Structural Elucidation of the Metabolome using Isotopic Ratio Outlier Analysis (IROA) in combination with UHPLC-QTOF and Data-Independent Acquisition

IROA TECHNOLOGIES

Chris Beecher[1]; Felice de Jong[1]; Amrita Cheema[2]; Tyrone Dowdy[2]; Giuseppe Astarita[3]

[1]IROA Technologies LLC, Ann Arbor, MI, [2]Georgetown University, Washington, DC, [3]Waters Corporation, Milford, MA

Waters THE SCIENCE OF WHAT'S POSSIBLE.

Abstract

Metabolite identification represents the bottleneck of most metabolomics studies. This is aggravated by the presence of noise signals, impurities due to sample collection and extraction procedures and other non-biological relevant information. Isotopic Ratio Outlier Analysis (IROA)[1,2] protocol mitigates several of these commonly encountered sources of variance by using specific isotopic signature. Once the biological relevant analytes have been identified, the characterization of their structure often relies only on accurate mass and isotopic pattern. Here, we propose a metabolomics approach using IROA in combination with UHPLC-QTOF in data-independent acquisition (DIA) mode for a rapid screening of the metabolome and the simultaneously collection of both qualitative and quantitative information of known and unknown metabolites.

Introduction

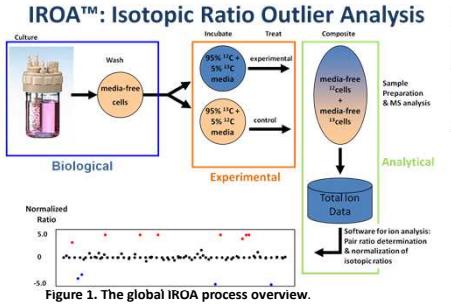


Figure 1. The global IROA process overview.

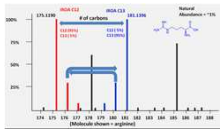


Figure 2. The IROA peaks. In the case of arginine, the ^{12}C M^{+} located at 175.1190 and its ^{13}C mate at 181.1396 clearly indicate a 6 carbon molecule. The corresponding M^{+1} and M^{-1} peaks are plus or minus the mass of a neutron. Natural abundance peaks do not have IROA patterns.

IROA Basic Application

The greatest challenge of most metabolic profiling experiments is the ability to differentiate peaks of biological origin from artifactual peaks, and to accurately identify and quantitate the peaks of interest. The IROA Basic labeling protocol (see Figure 1), utilizes isotopically-defined media in which all nutrients are labeled with either 5%^{13}C, "^{13}C IROA media" (experimental), or 95%^{13}C, "^{13}C IROA media" (control), so that all biological compounds carry a distinct molecular signature and molecules can be distinguished from each sample set, as they have differing masses. Control and experimental samples can be analyzed as a single composite sample by LC-MS with all biological peaks uniquely paired, and unlabeled natural abundance artifacts may be identified and discarded. All biological compounds will have two paired peaks; the peak from the ^{12}C-media is mirrored by a second peak from the ^{13}C-media (see Figure 2). The distance between these peaks readily identifies the number of carbons in the compound. In addition there are corresponding M^{+1} and M^{-1} peaks (and M^{+2} and M^{-2} etc. peaks) which are a mass difference of 1.00335 amu (the mass difference between a ^{12}C and ^{13}C isotope), giving the IROA peaks a characteristic U-shape "smile" pattern. Accurate mass together with the knowledge of the number of carbons in a molecule greatly facilitates metabolite identification.

Theory & Discussion

Using the IROA Basic Protocol[1,2] biological compounds from samples associated with 95% ^{13}C and 5% ^{13}C media are differentiatable and therefore control and experimental samples can be pooled and prepared simultaneously, removing sample-to-sample variance and ion suppression. Software algorithms can remove artifactual information identified by their absence of isotopic signature allowing for a very dramatic reduction in data size. The identification of each compound enabled by the use of ultra-high resolution mass measurement and the knowledge of the number of carbons in each molecule make it possible to determine the empirical formula, unambiguously for masses below 400.

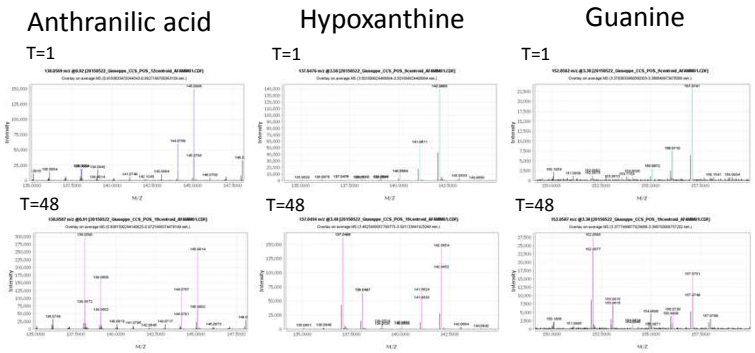
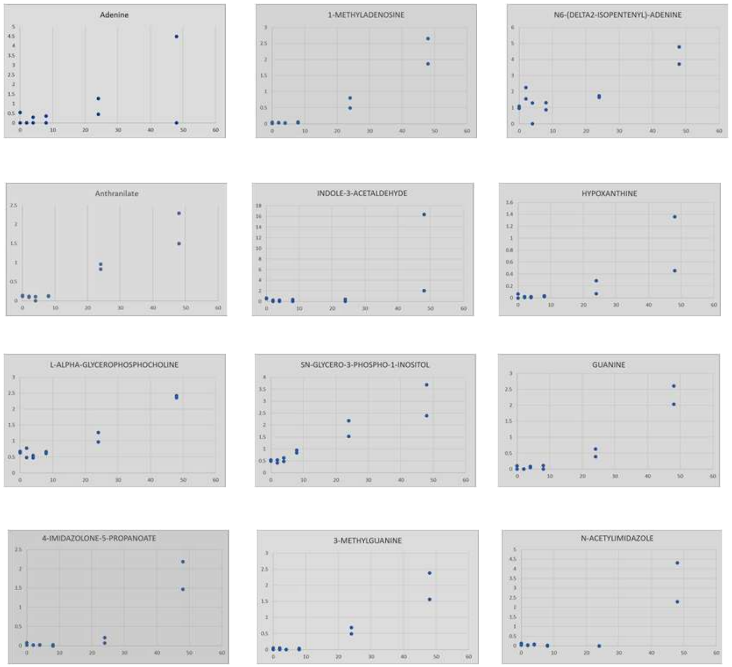


Figure 4. Representative IROA peaks. These peaks are representative of the 406 IROA peaks.

Results

We rapidly screened and identified metabolites produced by yeast using the analytical and bioinformatic metabolomics technology, "IROA Basic Protocol" (Figure 1) in combination with high resolution and mass accuracy SYNAPT G2-Si analysis[3]. Pooling medium from samples in which yeast were grown in 95% and 5% ^{13}C media allowed all artifacts (compounds not of biological origin) to be recognized by their absence of isotopic signatures and removed. IROA ClusterFinder™ software calculated the number of carbons in each IROA peak and with accurate mass, used to determine the molecular formula of each metabolite with high confidence. Peaks of biological origin were perfectly paired: each IROA envelope is half control and half experimental. The ratio of (95% 13C/ 5% 13C) paired peaks can be used to determine which metabolic pools are affected by treatments. Molecular formulae were calculated to provide tentative identification for previously unknown metabolites.



Methods

Experiment design and sample preparation: Following the IROA Basic protocol, two 1L fermentations of yeast (*Saccharomyces cerevisiae* S288C)[4] were grown in a 2L fermenter where the carbon source contained either 5% U-^{13}C glucose* ("experimental") or 95% U-^{13}C glucose* ("control") until all the original yeast metabolic pools were replaced by the labeled carbon. 2 mL aliquots were acquired from the fermenter at T= 0, 2, 4, 8, 24, and 48 hours. Samples were pelleted and the media supernatants collected. Pellets were washed by suspension in buffered saline, repelleted, flash frozen and stored at -80° C for subsequent experiments. A media Control Standard was created by mixing equal volumes of 95% U-^{13}C glucose* ("control") supernatant collected at T= 2, 24, 48 hours. Equal quantities of media control Standard were mixed with experimental samples collected at T= 0, 2, 4, 8, 24, and 48 hours. These pooled IROA media samples were frozen at -80° C until analysis. *Produced especially for IROA Technologies by Cambridge Isotope Laboratories (CIL).

LC-MS: Chromatographic separation was achieved using an ACQUITY UPLC system (UPLC ACQUITY, Waters Corporation, Milford, MA) and hydrophilic-interaction liquid chromatography (HILIC) using a 1.7-µm (2.1 mm × 150 mm) ACQUITY amide column (Waters Corporation). Samples and standard mixtures were analyzed in UPLC–HILIC-HDMSE, in positive ionization mode using acid chromatographic conditions. Solvent A was water+0.1% formic acid and solvent B was acetonitrile +0.1% formic acid. The gradient was: At 0 min, 99% B; 6 min, 40% A; 8 min, 99% B; 10 min, 99% B. The flow rate was 0.4 mL/min, the column temperature 45 °C, and the injection volume 1.5 µL[3].

The data were acquired on a SYNAPT G2-Si High Definition traveling-wave ion mobility mass spectrometer (Waters Corporation). In positive electrospray mode, the capillary and cone voltage were 2 kV and 25 V, respectively. The source and desolvation temperature were 100 and 450 °C, respectively, and the desolvation gas flow was 800 L/h. Nitrogen, the IMS gas, flowed at a rate of 90 mL/min (3.2 mbar), with a wave velocity of 650 m/s and wave height of 40 V.

Data processing and analysis: The dataset was analyzed by the IROA ClusterFinder software (K). The 406 peaks identified tentatively by ClusterFinder were compared against libraries of compounds for their IROA characteristics; ^{12}C base peak, ^{12}C M+1, ^{13}C base peak, ^{13}C M-1, and intervening peaks.

The complete dataset was subjected to a linear regression to identify all compounds that demonstrated time dependent patterns. Additional analyses will be performed, including analyses of the fragmentation and ion-drift datasets.

References

1. de Jong F, Beecher C, "Addressing the current bottlenecks of metabolomics: Isotopic Ratio Outlier Analysis (IROA®), an isotopic-labeling technique for accurate biochemical profiling." *Bioanalysis*, 2012, 4(18), 2303-14.
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.
3. Paglia G, Williams JP, Menikarachchi L, Thompson JW, Tyldesley-Worster R, Halldorsson S, Rolfsson O, Moseley A, Grant D, Langridge J, Palsson BO, Astarita G. "Ion mobility derived collision cross sections to support metabolomics applications." *Analytical Chemistry,* 2014, 86:3985-3993
4. Mortimer RK, Johnston JR. "Genealogy of principal strains of the Yeast Genetic Stock Center." *Genetics,* 1986, 113, 55-43.

Summary

We conducted metabolomics analyses of biological samples using a SYNAPT G2-Si high resolution mass spectrometer as an aid in the ability to separate and identity small biochemical compounds when coupled with the IROA protocol. Only a few of 406 molecules identified as being produced by the yeast are shown. Who would ever have guessed that yeast did this? Something to think about over a glass of wine . . .