabolites present [104].

Similar amine-focusing methods have evolved and, with these, have grown methods that can detect other functional groups in conjunction with amines [105]. For example, dansylation labeling has shown the ability to label primary amines, secondary amines, and the hydroxyl group of phenols [106]. Dansyl chloride and ^{13}C-dansyl chloride reagents are able to target amino groups and phenol groups within samples, specifically urine, for metabolic analysis. The derivatization chemistry for dansylation is well known since it has been used previously in pre-column derivatization for fluorescence or UV detection [106]. Dansylation provides the targeting of multiple functional groups while providing the potential for ESI enhancement (one- to three-fold), though not as strong as the enhancement seen in the methylation procedure above. Dansyl chloride was tested in 161 metabolite structures that were known aminocontaining compounds – 121 were found to be compatible with derivatization. Other than amide and indole nitrogens, dansyl chloride proved an effective tool for relative quantification of a wide range of amines and phenols when coupled with LC–MS.

Recently, dansylation labeling was successfully used to analyze the presence of metabolites in human CSF [107]. Identification of metabolites in CSF has been a crucial task because these biomarkers can be used to indicate problems in the central nervous system, but it has been difficult due to the small number and volume of metabolites in this complex body fluid. By using this method and comparing the results to already established standards, 347 unique ion pairs were found and 85 metabolites were identified (Box 1), more than have been identified using other, less-sensitive methods.

Recently, more targeted amine-labeling approaches have been developed. By developing reagents that have a more focused target, a metabolomic sample is simplified into smaller components for more accurate but less broad based metabolite analysis. For example, Walker *et al.* have developed a hydrazide reagent to target the reducing terminus of glycans leading to the formation of a hydrazone linkage [108]. Their work focused on N-linked glycans, as changes in glycosylation represents one of the most common PTMs. The derivatization reagents, 4-phenethylbenzohydrazide in the light and heavy form, were able to selectively label and quantify 27 glycans with analytical variability of ±30%. The ability to study changes in glycosylation could be applied to studies in both disease and cancer [108]. Though there is a need for methods that analyze the broad spectrum of the metabolome, the benefits of specialized targeting reagents are of great utility when a complex biological matrix is present and a more targeted approach is desired.

Many techniques have been developed in order to quantify the vast and important field of glycomics [109,110]. Several prevalent MIL techniques are now commonly used, including the use of MS glycoconjugate glycans [111], glycan-reductive isotope labeling [103], isotopic detection of aminosugars with glutamine as an isotopic-labeling technique [112], and isotopic labeling via permethylation using $^{13}CH_3I$ [113].

Carboxyl-containing molecules are another popular target for chemoselective tags. Compounds that contain carboxyl groups are one of the most abundant classifications of metabolites and play an important role in the understanding of metabolomics. Many of these compounds are known biomarkers and even more are potential biomarkers for disease analysis. Approximately 65% of metabolites are believed to contain at least one carboxylic acid group in their structure. For these reasons, carboxyl-selective reagents are one of the main focuses of metabolomics [86].

As mentioned above, targeted chemical labeling reagents are chosen based on their ability to not only target the functional groups of focus, but also improve analytical separation and detection. DmPA bromide is able to target carboxylic acid-containing metabolites for quantification while also improving LC–MS analysis through enhancement of ESI efficiency by two to four orders of magnitude. This technique has been used by Guo *et al.* to successfully detect and quantifiy 2671, 2546 and 2820 ion pairs from metabolites that contained carboxylic acids in a triplicate experiment [86,114]. Similar experimental designs have been developed, such as the use of CMP and CMP-d_3 to tag carboxylic acid-containing metabolites in mushroom samples in a method termed Group Specific Internal Standard Technology. The labeling reported with Group Specific Internal Standard Technology tackles some of the issues presented by labeling techniques, such as ensuring no chromatographic isotope effect and maintaining the ratio between the control and isotope sample concentrations and their respective MS peaks, while also demonstrating the ability to effectively label in differing matrices [115].

A current limitation in the global approach of the analysis of metabolites is the lack of standards for all functional groups. The isolation of functional groups, such as amines, thiols/phenols and carboxyl groups using their derivatives, has proven effective in the quantification of these groups (Table 3). In order to obtain a full view of the metabolome, derivatization methods for groups that currently do not have standards is essential. By developing these derivatives with specified targets, a better understanding of specific metabolic pathways may be obtained.

Conclusion & future perspective

Improving sensitivity and speed of MS as well as developing improved derivatization techniques for biomarkers has allowed for enhanced detection and accuracy, specifically for the analysis of complex samples. The recent advance of multiplexed analyses have also contributed to the quantification of biomarkers and have shown results comparable to binary analyses, minimizing the time and costs necessary to compare multiple samples.

The development of standards for all functional groups contained in biomarkers will be necessary for clinical applications of quantification and will allow for MIL direct comparisons of disease states, as well as precise comparisons between multiple laboratories. The usefulness of MIL in biomarker analysis is highlighted by the fact that challenging matrices and low abundance species can be identified and quantified with increasing regularity and speed. However, there is still much work to be done. Many MIL strategies are limited in their validation of biomarker analysis. As biomarker analysis approaches the clinical setting, it will be of critical importance that procedures are thoroughly investigated for their analytical validity. In addition to the need for greater clinical valid biomarker approaches, there still remains numerous compound classes that lack suitable isotopic labeling reagents and protocols for accurate analysis in complex biological systems.

New methodologies that involve two or more labeling approaches may expand the scope and accuracy of MIL. One such dual platform approach has been recently demonstrated for breast cancer research using a combination of multidimensional protein identification technology (MudPIT) coupled with TmT labeling [116]. The high resolving power of MudPIT coupled with the quantification abilities of TmT allowed for the complete profiling of 1700 proteins from cancerous interstitial fluid. The recently described IsoStamp [117] methodology, wherein a dibrominated chemical tag leads to enhanced protein identification, provides another opportunity for a dual platform approach. IsoStamp coupled to other MIL techniques, such as iTRAQ, could yield an approach that is both sensitive in detection (low

femtomole quantities in cell lystates using IsoStamp) and in quantification (eight-plex quantification possible using iTRAQ).

There are ample opportunities for new creative solutions to the challenges associated with quantifying the complexities contained within the proteome, lipidome, and metabolome. While some attempts have been made to combine several MIL approaches into one analysis, opportunities still remain for further conjugation between labeling systems to approach greater quantitative coverage within a particular 'omics' field. Creative solutions, such as MIL, to biomarker discovery will continue as a pressing need for all life scientists until a structured understanding of the complex chemical networks linked to the regulation of biological systems is achieved.

Acknowledgments

The mixed-isotope-labeling research by SM Lamos was supported by the Vermont Genetics Network through the IDeA Networks of Biomedical Research Excellence Program (grant number: 8P206M103 449) of the National Institute of General Medical Sciences and the National Center for Research Resources, components of the NIH. In addition, KL Schutt holds a Saint Michael's College Gianni Fund Scholarship.

Key Terms

Isotope labeling	Use of two chemically identical isotopically unique (heavy and light), labeling reagents for the quantification of biomarkers.
Multiplexed analysis	Multiple sample quantification completed in a single experiment.
Isobaric tags	Mass tags of the same nominal mass that once fragmented in MS, yield reporter ions with unique molecular weights that can be used for quantification.
Isotopomer	Set of labeling reagents that have the same chemical structure but differ in their isotopic composition.

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- ■■ of considerable interest

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Box 1. Identified metabolites from human cerebrospinal fluid using dansylation mixed-isotope labeling

Previously known	
Phophoethaolamine	Pipecolic acid
3-methylhistidine	Phenylalanine
Glucosamine	Isoleucine
Taurine	Leucine
1-methylhistidine	L-cystathionine
Arginine	L-norleucine
Homoarginine	Cystine
Asparagine	Hydroxyphenyllactic acid
Glutamine	Homocystine
L-citrulline	5-hydroxyindoleacetic acid
3-sn-phosphatidylethanolamine	Dimethylamine
Methylguanidine	Phenylpropanolamine
Serine	2,4-diaminobutyric acid
Homocitruline	L-omithine
Glutamic acid	Acetaminophen
Aspartic acid	Homovanillic acid
Folic acid	Homocarnosine
Threonine	Gentisic acid
Ethanolamine	Lycine
Glycine	4-hydroxybenzoic acid
Glycylproline	Histidine
Alanine	2-aminooctanoic acid
r-aminobutyric acid	1,3-diaminopropane
Hypoxanthine	L-tyrosinamide
5-hydroxymethyluracil	Tyrosine
2-aminobutyric acid	Cysteamine
Cysteine–glutathione disulfide	Phenol
Proline	4-nitrophenol
Methylamine	Serotonin
Valine	Pyrocatechol
Methionine	Spermidine
Tryptophan	Deoxyepinephrine
Previously unknown	
Homoserine	Methylcysteine
Methionine sulfoxide	3-hydroxypicolinic acid
4-hydroxyl-proline	3-hydroxymandelic acid
Aminoadipic acid	4-hydroxyphenylacetic acid
Iminodiacetic acid	1,4-diaminobutane
Diethanolamine	Cadaverine

Tyrosine methyl ester	Metoprolol
3-aminoisobutyric acid	Ocopamine
5-aminopentanoic acid	Tyramine
Sarcosine	Thymol

Adapted with permission from [107].

Executive summary

Background

- Mixed-isotope labeling is an effective tag for biomarkers and serves to play an important role in their identification and quantification.

Mixed-isotope labeling for biomarker analysis

- The use of chemical-labeling strategies with heavy and light forms has provided a targeted approach to biomarker analysis and relative quantification of various molecules to develop a better understanding of the chemical relationships that govern disease pathways.

Protein biomarkers

- The need to reduce sample complexity has led to the development of various techniques (isotope-coded affinity tags, isotope-coded protein labels, reductive methylation, tandem mass tag, global internal standard technology, isobaric tags for relative and absolute quantification [iTRAQ®], mass differential tags for relative and absolute quantification [mTRAQ™], deuterium isobaric aminereactive tags and *N,N*-dimethyl leucines) that target nearly every reactive site on a protein or peptide, increasing detection for quantification and identification.

Lipids

- The primary focus for the labeling of lipid compounds has been chemical derivatization at the carboxylic acid group. Several reagents have been developed for LC–MS that maintain appropriate mass shifts for quantification while adding a strong ionizing component to compensate for the weakly ionizing lipid. Recent advances in the conversion of lipid compounds to volatile substances has also allowed for the quantification of lipids using GC–MS.

Metabolites

- In order to analyze the vast assortment of molecules found in the metabolome, techniques for quantification must focus on the various functional groups found within the metabolites. These techniques, however, are limited by the standards available for comparison to the labeled functional groups.

Conclusion

- Although recent advances in mixed-isotope labeling have contributed to enhanced detection and quantification of biomarkers, these strategies are still limited in their validation of biomarker analysis. This step will be crucial if these strategies are to advance into a clinical setting.

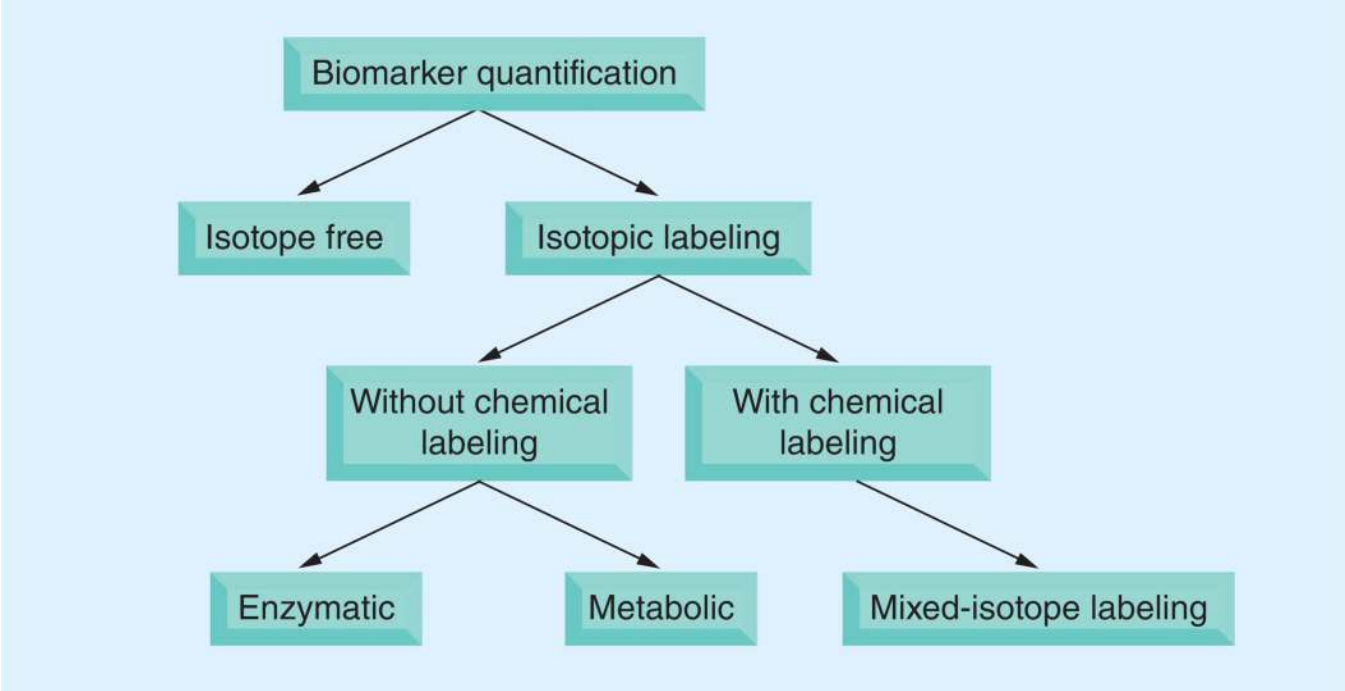

Figure 1.
Biomarker quantification methods.

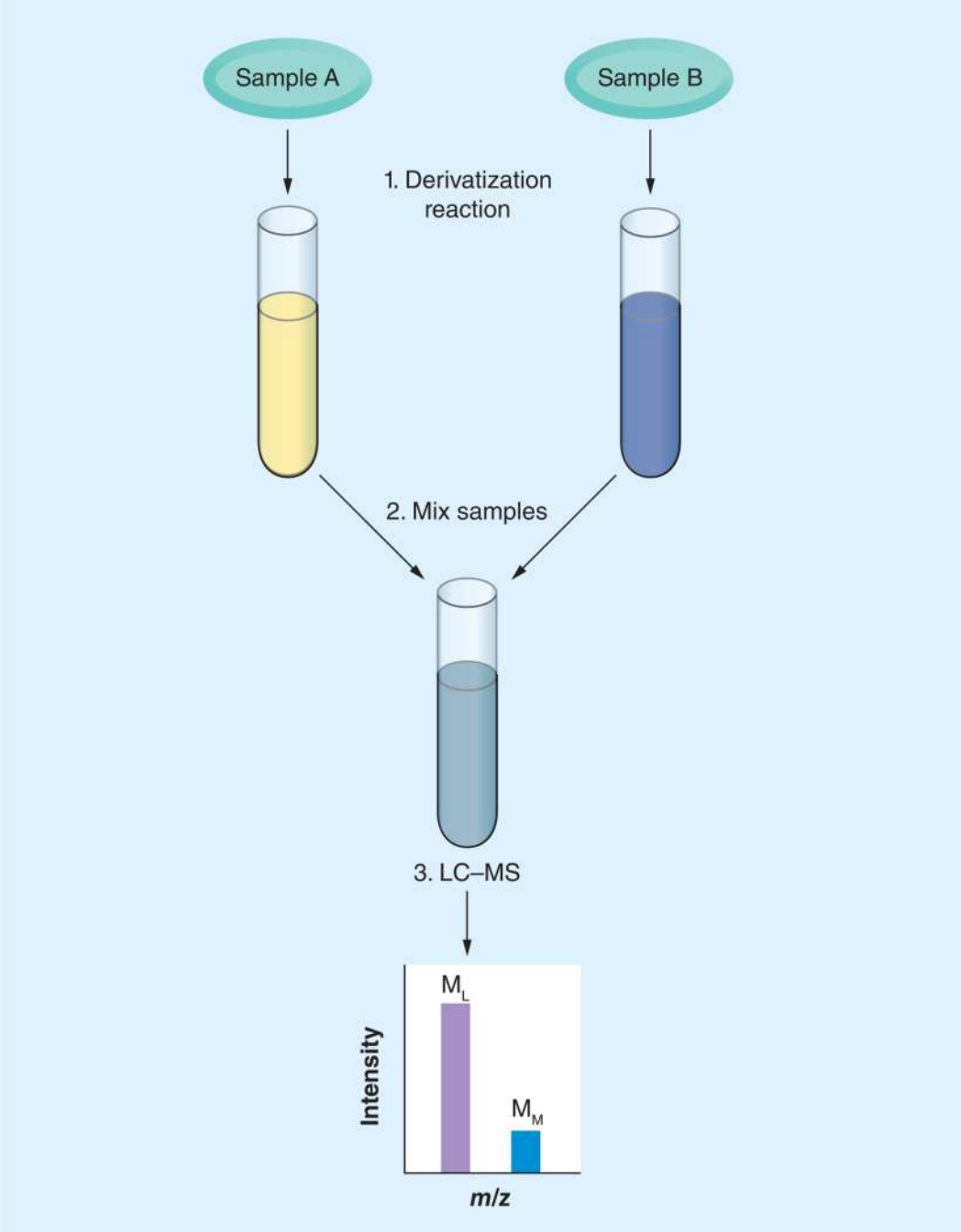

Figure 2.
Mixed-isotope chemical labeling strategy.

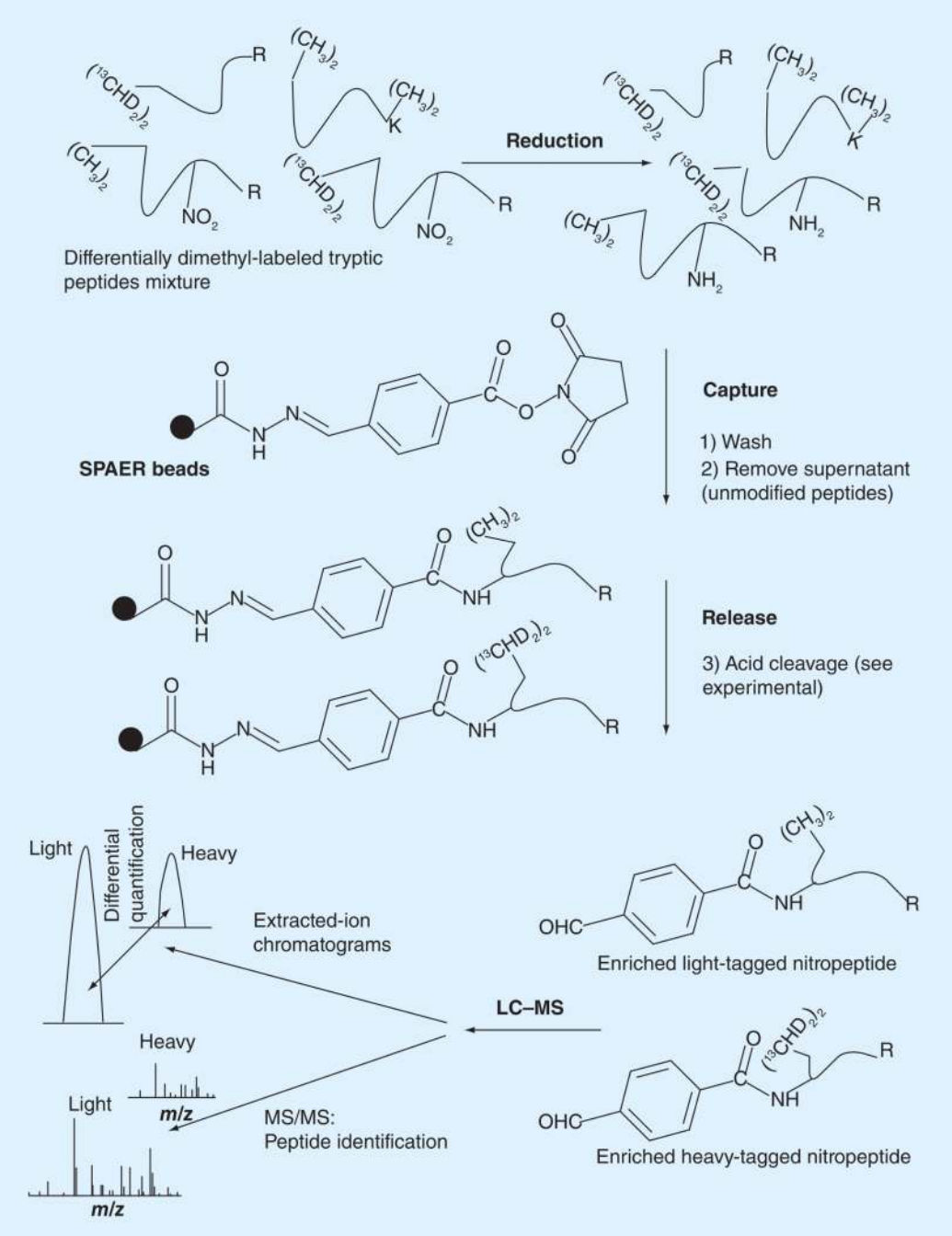

Figure 3.

Protocol for mixed-isotope-labeling analysis of human cerebrospinal fluid biomarkers. *N*-dimethylation of aliphatic amines is followed by the reduction of nitrotyrosines to their corresponding aminotyrosines. The resulting peptides are then captured via solid-phase active ester reagent and released upon acid-catalyzed hydrolysis. The relative abundance between light- and heavy-labeled peptides is determined from LC–MS analyses. The mass shift of this chemical tagging produces 28-Da shift for N-$(CH_3)_2$ or 34-Da shift for the N-$(^{13}CHD_2)_2$ tag yielding a mass difference of 6 Da per labeling site between the isotopically labeled peptides. Reproduced with permission from [58] 2012 © Elsevier.

Table 1

Mixed-isotope-labeling protein analysis approaches.

Method	Characteristics
Mixed-isotope tags	
ICAT	Allows identification of low abundance molecules
	Limited sequencing information
ICPL	Gives increased sequence information
	Allows three-plexed analysis
Reductive methylation	Low cost
	Fast reaction rate
	Mild reaction conditions
	High specificity to amino groups
	High labeling and ionization efficiency
	Stable post-derivative products
Isobaric tags	
iTRAQ®	Commercialized
	Allows analysis of low abundance kinases
	Higher sensitivity
	Less variability
	Better reproducibility
	Multiplex analysis
	Less accurate
mTRAQ™	Gives accurate quantification of low-abundance tags
	Multiplex analysis
	Redundant readings
Deuterium labeling	
DiART	Six-plexed analysis
	Greater response of reporter ions
	Better quantitative coverage of samples
DiLeu	Four-plexed analysis
	Low cost

DiART: Deuterium isobaric aminereactive tags; DiLeu: N,N-dimethyl leucines; ICAT: Isotope-coded affinity tags; ICPL: Isotope-coded protein labels; iTRAQ®: Isobaric tags for relative and absolute quantification; mTRAQ™: Mass differential tags for relative and absolute quantification.

Table 2

Mixed-isotope-labeling lipid analysis approaches.

Method	Derivatization reagent	Characteristics
LC–MS		
Mixed-isotope labeling (heavy and light labels)	DBD-PZ-NH2 (D)	Targets lipids in biofluids; allows sufficient separation for quantification by MS; gives isotope internal standards
Ionizing group partners with isotope labels	Charge group: quaternary ammonium group on labeling reagent	More sensitive to LC–MS in positive-ionization mode; better, more efficient chromatographic resolution
	CMP, CMP-d3	More sensitive (2500-times) than underivatized samples
	Cholamine	No co-elution of lipids
	DmPA bromide	Allows detection two- to four-times lower than unlabeled lipids; stronger signal; clearer peaks
GC–MS		
Silylation isotopic labeling	N/A	Creates volatile lipid compounds; needs anhydrous reaction conditions; heating of lipid samples
iFAT	N/A	Faster, simpler and less errors

CMP: 3-carbinol-1- methylpyridinium iodide; CMP-d3: 3-carbinol-1-methyl-d3-pyridinium iodide; DBD-PZ-NH2 (D): 7-(*N,N*-dimethylaminosulfonyl)-4-(aminoethyl) piperazino-2,1,3-benzoxadiazole (d_6); DmPA: *p*-dimethylaminophenacyl; iFAT: Isotope-coded fatty acid transmethylation.

Table 3

Mixed-isotope-labeling metabolite analysis approaches.

Functional group	Derivatization chemistry	Chemical tag	Method of detection	Advantages	Disadvantages
Amines (primary and secondary)	Reductive methylation	Formaldehyde, ^{13}C-formaldehyde	ESI	Gives information on amount and types of amine groups present; increases detection of labeled amines; sample more readily protonated for ESI detection	N/A
	Dansylation labeling	Dansyl chloride, ^{13}C-dansyl	Fluorescence or UV detection	Targets multiple functional groups	ESI enhancement not as effective as reductive methylation (only one- to three-times)
Carboxylic acids	Group specific internal standard technology	DmPA bromide CMP, CMP-d_3	LC–MS	Improves ESI enhancement (two- to four-times); no chromatographic isotope effect; maintains ratio between control and isotope sample concentrations	N/A N/A
Phenols	Dansylation labeling	Dansyl chloride, ^{13}C-dansyl	Fluorescence or UV detection	Targets multiple functional groups	ESI enhancement not as effective as reductive methylation (only one- to three-times)

CMP: 3-carbinol-1- methylpyridinium iodide; CMP-d3: 3-carbinol-1-methyl-d3-pyridinium iodide; DmPA: *p*-dimethylaminophenacyl.