Application Note

Metabolomics

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Monitoring of Mammalian Cell Culture Media with HILIC LC/MS

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Abstract

An Agilent InfinityLab Poroshell HILIC-Z column, in combination with an Agilent 6545 Q-TOF LC/MS was used to monitor nutrient uptake and excreted waste products in mammalian cell culture media collected over six days. The use of the HILIC-Z column provided resilience against high-salt-containing samples (for example, cell culture media) by maintaining excellent peak shape and retention time reproducibility. By optimizing the mobile phase, gradient, and instrument parameters, this method could be used to monitor growth media nutrients (that is, glucose and amino acids) and metabolite waste products (that is, lactate and other TCA cycle-related organic acids) in a single analytical run in negative ion mode.

Introduction

Mass spectrometry (MS) is routinely used as a highly sensitive analytical technique for metabolomics studies to target a wide variety of small molecules[1]. As mammalian cell metabolomics emerges as a promising tool with potential applications in many research areas, it has become increasingly important to study many of the metabolites consumed and secreted by the cells in cell culture media[2,3]. Some analytical challenges remain, including retention of anionic metabolites[4], sample matrix effect[5], and analytical performance of chelating organic acids and phosphorylated compounds[6].

To address these challenges, a robust and reproducible hydrophilic interaction chromatography (HILIC) LC/MS method was developed using the InfinityLab deactivator additive as a mobile phase modifier to enhance the peak shape and detection signal of metal-chelating organic acids and phosphorylated analytes. As part of the chromatographic optimization and testing of the HILIC column, the effect of salt concentration in the sample matrix was investigated. A method was developed to enable the profiling of metabolites consumed and excreted in the cell growth media.

Experimental

Method

Salt tolerance study: A set of metabolite standards covering phosphorylated analytes and sugar phosphate isomers was selected for HILIC LC/MS method development. Stock solutions of the analytes were made in Milli-Q purified water at 5 mg/mL. The sample was then diluted from the stock to 1 ng/µL (ppm) in 80:20 acetonitrile (ACN)/water. A high salt stock solution (4 M urea and 2 M sodium chloride (NaCl) in water) was made for the salt spike-in experiment. The high-salt solution was spiked into the metabolite standards (3 ng) at a concentration equivalent to 20 % (80 mM urea, 40 mM NaCl) and 40 % (160 mM urea, 80 mM NaCl) of human urine. Higher salt concentrations (>40 %) were not attempted because the salt was not soluble in the sample matrix (80 % ACN).

Cell culture study: K562 leukemia cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum. A portion of the cells and media were collected immediately (day 0) and at 24 hours (day 1), 48 hours (day 2), 72 hours (day 3), or six days later (day 6). Samples were centrifuged at 250 × g for five minutes to pellet the cells. The growth media (100 µL) was transferred to a second centrifuge tube, mixed with 400 µL of 50 % ACN, and centrifuged at 10,000 x g for five minutes. Then, 1 µL of the supernatant was subjected to HILIC LC/MS analysis. The results were analyzed with Agilent MassHunter Qualitative Analysis.

Analyte separation: Chromatographic separation was performed on an InfinityLab Poroshell 120 HILIC-Z column, 2.1 mm × 50 mm, 2.7 µm, PEEK-lined (p/n 679775-924). A 10X mobile phase buffered stock solution (100 mM ammonium acetate, pH 9.0) in water was first prepared, and the 1X solvents were made with either water (solvent A), or ACN (solvent B). The InfinityLab deactivator additive (p/n 5191-4506) was added to both aqueous and organic mobile phases to ensure a constant concentration during gradient elution.

Instrumentation

LC/MS analysis was performed using an Agilent 1290 Infinity LC coupled to an Agilent 6545 Q-TOF with an Agilent Jet Stream Source. The LC consisted of:

- Agilent 1290 Infinity binary pump with seal wash (G4220A)
- Agilent 1290 Infinity autosampler (G4226A) with thermostat (G1330B)
- Agilent thermostatted column compartment (G1316C)

Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution. Tables 1 and 2 summarize the optimized LC and MS conditions. Data acquisition and analysis was done using the Agilent MassHunter software suite.

Table 1. The optimal LC parameters.

1290 Infinity LC system	
Column	InfinityLab Poroshell 120 HILIC-Z, 2.1 mm × 50 mm, 2.7 µm (p/n 679775-924)
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 95 %B 2 to 12 minutes 95 to 50 %B 12 to 13 minutes 50 %B to 95 %B 13 to 21 minutes 95 %B
Column temperature	25 °C
Injection volume	1 µL
Autosampler temperature	10 °C

Table 2. The optimal MS parameters.

6545 Q-TOF LC/MS	
Ionization mode	Agilent Jet Stream
Ionization 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
MS acquisition rate	1 spectrum/sec
Reference mass	*m/z* 980.01638

Results and discussion

A simple and robust HILIC LC/MS method was developed taking advantage of the wide pH stability and high resolution of the InfinityLab Poroshell HILIC-Z column. In this study, a mobile phase at pH 9.0 provided the best overall results for chromatographic separation. Moreover, the addition of the InfinityLab deactivator additive greatly improved the peak shape and signal intensity of chelating organic acids and phosphorylated compounds. The two most common cell culture media, DMEM and RPMI 1640, contain a high concentration of inorganic salts including (NaCl, ~100 mM), potassium chloride (KCl, ~5 mM), and calcium nitrate ($Ca(NO_3)_2$, ~0.4 mM). These maintain osmotic balance, and help regulate the membrane potential of the cultured cells. To determine the effect of salts on retention time reproducibility and metabolite peak shape, samples spiked with increasing concentrations of urea and NaCl were analyzed with the HILIC LC/MS method (Figure 1). The results showed that the HILIC column maintained retention time reproducibility with minimal ion suppression detected for the target nucleotides (for example, AMP, ADP, ATP, and GTP) and sugar phosphate isomers (for example, glucose-1-phosphate and glucose-6-phosphate) in high salt samples (Figure 1).

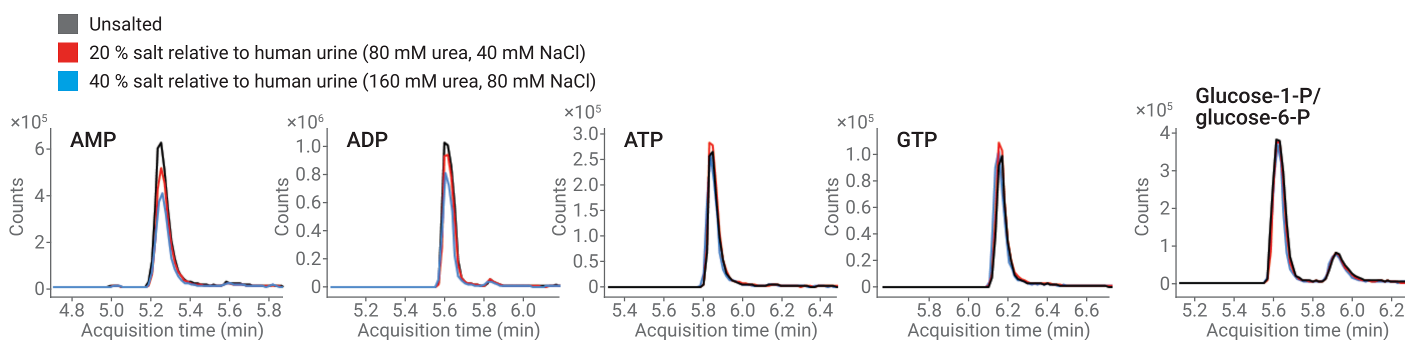


Figure 1. Retention time reproducibility of analytes in salty samples using HILIC-LC/MS.

Next, an experiment was designed to monitor nutrient consumption and excretion of metabolite waste products in the cell culture media over a period of six days (Figure 2). As expected, lactate accumulation was observed over time in the growth media as a metabolic waste product (Figure 3, column 1). Moreover, other organic acids associated with the TCA cycle (that is, malate, α-ketoglutarate (α-KG), glutamate, and citrate) were excreted and found to accumulate in the cell culture media over the monitored timeframe (Figure 3). In contrast, glucose levels decreased over time as the cells consumed the sugar for cellular metabolism (Figure 3, column 2) until glucose was completely depleted (Figure 3, column 2, day 6). Amino acid levels also decreased over time, which correlated with the consumption of the nutrients from the growth media by the cells (Figure 4). These results demonstrated that in a single HILIC LC/MS run, a wide range of metabolites including organic acids and amino acids could be profiled and monitored from mammalian cell culture media.

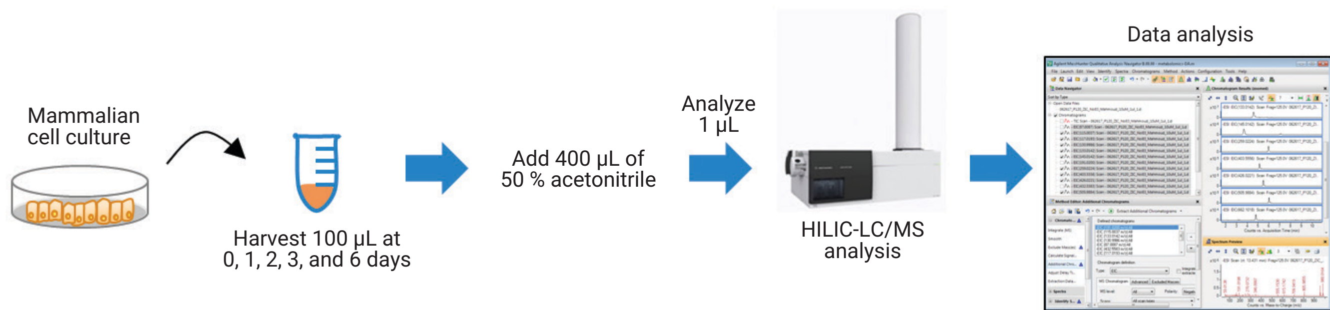


Figure 2. Experimental design for a time-course study to monitor metabolites in mammalian cell culture media.

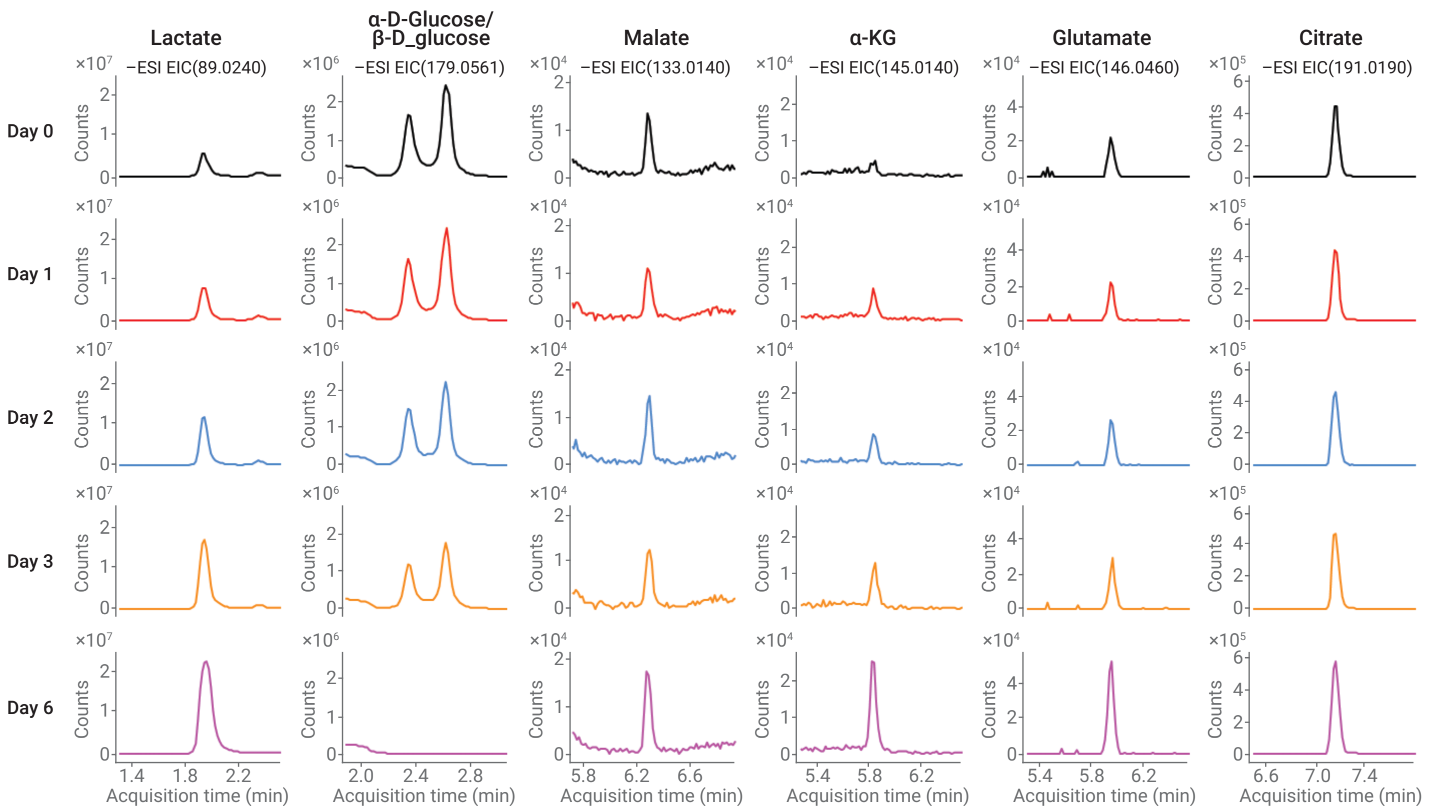
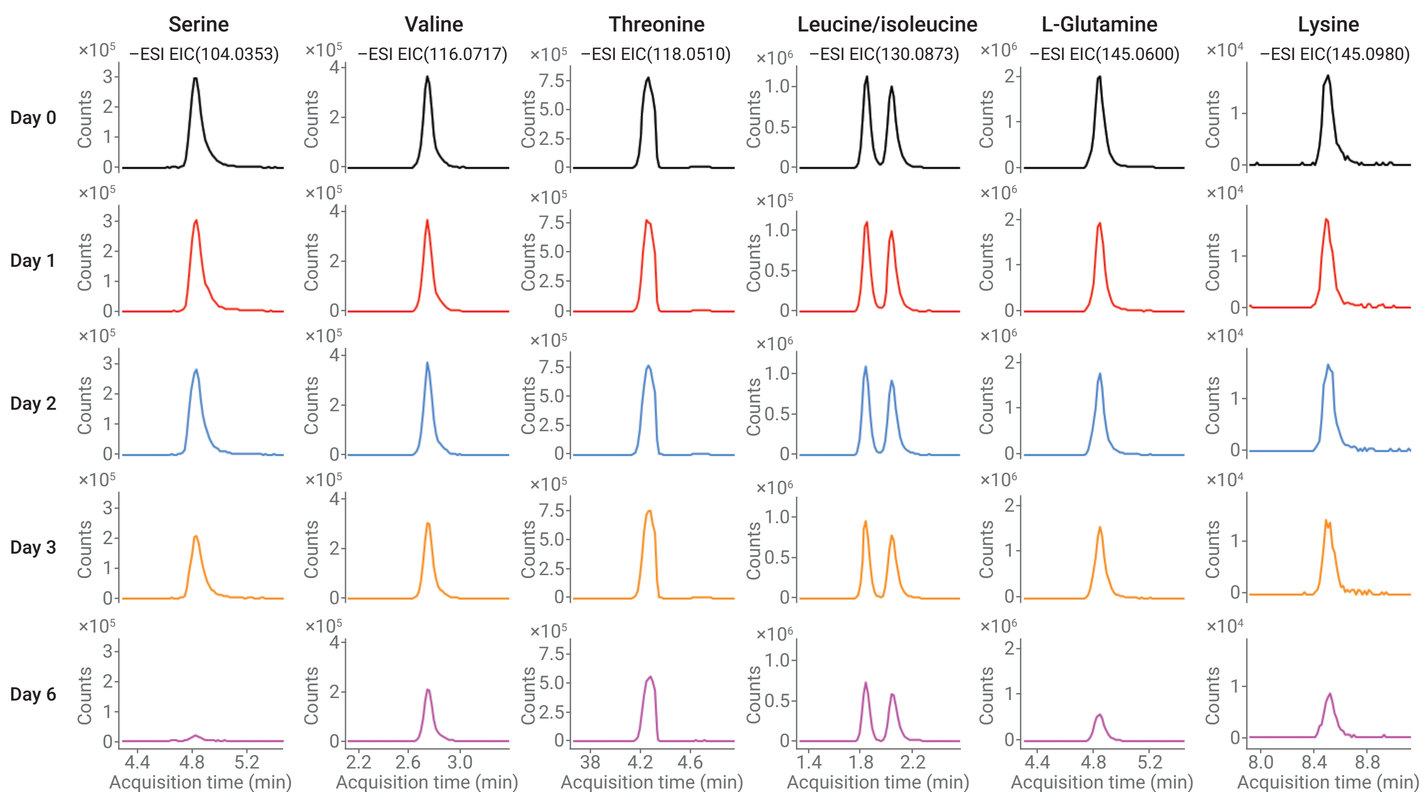


Figure 3. Tracking the secretion of cellular metabolism waste product accumulation in the growth media.



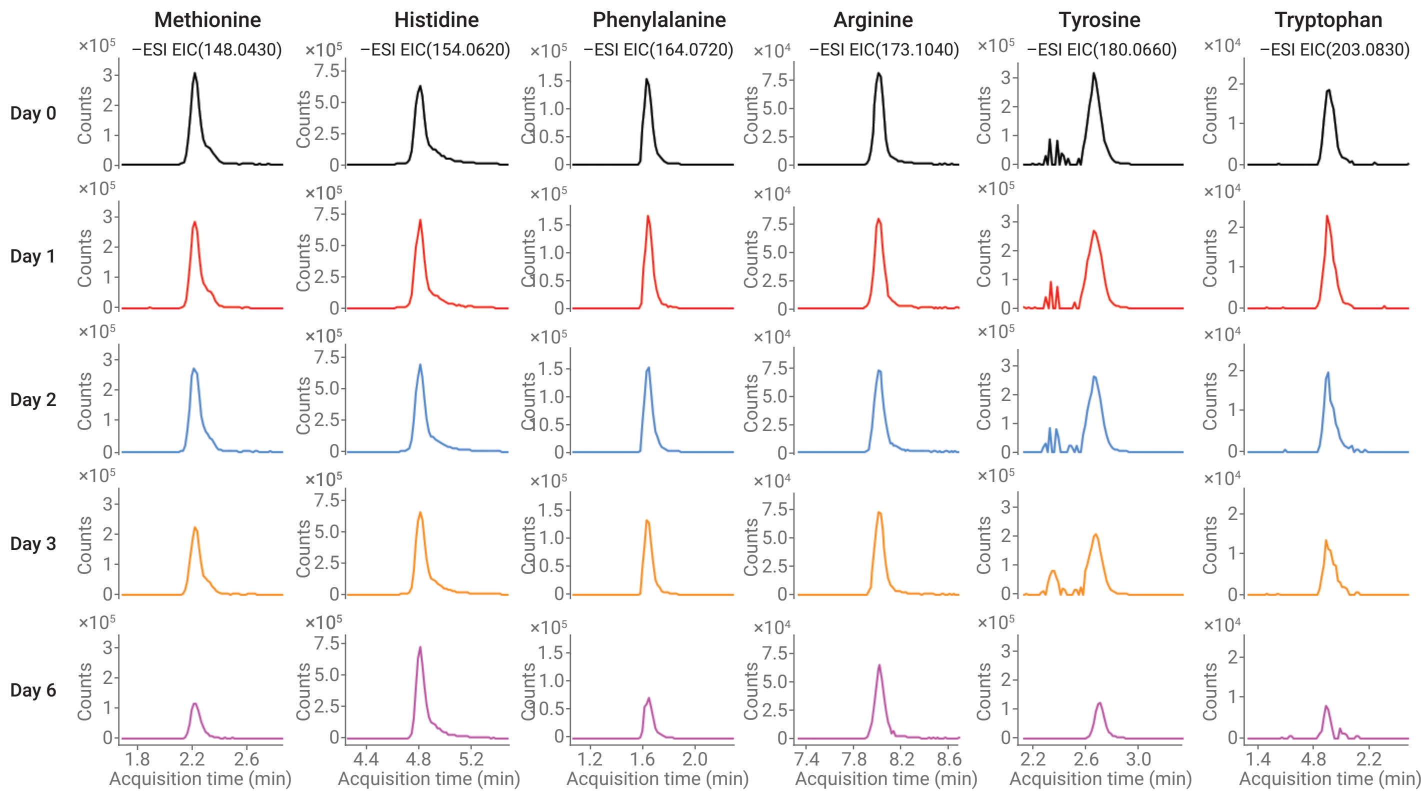


Figure 4. Monitoring the consumption of amino acid nutrients in the cell culture media.

Conclusions

A HILIC-based chromatographic method coupled to the 6545 Q-TOF LC/MS provides excellent analytical performance for profiling across a broad range of metabolite classes in mammalian cell culture media. The InfinityLab Poroshell HILIC-Z column in PEEK-lined hardware is shown to provide reliable retention time reproducibility and analytical sensitivity for high-salt samples. The PEEK-lined HILIC-Z column combined with the 1290 Infinity LC system and the 6545 Q-TOF system delivered excellent performance, making it an ideal platform for metabolomics profiling. Together, these features allow the detection and separation of both anionic and more hydrophobic metabolites in a single method, and could be used to monitor biomanufacturing processes.

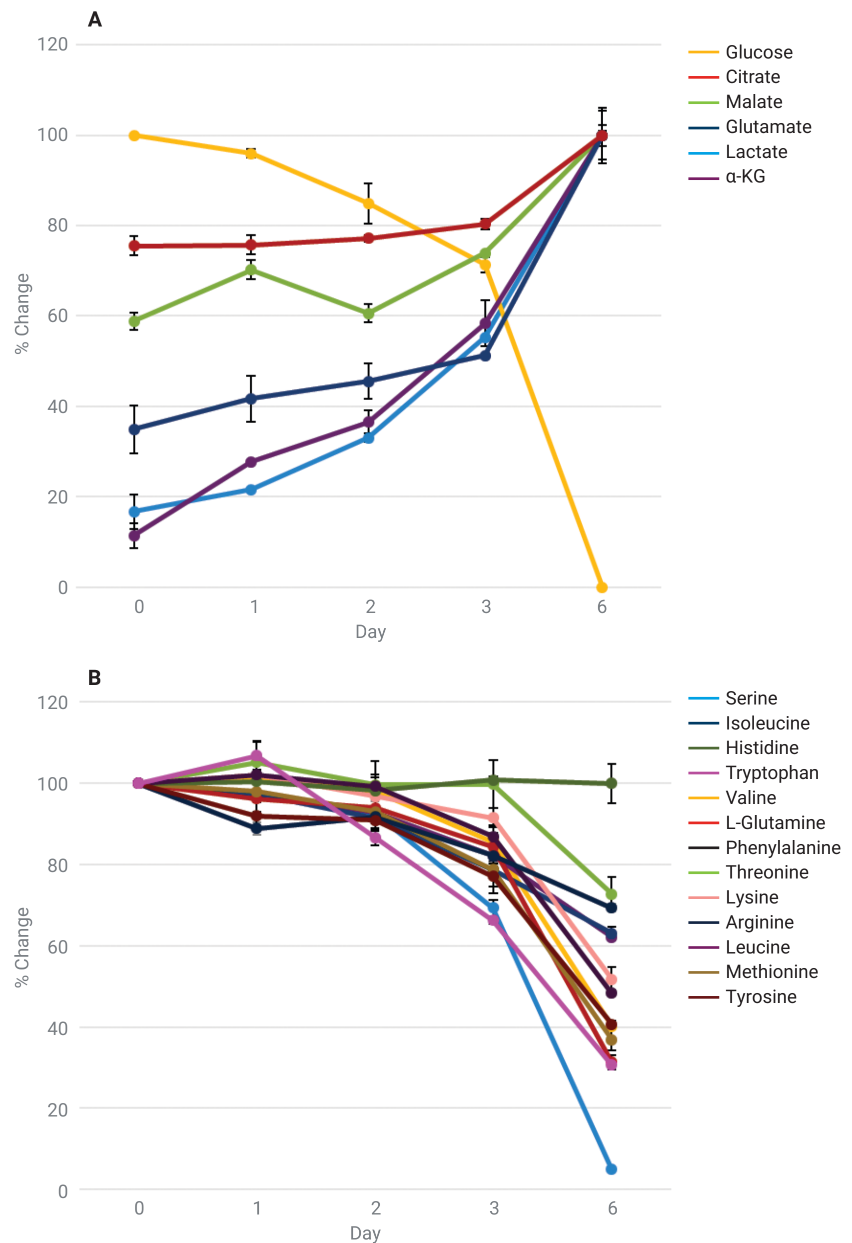


Figure 5. Quantitative analysis of nutrient consumption and metabolite secretion from mammalian cell culture media.

References

1. Wishart, D.S. Emerging applications of metabolomics in drug discovery and precision medicine. *Nat. Rev. Drug Discov.* **2016**, *15(7)*, 473-484.
2. Silva, L. P.; *et al.* Exometabolomics and MSI: deconstructing how cells interact to transform their small molecule environment. *Curr. Opin. Biotechnol*. **2015**, *34*, 209-216.
3. Garcia-Manteiga J. M.; *et al.* Metabolomics of B to plasma cell differentiation. *J. Proteome Res*. **2011**, *10(9)*, 4165-76.
4. Zhang, Z.; *et al.* Evaluation of coupling reversed phase, aqueous normal phase, and hydrophilic interaction liquid chromatography with Orbitrap mass spectrometry for metabolomic studies of human urine. *Anal. Chem*. **2012**, *84(4)*, 1994-2001.
5. Panuwet, P.; *et al.* Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring. *Crit. Rev. Anal. Chem*. **2016**, *46(2)*, 93-105.
6. Pesek, J. J.; *et al.* Improvement of peak shape in aqueous normal phase analysis of anionic metabolites. *J. Sep. Sci*. **2011**, *34(24)*, 3509-3516.

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