Application Note

Metabolomics

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^{13}C Glucose Qualitative Flux Analysis in HepG2 cells

Using an Agilent 6546 LC/Q-TOF and VistaFlux

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Abstract

The Agilent 6546 LC/Q-TOF is designed to simultaneously provide superior isotope ratio fidelity, wide dynamic range, and high mass resolution independent of acquisition speed. Flux analysis requires the combination of isotope ratio fidelity, high resolution, and dynamic range for good results. When combined with Agilent MassHunter VistaFlux software, a comprehensive workflow from data acquisition to data analysis is provided to help scientists perform qualitative flux analysis in a streamlined fashion. VistaFlux software is composed of tools for feature extraction, analysis of isotope incorporation and isotopologue abundance, natural abundance correction, statistical analysis, and visualization of metabolic pathways.

This Application Note demonstrates qualitative flux analysis in human carcinoma cell lines using U-^{13}C glucose as the tracer. Data were acquired on a 6546 LC/Q-TOF system, and analyzed with MassHunter VistaFlux. The study demonstrates the effect of pyruvate carboxylase knockdown on glucose flux in the TCA cycle in HepG2 cells.

Introduction

Stable isotope labeling provides a unique picture of intracellular metabolism. Although metabolomics profiling provides a great overview of the abundance of different metabolites, many metabolic changes do not result in an accumulation or a reduction at the metabolite level. Stable isotope tracing can provide insightful information not revealed by normal metabolomic profiling. In stable isotope tracing, isotopologues are normalized to each other to calculate the enrichment percentage. This self-normalizing approach makes stable isotope tracer studies a powerful option to probe metabolic changes in challenging biological systems that might differ in cell number.

There are many challenges to accurately identify small but significant isotope changes that alter normal metabolite isotope distribution. The mass spectrometer (MS) should maintain accurate isotope abundance and mass measurements for correct metabolite identification and quantitation in complex samples. In addition, the MS should simultaneously provide a wide dynamic range and high resolution. A wide dynamic range accommodates varying metabolite concentrations and isotope incorporation rates while high resolution reduces interference from coeluting compounds. Finally, the data analysis software should correctly assign isotopologues and perform natural isotope correction to provide an accurate net label incorporation.

The 6546 LC/Q-TOF and VistaFlux software provide a comprehensive workflow for qualitative flux analysis. The 6546 LC/Q-TOF simultaneously delivers the isotope ratio fidelity, wide dynamic range, and high mass resolution required for qualitative flux analysis. VistaFlux software provides full data analysis and visualization of flux data. This full workflow solution was applied to pyruvate carboxylase knockdown HepG2 cells treated with U-^{13}C glucose.

Pyruvate carboxylase (PC) is a mitochondrial anaplerotic enzyme that generates citric acid cycle intermediates for biosynthetic purposes. This enzyme is essential for gluconeogenesis in liver and kidney, *de novo* lipogenesis in adipose tissue, and glucose-induced insulin secretion in pancreatic beta cells. Recent studies show that PC is pivotal for several cancer forms where it supports various anabolic pathways. We have previously shown that PC suppression reduces the proliferation rate in highly invasive breast cancer cells as well as reducing glucose and glutamine anaplerosis[1]. This Application Note shows that PC inhibition exhibits a similar effect on human hepatic cancer cells (HepG2).

Experimental

Cell culture and reagents

Pyruvate carboxylase knockdown HepG2 cells were prepared using siRNA, as described previously[1]. Five different knockdown cell lines were generated with different levels of inhibition (PC179, PC2096, PC 3436, and PC847). Approximately 3 × 10^6 cells were plated into a 60 mm culture plate. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin, and were maintained at 37 °C with 5 % CO_2 for two days. The medium was replaced with complete DMEM containing 3 mM glucose, and cells were cultured for one more day. On the day of the experiment, cells were incubated for 30 minutes with 0 mM glucose DMEM, then the medium was replaced with 2 mL of DMEM containing 10 mM ^{13}C-labeled glucose, and cells were incubated for 30 minutes at 37 °C. Experiments were done in triplicate for each cell line.

Metabolite extraction

After a quick washing with 150 mM ammonium acetate, cells were extracted with 8:1:1 (v/v/v) methanol: chloroform: water, followed by sonication and centrifugation. The clear supernatant was then transferred to HPLC vials and injected.

LC/MS analysis

Data were acquired using a 6546 LC/Q-TOF coupled to an Agilent 1290 Infinity II LC. Chromatographic separation was performed on an Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm (p/n 675775-924), with a UHPLC Guard, HILIC-Z, 2.1 mm × 5 mm, 2.7 µm (p/n 821725-947). A 10× mobile phase buffered stock solution (100 mM ammonium acetate, pH 9.0) was first prepared in water, then final mobile phases were prepared with the addition of either water (A) or acetonitrile (ACN, B). To ensure a constant concentration during gradient elution, the InfinityLab deactivator additive (p/n 5191-4506) was added to both aqueous and organic mobile phases. Pooled samples and blank injections were analyzed to ensure chromatographic stability[2].

Software packages

- MassHunter Acquisition software version 10.0
- Agilent Profinder V10.0
- Omix Premium V1.9.30
- PCDL Manager B.08
- Agilent MassHunter Pathways to PCDL software B.08

Table 1. LC and MS parameters.

Parameter	Value
LC Conditions	
Column	InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm (p/n 675775-924) with a UHPLC Guard, HILIC-Z, 2.1 mm × 5 mm, 2.7 µm (p/n 821725-947)
Mobile Phase	A) 10 mM Ammonium acetate in water, pH 9 with 5 µm deactivator additive (p/n 5191-4506) B) 10 mM Ammonium acetate in water/ACN 10:90 (v:v), pH 9 with 5 µm deactivator additive (p/n 5191-4506)
Flow Rate	0.25 mL/min
Gradient	0 to 2 minutes 90 %B 2 to 12 minutes 90 to 60 %B 12 to 15 minutes 60 %B 15 to 16 minutes 60 to 90 %B 16 to 24 minutes 90 %B
Column Temperature	25 °C
Injection Volume	1 µL
Autosampler Temperature	6 °C

MS Conditions	
MS System	6546 Q-TOF LC/MS
Ionization Source	Agilent Jet Stream
Polarity	Negative
Gas Temperature	200 °C
Drying Gas	10 L/min
Nebulizer Pressure	40 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,000 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	65 V
Octopole 1 RF Voltage	750 V
Acquisition Range	*m/z* 50 to 1,000
Reference Mass	*m/z* 980.01638 and 119.0363

Data analysis

Using Agilent MassHunter Pathways to PCDL software, an Agilent Personal Compound Database and Library file (.cdb) was created for the tricarboxylic acid (TCA) cycle drawn from BioCyc. In addition, pyruvate, aspartate, and glutamate were added to the custom PCDL. Metabolite retention times were added to the custom PCDL, which was then used as the database for batch isotopologue extraction in Profinder. Results from Profinder were exported for visualization in Omix Premium software. Figure 1 summarizes an overview of the data analysis workflow.

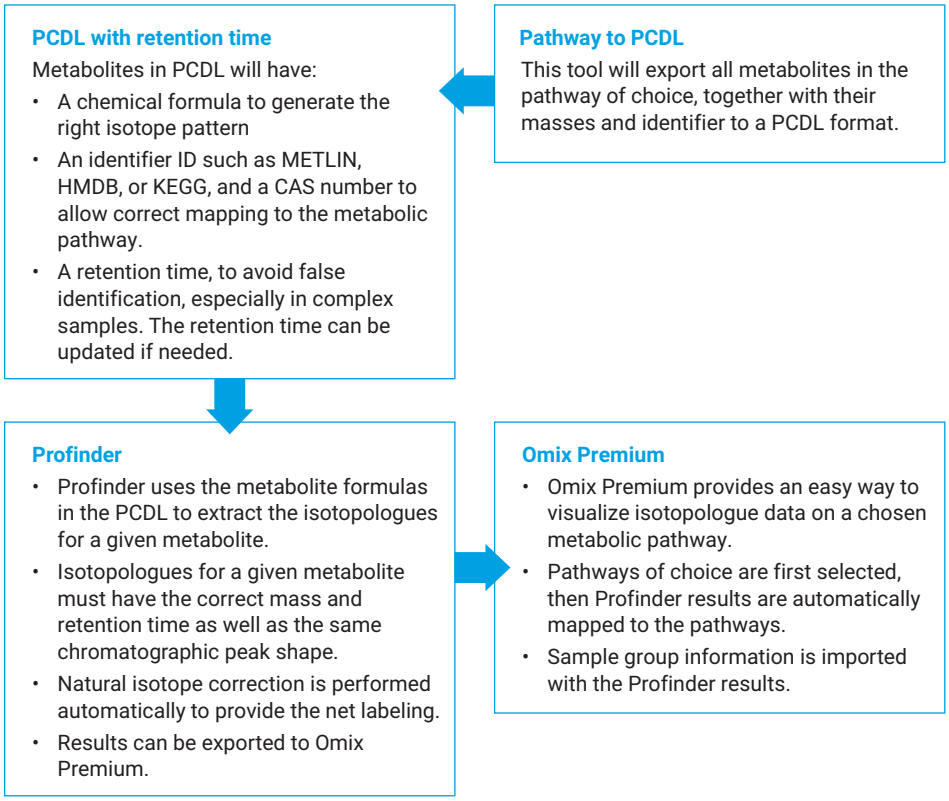

Figure 1. Overview of VistaFlux data analysis workflow.

Results and discussion

The flux samples were analyzed on the 6546 LC/Q-TOF MS. Figure 2 shows a representative MS spectrum for malate isotopologues with greater than 40,000 resolution while showing less than 5 % CV for all isotopologues among seven technical replicates (Figure 3). The high resolution of the 6546 Q-TOF reduces potential interferences, increasing confidence in the data analysis. The excellent reproducibility of the isotope ratios enables the detection of low incorporation rates in a qualitative flux experiment.

Batch isotopologue extraction in Profinder represents the easiest and most efficient way to analyze isotopologue data. It contains algorithms that identify isotopologues based on retention time, accurate mass, and chromatographic peak shape, reducing false positives. Data were manually reviewed for all isotopologues for every metabolite (Figure 4). The corrected abundance or percent enrichment for groups are shown individually or combined to evaluate reproducibility (Figure 4). U-^{13}C glucose shows substantial labeling in glutamic acid in the HepG2 control cells with m+2 labeling being the dominant isotopologue, while cells with pyruvate carboxylase knockdown show a much lower rate of labeling (Figure 4). To be able to visualize all metabolites on a pathway, results were exported to Omix Premium as Profinder Archive file (.PFA). The Profinder archive file contains the naturally occurring isotope corrected results together with sample group information and compound identifiers.

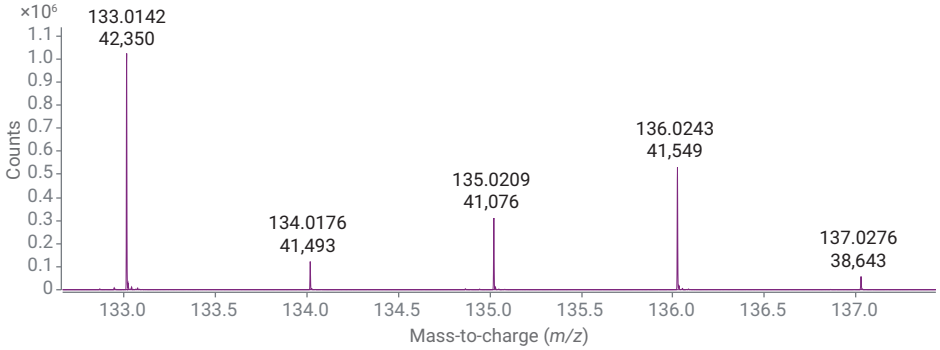

Figure 2. Mass spectra of malate isotopologues with *m/z* and resolution labeled on each ion peak.

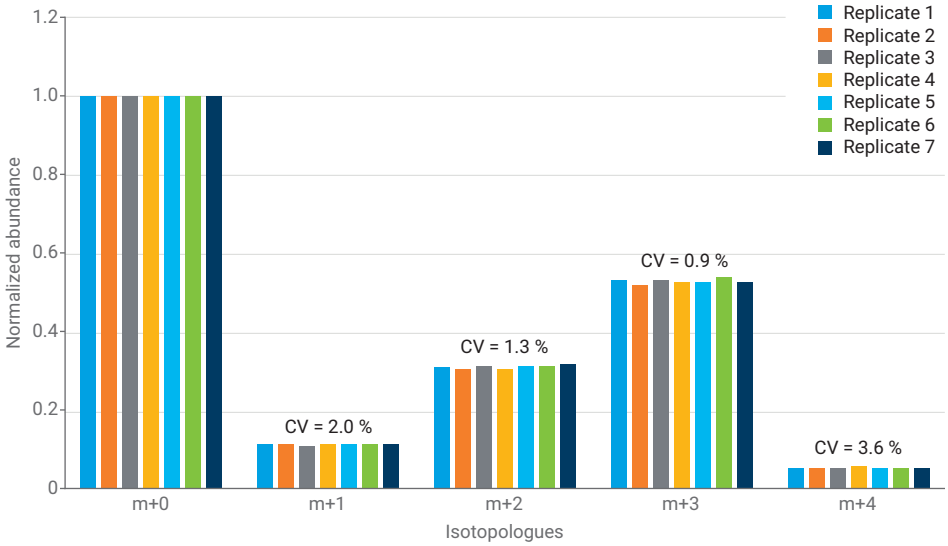

Figure 3. The coefficient of variation (CV%) of all malate isotopologues for seven technical replicates.

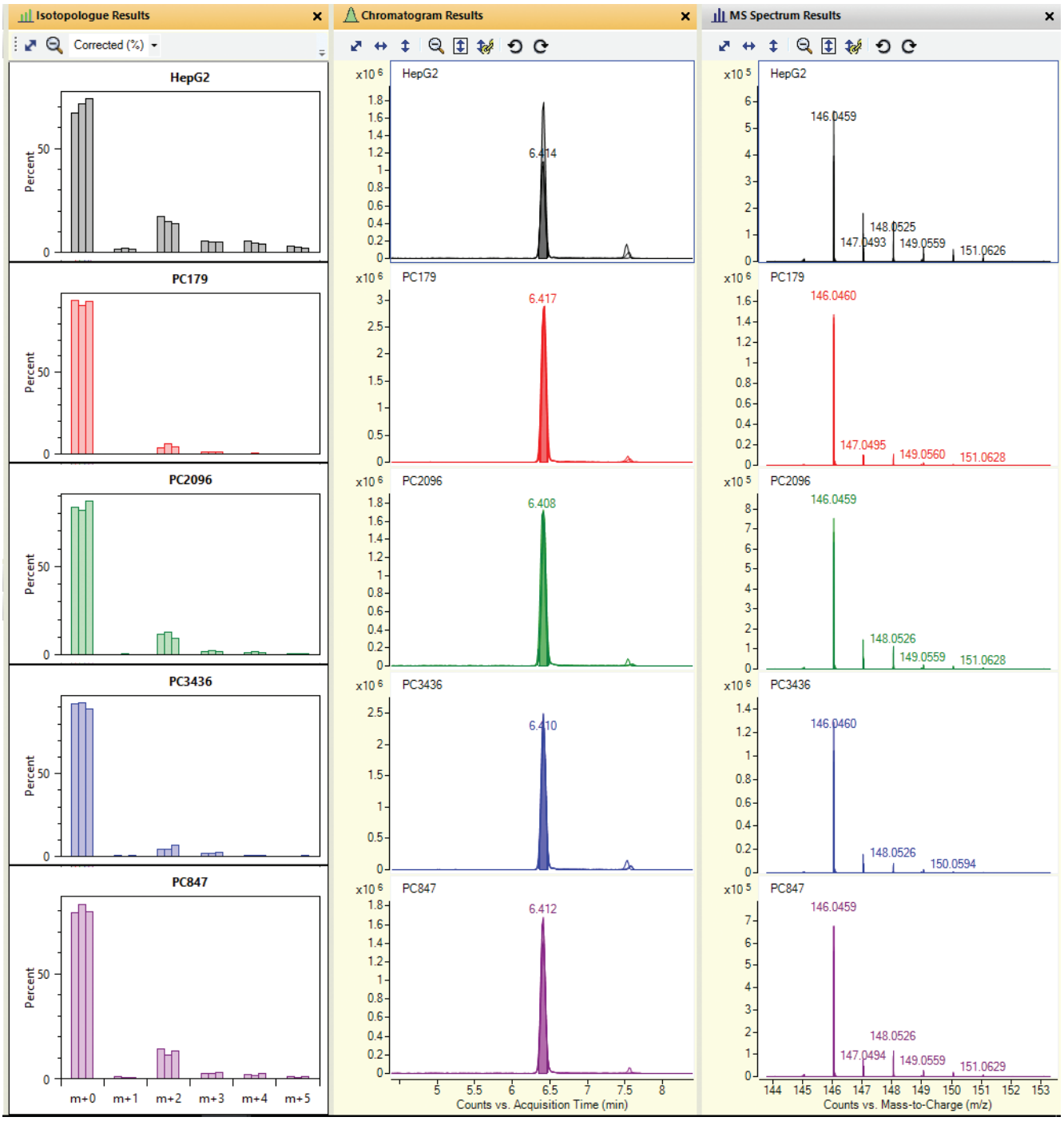

Figure 4. Profinder visualization showing isotopologue, chromatogram, and MS spectrum results.

Omix Premium offers graphical options designed for the visualization and interpretation of qualitative flux data. There are multiple ways of viewing the results either using absolute amount or isotope enrichment ratio. Citrate results show ~50 % enrichment in HepG2 control cells, while only ~25 % in PC-knockdown cells, suggesting that pyruvate carboxylase knockdown reduces glucose flux into the TCA cycle (Figure 5A). Total abundance of citrate was slightly lower in knockdown cells, but the abundance of labeled citrate was significantly lower (Figure 5B). This indicates that either the knockdown cells have reduced TCA cycle turnover, or the cells are relying on other anaplerotic sources such as glutamate to substitute for the reduction of glucose flux and replenish the TCA cycle.

To understand the detailed labeling pattern, other visualizations were used, such as the quilt plot, or using individual isotopologue enrichment plots (Figure 6). These plots clearly show that the most abundant labeling was in carbon number m+2 and m+3 during this short period of incubation with U-^{13}C glucose. Longer incubation leads to more turns in the TCA cycle and thus a higher number of carbons will be labeled. Most isotopologues, such as m+2, m+3, m+4, and m+5, were more enriched in HepG2 cells compared to the other cells, suggesting that knockdown cells have a lower turnover in the TCA cycle than control cells.

To evaluate if glutamate was replenishing the TCA cycle in PC knockdown cells, we looked at the abundance of glutamate and its isotopologues. Unlabeled glutamate (m+0) (Figure 7), shows more accumulation in the knockdown cells, suggesting that it was less consumed. In contrast, it was actively consumed in the more active TCA cycle of the control HepG2 cells, suggesting that glutamate acted as an anaplerotic

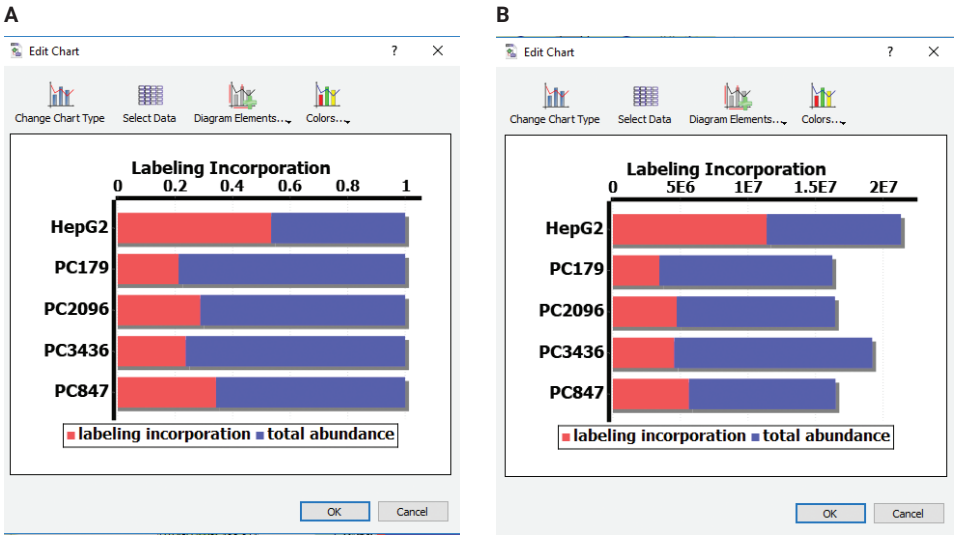

Figure 5. Omix Premium software displaying citrate results either using fractional labeling (A) or abundance (B).

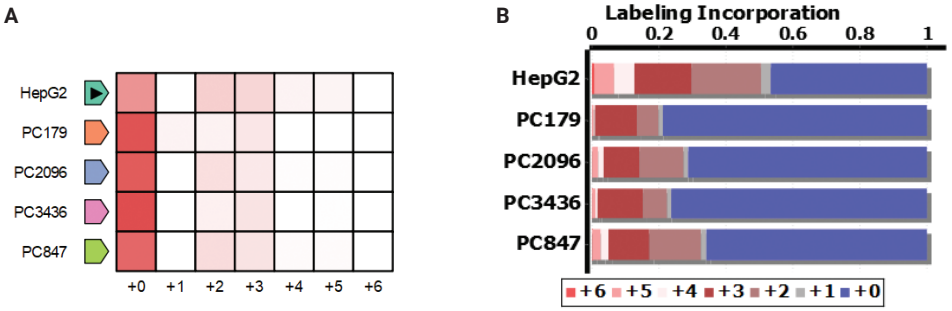

Figure 6. Omix Premium software showing citrate labeling as a quilt plot (A) or as individual isotopologues enrichment plot (B).

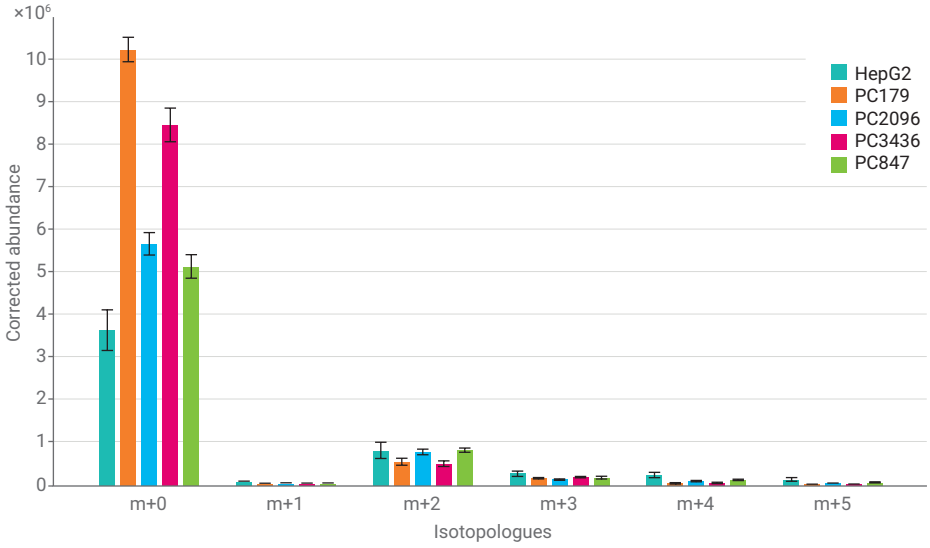

Figure 7. Omix Premium software showing glutamate isotopologue abundance.

substrate in HepG2 cells, and to a much lesser extent in knockdown cells. Thus, pyruvate carboxylase knockdown not only altered the glucose flux in the TCA cycle, but also reduced the anaplerosis of glutamate into the TCA cycle. These data agree with the effect of PC knockdown on glucose and glutamine anaplerosis in breast cancer cells[1]. Figure 8 shows a summary of metabolites mapped onto the TCA cycle with their corresponding enrichment.

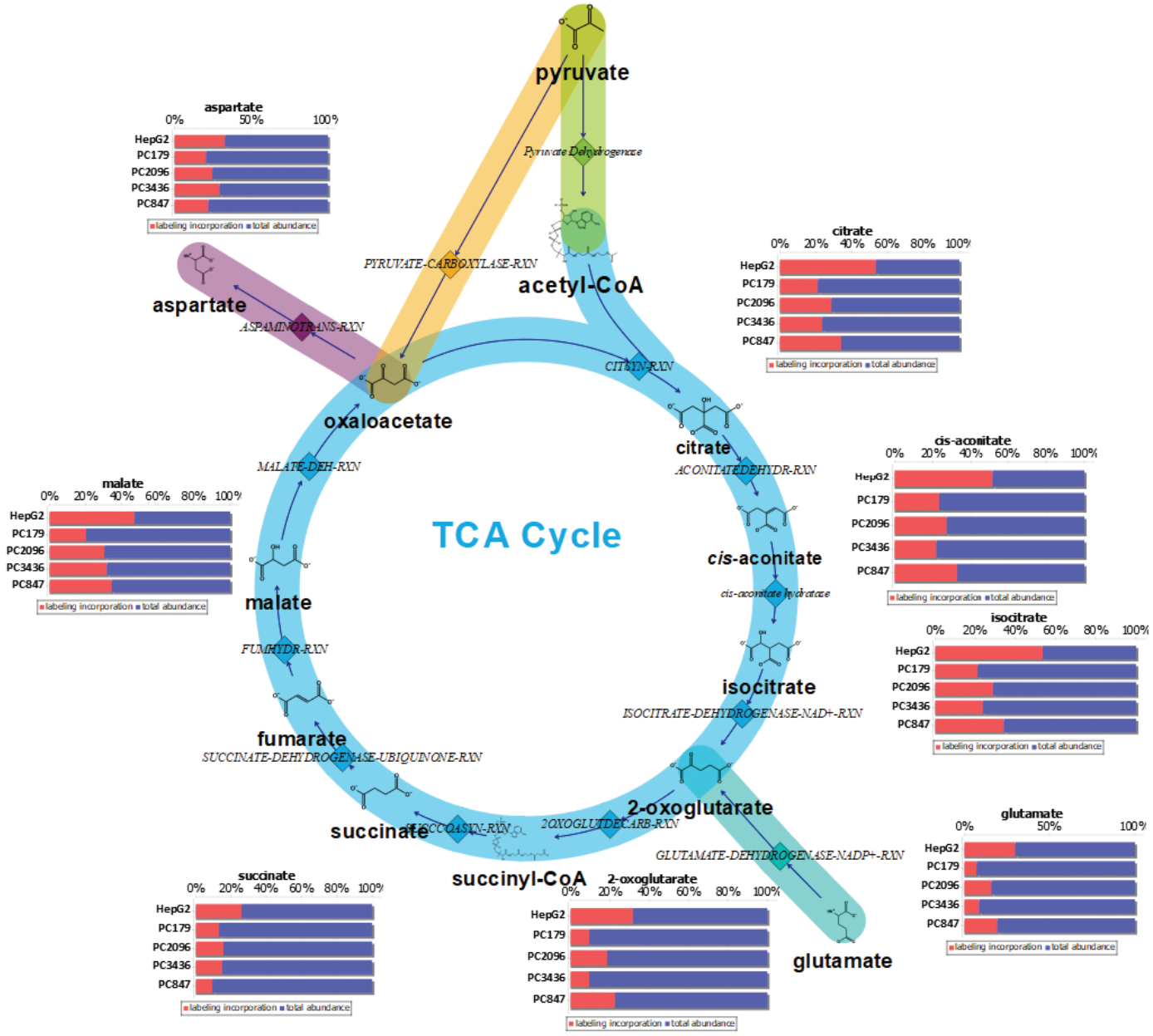

Figure 8. Omix Premium software showing the TCA cycle and related metabolites labeling.

Conclusion

The 6546 LC/Q-TOF offers the isotope ratio fidelity, high resolution, and dynamic range for stable label isotope tracing. Together with VistaFlux software, this platform provides a comprehensive workflow solution to perform qualitative flux analysis. Using this workflow, HepG2 cells with pyruvate carboxylase enzyme knockdown showed less glucose oxidation. This reduction in glucose oxidation subsequently reduced glutamate anaplerosis.

The VistaFlux workflow provides a fast, powerful solution for extracting isotopologue information, correcting natural isotope abundance and visualizing results.

References

1. Phannasil. P.; *et al.* Mass spectrometry analysis shows the biosynthetic pathways supported by pyruvate carboxylase in highly invasive breast cancer cells. *Biochimica et Biophysica Acta* **2017**, *1863(2)*, 537–551.
2. Hsiao, J. J.; *et al.* Monitoring of Mammalian Cell Culture Media with HILIC LC/MS. *Agilent Technologies Application Note*, publication number 5994-0024EN, **2018**.

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