Application Note

Metabolomics,
Small Molecule Pharma

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Underivatized Amino Acid Profiling in Yeast Fermentation Samples

Using the biocompatible Agilent Altura Poroshell 120 HILIC-Z and the Agilent 6230C time-of-flight LC/MS

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Abstract

During fermentation process development, changes in spent media composition can provide insight into nutrient utilization, metabolite release, and the changing metabolic activity of a culture over time. This application note details a workflow for the quantitation of underivatized amino acids directly from fermentation media using a fully biocompatible liquid chromatography (LC) system with an Agilent Altura Poroshell 120 HILIC-Z column, enabling the preservation of metal-sensitive analytes without loss of signal or peak shape degradation. Traditional protocols for amino acid analysis use chemical derivatization, which can impact relative yield, metabolite integrity, and increase sample preparation time. The method described herein includes a detailed sample preparation protocol, a hydrophilic interaction liquid chromatography (HILIC) method, liquid chromatography/mass spectrometry (LC/MS) parameters, and data processing using Agilent MassHunter Quantitative Analysis 12.1 and Agilent MassHunter Explorer 2.0 software. The Agilent 6230C time-of-flight (TOF) LC/MS system provides high-resolution accurate mass full scan detection of small molecules, including amino acids, organic acids, and carbohydrates within complex matrices. Together, targeted quantitation and untargeted metabolite profiling provide complementary information for characterizing fermentation processes as they develop over time.

Introduction

Fermentation process development requires an understanding of how nutrients are consumed and metabolites are released as cultures grow. Analysis of fermentation media can provide insight into nutrient utilization, metabolite accumulation, and changes in metabolic activity over time. *Saccharomyces cerevisiae*, commonly known as baker's yeast, is a well-characterized microbial fermentation system used across biotechnology and industrial production. During fermentation, *S. cerevisiae* metabolizes sugars, amino acids, vitamins, and other nutrients present in the environment, producing alcohol, carbon dioxide, organic acids, and other small metabolites while generating adenosine triphosphate (ATP) as an energy source.[1] Monitoring amino acid uptake and release using LC/MS can provide essential information on yeast fermentation and growth performance. Conventional workflows for amino acid analysis and quantitation frequently use derivatization to improve chromatographic retention in reversed-phase chromatography and boost ionization efficiency. However, derivatization can increase sample preparation time and complexity. Here, a previously described hydrophilic interaction liquid chromatography (HILIC) methodology[2] is used to quantify concentration changes of underivatized amino acids in fermentation media, using a biocompatible flow path and the Agilent 6230C TOF LC/MS system.

This method demonstrates robust detection and quantitation of amino acids and nutrient composition across a wide dynamic concentration range throughout fermentation process development. In addition to targeted amino acid quantitation, high-resolution full scan acquisition provides a broader view of metabolic changes and supports untargeted analysis as fermentation progresses. With a fully biocompatible Agilent 1290 Infinity II bio LC system and an Agilent Altura Poroshell 120 HILIC-Z column with Agilent Ultra Inert technology (Figure 1), metal-analyte interactions are mitigated for improved sensitivity and peak shape using an iron-free LC flow path.[3] Equivalent performance is expected with the Agilent 1290 Infinity III bio LC, and all methods described here are compatible with both systems. The 6230C TOF LC/MS provides high-resolution accurate mass detection across a mass range of *m/z* 25–20,000, supporting applications ranging from small-molecule metabolomics to macromolecular characterization and providing versatility across pharmaceutical research workflows. Updated electronics in the 6230C TOF LC/MS provide better performance over previous iterations, including improved mass accuracy and sensitivity. The inclusion of Agilent VacShield technology improves instrument productivity by allowing ventless capillary maintenance and reducing instrument downtime.

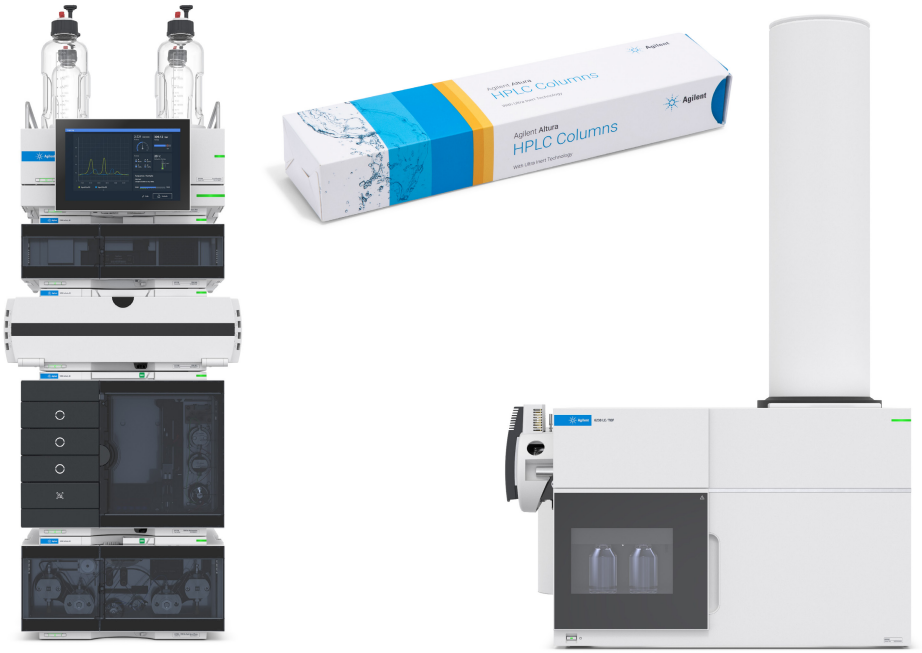


Figure 1. The workflow uses an Agilent 1290 Infinity II/III bio LC system, an Agilent Altura Poroshell 120 HILIC-Z column with Ultra Inert technology for an iron-free flow path, and an Agilent 6230C TOF LC/MS system (shown from left to right).

Experimental

Sample preparation

Baker's yeast (*S. cerevisiae*, ATCC 204508) was purchased from ATCC (Manassas, VA) and cultured at 30 °C in dilute (20x) Y5000 yeast peptone dextrose broth from Teknova (Hollister, CA). Fermentation samples were collected at discrete time points at two-hour increments from 0 to 12 hours and at 24 hours. To remove yeast and cellular debris, samples were centrifuged at 5,000 × rcf for five minutes. The resulting supernatant was collected and diluted 1:4 with 50:50 methanol:water before vortex mixing and centrifugation at 10,000 × rcf for 10 minutes to remove residual debris and insoluble material. Extracted supernatant was diluted 20-fold into 70:20:10 acetonitrile:water:methanol before LC/MS analysis. Quantitation was performed using a standard mixture (Agilent Amino Acid Analysis standard) supplemented with glutamine, asparagine, and tryptophan. A calibration curve (n = 4) was generated at concentrations from 50–50,000 nM, a range estimated to encompass the expected concentration range in the samples.

Data collection

Replicates from each fermentation time point (n = 6) were analyzed using HILIC chromatography with an Altura Poroshell 120 HILIC-Z column (2.1 × 150 mm, 2.7 µm) on a 1290 Infinity II bio LC coupled to the 6230C TOF LC/MS. Method parameters are provided in Tables 1 and 2.

Table 1. Chromatographic conditions.

LC Parameter	Value
Analytical Column	Agilent Altura Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 µm (p/n 227215-924)
Column Temperature	30 °C
Injection Volume	3 µL
Sampler Temperature	4 °C
Needle Wash	Multiwash, 3 s each: acetonitrile, isopropanol, water
Mobile Phase	A) 10 mM ammonium acetate, pH 9.0, in water B) 10 mM ammonium acetate, pH 9.0, in 90:10 acetonitrile:water
Flow Rate	0.25 mL/min
Gradient Program	Time (min) %B 0 90 2 90 12 40 13 20 16 20 17 90 25 90
Total Run Time	25 min

Table 2. Agilent Jet Stream technology source parameters on the Agilent 6230C TOF LC/MS.

Parameter	Value
m/z Range	60–1,000
Sheath Gas Temperature	300 °C
Sheath Gas Flow	12 L/min
Gas Temperature	200 °C
Gas Flow	10 L/min
Nebulizer	40 psi
Capillary Voltage	3,000 V
Nozzle Voltage	0 V
Polarity	Negative

Results and discussion

The HILIC chromatographic separation provides effective retention and resolution of underivatized amino acids, with the majority eluting within the first 10 minutes of the gradient (Figure 2). Good chromatographic separation of the isomers leucine and isoleucine is also observed (Figure 3). This separation performance supports reproducible monitoring of amino acids in complex fermentation media without requiring derivatization.

Method performance was evaluated through replicate injections of a calibration curve (n = 4), demonstrating strong linearity (R^2 > 0.998) over an extended dynamic range and reproducible peak areas (relative standard deviations [RSDs] < 10%) with high mass accuracy (< 2 ppm). These results are shown in Table 3 and Figure 4. Quantitation of the amino acid concentrations across different time points was performed using Agilent MassHunter Quantitative Analysis 12.1.

External calibration curves spanned concentrations from 50–50,000 nM and demonstrated linear responses across three orders of magnitude, supporting quantitation of low- and high-abundance amino acids within a single analytical run and the ability to analyze large changes in the concentration of a single amino acid throughout fermentation (Figure 4). This broad quantitative range is particularly useful for fermentation media analysis, where nutrient concentrations may vary substantially as fermentation progresses. The Calibration Curves-at-a-glance feature within MassHunter Quantitative Analysis 12.1 provides a straightforward evaluation of calibration performance.

To facilitate data processing and annotation, a custom personal compound database and library (PCDL) was developed for the target amino acids, including accurate mass, chemical formulas, and experimentally determined retention times obtained from the standards. This PCDL is imported directly into MassHunter Quantitative Analysis 12.1, enabling identification of amino acids within the matrix, peak integration, and quantitation with a calibration curve in a single software environment. Analysis of the fermentation media prior to yeast fermentation reveals a wide distribution of amino acid concentrations (Table 4). As fermentation progresses, changes in several amino acid concentrations reveal distinct nutrient-utilization patterns within the culture (Figure 5). Several amino acids are depleted quickly, metabolized to support cellular growth and protein synthesis. Others show relatively minor changes in concentration over the 24-hour time period.

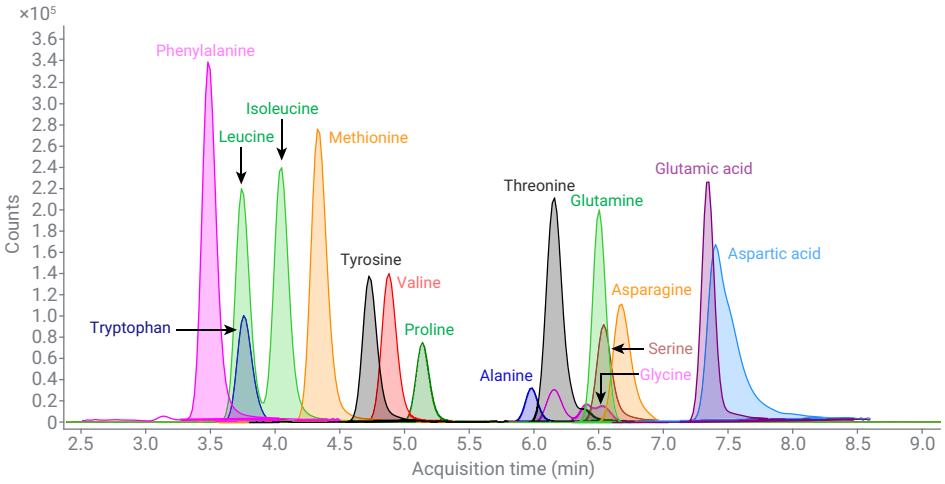


Figure 2. Extracted ion chromatogram (EIC) traces of amino acids across the gradient from a 2.5 µM standard.

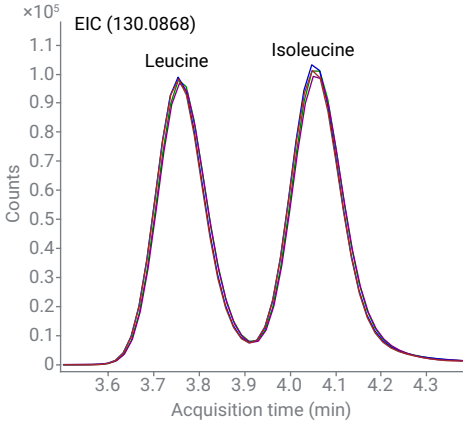


Figure 3. EIC traces of leucine and isoleucine (*m/z* 130.0868) overlaid from four replicate injections, showing separation of the isomers and peak reproducibility across injections.

Table 3. Summary of quantitative results for the amino acid standards in negative ionization mode.

Compound	Retention Time (min)	Calibration Curve Range (nM)	R^2
Alanine	6.0	250–25,000	0.999
Asparagine	6.7	100–10,000	0.999
Aspartic Acid	7.4	100–10,000	0.998
Isoleucine	4.1	50–50,000	0.999
Glutamic Acid	7.3	100–10,000*	0.999
Glutamine	6.5	250–25,000*	0.999
Glycine	6.5	250–5,000	0.998
Leucine	3.7	50–25,000	0.998
Methionine	4.4	50–10,000	0.999
Serine	6.5	250–25,000*	0.998
Phenylalanine	3.5	50–10,000	0.999
Proline	5.1	100–50,000	0.999
Threonine	6.2	100–10,000*	0.999
Tryptophan	3.8	250–10,000	0.999
Tyrosine	4.7	50–10,000	0.999
Valine	4.9	50–50,000	0.998

* Indicates best fit as quadratic

Table 4. Quantitation of the initial amino acids in the fermentation media at the 0-hour time point. The supernatant was diluted 20x before injection.

Compound	Starting Concentration (µM, Mean ± SD)
Alanine	135.6 ± 4.4
Asparagine	24.0 ± 0.4
Aspartic Acid	28.0 ± 0.6
Isoleucine	53.1 ± 0.9
Glutamic Acid	71.4 ± 2.3
Glutamine	4.8 ± 0.1
Glycine	33.24 ± 3.1
Leucine	63.3 ± 1.1
Methionine	1.5 ± 0.2
Serine	32.7 ± 1.6
Phenylalanine	31.3 ± 0.5
Proline	16.9 ± 0.3
Threonine	27.0 ± 0.8
Tryptophan	8.4 ± 0.3
Tyrosine	9.0 ± 0.4
Valine	39.1 ± 1.0

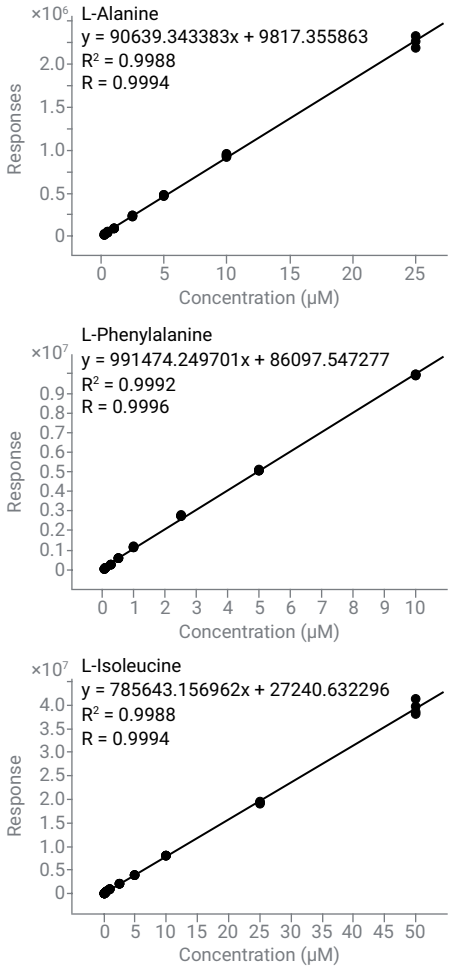


Figure 4. Agilent MassHunter Quantitative Analysis 12.1 generates Calibration Curves at-a-glance. Representative calibration curves for four amino acids are displayed here.

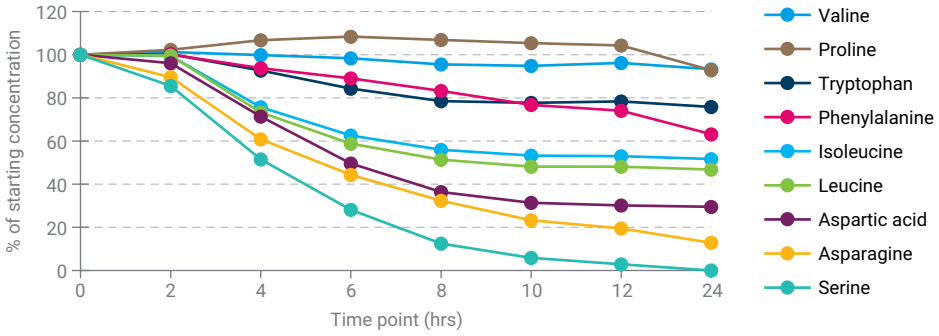


Figure 5. Depletion of amino acids in the fermentation media at discrete time points (mean, n = 6).

While targeted amino acid quantitation provides one view of nutrient utilization, the LC/MS workflow can also be used to examine other metabolite classes in the media, such as organic acids and carbohydrates. Use of a high-resolution system allows broad coverage of untargeted metabolites and retrospective analysis that would be difficult with a unit mass resolution instrument. High mass accuracy allows the annotation of organic acids such as lactic acid, a fermentation by-product. As fermentation progresses, a measurable increase in lactic acid peak area from 0 to 8 hours suggests excretion into spent media as the yeast grows (Figure 6A). The method also enables detection of hexose sugars, a critical source of energy for yeast that is extensively depleted across the 24-hour time frame (Figure 6B). The operation of the TOF instrument in negative mode detection allows simultaneous monitoring of amino acids, organic acids, and hexoses within a single injection. Together, these measurements provide a broader view of nutrient consumption and metabolite release during fermentation and can support characterization of changing metabolic activity during fermentation process development.

Yeast metabolism encompasses diverse biochemical pathways and many metabolites across chemical classes. Targeted quantitation of amino acids using a calibration curve and custom PCDL provides valuable insight into specific nutrient-utilization patterns, while untargeted metabolomics provides a complementary view of additional biochemical changes in the fermentation media. The 6230C TOF LC/MS provides an advantage for discovery workflows, as high-resolution accurate mass full scan data is acquired for every detectable peak within each injection. Unlike instruments that only provide nominal mass information or monitor specific transitions, this allows for peak annotation without knowledge of sample composition or retention times prior to sample injection. This data collection also allows older datasets to be revisited for newly relevant metabolites and enables higher-confidence annotations using molecular formulas and isotopic pattern matching.

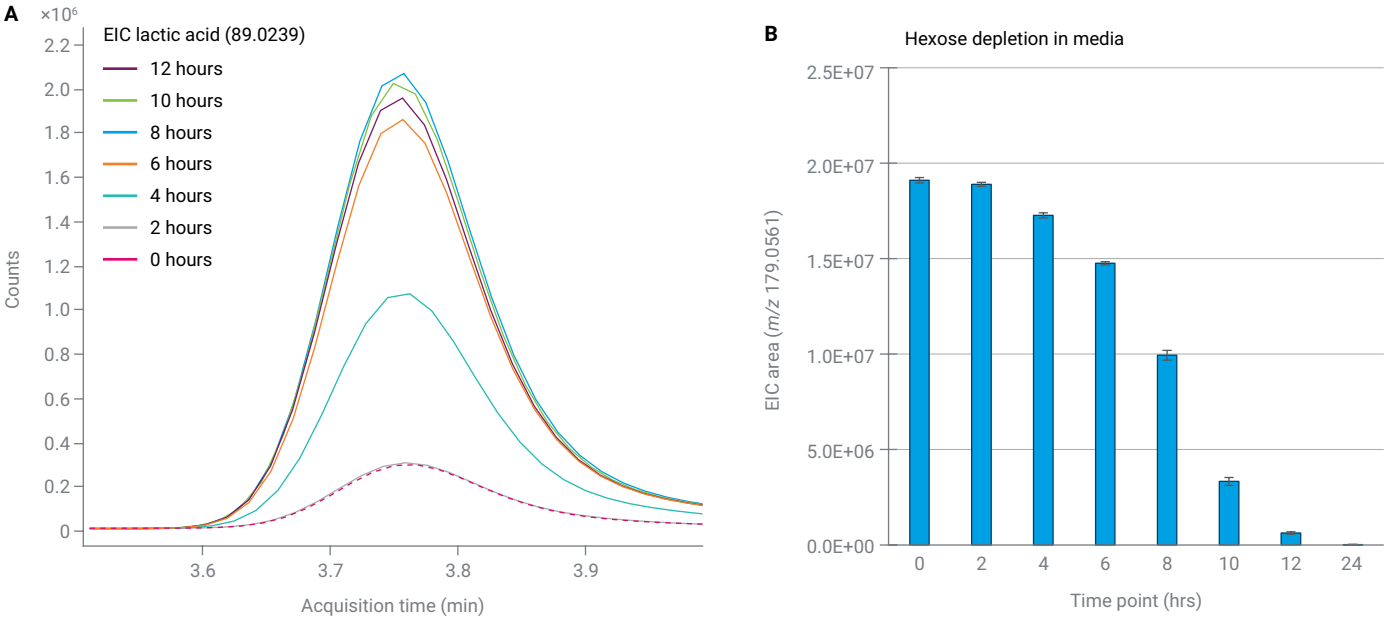


Figure 6. (A) Representative EIC traces of lactic acid show changes in abundance across fermentation time points. (B) Available hexose monosaccharides decrease in fermentation media over time. Data are presented as mean ± SD, n = 6.

Untargeted feature extraction and analysis were performed with Agilent MassHunter Explorer 2.0. Principal component analysis reveals clustering of samples according to fermentation time (Figure 7), demonstrating broader changes in metabolite composition across fermentation time points as yeast grow. Beyond visualization of overall trends in features, MassHunter Explorer 2.0 can identify statistically significant features between time points. Metabolite libraries can also be directly imported, allowing for annotation of these compounds within the sample. The combination of high-resolution TOF data and HILIC chromatography supports both quantitation of target amino acids and untargeted exploration of additional metabolic changes within a single analytical workflow.

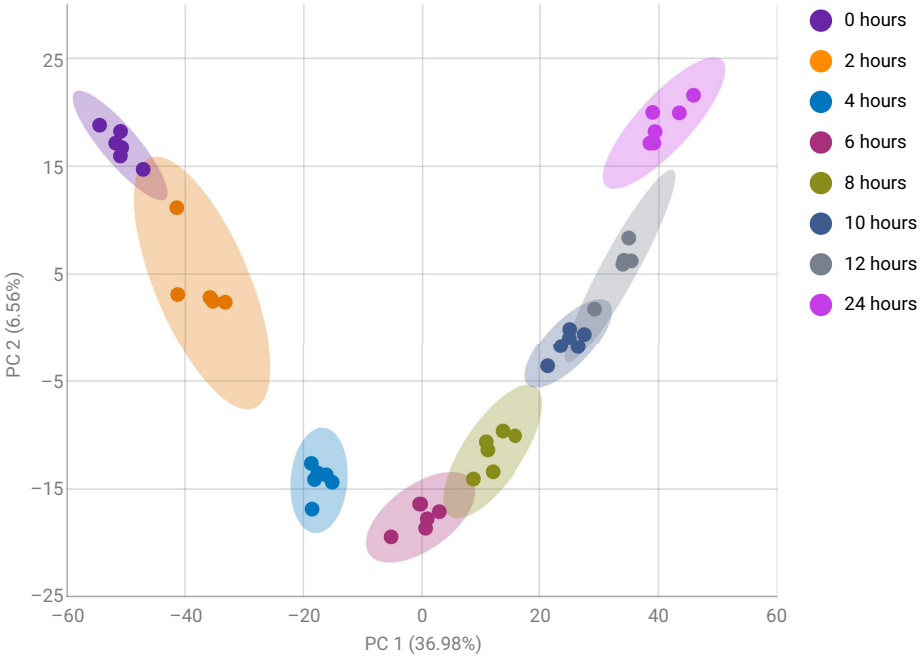


Figure 7. Principal component analysis of overall features across the different time points using Agilent MassHunter Explorer 2.0 shows clustering of samples according to fermentation time.

Conclusion

This application note demonstrates a HILIC-LC/TOF workflow for the detection and quantitation of underivatized amino acids directly from yeast fermentation media. The use of an iron-free flow path through the combination of the Agilent 1290 Infinity II bio LC system and Agilent Altura Poroshell 120 HILIC-Z column preserves metal-sensitive compounds, improving chromatographic peak shape and sensitivity. Coupled with the Agilent 6230C TOF LC/MS, the method provides high-resolution accurate mass detection of small molecules, including amino acids and other metabolites present in the fermentation media. With a mass range up to *m/z* 20,000, the 6230C TOF LC/MS demonstrates excellent versatility for both small- and large-molecule applications. Analysis of samples collected throughout fermentation reveals distinct amino acid depletion profiles over time. Beyond targeted analysis, high-resolution full scan acquisition also facilitates the detection and annotation of additional metabolites across several chemical classes. The use of negative mode detection allows for simultaneous analysis of organic acids released as fermentation byproducts and carbohydrate energy sources for the yeast. Untargeted feature analysis using Agilent MassHunter Explorer 2.0 demonstrates the overall changes in metabolite composition that can be detected directly from the fermentation media, highlighting the value for discovery-based platforms. Collectively, this HILIC-LC/TOF workflow provides a sensitive, reproducible, and versatile platform to monitor fermentation, combining targeted quantitation and untargeted metabolomics within a single analytical approach.

References

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3. Hsiao, J.; Masigol, M.; Closser, R.; Serge, L.; Hu, L.-C.; von-Doehren, N.; Tripodi, A. A. P.; Blackwell, A. Pushing the Boundaries of Chromatographic Separation with Inert HPLC Column Hardware; *Agilent Technologies white paper*, publication number 5994-8618EN, **2025**.

Products used in this application

Agilent products

6230C TOF LC/MS

Altura Poroshell 120 HILIC-Z

1290 Infinity III bio LC

MassHunter Quantitative Analysis

MassHunter Explorer

Jet Stream Technology ion source

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