

Pathway-Targeted Metabolomic Analysis in Oral/Head and Neck Cancer Cells Using Ion Chromatography-Mass Spectrometry

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Key Words

Ion chromatography, Q Exactive HF mass spectrometer, high resolution, accurate mass, TCA cycle, isotopic labeling, targeted metabolomics, oral cancer cells

Goal

To demonstrate ion chromatography (IC) coupling with high-resolution, accurate-mass (HRAM) MS for targeted metabolomics analysis.

Introduction

Metabolomics aims to measure a wide breadth of small molecules (metabolome) in the context of physiological stimuli or disease states.¹ The general problems encountered when characterizing the metabolome are the highly complex nature and the wide concentration range of the compounds. Separation science plays an important role in metabolomics by reducing the sample complexity to achieve a comprehensive profiling analysis.² The strength of mass spectrometry (MS)-based metabolomics is best realized when coupled to a separation technique such as capillary electrophoresis³, gas chromatography (GC)⁴, or liquid chromatography (LC)⁵. Ion chromatography (IC) or ion-exchange chromatography offers an excellent complementary platform for separation of charged and polar compounds.⁶ Because of its unique selectivity, IC has been coupled with MS for targeted screening and quantification of metabolites such as carbohydrates, organic acids, sugar phosphates, and nucleotides in biological samples.⁷⁻⁹

Metabolomics is now widely used in the characterization and diagnostic research of an ever-increasing number of diseases.¹⁰ An MS-based metabolomics approach has received increasing attention in oral cancer studies.¹¹ Recently, an analytically sensitive platform coupling capillary ion chromatography (CapIC) with a Thermo Scientific™ Q Exactive™ mass spectrometer has been successfully developed for nontargeted metabolic profiling of head and neck cancer cells.¹² The outstanding resolution of IC has led to the differentiation of many isobaric and isomeric polar metabolites. In addition,

IC has shown broad coverage of glycolysis and the tricarboxylic acid cycle (TCA cycle) intermediates. Significant changes of TCA cycle metabolites in cancer stem cells versus nonstem cancer cells were observed.¹²

Targeted metabolomics is a quantitative approach wherein a set of known targeted metabolites are quantified based on their relative abundance when compared to internal or external reference standards. The resulting data can then be used for pathway analysis or as input variables for statistical analysis. Because of the reliable measurement of metabolite integrals, targeted metabolomics can provide insight into the dynamics and fluxes of metabolites.

In this work, a high flow rate IC system was utilized for targeted analysis of the TCA cycle intermediates to shorten the run time and increase throughput and robustness to accommodate the hundreds of samples typically analyzed in a targeted metabolomics project. This work also validates our previous observations through global metabolomic analysis using the CapIC-MS method.¹²

Isotopically labeled standards are ideal internal calibration references to spike into the metabolomic samples for targeted quantitative analysis in an IC-MS experiment because of their similar ionization effect and chromatographic retention. Six stable-isotope-labeled standards available for the TCA cycle were used to generate the standard curves for targeted quantitative analysis of the corresponding metabolites. By using Thermo Scientific™ TraceFinder™ software, six TCA endogenous compounds in a large set of samples were quantified quickly and accurately.

Experimental

Sample Preparation

A spherogenic assay was used to enrich and isolate stem-like oral cancer cells, as described previously.¹³ UM1, UM2, UMSCC5, and UMSCC6 head and neck cancer cells were cultured in Dulbecco's modified eagle medium (DMEM) plus 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were maintained at 37 °C in a humidified 5% CO₂ incubator and passaged when they reached 90–95% confluence. Cell numbers were counted with a Beckman Coulter cell viability analyzer and the average numbers per plate were: 3.2 ± 0.2 million. Cellular metabolites were extracted using a liquid nitrogen snap-freezing method with methanol/water according the literature.¹⁴ Briefly, the cells were quickly washed twice with ice-cold phosphate-buffered saline solution (PBS) in a cold room to remove medium components and then quickly rinsed with 18 MOhm ultrapure water from Milli-Q®. After removal of water, the cells were flash frozen with liquid nitrogen, and 1.0 mL of ice-cold 90% methanol/chloroform was immediately added to each plate. Cells were scraped/suspended with a cell scraper. Extracts were transferred to microcentrifuge tubes and pelleted at 4 °C for 3 min at 16,100 x g. Supernatants were then transferred to new microcentrifuge tubes for IC-MS analysis. All experiments were performed in 3 to 12 replicates.

Six stable-isotope standards were supplied by Cambridge Isotope Laboratories. Sodium pyruvate (¹³C₃, 99%), malic acid (¹³C₄, 99%), fumaric acid (¹³C₄, 99%), succinic acid (¹³C₄, 99%), *alpha*-ketoglutaric acid (¹³C₅, 99%), and citric acid (2,2,4,4-D₄, 98%) were pooled together and diluted serially with deionized water at 11 concentrations of 10,000, 5,000, 1,000, 500, 100, 50, 10, 5, 1, 0.5, and 0.1 pg/µL. Then, 200 µL of each standard solution was used to reconstitute the dried cell lysate samples. In the end, each biological sample was spiked in triplicate at one calibration level.

Ion Chromatography

A Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ ion chromatography system was coupled to a Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap™ mass spectrometer for the analysis. The IC was equipped with a Thermo Scientific™ Dionex™ AERS™ 500 anion electrolytically regenerated suppressor to convert the potassium hydroxide mobile phase to water before the sample entered the MS. A 2 µL partial loop injection on a 5 µL loop of cellular metabolites was separated using a Thermo Scientific™ Dionex™ IonPac™ AS11-HC 2 × 250 mm, 4 µm particle size column. The total flow entering the MS was comprised of the IC flow rate of 0.38 mL/min and the post-column solvent flow of 0.060 mL/min. The post-column solvent was methanol with 2 mM acetic acid (Figure 1). The gradient conditions are shown in Figure 2 and Table 1.

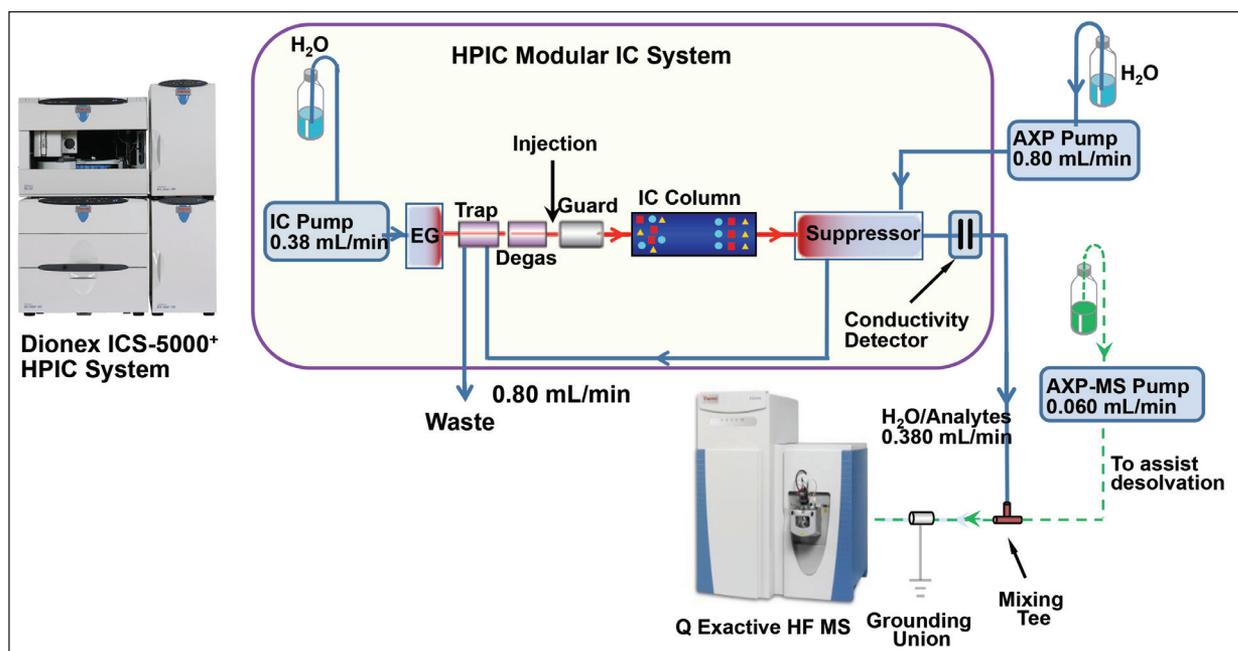


Figure 1. High-performance IC-Q Exactive HF MS platform.

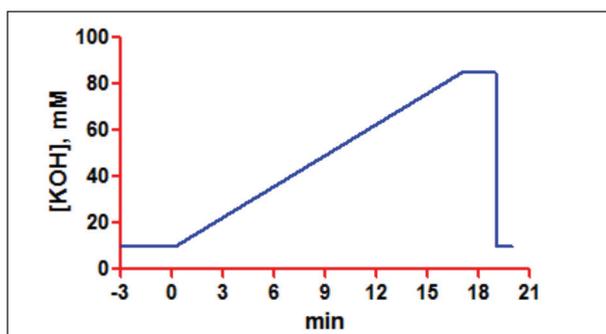


Figure 2. The IC gradient on the Dionex IonPac AS11-HC 2 x 250 mm, 4 μ m particle size column.

Table 1. IC gradient.

Retention [min]	Concentration of KOH [mM]
-3.0	10
0.20	10
17.0	85
19.0	85
19.1	10
20.0	10

Mass Spectrometry

The Q Exactive HF mass spectrometer (Figure 3) was operated in electrospray negative ion mode for all experiments.

Mass range	m/z 67–1000
Resolution	120,000
Scan rate	~3.5 Hz
Automatic gain control (AGC) target	1×10^6 ions
Maximum ion injection time (IT)	100 ms

Source ionization parameters were optimized with the following settings

Spray voltage	3.5 kV
Transfer temperature	320 $^{\circ}$ C
RF-Lens	50 V
Heater temperature	325 $^{\circ}$ C
Sheath gas	36 arb units
Aux gas	5 arb units

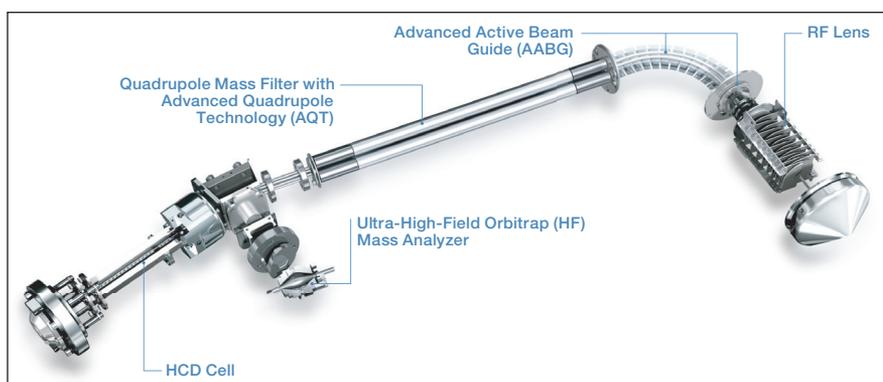


Figure 3. Q Exactive high-field Orbitrap analyzer mass spectrometer.

Data Processing

Data were acquired in full-scan mode. Differential analysis of profiling data was performed using the Thermo Scientific™ SIEVE™ software version 2.2. Targeted analysis of TCA compounds was performed using TraceFinder software version 3.2.

Results

IC-MS Optimization for Higher-Throughput Analysis

A high-pressure Dionex ICS-5000+ HPIC system was used in this study because a high-throughput separation system with high chromatographic reproducibility was needed for targeted quantitation. Using the IonPac AS11-HC 2 mm (i.d.) column with 4 μ m particles, the Dionex ICS-5000+ system can operate at flow rates up to 380 μ L/min below the pressure limit of 5000 psi. The flow rate was 15-fold higher than the previous application on the capillary IC system with a flow rate of 25 μ L/min.¹² Higher peak chromatographic reproducibility, including retention times and peak intensity, can be achieved with the current system.

The transfer line from the autosampler to the injection valve was red PEEK™ tubing (0.13 mm i.d.) with a volume of 14 μ L. This tubing was found to substantially improve the sensitivity and peak shape as compared to black (0.25 mm i.d.) or blue tubing (0.5 mm i.d.) with volumes greater than 40 μ L. Black PEEK tubing was used from the waste valve, which was found to reduce the carryover to negligible levels (<0.01%). PushPartial sample injection mode was used on a 5 μ L loop (0.13 mm i.d., red) with a 2 μ L injection and 5 μ L cut volume. This achieved a total sample consumption volume of ~12 μ L, which was better suited for the precise biological sample injection.

Compared to CapIC running at capillary flow rates, which used a very long gradient (45 min), the Dionex ICS-5000+ IC system was optimized for a short, 20 minute gradient. The separation of the six stable-isotope standards (Table 2) is shown in Figure 4, with pyruvate eluting first at 3.3 min and citric acid last at 11.9 min. The successful separation of sugar monophosphates and diphosphates is the most challenging among those isomer pairs; it is a good measure of the success of the IC method. As we can see from cancer cell lysates, 11 sugar monophosphates and nine sugar diphosphates were well resolved (Figure 5). More than 150 consecutive injections of cell lysates were run and the RT variation of Peak #9 (fructose 6-phosphate, identification based on RT and MS/MS) was 7.93 ± 0.03 min with p-value = 0.02 (Figure 6).

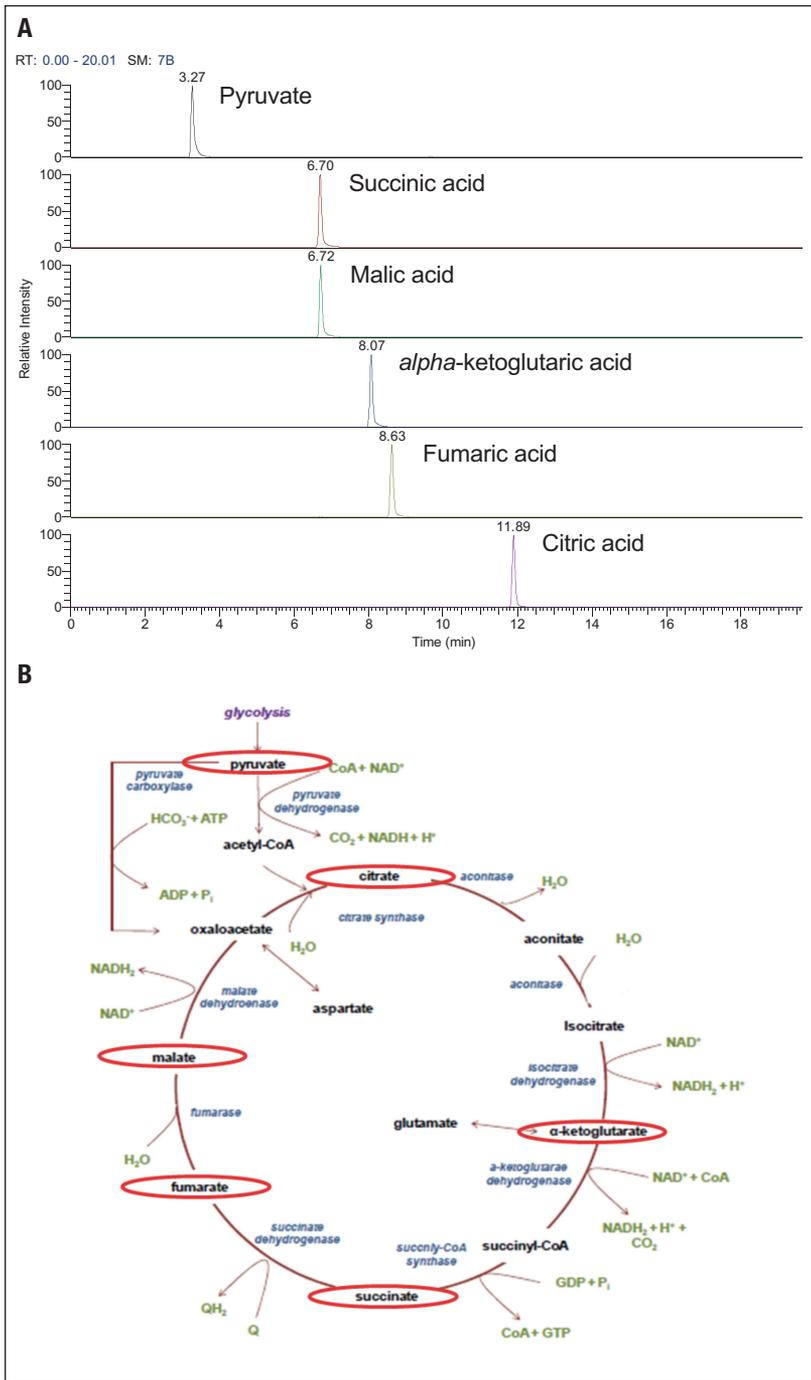


Figure 4. Detection of six stable-isotope-labeled standards with the IC-MS (A). These compounds are the intermediates of the tricarboxylic acid (TCA) cycle (B).

Table 2. The six stable-isotope-labeled internal standards.

#	Metabolite Name	Formula	Obs. m/z	Ion	RT (min)
1	Sodium pyruvate ($^{13}\text{C}_3$, 99%)	$[\text{C}_3\text{H}_4\text{O}_3]$	90.0188	$[\text{M}-\text{H}]^-$	3.27
2	Succinic acid ($^{13}\text{C}_4$, 99%)	$[\text{C}_4\text{H}_6\text{O}_4]$	121.0328	$[\text{M}-\text{H}]^-$	6.70
3	Malic acid ($^{13}\text{C}_4$, 99%)	$[\text{C}_4\text{H}_6\text{O}_5]$	137.0277	$[\text{M}-\text{H}]^-$	6.73
4	α -ketoglutaric acid ($^{13}\text{C}_5$, 99%)	$[\text{C}_5\text{H}_6\text{O}_5]$	150.0310	$[\text{M}-\text{H}]^-$	8.07
5	Fumaric acid ($^{13}\text{C}_4$, 99%)	$[\text{C}_4\text{H}_4\text{O}_4]$	119.0172	$[\text{M}-\text{H}]^-$	8.63
6	Citric acid (2,2,4,4- D_4 , 98%)	$\text{C}_6\text{H}_4[\text{D}_2]\text{H}_4\text{O}_7$	195.0449	$[\text{M}-\text{H}]^-$	11.89

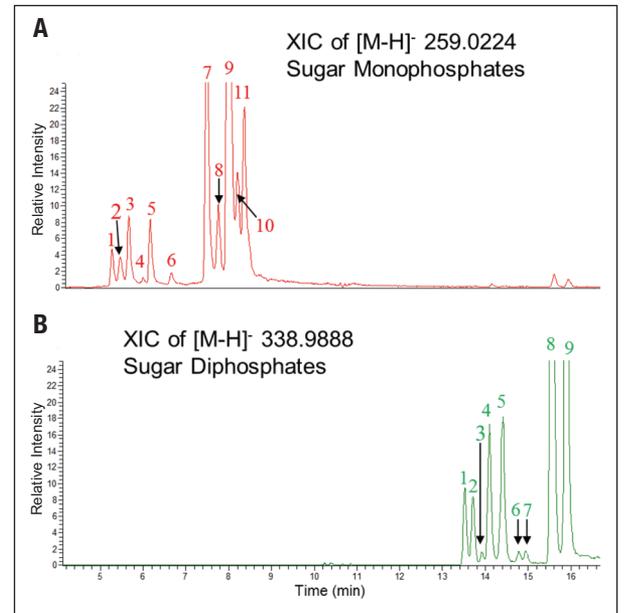


Figure 5. IC-MS for metabolite analysis of UM1 cancer cells, demonstrating the separation and analysis of sugar monophosphates (A) and sugar diphosphates (B). In A, peak #9 eluting at 7.94 min was identified as fructose 6-phosphate based on MS/MS and RT confirmation with standard. The Q Exactive HF MS offers very accurate mass measurement of the parent and product ions in negative ion mode (< 1 ppm).

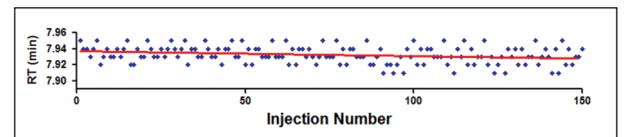


Figure 6. Reproducibility of analysis of fructose 6-phosphate (Peak #9) with a large scale injections. The RT averaged 7.93 ± 0.03 min ($n = 150$).

Biology of Interest and Workflow

The study design and workflow is shown in Figure 7. UM1 and UM2 cells were initially established from the same tumor of a tongue cancer patient. UM1 cells are highly invasive, whereas UM2 cells are less invasive. Similarly, UMSCC5 cells are significantly more invasive than UMSCC6 cells. A previous study¹² showed that the cancer stem-like cells (CSC) were present in the highly invasive UM1 cells.

The metabolic feature of CSC compared to nonstem cancer cells (NSCC) is also very interesting. In previous nontargeted analyses, metabolites in the TCA cycle showed significant changes between CSCs and NSCCs. The first half cycle (pyruvate/citrate/*cis*-aconitate/isocitrate/2-oxoglutarate) showed up-regulation in CSC cells, although the second half cycle (succinate/fumarate/malate) showed progressive down-regulation in CSCs versus NSCC.¹² This study investigated the metabolic difference between highly invasive and less invasive cancer cells and confirmed the previous observation regarding the changes in TCA metabolites between CSC and NSCC.

While the high-resolution, accurate-mass, full-scan data can be used for targeted quantitative analysis, the same data can also be revisited for nontargeted analysis and/or reprocessed for targeted quantitative analysis of additional compounds of interests.

Calibration Curves for Stable-Isotope-Labeled Standards

Six stable-isotope-labeled standards were pooled and diluted into eleven serial dilutions of 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000, and 10,000 pg/ μ L. Cell lysates containing the stable isotopes from UM1, UM2, UMSCC5, UMSCC6 cells, UM1-CSCs, and UM1-NSCCs were injected onto the IC-Q Exactive HF MS for analysis. There were at least three biological replicates for each concentration level of internal standards. The data was analyzed using TraceFinder software. The quantitative curves for the six stable-isotope-labeled standards were measured and are shown in Figure 8. The Q Exactive HF MS allows quantification to low fmol levels, with five orders of linear dynamic range and tight CV's (<10%). The dynamic range was within 10^{-9} to 10^{-5} M, which fits very well with the endogenous concentration level range from 10^{-10} to 10^{-3} M.

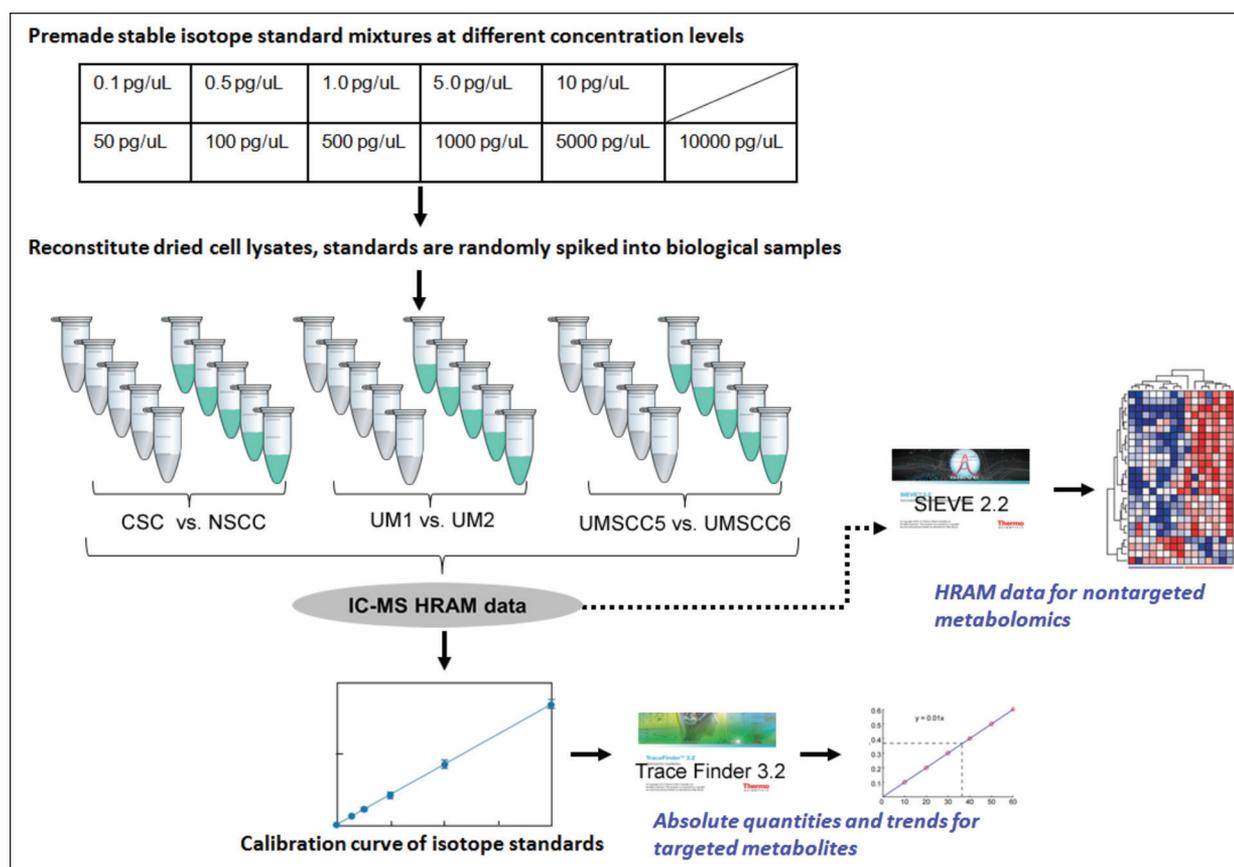


Figure 7. High-resolution, accurate-mass (HRAM) IC-MS approach in combination with stable-isotope-labeled standards as internal references for targeted metabolomics analysis of cancer cells.

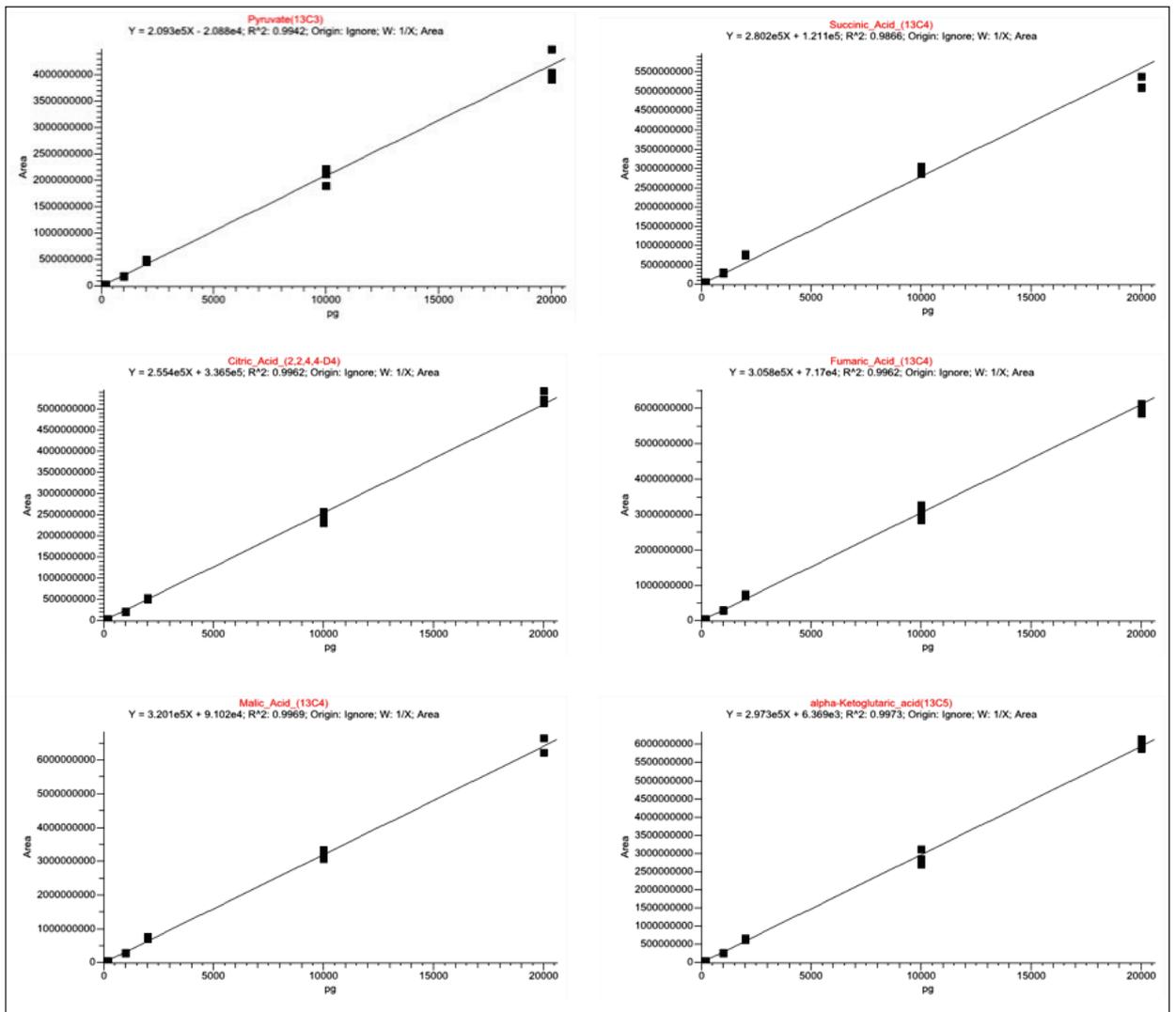


Figure 8. The IC-Q Exactive HF MS achieved five orders of magnitude dynamic range from 0.1 to 10,000 pg/ μ L with $R^2 \geq 0.99$ ($n=3$).

Quantify Targeted Endogenous Metabolites

There are several approaches, such as external calibration or a standard addition method, to quantify endogenous compounds in a biological matrix. Using an external calibration standard, the concentration-MS response curve is established using multiple concentrations of a neat compound standard without considering the sample matrix. On the other hand, standard addition using incremental addition of a standard to the sample, is a more widely used approach. However, this method is extremely labor intensive and unsuitable when measuring a large number of samples. Both methods have limitations that could significantly affect the accuracy.

This work used the methodology of directly spiking different concentration levels of stable-isotope standards into biological samples to generate the standard curve. The spiking solution was added to reconstitute the dried sample, thereby eliminating variations in sample volumes.

This approach can eliminate the variance by acquiring the standard response curve in each biological matrix. Endogenous metabolites having the same MS ionization efficiency will co-elute with their counter-reference compounds (the stable-isotope-labeled standards, or internal standards). TraceFinder software serves as a powerful tool for absolute quantitation by linking the target endogenous compound to its standard reference (which is called external standard in the software) and uses the reference calibration curve to automatically determine the concentration and amount of endogenous metabolite (Figure 9).

Calibration file last used: quanpart.calx

target compound linked reference compound

RT	Compound	Compound type	Standard type	Response via	Curve type	Origin	Weight	Units	ISTD	Amount	Linked compound	Estimation method
3.26	Pyruvate	Target Compo...	Estimated								Pyruvate(13C3)	Ext Curve
3.26	Pyruvate(13C3)	Target Compo...	External	Area	Linear	Ignore	1/X	pg				
6.70	Succinic_Acid_(13C4)	Target Compo...	External	Area	Linear	Ignore	1/X	pg				
6.71	Succinic acid	Target Compo...	Estimated								Succinic_Acid_...	Ext Curve
6.73	Malic Acid	Target Compo...	Estimated								Malic_Acid_(13...	Ext Curve
6.73	Malic_Acid_(13C4)	Target Compo...	External	Area	Linear	Ignore	1/X	pg				
8.07	alpha-Ketoglutaric aci...	Target Compo...	External	Area	Linear	Ignore	1/X	pg				
8.08	alpha-Ketoglutaric acid	Target Compo...	Estimated								alpha-Ketoglu...	Ext Curve
8.62	Fumaric_Acid_(13C4)	Target Compo...	External	Area	Linear	Ignore	1/X	pg				
8.63	Fumaric Acid	Target Compo...	Estimated								Fumaric_Acid_(...	Ext Curve
11.89	citrate	Target Compo...	Estimated					pg			Citric_Acid_(2,...	Ext Curve
11.89	Citric_Acid_(2,2,4,4-D4)	Target Compo...	External	Area	Linear	Ignore	1/X	pg				

Figure 9. TraceFinder software links the targeted endogenous compound to its external standard reference and uses the calibration curve to estimate the metabolite's quantity.

Based on the quantitative analysis results, UM1 cells displayed statistically significant higher levels of TCA metabolites including malic acid, fumaric acid, citric acid, and succinic acid than UM2. Both UM1 and UM2 were initially established from the same tumor of a tongue cancer patient. Similarly, UMSCC5 cells expressed dramatically higher levels of all metabolites than UMSCC6 cells and the other cell lines. These results indicate the rough quantities of metabolites in one plate (~3 million) cells range from 100 pg to 4 ng with citric

acid being the most abundant component. The quantities in UMSCC5 were observed to be 10 to 20 times higher than the other cell lines. It is known that UMSCC5 cells consume a very large amount of glucose and secrete a high concentration of lactate in cell culture. Therefore, it is not surprising that pyruvate and other metabolites levels were significantly higher in UMSCC5 cells. The results suggest that highly invasive HNSCC cells possess a more active TCA cycle than less invasive HNSCC cells, as shown in Figure 10.

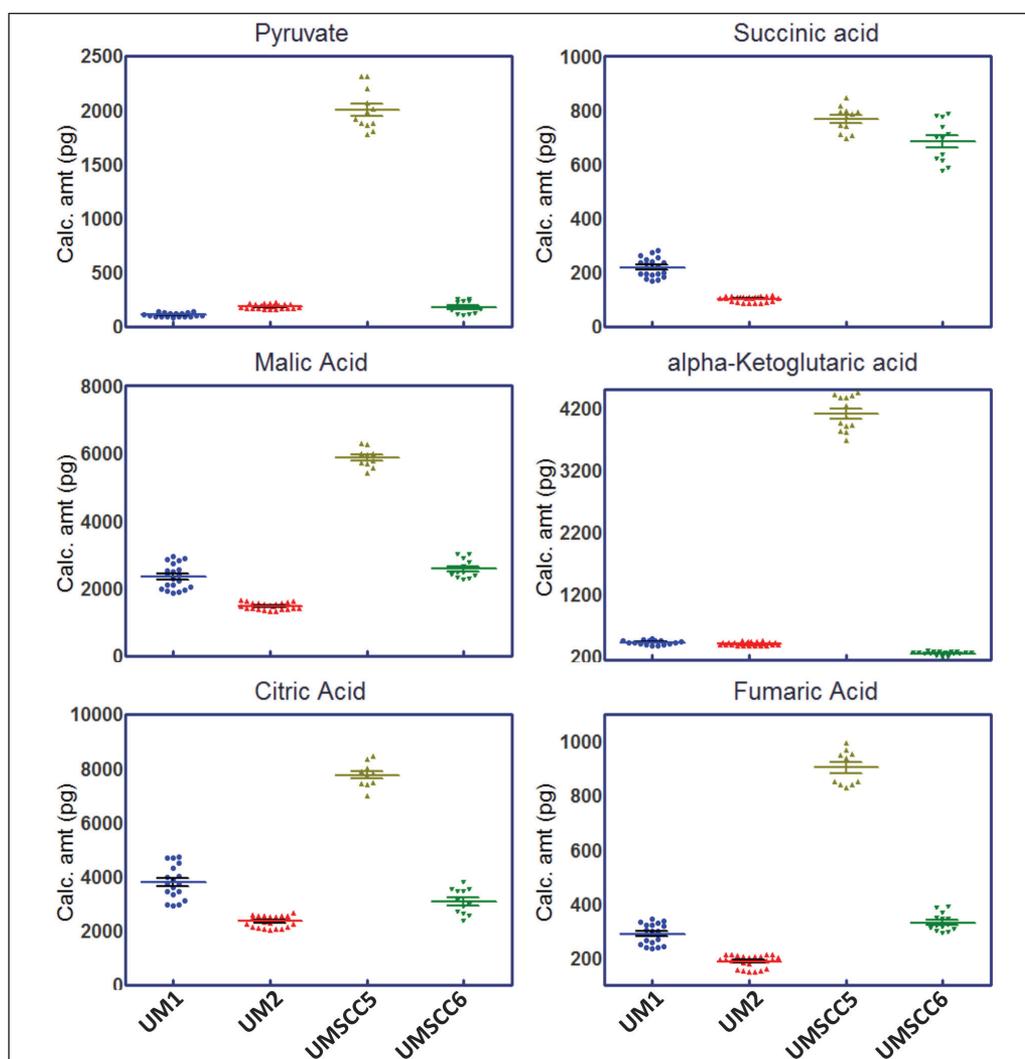


Figure 10. Calculated amounts of TCA metabolites per plate (avg. 3 million cells) and replicate numbers in UM1, UM2, UMSCC5, and UMSCC6 based on the IC-Q Exactive HF MS measurement.

The targeted quantitation results for the six TCA metabolites in CSC versus NSCC (Figure 11) match exactly with previous study using a nontargeted differential analysis. The first half cycle (pyruvate/citrate/*cis*-aconitate/isocitrate/2-oxoglutarate) was found to have an increasing up-regulation in CSC cells, while the second half cycle succinate/fumarate/malate) showed progressive down-regulation in CSCs versus NSCC.¹² The absolute quantitation results presented confirmed the quantitative trends with a new dataset, which leaves an interesting biological subject for future investigations.

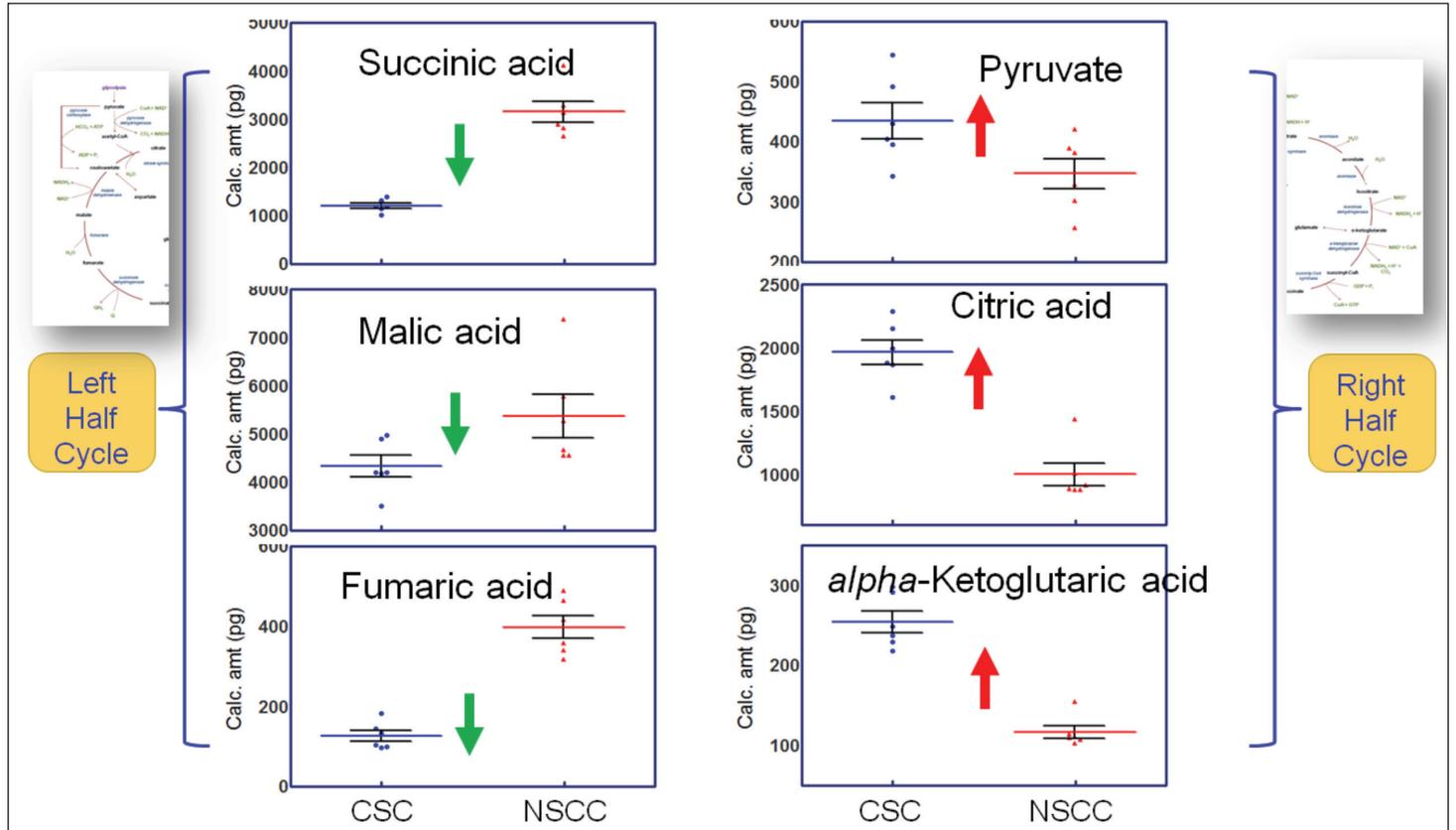


Figure 11. The targeted quantitation of six TCA metabolites in CSC versus NSCC. The results indicate that three metabolites (pyruvate/citric acid/*alpha*-ketoglutaric acid) in the first half cycle have an increasing up-regulation in CSC cells. The other three metabolites (fumaric acid/malic acid/succinic acid) in the second half cycle have an increasing down-regulation in CSC cells. The results are consistent with the previous findings obtained by the untargeted, global profiling approach.¹²

Conclusion

- Preliminary findings regarding the metabolic reprogramming in the sugar and TCA metabolism of cancer stem cells were achieved with the IC–high-resolution, accurate-mass Orbitrap MS-based system.
- IC provides efficient separation for polar metabolites. It covers almost all metabolic intermediates in glycolysis and the TCA cycle of the cancer cells and stem-like cancer cells. IC also provides excellent resolving power for many isobaric polar metabolites, such as mono- and di-phosphate sugar variants and *cis*- and *trans*-stereoisomers of organic acids and nucleotides, that are very challenging for RP and HILIC methods.
- The HRAM-based quantitation approach acquires targeted and global metabolomic data in one run. It offers great advantages such as high productivity, sensitivity, accuracy, and selectivity, as well as a “bird’s-eye view” of the sample. The HRAM full-scan data can be revisited for nontargeted analysis and/or reprocessed for targeted quantitative analysis of additional compounds of interests.
- The IC-Q Exactive HF MS achieved five orders of magnitude linear dynamic range with $R^2 > 0.99$ and very tight CV’s (< 10%). With this platform, metabolites can be quantified down to low fmol/ μ L and up to nmol/ μ L levels in biological samples. The high flow rate of the IC at 380 μ L/min has demonstrated excellent reproducibility for an extended period of time (150 injections) with minimal chromatographic shift ($RT < \pm 0.03$ min).
- The use of stable-isotope standards as internal references facilitates targeted quantitation in large-scale metabolomics analysis projects by eliminating variance caused by sample matrices.
- TraceFinder software facilitates data processing in absolute quantitation by linking the endogenous target compound to its external reference standard and uses the standard calibration curve to determine the concentration of the endogenous metabolite.

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