Carbon and Nitrogen Positional Isotopomer Determination in Metabolites using Orbitrap IQ-X and Stellar™ MS - a novel hybrid nominal mass instrument

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Abstract

Purpose: Development of a targeted MS^2/MS^3 approach for the determination of positional isotopomers.

Methods: Initial data acquired on Thermo Scientific™ Orbitrap IQ-X™ Tribrid Mass Spectrometer was used for the structural annotation. A targeted MS^n based assay utilizing the above data to get the structural information of the metabolites was developed on a novel hybrid nominal mass instrument.

Results: A targeted MS^2/MS^3 method was established for the determination of positional isotopomers in Adenosine Monophosphate (AMP). The method was used for evaluation of the effect of hypoxia on cellular metabolism in an *in-vitro* model.

Introduction

Fluxomics or flux analysis aims to quantify metabolism by computing the flow of carbon or nitrogen atoms through various metabolic pathways. Use of stable isotopes for flux analysis in biology has contributed greatly to the understanding of cellular metabolism and regulation. However there remains a challenge to determine accurate flux rates for metabolites which are formed from multiple precursors utilizing different enzymatic pathways. Positional isotopomer distribution of metabolites obtained by utilizing multiple tracers are invaluable pieces of information for the computation of these fluxes. Classically done by GC-MS and NMR, here we show the application of liquid chromatography-based separation of metabolites with the use of hybrid dissociation strategies using HCD and CID MSn fragmentation for positional isotopomer determination.
In this work we used the fragmentation data on a HRAM mass spectrometer for the structural annotation of the fragments to develop a targeted assay for the isotopomer determination. We chose the nucleotide AMP for our initial analysis due to its involvement in a wide variety of biological process and that its biosynthesis involves multiple pathways.

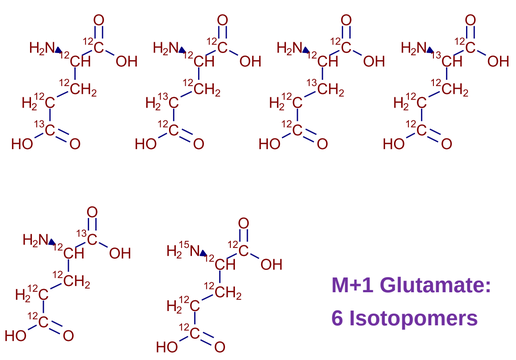


Figure 1. Example of positional isotopomers: The M+1 isotopologue of Glutamate can have 6 different isotopomers depending on the stable isotope label present and its position.

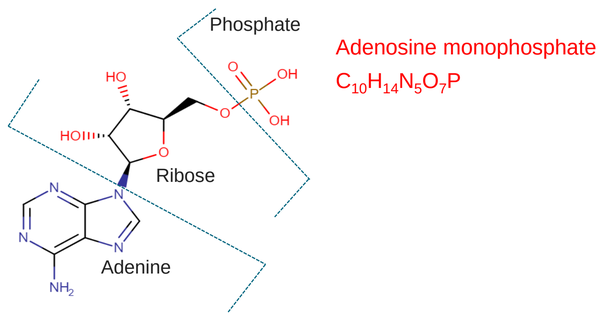


Figure 2. Nucleotides are required for a wide variety of biological processes and are building blocks of DNA and RNA. AMP biosynthesis involves multiple pathways that provide carbon and nitrogen precursors. Analytical method to delineate the contribution from each pathway can lead to a better understanding of biosynthetic regulation.

There are many pathological states that are characterized by low oxygen. In fact, three of the five leading causes of death in the US result from impaired oxygen and nutrient delivery – heart attack, stroke and respiratory failure. However, low oxygen is not always pathological. Chronic adaptation to hypoxia can even be beneficial. Here we investigated the *in-vitro* effect of hypoxia on the cellular metabolism.

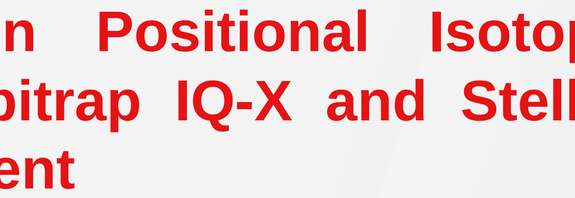


Figure 3. Textbook version of rewiring of tumor metabolism in hypoxic conditions.

Material and Methods

Sample

Credentialed *E-Coli* Cell Extract Kits (Cambridge Isotopes) consisting of U-^{13}C Labeled *E-Coli* and Unlabeled ^{12}C *E-coli* Extract were used for initial data acquisition and structural annotation of fragments.
KP4 Cells (a human pancreatic cancer cell line) was used for this study. Cells were grown in 21% Oxygen (normoxia) and 0.5% Oxygen to stimulate hypoxic conditions. Cells were grown in four different media:

1. Unlabeled Media
2. $^{13}C_6$ Glucose: To monitor the metabolism of carbon through glycolysis and other pathways
3. $^{15}N_2$ Glutamine: To monitor the nitrogen incorporation into various metabolites
4. $^{13}C_6$ Glucose and $^{15}N_2$ Glutamine: To show the utility of nominal mass instrument for experiments typically limited to higher resolution mass spectrometers (>120K).

Data Acquisition

Extracts were separated on a HILIC column and analyzed Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a Thermo Scientific™ Orbitrap™ IQ-X™ Tribrid™ mass spectrometer. In this method, a full scan was followed by the targeted fragmentation of AMP utilizing both HCD (higher energy collisional dissociation) and CID (collision-induced dissociation). MS^3 fragmentation was carried out for the higher intensity fragments.
A targeted MS^2/MS^3 assay was developed on Thermo Scientific™ Stellar™ MS, a hybrid quadrupole-radial ejection linear ion trap mass spectrometer.

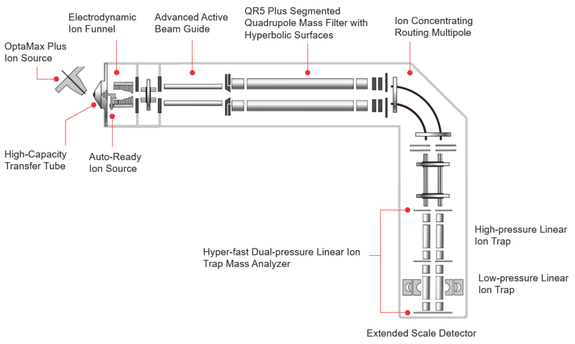


Figure 4. Thermo Scientific Stellar MS diagram

	QQQ	Stellar™ MS
Full Product Ions Scan (PRM)	-	+
Fast MS^n	-	+
Alternate Fragmentation Strategies (HCD, CID)	-	+
Stepped Collision Energies	-	+
Fast Method Development	-	+

Table 1. Advantage of using nominal mass Stellar MS for stable isotope workflows compared to triple-quadrupole nominal mass instruments

Data Analysis

HRAM data was processed using Thermo Scientific™ Mass Frontier™ 8.1 software for structural annotation of fragments. Targeted-MS^2/MS^3 method was processed using Thermo Scientific™ TraceFinder™ 5.2 Software.

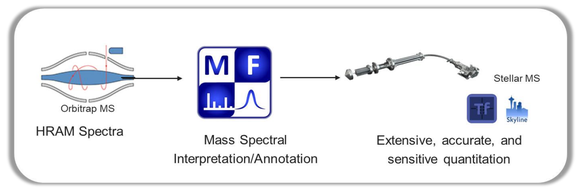


Figure 5. HRAM discovery to accurate and sensitive quantitation of stable isotope labeling pattern via Stellar MS

Results

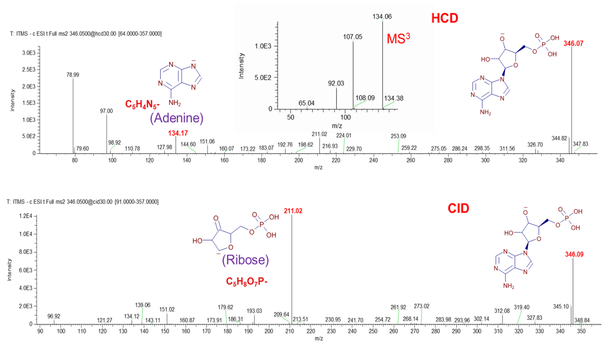
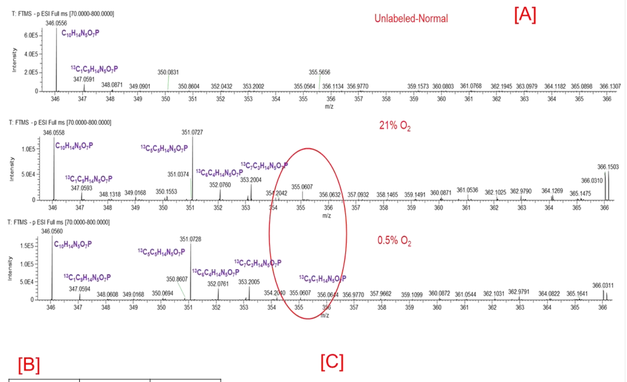


Figure 6. AMP fragmentation and annotation on the Stellar MS: The use of HCD and CID MS^2 Product scans as well as MS^3 of the fragment containing the adenine ring can provide valuable information regarding the nitrogen and carbon positional labeling.



[B]

Isotope	Parent	Product
M0	346.1	79.1
M1	347.1	79.1
M2	348.1	79.1
M3	349.1	79.1
M4	350.1	79.1
M5	351.1	79.1
M6	352.1	79.1
M7	353.1	79.1
M8	354.1	79.1
M9	355.1	79.1
M10	356.1	79.1

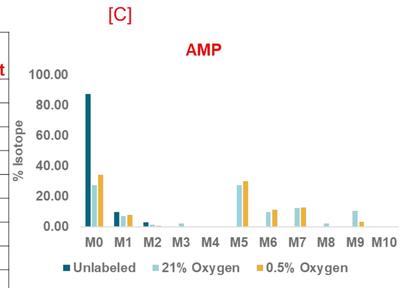


Figure 7. $^{13}C_6$ Glucose Cell Extract- AMP ($C_{10}H_{14}N_5O_7P$) Labeling: [A] Full Scan MS of AMP from HRMS shows difference in [M+9] abundances of hypoxia vs normaxia. [B] Stellar MS was used to quantify the isotopologues of AMP. From the PRM of each isotopologue, m/z 79.1 which is the fragment from the phosphate ion (which does not contain nitrogen or carbon) of AMP was chosen for quantification. PRM eliminates the need to set up transitions for multiple parent-product scans. [C] Quantification of AMP isotopologues using Stellar MS. M+9 is reduced in hypoxia compared to normaxia.

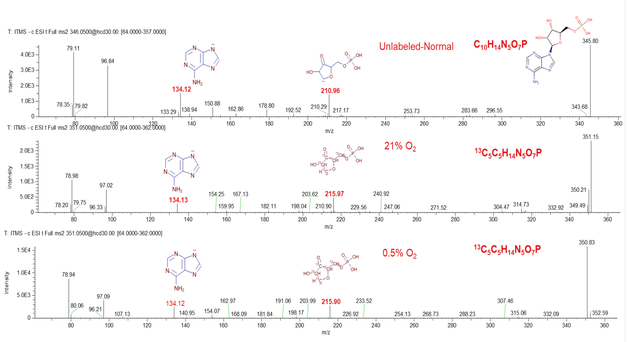


Figure 8. $^{13}C_6$ Glucose Cell Extract- AMP ($C_{10}H_{14}N_5O_7P$) Labeling: Fragmentation of [M+5] isotopologue shows that in both hypoxia as well as normoxia, the ribose ring gets labeled.

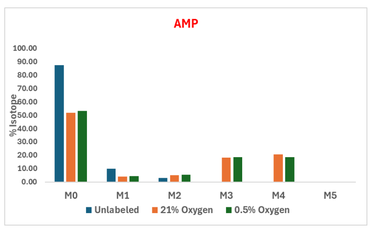


Figure 9. $^{15}N_2$ Glutamine Cell Extract- AMP ($C_{10}H_{14}N_5O_7P$) Labeling: Quantification using Stellar MS PRM shows no diference between hypoxia and normoxia. However not all nitrogen [M+5] are labeled.

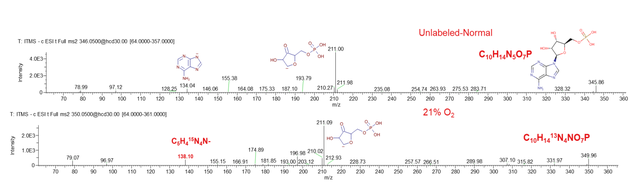


Figure 9. $^{15}N_2$ Glutamine Cell Extract- AMP Labeling: MS^2 Spectra of [M+4] isotopologue.

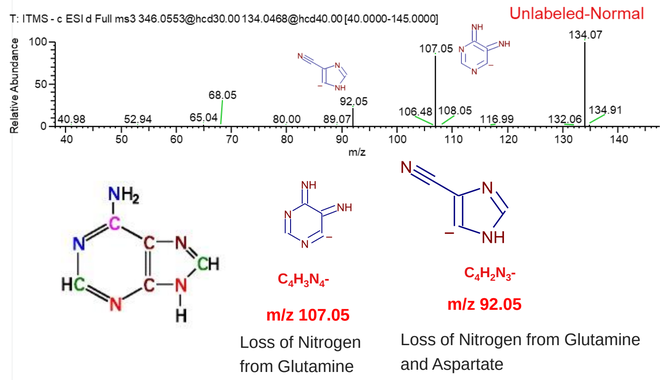


Figure 10. Cell Extract- AMP : MS^3 Spectra of adenine ring gives fragments indicating loss of nitrogen from glutamine and aspartate.

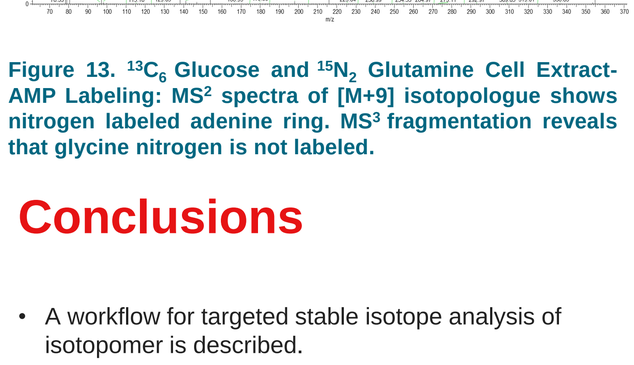


Figure 11. $^{15}N_2$ Glutamine Cell Extract- AMP Labeling: MS^3 Spectra of adenine ring. Both the fragments with the loss of glutamine and aspartate nitrogen still retain the unlabeled nitrogen. This indicates that the nitrogen in the ring coming from glycine is unlabeled.

Figure 12. $^{15}N_2$ Glutamine Cell Extract- Glycine Labeling: HRMS spectra of glycine confirming that nitrogen in glycine is unlabeled.

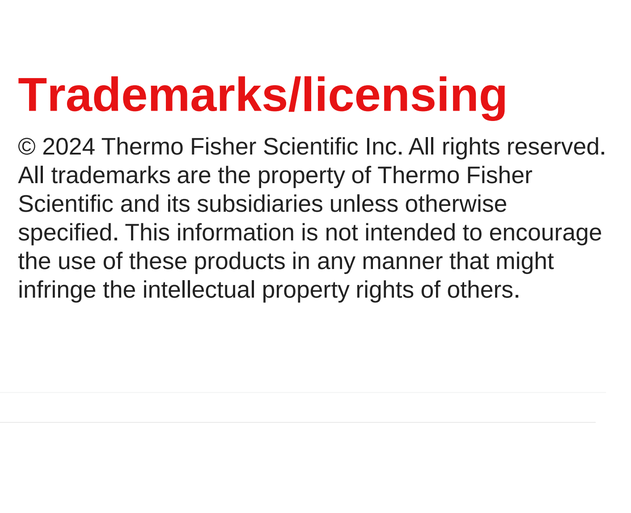


Figure 13. $^{13}C_6$ Glucose and $^{15}N_2$ Glutamine Cell Extract- AMP Labeling: MS^2 spectra of [M+9] isotopologue shows nitrogen labeled adenine ring. MS^3 fragmentation reveals that glycine nitrogen is not labeled.

Conclusions

- A workflow for targeted stable isotope analysis of isotopomer is described.
- The use of product scans with MS^n and alternate fragmentation can give positional information of label using nominal mass resolution instrument.

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