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A Novel Labeled Metabolomics Workflow applying Isotope Ratio Outlier Analysis (IROA) and SWATH® Acquisition for Unambiguous Compound Identification

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ABSTRACT

Metabolomics focuses on the chemical processes central to cellular metabolism. Mass spectrometry and specifically data dependent workflows tend 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.

INTRODUCTION

IROA® is an isotopic methodology in which all biological molecules are uniformly and randomly labeled to create informative isotopic patterns that are readily discriminated from artifacts (See Figure 1). The IROA protocol generates an IROA Internal Standard providing specific molecular information so that the small biochemical molecules within biological samples may be easily and more accurately identified (See Figure 2). Because of the uniform nature of the labeling these patterns show up in not only the ms, but also in all fragments in any subsequent MS/MS. SWATH® acquisition, a data independent acquisition (DIA) workflow is well adopted in quantitative discovery proteomics, but still not commonly used in discovery metabolomics. SWATH allows a user to collect MS and MSMS of every detectable peak in their sample thus creating a digital map of the metabolome. Variable Window SWATH (an enhanced way of collecting MSMS, using narrower mass windows in denser regions of the MS spectrum) allows for specificity. The use of IROA with a SWATH data collection system has allowed us to collect extremely advanced information for the biological components of a mixture, with significantly enhanced accurate identification and quantitation, clearly differentiating MS/MS SWATH-IROA peaks derived from artifacts. The MSMS IROA patter can be observed because we can vary the mass window overlap during SWATH acquisition. IROA always correctly assigns formula to all IROA peaks; this is the first example in which the correct formula for not only the parent peak but also all fragments is routinely found. Quantitation of any compound may be done at either the MS or MS/MS level.

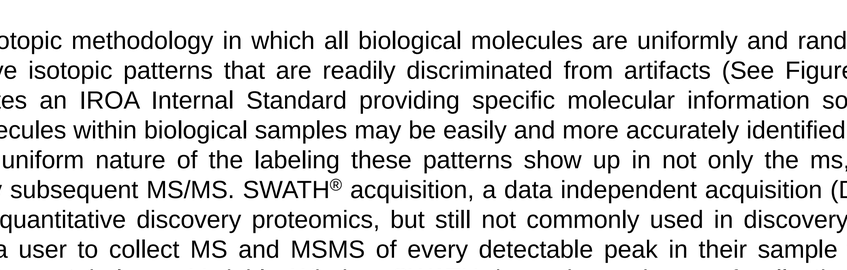


Figure 1: The IROA Basic Protocol.
The experimental and control samples are labeled differently. The control samples make up an internal standard against which every compound present in either sample may be quantified and identified. The heights and distances between IROA peaks are all mathematically calculable and IROA ClusterFinder algorithms are used to remove irrelevant data, identify and quantitate molecules of interest.

MATERIALS AND 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.

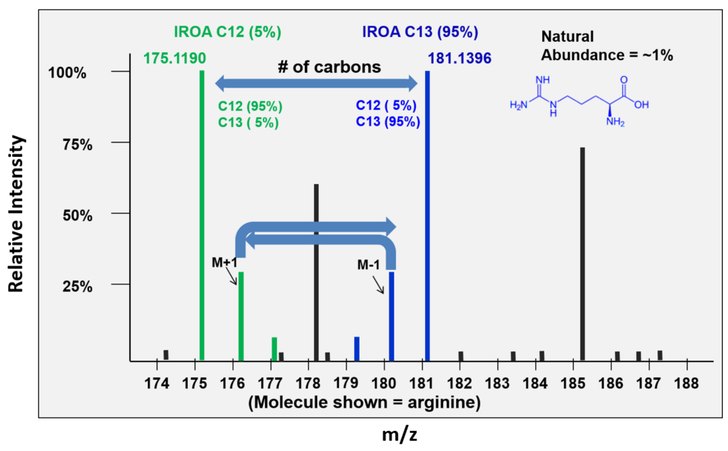


Figure 2: The IROA peaks
The IROA peaks shown in this figure are for the 6-carbon molecule arginine. There are both the peaks from the experimental samples (shown in green) at 5% U-13C, and the Internal Standard or control samples (shown in blue) at 5% U-13C. This system represents a triply redundant information system; the number of carbons is verified by the relative heights of the M+1 and M-1 and the distance between the monoisotopic peaks. The formula for the molecule is constrained by the number of carbons.

Chromatography: The reverse phase HPLC separation was performed using a Shimadzu LC System, operating at a flow rate of 350 µL/min. An Ace Excel C18-PFP column (100 x 1mm, 2 µm) was maintained at 30 ºC. A standard reverse phase gradient was used employing mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. The injection volume was 3 µL in positive ion mode and 5 µL in negative ion mode.

Mass Spectrometry: Raw data was collected on the TripleTOF® 6600 System (SCIEX) using SWATH® Acquisition with variable-width precursor isolation windows. The TOF-MS mass range analyzed was 50-1000 m/z and the MS/MS was acquired with a mass range of 40-1000 m/z with a 25 msec accumulation time. The mass window overlap was set across the mass range to reflect the diversity of labeled metabolites. The collision energy was set to 35 V with a 15 V collision energy spread.

Data Processing: The IROA ClusterFinder software was adapted to handle the SWATH® Acquisition data; specific SWATH windows for every IROA peak were identified and then the appropriate scans examined for their IROA fragments. In order to assure that all IROA peaks would be captured, a specific SWATH windowing protocol was developed (See Figure 3). This windowing protocol assured that, unlike other MS/MS selection protocols, for every IROA peak there was an optimal fragmentation scan. Once ClusterFinder identifies an IROA peak it automatically retrieves the correct SWATH scan. This is extremely efficient and accurate. If all IROA fragments are sorted by descending mass, since they all have formulae, the full fragmentation history of the molecule is known. This happens for all molecules and is the only method of doing this at this time.

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.

Max # carbons below center	center	window	overlap	count	min	max	SWATH Window	Overlap
3	59	10	5	1	49	69	Window = 20	= 10
4	74	15	8	2	59	89	Window = 30	= 15
5	89	15	8	3	74	104		
7	119	30	15	4	89	149		
9	149	30	15	5	119	179		
13	209	60	30	6	149	269		
17	269	60	30	7	209	329		
27	389	120	60	8	269	509		
36	509	120	60	9	389	629		
54	749	240	120	10	509	989		

Figure 3: SWATH® Acquisition Variable Window example.
Highlighting the window size in red arrows and the mass window overlap in green arrows.

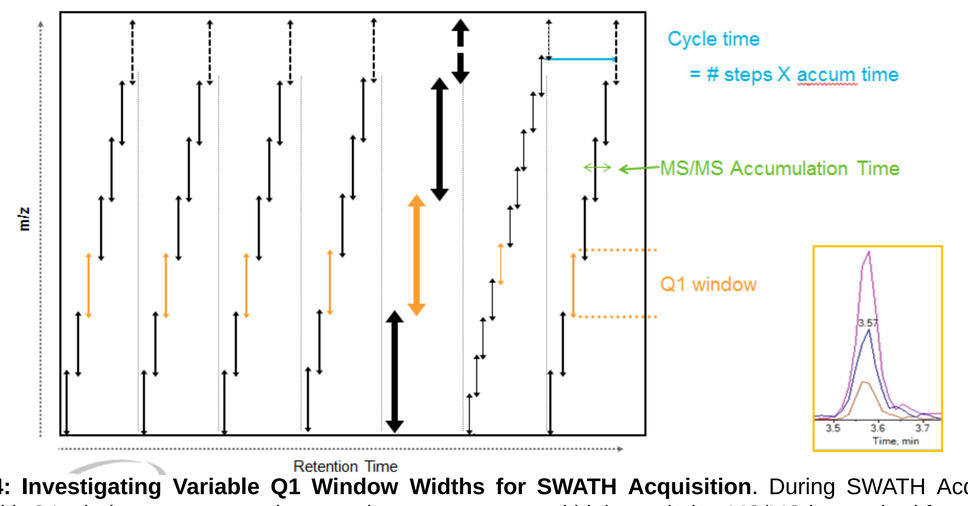


Figure 4: Investigating Variable Q1 Window Widths for SWATH Acquisition. During SWATH Acquisition, wider width Q1 windows are stepped across the mass range, and high resolution MS/MS is acquired for a specific accumulation time (top).To achieve better specificity in complex matrices, smaller Q1 windows are desirable especially in the m/z dense regions where many peptide precursors are measured. The m/z density histograms constructed from the TOF MS data for the proteome of interest (bottom, blue line) can be used to construct variable sized windows, where the density of precursors in each of the isolation windows is equalized across the m/z range.

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 artefactual (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.

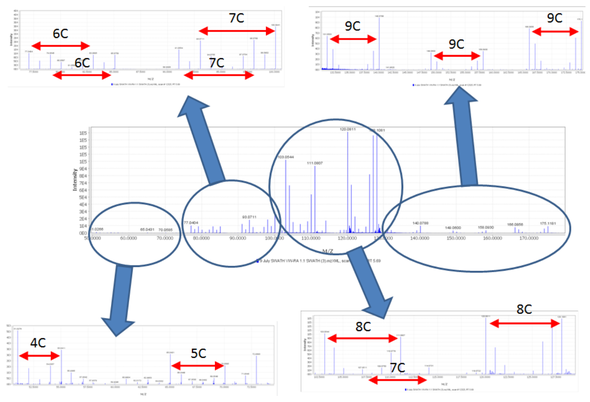
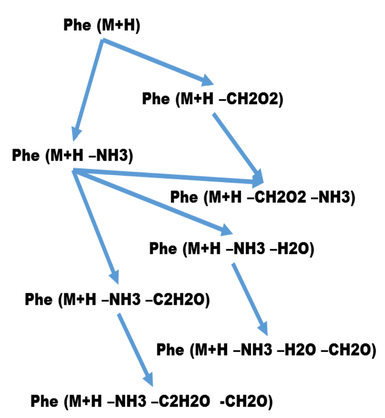


Figure 6: IROA/SWATH fragmentation.
The fragmentation of phenylalanine is seen in the central SWATH scan (A). ClusterFinder detected all of the IROA peaks in this scan (B & C). Note that for each peak the number of carbons in the fragment can be determined, and therefore the formula for the fragment is known (D). ClusterFinder Represents them both as (D) & (E). The actual fragmentation path (F) is easily determined from (E).

Figure 5: SWATH-IROA fragmentation.
The fragmentation of phenylalanine is seen in the central SWATH scan (A). ClusterFinder detected all of the IROA peaks in this scan (B & C). 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.

CONCLUSIONS

The IROA / SWATH protocol presented here is a powerful toolset for the assured identification of any biological compound, the determination of its structure, via fully identified fragmentation, and the complete quantification of all of the components of complex biological mixture. Because of the nature of the IROA and SWATH routines these processes are completely automatable and completely reproducible. Metabolites and their fragments may not be confused with artifacts, or noisy peaks. The fragmentation path attributable from the combination of IROA labeling and SWATH variable window acquisition reinforces the identity of the molecule and data quality. We see additional opportunity for improvement but already believe this system, in terms of assured quantitation and compound identification, will produce higher quality data that any other mass spectrometer-based system, and should be a useful adjunct in metabolomics and eventually clinical measurements.



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