Introduction

HPLC with post-column derivatization is a standard technique in the analysis of amino acids. Pre-column derivatization of free amino acids in solution for HPLC separations with UV or fluorescence detection is at times done offline, manually. Some immediate drawbacks to offline derivatization are sources of error due to operator skill, competence, and laboratory technique; extra sample manipulation; additional time required; and increased risk of contamination. Automated online derivatization minimizes these error sources, immediately improves precision, and saves time. A rugged high-resolution HPLC method including online derivatization, therefore, can increase productivity compared to offline methods. Consistent automated OPA (ortho-phthalaldehyde) and FMOC (9-fluorenemethyl chloroformate) derivatization using injector programming of the HPLC’s autosampler, and highly efficient AdvanceBio columns generate a rapid, reproducible amino acid method ideal for cell culture media. This method is convenient because the cell media samples are simply transferred to autosampler vials and analyzed. The selectivity of the AdvanceBio AAA column and the gradient mobile phase provide high resolution of 22 amino acids. A method is presented for the primary amino acids in cell culture media using an Agilent 1200 and 1290 system with a AdvanceBio AAA column with absorbance detection. Amino acids are derivatized with OPA/FMOC using an online injector program, which decreases preparation time while increasing reproducibility over traditional offline methods. The method is rapid and highly reproducible, with an excellent %RSD of peak area and retention time for all amino acids, with most between 1 and 2 percent. Furthermore, we also used this technique to determine the LOD, LOQ and system suitability requirements

Experimental

Instrumentation

The recommended chromatographic system is the Agilent 1200 Infinity LC, 5425A binary pump with 5412A Diode Array Detector (DAD), 6-mm × 1.0-mm flow cell and/or D1315A Fluorescence Detector (FLD). While the results shown here were obtained with the binary pump, this procedure has also been used with the Agilent 1200 Infinity Bio-inject LC, Agilent 1200 Infinity LC, and Agilent 1200 Infinity II LC.

Mobile Phase:

Mobile phase A contained 10 mM Na2HPO4 and 10 mM Na2HPO3H, pH 8.2. Mobile phase B contains acetonitrile/methanol/water (45:45:10, v/v/v).

Stationary Phase:

AdvanceBio AAA, 4.6 × 100 mm, 2.7 μm column

Flow:

The flow rate used with the column was 1.5 mL/minute

TCC:

40°C. In all cases, the low-volume heat exchanger was used with short red tubing to minimize extra column volume

Detector Settings (DAD):

Signal A: 238 nm, 10 nm bandwidth, and reference wavelength 399 nm, 20 nm bandwidth. Signal B: 282 nm, 16 nm bandwidth, and reference wavelength 324 nm, 8 nm bandwidth. Signal C: 338 nm, 10 nm bandwidth, and reference wavelength 399 nm, 20 nm bandwidth. The DAD was programmed to switch to 282 nm, 16 nm bandwidth, reference wavelength 324 nm, 8 nm bandwidth, after isocitrillate elute, and before hydroxyproline elute. Signal D was determined by examining signal A and B timeframes between peaks 20 and 21, then choosing a suitable point to switch wavelengths. Once the time switch was established and programmed into the method, signals A and B were optional. Peak width settings of +0.01 minutes used for all columns

Sample Preparation:

The injection aliquot was 100 μL mobile phase A, plus 0.4 μL concentrated H3PO4 in a 100 μL bottle, stored at 4°C. To prepare 0.1 N HCl, and 4.2 M concentrated HCl (38%) to a 500 mL volumetric flask that is partially filled with water, mix, and fill to the mark with water. This solution is for making extended amino acid and internal standard stock solutions. Store at 4°C.

Derivatization reagents:

Buffers, OPA, and FMOC are made-ready solutions supplied by Agilent. They simply need to be transferred from their container into an autosampler vial.

Simplified Operation:

Just Add Samples and Reagents in separate vials, instrument autosampler draws, mixes, does derivatization in sample loop, then injects. Automated derivatization reduces offline container transfer, measurement, and operator error.

The Agilent Amino Acid Analysis solution

Ready to use AdvanceBio AAA kit (Standards and Reagents)

All Agilent LC systems including Infinity II systems

AdvanceBio AAA Columns

Fast and rugged

Results and Discussion

Fast and Rugged Amino Acids Separation

Table 1: Retention time and area RSD precision for amino acids (100 pmol) separated on an Agilent AdvanceBio AAA, 4.6 × 100 mm, column (n = 5)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>RT (min)</th>
<th>Area (mAU)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>2.2</td>
<td>1.969</td>
<td>0.81</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.01</td>
<td>1.262</td>
<td>0.89</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.03</td>
<td>1.074</td>
<td>0.73</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.69</td>
<td>1.40</td>
<td>0.54</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.91</td>
<td>0.846</td>
<td>0.25</td>
</tr>
<tr>
<td>Methionine</td>
<td>9.25</td>
<td>1.4</td>
<td>0.45</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>12.13</td>
<td>0.47</td>
<td>0.21</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.26</td>
<td>0.75</td>
<td>0.47</td>
</tr>
<tr>
<td>Arginine</td>
<td>14.97</td>
<td>1.59</td>
<td>0.77</td>
</tr>
<tr>
<td>Glutamate</td>
<td>14.98</td>
<td>1.59</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Figure 1: Separation of Amino acid standard on various dimensions of AdvanceBio AAA columns using amino acid method

Precision of retention time and area

Figure 2: Linearity curve with 10 standard concentrations of asparagine, glutamic, and tryptophan ranging from 0.8 to 10.0 pmol, showing excellent coefficient values

Concentration (pmol) S/N ratio

Aspartic acid

0.8 (LOQ) 5.3
1.8 (LOQ) 10.8
Glutamic acid

0.8 (LOQ) 3.0
1.8 (LOQ) 13.8
Tryptophan

0.8 (LOQ) 4.5
1.8 (LOQ) 20.5

Figure 3: Comparison of Amino acid of MEM media (blue trace) with AA standard on 4.6 × 100 columns using amino acid method

Cell Media Analysis

Figure 4: Comparison of Amino acid of NEAA media (blue trace) with AA standard on 4.6 × 100 columns using amino acid method

System suitability as per the European Pharmacopoeia (Ph. Eur.)

Baseline resolution of tyrosine and leucine with the Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 μm column is much higher than the reported value for system suitability requirement for this pair

Figure 5: Comparison of Amino acid of RPMI1640 media (blue trace) with AA standard on 4.6 × 100 columns using amino acid method

References

Automated Amino Acid Analysis For Biopharma Applications Using Advancebio AAA Superficially Porous High pH Stable Reversed-phase Columns.

Sundaram Palaniswamy M. and Linda Lloyd; Agilent Technologies, Inc.

• European Pharmacopoeia 9.2 (2.3.6) Amino Acid Analysis.
• Determination of Amino Acid Composition of Cell Culture Media and Protein Hydrolysate Standard. The Agilent AdvanceBio Amino Acid Solution. 2017, Agilent Application Note Publication number 5991-7502EN