Abstract
The greatest challenge of most metabolomic profiling experiments is the ability to differentiate peaks of biological origin from artifactual peaks, and to accurately identify and quantify the peaks of interest. The IRoA® (Isotopic Ratio Outlier Analysis) method allows the identification of the isotopically labeled peaks and their C isotopomers, and the removal of artifacts, noise and extraneous peaks. By pooling experimental and Standard samples, variances introduced during sample-preparation and analysis are controlled.

Introduction
This study describes the use of an analytical and bioinformatic metabolomics technology, “IROA Phenotypic Protocol” (Figure 3), applied to the understanding of controlled greenhouse-grown wheat profiles under well-watered or one of two water-stress conditions. The IROA-labeled Standard used was wheat leaves derived from 7 week old Triticum aestivum seedlings grown using 13CO2 as the only carbon source. IROA analysis was performed using ClusterFinder™ software. The 13C IROA peaks in the labeled IROA control Standard were used to identify and quantify their correspondent natural abundance peaks in the greenhouse-grown plants, allowing for a complex targeted analysis. The resulting database was analyzed as discussed in the Methods.

Theory & Discussion
Using the IRoA Phenotypic Protocol, both control Standard and experimental samples are analyzed as a pooled sample so that variability and ion suppression are accounted for, and fewer samples are required to be analyzed. Since the Standard is labeled, and even though the experimental samples do not carry any isotopic labeling, their exact mass and position are established relative to the Standard and noise and artifacts can be removed using software algorithms allowing for a very dramatic reduction in data size. The number of carbons are calculated for each IROA peak and this information together with accurate mass for either of the monoisotopic peaks makes it possible to unambiguously determine the empirical formula for each metabolite below FWHM.

In this experiment a total of 724 IROA peaks were identified over 72 samples analyzed in both positive and negative mode; 8 time points, 3 conditions - well-watered and two different water-stress conditions, using biological triplicates.

Results
This IRoA Phenotypic Protocol was applied to understand the metabolic profile of wheat grown under water-stress conditions. The 13C IROA peaks identified by IRoA control Standard were used to identify and quantify their correspondent natural abundance peaks in the greenhouse-grown plants, allowing for a complex targeted analysis. The resulting database was analyzed as discussed in the Methods.

Summary
This IRoA Phenotypic study was performed in wheat to understand key growth processes including the Krebs cycle, amino acids and lipid metabolism. 724 significantly differentially expressed metabolites were identified, contributing to understanding the unique metabolic profile of drought-stressed wheat.

Methods

Experiment design: Seeds of wheat variety SS8641, developed by Jerry Johnson, University of Georgia were grown at day-night temperature of 20-25°C, relative humidity of 40-42%, 16 day lengthful day until anthesis period. A control group was maintained watered at Field Capacity while two groups (W1, W2) were subjected to water-stress (25%, 50%, FC, respectively). Leaves were harvested 2 weeks post-anthesis every 3hrs during a 24h period, flash frozen in LN2, lyophilized and ground for each sample (24 samples in triplicate, total of 72 samples), 10 mg dry weight was mixed with an equal quantity of dry wheat leaf ground under CO2 maintained at 97.5% 13C and 2.5% 12C (IROA control Standard). The combined wheat leaf samples were held at −80°C until preparation.

Sample Preparation: An aliquot of mixed sample in a small round-bottomed vial, containing approximately 270 mg 0.7 mm zirconia beads (Bio spec Products), two 3 mm borosilicate glass beads (Kimble Chase), was suspended in 800 µl of MAA (MeOH:ACN:acetone, 1:1:1). The vial was capped and homogenized on a bead beater for 10 minutes at maximum speed. After homogenization the vials were centrifuged for 10 min at 4°C, 4,750 RPM. 500 µl of the supernatant was recorded, filtered, and dried under nitrogen. The dried sample was re-dissolved in 50 µl of distilled water for analysis.

LC-MS: Samples were stored in freezer and at 4°C on the instrument autosampler. All data were acquired on the Thermo Scientific Q Exactive using external mass calibration. Sample analyses were conducted in both positive and negative ESI modes as separate LC/MS/MS runs. Full-scan MS were collected at a mass resolution of 70,000 FWHM from m/z 70 – 1000. Data-dependent MS/MS scans were acquired at 17,500 FWHM.

Data processing and analysis: The dataset was analyzed by the IRoA ClusterFinder software (K). The 724 peaks identified tentatively by ClusterFinder were compared against libraries of compounds for their IROA characteristics; 13C base peak, 13C M+1, 13C base peak, 13C M-1, and intervening peaks. The metabolic phenotype of each experimental sample consists of the relative contribution of a large number of metabolites. In the IRoA method, all compound measurements are made relative to a 13C Standard; in this case, an isotopically-labeled wheat Standard (97% 13C enriched, Isotrac). Therefore, these measurements represent the derivation of each metabolite relative to the isotopically-labeled Standard.

References