IROA TECHNOLOGIES
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Challenges in Metabolomics Analysis

AND A SOLUTION

WHITEPAPER

Metabolomic Analytical Measurements

Challenges in Metabolomics Analysis and a Solution

3 Challenges

Challenge 1: Reproducible measurements across time and instruments

Metabolomic techniques can identify and crudely quantify several hundred or even thousands of compounds in a biological sample. However, unless the samples are analyzed in a single batch relative to a known standard, reproducibility cannot be assured because accumulated variances (from instrument drift, chromatographic drift, sample preparation etc.) can alter results sufficiently. Further, it is not a trivial task to achieve reproducible measurements when directly comparing data generated on the same instrumentation several days apart, or even more problematic, different instruments based on the same or different methods[1].

Challenge 2: Reliable QC Standards and a reliable scoring method to validate metabolomic data

Quality control standards are essential to measure matrix effects and validate metabolomic data. Human plasma and serum are widely used matrices and there has been a concerted effort to develop QC standards that represent these matrices[2]. A pooled aliquot of every sample to be analyzed can be collected so as not to miss any compounds unique to the treatment as compared to control[3,4], however without sufficient material this is not practical for long-term projects[5]. A huge issue is discerning the real compounds from artefacts, or otherwise determining which of the multitude of peaks to use as standards. If large pooled samples can be obtained as reference standards, then safe handling precautions are necessary, and samples must be carefully monitored as storage conditions, and sample preparation will create artefacts and further potential sources of variability that will obscure metabolite identification[6]. Furthermore, plasma is unsuitable for cross-platform analysis, because the number of differences that will be encountered when using different sources, chromatographic systems, or even detectors will preclude any point of comparison[7].

Along with reliable QC standards, it is essential to have appropriate bioinformatic tools to efficiently process huge datasets and scoring algorithms that compare metabolomic information, such as orthogonal physiochemical characteristics, against biochemical databases to validate data.

Challenge 3: Accurate identification of compounds

The identification of any compound across different mass spectral techniques is unlikely to be successful without careful calibrations and authentic standards. Multiple biological compounds may be confused because they have the same exact mass. Even more problematic are unknown artefactual or fragmentary compounds that are structurally and chemically different from their biological isobaric equivalents, but may share the same mass, or even formulae. These artefacts typically outnumber the known metabolites in metabolomics studies. MS-based metabolomics methods use isotopically labeled internal standards to ensure accurate identification, as they behave physically and chemically (including ionization efficiency) identical to the analytes under measurement[8]. However, for untargeted or

complex targeted analyses, it becomes impractical (and unaffordable) to match internal standards to large numbers of unlabeled compounds and their fragments, or to achieve the clean baseline separations that would be needed to do so.

A Solution

To develop a reference material that solves these 3 challenges, encompasses primary metabolism, and is adaptable to a wide range of analytical platforms and applications, our approach was to construct a ***fully labeled Standard*** labeled at 5% and 95% U-^{13}C and mixed 1:1. The Standard contains a broad spectrum of metabolites including amino acids, peptides, vitamins, carbohydrates, and lipids. Over five hundred metabolites have been characterized in the Standard using ClusterFinder software. The final number is expected to reach between 700-800 as different analytical approaches enable the identity of more compounds.

a) The fully labeled yeast extract reference standard called Matrix

Matrix is a fully labeled yeast extract IROA[9] labeled at 5% and 95% U-^{13}C and mixed 1:1

i. *How are* 5% and 95% U- ^{13}C *compounds created?*

Compounds are randomly and universally labeled. The relative abundances of the isotopes of carbon are altered; i.e. enriched in one isotope and depleted in its other isotopic form. Compounds such as 95% and 5% U-^{13}C glucose, amino acids, etc. were produced especially for IROA Technologies LLC by Cambridge Isotope Technologies, Inc.

ii. *Why* 5% and 95% U- ^{13}C *and why mixed?*

Yeast extract was fully labeled at 5% and 95% U-^{13}C and mixed 1:1 to create a **triply redundant QC Standard** containing many 100's of metabolites, each with unique IROA signatures that are all mathematically calculable to assure reproducibility and accuracy.

iii. *What is meant by a triply redundant QC Standard?*

Figure 1 below is an example of a molecule (arginine) represented in the Standard that is labeled with both 5% and 95% U- ^{13}C and mixed 1:1. The mixture gives rise to a unique "U-shaped smile" pattern of peaks which contains both the 95% envelope (95% U-^{13}C peaks; M-1, M-2 etc.) and its mirror-image envelope (5% U-^{13}C peaks; M+1, M+2 etc.). Every molecule in the Standard presents itself as a collection of isotopomeric set of peaks with the mass distance between each peak being exactly one carbon neutron, or approximately 1.00335 AMU.

The height of the M+1 and M-1 peaks differ directly according to the number of carbons in a molecule; for arginine in the Figure, the heights of M+1 and M-1 are 32% the height of their respective monoisotopic peaks, representing a six-carbon molecule. The heights of the M+1 and M-1 peaks and the shape of the entire isotopic envelope is indicative of

the number of carbons in the molecule. The number of carbons in an IROA molecule can be also determined by the distance between the two monoisotopic peaks. Therefore, three factors provide confirmation of the number of carbons in a basic IROA peak: 1) The relative height of the M+1, 2) the relative height of M-1, and 3) the distance between the monoisotopic peaks; demonstrating that a Matrix IROA peak, such as shown in Figure 1 below, is a **triply redundant structure and the basis for extremely strong, reproducible quality control** measurements. Furthermore, the number of carbons together with the mass of the monoisotopic peak can be used to reliably determine the molecular formula of each molecule.

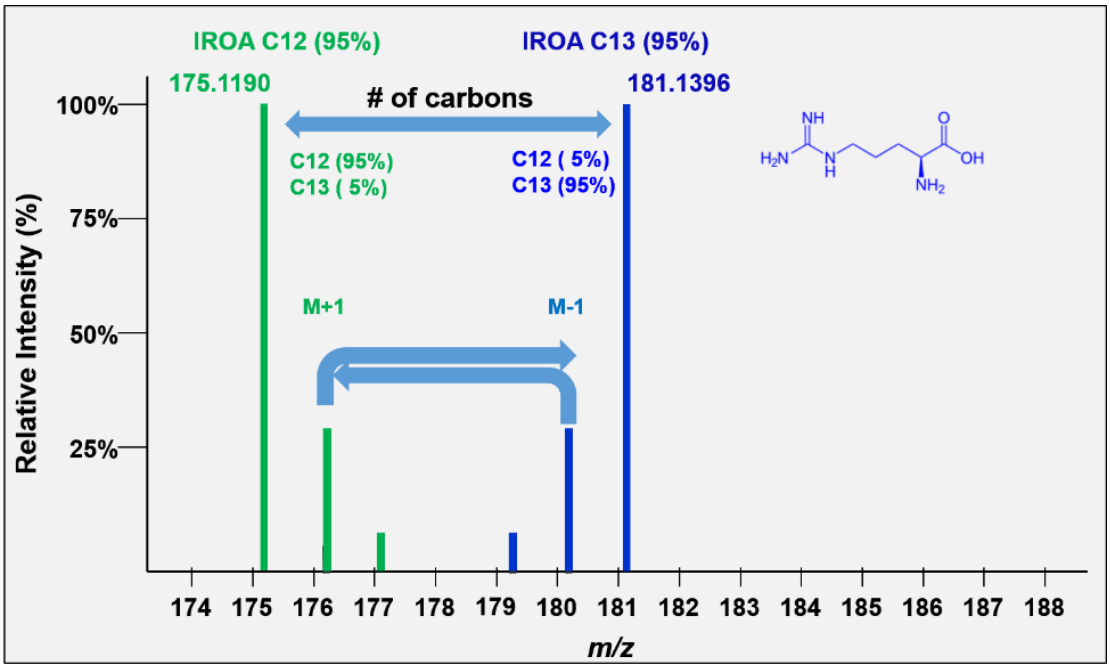


Figure 1: Representation of the IROA-Matrix molecule arginine; IROA labeled with 5% and 95% U- ^{13}C and mixed 1:1. The relative height of the M+1, the relative height of M-1, and the distance between the monoisotopic peaks all provide confirmation of the number of carbons = **triply redundant quality control** measurements.

b) How is Matrix used?

Matrix is analyzed by LC-MS and ClusterFinder™ software to automatically build a "dictionary" of RT, m/z, formula and physical characteristics. The triply redundant nature of these identification measurements allows algorithms to go deep into mass spectral noise to find a broad assortment of compounds and ensures that the dictionary is both reproducible and accurate for all the compounds in Matrix. The isotopic signatures in the Matrix compounds are all mathematically calculable enabling ClusterFinder algorithms to use the redundant information to easily characterize peaks (as either artefacts, or Standard compounds), remove unlabeled artefacts, calculate carbon number and molecular formula. For every Matrix IROA

peak, the physical information collected from primary scans i.e. retention time, 12C and 13C monoisotopic masses, number of carbon in the molecule; in-source and post-source fragmentation characteristics i.e. orthogonal data from second-stage analyses such as an Ion Mobility, SWATH fragmentation etc. The information used to find these same features in experimental samples. Run daily, these Matrix features are monitored to look for any disappearances or changes and are the basis of instrument performance.

c) How is the challenge of accurately identifying compounds in experimental samples solved?

Prior to analysis, experimental samples are spiked with IROA **Internal Standard (IS)** – this is the same 95% U- ^{13}C component of the Matrix and at the same concentration. Experimental/IS samples and Matrix are randomly interspersed into a single sample set (e.g. one Matrix injection for every 10 experimental/IS samples) and analyzed using LC-MS, see **Figure 2**.

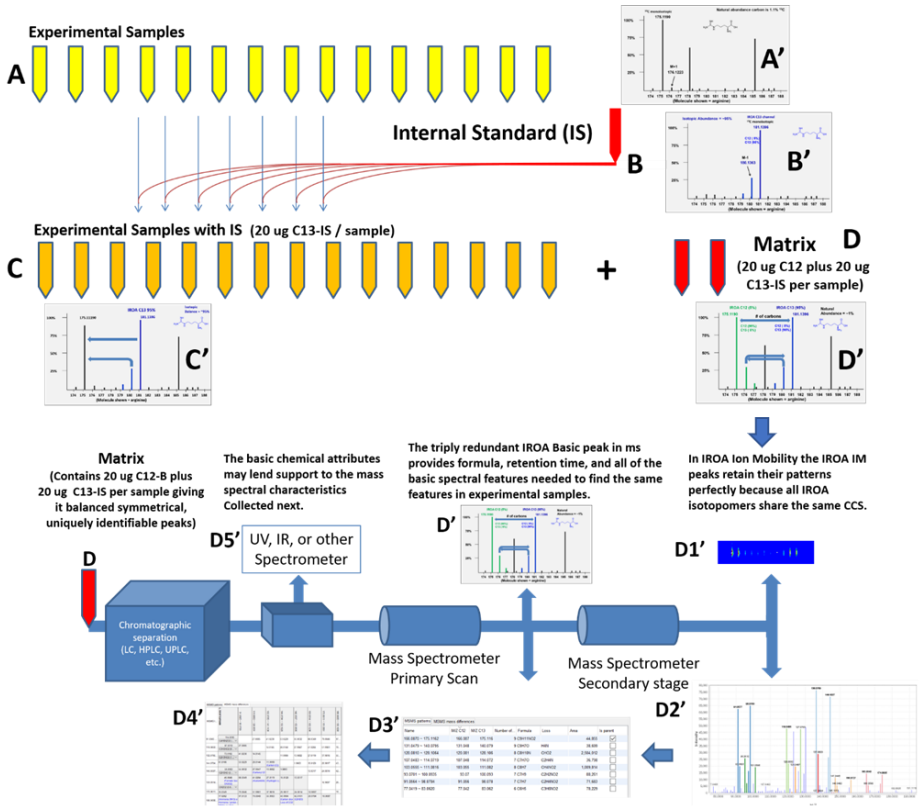
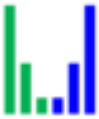


Figure 2. Experimental samples (A) contain compounds where the isotopic balance is present only at natural abundance, i.e. 1.1% ^{13}C (A'). A fully labeled 95% ^{13}C Internal Standard- IS (B') is added to experimental samples. Experimental/IS samples and Matrix (D; fully labeled 95% and 5% ^{13}C, mixed 1:1) are randomly interspersed into a single sample set (e.g. one Matrix injection for every 10 experimental/IS samples) and analyzed using LC-MS. The same 95% 13C isotopomeric IROA signal is present in both the Matrix (D) and experimental/IS samples (C) and the chromatography is consistent across both, the Matrix is mapped directly to the experimental samples. The catalog of IROA peaks found in each daily Matrix analysis (D') provides a mechanism for correcting instrument error. Since the amount of IS is identical to that in the Matrix and is the same across all samples, the sum of all signals in the IS is constant and may be used to normalize samples. Natural abundance peaks are easily located and quantitated as they co-locate with their corresponding IROA peaks (C). In IROA MSMS fragmentation such as SWATH (D2') the IROA peaks retain their patterns (D3') because wide windows are used. Since all fragments retain their IROA character, their formulae (D4') and their relationships between them are determinable (D5'). With the inclusion of second stage analysis (D1-D5) the compounds found in the Matrix samples run under different analytical conditions i.e. HPLC system 1 and HPLC system 2, may be unequivocally mapped from one to the other and will provide for the quantitative comparison of experimental samples associated with their respective Matrix.



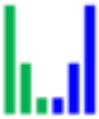
The concentration of the compounds in Matrix and IS and their chromatography are identical. The software used the information stored in the Matrix dictionary (catalog of peak pairs, their RT, number of carbons, and IM and fragmentation characteristics) to identify where each of these same IROA peaks will be found in the experimental samples using the IS. The IS serves as a yardstick and provides enough information for complete identification and quantitation of samples without the need for chromatographic base-line correction, and without the need for using the same orthogonal identification system in the experimental samples. (This is critical because these secondary systems may lower the temporal resolution and thereby lower the precision of the analytical measurement). To compare samples across different chromatographic systems the software can rely on the physical characteristics stored in the dictionary to accurately ID compounds.

The experimental natural abundance peaks are easily located and quantitated as they will co-locate with their corresponding IROA peaks at a mass that is the mass of the IROA ^{13}C monoisotopic peak less the number of carbons it contains times the mass of a neutron.

i. How are compounds identified?

During LC-MS, a metabolite is often seen multiple times. Most frequently these are neutral loss fragments of structurally-related parents due to in-source fragmentation. Post-source fragmentation also occurs using SWATH and other MSMS techniques. IROA-formatted peaks maintain their integrity through MSMS; fragments show as IROA fragmentation, and similarly through IM where all the IROA-peaks share a common CCS. Because fragments share the same unique IROA isotopic signatures as their parent compounds, ClusterFinder peak correlation analysis associates both in- and post-source fragmentation sets, greatly aiding in peak identification.

Each Matrix has a data-base library of validated compounds associated with it when it is analyzed. These are compounds that may be seen reproducibly when Matrix is chromatographically separated and the IROA peaks in it are examined. Given the diversity of possible chemical structures, standard mass spectral data generated after chromatographic separation alone is not sufficient to identify most compounds, nor usually sufficient to identify a unique molecular formula for most molecules. However, the monoisotopic mass and the exact number of carbons in the molecule are known for all IROA peaks, and this is sufficient to provide a unique molecular formula. In some cases a molecular formula may be shared by a large number of compounds, so that while IROA provides assured formula it does not alone provide assured identification. If in addition to the molecular formula for each IROA peak, we add collisional cross-section (CCS from IM), Fragmentation data (MSMS from SWATH or other techniques), UV, IR or any other physical characteristic of each compound as determined in the Matrix sample and the library of compounds known to be contained in it, **then the combination of assured molecular formula and these physical attributes become unique identifiers for**



each compound. This information is added to the "dictionary" and becomes the basis of completely reproducible accurate identification and quantitation, and the Matrix sample provides a complete QA-QC of instrument performance on a daily basis.

ii. How does ClusterFinder identify fragments?

During LC-MS based analysis, a metabolite is often represented by multiple peaks due to the presence of adducts and neutral loss fragments. For example, using positive mode ionization method a metabolite can be detected as $[M+H]^+$ and also $[M+Na]^+$, $[M+NH_4]^+$, $[M+K]^+$ etc. In addition to in-source fragmentation, there is post-source fragmentation that arises from SWATH and other MSMS techniques.

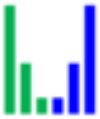
All IROA-based fragments will have the IROA ratio pattern of their parent peaks, as full IROA peaks. The ClusterFinder software includes a module for the analysis of peak correlation. In the module the user can specify the correlation cutoff for chromatographic peaks, the retention window and mass error parameters to use in considering peaks for correlation. Results of correlation analysis allows the user to evaluate the relationships between correlated peaks and the reproducibility of the correlation of different peaks between samples.

Conclusion

Challenges 1 and 2 addressed

Matrix serves as a reliable QC standard and is separately analyzed, interspersed within the collection of randomized experimental samples, to account for fluctuations in mass and retention time drift **ensuring reproducible measurements across time and even instruments**.

a) Matrix is always the same and the catalog of all IROA peaks found in each daily Matrix analysis provides a way to quantitate the performance characteristics for the instrumentation for every day's analysis and a mechanism for correcting any instrumental error.
b) Since the amount of IS introduced to every sample is identical to that of the Matrix and is the same across all samples, the sum of all signals in the IS should be constant and may be used to normalize samples.
c) With the inclusion of an orthogonal, second-stage analysis and the collection of data detailing additional physical characteristics, such as an Ion Mobility (see Figure 2 below D1'), fragmentation such as SWATH (D2' through D4'), UV, or IR (D5') etc., the compounds found in two sets of Matrix samples which have been analyzed under very different analytical conditions may be unequivocally mapped from one to the other and will therefore provide for the quantitative comparison of the clinical or experimental samples associated with their respective Matrix samples.



d) In addition to its triply redundant QC properties, unlike other QC standards such as plasma, IROA-Matrix dried yeast extract has the added advantage of a stable long shelf life.

Challenge 3 addressed

The Matrix and IS workflow provides a mechanism to assure **consistent identification** of all compounds across time and analytical platforms

a) The Matrix (yeast extract labeled with 5% and 95% U- ^{13}C and mixed 1:1) is randomly interdispersed into a single sample set (one Matrix injection every 10 experimental injections or so). It is analyzed separately to build a triply redundant "dictionary" of information: RT, m/z, C1 monoisotopic mass, C13 monoisotopic mass, number of carbons in the molecule, ion mobility characteristics, fragmentation characteristics (in-source and post-source), amplitude of each peak in the IROA molecule, the relationships between all IROA peaks, and any other physical characteristics which is then stored in the ClusterFinder program.
b) The IROA-IS (yeast extract labeled with 95% U- ^{13}C only) is added to every natural abundance experimental sample. Because the same 13C isotopomeric IROA signal is present in both the Matrix and experimental sample, the chromatography is consistent across both, the chemical compound identification and physical characteristics verified in the Matrix may be mapped directly to the experimental samples.
c) Because of the unique IROA-IS signal placed into the experimental samples, the mapping does not require that the experimental samples also have the same secondary physical characteristics, but rather can infer these based on references to a co-incidentally analyzed Matrix sample.
d) Importantly ClusterFinder algorithms uses a scoring system based on calculated-observed isotopic patterns against expected, derived from compound databases. These scores provide another quality check in assuring reliable data.

The IROA Matrix and IROA-IS system (named **IROA ID-QUANT-QC Workflow)** provides: 1) a method for the reproducible accurate identification of a very large number of compounds, 2) a means to generate and correct errors in quantitation irrespective of the analytical systems used, and 3) a process to validate instrument and analytical procedures across time and platforms.

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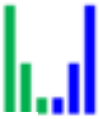

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