

Integration of Standards for Ion Suppression Correction and QC in an Untargeted Metabolomics Workflow



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Introduction

The lack of reproducibility in untargeted metabolomics has been an on-going major concern. We all know it is not worth the time to work up bad data. As described in this report, we have developed a workflow to minimize variability and to correct ion suppressed data that could be effectively normalized to achieve reproducible measurements. The process is based on internal standards, provides QC of instrument performance, and will withstand day-to-day variation.

Metabolomics Standards should be well-characterized, stable, readily available, and ideally provide the means for the following:

- Compound identification and quality control
 - Accurate identification of compounds and unknowns, and removal of false data
 - Assessment of platform performance – injection accuracy, in-source fragmentation activity, chromatography, sensitivity, matrix effects
 - Inter-study and inter-laboratory assessment of data
- Suppression-corrected quantitation and sample-to-sample normalization
- Standardization of data across batches and datasets

Reference Standards for compound identification and quality control

Which types of Standards should be considered for untargeted metabolomics?

Process blank samples

Chemical contaminants from sample collection handling and processing include plasticizers, and extraction solvents, or column carry-over from previous samples. Carefully positioned blanks within the analytical run provide information which can be used to eliminate background noise from the dataset.

Pooled experimental samples and standards

A pooled aliquot of every sample can be collected and analyzed to observe repeated measurements of a representative sample and so as not to miss any compounds unique to the treatment as compared to control. However, this may be problematic if not economical or impractical for long-term projects if the samples are limited. When pooled study samples are not possible a single large sample, often pooled plasma or urine, may be acquired, sub-aliquoted, and stored at -80°C to be used as a daily QA/QC “standard”. Individual aliquots are then retrieved and analyzed daily. However, such aliquots are so chemically complex and undefined that only a very small percentage of their components offer any insight into compound identification, system performance, or QA/QC. Further, plasma may be unsuitable for cross-platform analysis because the number of differences that will be encountered when using dissimilar sources, chromatographic systems, or even detectors, will preclude most points of comparison.

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If large pooled samples can be obtained as reference standards, stability and safe handling precautions must be considered. Samples must be carefully monitored as storage conditions and sample preparation will create artifacts and degraded samples are sources of variability that will obscure metabolite identification.

Long-Term Reference Standard Materials

Long-Term Reference Standard Materials provide a means for intra-laboratory and inter-study assessment across differing studies; namely long-term assessment of data within and between laboratories. LTRS materials should be stored as such for long-term stability and should not suffer from freeze-thaws. As sample stability here is critical, pooled plasma may not be an ideal LTRS material.

The optimal LTRS material will be chemically complex yet very well characterized prior to distribution, and most of its components should be re-analyzed during its daily analytical use. Simple mixtures of compounds are sometimes used however they lack the usefulness of more complex mixtures.

Internal Standards/External Standards

Discerning compounds from isobaric artefacts, or otherwise determining which of the multitude of peaks to use as standards or reference points is almost impossible without extensive use of internal and/or external standards. The use of libraries of authentic compounds, injected to determine instrument-specific responses will be required for “verified” chemical identification.

MS-based targeted metabolomics methods typically use isotopically-labeled internal standards to ensure accurate identification and quantitation. Carbon-13 based standards behave physically and chemically (including ionization efficiency) more similar to the analytes under measurement, and are therefore preferred, however deuterium based standards may be used when the chromatographic conditions are carefully prescribed. In both cases, up until now it has been impractical (and unaffordable) to match internal standards to large numbers of unlabeled compounds and their fragments for untargeted or complex targeted analyses.



Consider a fully labeled biological sample universally labeled at both 5% and 95% U-13C and mixed 1:1 as a Long-Term Reference Standard (LTRS) that can be used both for compound identification and QC.

Compound identification

The identification of any compound across different mass spectral protocols is unlikely to be successful without careful calibrations and authentic standards. Multiple biological compounds may be confused because they are isobaric, i.e. have the same formula or mass. Even more problematic are unknown artifactual or fragmentary compounds that are structurally and chemically different from their biological isobaric equivalents, but may share the same mass, or even formulae. These artifacts typically outnumber the known metabolites in metabolomics studies.

The IROA Long-Term Reference Standard (IROA LTRS or LTRS) is an isotopically-labeled complex standard, universally labeled at **both 5% and 95% U-13C** and mixed 1:1, that has been economically produced in bulk and has been characterized to measure hundreds of primary and secondary metabolites. When analyzed by LC-MS, the LTRS exhibits unique labeling patterns for all biological compounds (represented by the **peak pairs** of the molecule arginine - see Figure 1) which discriminates them from all artifacts. These peak pairs provide many useful benefits:

- *They are computationally easy to find and characterize.*
- *For masses below 500 amu the C12 or C13 monoisotopic mass plus the number of carbons almost always uniquely identifies the molecular formula.*
- *Unique isotopic patterns discriminate peaks of biological origin allowing removal of false data.*
- *Isobaric formulae are distinguished using secondary MS scans either by collecting Collisional Cross Section (CSS), or fragmentation.*
- *Fragments and adducts of the M+H, e.g. [M+Na]⁺, [2M+H]⁺, [M+H-CH₂O₂]⁺, etc., are most commonly considered “unknowns”. As peak pairs with artifacts excluded, the isotopic pattern of fragments derived from their parent peaks may be analyzed, correlated, and named.*
- *The IROA LTRS is stable. The exact same compounds that are measured today will be remeasured 5 years from now.*

Based on their IROA chemical characteristics, LTRS metabolites, plus their fragments and adducts, can be readily identified and a “library” of RT, *m/z*, formula and physical characteristics can be generated to measure the same hundreds of metabolites in any LTRS sample using any chromatographic system on any instrument or interface. With the inclusion of an orthogonal, second-stage analysis, such as an Ion Mobility, fragmentation (such as DDA, DIA, SWATH, etc.), UV, or IR, the compounds found may be unequivocally identified. In addition, when two sets of LTRS samples have been analyzed separately

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under very different analytical conditions, the resulting datasets will be unequivocally identified, and may therefore be mapped from one to the other.

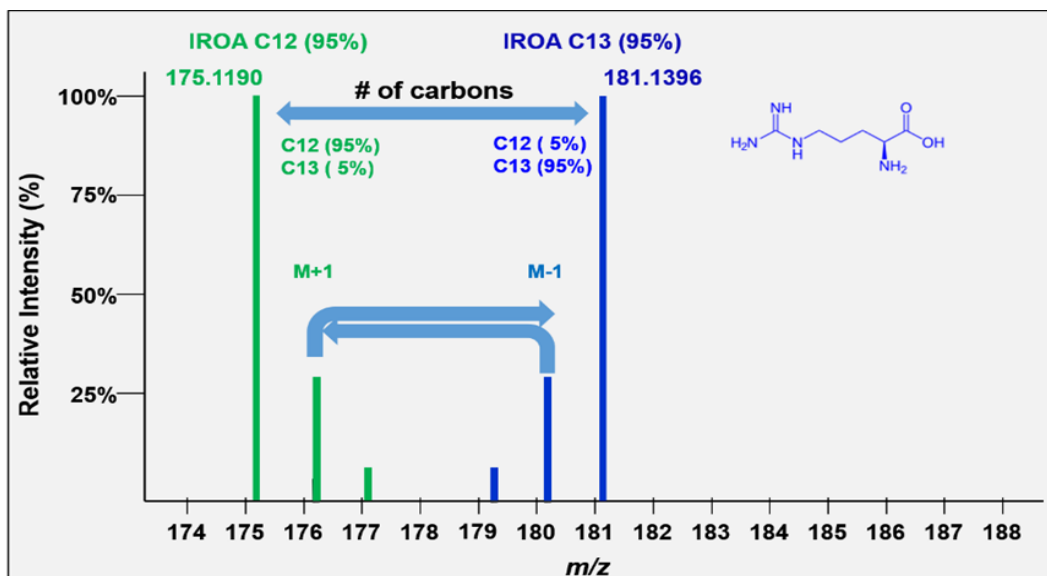


Figure 1. Representation of the molecule arginine contained in the LTRS, universally labeled with 5% and 95% U-¹³C and mixed 1:1. The mixture gives rise to a unique “U-shaped smile” pattern of peaks which contains both the 95% envelope (95% U-¹³C peaks; M-1, M-2 etc.) and its mirror-image envelope (5% U-¹³C peaks; M+1, M+2 etc.). All of these peaks are mathematically calculated to assure reproducibility and accuracy. Every molecule in the LTRS 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 basic IROA peak, such as shown here in Figure 1, is a **triply redundant structure and the basis for 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.

Quality Control

The use of affordable, stable, homogenous QA/QC Internal Standards is critical to assess measurement variability and platform performance during the metabolomic workflow. The Long-Term Reference Standard (LTRS) is derived from a large supply of stable material and is always the same collection of compounds at the same concentrations. When analyzed on a daily basis, the LTRS will provide QA/QC of instrument performance.

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- The total of paired peaks is a measure of instrument sensitivity.
- The retention time for each compound is a measure of chromatographic performance.
- The relative strength of signal/compound is an important measure of in-source fragmentation.
- The total signal found for all compounds is a measure of injection accuracy.
- When interspersed within the collection of randomized experimental samples, the LTRS accounts for fluctuations in mass and retention time drift ensuring reproducible measurements across time and even instruments.

The LTRS is a stable chemically complex reference standard. Measurements made on every LTRS sample are always based on exactly comparable data to achieve QC instrument performance for daily metabolomic measurements, across studies and for inter-laboratory comparisons.

Consider a chemically complex Internal Standard (IS) that is added to every experimental sample that is chemically identical to the IROA LTRS but universally labeled at only 95% U-¹³C. The amount of IS added to the experimental sample is the same amount as present in the LTRS so that the concentration of all compounds in the IS is mirrored in the LTRS. With a verifiably identified internal standard the natural abundance compounds in the experimental sample may be identified, suppression corrected, and sample-to-sample normalized for accurate quantitation.

Compound quantitation

Mass spectrometers have become so user friendly and good at producing copious data that we tend to forget their problems. Source related issues such as tuning issues, ionization efficiencies, and ion suppression and adduct formation, mean that the strength of the mass spectral signal that is detected is never really a quantitative measure of any compound. This is further complicated by in-source fragmentation, or later fragmentation in the instrument lensing. This is not a new or brilliant observation, just one we tend to forget. The classical solution to these problems is the use of an internal standard of known characteristics which enables the experimenter to overcome or correct for these issues. The best internal standard for any compound is an isotopomer, preferably a ¹³C isotopomer, that will mirror as closely as possible the chemical properties of the compound. Historically, the use of isotopic internal standards was an expensive option and would only be used in specific targeted assays; thus, non-targeted metabolomics tended to “get by” as an exploratory, non-quantitative technique based on assumptions that suppression, adduct formation, and fragmentation would be consistent for any compound across all samples. This has never been true, but generally

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accepted as good enough for “hypothesis generation” with true quantitation being required for all follow up work.

The IROA Internal Standard (IS) is a biological extract of 100s of primary and secondary metabolites which mirror the chemistry of most living things. All of these compounds are always present at the same “standard” concentration. Thus, when the IS is added to an analytical sample it provides an internal standard for identification and quantitation of many hundreds of compounds. More importantly, each compound in the IS will form adducts, fragments, and be suppressed to same extent as its natural abundance equivalent. This IS makes it possible to correct for almost all the sources of error (or variances) in a mass spectral measurement. Where the concentration of a compound in the IS is quantitated the quantitation of the analyte is exact, but even where the IS concentration is not yet known the amount of the compound in the analytical sample is relative to its Internal Standard peak, which is constant. In all cases the resulting data will be suppression-correctable and normalized assuring completely comparable results across all samples.

Sample normalization

While the corrections to mass spectral signals discussed in the previous section will correct for instrumentation issues, there will still be variance in any measurement devolving from the nature of the samples, their preparation, and injection. Unlike the source and instrument-based issues which vary on a compound-by-compound basis, these three issues are sample-based and therefore the variance produced by them is sample wide. This means that the correction of these problems requires a different solution, but one that is available given that it is now possible to correct for instrument-based errors; namely we need to do a sample-to-sample normalization.

While many options are available, we have implemented a variation of the MSTUS (MS Total Usable Signal) normalization protocol of Warrack et al. The TruQuant protocol provides a normalization mechanism that uses the “instrument” corrected data to achieve a single normalization strategy in two different ways based on a “dual MSTUS” strategy. The dataset relies on the fact that there are, in fact, two different comingled samples in each physical sample that is analyzed, i.e. there is 1) the natural abundance sample, and 2) the Internal Standard (IS) sample. The IS is unique in that it is always derived from the same source and is always present at the same concentration, while the natural abundance sample (NA) is likely to vary in size and concentration and therefore it will need normalization. In the original MSTUS method all of the “known” compounds would be summed and the sum normalized to an arbitrarily selected number. Using the IROA Dual MSTUS method the sum of the C12 IROA paired peaks is always normalized to the sum of the C13 IROA paired peaks, i.e. rather than using an arbitrary value the number used is a constant that will always be the same, every day. Thus, making the normalization common across multiple days.

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The IROA normalization protocol is the most robust and accurate normalization currently available because it is based on suppression-corrected data and the normalization is to a standard value.

The WORKFLOW – putting together all the pieces

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

I. How is the LTRS used?

The LTRS is analyzed by LC-MS and ClusterFinder™ software to automatically build a “dictionary” of RT, m/z, formula and physical characteristics. The information is also used to find these same features in experimental samples. The LTRS “Dictionary” (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) stored in the ClusterFinder program provides information of where each of these same IROA peaks will be found in the experimental samples using the IS. To compare samples across different chromatographic systems the software can rely on the physical characteristics stored in the dictionary to accurately ID compounds.

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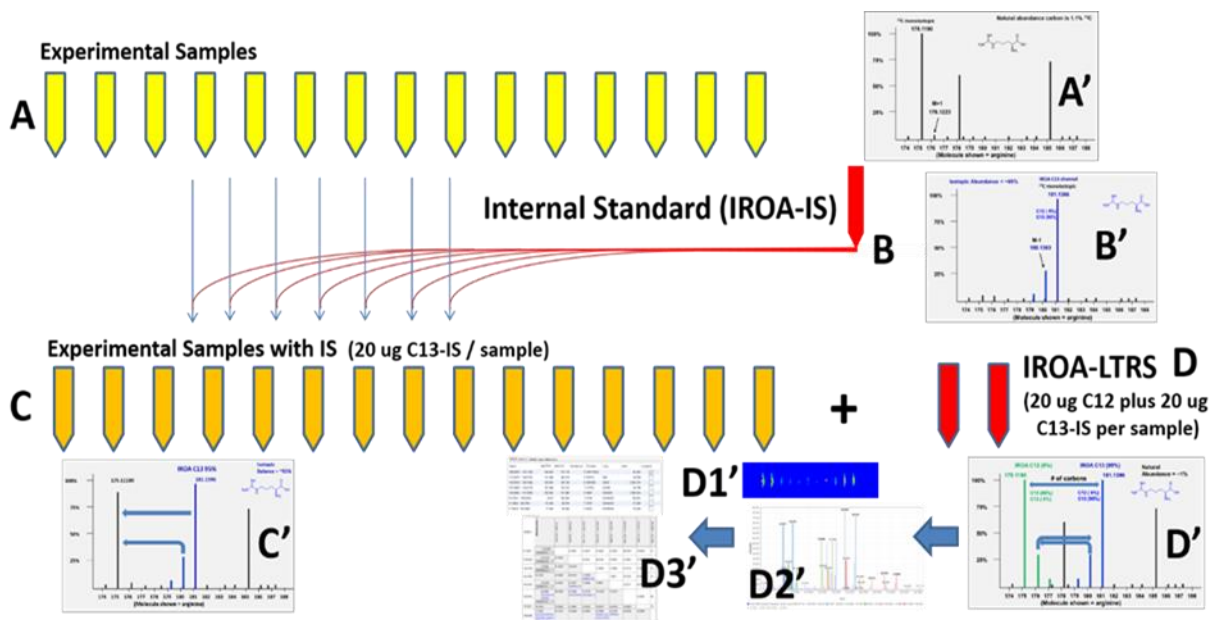


Figure 2. The WORKFLOW is based on a well characterized Long-Term Reference Standard (D = LTRS) and a carefully matched Internal Standard (B = IS) to provide instrument and process QA/QC, validated compound identification, and quantitation. These standards allow for suppression-correction and normalization to achieve intra- and inter-laboratory reproducible datasets.

(A) Experimental samples (plasma, urine, cells, etc.) are mixed with (B) the Internal Standard (IS), fully labeled with U-95% ^{13}C (B'). Experimental/IS samples are analyzed using LC-MS, injecting the LTRS (D) intermittently, approx. every 10 samples. The LTRS has the same concentration and is chemically identical to the IS but is a 1:1 mixture of fully labeled U-5% and 95% U- ^{13}C metabolites, producing a U-shaped pattern of carbon envelopes (D'). 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 in each biological molecule resulting in a triple redundant quality control check point. The IROA peaks represent actual biological compounds, fragments and adducts easily discriminated from unsigned artefacts and noise which then can be removed from the data, eliminating false discoveries. As a chemically identical composite sample, sample-to-sample and analytical variance is removed, and during MS analysis the identical compounds (labeled with either U-95% ^{13}C or U-5% ^{13}C) experience the same ionization efficiency and suppression.

Over 1000 peaks can be detected in the IROA-LTRS. Following analysis, the resulting IROA-LTRS dictionary of compounds is used to identify compounds in the IROA-IS saving time, effort and related costs. The IROA-IS together with ClusterFinder software are used to co-locate, identify, and quantify 400 to 600 biochemicals in experimental samples, depending upon chromatographic mode(s) employed. The characteristics of each compound in the IROA-IS allow for a ClusterFinder-assisted suppression correction factor to be generated for each compound for more accurate quantitation.

Unambiguous ID and quantitation (ms2) in a single injection. Complete identification of compounds is achieved with the addition of IM or SWATH. The IROA IM peaks retain their patterns perfectly because all IROA isotopomers share the same CCS (D'). In IROA msms fragmentation, such as SWATH, the IROA peaks retain their patterns (D2') because wide windows are used. Since all fragments retain their IROA character, their formulae and the relationships between them (D3') are determinable.



II. How are compounds identified?

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.)

The experimental natural abundance peaks will co-locate with their corresponding IS 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.

Given the diversity of possible chemical structures, standard mass spectral data generated after chromatographic separation alone is not sufficient to identify most compounds and not even 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 in and of itself 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, 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.

III. How does ClusterFinder identify fragments?

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. There is also post-source fragmentation that rises from using SWATH and other MSMS techniques. The IROA fragments will maintain the general IROA ratio pattern of their parent peaks, 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 can be performed to associate both in- and post-source fragmentation sets, greatly aiding in the identify of compound peaks. 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 allow the user to evaluate the relationships between correlated peaks and the reproducibility of the correlation of different peaks between samples. Importantly ClusterFinder algorithms use 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.

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IV. How does ClusterFinder test and correct for ion-suppression and normalize samples?

Ion suppression is the result of interference from coeluted chemical components that negatively impact detection capacity. It is often observed as the loss of analyte ionization response and negatively affects precision, accuracy, and reproducibility (over- or underestimation of analyte levels). These suppression effects are not fully understood and may include both endogenous substances from the sample matrices, exogenous contaminating molecules introduced during sample preparation, or both, but the detection of ion suppression is an important consideration for quality analysis in research and clinical applications.

Testing for Ion-suppression

We used the standard WORKFLOW (Figure 2) to test for ion suppression in a common human plasma sample, data shown in Figure 3. The LTRS was used for compound identification and Quality Control, and the IS was spiked into every experimental plasma sample. Triplicate aliquots were made from a serial dilution of common plasma, dried down and resolvated with a fixed volume of IS. The natural abundance compounds in the plasma samples all varied but were of a known relative concentration, and the C13 IS compounds were present at the same concentration in all samples. These samples were then subjected to LC-MS analysis and the resulting data examined for all compounds seen in both the natural abundance sample (C12) and C13 IS.

When the concentrations are plotted at concentration expected vs. amplitude of MS signal (either AUC or height) seen, we expect the theoretical C12 levels to be sloped (see Figure 3A) and the C13 IS amplitudes should be constant (see Figure 3B). Since the concentrations of all compounds are fixed by the experimental design, deviations from these theoretical linear forms would indicate inefficiencies in ionization, largely due to ion suppression.

Based on this experiment an R script (version 3.4.2) was originally used to model the calculations and develop the algorithms for both the suppression correction and MSTUS normalizations required to remove these variances. Once developed these algorithms were incorporated into IROA's ClusterFinder software (V 4.0) and both are automatically applied to all appropriate samples and this additional output is made available together with the original raw data.

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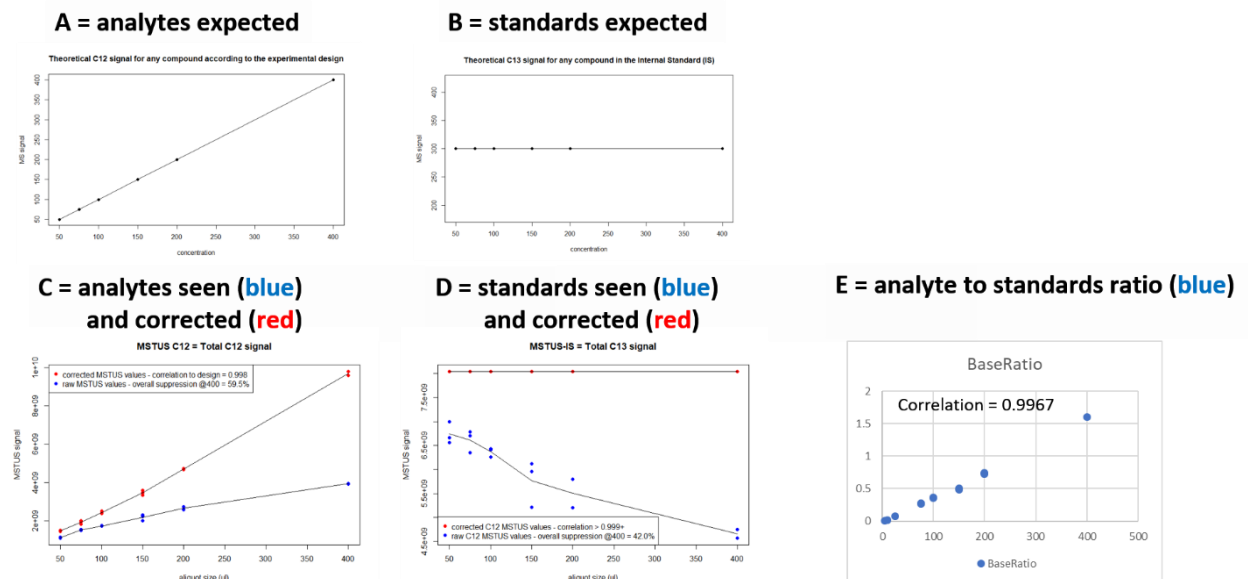


Figure 3 - Expected and observed data outcomes

Suppression correction

As noted above, the experimental design for the experiment implied two linear graphs (see Figure 3 A & B) were expected; however, the raw data presented in blue in Figure 3 graphs C & D were observed. The difference between the observed and expected is mostly due to ion suppression. As noted above, when the isotope involved is C13 then the physical and chemical behavior of the C12 and C13 isotopomeric ions should be similar except for mass. We can determine that this is true if we look at the ratio of the C12 ion to its C13 companion (using either AUC or height). In Figure 3 E we can see that the graph of this ratio yields exactly the expected linear relationship, which implies that the ions formed from all isotopomers of a given compound are equally and commonly suppressed. If we determine the non-suppressed, or the least suppressed value for a given compound we can substitute it for all of the C13 values and obtain the appropriate C12 values by multiplying the non-suppressed value by the ratio. The corrected values are represented in Figure 3 graphs C & D as red dots.

Having access to suppression-corrected data allowed us to understand the level of suppression in every compound. As shown in Figure 4, the average rate of suppression in the most concentrated samples is 40 percent with almost 50% of the compounds being suppressed between 20% to 60%. (When we look at the plots of the sample with the highest concentration (largest aliquots), no compounds appear to be natively suppression-free.)

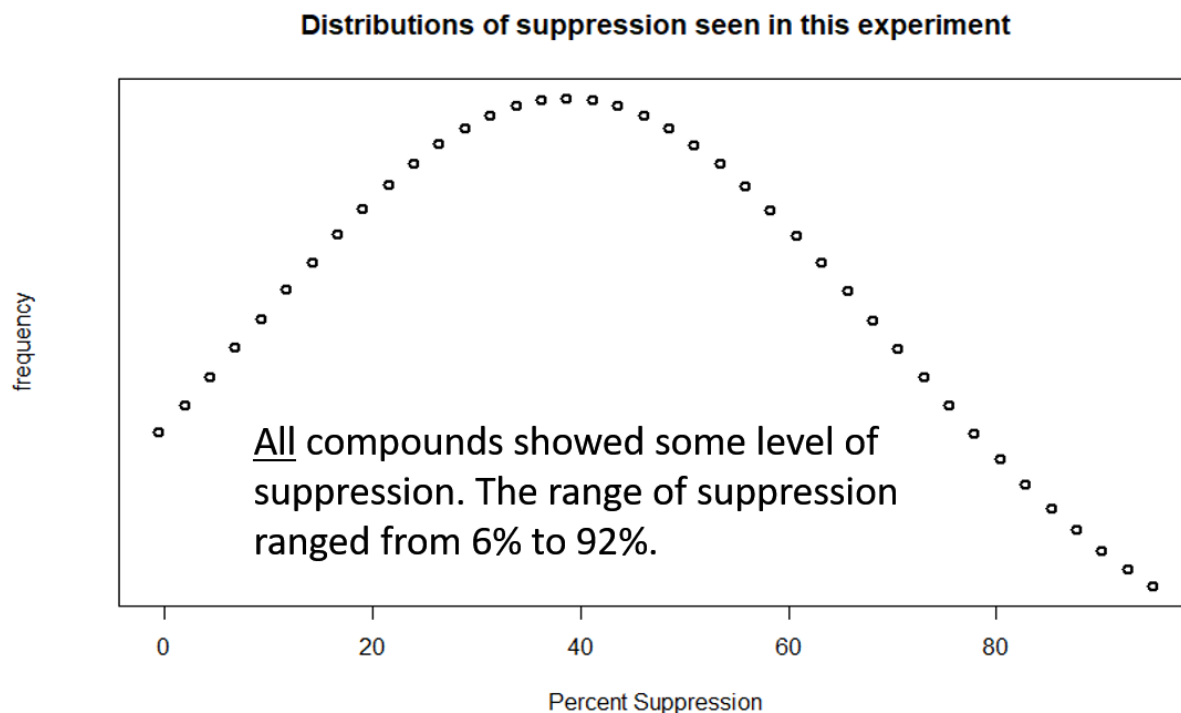


Figure 4 - The distribution of suppression among the compounds in this experiment

A Dual MSTUS Normalization

The problem of mass spectral sample-to-sample normalization is frequently confronted, especially in metabolomics where the samples tend to be “messy”, for instance urines, plasmas, and even biopsies all tend to differ in concentrations, sizes, etc., normalization is important but it is very difficult. Once we had achieved suppression correction we decided to see if the suppression correction had removed enough variance to make a total area under the curve normalization more effective. We decided to extend a MSTUS type of normalization based on two special properties we could achieve in our experiments. The algorithm we use, described below, is based on suppression-corrected data, and the presence of two full samples (the C13 IROA-IS plus the experimental sample) contained in each analytical sample, hence we call it a “Dual MSTUS” algorithm. Like suppression correction, the Standard qualities of the IS are critical to its use.

The original Warrick et al. MSTUS algorithm has been typically used to normalize NMR and LC/MS-based metabolomics data, especially in highly variable samples such as urine. The classic MSTUS normalization has always also used only a subset of the entire dataset of “validated compounds” to generate the total area under the curve, i.e. it does not sum any possible artifacts or noise; it uses only a subset of identified compounds. In theory, the more compounds it can validate the better the normalization. In

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practice it sums all the validated compounds in all samples and develops a “normalization factor” that would be required to make all these sums equal, but the number that they are normalized to is arbitrary.

Similar to the Warrick validation step all the peaks we used in computing a normalization factor had **minimum criteria to qualify**: 1) both the C12 and C13 isotopic clusters have to be present in all samples, 2) they both have to be above a minimum peak area, and 3) and the ratio between the C13-IS and the C12 monoisotopic peaks has to be greater than 0.001. The original dataset had 389 IROA peaks found in all 16 samples, 232 of these peaks met the acceptance criteria and were used for normalization. Since they are all IROA peaks they were all assured to be of biological origin.

In the IROA TruQuant IQQ WORKFLOW since we add an identical amount of the IROA-Internal Standard (IS) to every sample (the same across all samples) the sum of all signals in the IS should be constant and may be used to normalize samples. Furthermore, the unique carbon envelope of associated peaks for each analyte in the IROA-IS (95% C13) ensures removal of artefactual data, so only real compounds are considered.

The sum of all qualified C12 compounds was determined, and the sum of all qualified C13 (IS) compounds was determined. Therefore, within each sample two sums were ascertained, hence “Dual MSTUS”, and the normalization factor would be a multiplier that could be applied to all compounds to make the sums equal. Since the normalization factor is sample specific the normalization factor could be applied to both qualified and non-qualified compounds. Therefore, while compounds in the IS may be both suppression-corrected and normalized, compounds that do not have an associated IS may still be normalized (See Figure 5).

Compound status	Correction Suppression / Drift	Normalization
With IS	Yes	Yes
No IS	No	Yes

Figure 5 - Suppression correction and Normalization

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In a normal metabolomic experiment, where sample sizes and chemical constituents vary, the normalization will correct for differences in sample sizes or preparation, i.e. changes that effect the entire preparation, and the normalized sums will differ because of the differences in chemical composition. In the design of this experiment, and only in this experiment, since the samples were chemically the same the normalization should normalize all samples to be identical. Figure 6 demonstrates that this is true and summarizes all of the data presented.

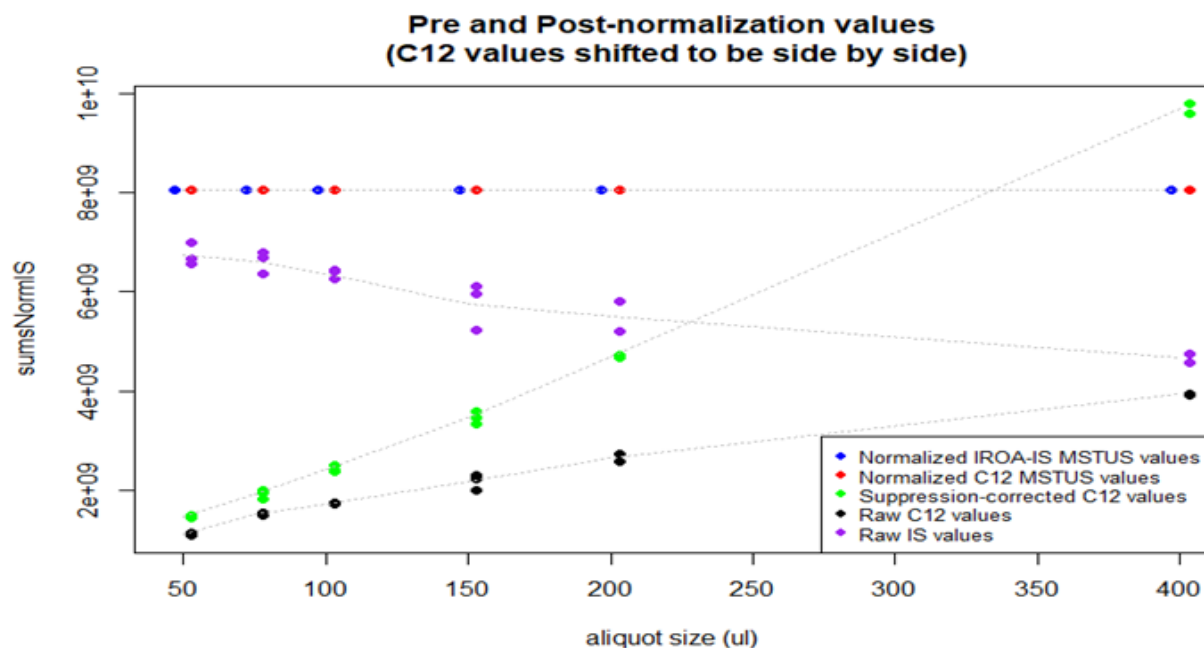


Figure 6. Summary of data. Since the samples were chemically the same, all samples were normalized to be identical.

The combination of the WORKFLOW and the dual MSTUS approach provides a reproducible means to generate ion-suppression-corrected data that can be effectively normalized to achieve accurate measurements that are based on standards and will withstand day-to-day variation.

Conclusion

The IROA TruQuant IQQ WORKFLOW provides: 1) a method for the reproducible accurate identification of 100's of compounds, 2) a means to generate suppression-corrected quantitation and sample-to-sample normalization irrespective of the analytical systems used, and 3) a process to validate instrument and analytical procedures across time and platforms (QA/QC). Better data always yields better results. Why waste time working up bad data?



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