Correction of Ion Suppression and Normalization for Improved Quantitative Rigor and Reproducibility Using IROA

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ABSTRACT

Ion suppression and ion enhancement are well-known phenomena in mass spectrometry, and spiking stable isotope-labeled internal standards into a sample at known concentrations is generally thought to represent the only method for dealing with the problem. Here we show that a chemically complex Internal Standard (IROA-IS) containing hundreds of stable isotope-labeled metabolites can correct for loss or gain of corresponding targeted metabolite signals during sample preparation and data acquisition, yielding more accurate measurements. Notably, we demonstrate that the strategy is effective over a wide range of IS:analyte ratios. The resulting, suppression-corrected measurements, in most cases, correlated strongly ($r^2 > 0.98$) with the concentration of each metabolite. Furthermore, from the ratio both suppression-corrected values and sample normalization may be calculated[1], correcting for variation introduced by sample preparation and analytical sources of error. In summary, incorporation of a chemically complex IS into targeted and non-targeted metabolomic workflows provides a viable strategy for increasing quantitative rigor and reproducibility.

INTRODUCTION

Normalization of ion-suppressed mass spectrometry data using ratio-based correction
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. In general, the results of this investigation indicate that 1) ion suppression is both much more common and variable than currently presumed, 2) it may be mathematically overcome, 3) it is possible to generate suppression-corrected data, and 4) since suppression is the largest problem in most sample normalization schemes, once suppression-corrected data are available, sample normalization by MSTUS[2], or similar protocols, becomes more effective.
The IROA[3,4] Workflow (TruQuant™) protocol was employed to test for ion suppression (see Figure 1). A biochemically complex IROA Matrix sample (see Figure 1D), containing equal concentrations of both a 5% and 95% biochemical mixture was used for compound identification and Quality Control, and a 95% Internal Standard (IS – see Figure 1B); identical to that contained in the Matrix was spiked into every experimental sample (Figure 1A). Ion suppression was determined by making a set of samples in which the concentration of every compound increases or decreases by a known amount. This may be done by either making a series of dilutions of a very concentrated but homogeneous sample that has all isotopes present at their natural abundance (C13 natural abundance is approximately 1.1%; referred to as the C12 samples) and delivering each sample as a fixed volume of each dilution, or delivering varying volumes of a less concentrated homogeneous natural abundance sample. In either case every compound present in the original solution will be present in known but varying relative concentrations in the final sample. The C12 samples are then dried and resolvated with a fixed volume of a homogeneous C-13 (isotopic balance of 95% C13) Internal Standard (C13 IS or IROA-IS) of similar or identical chemical composition to the natural abundance sample. Under either scenario all the natural abundance compounds in the C12 samples will each be of varying, but known, relative concentration, and the C13 IS compounds will each be present at the same concentration in all samples. These samples are then subjected to LC/mass spectral 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 seen, both the C12 and the C13 IS amplitudes should be constant (see Figure 2B) and C12 levels be sloped (see Figure 2A). Since the concentrations of all compounds are fixed by the experimental design, deviations from these linear forms would indicate inefficiencies in ionization, largely due to ion suppression.

METHODS

Sample Preparation
For each of three replicates (n=3), we added 75 µL of a common human plasma sample (containing K2-EDTA as anti-coagulant) to a polypropylene microcentrifuge tube followed by 1.5 mL of dry ice-cooled methanol, and the solution was centrifuged at 16,100 *g* at 4 °C for 10 min. The supernatant was transferred to a new tube from which aliquots of 400, 200, 150, 100, 75, and 50 µL were prepared. The methanolic aliquots were then dried using a centrifugal vacuum concentrator and stored at -80 °C. Immediately before LC-MS analysis, the dried residues were resuspended in 40 µL of IROA-IS (C13 IS) solution. The C13 solution was prepared by dissolving the contents of an IROA-IS vial containing 20 ng of a biologically complex mixture fully labeled at 95% C13 in 1.2 mL of H_2O, vortexed, and briefly centrifuged. The contents of the tube were then transferred to a polypropylene autosampler vial. IROA-Matrix was prepared by dissolving the contents of an IROA-Matrix vial containing 20 ng each of a biologically complex mixture fully labeled at both 5% and 95% C13 in 40 µL of H2O. Three µL of matrix and 5 µL of each prepared plasma sample were analyzed by making triplicate injections using both positive and negative ionization and hydrophilic interaction chromatography (HILIC) and reverse phase liquid chromatography (RPLC).
Liquid Chromatography
A Phenomenex Kinetex C18 column was used (2.6 µm particle size, 100 Å pore size, 150 x 2.1 mm) and the column compartment was kept at 40 °C. Solvent A consisted of water containing 10 mM positive ammonium acetate and 0.1 % formic acid and solvent B was methanol containing 10 mM ammonium acetate and 0.1 % formic acid. A flow rate of 200 µL/min was used throughout the duration of the chromatographic run. From 0 to 5 min the solvent composition was held constant at 5% solvent B. The solvent composition then ramped from 5 to 95% solvent B from 5 to 30 min. A solvent composition of 95% solvent B was next maintained from 30 to 40 min. Finally, the solvent composition was returned to 5% solvent B and held constant from 40 to 45 min.
Mass Spectrometry
A Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer was operated in FTMS full scan mode using the Orbitrap with a scan range of 70-650 *m/z* and a resolution of 240,000. For heated electrospray positive ionization, a spray voltage of 3500 was used and the vaporizer and capillary temperatures were set at 250 and 375 °C, respectively. The sheath, auxiliary and sweep gas pressures were 35, 10, and 0 (arbitrary units), respectively.
Data processing
An R script (version 3.4.2) was used to model the calculations and develop the algorithms for suppression correction and MSTUS normalization. Briefly, all the peaks used in the algorithms had minimum criteria: 1) they had to be present in all samples, 2) they had to be above a minimum peak area, and 3) and the ratio between the C13-IS and the C12 monoisotopic peaks had 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 MSTUS normalization. Note that the MSTUS normalization has always used a subset of the entire dataset for normalization with the current algorithm we continue using a subset and even a subset of the found IROA peaks but since they are all IROA peaks they are all assured to be of biological origin, and with 232 compounds being considered there are plenty to determine the MSTUS ratios needed. For all the peaks the suppression-corrected C12 values used as they are a better reflection of the true concentration found in the sample. These suppression corrected values were determined (see discussion) by multiplying the least suppressed C13-IS value by the ratio of the C12 and IROA AUCs (These ranged from 0.001 to 98.5). The C13-IS values were fixed at the least suppressed value seen for that particular IS compound. For normalization, the MSTUS sum for the C12 side was divided by the MSTUS sum for the C13 side to obtain a correction factor in that when every compound was multiplied by this factor the sum for every compound in the original sample (MSTUS C12) would be equal to the sum of all the suppression corrected IS values. Because these samples are chemically identical, the proof of the normalization is that the values once normalized will be the same. Fora "normal" sample in which the chemical balance varies, the normalization will make the two sides equal but not disturb the concentration relationships between the compounds.

Figure 1 – ClusterFinder TruQuant™ workflow

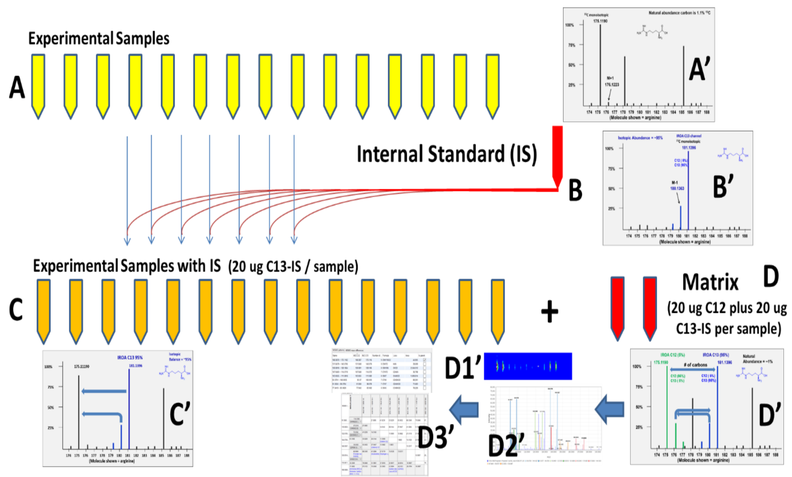


Figure 2 – Theoretical and MSTUS patterns to match

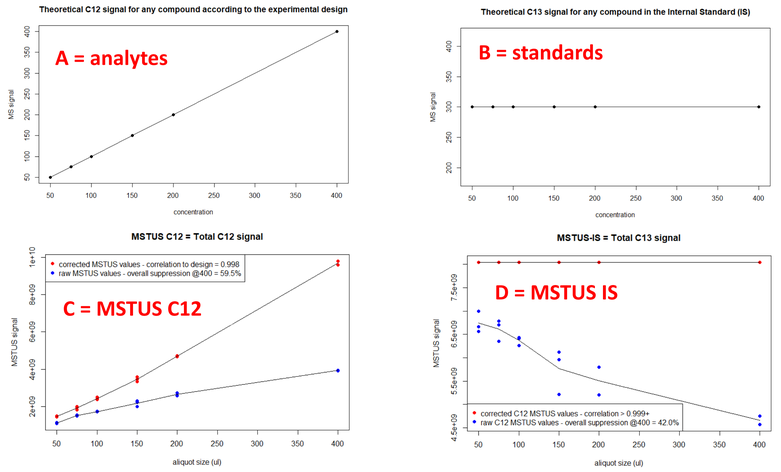


Figure 3 – Individual compound profiles

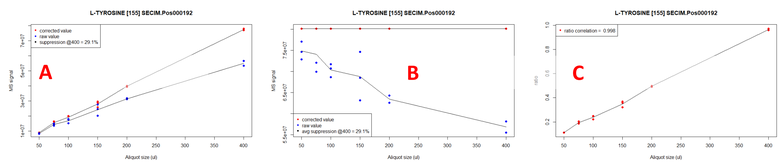


Figure 4 – Suppression distribution

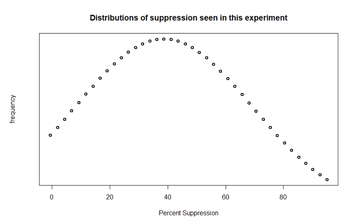


Figure 5 – Suppression outliers

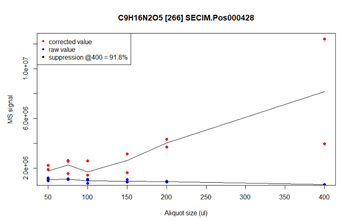
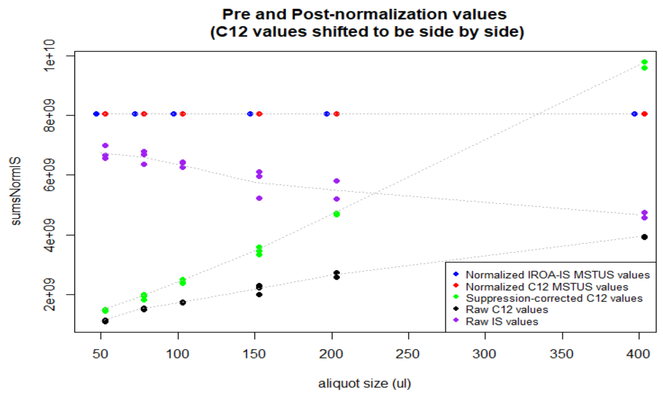


Figure 7 – Summary



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THEORY AND DISCUSSION

Because the IROA Internal Standard (IROA-IS) is always present at the same concentration, the plot of the total area of all IS peaks (MSTUS*-IS) should match the theoretical plot shown in Figure 2B, and the sum of the suppression-corrected values for the MSTUS-IS (Figure 2D, red dots) confirm this. However, the MSTUS-IS raw values (Figure 2C, blue dots) show significant cumulative effect from the overall suppression of its constituent peaks as concentration increases (as shown, the average suppression for the MSTUS-IS in the 400 ul aliquots is 42%.). Similarly, according to the experimental design, the raw natural abundance MSTUS-C12 signal should increase predictably, as demonstrated in Figure 2A. However, in this case, again the actual raw data (Figure 2C; blue dots) deviate because of the overall suppression affecting most of the compounds in the sample, resulting in a value that is almost 60% lower than it should be. Note that in both Figures 2C & 2D, the red dots not only correspond to the desired theoretical value, but the data used to create these graphs represents the real suppression-corrected values that have been computed by a suppression-correction process. Clearly the suppression-corrected values are more accurate values compared with the original raw numbers.
The above discusses the MSTUS values or the sums across all compounds in the MSTUS set. When individual compounds are examined, for instance L-tyrosine (Figure 3A), suppression is evident in all samples except the lowest concentration. In the highest concentration samples a suppression of 29.1% appears to be evident using the equation:

(SC-C12 - rawC12)/SC-C12

Where SC-C12 is the average value of the Suppression-Corrected C12, and rawC12 is the average value of the uncorrected peaks in the same samples. It is clear from this dataset that virtually every compound exhibits some suppression, and obviously so do their internal standards (see Figure 3B). Therefore, it is no surprise that the IROA tyrosine internal standard is not only suppressed but shows exactly the same level of suppression as its natural abundance counterpart (a number independently determined). Despite the suppression of both the analyte and the Internal standard, the ratio of the IROA IS area to the analyte area is exactly consistent with experimental design (see Figure 3C). This is not surprising since they are subject to the same environment during ionization process, and the carbon isotopes the IROA employs should, in theory not have any significant effect on ionization properties. (Note: We have recently shown that the IROA isotopomer collections behave identically in Ion Mobility, and predictably similar in fragmentation; except for mass, the IROA isotopomeric peaks for any given peak will behave uniformly.
As discussed in the experimental, the 232 compounds examined and used in our calculations had to have a base peak larger than 250,000, and a ratio at the lowest concentration of at least 0.001. The range of suppression for all of the compounds in this collection is from ~6% to 92% (Figure 4), and the majority of compounds appear to be suppressed between 20 and 60 percent (in the most concentrated samples). At very high levels of suppression, some compounds were so severely suppressed they were negatively correlated to their actual concentration (Figure 5).
Suppression correction
Since the analyte to ROA-IS ratio for every compound correctly reflects the original concentration of the analyte relative to the IROA-IS, a suppression-corrected value can be determined by setting the IS equal to it's least suppressed value (accepted to be the highest value seen experimentally) and multiplying this value by the ratio to determine what a reasonable suppression-corrected value may be. While any number may be used to act as the constant in place of the "least suppressed value", the use of the actual least suppressed value has the benefit of retaining the approximately correct amplitude for the peaks in question. Therefore, it is relatively easy to compute suppression-corrected values for all compounds found. The formula for C12 suppression correction is:

X * C12/IROAIS ratio

(where x may be any value, but the least suppressed observed value is of the normal amplitude)
Similarly, in the absence if suppression the IROA-IS will be a constant and therefore it is always assigned the same value.
Normalization
The MSTUS algorithm is typically used to normalize NMR and LC/MS-based metabolomics data, especially highly variable samples such as urine. However, it is clear that MSTUS is not a perfect solution as it does not account for noise in the dataset. This noise is the result of the inclusion of artifactual data, causing extreme suppression which in turn strains the underlying rational for MSTUS itself. In IROA Noise is automatically removed as it will never exhibit an IROA profile, so using a suppression-corrected value for all IROA compounds a MSTUS algorithm can now be applied to this corrected dataset to achieve a better result. Furthermore, because an IROA internal standard has been added, there are two physical samples present (the natural abundance sample, which constitutes the analytes, and the reference IROA-IS sample), and a true normalization relative to the internal standard can be performed, with the removal of noise which was not previously possible. The unique carbon envelope of associated peaks for each analyte in the IROA-IS (95% C13) ensures removal of artefactual data. Specific (ClusterFinder) algorithms are used to search for these envelopes and then identify their associated peaks in the natural abundance peak samples. Any features without a match will be eliminated, removing noise and artifactual data. The calculation for the MSTUS normalization correction factors is:

sumSCC12/sumSCIS

where sumSCC12 is the total suppression-corrected area of all considered C12 compounds, and sumSCIS is the total suppression-corrected area of all considered IS compounds
The suppression-corrected values are multiplied by these factors to normalize them all to the same base. The normalized MSTUS value is the sum of all normalized values. In a normal metabolomic experiment, where sample sizes and chemical constituents are varying 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 7 demonstrates that this is true and summarizes all of the data presented. The combination of the IROA and 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.

SUMMARY

- Almost all compounds exhibited suppression; ranging from 6% to 92%. The majority were in the range of 30% to 40%.
- IROA ratios are linear as a function of concentration, and show no effect from suppression.
- Suppression-corrected values for both the IS and analyte may be estimated using IROA ratios.
- Suppression-corrected values may be used for MSTUS, or similar, normalization protocols.
- If the analyte MSTUS is normalized to the "Standard" IROA-IS MSTUS normalization then day-to-day variation may be eliminated.

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