Application Note

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Analysis of Underivatized Amino Acids by LC/MS for Bioreactor Cell Culture Monitoring

Authors

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Abstract

This Application Note presents a solution for LC/MS analysis of amino acids in fermentation media. The polar nature of amino acids makes analysis by reversed-phase liquid chromatography challenging, so derivatization is often used to improve retention. However, hydrophilic interaction chromatography (HILIC) is capable of retaining and separating complex amino acid mixtures without derivatization, while still offering a similar workflow to traditional reversed-phase. The combination of HILIC and mass spectrometry offers a particularly simple and powerful solution for underivatized amino acid analysis.

Introduction

Monitoring the wide range of polar compounds found in bioreactors and fermenters is a challenging real-world application for hydrophilic interaction chromatography (HILIC). Using high pH conditions with negative mode LC/MS detection, amino acids, feedstock, and waste products can be monitored in a single analysis. The high pH stability of Agilent AdvanceBio MS Spent Media chemistry makes it ideal for separating the mixture.

Repeatability from injection to injection was excellent despite the challenging matrix. Testing of a hollow fiber and rolling bottle reactor revealed the consumption of glucose and amino acids, and secretion of lactate by the cultured cells.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher. Ultra LC/MS grade acetonitrile was bought from J.T. Baker (Center Valley, PA, USA). Water was purified using an EMD Millipore Milli-Q Integral System (Darmstadt, Germany). Reagent-grade formic acid (FA, p/n G2453-85060) was from Agilent Technologies. Ammonium formate, ammonium acetate, ammonium hydroxide, and amino acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM media was purchased from Thermo Scientific (Waltham, MA, USA). Amino acids were stored at –70 °C until day of use.

Equipment and materials

- Agilent InfinityLab Fittings
  - **Column front:** Agilent Quick Connect (p/n 5067-5965)
  - **Column back:** Agilent Quick Turn (p/n 5067-5966)
- Agilent Vial, screw top, amber, write-on spot, certified, 2 mL, 100/pk. (p/n 5182-0716)
- Agilent bonded screw cap, PTFE/red silicone septa (p/n 5190-7024)
- Agilent vial insert, 250 µL, deactivated glass with polymer feet (p/n 5181-8872)
- Eppendorf pipettes and repeater
- Ultracentrifuge (VWR, Radnor, PA, USA)
- Vortexer and multitube vortexers (VWR, Radnor, PA, USA)
- HDPE solvent bottles (VWR, Radnor, PA, USA)
- Rolling bottle bioreactor (Sigma-Aldrich, St. Louis, MO, USA)
- Hollow fiber bioreactor (FiberCell Systems, New Market, MD, USA)

Instrumentation

- Agilent 1290 Infinity II binary pump (G7120A)
- Agilent 1290 Infinity II Vialsampler (G7129B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Ultra-low dispersion kit for Agilent 1290 Infinity LC Series (5067-5189)
- Agilent 6545 Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometer
- Agilent Jet Stream Electrospray Ionization source

Software

Agilent MassHunter workstation software B.08.00

Sample preparation

Cell culture samples were diluted 1:4 with 50 % ACN, and centrifuged at 10,000 x g for 10 minutes. The supernatant was collected and injected with no further sample preparation.

Mobile phase

A 100 mM ammonium acetate stock was made in water, and adjusted to pH 9 with ammonium hydroxide. Mobile phase A was made by diluting the stock solution 9:1 in water. Mobile phase B was made by diluting the stock solution 9:1 in ACN. The final concentration in both mobile phases was 10 mM ammonium acetate.

Extended exposure of mobile phase to glassware was found to introduce ionic species that interfere with and suppress MS detection. Mobile phases stored in glass should be changed regularly, or transferred to HDPE bottles.

Instrument conditions

HPLC Conditions	
Column	Agilent AdvanceBio MS Spent Media, 2.1 × 150 mm (p/n 673775-901)
Mobile phase A	10 % (100 mM ammonium acetate in water at pH = 9)/ 90 % water
Mobile phase B	10 % (100 mM ammonium acetate in water at pH = 9)/ 90 % acetonitrile
Gradient	Time (min) %B 0 90 2 90 12 40 13 20 16 20 17 90 25 90
Flow rate	0.25 mL/min
Column temperature	30 °C
Injection volume	1 µL
Total runtime	25 minutes
MS Conditions	
Ionization mode	ESI Negative
Gas temperature	200 °C
Gas flow	10 L/min
Nebulizer	40 psi
Sheath gas temperature	300˚C
Sheath gas flow	12 L/min
Capillary voltage	3,000 V
Nozzle voltage	0 V
Fragmentor voltage	125 V
Skimmer voltage	65 V
Oct RF Vpp	750 V
Acquisition parameters	Data were acquired at 2 GHz extended dynamic range MS mass range 50–1,000 *m/z*

Bioreactor conditions

Parameter	Value
Bioreactor formats	Rolling bottle bioreactor Hollow fiber bioreactor
Cell line	Chinese hamster ovary (CHO) cells
Culture media	DMEM growth media
Temperature	30 °C

Table 1. Composition of cell culture matrix (DMEM growth media).

Component	Concentration (mg/L)
Glycine	30.0
L-Arginine hydrochloride	84.0
L-Cystine 2HCl	63.0
L-Glutamine	584.0
L-Histidine hydrochloride-H_2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine hydrochloride	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Serine	42.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine disodium salt dihydrate	104.0
L-Valine	94.0
Choline chloride	4.0
D-Calcium pantothenate	4.0
Folic acid	4.0
Niacinamide	4.0
Pyridoxine hydrochloride	4.0
Riboflavin	0.4
Thiamine hydrochloride	4.0
i-Inositol	7.2
Calcium chloride ($CaCl_2$)(anhydrous)	200.0
Ferric nitrate ($Fe(NO_3)_3$*$9H_2O$)	0.1
Magnesium sulfate ($MgSO_4$)(anhydrous)	97.67
Potassium chloride (KCl)	400.0
Sodium bicarbonate ($NaHCO_3$)	3,700.0
Sodium chloride (NaCl)	6,400.0
Sodium phosphate monobasic (NaH_2PO_4*H_2O)	125.0
D-Glucose (Dextrose)	4,500.0
Phenol red	15.0
Sodium pyruvate	110.0

Results

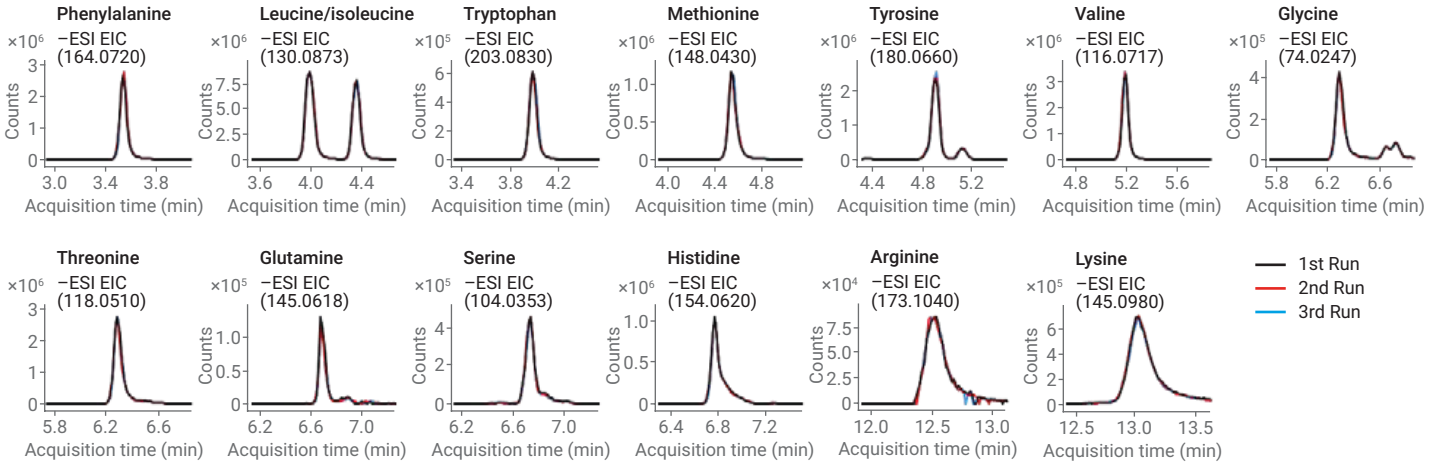

Figure 1. Reproducibility test.

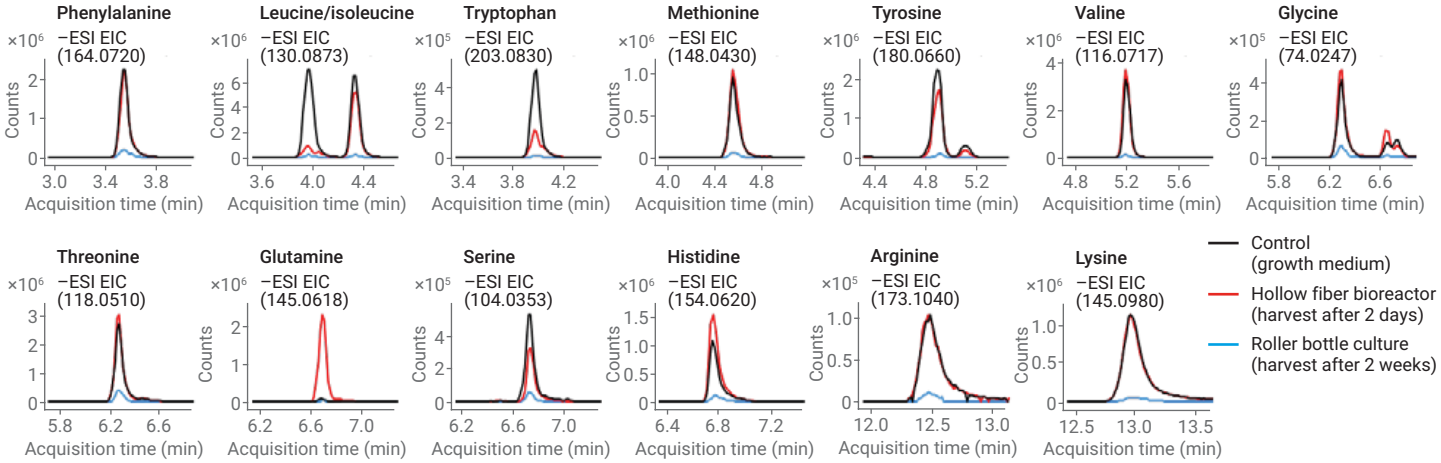

Figure 2. Analysis of cell culture media, showing consumption of amino acids. The growth media was supplemented with 6 mM glutamine in the hollow fiber bioreactor, which is why the glutamine level was higher in the hollow fiber bioreactor than in the control growth media and the nutrient-depleted roller bottle bioreactor.

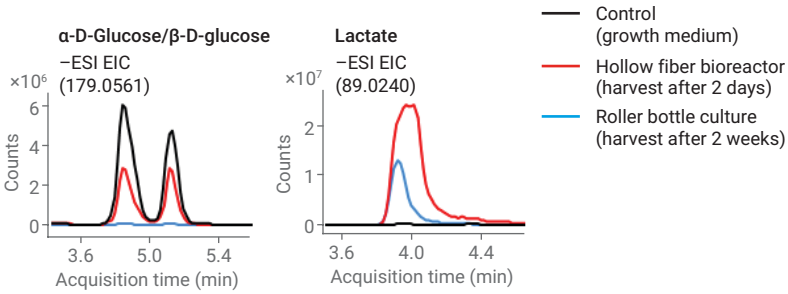

Figure 3. Analysis of cell culture media, showing consumption of glucose feed and secretion of lactate.

Table 2. Cell culture media retention times and predominant ion for each analyte.

Monitored analyte	Retention time (min)	Precursor ion (*m/z*)
Phenylalanine	3.55	164.072
Lactate	3.95	89.024
Leucine	3.98	130.087
Tryptophan	3.98	203.083
Isoleucine	4.35	130.087
Methionine	4.53	148.043
D-glucose (*alpha*)	4.87	179.056
Tyrosine	4.91	180.066
D-glucose (*beta*)	5.13	179.056
Valine	5.19	116.071
Glycine	6.28	74.0247
Threonine	6.29	118.051
Glutamine	6.67	145.06
Serine	6.73	104.03
Histidine	6.75	154.062
Arginine	12.53	173.104
Lysine	13.01	145.098

Conclusions

Amino acids in spent media were successfully analyzed by HILIC-MS in negative ion mode. The normally challenging leucine/isoleucine isobars were baseline-separated, with a resolution of 1.6. Being zwitterionic, amino acids readily ionize in both positive and negative ion mode, but the high pH negative mode allowed for cell media, feedstock, and cell waste products to be monitored simultaneously.

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This information is subject to change without notice.

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