A NOVEL LABELED METABOLOMICS WORKFLOW APPLYING ISOTOPE RATIO OUTLIER ANALYSIS (IROA) AND SWATH® ACQUISITION FOR UNAMBIGUOUS COMPOUND IDENTIFICATION

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Introduction:
Metabolomics focuses on the chemical processes central to cellular metabolism. Mass spectrometry and specifically data dependent workflowstend to be the choice for the measurement of these metabolites. Data independent techniques such as SWATH® Acquisition are different in that they allow for unbiased data collection and MSMS of every single mass precursor can be collected allowing for information rich datasets. However, unambiguous metabolite identification can be increasingly challenging due to the lack of databases, chemical noise and isobaric compounds. The SWATH analysis of the Isotope Ratio Outlier Analysis (IROA) labeled Internal Standard (IS) provides the first mechanism for simultaneous and unambiguous compound identification and quantitation for unbiased metabolomics analysis.

Methods:
A biochemically complex Internal Standard (IS) which contains 100's of biochemicals, each with an IROA isotopic pattern, was added to clinical samples to accurately identify and quantitate complex mixtures without the need for baseline separation, and overcome variances introduced sample-to-sample or by ion suppression. SWATH fragmentation of the IROA peaks completely differentiates fragments, and artifacts. The identification of all IROA compounds and their fragments by ultra-high-resolution mass measurement make it possible to determine the empirical formula for all fragments. Data were collected a TripleTOF® 6600 System in SWATH® Acquisition using a variable window strategy which defined windows of varying mass ranges to be applied in areas of the chromatogram where there are many ions co-eluting.

Results:
Applying a variable window SWATH Acquisition strategy to an IROA Internal Standard (IS) spiked sample made it possible to unambiguously identify and accurately quantify hundreds of biochemicals in a single unbiased metabolomics analysis using a TripleTOF® high resolution mass spectrometer. The IS contains 500+ well characterized compounds, which migrated in an HPLC separation with their natural abundance isotopomers, to provide for both identification and standard quantities for accurate measurement even in a non-baseline separated, "unbiased" metabolomics separation. Using traditional DIA, all compounds with the same retention time are fragmented without selection. SWATH DIA subjects all ions within a discrete m/z window to fragmentation allowing specific precursor ions to be selected, making it easier to analyze fragmentation spectra. However, a corresponding spectral library of metabolites is required for accurate identification. Here, we present SWATH-IROA DIA whereby uniquely-labeled IROA metabolites were captured within discrete SWATH windows and subjected to fragmentation. IROA fragments and adducts were shown to have the identical labeling patterns of their precursor ions, with defined formulae. All artifactual (non-IROA) peaks from the SWATH window were eliminated and data was quantitated based on MSMS peaks. The combination of IROA and SWATH allow a path in which a basic metabolomic-style system may be used for the accurate clinical quantitation of several hundred compounds in a single sample without the need for a base-line separation. Specific software was developed to automatically find, quantitate and identify all natural-abundance peaks that corresponded to their known IROA isotopomers. The identification of compounds of unknown identity is simplified because all fragments are identified by their complete formula making the mode of fragmentation of the parent compound clear. Fragments are identified as they share the daughter fragments of their parent compounds.