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Addressing the current bottlenecks of metabolomics: Isotopic Ratio Outlier Analysis™, an isotopic-labeling technique for accurate biochemical profiling

Felice A de Jong[*] and **Chris Beecher**
NextGen Metabolomics, Inc., Ann Arbor, MI 48105, USA

Abstract

Metabolomics or biochemical profiling is a fast emerging science; however, there are still many associated bottlenecks to overcome before measurements will be considered robust. Advances in MS resolution and sensitivity, ultra pressure LC MS, ESI, and isotopic approaches such as flux analysis and stable-isotope dilution, have made it easier to quantitate biochemicals. The digitization of mass spectrometers has simplified informatic aspects. However, issues of analytical variability, ion suppression and metabolite identification still plague metabolomics investigators. These hurdles need to be overcome for accurate metabolite quantitation not only for *in vitro* systems, but for complex matrices such as biofluids and tissues, before it is possible to routinely identify biomarkers that are associated with the early prediction and diagnosis of diseases. In this report, we describe a novel isotopic-labeling method that uses the creation of distinct biochemical signatures to eliminate current bottlenecks and enable accurate metabolic profiling.

Metabolite profiling & stable isotopes

Standardization of current practices in MS-based **metabolic profiling** is required to ensure laboratory-to-laboratory comparative analysis for the development of precise discriminatory diagnostic tests and therapeutic treatments. In order to accurately compare data and ratios of individual metabolite concentrations, several analytical challenges must be overcome. The ability to measure inherent biological variation is directly compromised by the introduction of variability through sample handling, extraction, ionization efficiency (including suppression), or variation of analytical parameters (including instrumentation conditions). Standardization techniques to remove introduced variability in the determination of biological information have historically been addressed either through the use of multiple internal standard compounds to normalized data [1] or through stable-isotope-assisted methods [2,3].

When employing multiple internal standards as a means to standardize, the choice of which standard to apply to a particular metabolite is difficult. The retention time and mass-to-charge ratio of a metabolite compound is dependent on its chemical properties, and the use

[*]Author for correspondence: Tel.: +1 734 265 0884, Fax: +1 978 779 2947, felice@nextgenmetabolomics.com.

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of a single standard or standard normalization factor for multiple chemical species may not be practical or appropriate as the best standard is almost always an isotopomer of the compound itself. This can be prohibitively expensive when the number of compounds to be measured is significant. For this reason there are many techniques for the creation of assemblages of isotopic standards used in NMR and LC–MS data analysis [1,4–8].

Stable-isotope assisted methods employ either an *in vivo* or *in vitro* (chemical) incorporation of an isotopically enriched element (e.g., ^{13}C or ^{15}N), an amino acid or salt into a biological sample. The *in vivo* methods are the oldest and most frequent usage of stable isotopes in studies probing metabolism, to understand flux [9,10] and also for biomarker discovery [11,12]. In these studies, a stable isotope is incorporated into a specific molecule at a specific location in order to achieve a precision in understanding the metabolic fate of that molecule or its substituent. Investigators employ wholly labeled molecules where >99% of an atom is replaced with an isotopic equivalent, and universally labeled isotopes where the isotope is universally distributed within the target molecule at less than saturation levels. The isotopically labeled molecule, or 'precursor', is fed to an *in vivo* organism, *in vitro* cell system or *in vitro* cell-free system for either a brief or extended period of time, after which the fate of the isotope is determined either by the use of NMR, MS, chemical degradation or other detection technique. There are many studies in which more than one isotope is incorporated into a target molecule, and all of the isotopic fragments are examined for their differential fates. In all cases, these methods are targeted analyses; that is, they seek the incorporation of a specific labeled atom into other specific molecules [2]. *In vivo* tracer labeling or metabolic-flux analysis typically measures metabolic fluxes or the rate of turnover of molecules through a metabolic pathway [13,14]. Other commonly used *in vivo* **stable-isotopic-labeling** approaches include stable-isotope labeling with amino acids [15], and stable-isotope labeling by amino acids in cell culture (SILAC) [16]. In both of these, the experimental and control samples are differentially labeled so that the pooled sample may be analyzed as a single sample in order to get comparative information on the experimental relative to the control. These approaches are primarily used in proteomic studies. The benefits of these techniques are that they require fewer analyses because both the control and experimental samples are analyzed at once, and data quality is enhanced as there can be no sample-to-sample variance between the commonly prepped and analyzed experimental and control. One of the biggest problems in the interpretation of mass spectral data is the variability of ionization efficiency, commonly known as 'ion suppression'. This is one of the major sources of sample-to-sample variance. Where an isotopically labeled standard is chemically identical to the analyte, both the standard and the analyte are measured in an identical environment and, therefore, share identical ionization efficiencies making the measurements more accurate. This reduction of the impact of suppression is common to many of the isotopic techniques discussed below.

Chemical or *post vivo* labeling techniques include isotope-coded affinity tag [17], isotope-coded protein labeling [18], or the utilization of isobaric mass tags for labeling, such as isobaric tags for relative and absolute quantitation [19] and tandem mass tag [20]. These techniques also run as a single, pooled sample composed of multiple differentially tagged samples, and also demonstrate improved data quality due to the reduction of sample-to-sample variance.

Stable-isotopic internal standards

Another use of stable-isotopically labeled compounds is as internal standards for their nonlabeled counterparts, referred to as stable-isotope dilution methodology [21]. In such an experiment, an isotopically enriched molecule is added to a sample or extract at a known concentration prior to an analysis, and the final measurement determines the exact

concentration of the nonlabeled material by comparison. In this type of study, it is not uncommon for a researcher to add more than one isotopically-distinct standard if more than one molecule is to be quantified. In this situation, the same standard is introduced into all samples, but there is no information carried by the standard other than for purposes of relative quantitation; that is, the standard has no relation to the experiment at hand.

More recently, there have been a number of variants of the above whereby an organism is grown on an isotopically defined feedstock, such that the entire organism is heavily, if not entirely, composed of molecules consisting of only one or two isotopes [22,23]. Historically, such standards are carefully constructed to differ from any other analyte by a specific mass difference. For example, by labeling one cell population with a cell-culture component or components containing isotopically light atoms, for example ^{12}C, and another cell population with isotopically heavy atoms, for example ^{13}C. Once a stressor is applied to the experimental group – for example, drug or chemical treatment – equal amounts of both populations are combined and analyzed by LC–MS. Again, the composite sample is mass-spectroscopically analyzed for analyte peaks, with a reduction of sample-to-sample variance. The ratio of the first to second isotope (isotopic ratio) for each compound is determined. While the practice of isotopic-labeling standardization to introduce a specific stable-isotope-labeled compound for each metabolite has provided a means to effectively reduce variability introduced during analysis and sample processing, the fact that the peaks carry no inherent means of identification, artifactual confounding peaks and metabolite misidentification remain unsolved sources of error.

Metabolomics & stable-isotope labeling

The diverse chemical nature of the metabolome makes the comprehensive chemical tagging methods unfeasible. Until recently, other than metabolic-flux analysis and simple targeted analyses, very few additional methods that employ stable isotopes were used for the full quantitation of biochemical metabolites. In 2004, Mashego *et al.* reported the use of stable-isotope dilution for the metabolome characterization of *Saccharomyces cerevisiae*, termed 'Mass Isotopomer Ratio Analysis of U-^{13}C-Labeled Extracts' (MIRACLE), in which metabolites were quantitated relative to their U-^{13}C-labeled equivalents [24]. Using the same isotope ratio-based quantitation approach, both Birkemeyer *et al.* [23] and Bennett *et al.* [25,26] describe the quantitation of microbial metabolites using uniformly labeled U-^{13}C carbon sources. Others have also utilized this approach for specific classes of metabolites, including nitrogen-containing metabolites [27] and microbial folates [28]. Other unique metabolite labeling approaches have been described by Nagai *et al.*, namely, an isotopic tracing method for the metabolic assessment of lipogenesis, lipolysis and glucose uptake in adipocytes using LC–MS through the absorption of ^{13}C-labeled compounds [29], while Huang and Regnier differentially derivatized control and experimental samples with isotopically light and heavy (D6) forms of the silylation reagent *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide, which were then mixed and analyzed using 2D GC–MS (GC × GC–MS) [30]. These methods are useful for the simultaneous analysis of multiple compounds, as metabolites are quantitated relative to their U-^{13}C-labeled equivalents. They are targeted methods, either to specific classes of compounds or individual metabolites and their pathways; however, if a new pathway is triggered and metabolites synthesized in the unlabeled samples, then these compounds will not be identified by the mass ratio-based analysis of the type described here, since the U-^{13}C equivalents will be absent.

Isotopic Ratio Outlier Analysis™ as a protocol for metabolic profiling: quantitation & identification

Metabolic profiling is rapidly gaining adoption as a methodology to measure metabolic phenotypes associated with disease and drug therapies, and MS is excellently suited for the analysis of labeling patterns of individual atoms (i.e., isotopomers) in a wide range of metabolites. The **Isotopic Ratio Outlier Analysis™** (IROA) method is not a targeted analysis [101], it utilizes full metabolic labeling instead, such as SILAC in cell culture, but uses specifically labeled materials with random, universal enrichments centered on either 5 or 95% ^{13}C. The reduction in purity of the label from either natural abundance, or the high purity ^{13}C (generally >98%) creates an isotopic signature of such molecules that is dramatic and imparts many analytical advantages compared with standard isotopic labeling, as outlined in Table 1. With the creation of distinct chemical signatures in every biological molecule, it is possible to develop software algorithms to sort these biological signals. Using comprehensive (>98%) labeling, the base peak can usually be detected, but the M+1 peak that provides important diagnostic information is generally less useful for data interpretation because of its small size (e.g., the M+1 peak is 6% of the height of the base peak for a six-carbon molecule). If the percentage of ^{13}C is increased to 5%, then the M+1 for a six-carbon molecule is significantly larger, namely 32%. With the IROA ^{13}C-labeling approach, the number of carbons in a biological molecule can be determined by the distance between the two base peaks, ^{12}C and ^{13}C, and the relative height of the M+1 and M-1 provide confirmation of this fact, providing triply redundant information. Isotopically labeled compounds for the IROA are made for NextGen Metabolomics, Inc. (MI, USA) under patent protection and through special contract by Cambridge Isotope Laboratories (MA, USA).

When considering the IROA experiment, the use of an isotope that exhibits minimal biological isotope effect is of importance. For instance, the use of deuterium isotopes with a mass twice that of hydrogen is known to cause a reduction in the kinetics of some enzyme mechanisms, but not in others [31]. On the other hand, the carbon ^{13}C isotope effect is minimal, being an increase of only 8%, not 100% as in the case of deuterium. In the majority of biological systems, except in Crassulacean acid metabolism plants [32], no significant isotopic effects have been shown for ^{13}C in most commonly used model systems. In an IROA experiment, described below, in order to accommodate for any minor ^{13}C isotope effects, the ^{13}C-labeled samples are always used as the control and directly compared with their ^{12}C-labeled equivalents to identify any ^{13}C effects. Furthermore, during the analysis of the IROA samples, it is important to select injection volumes to minimize detector saturation while allowing optimal compound identification.

In the IROA experiment (Figure 1), as in other *in vivo* experiments, an isotopically-defined media is biotransformed by experimental systems to induce identifiable isotopic-labeling patterns (signatures) in all of their biological compounds. In brief, a homogenous cell population is divided into two populations: a ‘control’ population and an ‘experimental’ population, which will be subsequently treated with vehicle or a stressor, respectively. All of the biological compounds in the control sample are labeled with ‘^{13}C media’ signatures and all of the biological compounds in the experimental sample are labeled with ‘^{12}C media’ signatures. The key element is that both the ^{12}C and ^{13}C media are chemically identical but isotopically different. Unlike the previously discussed techniques, all of the carbon sources in the ^{12}C media are randomly and universally composed of approximately 95% ^{12}C with 5% ^{13}C. Conversely, all of the carbon sources in the ^{13}C media are composed of 95% ^{13}C with 5% ^{12}C, again, and importantly, the isotopes are universally and randomly incorporated into all carbon positions.

If both the control and the experimental cell samples are grown in the IROA isotopically-defined media for a sufficient time to replace their original natural-abundance carbon, then all of their contents will demonstrate distinctive isotopic patterns (orange box, Figure 1). Once labeled, the experimental sample is treated with a stressing regimen and the control sample is treated with only the vehicle for the same period of time. The stressing agent may be chemical, genetic, environmental, or any element or combination of elements that induce physiological alteration. At the conclusion of the experiment, the experimental sample is mixed with a control sample and the pooled sample prepped and analyzed in a single analysis. This has the effect of, as noted above, reducing sample-to-sample variance and increasing data quality.

In natural abundance, biochemicals or metabolites, the M+1 secondary peak is quite small. In the IROA experiment, all biologically derived molecules may be easily distinguished from artifacts (which are present at only natural abundance), and each peak carries a ready identifier of its origin, a diagnostically enhanced M+1 for the ^{12}C-derived sample and an identical diagnostically enhanced M-1 for the ^{13}C-derived sample. The compounds associated with the IROA ^{13}C control and IROA ^{12}C experimental samples are both differentiatable (see the IROA peaks, Figure 2) and carry a tremendous amount of additional information; namely, the height of the M-1 relative to the ^{13}C-base peak is a clear step function indicating the number of carbons in the molecule; unlike the ^{12}C-base peaks, only the noncarbon components contribute to the M+1 and M+2 of the ^{13}C-base peak, and may be examined without interference from carbon; and the exact mass of the ^{13}C-base peak may be used to calculate the probable formula of the molecule. When armed with the number of carbons in the molecule, the ability to closely examine the remaining elements is generally quite discriminatory, and not only affords correct molecular formulae with a great deal of assurance, but allows the prediction of the characteristics of the ^{12}C-based isotopomer of the same compound. Once labeled with the ^{12}C and ^{13}C media, all of the biological components in the control sample and experiment sample will carry unique signatures, and this means that the control sample can be directly embedded into and prepared simultaneously with an experimental sample and analyzed as a composite (green box, Figure 1). As noted previously, this not only reduces the number of samples to be analyzed by half, but also removes the sample-to-sample variance (or noise) that will normally be present in most metabolomics experiments, with not only no loss of information, but actually improved information content. IROA also fully removes the problem of ion suppression because a compound's ability to ionize is a function of its structure and is not significantly altered by its isotopic distribution, similar to the technique noted above.

Another advantage of the IROA patterning is that the M+1 and M-1 peak height, relative to the base peak of each molecule, generally indicates the number of carbons in the molecule. Since the height of the peaks of an isotopically defined compound may be effectively calculated (this is technically a polynomial expansion in which the dominance of carbon makes the remaining terms less important) by the binomial expansion of the expression: $(^{12}C\% + ^{13}C\%)^{N}$ where N equals the number of carbons and $^{12}C\%$ and $^{13}C\%$ equals the relative isotopic abundances [33]. This is not sufficiently accurate when all atoms are present at their natural abundance, but at 5% the M+1 is diagnostic. For the ^{12}C (5% ^{13}C) peaks, if there are: seven carbons the M+1 will be approximately 36.8%; six carbons the M +1 will be approximately 31.6%; five carbons the M+1 will be approximately 26.3%; four carbons the M+1 will be approximately 21.1%; three carbons the M+1 will be approximately 15.8% and so on. This calculation indicates exactly where the ^{13}C-paired mate will be located. Together with accurate mass, the identification of metabolite entities in the sample is made possible.

The distinctive patterns are important in the interpretation of the resulting composite spectra and because it is possible to discriminate ^{12}C-derived molecules, ^{13}C-derived molecules, artifacts and derivatives of exogenously applied compounds, and the origin of every peak in the composite (see the IROA peaks, Figure 2).

The IROA peaks are all mathematically calculable and each set (^{12}C and ^{13}C) of carbon isotopomers will reliably and accurately account for the other set providing a redundant QC check point. As such, it is possible to interpret the analytical results of the composite sample to an even greater extent through the creation of IROA software algorithms that achieve data reduction of complex raw data, to concise, high-value information, as follows:

- Characterize all peaks according to source (artifact, experimental [^{12}C], control [^{13}C], or standard);
- Remove all artifacts;
- Align and pair all remaining peaks across all scans;
- Normalize and identify all pairs;
- Determine the relative $^{12}C/^{13}C$ ratios of analytes in each sample;
- Determine the statistical variance of the sample ratios.

Any experimental analyte compound that has a ratio that is a significant deviation (two or more standard deviations) from the average ratio will indicate a point where the biochemistry was altered. For instance, if the average ratio for all of the analytes is 1 (1:1 $^{12}C/^{13}C$ ratio), but some analytes have ratios of 10 (10:1) or 0.1 (1:10) then the analytes that are outliers to the general population are those most strongly affected by the stressor.

A software package named ClusterFinder™ has been written in Java (JRE 1.7). To achieve step 1, ClusterFinder does a scan-by-scan analysis of the complete dataset and identifies all IROA peaks based on their extended isotopic envelopes. The removal of all non-IROA peaks follows logically upon the conclusion of step 1, and results in a significantly simplified dataset that may then be aligned based on mass across adjacent scans. The mirrored symmetry of the ^{12}C and ^{13}C halves of each IROA cluster allow the isotopic peaks to be correctly associated with their appropriate ^{12}C- or ^{13}C-base peak. As the isotopic dilution distributes molecules over a number of masses, the sum of the base peaks and their associated isotopic peaks must be used to calculate their respective areas accurately. Once the summed areas are available their ratio is simply calculated. On the whole, because the isotopic patterns carry so much information, the tasks the software performs are well defined but not overly difficult. The software has two modes of use; where both the ^{12}C and ^{13}C media are utilized, the software can find all IROA peaks regardless of their source. This represents a fully unbiased analysis as compounds of biological origin present or absent in either samples (control or experimental) will be identified by the software and artifacts will be simultaneously identified and removed from the dataset. The software will also find all IROA-labeled compounds where the IROA peak is present in one sample but not the other. This allows for targeted analysis whereby the control may contain ^{13}C IROA peaks that may be used to identify their associated natural abundance peaks. This mode of analysis has been called the ‘phenotypic mode’ (see section titled ‘The use of IROA phenotyping application for the metabolic profiling of tissues or biofluids’ later in this article).

Normalization of IROA datasets

Various normalization techniques to account for matrix and analytical variances in transcriptomics, proteomics and metabolomics have been explored extensively. Methods that account for dilution or cell number have included total ion count (TIC), a standard

'housekeeping' compound or other physical characteristics of the sample (e.g., DNA, protein and osmality).

In metabolomics studies there have not yet been any molecules identified as good candidates for 'housekeeping' compounds. Creatinine has been proposed as such a 'housekeeping' compound in urine, but it has been discounted as it is, in itself, quite variable [34]. The physical measurements of the sample are often destructive and performed on like aliquots and not the samples to be analyzed, which lowers its precision. In a recent publication, Warrack *et al.* demonstrated the benefits of normalization with respect to the TIC of a spectrum, utilizing only the total intensity of components that are common to all samples after baseline correction – the 'MS total useful signal' (MSTUS) approach [35]. They noted the superiority of this approach in avoiding the influence of matrix- and solvent-related intensity measurements. This approach is similar to the common practice used in ^{1}H NMR-based metabonomics analyses wherein each spectrum is normalized to the total integrated proton signal, after removal of regions corresponding to xenobiotics, internal standards and artifacts [36].

The IROA technology is designed to identify and remove noise and artifacts, which has the advantage in that an artificial cut-off for noise removal or baseline correction is not required, so that any low abundance metabolites are not artificially removed and can be considered the data-set. As such, IROA metabolomics utilizing TIC normalization follows the MSTUS approach; namely, normalizing only the components that are common to all signals. Since artifacts and non-biological compounds may be identified as they carry no IROA signatures, their removal further strengthens the MSTUS normalization approach.

Application of the IROA approach in *S. cerevisiae*

To demonstrate the application of IROA in the biological system of *S. cerevisiae* S288C, the bio-synthetic pathways surrounding glutathione in an aerobic culture were measured over a 72-h time course (Figure 3). The fermentation process of *S. cerevisiae* was measured using isotopically defined media (whereby all carbon sources were labeled with either 5% ^{13}C/95% ^{12}C or 95% ^{13}C/5% ^{12}C) and analyzed using LC–MS. The dataset produced was analyzed by the IROA ClusterFinder software (not shown). Figure 3 shows the representative IROA peaks for the compounds glutamine, γ-l-glutamyl-l-cysteine and glutathione and the normalized concentrations of these biochemical metabolites measured in culture samples collected at 4, 8, 24, 48 and 72 h. The glutathione pathway metabolites, some of the most important biological metabolites controlling many aspects of physiological response, are shown to be increasing over the 72-h time course, which is most likely a result of glucose levels being depleted over the 72-h time period (not shown) [37].

The use of IROA phenotyping application for the metabolic profiling of tissues or biofluids

The clinical usefulness of metabolic profiling is just beginning to take shape [38,39]. In the basic IROA methodology described above (Figure 1), both the control sample and experimental samples are fully isotopically labeled so both may be easily found with absolute assurance of their identity and any artifacts may be removed. This leads to very clean, high-resolution datasets that clearly define the biological response of a biological system. Where it is not possible to isotopically label the biological sample, the IROA phenotyping application is applied and the sample is collected at natural abundance and mixed with a fully predefined 'standard' that has been isotopically labeled using IROA ^{13}C media (Figure 4). An ideal standard would be one that represented the entire metabolome of the fluid or tissue under study. While this could be generated in a number of ways, including

the use of IROA grown cells lines (the IROA standard) to achieve accurate quantification, this aspect of the application will not be considered here.

Using an IROA-labeled standard, all of the peaks may be easily identified according to the presence of their characteristic M-1 peak. The natural abundance peaks corresponding to each standard peak may be readily identified because, although they do not carry any isotopic labeling, their exact mass and position are established relative to the standard. If the pooled standard is well characterized and the compounds that are present in it have already been identified, then it can be used as a point of comparison for all of its contained compounds. Artifacts will have no match in the standard and, thus, need not be considered in a final dataset. Whereas, in a basic IROA dataset the ratio of the peak areas represents the relative deviation of the metabolic pool sizes brought about by the experimental condition, in a phenotyping experiment the overall pattern of deviations from the standard will define phenotype by difference from the standard. It is possible to consider the phenotyping experiment a complex targeted analysis relative to the unbiased analysis of the full IROA experiment.

Conclusion & future perspective

Isotopic techniques have proven valuable in improving the quality of data for many experimental systems. In general, where a standard can be embedded into an experimental sample, the measurements of the experimental analytes are greatly improved. In metabolomics, there have been many uses of isotopic standards for understanding flux and biomarker discovery. The use of high purity isotopic labels has been a common technique in many other omics sciences and has just recently been used in metabolomics [24–30,40]. The IROA technique has advanced this by using specific isotopic balances to create definable patterns in metabolites. This makes the mass-spectral signals complex but increases the information content that can be extracted from these signals. It is clear that metabolomics has just begun to explore the potential of using isotopic labels to further its effectiveness.

Key Terms

Metabolic profiling	Quantitative analysis of metabolites, generally performed by either NMR or MS, to measure a change of the metabolome in response to a stressor
Stable-isotopic labeling	A technique that incorporates a stable (nonradioactive) isotope into a molecule creating a higher molecular weight compound
Isotopic Ratio Outlier Analysis™	MS method of metabolic profiling that utilizes stable isotopes to create distinct molecular signatures in biological compounds

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Executive summary

Metabolite profiling & stable isotopes

- Standardization techniques through the use of multiple internal standard compounds and stable-isotope-assisted methods have been historically employed to overcome analytical challenges in metabolite profiling.
- These analytical challenges include ion suppression, sample-to-sample variability, noise and artifactual confounding peaks and metabolite identification.
- Several approaches go a long way in rectifying most of these issues, but they all employ targeted methods and fall short of solving all analytical problems.
- The use of an internal standard for each metabolite measured is optimal; however, for broad metabolic profiling when using multiple internal standards to normalize, the choice of which standard to apply to a particular metabolite can be problematic.
- *In vivo* stable-isotope-assisted methods include flux analysis, stable-isotope labeling with amino acids and stable-isotope labeling by amino acids in cell culture. Stable-isotope labeling with amino acids, stable-isotope labeling by amino acids in cell culture and various chemical labeling techniques improve data quality by removing ion suppression and sample-to-sample variability, and also provide the benefit of requiring fewer analyses (as the control and experimental samples are analyzed simultaneously).

Stable-isotopic internal standards

- Rather than the use of natural abundance isotopic standards, stable isotopes can also be applied as internal standards through the use of stable-isotope dilution methodology.
- While this method also utilizes a composite sample for analysis resulting in the removal of ion suppression and sample-to-sample variability, the problem of confounding artifactual peaks and metabolite misidentification remains.

Metabolomics & stable-isotope labeling

- Stable-isotope dilution labeling was initially applied to proteomics and was later introduced to metabolomics through the use of ‘Mass Isotopomer Ratio Analysis of U-^{13}C-Labeled Extracts’, an isotope ratio-based quantitation approach.
- While many researchers have applied this protocol to quantitate specific classes of metabolites, the protocol does not eliminate issues of metabolite identification and confounding artifactual peaks.

Isotopic Ratio Outlier Analysis™ as a protocol for metabolic profiling, quantitation & identification

- Similar to other stable isotope approaches, the same cell pool can be used to create both control and experimental samples using Isotopic Ratio Outlier Analysis™ (IROA) technology, so that variability is removed, fewer samples are required and ion suppression is reduced.
- Artifacts and noise are removed using the IROA protocol. Since all material from either biological system (experimental or control) has a unique isotopic signature, all unsigned molecules may be discarded or considered separately.

This means all artifactual signals can be removed from the dataset (usually the largest portion of the dataset), which is not possible in current metabolomics datasets where the high number of 'observations' increases the false discovery rate. IROA ClusterFinder™ software algorithms sort through the dataset, removing irrelevant noise and artifacts, allowing for a very dramatic reduction in data size.

- When employing IROA, because samples are isotopically labeled, the number of carbons for each biochemical compound can be calculated from its mass spectra and the compound readily identified. The IROA technology uses a central point of reference (biochemical) embedded in every sample, thereby enabling measurements of samples with quantitative exactness.

Normalization of IROA datasets

- Since the IROA technology is designed to identify artifacts and nonbiological peaks, normalization can be achieved using 'MS total useful signal' normalization using only the components that are common to all signals, following the removal of xenobiotics, noise and artifacts.

Application of the IROA approach in *Saccharomyces cerevisiae*

- To illustrate the IROA methodology, the metabolite profiles of the biosynthetic compounds surrounding glutathione during the fermentation process of *Saccharomyces cerevisiae* were measured using isotopically defined media (whereby all carbon sources were labeled with either 5%^{13}C/95%^{12}C or 95%^{13}C/5%^{12}C) and LC–MS analysis.

The use of IROA phenotyping application for the metabolic profiling of tissues or biofluids

- Where it is not possible to isotopically label experimental samples, such as biopsies or biological fluids, a 'phenotypic' approach to the IROA technique can be applied. Here, a complex internal standard that represents the metabolome of the fluid or tissue under study is isotopically labeled using IROA ^{13}C media and then spiked into the experimental sample prior to analysis.
- While this is a targeted approach, all labeled metabolites will have a characteristic M-1 peak and the experimental natural abundance equivalents can be identified relative to the labeled standards and artifactual peaks can be removed as they will have no match.

Conclusion & future perspective

- The IROA technology has analytical advantages over existing isotopic metabolite profiling methods. However, the application of these methods for metabolomics is still in its infancy.

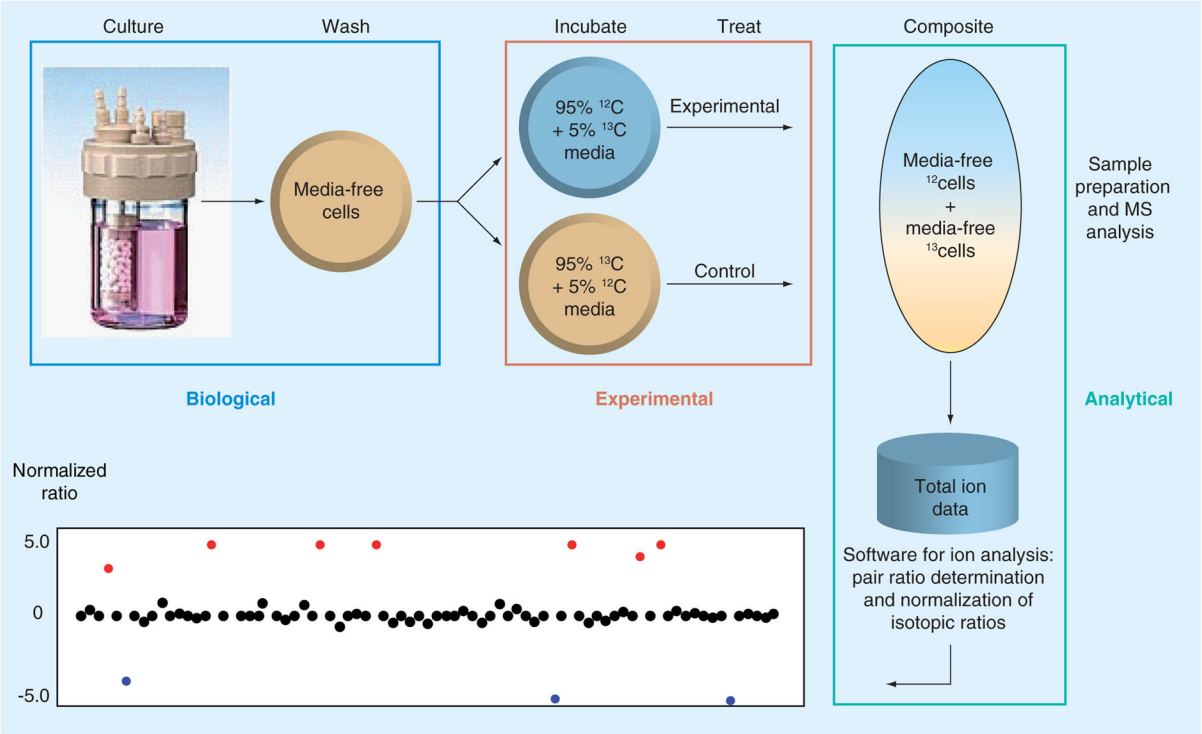

Figure 1. The Isotopic Ratio Outlier Analysis™ method for determining the biological response to drugs, toxins or other stressors

Isotopic Ratio Outlier Analysis isotope ratios of $^{12}C/^{13}C$ 95/5% and 5/95% allow for control and experimental samples to be run simultaneously, eliminating sample-to-sample variability.

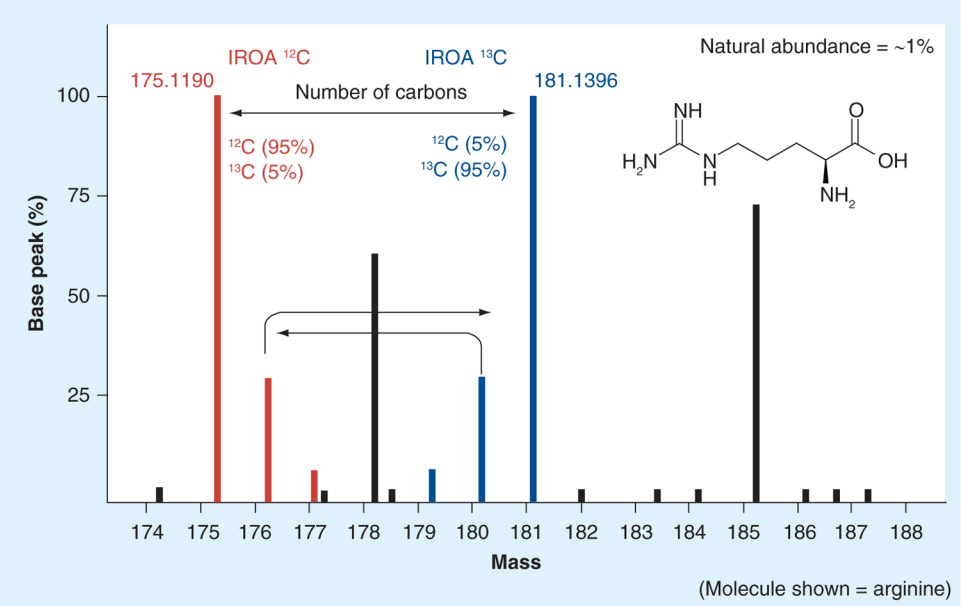

Figure 2. The Isotopic Ratio Outlier Analysis™ peaks

In the case of arginine, the ^{12}C M+ located at 175.1190 and its ^{13}C mate at 181.1396 clearly indicate a six-carbon molecule. The corresponding M+1 and M-1 peaks are a mass difference of 1.00335 amu (the mass difference between a ^{13}C and ^{12}C isotope). Natural abundance peaks (in black) from exogenous sources do not have a ^{13}C counterpart and are not considered in the analysis. IROA ^{12}C peaks (red) and IROA ^{13}C peaks (blue) are from biologically derived compounds.

IROA: Isotopic Ratio Outlier Analysis™.

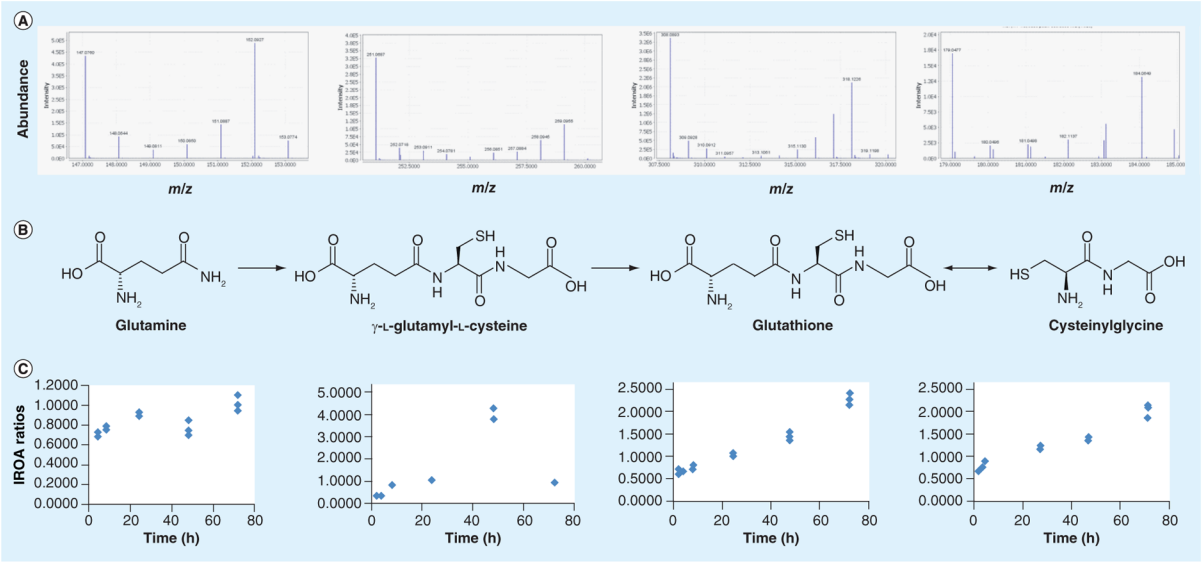

Figure 3. The biosynthetic pathways surrounding glutathione in *Saccharomyces cerevisiae* during a 72-h time course

(A) IROA peaks, **(B)** Biological compounds and **(C)** Isotopic Ratio Outlier Analysis ratios. IROA: Isotopic Ratio Outlier Analysis™.

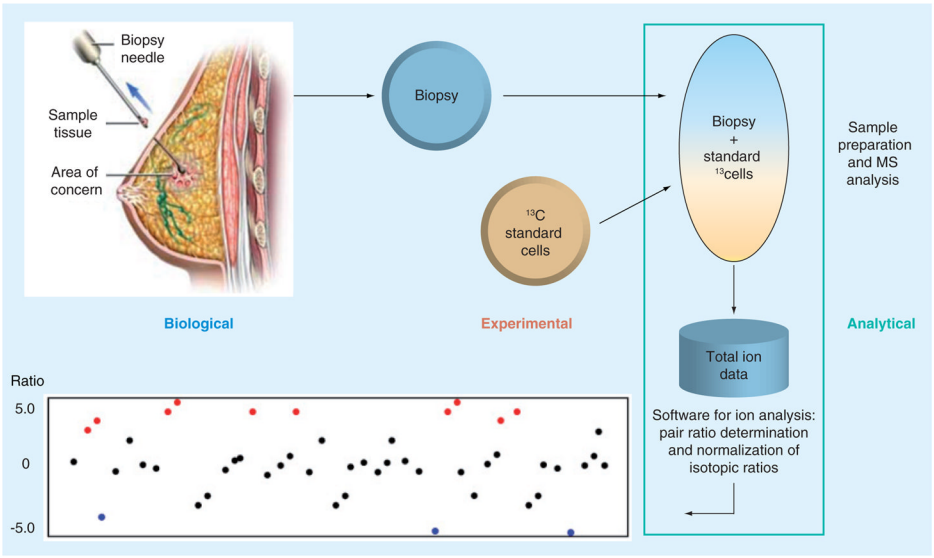

Figure 4. The Isotopic Ratio Outlier Analysis™ phenotyping application

The material to be phenotyped is mixed with ^{13}C (Isotopic Ratio Outlier Analysis) cells and/or standard compounds that allow one to find and pair all peaks. The deviation from the standard is diagnostic of the sample's biochemical phenotype.

Table 1

Features of standard isotopic labeling using U-^{13}C versus Isotopic Ratio Outlier Analysis™.

Feature	U-^{13}C (99 atom %)	IROA
Sample throughput	High to medium	High to medium
Removal of sample-to-sample variance	Yes – control and experimental samples are prepped and analyzed together, removing variability	Yes – control and experimental samples are prepped and analyzed together, removing variability
Removal of ion suppression	Yes – ionization is similar for isotopomers	Yes – ionization is similar for isotopomers
Reduction of the number of samples to be analyzed	Yes – control and experimental samples pooled and run as a single sample, reducing the number of samples to be analyzed by half	Yes – control and experimental samples pooled and run as a single sample, reducing the number of samples to be analyzed by half
Distinguishes artifacts and noise	No	Yes – allows classification of all peaks as compounds or artifacts
Metabolite coverage	Targeted	Global
Metabolite Identification	No	Number of carbon atoms and residual mass defined

IROA: Isotopic Ratio Outlier Analysis™.