Correcting metabolomic data for source variances using IROA

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OVERVIEW

Experimental LC-MS runs, which extend for several days, may have significant source variance coming from sensitivity, in-source fragmentation, transport efficiency, etc. This variation makes it difficult to analyze and quantitate data from very large experiments. To address this issue, several strategies have been developed to "normalize" the datasets as a function of time, but there is no universally accepted method.

In this presentation we will discuss a novel scheme that allows for data normalization even after significant source changes. The system relies on the use of a biochemically complex IROA Internal Standard (IS), and a closely related IROA Long-Term Reference Sample (LTRS).

INTRODUCTION

LC-MS experiments extending for several days may have significant source variance originated from sensitivity, in-source fragmentation, transport efficiency, etc. Such variation makes data analysis and quantification in large experiments very challenging.

Several strategies have been developed to "normalize" the datasets as a function of time, but there is no universally accepted method. Here we demonstrate a novel system which uses a biochemically complex IROA Internal Standard (IS), and a closely related IROA Long-Term Reference Sample (LTRS) that makes it possible to normalize samples even after significant source changes.

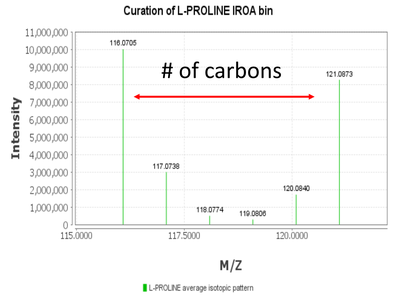
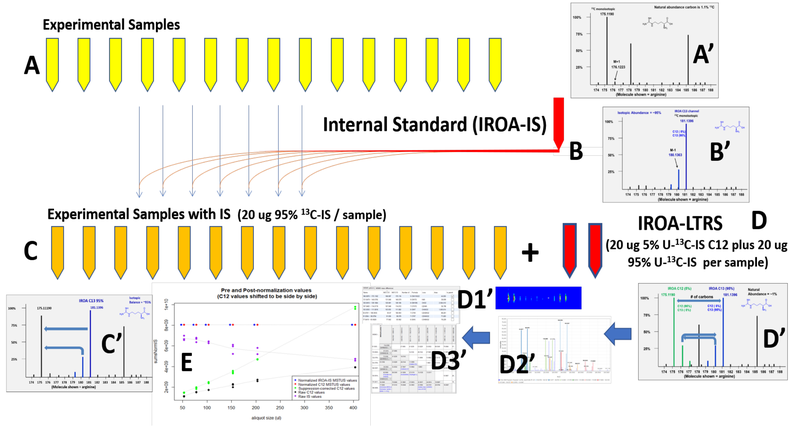


Figure 1: The IROA pattern for L-proline which is representative of a five carbon molecule with a C12 base mass at 116.0705. Note the spacing of peaks is exactly a neutron mass apart, and the peaks are symmetrical.

Figure 2: The IROA TruQuant workflow and protocol



METHODS

Pooled human plasma was extracted with 8 volumes of cold acetonitrile:acetone:methanol 8:1:1, followed by centrifugation at 18300g for 10 minutes at 4 °C. Supernatants were aliquoted at 100, 200, 400, and 600 µl in triplicate, dried under nitrogen at room temperature and stored at -80°C until analyses.

Each aliquot of plasma extract was resolvated with 40 µl of dissolved IS, which contains 95% U-13C labeled metabolites. LTRS, which contains a paired 1:1 mix of U-5% and - 95% U-13C labeled metabolites, was dissolved in 40 µl water. The dissolved LTRS and IS have equivalent U-13C concentrations for approx. 600 compounds. LTRS and IS spiked samples were analyzed before and after a source "retuning," which changed ionization characteristics.

LC-MS analysis was performed with an AB Sciex 5600 QTOF coupled with a Shimadzu HPLC system in both positive and negative modes. A Kinetex 2.6 um F5 100A, 100x2.1mm column (Phenomenex) was used. Mobile phases consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Injection volume was 6µl and run time was 23 min. %organic phase started at 0%, increased to 95% from 2.1 to 14 min, maintained at 95% until 15 min and decreased to 0% at 15.1 min. Column was then allowed to re-equilibrate. Variable window SWATH was applied to LTRS injections, while only single scans were acquired on the aliquots. Data were processed using ClusterFinder software.

The TruQuant protocol uses the Long-Term Reference Standard (LTRS) for compound identification, and a daily measure of instrument performance. The LTRS, a defined Standard Composition that is chemically but not isotopically identical to the Internal Standard (IS), provides a record of chromatographic-behavior for all the compounds in it, a sensitive window into source behavior, in-source fragmentation, and instrument sensitivity.

In this experiment, the LTRS was used to determine the chromatographic-behavior of every compound present in the IS and verify identity according to their IROA peaks, and IROA SWATH (fragmentation scans). Once the chromatographic behavior and identity were determined in the LTRS, a targeted library was created and used to interrogate the analytical samples. LTRS injections were obtained before and after source retuning/modification.

Figure 3: Overall Results

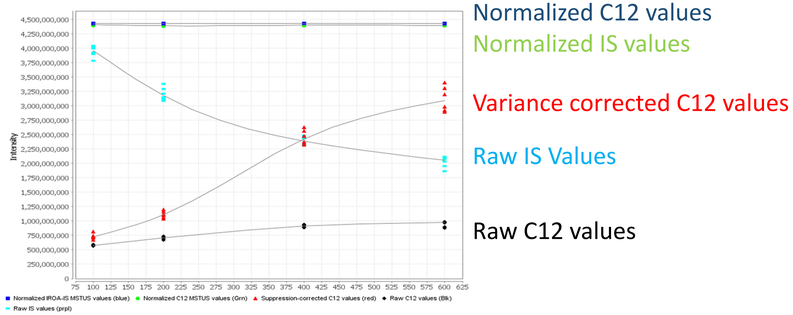
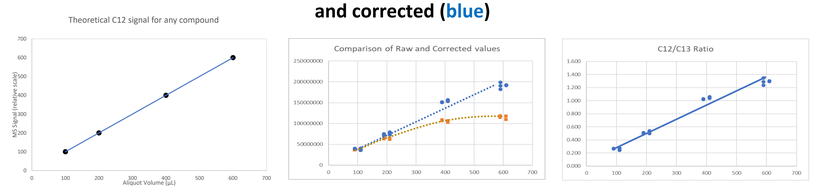


Figure 4: Detailed Results

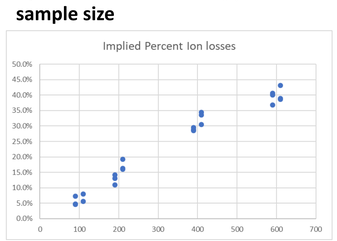


RESULTS

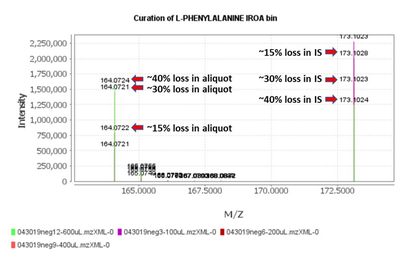
LC-MS chromatograms for the analytical samples collected before and after source modification were different, as expected. These differences could be seen in the EIC, BPC and TIC indicating that the source modification had altered the overall source-response characteristics. Compounds identified in the analytical samples are potentially present as IROA IS peaks plus their plasma natural abundance isotopomer. This pair of peaks presents itself as the ratio between the isotopomer collection of the C13 IROA Internal Standard (IS) and the isotopomers of the natural abundance monoisotopic peak (C12). This ratio is highly correlated with the original aliquot size and unaffected by the source variance; thus demonstrating that the Internal Standard peaks and the natural abundance peaks for each compound are subject to identical ionization degradations. Since the Internal Standard is always at the same concentration and the ratio between the IS (C13) and C12 peaks correctly reflects the original concentration, correction of the monoisotopic peaks value (height or area) is possible. This experiment demonstrates that this correction applies to source modification as well.

Figure 5: Variances corrected for as:

A) Percent loss to ion suppression vs sample size



B) Loss of peak height



CONCLUSIONS

- **Use of a Long-Term Reference Sample and IROA Internal Standards to correct source variation in large sets of metabolomics runs.**
- **The correction for source variances makes it possible to normalize samples.**
- **The normalization to a "Standard Sample", in this case an Internal Standard of defined size and composition makes it possible to normalize samples across differing source variations.**

REFERENCES

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2. **Stupp GS, Clendinen CS, Ajredini R, Szewc MA, Garrett T, Menger RF, Yost RA, Beecher C, Edison AS. "Isotopic Ratio Outlier Analysis Global Metabolomics of Caenorhabditis elegans." *Analytical Chemistry* 2013 *85*(24), 11858-11865. doi: 10.1021/ac4025413.**

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