Agilent

Trusted Answers

Consumables Workflow Ordering Guide

Cell Culture Media Analysis in Biopharma by Liquid Chromatography

Process understanding and control is essential to the production of a consistent biotherapeutic product, and a significant aspect of that process is the cell culture conditions, including the nutrients and metabolites available to the cells. The composition of the cell culture media is fundamental to the product yield and the health and survival of the cells used to produce the biotherapeutic. Additives to the cell culture media can also impact critical properties of the biotherapeutic such as glycosylation patterns.

Speed of analysis is often a vital need for amino acid analysis, with increasing desire for online monitoring directly at the bioreactor for rapid decision making.[1] Reproducibility, robustness, and column lifetime are also common challenges faced in amino acid analysis, and Agilent offers two solutions to meet these challenges in different ways.

The AdvanceBio Amino Acid Analysis column and reagents kit yields highly reliable and reproducible results. The amino acid derivatization is fully automated in the autosampler of an LC eliminating both the variability of manual sample preparation as well as any delay between preparation and analysis that could lead to sample degradation. Derivatization is necessary in order to effectively retain amino acids on a reversed-phase column and to detect them via UV or fluorescence. The AdvanceBio Amino Analysis column is a reversed phase column that has been specially treated to protect it at the high pH preferred for amino acid separations, resulting in a robust column with long lifetime.

Agilent's second amino acids separations solution, the AdvanceBio MS Spent Media column, is a HILIC separation paired with mass spectrometry (MS) detection. This alternative approach to retention makes derivatization unnecessary and enables more comprehensive cell culture analysis with a single method. Samples can be taken from the bioreactor and promptly analyzed after only a short centrifugation to precipitate any cellular debris. HILIC method development has its own unique challenges, but by following the best practices described below, robust and reliable results are within reach.

Choosing a workflow for spent media analysis depends upon a combination of analytical needs and in some cases, preferences:

- **Is MS detection available or preferred?**
  If yes, HILIC-MS enables monitoring of a broad array of analytes. If only UV or fluorescence detection is available, then a reversed phase method for amino acid analysis is recommended.
- **Is it only necessary to monitor amino acids, or is it necessary to monitor other cell culture media components?**
  If other nutrients or cellular waste products such as B vitamins, sugars, nucleotides, polyamines, or lactate need to be monitored, it can be more efficient to develop a multiplexed assay using HILIC-MS in which those metabolites are measured simultaneously with amino acids. If only amino acid analysis is required, then a reversed-phase LC/UV method with derivatized amino acids would meet your needs.
- **Do you prefer to derivatize or not derivatize amino acids?**
  Barring other circumstances, that can be the basis for choosing between reversed-phase LC/UV or LC/FLD with sample derivatization or HILIC-MS without derivatization.

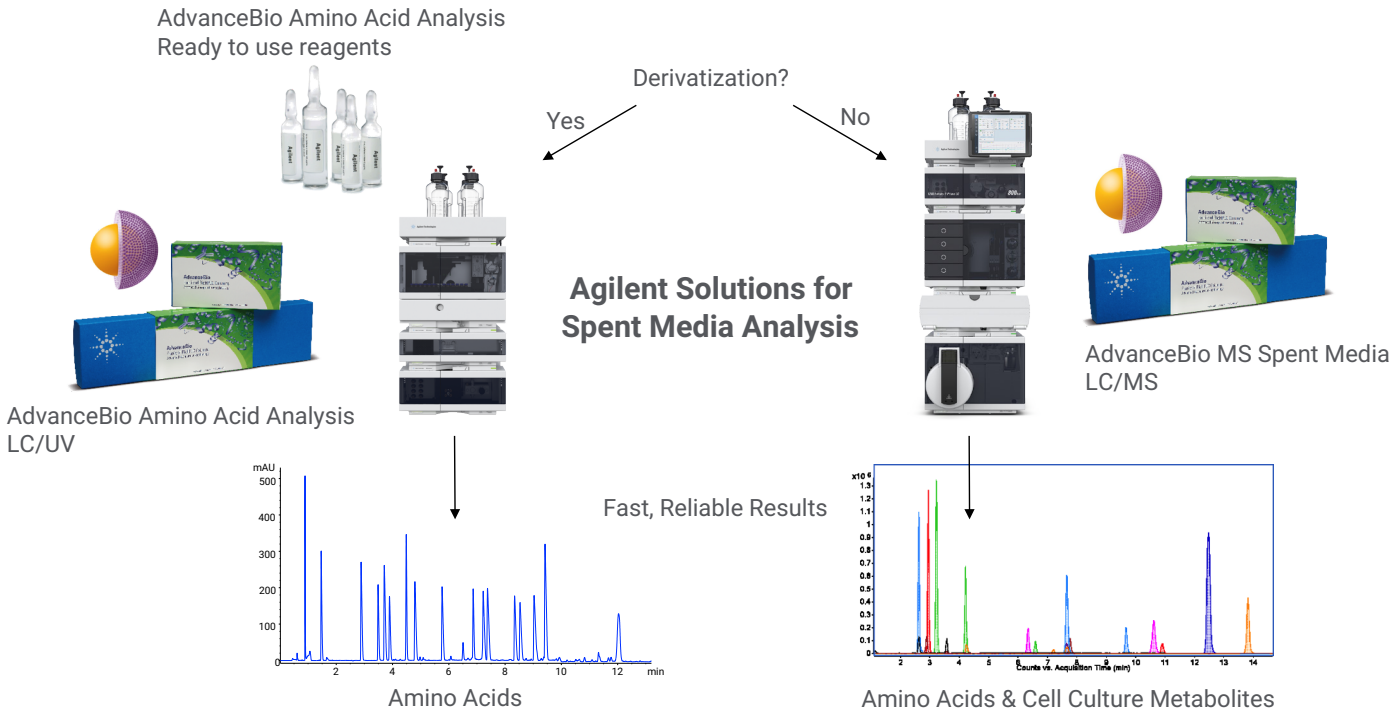

Figure 1. Choosing a spent media workflow depends upon which analytes must be monitored, preference for sample derivatization, and available detector options

Best practices for effective Amino Acid analysis

Sample preparation

- Centrifuge samples to precipitate any particulate matter from bioreactor samples.
- For labeled amino acids, replace derivatization reagent, borate buffer, and amino acid standards daily.
- For HILIC separations, dilute samples with acetonitrile for best chromatographic peak shape. For further discussion of the impact of sample solvent and injection volume on chromatographic peak shape, please see this HILIC Method Development Technical Overview.[2]

Chromatographic separation – General

- Lower the flow ramp rate from the default to 1 mL/min or lower. The gradual increase in flow rate will prolong column lifetime and help prevent sudden over pressuring. In Agilent software this setting can be found in the Advanced section of the LC pump controls.
- Set the maximum pressure limit in the LC method to match that of the column (600 bar for all columns recommended here). This is key for any instance in which the maximum pressure capabilities of the LC exceeds that of the column.

Chromatographic separation – Reversed-phase

- Recalibrate for retention times and response factors weekly.
- Monitor column and guard column performance by choosing a couple of specifications and tracking them regularly, for example resolution between leucine and isoleucine.
- Avoid using the maximum mixing speed during derivatization to avoid excessive wear on the autosampler.

Never leave the column in mobile phase A (Table 1: 10 mM Na_2HPO_4, and 10 mM $Na_2B_4O_7$, pH 8.2), even only overnight! For short term storage, always store the column in mobile phase B (Table 1: Acetonitrile, methanol, and water (45/45/10, v/v/v). For long term storage, store the column in 50/50 acetonitrile/water.

Chromatographic Separation - HILIC

- Amino acids are not sensitive to metal, however other analytes such as phosphate-containing molecules or polyamines can be extremely sensitive to the presence of metal in the LC system. To analyze non-amino acids, it is recommended to consider a Bio-Inert LC, or to otherwise minimize the presence of metal in the sample flow path by replacing metal tubing with PEEK, replacing glass solvent bottles with plastic, or following a deactivation protocol as outlined in the HILIC Method Development Technical Overview.[2] The AdvanceBio MS Spent Media column has PEEK-lined stainless steel hardware, and so is already a metal-free flow path.
- It is recommended to prepare HILIC mobile phases from a stock buffer solution, as described in the AdvanceBio MS Spent Media user guide[3] and the sample method below. This minimizes solubility challenges of salts in acetonitrile and increases consistency of ionic strength between Mobile Phase A and B.
- Mobile phase pH should be controlled for consistent column chemistry and therefore reproducible separations. Operating at a mobile phase pH within the buffer capacity of the chosen buffer system ($pK_a \pm 1$) will have better reproducibility.
- The higher the aqueous content of the sample matrix, the lower the injection volume should be to avoid peak splitting.
- HILIC columns take longer to re-equilibrate between injections than reversed-phase columns. Adequate re-equilibration is critical to reproducibility. Always maintain >3% H_2O to maintain an aqueous layer on the solid stationary phase. Consider starting the gradient at the highest % aqueous that still retains the least polar analyte for faster re-equilibration.

Mass spectrometry

- Do not use phosphate-containing buffers with MS detection!
- Choose volatile buffers such as ammonium acetate or ammonium formate for MS detection. Note that you won't be able to detect formate when using a formate-containing mobile phase, and likewise for acetate.
- Divert the LC stream to waste outside of the retention time(s) of interest, especially during a high organic rinse at the end of the method and, if possible, as the void volume elutes.
- Use HPLC-grade or higher solvents.
- Establish a regular cleaning routine for the MS source.

Getting started

Reversed-phase analysis of derivatized amino acids

Amino acid analysis with automated derivatization and LC/UV or fluorescence analysis is thoroughly described in this "how-to" guide.[4] This guide contains instructions for preparation of standards, programming the autosampler to execute the sample derivatization, and the chromatographic method

Parameter	Value	
Column	AdvanceBio Amino Acid Analysis 4.6 x 100 mm or 3.0 x 100 mm	
Instrument	Agilent 1290 Infinity II LC System	
Flow Rate	1.5 mL/min for 4.6 mm id columns 0.62 mL/min for 3 mm id columns	
Mobile Phase A	10 mM Na_2HPO_4, and 10 mM $Na_2B_4O_7$, pH 8.2	
Mobile Phase B	Acetonitrile, methanol, and water (45/45/10, v/v/v)	
Gradient	Time (min)	%B
	0	2
	0.35	2
	13.4	57
	13.5	100
	15.7	100
	15.8	2
	18	end
Column Temperature	40 °C	
Detector	Signal A: 338 nm, 10 nm bandwidth; reference wavelength 390 nm, 20 nm bandwidth Signal B: 262 nm, 16 nm bandwidth; reference wavelength 324 nm, 8 nm bandwidth	

Table 1. LC method for reversed-phase analysis of labeled amino acids using the AdvanceBio Amino Acid Analysis column

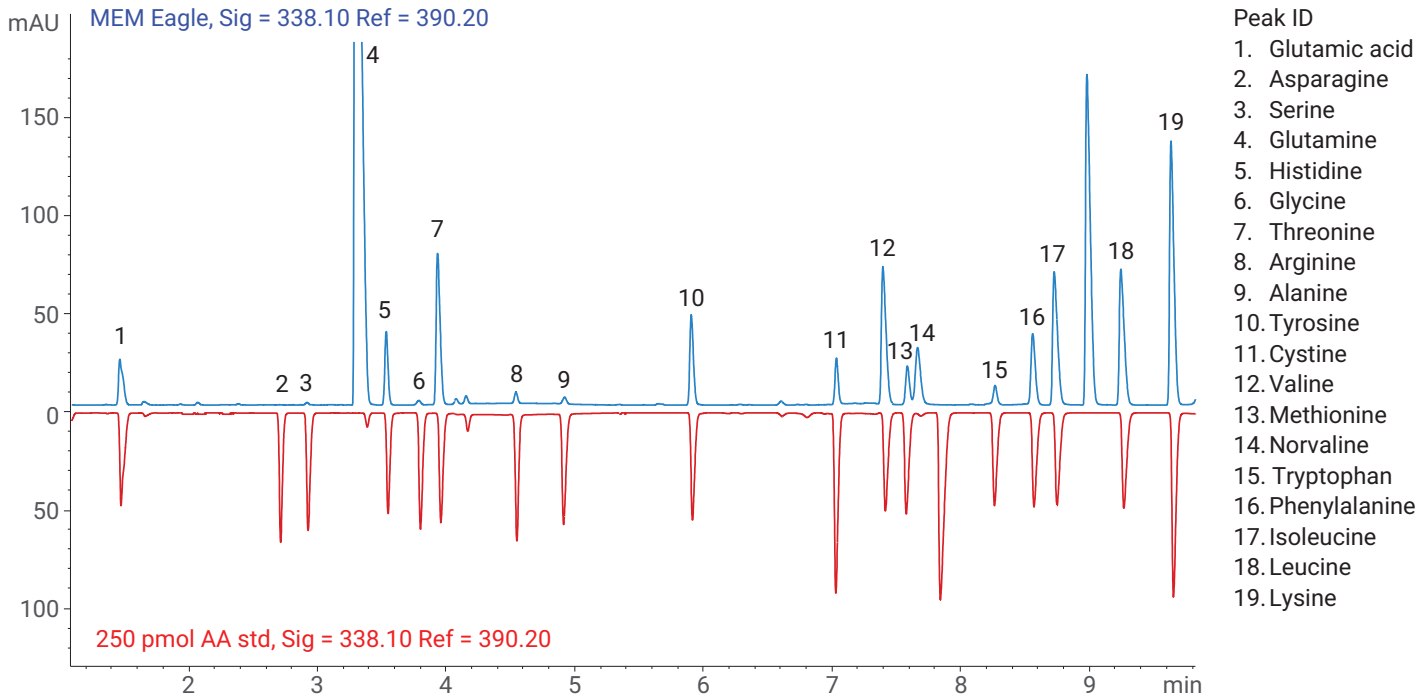

Figure 2. Example separation of OPA and FMOC labeled amino acids using the AdvanceBio Amino Acid Analysis column.[5]

HILIC analysis of underivatized amino acids

A sample method used for a variety of metabolites in addition to amino acids is shown below. For a sample method focused on amino acids, please see this application note[6] or this brochure[7].

Parameter	Value	
Column	AdvanceBio MS Spent Media, 2.1x100 mm	
Instrument	Agilent 1260 Infinity II Bio-Inert LC System	
Flow Rate	0.5 mL/min	
Mobile Phase	**Low pH, Positive Ion Mode MS Detection:** A = 10% 200 mM ammonium formate in water pH 3, 90% water B = 10% 200 mM ammonium formate in water pH 3, 90% acetonitrile *Final salt concentration is 20 mM.*	
Gradient	Time (min)	%B
	0	100
	10	75
	20	20
	21	20
	21.1	100
	28	100
Column Temperature	40 °C	
Detector	Agilent 6230 TOF	

Table 2. LC method for HILIC analysis of amino acids and other cell culture media analytes using the AdvanceBio MS Spent Media column

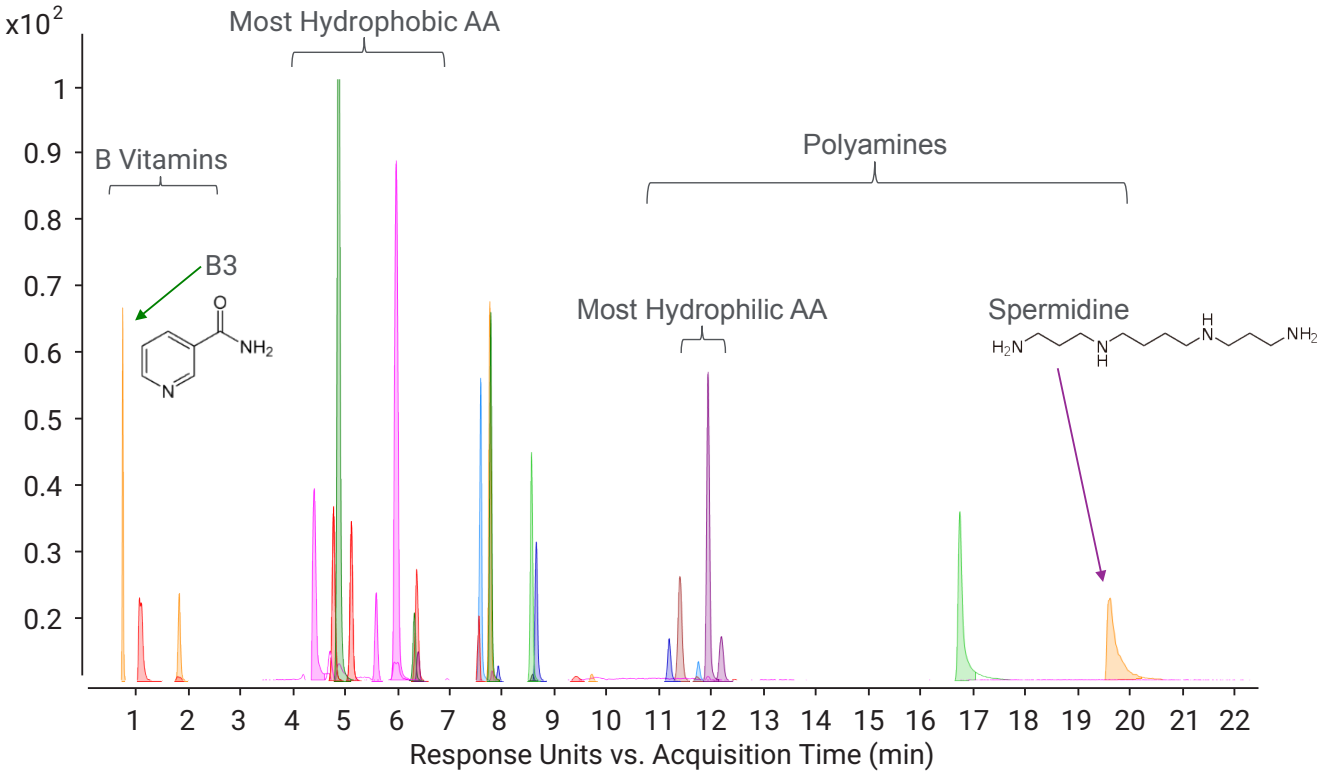

Figure 3. Sample separation of amino acids, B vitamins, and polyamines using the AdvanceBio MS Spent Media column with TOF detection.[8]

Easy selection and ordering information

To order items listed in the tables below from the Agilent online store, add items to your Favorite Products list by clicking on the MyList # header links. You can then enter the quantities for the products you need, add the products to your Cart and proceed to checkout. Your list will remain under Favorite Products for your use with future orders.

If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. However, if you don't have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled. All items can also be ordered through your regular sales and distributor channels.

Description	Part No.
MyList 1: AdvanceBio Amino Acid Analysis (AAA) Columns	
AdvanceBio Amino Acid Analysis (AAA), 3.0 x 100 mm, LC columnA	695975-322
AdvanceBio Amino Acid Analysis (AAA), 4.6 x 100 mm, 2.7 µm LC column	655950-802
AdvanceBio Amino Acid Analysis (AAA), 3.0 x 5 mm, guard column, 3/pk	823750-946
AdvanceBio Amino Acid Analysis (AAA), 4.6 x 5 mm, guard column, 3/pk	820750-931
MyList 2: AdvanceBio MS Spent Media Analysis Columns	
AdvanceBio MS Spent Media 100 Å, 2.1 x 50 mm, 2.7 µm	679775-901
AdvanceBio MS Spent Media 100 Å, 2.1 x 100 mm, 2.7 µm	675775-901
AdvanceBio MS Spent Media, 100 Å, 2.1 x 150 mm, 2.7 µm	673775-901
MyList 3: AdvanceBio AAA Standards and Reagents	
AdvanceBio amino acid reagents kit; 1-250 pmol/µL	5190-9426
Borate Buffer 100 mL	5061-3339
FMOC reagent, 2.5 mg/mL in acetonitrile, 10 x 1 mL	5061-3337
Dithiodiproprionic, 5 g	5062-2479
AA standard, 1 nmol/µL, 10 x 1 mL	5061-3330
AA standard, 250 pmol/µL, 10 x 1 mL	5061-3331
AA standard, 100 pmol/µL, 10 x 1 mL	5061-3332
AA standard, 25 pmol/µL, 10 x 1 mL	5061-3333
AA standard, 10 pmol/µL, 10 x 1 mL	5061-3334
Amino acids supplement kit	5062-2478
MyList 4: HPLC supplies	
Agilent InfinityLab Quick Connect Fitting assembly (for connection on column inlet)	5067-5965
Agilent InfinityLab Quick Connect Capillary MP35N 0.12 x 105 mm (BioInert; for Quick Connect fitting)	5500-1578
Agilent InfinityLab Quick Connect Capillary SS 0.12 x 105 mm (for Quick Connect fitting)	5500-1173
Agilent InfinityLab Quick Turn Fitting (for connection on column outlet)	5067-5966
Agilent InfinityLab Quick Turn Capillary MP35N 0.12 x 280 mm (for Quick Turn fitting)	5500-1596
Agilent InfinityLab Quick Turn Capillary SS 0.12 x 280 mm (for Quick Turn fitting)	5500-1230
Mounting tool for quick turn fittings	5043-0915

Description	Part No.
MyList 5: Sample Containment	
High recovery vial, screw top, with fixed insert, clear, 300 µL insert volume, 100/pk. Vial size: 12 x 32 mm (12 mm cap)	5188-6591
Cap, screw, blue, PTFE/red silicone septa, 100/pk. Cap size: 12 mm	5182-0717
Vial, crimp/snap top, polypropylene, 250 µL, 1,000/pk. Vial size: 12 x 32 mm (11 mm cap)*	5190-3155
Cap, snap, clear, PTFE/silicone/PTFE septa, 100/pk. Cap size: 11 mm (for 5190-3155)	5182-0566
InfinityLab Well-plate 96/0.5 mL, 30/pk	5043-9310
InfinityLab Well-plate closing mat, 50/pk	5042-1389
MyList 6: Solvents and Additives	
InfinityLab Ultrapure LC/MS Water, 1L	5191-4498
InfinityLab Ultrapure LC/MS Acetonitrile, 1L	5191-4496
Formic acid, 5 mL	G2453-85060
InfinityLab Deactivator Additive, 25 mL	5191-3940
InfinityLab Deactivator Additive, 50 mL	5191-4506
MyList 7: Solvent Filtration	
InfinityLab solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, pore size 0.2 µm, 100/pk	5191-4341
Filter membrane, Regenerated Cellulose 47 mm, pore size 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168
MyList 8: Solvent Handling	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe purging bottle	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap	5043-1221
InfinityLab charcoal filter with time strip, 58 g	5043-1193

References:

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   5994-4931EN
2. Hydrophilic Interaction Chromatography Method Development and Troubleshooting
   5994-9271EN
3. Agilent AdvanceBio MS Spent Media Column User Guide
   820120-015
4. Amino Acid Analysis, “How-to” Guide
   5991-7694EN
5. Determination of Amino Acid Composition of Cell Culture Media and Protein Hydrolysate Standard
   5991-7922EN
6. Analysis of Underivatized Amino Acids by LC/MS for Bioreactor Cell Culture Monitoring
   5991-8816EN
7. Agilent AdvanceBio workflows for spent media analysis
   5991-8817EN
8. Analysis of Underivatized Amino Acids and Metabolites in Cell Culture Media by HILIC-LC/MS
   ASMS 2018 MP-566

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