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UHPLC-Q-TOF MS-Based Profiling of Cell Culture Medium and Post-Culture Supernatant Combined with a Retention Time-Integrated Database

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Introduction

Cell culture medium is the fundamental microenvironment governing the survival, proliferation, differentiation and functional phenotypes of in vitro cells, with its composition underpinning basic biological research, biopharmaceutical production and cell therapy reliability. Therefore, clarifying the components of cell culture media holds the following significance:

- Quality Assurance — Ensures batch consistency and reproducibility
- Formula Optimization — Understands component functions to improve culture efficiency
- Cost Control — Optimizes ingredient ratios to reduce production costs
- Troubleshooting — Quickly identifies causes of culture problems

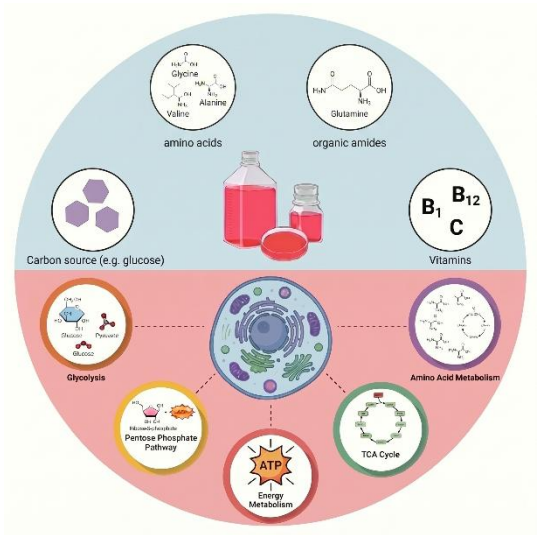

Figure 1. Major nutritional components in cell culture medium (Upper). Metabolic pathways and metabolites in cells (Lower).

Meanwhile, the correlation analysis between culture medium component changes and cell metabolic status is also very important for the optimization of cell culture medium components. Therefore, a method that can comprehensively analyze the components in culture medium and cell metabolites is needed. Such a method faces several challenges, for example: the separation of isomers (such as leucine and isoleucine), compounds with phosphate groups that are difficult to produce good peak shapes (such as ATP, ADP, etc.), and certain compounds that require retention time to assist in identification (such as malic acid and fumaric acid). Therefore, we have established a database with retention times, which can be used for comprehensive analysis of sugars, amino acids, vitamins, organic amines in culture medium, as well as metabolites produced through cell glycolysis pathway, pentose phosphate pathway, TCA cycle, amino acid metabolism, and energy metabolism.

Experimental

Sample Information and Pretreatment

A total of four samples (two media: 1-1-M, 6-4-M; two supernatants: 1-1-S, 6-4-S) were diluted with 80% acetonitrile/water, centrifuged, and the resulting supernatants used for UHPLC-Q-TOF MS analysis.

UHPLC-Q-TOF MS Parameters

LC Mode	HILIC
Ion Source	AJS
Polarity	Pos/Neg
Gas Temp （℃）	300
Drying Gas （L/min）	8
Nebulizer （mL/min ）	0.3
Vcap （V）	3500
Fragmentor （V）	110
Sheath gas temp （℃）	325
Sheath gas flow （mL/min ）	11
Nozzle Voltage （V）	500 (pos)/1000 (neg)

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Data processing

A PCDL library containing more than 220 compounds with retention time information was used for target component confirmation, including amino acids, vitamins, nucleotides, sugars related, organic amines. Agilent Masshunter Profinder B.10 was employed as the components analysis software.

Component confirmation in cell medium and method validation

- Amino acids : A total of 21 amino acids were identified, including all 8 essential amino acids (Leu, Ile, Phe, Val, Trp, Met, Thr, Lys)） and 13 non-essential amino acids (His, Arg, Pro, Glu, Gln, Asp, Tyr, Ala, Gly, Asparagine, Cysteine, Cystine, Hydroxyproline). Figure 2 demonstrates that the isomers of leucine (Leu) and isoleucine (Ile) can be well separated under the established experimental conditions.
- Vitamins and coenzymes : 14 vitamins (Riboflavin, Nicotinamide, Niacin, Pantothenate, PL, PM, Pyridoxine, Biotin, Folate) and coenzymes (NAD+, 7,8-Dihydrobiopterin, PLP, PMP, FAD) were detected.
- Carbon source: Three sugar types were identified: glucose, pentose, and sucrose.
- Organic amines : Other organic amines detected in the medium included Ethanolamine/Monoethanolamine (MEA), Diethanolamine (DEA), and Trimethylamine.

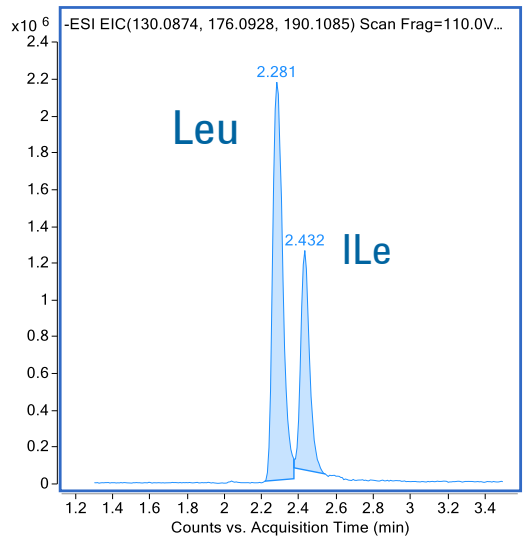

Figure 2. Chromtogram of leucine（Leu） and isoleucine（Ile）

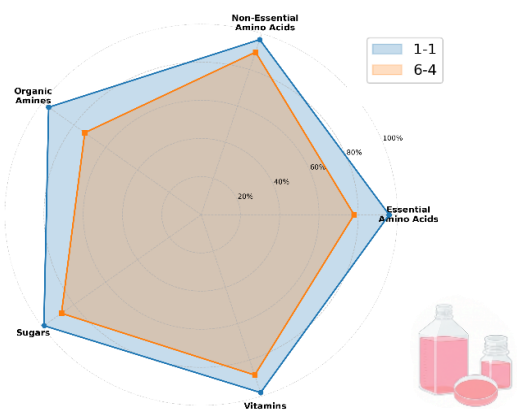

Figure 3. comparison of components: 1-1, 6-4. (normalized to maximum values)

Overall, 1-1 and 6-4 culture media exhibit similar composition patterns in four nutrient categories: amino acids, vitamins, sugars, and organic amines. However, two key differences were observed: (1) 1-1 medium contains higher levels of all four nutrient categories compared to 6-4; (2) 1-1 shows more prominent proportions in organic amines and essential amino acids. (Figure 3)

Cell metabolites component confirmation and method validation

A total of 99 metabolites were detected in the cell culture supernatant, classified into the following categories:

- Essential Amino Acids (8)
- Non-Essential Amino Acids (13)
- Amino Acid Metabolites (18)
- Vitamins and Coenzymes (15)
- Glycolysis Metabolites (5)
- Pentose Phosphate Pathway (PPP) Metabolites (5)
- TCA Cycle Metabolites (7)
- Energy Metabolism Compounds (8)
- Nucleosides and Nucleic Acid Bases (16)
- Sugars and Others (3)

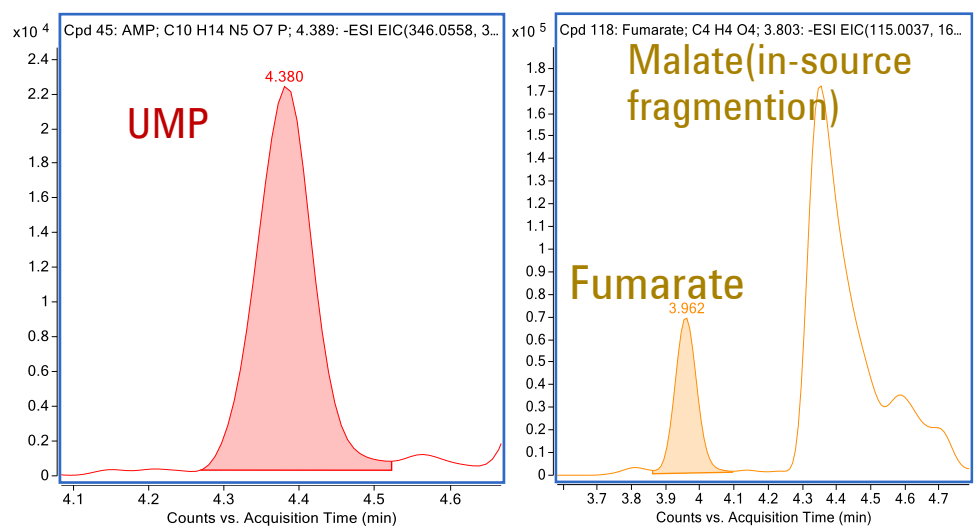

Figure 4. EIC of UMP

Retention time information is critical for compound identification. For instance, when extracting the EIC (Extracted Ion Chromatogram) of fumarate, two peaks appeared at 3.8 min and 4.5 min, respectively, which could lead to misidentification. Standard verification confirmed that the 4.5 min peak resulted from in-source fragmentation of malate (Figure 5).

Phosphate-containing compounds often exhibit severe peak tailing and poor peak shapes during chromatographic separation, compromising sensitivity and quantitative accuracy. This limitation poses significant challenges for studying cellular energy metabolism and compounds involved in glycolysis and the pentose phosphate pathway. Gratifyingly, our method achieved well-defined peak shapes for phosphate-containing compounds, as exemplified by UMP (Figure 4).

Results and Discussion

From the perspective of compound categories, Metabolites distribution in 1-1 and 6-4 supernatant are as Figure 6. It can be observed that the 1-1 and 6-4 cell culture supernatants exhibit similar coverage of metabolite categories. However, there are notable differences in the number of metabolites detected between the two samples, for instance energy and glycolysis metabolites.

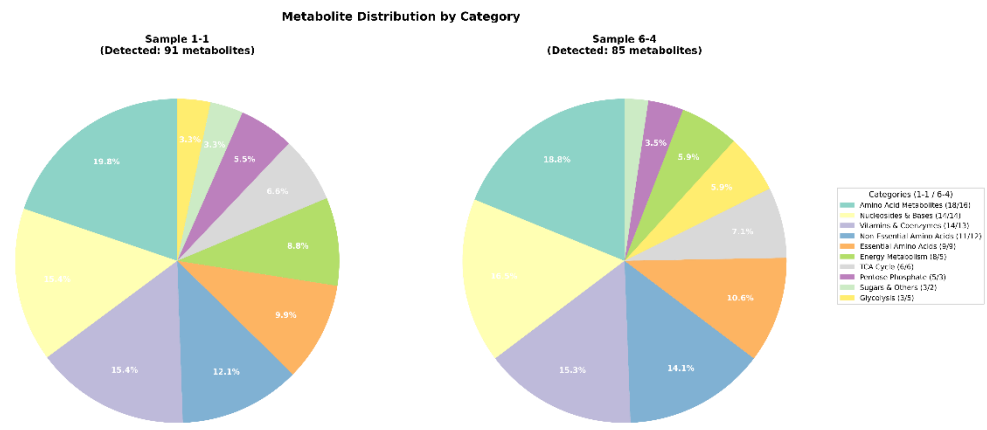

Figure 6 Metabolites category distribution in 1-1 and 6-4 cell culture supernatant

Differential metabolite analysis revealed that the 1-1 sample exhibited significantly elevated metabolites across multiple pathways compared to the 6-4 sample. Pyrimidine metabolism showed the most dramatic changes, with dU (70.22-fold) and cytosine (42.97-fold) being the most significantly increased. The citric acid cycle was markedly upregulated, with citrate elevated by 68.09-fold, suggesting enhanced energy metabolism. Glycolysis/pentose phosphate pathway and amino acid metabolism were also increased. Collectively, these findings indicate that the 1-1 sample displays a metabolically active phenotype with robust nucleotide biosynthesis and elevated TCA cycle activity. (Figure 7)

The 6-4 sample exhibited elevated metabolites associated with distinct pathways compared to the 1-1 sample. Purine metabolism was notably activated (inosine, 6.33-fold; cAMP, 3.10-fold), along with enhanced glycolysis/gluconeogenesis (PEP, 4.43-fold) and amino acid metabolism (aspartate, 3.10-fold). Biotin was substantially elevated (4.97-fold), while TCA cycle activation was moderate (malate, 2.96-fold). These findings indicate that the 6-4 sample displays a distinct metabolic phenotype characterized by heightened purine metabolism and glycolysis, contrasting with the TCA cycle and pyrimidine metabolism dominance observed in the 1-1 sample. (Figure 7)

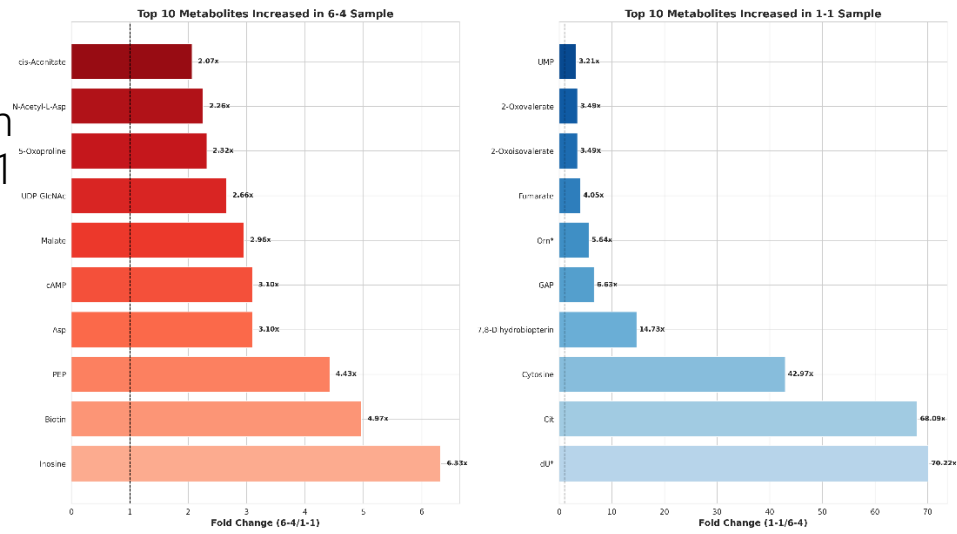

Figure 7. Top 10 Metabolites with Significant Fold Change Differences Between 6-4 and 1-1 Samples

Notably, UDP-Glc was found at significantly higher levels in the 6-4 cell culture supernatant compared to the 1-1 sample (Figure 8). UDP-Glc is closely associated with the glycosylation process during antibody synthesis. This finding may provide valuable insights for optimizing antibody production. Therefore, comprehensive analysis and optimization of cell culture medium components, coupled with metabolomic profiling of cellular metabolites, can effectively guide the development of improved cell culture strategies for enhanced production of biotherapeutics such as antibodies.

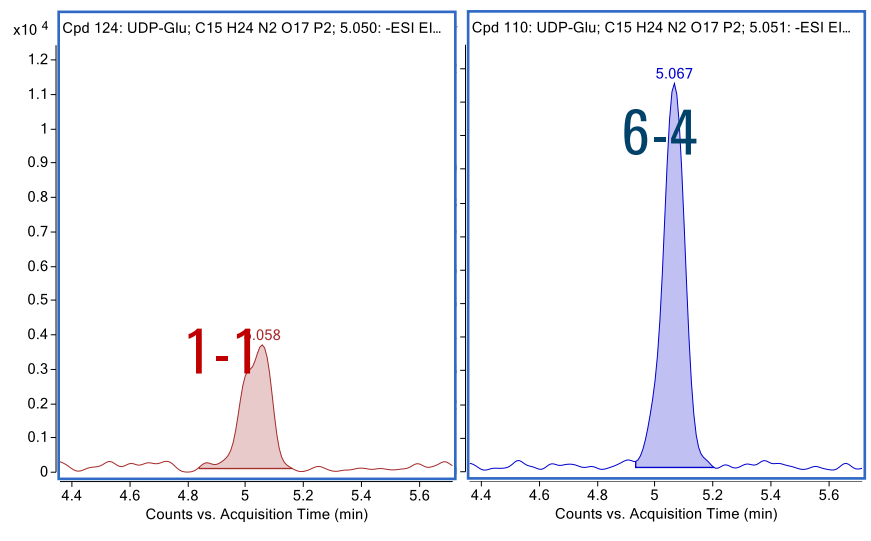

Figure 8. EIC of UDP-Glc in 1-1 and 6-4

Conclusions

- A metabolomics database containing 220 compounds (with RT information), covering nutrients (carbohydrates, amino acids, vitamins, amines) and key metabolites from central carbon pathways, was established.
- Metabolomic profiling of cell culture supernatants revealed distinct metabolic profiles between media formulations, demonstrating that medium composition substantially affects cellular metabolic states.
- Integrating antibody titer data in the future with metabolomics enables correlation analysis across media composition, metabolic phenotype, and product yield, providing a rational framework for culture optimization and antibody manufacturing.

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