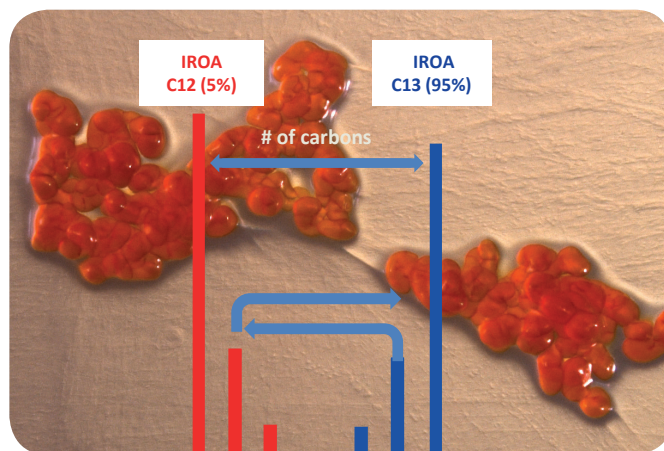




Application Note LCMS-88

Isotopic Ratio Outlier Analysis (IROA®) coupled with the Bruker maXis 4G QTOF to investigate changes in the secondary metabolite profiles of Myxobacteria

Introduction

Metabolite profiling is increasingly becoming a standard tool to analyze the biochemical composition of organisms between different individuals or populations in many applications. Strategic “metabolome mining” [1] has become the new frontier for discovering novel natural compounds as a source for potential pharmaceutical lead molecules. However, there are challenges in measuring the entire metabolome in any sample to find unique metabolites. Analytically, it is critical to eliminate sample-to-sample variance and to avoid detecting artificial differences related to ion suppression. The most significant problems in data analysis stem from the inability to discriminate biological data from noise and artifacts, and the clear identification of unknown compounds.

The Isotopic Outlier Analysis (IROA) tools were specifically developed to address many of the bottlenecks associated with metabolite profiling. The novel IROA isotopic labelling system and IROA ClusterFinder™ software provide a unique method to create distinct biochemical signatures in every molecule, providing a full metabolome complement of labeled internal standards enabling global metabolic identification and quantitation [2].

Authors

Daniel Krug^{1,2}, Ullrich Scheid¹, Aiko Barsch³, Sandy Yates³, Gabriela Zurek³, Rolf Müller^{1,2}, Chris Beecher⁴, Felice de Jong⁴

¹Helmholtz Institute for Pharmaceutical Research, Saarland (HIPS), Saarbrücken, Germany

²Saarland University, Saarbrücken, Germany

³Bruker Daltonik GmbH, Bremen, Germany

⁴IROA Technologies, Ann Arbor, Michigan, USA

Keywords	Instrumentation and Software
IROA	IROA
Metabolomics	ClusterFinder™
Quantitation/Identification of Unknowns	SmartFormula
Myxobacteria	maXis 4G

Myxobacteria represent an important source of novel natural products exhibiting a wide range of biological activities [1]. As a proof of concept study, we employed the IROA tools coupled with the Bruker Daltonik UHR-QTOF instrument (maXis 4G) and complementary SmartFormula™ software to analyze the secondary metabolome of myxobacteria to detect changes triggered by differential iron supply. The task was to clearly and unambiguously identify novel compounds on the basis of their differential appearance in sample groups. These newer metabolic profiling methods hold great promise for uncovering novel secondary metabolites from myxobacterial strains, as the number of known compounds identified to date is often significantly lower than expected from genome sequence information [3, 4].

IROA as a protocol for metabolic profiling

The IROA method is not a targeted analysis, but instead utilizes full metabolic labeling, like SILAC, but uses specifically labeled materials with universal enrichments centered on either 5% or 95% ^{13}C creating an isotopic signature in every molecule that imparts many analytical advantages compared to standard isotopic labeling. The monoisotopic peak in non-labeled samples can usually be detected even if its intensity is low, but for these minor peaks the M+1 peak can be lost in the noise because of its small size (e.g. the M+1 peak is only 6% of the height of the monoisotopic peak for a 6 carbon molecule). When the percentage of ^{13}C is increased to 5% using comprehensive (>98%) labeling, then the M+1 for a six-carbon molecule is significantly larger, namely 32% (Figure 1). Likewise, when the percentage of ^{13}C is increased to 95%, then the M-1 for a six-carbon molecule is also 32% the height

of the monoisotopic peak. With the IROA- ^{13}C - labeling approach, the number of carbons in a biological molecule can be determined by the distance between the two monoisotopic peaks, ^{12}C and ^{13}C , and the relative height of the M+1 and M-1 provide confirmation of this fact, resulting in a triply redundant quality control check point. When armed with the knowledge about the number of carbons present in an unknown molecule the ability to calculate the remaining elements is significantly enhanced. A further advantage of the IROA approach for the analysis of complex metabolomics samples is the fact that artifacts and noise will contain no labeling signatures and are easily removed from the final analysis. Biological compounds from samples associated with 95% ^{13}C and 5% ^{13}C media are differentiable by MS analysis and therefore control and experimental samples can be pooled and prepared simultaneously, removing sample-to-sample variance and ion suppression effects.

On the analytical side, it is obvious that the IROA approach relies on instruments providing accurate measures for isotopic abundances across a wide linear dynamic range. This is important not only for the identification of all “non-carbon” atoms in unknown compounds but also for providing reliable quantitative information (the IROA ratio). Here, the IROA approach can benefit from the well-known True Isotopic Pattern (TIP™) provided by all of Bruker’s QTOF instrument platforms – isotopic intensities are measured with high accuracy across a wide dynamic range of intensities [5]. As shown in Figure 2 Phenalamid A1, a compound contained in a non-labeled myxobacterial extract can be reliably identified based on the accurate mass and isotopic fit (expressed in a low mSigma value).

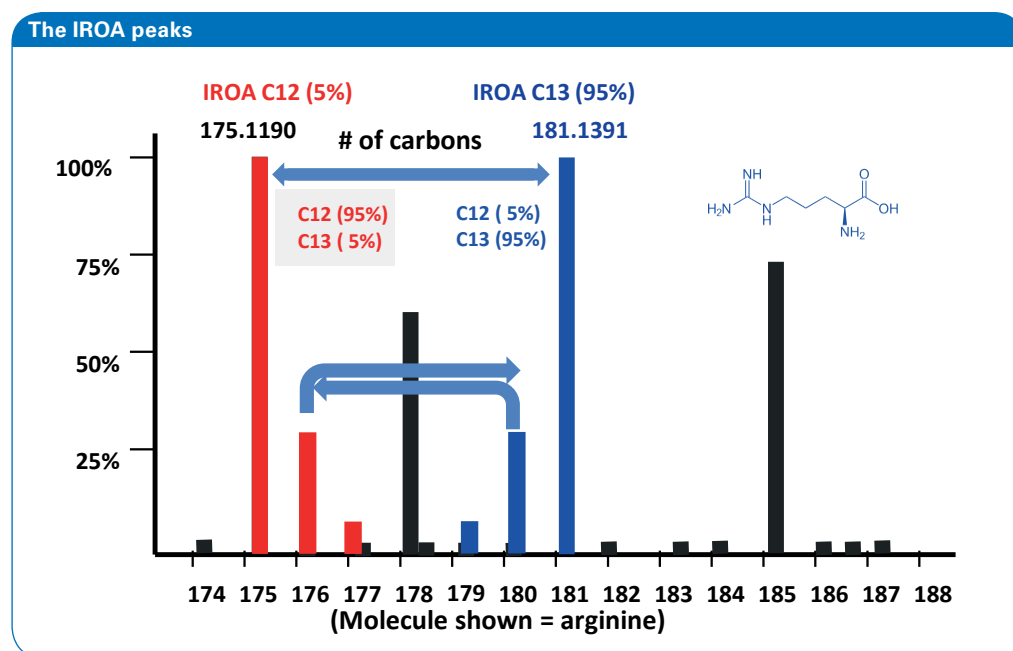


Figure 1: The IROA peaks are calculable. In the case of arginine, the ^{12}C [M+H]⁺ located at 175.1190 and its ^{13}C mate at 181.1391 clearly indicate a 6 carbon molecule. The corresponding M+1 and M-1 peaks show a mass difference of 1.00335 amu (the mass difference between a ^{13}C and ^{12}C isotope). Natural abundance peaks from exogenous sources, shown as black peaks, do not have a ^{13}C IROA counterpart and are not considered in the analysis. (picture adapted from [2]).

Myxobacteria secondary metabolite extract analyzed by maXis 4G UHR-QTOF

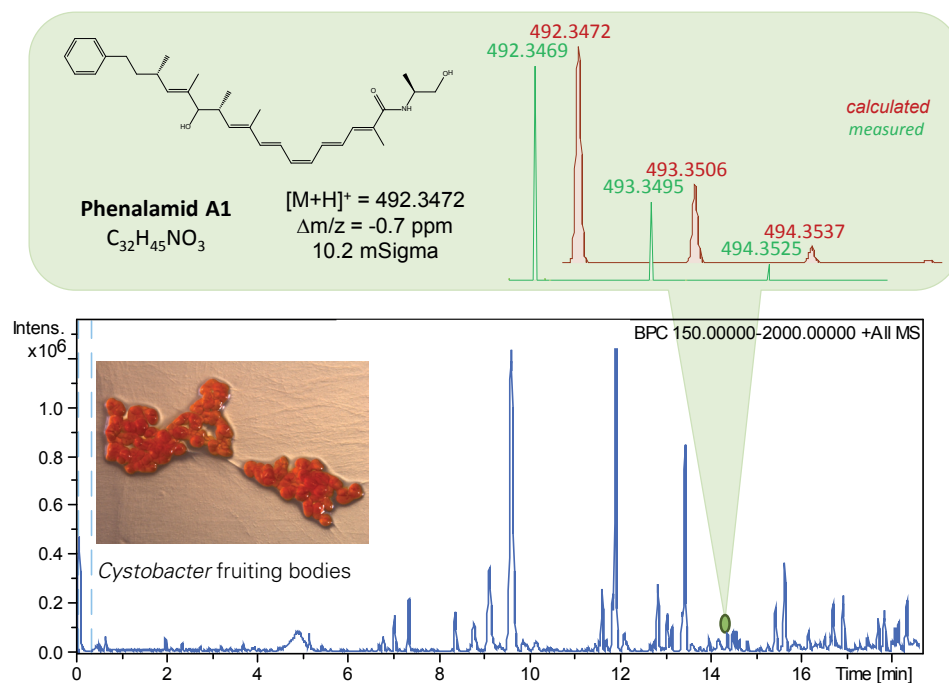


Figure 2: Separation of a crude extract from the *Myxobacteria* strain used in this study. Highlighted is a comparison of measured and calculated spectra for the myxobacterial secondary metabolite Phenalamid A (with a natural content of carbon, i.e. a sample that was not IROA labeled).

Experimental

Myxobacteria strains of control or experimental culture were maintained in yeast-based VY/2 media in which all of the carbon was isotopically defined (95% or 5% ^{13}C , respectively) [Figure 3]. Two successive rounds of cultivation were performed (96 hours each) in order to transform bacterial cell mass to the isotopic balance of the media. Cell pellets and adsorber resin were harvested, equal aliquots of 95%- and 5%- ^{13}C material were pooled and extracted.

Extracts were separated using a Waters BEH C18 column on an UltiMate 3000TM RSLC system (Dionex). Gradient elution was at 0.6 ml/min (45 °C) using H_2O + 0.1 % formic acid (A) and acetonitrile + 0.1 % formic acid (B). The gradient started at 5 % B for 0.5 min, increasing to 95 % B in another 19 min. MS analysis was performed with a UHR-QTOF instrument (Bruker Daltonik maXis 4G).

The IROA ClusterFinderTM software (IROA Technologies) was used to perform a scan-by-scan analysis of the complete dataset and identify all IROA peaks based on their extended isotopic envelopes. SmartFormulaTM (part of DataAnalysis 4.1 software; Bruker Daltonik) was used to confirm the molecular formulae generated by the ClusterFinderTM software.

Results and Discussion

IROA as a protocol for metabolic profiling

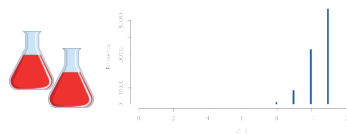
The IROA protocol [2] was applied to the analysis of myxobacterial secondary metabolomes. Biological compounds from samples associated with 95% ^{13}C and 5% ^{13}C media are differentiable by MS and therefore control and experimental samples could be pooled and prepared simultaneously, removing sample-to-sample variance and artifacts caused by ion suppression. Background ions could be identified by their absence of an IROA isotopic signature and were automatically removed. Compound identification was enabled by the use of ultra-high resolution mass measurements and the knowledge of the number of carbons in each molecule.

Quantitation of changes in myxobacterial secondary metabolomes – IROA ClusterFinder software

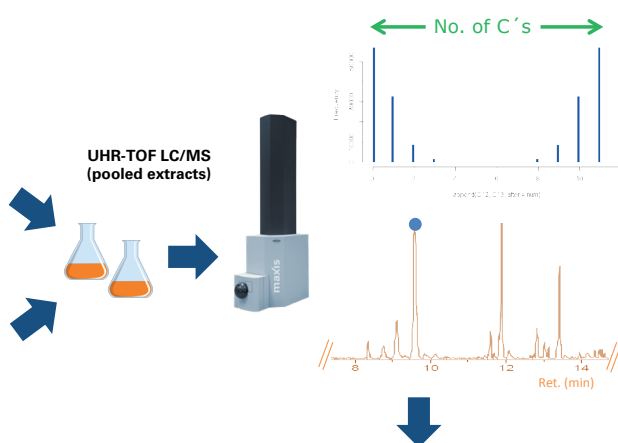
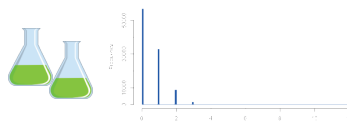
As the IROA peaks are all mathematically calculable, the IROA ClusterFinder software algorithms were generated to achieve a data reduction of complex raw data, resulting in concise, high value information. This is achieved by characterizing all peaks according to source (artifact, experimental (^{12}C); control (^{13}C), or standard), removing all artifacts, aligning and pairing all remaining peaks across all scans and identifying and determining the relative $^{12}C/^{13}C$ ratios of analytes in each sample.

The workflow for IROA analysis

A 95%-¹³C media & high Fe



5%-¹³C media & low Fe



B Myxovalargin A

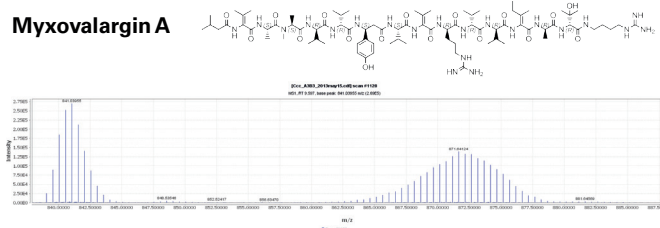


Figure 3: A) The *Myxobacteria* control and experimental strains under investigation are grown using yeast-based media with defined 95%-¹³C and 5%-¹³C isotope signatures, respectively. Experimental and control groups may differ by cultivation conditions or genetic context. Here high and low iron supplies were compared. B) Myxovalargin A, a peptide antibiotic known from myxobacteria, was detected based on the IROA pattern by the ClusterFinder software. The IROA isotopic ratios also enabled to readily identify the correct number of carbon atoms in the molecule; the first step for unambiguous molecular formula generation.

Here we used differential iron supply as a test case for IROA with secondary metabolite producing myxobacteria (Figure 3). In this experiment all compounds known to be present in the bacterial strain from previous results (data not shown) were identified, and a number of novel molecules were identified by examination of their IROA patterns (Figure 5). The formulae of these peaks have been tentatively determined and the identity of these molecules is currently being examined.

Identification of novel metabolites through generation of reliable molecular formulae

The IROA protocol enforces that the number of carbons in any mass spectral object is always known. When this is combined with high resolution mass spectrometry the formula for small molecules may be unambiguously determined. Since only compounds of biological origin can develop IROA patterns, all IROA peaks are of biological origin, and all artifacts are correctly identified as artifacts. The ClusterFinder software identified 160 IROA peaks, at the most stringent level in the *Myxobacteria* metabolite extract (an additional 200+ were tagged for manual curation).

The carbon envelope shapes are exactly calculable. While despite containing the most abundant isotope for each atom, the monoisotopic peak is not always the most abundant isotopic peak in the spectrum. This is observed because as the number of atoms in a molecule increases, the probability that the entire molecule contains at least one heavy isotope also increases. Thus the shape of the carbon envelope changes dramatically as they become more complex with size as seen when comparing the carbon envelope for myristic acid, 16 carbons (Figure 4), with that of the carbon envelope for the 81 carbon molecule Myxovalargin A in Figure 3.

The iron limitation in the media (5%-¹³C culture) significantly changed the levels of many compounds. An example of an unknown compound with higher abundance in high-Fe conditions is shown in Figure 5. The IROA ratio is the difference between the metabolic pool sizes of the control and experimental. Here the ratio is 0.16, clearly demonstrating a significant down regulation in low-Fe conditions. The distance between the monoisotopic peaks in the IROA carbon envelope provides the exact number of 24 C atoms contained in the molecule.

Complementary to the IROA ClusterFinder, the analysis tool SmartFormula™ allowed for the verification of molecular formula suggestions by combining accurate mass and isotopic pattern information. Typically the natural abundance of carbon is utilized by SmartFormula to calculate the elemental composition for unknown compounds. Using the Bruker PeriodicTable Editor the non-natural IROA carbon isotopic ratio was defined (95% ^{12}C , 5% ^{13}C). Using this new "IROA *C12" element and exactly 24 C atoms as input for molecular formula generation one hit is returned: $\text{C}_{24}\text{H}_{49}\text{NO}_7\text{P}$. A public database query indicates that this unknown compound could be a phospholipid. Since IROA compounds are universally, and uniformly labeled the fragmentation of an IROA peak yields IROA fragments that are equally easy to apply an exact formula to.

Summary

- Comprehensive secondary metabolome analysis of myxobacteria using Isotopic Ratio Outlier Analysis (IROA) and maXis 4G UHR-QTOF
- Reliable relative quantitation of known and unknown myxobacterial metabolites in response to differential iron supply
- Avoiding ion suppression artifacts and easily removing background ions by the IROA approach
- Compound identification facilitated by the use of ultra-high resolution MS and the knowledge of the number of carbons in each molecule due to IROA

IROA pattern: curation of myristic acid

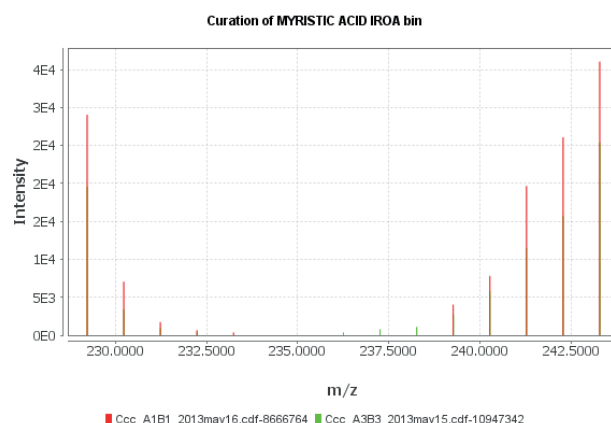
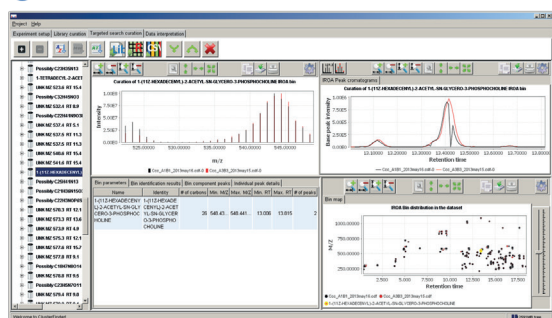


Figure 4: Curation of myristic acid reveals a typical carbon envelope ('smile') shape for a 16 carbon molecule. This carbon envelope shape changes with more complex molecules although it can always be calculated.

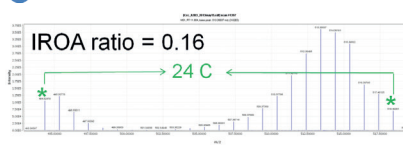
Figure 5: A) IROA Technologies ClusterFinder: Detecting changes in the secondary metabolome of the myxobacterial strain under investigation triggered by differential iron supply in high-Fe cultivations (95% ^{13}C) and low-Fe (5% ^{13}C). B) Example of unknown compound with higher abundance in high-Fe conditions. The IROA ratio is the difference between the metabolic pool sizes of the control and experimental. IROA Peak difference provides the exact number of 24 C atoms contained in the molecule. C) Molecular formulae generation by Bruker's SmartFormula: In the PeriodicTable Editor the non-natural IROA carbon isotopic ratio is defined (95% ^{12}C , 5% ^{13}C). Using this new IROA *C12 element and exactly 24 C atoms as input for molecular formula generation one hit is returned: $\text{C}_{24}\text{H}_{49}\text{NO}_7\text{P}$. A public database query indicates that this unknown compound could be a phospholipid.

Data analysis

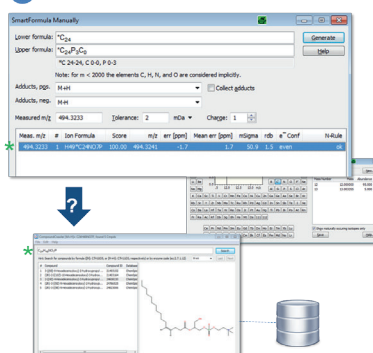
A ClusterFinder



B



C SmartFormula



Acknowledgement

Dr. Chris Beecher, IROA Technologies
Board Member, Founder and Chief Scientific Officer

We have found a win-win situation working with this instrument. The combination of extreme resolution and outstanding isotopic pattern accuracy found in the Bruker instrumentation allows the researcher to obtain high precision in finding peaks, which is also ideal for the IROA protocol.



References

- [1] "Discovering the hidden secondary metabolome of *Myxococcus xanthus*; a study of intraspecific diversity", Appl. Environ Microbiol., 2008, 74, p. 3058-3068
- [2] "Addressing the current bottlenecks of metabolomics: Isotopic Ratio Outlier Analysis (IROA®), an isotopic-labeling technique for accurate biochemical profiling", Bioanalysis, 2012, 4(18), p. 2303-14
- [3] "Myxoprincomide; a natural product from *Myxococcus xanthus* discovered by comprehensive analysis of the secondary metabolome", Angew. Chem. Int. Ed., 2012, 51(3), p. 811-816
- [4] "The biosynthetic potential of myxobacteria and their impact on drug discovery", Curr. Opin Drug Disc Dev., 2009, 12(2), p. 220-230
- [5] "SmartFormula 3D – the New Dimension in Substance Identification – From Mass Spectrum to Chemical Formula" Bruker Technical Note #TN-26

For research use only. Not for use in diagnostic procedures.

● Bruker Daltonik GmbH

Bremen · Germany
Phone +49 (0)421-2205-0
Fax +49 (0)421-2205-103
sales@bdal.de

www.bruker.com

Bruker Daltonics Inc.

Billerica, MA · USA
Phone +1 (978) 663-3660
Fax +1 (978) 667-5993
ms-sales@bdal.com

Fremont, CA · USA
Phone +1 (510) 683-4300
Fax +1 (510) 687-1217
cam-sales@bruker.com