Application Note

Metabolomics

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Monitoring of 20 Amino Acids and Other Key Compounds in Fermentation Processes

Authors

Floriant Bellvert and
Hanna Kulyk Barbier
MetaToul, Toulouse, France

Olivier Galy, Xavier Amoyal,
Jean-Marie Francois, and
Yoann Malbert
Toulouse White Biotechnology,
Ramonville Saint-Agne, France

Daniel Mobbs, Steffen Mueller,
and Adam Bivens
Agilent Technologies, Inc.

Introduction

This Application Note presents an LC/MS method optimized for the analysis of underivatized amino acids in various broth cultures.

Amino acids are the basic building blocks of proteins. They constitute all proteinaceous material of the cell including the cytoskeleton and the protein component of enzymes, receptors, and signaling molecules. In addition, amino acids are used for the growth and maintenance of cells.

The monitoring and control of the optimal concentration of amino acids is important in fed-batch and perfusion culture. Therefore, the design of an amino acid supplementation strategy might be streamlined by identifying the amino acid demands of a cell culture due to host cell growth and product production. It is true when harvesting micro-organisms with engineered metabolic pathways where the metabolism of amino acid is altered.

This Application Note addresses the development and validation of an LC/MS/MS method on an Agilent 6490 triple quadrupole LC/MS system for the quantitation of 20 amino acids (AA). This LC/MS/MS method has the advantage of simpler sample preparation, without derivatization, compared to standard RP-HPLC with fluorescence detection and GC/MS methods.

Reagents and Standards

- Agilent Amino Acid Standard (16 AAs) in HCl 0.1 N
  - Agilent Amino Acid Standard, 250 pmol/µL, each, (p/n 5061-3331)
  - Agilent Amino Acid Standard, 10 pmol/µL, each, (p/n 5061-3334)
- Water provided by an Elga Pureflex 1&2
- Acetonitrile LC/MS grade, certified, purchased from Fisher Chemicals
- Agilent InfinityLab fittings
  - Column front: InfinityLab Quick Connect LC fitting (p/n 5067-5965)
  - Column back: InfinityLab Quick Turn LC fitting (p/n 5067-5966)
- Agilent vial, screw top, amber, write-on spot, certified, 2 mL (p/n 5182-0716)
- Agilent bonded screw cap, PTFE/red silicone septa (p/n 5190–7024)
- Eppendorf pipettes and repeater
- Complementary amino acids: L-asparagine, L-cysteine, L-glutamine, L-tryptophan, and homoserine and L--hydroxyproline were purchased from Sigma-Aldrich

Instrument

The analysis of amino acids in culture media were performed on an Agilent Infinity 1290 LC composed of a 1290 Infinity Binary Pump (G4220A, equipped with an Agilent Jet Weaver V35 mixer), a 1290 Infinity Multisampler (G7167B), and a 1290 Infinity Thermostatted Column Compartment (G1316C), coupled to an Agilent 6490 Triple Quadrupole LC/MS (with Agilent Jet Stream and ion funnel technology).

Data analysis

Agilent MassHunter quantitative analysis software was used for data analysis. For the calibration curve, all replicates' results were loaded into the software database, and the automatic quantification method was then used to obtain the appropriate calibration curve type with the best fit to the experimental data.

Agilent MassHunter optimizer software was used for optimizing the parameters in the method validation.

Agilent integration parameters were rugged, highly efficient, and accurate, and were mostly used during this study except for rare exceptions.

For sample analysis, quadratic fits including the origin were used, without weighting and R^2 greater than 0.99 depending on the amino acid and its MRM response.

Linear range can be extended to 100 µM (100 pmol/µL) using this mode.

Table 1. Chromatigraphic parameters.

Column	InfinityLab Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 µm (p/n 683775-924)
Mobile phase A	20 mM ammonium formate in water at pH 3
Mobile phase B	20 mM aqueous ammonium formate at pH 3 in 9:1 acetonitrile:water
Flow rate	0.60 mL/min
Column temperature	30 ˚C
Injection volume	5 µL

Table 2. Elution gradient.

Time (min)	%A	%B	Flow (mL/min)
0	0	100	0.6
7	11	89	0.6
10	12	88	0.6
16	25	75	0.6
16.5	0	100	0.6
20	0	100	0.6

Method Validation

Chromatography

The chromatographic separation for amino acids was carried out on an Agilent InfinityLab Poroshell 120 HILIC-Z column (2.1 × 150 mm, 120 Å). The column was maintained at a temperature of 30 °C, and the sample volume injected was 5 µL. Optimal chromatographic separation was achieved at a flow rate of 0.6 mL/min using a gradient with solvent A (10 % 200 mM ammonium formate at pH 3 (formic acid) + 90 % water) and solvent B (10 % 200 mM ammonium formate at pH 3 (formic acid) + 90 % acetonitrile), as shown in Tables 1 and 2, respectively.

Instrument parameters

The mass spectrometer was operated in AJS ESI-Positive mode, capillary voltage 4.00 kV, nozzle voltage 1.00 kV, desolvation temperature 400 °C, source temperature 250 °C, cone gas flow 17 L/min, desolvation gas flow was 12 L/min, nebulizer 40 psi, iFunnel parameters (high-pressure and low-pressure RF) were set to 140 and 60, respectively. Collision energy, iFunnel parameters, and MRM transitions for the 20 amino acids and nozzle voltage were optimized for each amino acid using MassHunter optimizer software. Dwell time was set to 45 ms, and delta EMV(+) to 200 V. The MRM transitions and respective collision energies for the amino acids are shown in Table 3.

Amino acid stock solution

The Agilent Amino Acid Standard was used as purchased. A stock solution of L-asparagine, L-cysteine, L-glutamine, L-tryptophan, and homoserine and L-hydroxyproline was made by weighing 15 mg of each into a 100-mL volumetric flask. Next, 25 mL 0.1N HCl was added, followed by sonication for two minutes, and finally the solution allowed to cool to room temperature and made up to volume with 0.1N HCl.

Validation studies were performed using mixtures of amino acid standards (11 levels of concentration from 0.001 to 25 µM). They were prepared in 100 %B five times, the QC samples at low-, middle-, and high-concentration levels prepared in triplicates. These sets of samples were analyzed by LC/MS/MS for method validation studies.

Validation studies included linearity and calibration model fits, precision and accuracy, and sensitivity measured by the limit of detection (LOD) and limit of quantitation (LOQ). The method was found to be a reliable method for routine amino acid analysis in biotech studies.

Table 3. Ion transitions, instrument settings, and retention times for amino acid detection.

	Compound		Precursor (*m/z*)	Product (*m/z*)	Frag (V)	CE (V)	RT (min)
1	Phenylalanine	Phe	166.1	120	380	13	3.34
2	Tryptophan	Trp	205.1	188.1	380	9	3.56
3	Leucine	Leu	132.1	86	380	9	3.73
4	Isoleucine	Ile	132.1	86.1	380	9	4.05
5	Methionine	Met	150.1	104	380	9	4.46
6	Tyrosine	Tyr	182.1	136	380	9	5.08
7	Valine	Val	118.1	72	380	9	5.18
8	Proline	Pro	116.1	69.9	380	17	5.34
9	Alanine	Ala	90.1	44.1	380	12	6.67
10	Threonine	Thr	120.1	73.9	380	9	6.91
11	Glycine	Gly	76	30.1	380	9	7.44
12	Glutamine	Gln	147.1	83.9	380	17	7.76
13	Serine	Ser	106.1	60	380	8	7.83
14	Asparagine	Asn	133.1	73.9	380	13	7.91
15	Glutamate	Glu	148.1	83.9	380	17	8.53
16	Aspartate	Asp	134.1	87.9	380	8	9.92
17	Histidine	His	156.1	110.1	380	17	11.26
18	Arginine	Arg	175.1	70	380	25	12.36
19	Lysine	Lys	147.1	84	380	17	13.4
20	Cystine	Cys-Cys	240.9	151.9	380	16	13.68

Frag = fragmentor voltage; CE = collision energy; RT = retention time

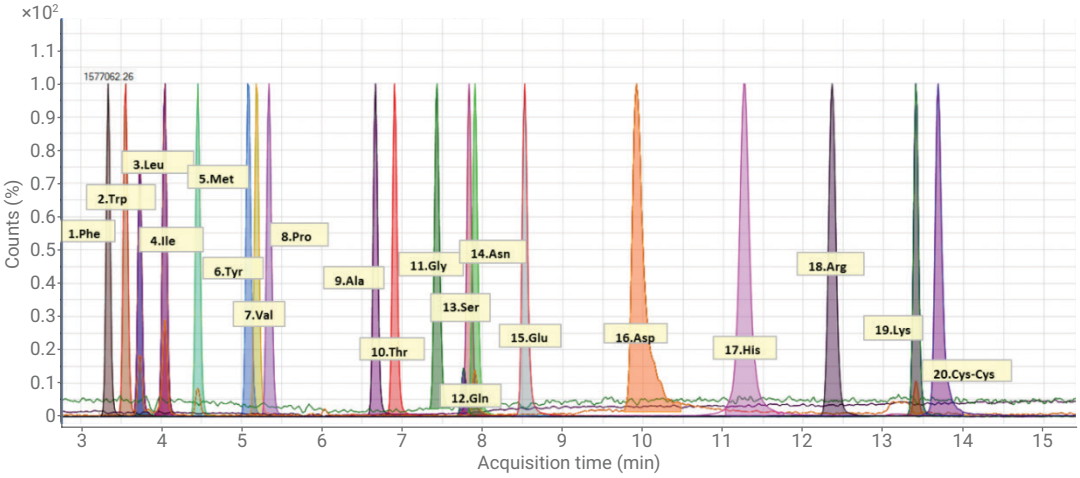

Figure 1. Amino acid chromatogram from a standard mixture at the 0.05 nmol level.

LOD and LOQ

The standard criteria used for LOD were ±5 % for retention time, accuracy between 80 and 120 %, and a minimum signal-to-noise (S/N) level of 3:1. The standard identification criteria for LOQ were ±5 % for retention time, accuracy between 80 and 120 %, and a minimum S/N level of 10:1.

Precision and linearity

The standard acceptance criteria for interrun and intrarun precision was ±20 % at each concentration level.

The within-run precision was ≤20 % for all amino acids, and for most of them within 0.06 to 25 µM range of concentration, with an average overall correlation of 0.996. For the following amino acids: aspartate, arginine, and lysine, the calibration curve types were set to quadratic to cover the largest concentration range.

The method shows an excellent stability in retention time for all amino acids, and the instrument remained stable without the need for extra cleaning or maintenance.

Table 4. Linearity data for amino acid standard mixture.

	Compound	Linear regression	R^2	LOD (µM)	LOQ (µM)
1	Phenylalanine	Type: Linear, Origin: Ignore, Weight: 1/x	0.993	0.006	0.01
2	Tryptophan	Type: Linear, Origin: Ignore, Weight: 1/x	0.991	0.006	0.01
3	Leucine	Type: Linear, Origin: Ignore, Weight: None	0.995	0.006	0.01
4	Isoleucine	Type: Linear, Origin: Ignore, Weight: None	0.995	0.006	0.01
5	Methionine	Type: Linear, Origin: Ignore, Weight: None	0.994	0.006	0.01
6	Tyrosine	Type: Linear, Origin: Ignore, Weight: 1/x	0.999	0.01	0.05
7	Valine	Type: Linear, Origin: Ignore, Weight: None	0.996	0.006	0.01
8	Proline	Type: Linear, Origin: Ignore, Weight: None	0.999	0.002	0.006
9	Alanine	Type: Linear, Origin: Ignore, Weight: 1/x	0.999	0.05	0.2
10	Threonine	Type: Linear, Origin: Ignore, Weight: None	0.998	0.006	0.01
11	Glycine	Type: Linear, Origin: Ignore, Weight: 1/x	0.999	0.4	1.5
12	Glutamine	Type: Linear, Origin: Ignore, Weight: None	0.999	0.01	0.05
13	Serine	Type: Linear, Origin: Ignore, Weight: None	0.998	0.01	0.05
14	Asparagine	Type: Linear, Origin: Ignore, Weight: None	0.999	0.01	0.05
15	Glutamate	Type: Linear, Origin: Ignore, Weight: 1/x	0.999	0.2	0.4
16	Aspartate	Type: Quadratic, Origin: Ignore, Weight: None	0.995	1.5	3.0
17	Histidine	Type: Linear, Origin: Ignore, Weight: 1/x	0.990	0.05	0.2
18	Arginine	Type: Quadratic, Origin: Ignore, Weight: None	0.996	0.006	0.01
19	Lysine	Type: Quadratic, Origin: Ignore, Weight: None	0.997	0.006	0.01
20	Cystine	Type: Linear, Origin: Ignore, Weight: 1/x	0.998	0.4	1.5

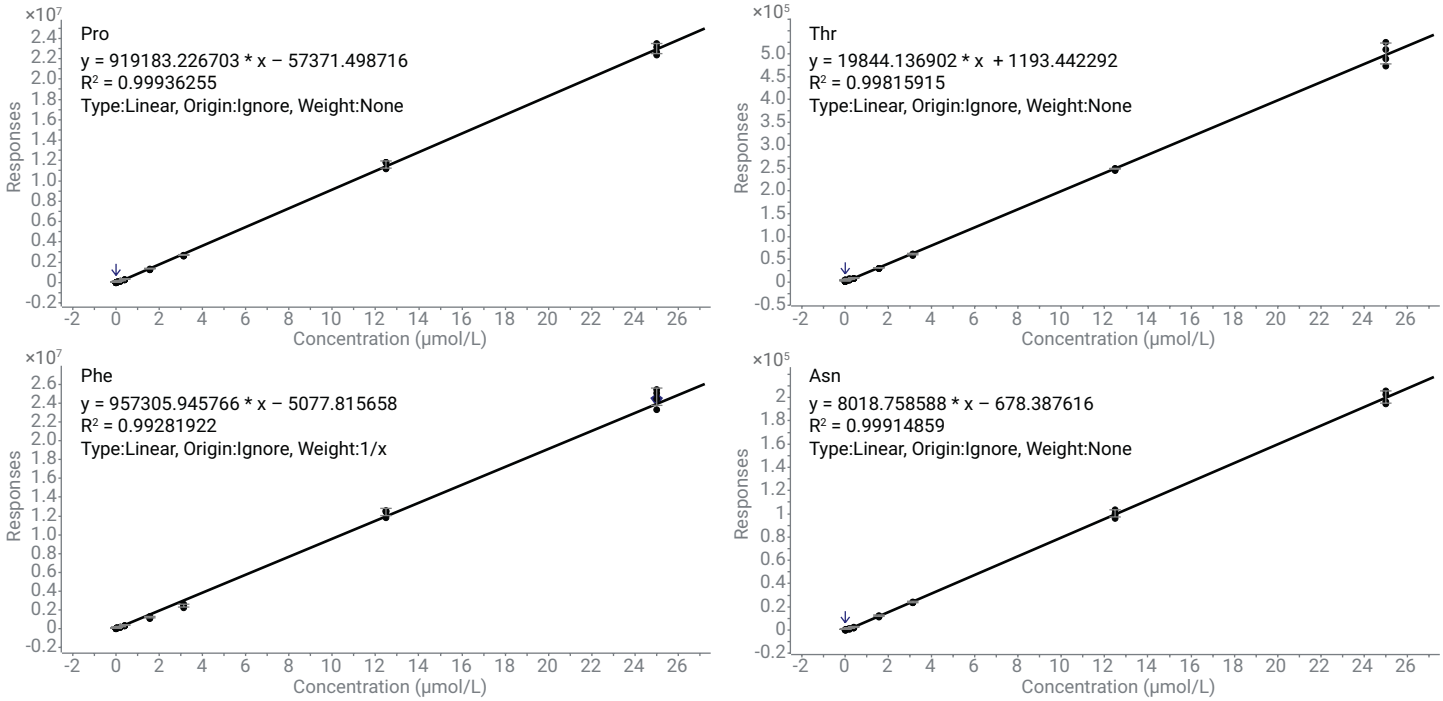

Figure 2. Linearity data for proline, phenylalanine, threonine, and asparagine.

Sample analysis

A modified strain of *E. coli* was grown in Modified Minimum Medium M9 40 g/L glucose 2018s05 (Sigma M9, Minimal Salts, 5X powder, minimal microbial growth medium).

Oligo elements	Nitrogen salts	Other solute
$CoCl_2$, $6H_2O$	Na_2HPO_4, $12\ H_2O$	$MgSO_4$, $7H_2O$
Na_2EDTA, $2H_2O$	K_2HPO_4	$CaCl_2$, $2H_2O$
$MnSO_4$, H_2O	$(NH_4)2HPO_4$	$FeCl_3$
$CuCl_2$, $2H_2O$	$(NH_4)_2SO_4$	Citrate Na
H_3BO_3	NH_4Cl	L-threonine
Na_2MoO_4, $2H_2O$		L-methionine
$ZnSO_4$, $7H_2O$		Glucose

Sample preparation

Samples were prepared according to the expected level of amino acids.

Samples were collected in plastic tubes. They were then centrifuged at 5,000 rpm for 10 minutes, filtered through 0.2 µm Filter 16534K SUPELCO Minisart filters of pore size 0.2 µm, then diluted.

Sample dilution was always done in two phases (as for the standards), first with HCl 0.1 N, then in ACN, followed by direct injection onto the LC/MS system.

Dilution pattern:

- Dilution 1:500
- To 10 µL sample, add 990 µL HCl 0.1 N
- To 100 µL of this solution, add 400 µL ACN.

Clear separation of leucine and isoleucine is observed, and both can be identified in the MassHunter quantitative analysis software.

At growth start, threonine overwhelms homoserine, and conversely at the end of the culture, homoserine is one hundred times more concentrated than threonine, which tends to disappear. MassHunter quantitative analysis software enables integration and quantitation of these dynamic samples without major effort.

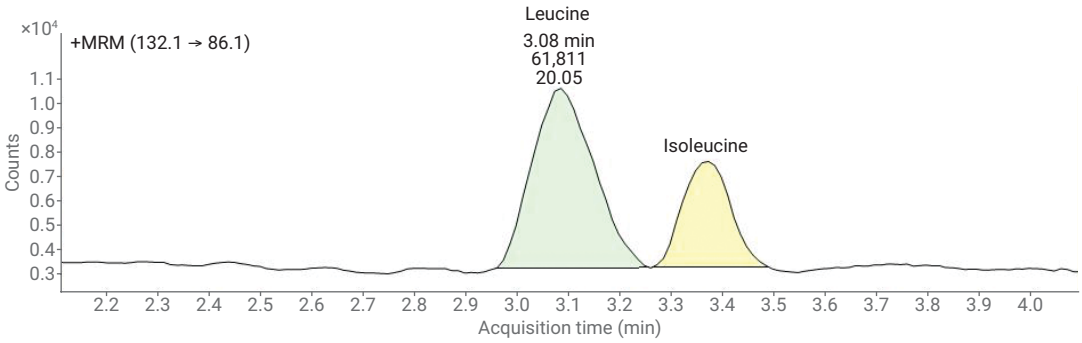

Figure 3. Leucine-isoleucine separation and identification in MassHunter quantitative analysis software.

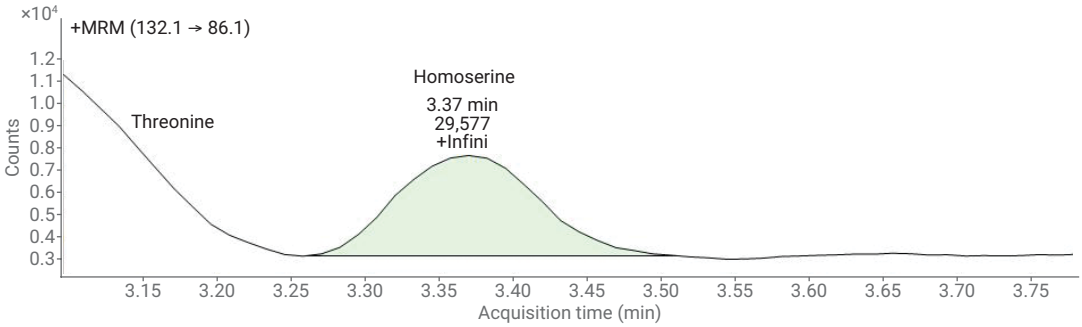

Figure 4. Identification of two isobars threonine and homoserine in MassHunter quantitative analysis software.

The next two figures represent analysis results from two different culture broths.

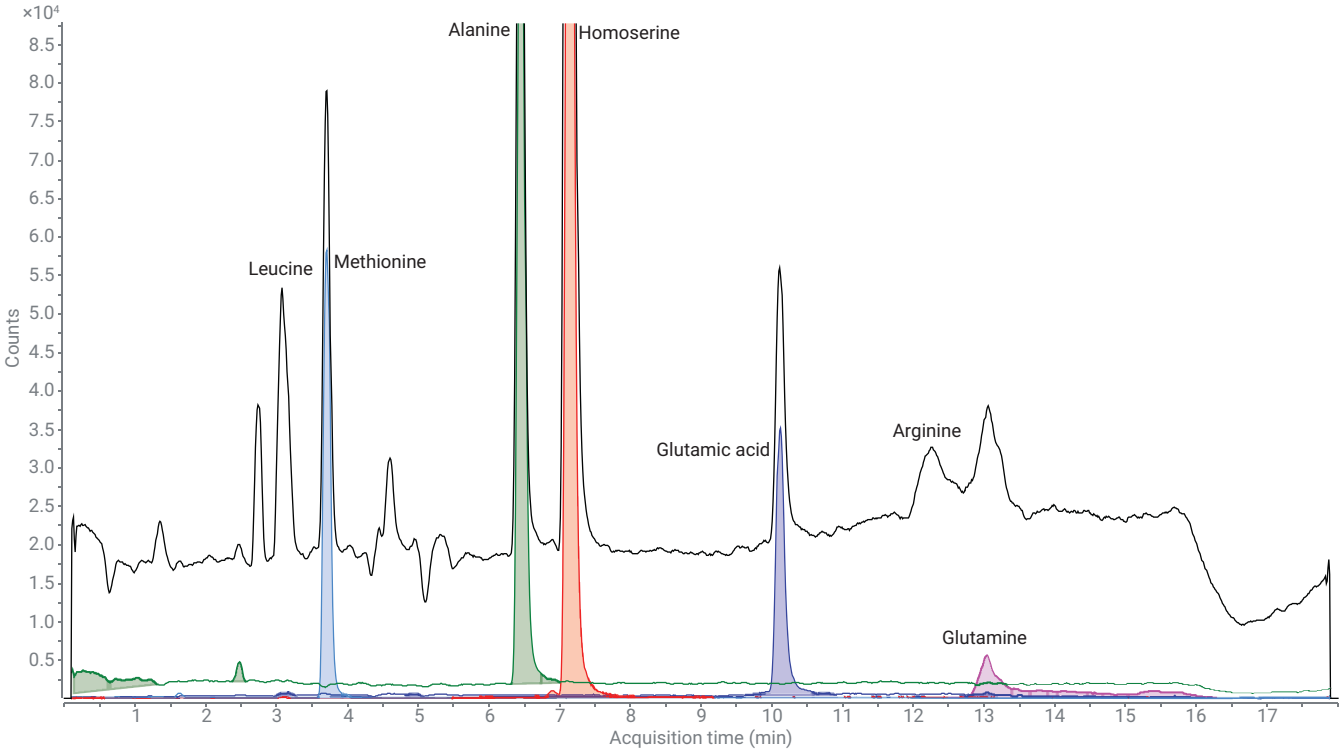

Figure 5. Culture broth study A.

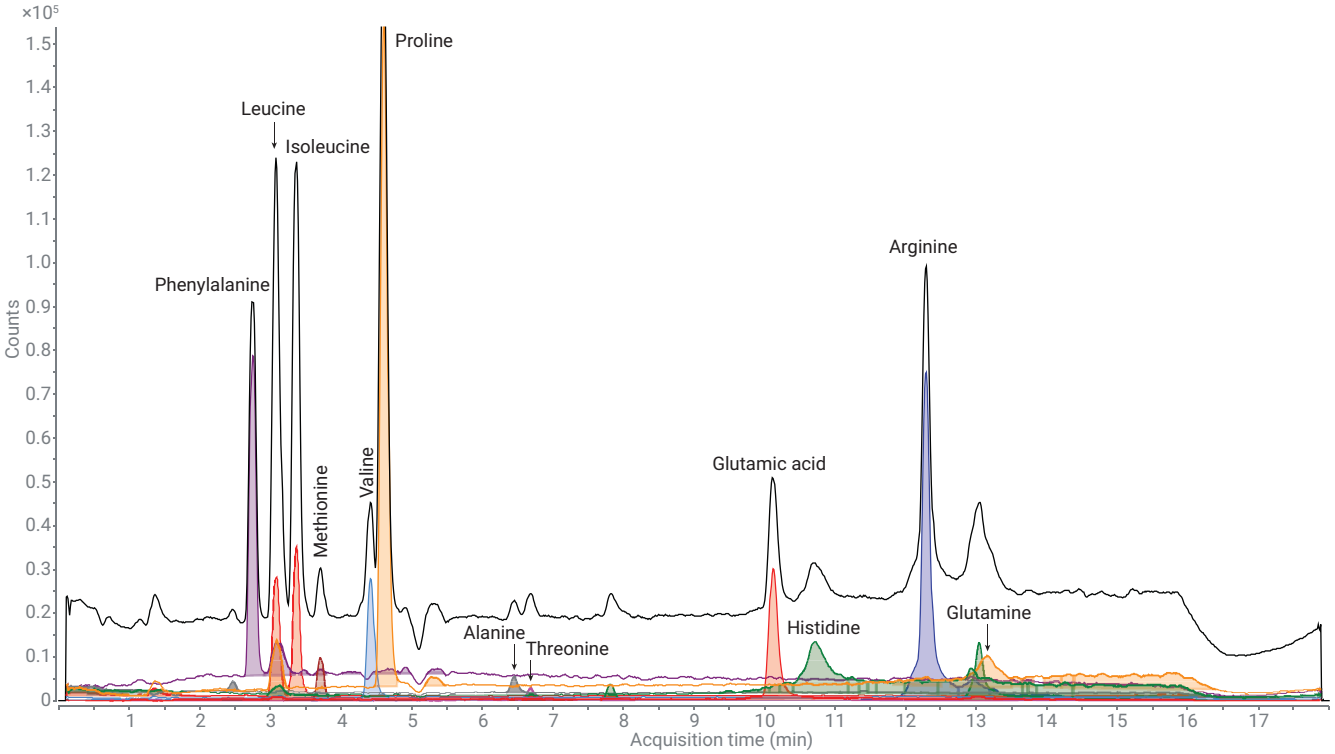

Figure 6. Culture broth study B.

Results and discussion

Amino acid evolution during a fed-batch fermentation of an *E. coli* engineered for the production of a chemical synthon from glucose

The purpose of this study was to monitor the consumption and production of amino acids along the fermentation of an *E. coli* strain engineered in a pathway leading to the production of 2,4-dihydroxybutyric acid (DHB) from glucose. The pathway has been described in a recent publication[1]. As the pathway departed from aspartate to homoserine, and that the engineered strain has been so far auxotroph for threonine and methionine, we wished to investigate both the rate of consumption of these two amino acids that had to be added for growth, and which type of amino acids the strains would eventually produce in response to the engineered pathway. We anticipated a high production of alanine and homoserine together with the production of our molecule of interest, DHB. As shown in Figure 7, the strain is consuming the two added amino acids with different kinetics, with threonine being consumed faster, suggesting that the latter is the limiting factor in the growth of this strain.

Figure 8 shows the production of amino acids by the engineered strain. As we anticipated, there was a high accumulation of alanine and homoserine along the production of DHB. The accumulation of these amino acids is highly important and not solely accounted for the loss of carbon but also justifies re-engineering our strain to reduce that production.

Figure 9 shows another less obvious production of amino acids but at levels 50 to 100-fold lower than alanine. In terms of carbon loss, this does represent less than 1 % which is negligible, but is anyway interesting to know.

In conclusion, the use of this methodology was helpful for a better characterization of the physiological behavior of our engineered strain.

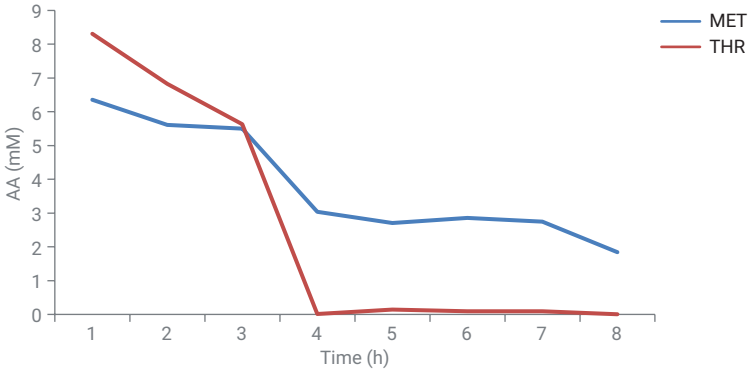

Figure 7. Consumption of methionine and threonine during fed-batch culture of *E. coli* auxotroph for Met and Thr on glucose.

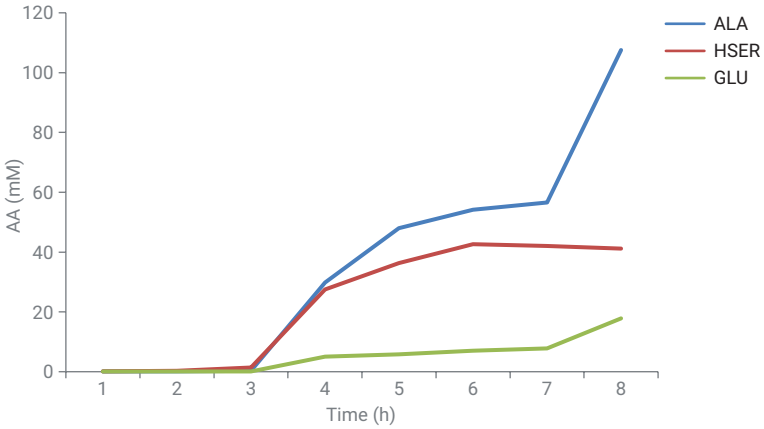

Figure 8. Production of amino acids during fed-batch culture of *E. coli* auxotroph for Met and Thr on glucose.

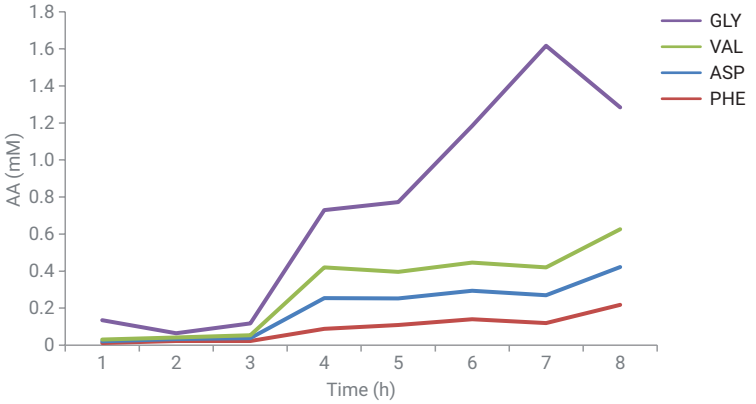

Figure 9. Identification of other amino acids produced during fed-batch growth of *E. coli* on glucose.

Yeast media

Alternatively, to extend the range of applications of this analytical method, a modified yeast strain (baker's yeast *saccharomyces* sp.) growth was studied in two YNB G30-based media: a minimal broth supplemented with essential amino acids and a rich broth (YNB G30 supplemented with autolytic yeast extract). Liquid culture media for baker's yeast often integrate proteins and amino acids at undefined concentrations. Matrix effects may occur when using classical derivatization methods. This method enabled detecting free amino acid composition in the culture broth and their variation during the culture course (data not shown) with no incidence of the matrix on sensitivity nor amino acid profile.

Other compounds

Concomitantly to the amino acid analysis, other compounds (free purine and pyrimidine, DHB, 2-oxo-4-hydroxybutyric acid, and so on) can be monitored and quantified accurately. The use of segments not shown here is mandatory to alleviate for Pos/Neg switching and overall sampling rate that must take peak width into account. This is done effortlessly in the method acquisition software.

Due to the inherent capabilities of the triple quadrupole LC/MS, allowing transition dwell times down to a few milliseconds, the tandem HILIC/triple quadrupole is versatile enough to accommodate additional compounds such as water-soluble vitamins, free purine, and pyrimidine that may be expelled in the medium.

Using the same mobile phase composition, tuning the collision energy of the MRM in a few steps for new compounds enables adding compounds of interest to the list of those mentioned earlier. The use of segments is mandatory to alleviate for Pos/Neg switching and the overall sampling rate, taking into account the peak width, which is done effortlessly in the method acquisition software.

A preliminary work was done on DHB mentioned earlier, uracil, and 2-oxo-4-hydroxy butyric acid, that were monitored and quantified accurately but are not shown here for the sake of clarity, and may be subject to another study in the future.

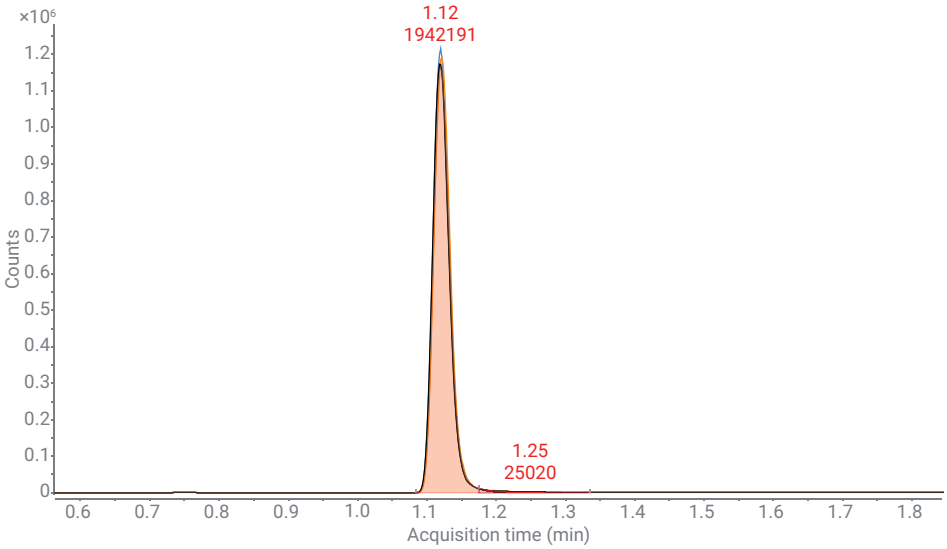

Figure 10. Example of uracil measured in negative mode.

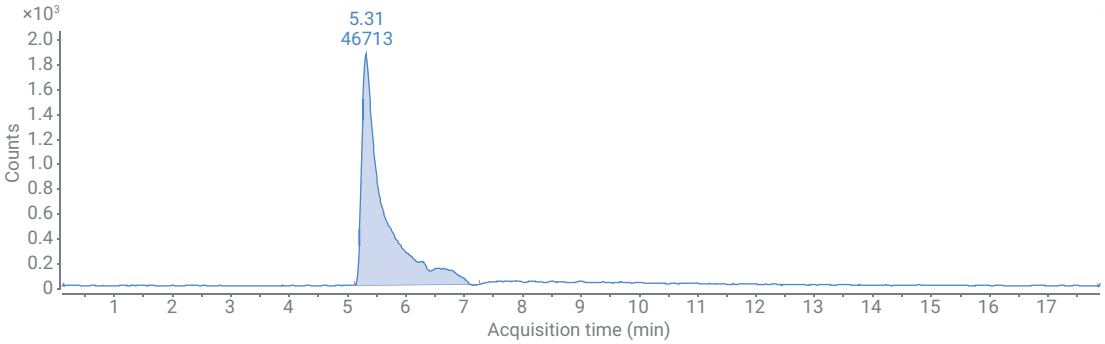

Figure 11. 2,4-Dihydroxybutyric acid (note typical diol peak shape probably due to gem-diol conversion).

Conclusion

The developed method enables analysis of more than 20 amino acids and other products of interest precisely and in a relatively fast way (approximately 18 minutes run time with simple sample preparation). This Application Note, using a 6490 triple quadrupole LC/MS, facilitates precise study of amino acid kinetics during a cell or microbial culture. In the case of a bioreactor culture, it has a real advantage knowing the evolution of compound concentration in the culture medium—on the one hand, exploring cellular metabolism, and on the other hand, being able to establish a precise carbon balance. This information helps to optimize the genetic background of an engineered microbial strain, for example by identifying a carbon loss in an amino acid production pathway. Finally, sensitivity level makes it possible to quantify low concentrations, which extends the application to intracellular kinetic monitoring. Understanding the intracellular accumulation and excretion of compounds in the medium generates the necessary feedback to engineering of the microbial strain studied. The combined InfinityLab Poroshell 120 HILIC-Z column/6490 triple quadrupole LC/MS is a powerful tool in a biotech analytical laboratory environment.

Reference

1. Walther, T.; Calvayrac, F.; Malbert, Y.; Alkim, C.; Dressaire, C.; Cordier, H.; Francois, J. M. Construction of a synthetic pathway for the production of 2,4-dihydroxybutyric acid from homoserine. *Metab. Eng.* **2018**, *45*, 237-245. DOI/10.1016/j.ymben.2017.12.005

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