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Review Article

Quantitative analysis with modern bioanalytical mass spectrometry and stable isotope labeling†

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Abstract: The invention of new ionization techniques namely electrospray ionization and matrix-assisted laser desorption/ionization combined with the development of novel mass spectrometer analyzers and evolving isotope-ratio mass spectrometry have fueled the presence and use of modern mass spectrometric methodologies in many bioanalytical laboratories. Consequently, over the past two decades, a steadily increasing number of quantitative methods employing stable isotope labeling techniques have been reported, including prominent examples of methods to determine differential expression of proteins in disease studies, new-born screening for metabolic disorders, and tracing drugs or dietary compounds and their respective metabolites. Labeling biomolecules for quantitative studies using mass spectrometry has several challenges, including potentially insufficient labeling efficiency, ionization suppression, chromatographic separation of labeled and non-labeled compounds, and isotope exchange with the environment. It is not surprising that method development to minimize or eliminate existing limitations represents a very active and dynamic research area.

Keywords: stable isotope labeling; mass spectrometry; ESI; MALDI; protein quantitation; twin-ion technique

Introduction

The discovery of stable isotopes and their subsequent use as labels are tightly connected with mass spectrometry's evolution: early mass spectrographs developed by Thomson and Aston enabled their discovery.[1] The first reported metabolic tracer studies with deuterium in the mid-1930s by Schoenheimer and Rittenberg[2] were soon complemented with '*unusually sensitive*' mass spectrometric procedures,[3,4] and expanded to the use of ^{15}N.[5,6] The combination of gas chromatography with mass spectrometric detectors (GC–MS) led to a tremendous breadth of stable isotope labeling applications,[1] similar to those witnessed after the inventions of electrospray ionization (ESI) by Fenn *et al.*[7] and matrix-assisted laser desorption/ionization (MALDI) by Tanaka *et al.*[8] and independently by Karas and Hillenkamp[9,10] during the late 1980s. The combination of separation techniques like liquid chromatography (on- or offline) with ESI and MALDI and the introduction of a variety of novel tandem or hybrid mass spectrometers, allowing the isolation and controlled fragmentation of gas-phase biomolecule ions, have further accelerated the progression of stable isotope labeling techniques in bioanalytical research. The steep incline in publications related to stable isotope labeling and mass spectrometry starting in the early 1990s coincides with the introduction of MALDI and ESI (see Figure 1) and these techniques are, besides other technological advancements, responsible for an increased interest in this subject. This selective review will summarize a number of basic principles underlying stable isotope labeling techniques in quantitative modern bioanalytical mass spectrometry, present an evaluative overview of the potentials and limitations of labeling methods, and demonstrate their performance with particular applications.

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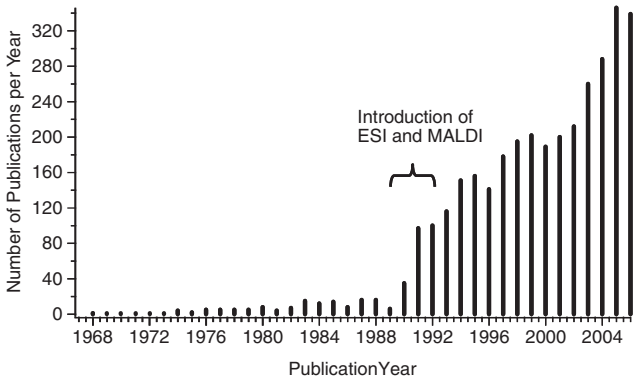

Figure 1 Number of publications per year versus publication year for publications related to mass spectrometry and stable isotope labeling. The literature search was done with the keywords 'stable isotope label*' and 'mass spectrometry' individually for every publication year with the ISI Web of Knowledge database search program (portal.isiknowledge.com).

Differential protein expression measured by mass spectrometry

In 1999, Gygi *et al.* demonstrated that results from gene expression studies cannot necessarily be employed to deduce respective protein expression levels.[11] Modern mass spectrometry had been previously applied for protein identification but generally not for quantitative studies, mostly due to lack of adequate standards. Protein quantitation in biological samples faces several challenges: ionization efficiency variation for even slightly structurally different biomolecules (e.g. different amino acid sequence in peptides[12]) and the complexity of the ionization process (ESI or MALDI), potentially resulting in ionization suppression,[13,14] generally do not permit the use of external calibration methods. Figure 2 shows a general schematic for sample preparation procedures in comparative protein quantitation studies. Table 1 summarizes some important labeling reaction schemes typically employed in protein quantitation studies. An overview follows with the main stable isotope labeling methodologies in quantitative proteomics.

Isotope-coded affinity tags (ICAT)

Described in 1999 by Gygi *et al.*, isotope-coded affinity tags (ICAT) are chemical reagents that show the popularity of mass spectrometry for proteomic analysis and are alternatives to ^{15}N labeling with 2D gels[15] with three elements: a specific chemical reactivity (directed toward free cysteine thiols), an isotopically coded linker (heavy deuterium or light hydrogen), and an affinity tag (biotin-based for avidin affinity isolation). The system is simple in principle[31]: protein samples from two separate states are reduced, derivatized with light or heavy reagent, samples are combined and digested into peptides, tagged peptides are isolated via an avidin affinity column and then separated and analyzed by liquid chromatography–mass spectrometry (LC–MS) and LC–MS/MS. Relative quantities of eluted peptides are determined by calculating the ratio of the peptide pairs' signal intensities that have the same sequence and are identified by their heavy and light ICAT mass difference.

In 2002, Zhou *et al.* reported the development of a similar system with a solid phase capture and release[32]: solid phase synthesis attaches aminopropyl-coated glass beads to a photocleavable linker, which is attached to an isotopically labeled leucine molecule (containing either hydrogen or deuterium atoms, with a mass difference of 7), and a sulfhydryl-specific iodoacetyl group is attached. A direct comparison to ICAT using protein from the yeast *Saccharomyces cerevisiae* in response to galactose induction showed several advantages: one step to combine peptide isolation and stable isotope incorporation; removal of non-covalently associated molecules through a stringent solid phase wash; robustness to proteolytic enzymes or strong denaturants or detergents; potential to use multiple tags with many samples in one experiment (i.e. amenable to other amino acid tags); uncomplicated MS/MS fragmentation due to the tag's small size and chemical nature; and the ability to perform additional chemical and enzymatic reactions to the solid-phase bound peptides. Similarly, there are reagents that incorporate a visible tag, allowing electrophoretic monitoring, a photocleavable linker removed prior to MS analysis, and an isotope tag incorporating ^{13}C or ^{15}N atoms to ensure precise comigration of light and heavy tagged peptides in HPLC.[33] These reagents reduce the tagged peptide's size, providing a preferred mass range for more efficient MS detection.

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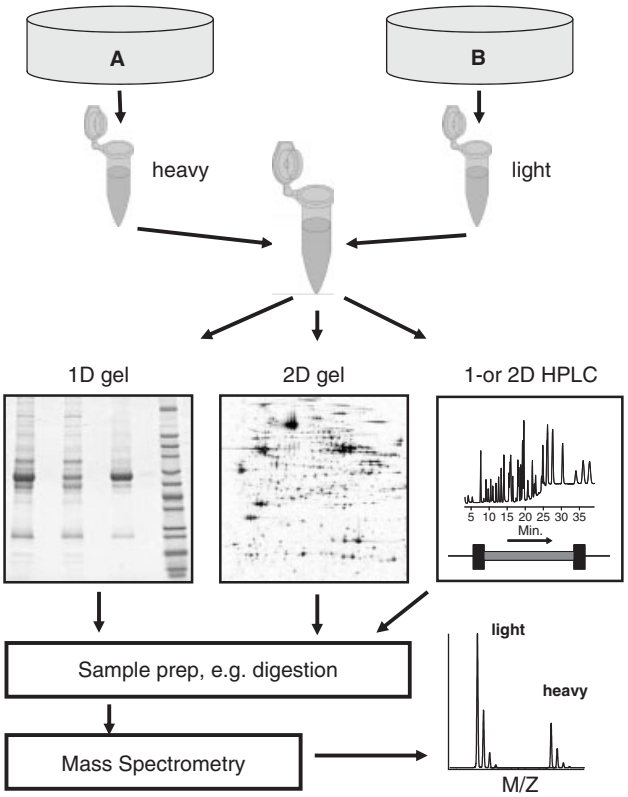

Figure 2 General schematic of protein or peptide labeling in quantitative proteomics. Two equal amounts of tissue (A + B), e.g. healthy versus diseased, to be compared in terms of specific protein abundances are labeled with the chemically identical labeling reagents differing in stable isotope content. Signals arising from the same protein or peptides labeled with heavy or light labeling reagents are utilized for comparative quantitation. Housekeeping proteins that should be equally abundant in both tissue samples can be used for control or adjustment purposes. The digestion step can also be administered before HPLC separation ('shotgun' peptide analysis).

ICAT provides a 'gel free' alternative to the use of ^{15}N-labeling with 2D gel separation coupled to mass spectrometry[34]; a 'one shot' analysis of control and experimental samples, eliminating errors introduced when samples are not treated identically, allowing for relative quantitative comparisons to be made,[35] and providing quantitative time course-based studies of biological processes.[36] However, only cysteine residue-containing peptides are labeled,[37] creating various limitations: inability to detect post-translational modifications[38] and failure of complete alkylation by iodoacetamide-based reagents.[39–41] Peptides of interest must co-migrate with the standards within the LC portion of the LC MS/MS method, presenting significant problems with the original d_0/d_8 ICAT reagents but nearly completely circumvented by the use of a $^{13}C_9$-coded reagent.[38,42] The label's biotin portion causes complicated MS/MS fragmentation spectra addressed by the creation of an acid-cleavable site to remove the biotin prior to MS/MS analysis.[38] The protocol must be optimized on a per-sample basis for ICAT reagent concentration, influence of protein, sodium dodecyl sulfate (SDS), urea concentrations on the labeling reaction and reaction time[43] all requiring significant technical competency, not to mention access to suitable, often very expensive, research infrastructure.[34] ICAT technology provides relative quantitation of one peptide under two conditions, not the concentration of one protein relative to another in a single state.[35]

A great testament to a technique's robustness and acceptance is its application to biological questions – ICAT's applications are too numerous to itemize here. In Gygi *et al.*'s seminal ICAT paper, protein expression differences were measured in *S. cerevisiae* grown with different carbon sources – ethanol versus galactose.[15] Han *et al.* used ICAT to identify and measure abundance of 491 proteins in microsomal fractions of human myeloid leukemia cells in naïve and *in vitro* differentiated states.[44] The ICAT technique demonstrated a method to determine composition, changes in composition, and changes in the abundance of protein complexes via use of RNA polymerase II pre-initiation complex and STE12 protein complexes from yeast cells

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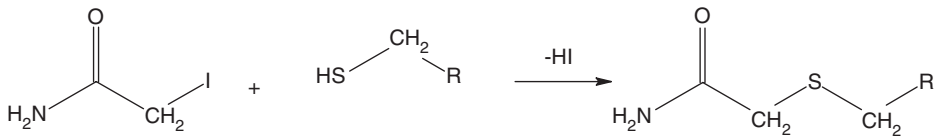
Table 1 Reaction schemes of labeling methods for protein quantitation

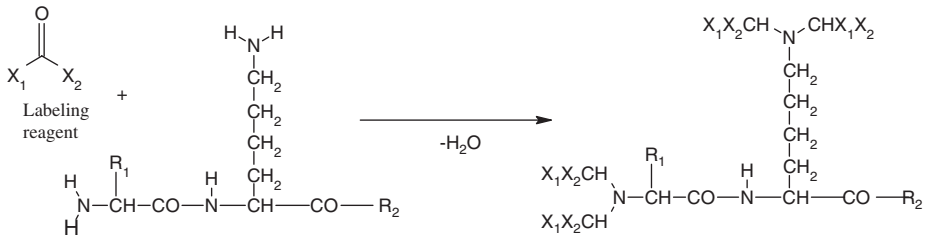
I. *Alkylation of free thiols on cysteine residues*:



Labeling reagent — Cysteine residue with free thiol in protein/peptide backbone (also reduction of disulfide linkages)

Examples for labeling reagents: ICAT,[15] acrylamide[16] and iodoacetamide.[17]

II. *Labeling of primary amines in proteins or peptides*:



Primary amine (either N-terminus of protein/peptide or sidechain of lysine residue)

Examples for labeling reagents: aldehydes,[18,19] *N*-acyloxy-succinimide esters of acids,[19] isothiocyanates,[19] guanidating reagent and imidazole-based reagents,[19] tandem mass tags (TMT)[20] and iTRAQ[21].

III. *Enzymatic C-terminal incorporation of* ^{18}O *during/after protein proteolysis*[22–26]:
First incorporation of ^{18}O:
(a) R_1–CO–NH–R_2+HO–Enzyme → R_1–CO–O–Enzyme+H_2N–R_2
(b) R_1–CO–O–Enzyme+H_2 ^{18}O → R_1–CO–^{18}OH+HO–Enzyme

Second incorporation of ^{18}O: (occurs only with certain enzymes, e.g. trypsin, and under specific conditions)
(c) R_1–CO–^{18}OH ← → R_1–C^{18}O–OH
(d) R_1–C^{18}O–OH+HO–Enzyme → R_1–C^{18}O–O–Enzyme
(e) R_1–C^{18}O–O–Enzyme+H_2 ^{18}O → R_1–C^{18}O–^{18}OH+HO–Enzyme

IV. *Derivatization of carboxyl groups of proteins or peptides to methyl esters*[18,27]:
R–COOH+HO–CX_3 (e.g. methanolic HCl) $\rightarrow_{-H_2O}$ R–COOCX_3

V. *Other methods*:
Metabolic introduction of labeled amino acids into proteins *in vivo* (e.g. ^{15}N-labeling,[28] SILAC[29])
Total synthesis of peptide standards with introduced labels (AQUA[30])

in different states.[45] Oda *et al.* used ICAT methods to systematically identify drug targets for a novel class of anticancer agents undergoing phase II clinical trials.[46] The technique has also been applied to proteomic profiling of cellular extracts and organelles.[34]

Absolute quantification standards (AQUA)

Absolute quantification (AQUA) of proteins and peptides[30] uses internal standard peptides synthesized strategically to correspond to proteolytic peptides of a protein and may be prepared with covalent modifications such as phosphorylation, acetylation, methylation, etc. AQUA peptides are the ideal quantitative internal standard being chemically identical to their naturally occurring counterparts. The procedure involves internal standard selection, synthesis with incorporation of a stable isotope (^{13}C, ^{15}N, etc.), and analysis by MS/MS to deduce its fragmentation pattern and set the single reaction monitoring (SRM)

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requirements to measure a precursor to product ion transition. The internal standard quantifies precisely being part of the entire sample preparation process, from protein removal from a sample, digestion, and LC-SRM. A specified fragment ion's abundance is measured by ultimately comparing abundance of known AQUA peptide to the native peptide. Validation experiments demonstrated that protein migration and trypsinization in polyacrylamide gels were efficient and essentially complete while partial digestion or missed trypsin cleavages must be considered in internal standard design. This issue is addressed by utilizing synthesized internal standards that concomitantly account for fully or partially tryptic native peptides. Although a very time and technically intensive procedure, AQUA provides powerful data.

Stable isotope labeling by amino acids in cell culture (SILAC)

In 2002, Ong *et al.*[29] described SILAC, which capitalizes on mammalian cells' inability to synthesize a number of amino acids naturally, hence cell culture medium is enriched with 'essential' amino acids, supplying isotopically labeled deuterated leucine (Leu-d_3). Leucine is the most abundant amino acid, is readily available, and provides distinction between leucine and isoleucine. Two batches of the same cell line are cultured with non-deuterated versus deuterated Leu media, complete Leu-d_3 incorporation occurs after five doublings in the cell lines, and labeled and unlabeled cultures are harvested and mixed together, digested enzymatically, and quantitated by MS.[47] MS-based identification and relative quantitation are uncomplicated because each Leu-containing peptide either incorporates all Leu or all Leu-d_3 and the corresponding heavy and light peptide pairs co-elute chromatographically.[48] SILAC is based on a similar principle, so-called 'residue-specific' mass tagging, previously demonstrated in *Escherichia coli* and *S. cerevisiae* for protein identification purposes[49–51] and has been demonstrated with numerous amino acids: fully ^{13}C-labeled arginine,[52] ^{13}C-methionine-d_3,[53] lysine-d_4,[54] $^{13}C_9$-tyrosine,[55] methionine-d_3, serine-d_3, and tyrosine-d_2,[50] and glycine-d_2.[49]

The approach is simple, inexpensive, and accurate, and can be used with any cell culture system or lab and standard equipment in a proteomics lab.[29] Incorporating the essential labeled amino acids into cell cultures has numerous advantages: no peptide labeling steps (maximizes initial starting material, minimizes manipulations after harvesting), no differences in labeling efficiency between one sample to another (nearly 100% incorporation of the labeled or unlabeled amino acids), numerous peptides from the same protein confirm relative change in the protein, peptide-labeling is sequence-specific since the tag arises from a stable isotope-containing amino acid rather than isotopic nuclei, and observation and quantitation of changes in small proteins are possible.[29] MS/MS spectra are easy to interpret since fragmentation patterns of the labeled and unlabeled peptide pairs are identical except for the predicted mass shift specific to the labeled amino acid's location.[47] Relative to ICAT, SILAC (with Leu) labels a much larger degree of tryptic peptides – estimated to be up to 50% based on relative abundance of cysteine to leucine, and approximately 14 amino acids/peptide that is sequenced by MS.[29]

However, it is simply impossible to completely label all of a proteome's proteins,[49,56] spectral interpretation can be complicated by mixtures and sequence-dependent mass shifts (generally solved by software),[39] and conversion of the incorporated amino acid into another by the cell's own metabolism leads to unpredictable isotope dilution, partial loss of labeling, and different mass shifts[39,52,57] (circumvented by using amino acids that do not 'isotope scramble',[57] or a cell-free protein synthesis system that incorporates amino acids *in vitro*[58]). A significant clinical impediment is that SILAC cannot be used *in vivo*.[39]

SILAC provides comparisons of closely related cell states, for example, ^{13}C-methionine-d_3 to observe methylation *in vivo*[53] or $^{13}C_9$-tyrosine-labeled media to quantitate changes in a chronic myeloid leukemia kinase's (BCR-ABL) phosphorylation state and its substrates in response to Imatinib treatment.[55] One study quantitated >700 phosphopeptides in yeast treated with a $^{13}C_6$-arginine and $^{13}C_6$-lysine-labeled pheromone, including 139 differentially regulated by a factor of at least 2.[59] Other applications include labeling plant cells from *Arabidopsis thaliana* to study glutathione S-transferase expression in response to salicylic acid-induced stress,[54] and the effect of miRNA-1 on the HeLa cell proteome, which demonstrated that 12 out of 504 investigated proteins were repressed by miRNA-1 transfection.[60]

Isobaric labeling reagents

The idea of isobaric labeling reagents was first demonstrated by Thompson *et al.* in 2003, employing small peptide groups with guanidino functionality (tandem mass tags or TMTs).[20] Figure 3 shows the basic isobaric label scheme – the clever isobaric design requires MS/MS analysis to create the reporter ions that are essential for quantitative comparison of samples as is described in more detail below.

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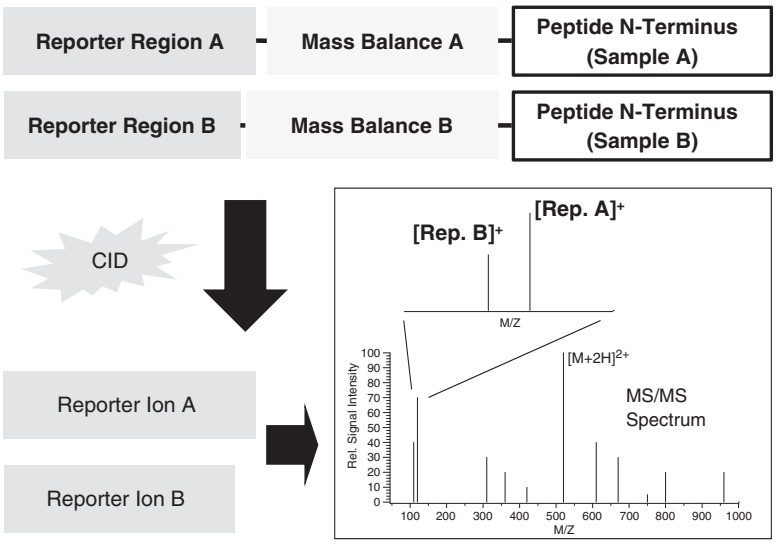

Figure 3 Basic principle of isobaric labeling reagents (TMTs[20] or iTRAQ[21]). Analog peptides from two different samples (A + B) labeled with the isobaric labeling reagents differ only in their reporter region and have the identical precursor ion (e.g. $[M + 2H]^{2+}$) m/z value. Upon collision-induced dissociation (CID) they form different mass reporter fragment ions. For peptides of differing abundances in the two samples, the relative intensity of reporter ion A ($[Rep.A]^+$) to reporter ion B ($[Rep.B]^+$) will differ accordingly, provided that the ionization efficiency of the reporter fragments A and B is similar.

A different set of four isobaric tagging reagents[21] (iTRAQ) were introduced in 2004 by Ross *et al.* to measure proteins relatively and quantitatively in multiplex from various complex mixtures. An iTRAQ molecule is composed of a reporter group, based on *N*-methylpiperazine and of m/z 114.1, 115.1, 116.1, or 117.1 moieties and a balance group of mass 28–31, so that the combined mass of these two groups is a constant 145.1 Da (achieved utilizing different isotopic enrichment with ^{13}C, ^{15}N, and ^{18}O), and a peptide-reactive (NHS ester) group. The isobaric tag reacts to form an amide bond with any peptide amines (either the N-terminal or ε-amino group of lysine) which on collision-induced dissociation (CID) fragments similarly to form backbone peptide bonds. The technique allows for comparisons between four different states: a mixture of four identical peptides (e.g. each sample subjected to a different growth condition) each labeled with a different isobaric label subjected to MS, appears as one unresolved precursor ion with the same m/z, while upon CID, the four reporter group ions appear as their distinct masses, allowing for relative concentration determination of each of the four peptides from their respective reporter ions' intensity. Upon CID, all sequence-informative fragment ions remain isobaric because along with the loss of the reporter group, the balance group (a carbonyl) is lost as a neutral loss. iTRAQ is distinct as quantitation is performed at the MS/MS, rather than the MS stage. The reporter group masses can be minimally contaminated by background low-mass fragments that accompany peptide fragmentation either by MALDI or ESI-based tandem mass spectrometers.[21] The MS spectra and MS/MS ion series of the derivatized peptides are indistinguishable, relying on the low-mass MS/MS signature ions for identification and providing exquisitely simple MS/MS interpretation and quantitation.[21] The method currently allows for multiplexing up to four sample states simultaneously, with a soon to be released kit for eight states.[21,61] This technique is highly robust and reproducible, with intra-protein peptide mean and standard deviations between 15–17% and highly consistent measured expression ratios in samples of multiple yeast strains.[21] Quantitative variability arising from separate peptide mixtures analyzed by sequential 2-D LC–MS analysis is eliminated,[62] and compared to ICAT, peptide coverage is significantly increased, the chemistry used to tag can be applied to any peptide with a free amine,[21] and to peptides that are sterically hindered. iTRAQ studies have incurred problems with the time-ion selector resolution of the MALDI-TOF/TOF and there is a greater likelihood of potential error sources during sample processing since samples from different states require separate processing up to tryptic digestion.[63] The seminal iTRAQ paper by Ross *et al.* demonstrated the technique's use for global protein expression of a wild-type versus two mutant yeast strains.[21] Discarding protein identifications made on single, unique peptides resulted in analysis of 685 proteins identified by two or more significant

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peptides, with high concordance level between individual peptides measuring relative quantitation for the same protein, and further statistical impositions displayed 62 and 48 up-regulated proteins and 23 and 29 down-regulated proteins for the mutant strains.[21] Sachon *et al.* report the use of iTRAQ to quantitate synthetic phosphopeptides and those of α- and β-casein, showing iTRAQ's compatibility with phosphopeptides and phosphoproteomic separation techniques, but raising a potential bias against large, multiply phosphorylated peptides.[64,65] Other groups utilized iTRAQ to measure changes in *E. coli* protein expression in response to rhsA induction[66] to discover and identify potential markers for endometrial cancer from clinical samples,[67] identify and quantitate bone marrow stromal cell proteins from individuals considered 'normal' versus leukemic,[68] and time course effects of a drug candidate on a protein.[69] iTRAQ's infancy means many novel studies are still being explored.

Oxygen-18 labeling in comparative protein quantitation

Fenselau's group introduced ^{18}O-labeling for comparative protein expression studies in 2001.[22] ^{18}O-labeling is attractive due to relative low costs and no need for an additional specific labeling reaction. One of the two protein mixtures to be compared is simply enzymatically digested in ^{18}O-enriched water ($H_2{}^{18}O$), the proteolytic hydrolysis reaction incorporates one or two ^{18}O atoms (depending on the employed enzyme and other factors, see Table 1) into the C-terminal residue's carboxyl group of the proteolytic peptide[22] and the resulting peptides are 2 or 4 Da higher in mass than their equivalent non-labeled counterparts. ^{18}O-labeling has no significant effect on chromatography retention properties, i.e. labeled and non-labeled peptides co-elute, so after combining the labeled and non-labeled peptide samples, ion intensities can be directly compared in LC–MS and LC–MS/MS experiments.[22] Studies revealed that even after digestion completion, the C-terminus is still recognized as a substrate by the proteolytic enzyme and $^{18}O/^{16}O$ back-exchange reactions can occur,[23] especially when using trypsin.[24] This characteristic can be advantageously exploited since it allows post-digestion labeling, eliminating the requirement to dry samples before digestion and allowing for optimization of ^{18}O-labeling with a minimum of $H_2{}^{18}O$.[23] Storms *et al.* recently reported heat deactivation of trypsin to avoid back exchange after ^{18}O-labeling.[25] A very detailed and comprehensive review on the subject of ^{18}O-labeling strategies in quantitative proteomic projects has been published by Miyagi and Rao.[26]

Other stable isotope labeling methods for quantitative proteomics

Numerous other labeling methods useful in quantitative proteomics and with similar challenges and limitations as described above have been recently published. These include (with no intention to be complete) the differential dimethyl labeling of free amino groups,[18,70–74] esterification of carboxylic groups,[18,27] or acrylamide labeling methods for free cysteine thiols in peptides or proteins.[16]

Further reading on quantitative techniques in proteomics

For more detailed and extended information on the important topic of stable isotope labeling methods in quantitative proteomics, we would like to bring the recent reviews of Julka and Regnier,[19,39,75] Yan and Chen,[76] MacCoss and Matthews,[77] Righetti *et al.*,[17] Ong and Mann,[78] Leitner and Lindner,[79] Moritz and Meyer,[28] and Fenselau[80] (which includes a section of metabolic protein labeling via nutrients) to the reader's attention.

Drug screening, metabolite tracing and quantitation using stable-labeled isotopes

The drug discovery process is lengthy and costly. Once a target mechanism for a particular disease has been identified and validated, the selection of potential drug candidates to regulate such target starts earnestly. Most standard drug development programs consist of first assessing toxicity and therapeutic ranges of selected compounds. These pre-clinical activities involve animal studies in which preliminary toxicity tests are carried out within the same protocol testing a compound's pharmacokinetic (PK) properties. A compound with the desired efficacy and therapeutic range with low or no toxicity and suitable PK behavior then undergoes investigation into its absorption, metabolism, distribution, and excretion (ADME). During the ADME phase, stable isotopes provide a clear advantage over other means of drug screening and metabolism studies.

One of the first surveys on the applications of stable isotopes in drug metabolism studies was carried out by Baille,[81] followed by three reviews by Browne,[82,83] Pons and Rey,[84] and Abramson.[85] Although these reviews are now dated and looked at selected aspects such as the economics of drug development, pediatric clinical

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pharmacology, and drug metabolism, they summarized the main uses of stable isotope labeling studies in mass balance and general ADME studies. Three main approaches have been put forward through the years including: tracer studies, isotope ratio monitoring studies, and isotopic pattern recognition. Lately, these approaches have been applied to measuring intact metabolic fluxes in toxicology[86,87] and functional genomic and biochemical phenotyping.[87] These developments have been only made possible by the advent of modern mass spectrometry, particularly, advances in atmospheric ionization techniques and associated technologies such as high-throughput hyphenated chromatographic methods.[88] The initial and ever present advantage of using non-radioactive isotopes in ADME and mass balance studies enables applications to *in vivo* human studies including topics in pregnancy and pediatric pharmacology.

Tracer studies

Examples of tracer studies can be found as early as 1972 when Knapp *et al.* introduced the use of the so-called 'twin-ion' technique to identify the metabolites of nortriptyline (see Figure 4).[89] Here, a mixture of labeled and non-labeled drug is administered to the subject (*in vivo*) or added to a cell culture or microsomal preparations (*in vitro*). Subsequent chromatographic and mass spectrometric analysis of the various pools (plasma, liver tissues, urine, etc.) permits the identification of the parent drug and its metabolites. The selection of the labeled sites on the drug is critical in this approach. Based on the molecular structure of the drug, deuterium (D)- or carbon (^{13}C)-appropriate labels can be made. Detection of sample ion clusters separated by the same mass difference as that between the drug and its labeled analog (twin ions) permits the confirmation of a metabolite's presence. The key to this technique is that the molecular structure resulting from the metabolic transformation must keep the same labeled site or sites as on the labeled drug. A similar technique was employed by Brazier *et al.*[90] where ^{15}N- and ^{13}C-labeled and non-labeled theophylline was administered to infants and the labeled and non-labeled metabolites (caffeine) were detected as a twin molecular ion clusters at 194 and 197 *m/z*,

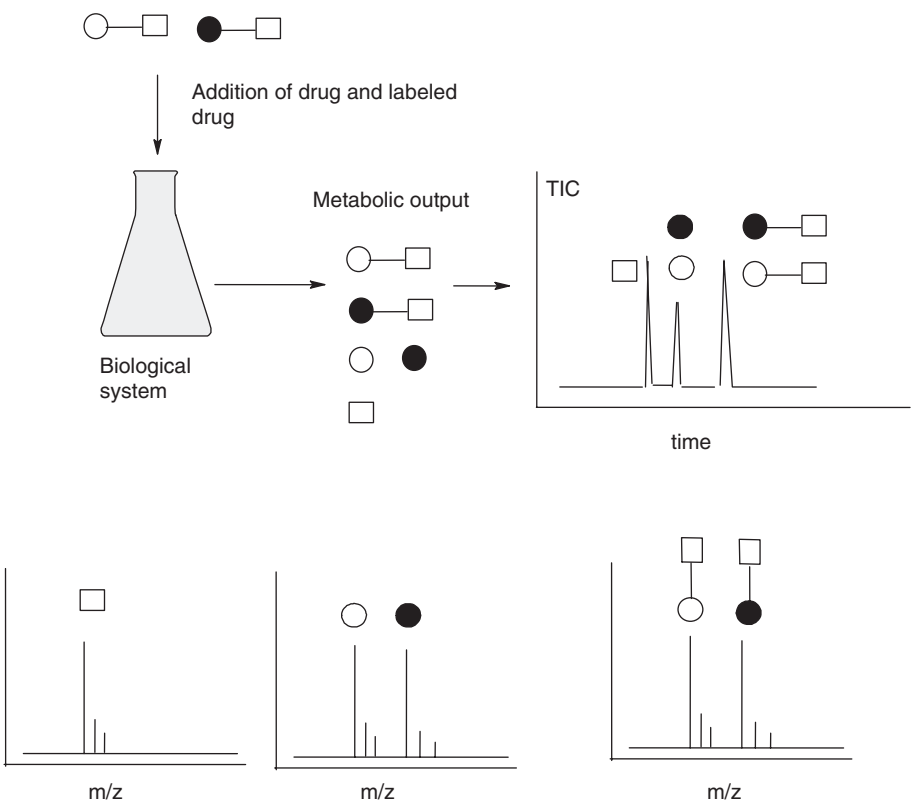

Figure 4 Example of the twin-ion technique for metabolite identification using labeled (e.g. with deuterium) and non-labeled drug applied to a biological system. The hypothetical drug is metabolized by the biological system by clipping the molecule in half. Non-labeled drug; (○ and □), labeled drug (● and □), suspected metabolic product 1(○), suspected metabolic product 2(□), twin ion to suspected metabolic product 1(●). Mass difference between non-labeled and labeled drug and mass difference between twin ions are the same as the number of deuterium on the labeled portion of the drug.

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corresponding to the native and tri-labeled caffeine, respectively. More recently, Weidolf and Covey[91] used this approach to unravel the metabolism of the drug omeprazole in the rat. Taylor *et al.*[92] demonstrated that stable isotopes could effectively track and accurately quantify metabolites in ADME studies. Although stable isotopes provide a powerful approach to metabolite identification, their application in metabolite characterization is not as popular as it is in the study of the metabolic fate of nutrients and other endogenous substances such as lipids[93] – one possible explanation is the significant expense of the synthetic labeling process.

Alternatives to the 'twin-ion' technique are needed when the metabolic process leads to a molecular structure that does not retain the same labeled sites as the labeled parent drug, such as continuous flow isotope-ratio mass spectrometry (CF-IRMS). CF-IRMS has been successfully used in mass balance studies,[94] was initially proposed by Browne *et al.*,[95] and consists of carrying out mass balance studies by administering stable isotope-labeled (^{13}C or ^{15}N) drug and measuring the (above background) isotopic pattern of ^{13}C or ^{15}N in biological fluids. Typically, samples of various biological fluids or tissues are extracted and analyzed by gas chromatography. Prior to introduction into the high-resolution mass spectrometer, the effluent from the chromatograph is directed to a reaction chamber in which it is combusted into CO_2, SO_2, and N_2 where applicable. The isotopic composition of each of these gases is monitored by a magnetic sector mass spectrometer and compared with that of a standard of each gas. The difference in the isotopic ratios between a CO_2 peak generated from a sample and that of standard CO_2 indicates that the detected peak corresponds to a metabolite. Recent developments in this approach include the design of an interface for direct introduction of the liquid chromatographic effluent into the mass spectrometer, extending application to drugs and metabolites not suitable for gas chromatographic analysis.[96] This new modification, also known as chemical reaction ion mass spectrometry (CRIMS), has shown to be as accurate, if not better, than radioisotope scintillation counting.[97–100] The attractiveness of CF-IRMS or CRIMS is that each compound eluting from the chromatographic system is detected as CO_2, SO_2, or NO_2. Although useful in mass balance studies, it reduces the mass spectrometer's ability to provide structural information.

H/D exchange studies

H/D exchange approaches have been popular in drug development for quite some time, with most recent developments involving application to *in vivo* studies, assessing metabolic fluxes or kinetics.[86] Most drugs undergo Phase I and Phase II metabolism. During Phase I, mostly oxidative processes involving P450 enzymes result in hydroxylation, aliphatic hydroxylation, *N*-, *O*- and *S*-dealkylation, *N*-hydroxylation, *N*-oxidation, sulfoxidation, deamination and dehalogenation, most of which involve the loss of exchangeable hydrogens. The determination of metabolite structural information is assisted by deducing the number of remaining exchangeable hydrogen atoms after metabolic transformation. Under specific conditions, exposure of a given sample to a deuterium source such as ^2H_2O can result in H/D exchange. Modern approaches include exposure of the liquid chromatographic effluent of the sample extract to an on-line H/D exchange system prior to entrance into the mass spectrometer. A common configuration uses ^2H_2O as part of the mobile phase as a H/D exchange system.[101–105]

An exciting application of H/D exchange includes cell proliferation studies for assessing drug efficacy. In these applications a bolus of ^2H_2O is administered concurrent with the application of the drug being tested for *in vivo* or *in vitro* studies and pure water is replaced with ^2H_2O throughout the study. At the experiment's end, tissues and/or cells are harvested and a selected target marker (e.g. genomic DNA) is isolated. The ^2H enrichment of the selected marker is determined by mass spectrometry and compared to a non-enriched standard to assess enrichment level, a factor that can be used to deduce the level of cell proliferation.[106–108]

Reactive metabolites trapping using labeled trapping agents

Stable isotopes are also used to identify metabolites through labeled trapping reagents that permit the capture of fast reactive metabolites as adducts, which are then analyzed by mass spectrometry. A labeled trapping reagent allows the unique identification of these adducts by the 'twin-ion' approach. Recent examples of this application include the use of labeled glutathione as a trapping agent.[109,110] Yan and Caldwell[111] used this approach to detect, for the first time, transient metabolites of drugs such as clozapine and troglitazone. The labeling of the trapping agent enables unequivocal detection of the transient metabolites by means of neutral loss experiments. Although trapping can be done with non-labeled trapping agents, trapping with mixture of labeled and non-labeled agents provides the aforementioned advantage of the twin-ion method.

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Use of stable isotopes as internal standards in quantitative bioanalytical methods

By far the most popular application of stable isotopes in the drug discovery process is as internal standards in LC–MS methods for the determination of drugs and their metabolites in biological fluids. Although the use of this technique in quantitative analytical chemistry is not new, initially used in gas chromatographic–mass spectrometric methods, its use in LC–MS methods is relatively new.[112] This is a natural consequence of atmospheric pressure ionization techniques allowing mass spectrometry to be adapted to analyze compounds not amenable to GC–MS analysis with electron impact ionization, due to their limited thermal stability and/or relatively high polarities. Although potentially costly, the synthesis of labeled analogs has the advantage of providing highly suitable internal standards with very similar physical–chemical properties to the analytes.[112] Although modern LC–MS instruments show improved reproducibility over their predecessors, issues such as matrix effects, due to co-elution of compounds not detected by the mass spectrometer, can introduce unwanted analytical variability. Internal standard is customarily added prior to sample preparation to compensate for losses during this and the chromatographic–mass spectrometric processes and to account for matrix effects. Because mass spectrometers can distinguish between non-labeled analytes and the labeled analytes used as internal standard, stable isotopes make the ideal internal standards in LC–MS-based bioanalytical determinations. The literature demonstrates this application on numerous occasions and it is the method of choice for rugged LC–MS bioanalytical determination of drug and their metabolites in body fluids.[113–118] Issues remain with the use of stable isotopes as internal standards in LC–MS methods: labeled compounds should not contain detectable traces of the non-labeled compound, as the method's detection limit is affected by cross-contamination from the internal standard, and potential signal suppression of the co-eluting internal standard by its respective analyte, relating directly to ESI, the most common ionization technique used in LC–MS.[119] Consideration of these issues when using stable isotopes as internal standard can improve the ruggedness and reliability of LC–MS determinations.

Metabolic fluxes and isotopomers

An interesting and stirring development is the use of stable isotopes in tracking metabolic fluxes by detecting *in vivo* changes in the isotopomer configuration of a drug or xenobiotic with time. The already-mentioned work by Turner[86] and Hellerstein[87] are concrete examples of this technique and the approach consists of determining the isotope pattern of the target entity undergoing metabolic modifications. Combinatorial approaches to mass isotopomer distribution analysis (MIDA) have been successful in assessing changes during the biosynthesis of polymers and other entities.[87] The work of Turner[86] has included the MIDA type of analysis of xenobiotic transformation *in vivo*, but to our knowledge, this promising approach has not been applied to drug and/or pharmaceuticals.

Further reading on quantitative techniques in metabolite tracing

For more detailed and extended information on the important topic of stable isotope labeling methods in quantitative metabolite tracing, we would like to bring the recent reviews of Dettmer *et al.*[120] and Ando and Tanaka,[121] to the readers' attention.

Finally, it should be mentioned that novel quantitative methods have been developed both for quantitative proteomics and metabolite quantitation that do not rely on stable isotope labeling. For example, these include statistical methods in combination with ion or signal intensities[122–124] or accurate mass measurements in combination with retention times.[125,126] However, due to generally higher variability in the data acquisition with these methods, compromises have to be made in terms of accuracy.[24]

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REFERENCES

1. Grayson MA (ed.). *Measuring Mass*: *From Positive Rays to Proteins*, vol. 1. American Society for Mass Spectrometry: USA, 2002.
2. Schoenheimer R, Rittenberg D. *Science* 1935; **82**: 156–157.
3. Schoenheimer R, Rittenberg D. *J Biol Chem* 1937; **127**: 285.
4. Schoenheimer R, Rittenberg D. *J Mass Spectrom* 1998; **33**: 1164–1166.
5. Rittenberg D, Keston AS, Rosebury F, Schoenheimer R. *J Biol Chem* 1937; **127**: 291.
6. Rittenberg D, Keston AS, Rosebury F, Schoenheimer R. *J Mass Spectrom* 1998; **33**: 1167–1170.
7. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. *Science* 1989; **246**: 64–71.

8. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, Matsuo T. *Rapid Commun Mass Spectrom* 1988; **2**: 151–153.
9. Karas M, Bachmann D, Bahr U, Hillenkamp F. *Int J Mass Spectrom Ion Proc* 1987; **78**: 53–68.
10. Karas M, Hillenkamp F. *Anal Chem* 1988; **60**: 2299–2301.
11. Gygi SP, Rochon Y, Franza BR, Aebersold R. *Mol Cell Biol* 1999; **19**: 1720–1730.
12. Krause E, Wenschuh H, Jungblut PR. *Anal Chem* 1999; **71**: 4160–4165.
13. Knochenmuss R, Stortelder A, Breuker K, Zenobi R. *J Mass Spectrom* 2000; **35**: 1237–1245.
14. Buhrman DL, Price PI, Rudewicz PJ. *J Am Soc Mass Spectrom* 1996; **7**: 1099–1105.
15. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. *Nat Biotechnol* 1999; **17**: 994–999.
16. Faca V, Coram M, Phanstiel D, Glukhova V, Zhang Q, Fitzgibbon M, McIntosh M, Hanash S. *J Proteome Res* 2006; **5**: 2009–2018.
17. Righetti PG, Campostrini N, Pascali J, Hamdan M, Astner H. *Eur J Mass Spectrom* 2004; **10**: 335–348.
18. Ji C, Li L. *J Proteome Res* 2005; **4**: 734–742.
19. Regnier FE, Julka S. *Proteomics* 2006; **6**: 3968–3979.
20. Thompson A, Schaefer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Hamon C. *Anal Chem* 2003; **75**: 1895–1904.
21. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. *Mol Cell Proteomics* 2004; **3**: 1154–1169.
22. Yao X, Freas A, Ramirez J, Demirev PA, Fenselau C. *Anal Chem* 2001; **73**: 2836–2842.
23. Yao X, Afonso C, Fenselau C. *J Proteome Res* 2003; **2**: 147–152.
24. Angel PM, Orlando R. *Anal Biochem* 2006; **359**: 26–34.
25. Storms HF, van der Heijden R, Tjaden UR, van der Greef J. *Rapid Commun Mass Spectrom* 2006; **20**: 3491–3497.
26. Miyagi M, Rao KC. *Mass Spectrom Rev* 2006; **26**: 121–136.
27. Goodlett DR, Keller A, Watts JD, Newitt R, Yi EC, Purvine S, Eng JK, von Haller P, Aebersold R, Kolker E. *Rapid Commun Mass Spectrom* 2001; **15**: 1214–1221.
28. Moritz B, Meyer HE. *Proteomics* 2003; **3**: 2208–2220.
29. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. *Mol Cell Proteomics* 2002; **1**: 376–386.
30. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. *Proc Natl Acad Sci USA* 2003; **100**: 6940–6945.
31. See ICAT product information at www.appliedbiosystems.com
32. Zhou H, Ranish JA, Watts JD, Aebersold R. *Nat Biotechnol* 2002; **20**: 512–515.
33. Bottari P, Aebersold R, Turecek F, Gelb MH. *Bioconjug Chem* 2004; **15**: 380–388.
34. Flory MR, Griffin TJ, Martin D, Aebersold R. *Trends Biotechnol* 2002; **20**: S23–S29.
35. Greenbaum D, Colangelo C, Williams K, Gerstein M. *Genome Biol* 2003; **4**: 117.
36. Toigo M, Donohoe S, Sperrazzo G, Jarrold B, Wang F, Hinkle R, Dolan E, Isfort RJ, Aebersold R. *Mol Biosyst* 2005; **1**: 229–241.
37. Rabilloud T. *Proteomics* 2002; **2**: 3–10.
38. Julka S, Regnier F. *J Proteome Res* 2004; **3**: 350–363.
39. Galvani M, Rovatti L, Hamdan M, Herbert B, Righetti PG. *Electrophoresis* 2001; **22**: 2066–2074.
40. Galvani M, Hamdan M, Herbert B, Righetti PG. *Electrophoresis* 2001; **22**: 2058–2065.
41. Hamdan M, Righetti PG. *Mass Spectrom Rev* 2002; **21**: 287–302.
42. Hansen KC, Schmitt-Ulms G, Chalkley RJ, Hirsch J, Baldwin MA, Burlingame AL. *Mol Cell Proteomics* 2003; **2**: 299–314.
43. Smolka MB, Zhou H, Purkayastha S, Aebersold R. *Anal Biochem* 2001; **297**: 25–31.
44. Han DK, Eng J, Zhou H, Aebersold R. *Nat Biotechnol* 2001; **19**: 946–951.
45. Ranish JA, Yi EC, Leslie DM, Purvine SO, Goodlett DR, Eng J, Aebersold R. *Nat Genet* 2003; **33**: 349–355.
46. Oda Y, Owa T, Sato T, Boucher B, Daniels S, Yamanaka H, Shinohara Y, Yokoi A, Kuromitsu J, Nagasu T. *Anal Chem* 2003; **75**: 2159–2165.
47. Ong SE, Foster LJ, Mann M. *Methods* 2003; **29**: 124–130.
48. Everley PA, Krijgsveld J, Zetter BR, Gygi SP. *Mol Cell Proteomics* 2004; **3**: 729–735.
49. Chen X, Smith LM, Bradbury EM. *Anal Chem* 2000; **72**: 1134–1143.
50. Hunter TC, Yang L, Zhu H, Majidi V, Bradbury EM, Chen X. *Anal Chem* 2001; **73**: 4891–4902.
51. Pan S, Gu S, Bradbury EM, Chen X. *Anal Chem* 2003; **75**: 1316–1324.
52. Ong SE, Kratchmarova I, Mann M. *J Proteome Res* 2003; **2**: 173–181.
53. Ong SE, Mittler G, Mann M. *Nat Methods* 2004; **1**: 119–126.

DOI: 10.1002.jlcr

54. Gruhler A, Schulze WX, Matthiesen R, Mann M, Jensen ON. *Mol Cell Proteomics* 2005; **4**: 1697–1709.
55. Liang X, Hajivandi M, Veach D, Wisniewski D, Clarkson B, Resh MD, Pope RM. *Proteomics* 2006; **6**: 4554–4564.
56. Zhu H, Pan S, Gu S, Bradbury EM, Chen X. *Rapid Commun Mass Spectrom* 2002; **16**: 2115–2123.
57. Engen JR, Bradbury EM, Chen X. *Anal Chem* 2002; **74**: 1680–1686.
58. Yabuki T, Kigawa T, Dohmae N, Takio K, Terada T, Ito Y, Laue ED, Cooper JA, Kainosho M, Yokoyama S. *J Biomol NMR* 1998; **11**: 295–306.
59. Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen ON. *Mol Cell Proteomics* 2005; **4**: 310–327.
60. Vinther J, Hedegaard MM, Gardner PP, Andersen JS, Arctander P. *Nucleic Acids Res* 2006; **34**: e107.
61. Ross P, Dey S, Pillai S, Adams D, Daniels S, Williamson B, Guertin S, Minkoff M, Chen X, Purkayastha B, Pappin D. *Proceedings of the 54th ASMS Conference on Mass Spectrometry Allied Topics,* 28 May–1 June 2006, Seattle, WA, USA, 2006.
62. Washburn MP, Ulaszek RR, Yates 3rd JR. *Anal Chem* 2003; **75**: 5054–5061.
63. Wu WW, Wang G, Baek SJ, Shen RF. *J Proteome Res* 2006; **5**: 651–658.
64. Sachon E, Mohammed S, Bache N, Jensen ON. *Rapid Commun Mass Spectrom* 2006; **20**: 1127–1134. DOI: 10.1002/rcm.2427 [doi]
65. Zhang Y, Wolf-Yadlin A, Ross PL, Pappin DJ, Rush J, Lauffenburger DA, White FM. *Mol Cell Proteomics* 2005; **4**: 1240–1250.
66. Choe LH, Aggarwal K, Franck Z, Lee KH. *Electrophoresis* 2005; **26**: 2437–2449.
67. DeSouza L, Diehl G, Rodrigues MJ, Guo J, Romaschin AD, Colgan TJ, Siu KW. *J Proteome Res* 2005; **4**: 377–386.
68. Seshi B. *Proteomics* 2006; **6**: 5169–5182.
69. Petti F, Thelemann A, Kahler J, McCormack S, Castaldo L, Hunt T, Nuwaysir L, Zeiske L, Haack H, Sullivan L, Garton A, Haley JD. *Mol Cancer Ther* 2005; **4**: 1186–1197.
70. Hsu JL, Huang SY, Chow NH, Chen SH. *Anal Chem* 2003; **75**: 6843–6852.
71. Ji C, Guo N, Li L. *J Proteome Res* 2005; **4**: 2099–2108.
72. Ji C, Li L, Gebre M, Pasdar M, Li L. *J Proteome Res* 2005; **4**: 1419–1426.
73. Huang SY, Tsai ML, Wu CJ, Hsu JL, Ho SH, Chen SH. *Proteomics* 2006; **6**: 1722–1734.
74. Hsu JL, Huang SY, Chen SH. *Electrophoresis* 2006; **27**: 3652–3660.
75. Julka S, Regnier FE. *Brief Funct Genomic Proteomic* 2005; **4**: 158–177.
76. Yan W, Chen SS. *Brief Funct Genomic Proteomic* 2005; **4**: 27–38.
77. MacCoss MJ, Matthews DE. *Anal Chem* 2005; **77**: 294A–302A.
78. Ong SE, Mann M. *Nat Chem Biol* 2005; **1**: 252–262.
79. Leitner A, Lindner W. *Proteomics* 2006; **6**: 5418–5434.
80. Fenselau C. *J Chromatogr B* 2007, in press (doi:10.1016/j.jchromb.2006.10.071).
81. Baillie TA. *Pharmacol Rev* 1981; **33**: 81–132.
82. Browne TR. *J Clin Pharmacol* 1986; **26**: 485–489.
83. Browne TR. *J Clin Pharmacol* 1998; **38**: 213–220.
84. Pons G, Rey E. *Pediatrics* 1999; **104**: 633–639.
85. Abramson FP. *Semin Perinatol* 2001; **25**: 133–138.
86. Turner SM. *J Pharmacol Toxicol Methods* 2006; **53**: 75–85.
87. Hellerstein MK. *Metab Eng* 2004; **6**: 85–100.
88. Papac DI, Shahrokh Z. *Pharm Res* 2001; **18**: 131–145.
89. Knapp DR, Gaffney TE, McMahon RE, Kiplinger G. *J Pharmacol Exp Ther* 1972; **180**: 784–790.
90. Brazier JL, Ribon B, Desage M, Salle B. *Biomed Mass Spectrom* 1980; **7**: 89–192.
91. Weidolf L, Covey TR. *Rapid Commun Mass Spectrom* 1992; **6**: 192–196.
92. Taylor EW, Jia W, Bush M, Dollinger GD. *Anal Chem* 2002; **74**: 3232–3238.
93. Parks EJ, Hellerstein MK. *J Lipid Res* 2006; **47**: 1651–1660.
94. Nakagawa A, Kitagawa A, Asami M, Nakamura K, Schoeller DA, Slater R, Minagawa M, Kaplan IR. *Biomed Mass Spectrom* 1985; **12**: 502–506.
95. Browne TR, Szabo GK, Ajami A, Browne DG. *J Clin Pharmacol* 1998; **38**: 309–314.
96. Abramson FP. *Mass Spectrom Rev* 1994; **13**: 341–356.
97. Abramson FP, Teffera Y, Kusmierz J, Steenwyk RC, Pearson PG. *Drug Metab Dispos* 1996; **24**: 697–701.
98. Goldthwaite Jr CA, Hsieh FY, Womble SW, Nobes BJ, Blair IA, Klunk LJ, Mayol RF. *Anal Chem* 1996; **68**: 2996–3001.
99. Chen P, Teffera Y, Black GE, Abramson FP. *J Am Soc Mass Spectrom* 1999; **10**: 153–158.
100. Osborn BL, Abramson FP. *Biopharm Drug Dispos* 1998; **19**: 439–444.
101. Eichhorn P, Ferguson PL, Perez S, Aga DS. *Anal Chem* 2005; **77**: 4176–4184.
102. Lam W, Ramanathan R. *J Am Soc Mass Spectrom* 2002; **13**: 345–353.

103. Liu DQ, Hop CE. *J Pharm Biomed Anal* 2005; **37**: 1–18.
104. Olsen MA, Cummings PG, Kennedy-Gabb S, Wagner BM, Nicol GR, Munson B. *Anal Chem* 2000; **72**: 5070–5078.
105. Pfeifer T, Tuerk J, Fuchs R. *J Am Soc Mass Spectrom* 2005; **16**: 1687–1694.
106. Busch R, Cesar D, Higuera-Alhino D, Gee T, Hellerstein MK, McCune JM. *J Immunol Methods* 2004; **286**: 97–109.
107. Cavaletti G, Cavalletti E, Montaguti P, Oggioni N, De Negri O, Tredici G. *Neurotoxicology* 1997; **18**: 137–145.
108. Dufner D, Previs SF. *Curr Opin Clin Nutr Metab Care* 2003; **6**: 511–517.
109. Yan Z, Maher N, Torres R, Caldwell GW, Huebert N. *Rapid Commun Mass Spectrom* 2005; **19**: 3322–3330.
110. Mutlib A, Lam W, Atherton J, Chen H, Galatsis P, Stolle W. *Rapid Commun Mass Spectrom* 2005; **19**: 3482–3492.
111. Yan Z, Caldwell GW. *Anal Chem* 2004; **76**: 6835–6847.
112. Garland WA, Barbalas MP. *J Clin Pharmacol* 1986; **26**: 412–418.
113. Yates 3rd JR. *J Mass Spectrom* 1998; **33**: 1–19.
114. Grey L, Nguyen B, Yang P. *J Chromatogr A* 2002; **958**: 25–33.
115. Kuklenyik Z, Ashley DL, Calafat AM. *Anal Chem* 2002; **74**: 2058–2063.
116. Magni F, Pereira S, Leoni M, Grisenti G, Galli Kienle M. *J Mass Spectrom* 2001; **36**: 670–676.
117. Sennbro CJ, Olin M, Edman K, Hansson G, Gunnarsson PO, Svensson LD. *Rapid Commun Mass Spectrom* 2006; **20**: 3313–3318.
118. Oe T, Ackermann BL, Inoue K, Berna MJ, Garner CO, Gelfanova V, Dean RA, Siemers ER, Holtzman DM, Farlow MR, Blair IA. *Rapid Commun Mass Spectrom* 2006; **20**: 3723–3735.
119. Sojo LE, Lum G, Chee P. *Analyst* 2003; **128**: 51–54.
120. Dettmer K, Aronov PA, Hammock BD. *Mass Spectrom Rev* 2006; **26**: 51–78.
121. Ando S, Tanaka Y. *Mass Spectrom Rev* 2005; **24**: 865–886.
122. Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR, Norton S, Kumar P, Anderle M, Becker CH. *Anal Chem* 2003; **75**: 4818–4826.
123. Liu H, Sadygov RG, Yates 3rd JR. *Anal Chem* 2004; **76**: 4193–4201.
124. Radulovic D, Jelveh S, Ryu S, Hamilton TG, Foss E, Mao Y, Emili A. *Mol Cell Proteomics* 2004; **3**: 984–997.
125. Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, McKenna T, Nold MJ, Richardson K, Young P, Geromanos S. *Anal Chem* 2005; **77**: 2187–2200.
126. Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ. *Mol Cell Proteomics* 2006; **5**: 144–156.

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