Isotopic Ratio Outlier Analysis improves metabolomics prediction of nitrogen treatment in maize

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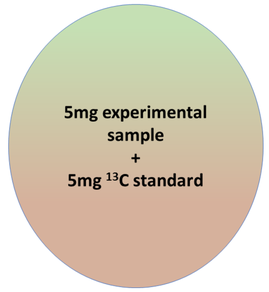
Introduction

We evaluated Isotope Ratio Outlier Analysis (IROA) as a metabolome-wide internal standard approach to improve the quality of LC/MS data. A large-scale greenhouse experiment was designed to metric the ability of metabolomics to model quantitatively nitrogen treatments. We compared IROA processed data with that generated without the benefit of metabolome-wide internal standards using our current tool, Genedata Expressionist, from the same raw LC/MS data files. Corn plants were treated from germination on with varying concentrations of nutrient nitrate. Metabolomics analysis of leaves was performed by LC/MS positive and negative electrospray ionization modes, and raw data were processed with both our routine Genedata software and IROA protocols. Genedata analysis without IROA yielded 281 metabolites in positive ionization mode and 172 in negative ionization mode. IROA data analysis detected 184 metabolites in each ionization mode. We demonstrate that the IROA protocol improve predictive modeling of nitrogen treatment. In addition, IROA corrected for detector saturation for several high abundant peaks.

IROA Processing Workflow

Kernels germinated and plants grown in growth chamber with 95% $^{13}CO_2$

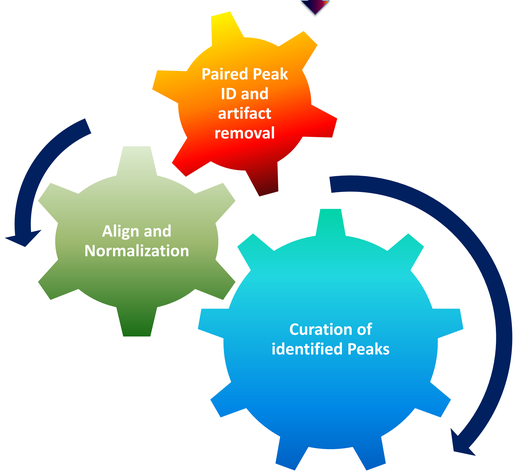
Tissue harvested and stored for analysis



Combine experimental sample and ^{13}C standard in equal amounts and prepare samples

Combined sample extract

Analysis by reversed-phase uHPLC/High Resolution Accurate Mass Spectrometry

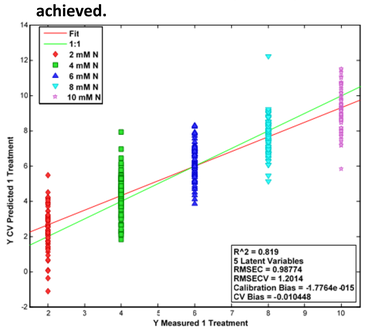


Data Processing with ClusterFinder software

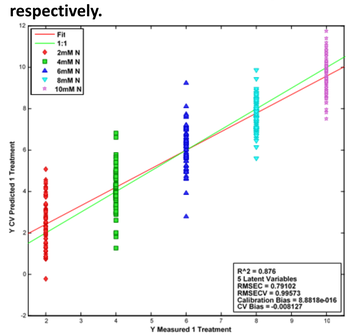
Results for Nitrogen Treatment

Analysis using Genedata yielded 281 metabolites in positive ionization mode and 172 in negative ionization mode. Data from both protocols were normalized for sample dry weight, location in the greenhouse, extraction batch, sample run order, and internal standard. Normalized results were subjected to partial least squares (PLS) analysis to model the relationship between the metabolome and nitrogen treatment.

Without IROA, regression coefficients of 0.819 and 0.849 for positive (shown) and negative modes, respectively were achieved.



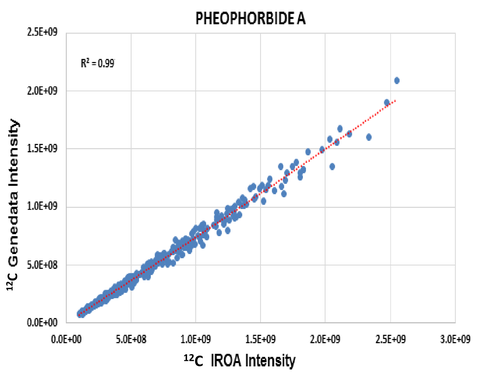
The IROA protocol improved on the values, yielding regression coefficients of 0.876 and 0.879 for positive (Shown) and negative modes, respectively.

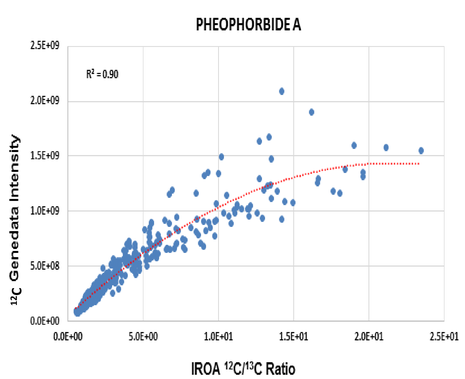


The improved relationship between amount of nitrogen applied and metabolome predicted amount of nitrogen applied can be explained by the very nature of the IROA concept. Since similar maize material was grown in the $^{13}CO_2$ environment, all likely biological metabolites were labeled with a stable isotope. When IROA labeled tissue was mixed with unlabeled experimental tissue, the $^{12}C/^{13}C$ isotopic pairs of metabolites are identified, and their intensity ratios are used for relative quantitation. Therefore, only true biological metabolites are detected.

Evaluation of Detector Saturation

It is well established in the literature that using a stable isotope as an internal standard can account for various deviations in ionization efficiency of electrospray ionization. Stable isotope internal standards are used widely for mass spectrometry based targeted analyses, and as such renders similar benefits to metabolomics. We evaluated very intense peaks to determine if we could account for potential detector saturation using the $^{12}C/^{13}C$ ratios from the IROA workflow when compared to the ^{12}C intensity from the Genedata workflow. Positive ionization mode detection of pheophorbide A is an example of this situation. We correlated ^{12}C intensities from Genedata against ^{12}C intensities from IROA. We found a very high linear correlation (r^2=0.99), indicating that both workflows are consistent in their respective peak detection and integration algorithms. When we correlated the Genedata intensities to the IROA $^{12}C/^{13}C$ ratios, this linear correlation fell to r^2=0.86. This saturation detector signal reduction is better accounted for by the $^{12}C/^{13}C$ ratio as shown using a second order polynomial curve (r^2=0.90).





Conclusons

The IROA workflow improved PLS modeling of nitrogen treatment observed in our study. This enhanced knowledge of the plants' response to applied nitrogen represents a step change in phenotyping the effect of this nutrient stress. In addition, accounting for both detector saturation and electrospray ion suppression by the $^{12}C/^{13}C$ ratios and removal of non-biological signatures improved the quantitative nature of LC/MS metabolomics data.