Automated Amino Acid Analysis using 2.7 µm Superficially Porous High pH stable Reversed-Phase Columns

Sundaram Palaniswamy[1] and Timothy Rice[2]

[1]Agilent Technologies, Inc. Bangalore, India, [2]Agilent Technologies, Inc. Wilmington, DE 19808,

Agilent Technologies

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Introduction

HPLC with pre-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 analytical 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-fluorenylmethyl 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 23 amino acids.

A method is presented for analyzing primary amino acids in cell culture media using an Agilent 1290 and 1260 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

Experimental

Instrument

The recommended chromatographic system is the Agilent 1290 Infinity Binary LC: G4220A binary pump with G4212A Diode Array Detector (DAD), 6-mm or 10-mm flow cell, and/or G1315A Fluorescence Detector (FLD). While the results shown here were obtained with the binary pump, this procedure has also been used with the Agilent 1260 Infinity Bio-inert LC, Agilent 1260 Infinity Binary LC and Agilent 1260 Infinity II LC.

Results

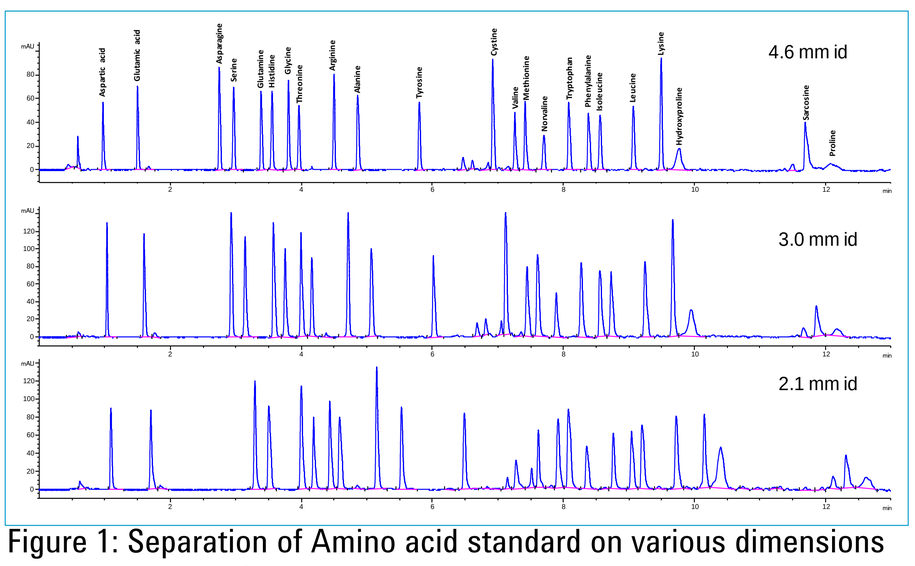

Figure 1: Separation of Amino acid standard on various dimensions of AdvanceBio AAA columns using amino acid method

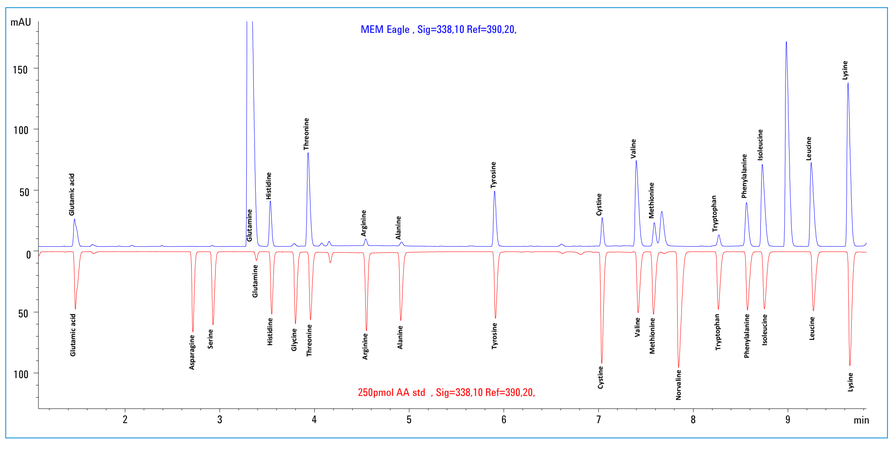

Figure2 : Comparison of Amino acid of **MEM media** (blue trace) with AA standard on 4.6 x 100 columns using amino acid method

Experimental

Mobile Phase: Mobile phase A contained 10 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$, pH 8.2. Mobile phase B contains acetonitrile:methanol:water (45:45:10, v:v:v).

Stationary Phase: AdvanceBio AAA, 4.6 × 100 mm, 2.7µm 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: 338 nm, 10 nm bandwidth, and reference wavelength 390 nm, 20 nm bandwidth. Signal B: 262 nm, 16 nm bandwidth, and reference wavelength 324 nm, 8 nm bandwidth. Signal C: 338 nm, 10 nm bandwidth, and reference wavelength 390 nm, 20 nm bandwidth. The DAD was programmed to switch to 262 nm, 16 nm bandwidth, reference wavelength 324 nm, 8 nm bandwidth, after lysine elutes, and before hydroxyproline elutes. Signal C was determined by examining signal A and B timeframes between peaks 20 and 21, then choosing a suitable point to switch wavelengths. Once the switch time was established and programmed into the method, signals A and B were optional. Peak width settings of > 0.01 minutes were used for all columns.

Sample Preparation: The injection diluent was 100 mL mobile phase A, plus 0.4 mL concentrated H_3PO_4 in a 100 mL bottle, stored at 4 °C. To prepare 0.1 N HCl, add 4.2 mL concentrated HCl (36%) 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: Borate buffers, OPA, and FMOC are ready-made 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 errors.

Results

Figure 3. Linearity curves of selected amino acids from

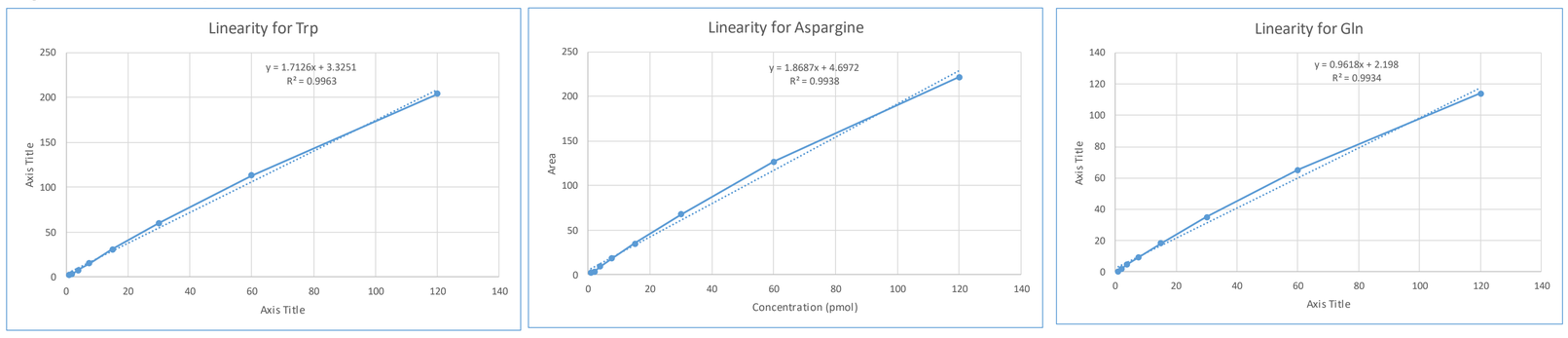

Table 1. LOD, LOQ and S/N ratio

Concentration (pmol)	S/N ratio	Concentration (pmol)	S/N ratio	Concentration (pmol)	S/N ratio
Asparagine		**Glutamine**		**Tryptophan**	
0.9 (LOD)	5.3	0.9 (LOD)	3.0	0.9 (LOD)	4.5
1.9 (LOQ)	10.8	3.8 (LOQ)	13.8	3.8 (LOQ)	20.5

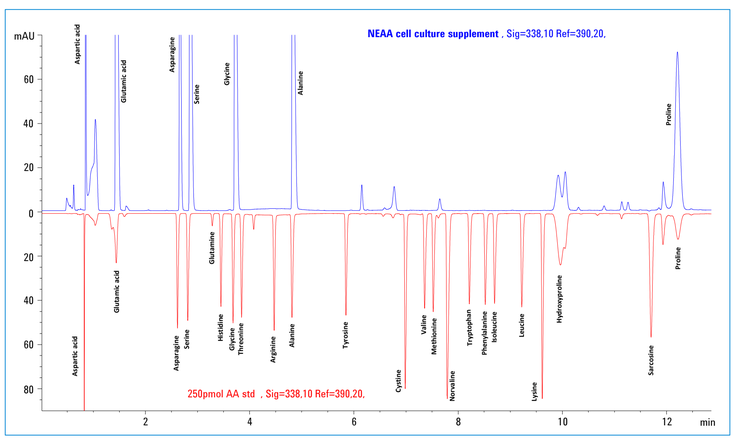

Figure 4 : Comparison of Amino acid of **NEAA media** (blue trace) with AA standard on 4.6 x 100 columns using amino acid method

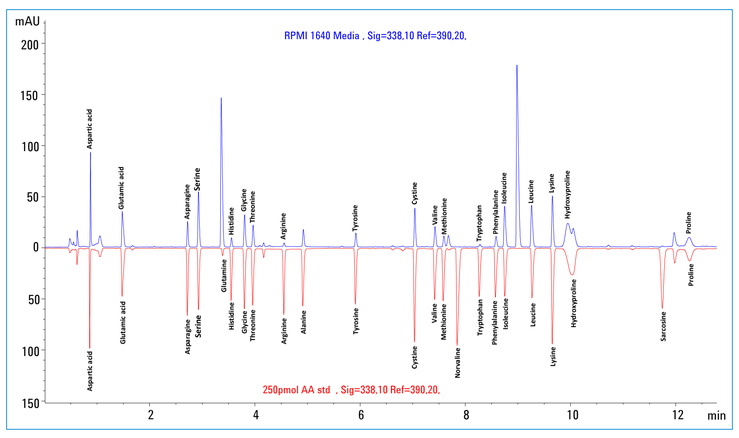

Figure 5: Comparison of Amino acid of **RPMI 1640 media** (blue trace) with AA standard on 4.6 x 100 columns using amino acid method

Conclusions

- An automated method for the analysis of pre-column derivitized amino acids using AdvanceBio Amino Acid Solution is presented here. Reproducibility of the method (RT and Area precision) of all the 23 amino acids were exceptional (data not shown).
- The method was found to be linear ranging from 0.9 pmol to 1,000 pmol showing excellent coefficient values. LOD and LOQ were about 0.9 pmol and 3.8 pmol, respectively, indicating that the method was sensitive.
- This solution enabled accurate amino acid compositional analysis of cell culture media, thus enabling to have a control over the supplementation requirement during upstream recombinant protein production.