Improved Amino Acid Methods using Agilent ZORBAX Eclipse Plus C18 Columns for a Variety of Agilent LC Instrumentation and Separation Goals

Application Note

Foods, Pharmaceuticals

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Introduction

The continuous improvement in HPLC columns and instrumentation presents an opportunity to improve HPLC methods. A proven ortho-phthalaldehyde/9-fluorenylmethyl chloroformate (OPA/FMOC) derivatized amino acid analysis method developed on the HP 1090 Series HPLC Systems, and later updated for the Agilent 1100 Series HPLC Systems, has now evolved further taking advantage of the Agilent 1200 Series SL and Agilent ZORBAX Eclipse Plus C18 stationary phase columns. [1-5]

Benefits of this protocol over previous iterations include:

- Better retention of the first two eluting amino acids, aspartic and glutamic acid.
- Higher resolution of several closely eluting amino acid pairs depending on the column configuration used.
- Ten column configurations, including three particle sizes and several column lengths and diameters, allowing the analyst to customize the separation to his specifications and constraints (for example, available Agilent HPLC model, desired throughput, or desired resolution).
- A quaternary pump option.

Of course, the previous protocols' benefits are still retained, especially the automatic online OPA/FMOC derivatization.

A chromatogram is presented for each column or method. They are grouped into three categories:

- Traditional 5-µm particle size columns that offer high resolution
- Practical 3.5-µm particle size columns that combine high resolution and speed of the 5 and 1.8-µm particle size columns (Rapid Resolution)
- Highest throughput 1.8-µm particle size columns that offer (Rapid Resolution High Throughput) excellent resolution

The 2.1 × 150 mm, 3.5 µm Rapid Resolution method is a preferred replacement method for the original AminoQuant method that used column p/n 79916AA-572.

All of the methods use the same chemicals and OPA/FMOC derivatization based on previous Agilent methods [1-6]. The differences among these methods are the mobile phase gradient programs and the flow path. These two parameters are optimized according to column dimensions and LC instrument models.

The ten method choices range from high-throughput analyses (<10 min cycle time) with R_s factors of at least 1.5 using 50-mm columns with 1.8-µm particles to 40-minute high-resolution methods (R_s >2 for all peak pairs) using 250-mm columns with 5-µm particles. Options in between include using 150-mm length columns with 3.5-µm particles or 100-mm columns with 1.8-µm particles also with R_s > 2. See Table 1.

Which LC is available?

Your method choice will likely depend on your LC model and configuration. An Agilent ZORBAX Eclipse Plus C18 method was developed for:

- Agilent 1100 with a quaternary pump
- Agilent 1100 with a binary pump
- Agilent 1200 with a binary pump (400 bar)
- Agilent 1200 SL with a binary pump (600 bar)

The ten methods are unique due to their column dimensions, and specific LC modules. Most of the other method parameters are the same. The 2.1 × 150 mm column method is a preferred replacement for existing AminoQuant users because the 2.1 × 150 mm, 3.5 µm column method has an analysis time comparable to the AminoQuant method and has nearly similar column dimensions.

Experimental:

Similar method parameters: chemical preparation

Mobile phase A: 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 5 mM NaN_3
For 1 liter: (1.4 g anhydrous Na_2HPO_4 + 3.8 g $Na_2B_4O_7 \cdot 10H_2O$ in 1 L water + 32 mg NaN_3). Adjust to about pH 8.4 with 1.2 mL concentrated HCl, then add small drops until pH 8.2. Allow stirring time for complete dissolution of borate crystals

Table 1. Agilent ZORBAX Eclipse Plus C18 Columns Meet a Range of Separation Goals

Method Number	Method Catagory	Column/ Method Name	Part Number	Analysis time with re-equilibration	Typical Minimum Resolution Factor	Approximate mL Solvent/ analysis*	Agilent HPLC Instrument
1	Traditional High Resolution	4.6 × 250, 5 um	959990-902	40 min	2.4	64	1200 or 1200 SL
2		3.0 × 250, 5 µm	custom	40 min	2.4	28	1200 or 1200 SL
3	Rapid Resolution	4.6 × 150, 3.5 µm	959963-902	25 min	2	42	1200 or 1200 SL
4		3.0 × 150, 3.5 µm	959963-302	25 min	2	18	1200 or 1200 SL
5		2.1 × 150, 3.5 µm‡	959763-902	25 min	2	12	1200 or 1200 SL†
6	Rapid Resolution High Throughput	4.6 × 100, 1.8 µm	959964-902	16 min	2.4	28	1200SL
7		2.1 × 100, 1.8 µm	959764-902	16 min	2.4	8	1200SL
8		4.6 × 50, 1.8 µm	959941-902	9 min	1.7	23	1200SL
9		3.0 × 50, 1.8 µm	959941-302	9 min	1.7	10	1200SL
10		2.1 × 50, 1.8 µm	959741-902	9 min	1.7	5	1200SL

* includes injector program and prerun DAD autobalancing (2.42 min), and re-equilibration time.
‡ preferred replacement method for AminoQuant method
† A 1200SL is needed for the preferred AminoQuant replacement method.

before adjusting pH. Filter through 0.45-µm regenerated cellulose membranes (Agilent p/n 3150-0576)

Mobile phase B: acetonitrile: methanol: water (45:45:10, v: v: v)
All mobile-phase solvents are HPLC grade. Since mobile phase A is consumed at a faster rate than B, it is convenient to make 2 L of A for every 1 L of B.

Injection diluent: 100 mL of mobile phase A + 0.4 mL concentrated H_3PO_4 in a 100-mL bottle. Store at 4°C.

0.1 N HCl: Add 4.2 mL of concentrated HCl (36 %) to a 500-mL volumetric flask that is partially filled with water. Mix, and fill to mark with water. 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. Some precautions include:

- OPA is shipped in ampoules under inert gas to prevent oxidation. Once opened, the OPA is potent for about 7-10 days. It is recommended to transfer 100-µL aliquots of OPA to micro vial inserts, label with name and date, cap, and refrigerate. Replace the OPA autosampler micro vial daily. Each ampoule lasts 10 days.
- FMOC is stable in dry air but deteriorates in moisture. It is also recommended to transfer 100-µL aliquots of FMOC to micro vial inserts, label with name and date, cap tightly, and refrigerate. Like the OPA, an open FMOC ampoule transferred to 10 micro vial inserts should last 10 days (one vial/day).
- Borate buffer can be transferred to a 1.5-mL autosampler vial without a vial insert. It can be replaced every 10 days.

Preparing the amino acid standards

Amino acid standard preparation remains unchanged from previous Agilent procedures or application notes. [4,5,6] See Table 7 on the back page for a list of part numbers.

Amino acid standards (10 pmol/µL to 1 nmol/µL): Solutions of 17 amino acids in five concentrations are available from Agilent for calibration curves. Divide each 1-mL ampoule of standards p/n 5061-3330 through 5061-3334 into 100-µL portions in conical vial inserts. Cap and refrigerate aliquots at 4 °C.

Extended amino acid (EAA) stock solution

Weigh 59.45 mg asparagine, 59.00 mg hydroxyproline, 65.77 mg glutamine, and 91.95 mg tryptophan into a 25-mL volumetric flask. Fill halfway with 0.1 N HCL and shake or sonicate until dissolved. Fill to mark with water for a total concentration of 18 nmol/µL of each amino acid.

For high-sensitivity EAA stock solution, take 5 mL of this standard-sensitivity solution and dilute with 45 mL water (1.8 nmol/µL).

Solutions containing extended standards are unstable at room temperature. Keep them frozen and discard at first signs of reduced intensity.

Internal standards (ISTD) stock solution

For primary amino acids, weigh 58.58 mg norvaline into a 50-mL volumetric flask. For secondary amino acids, weigh 44.54 mg sarcosine into same 50-mL flask. Fill halfway with 0.1 N HCl and shake or sonicate until dissolved, then fill to mark with water for a final concentration of 10 nmol each amino acid/µL (standard sensitivity).

For high-sensitivity ISTD stock solution, take 5 mL of standard sensitivity solution and dilute with 45 mL of water. Store at 4°C.

Calibration curves may be made using two to five standards depending on experimental need. Typically 100 pmol/µL, 250 pmol/µL, and 1 nmol/µL are used in a three-point calibration curve for "standard sensitivity" analysis.

The following tables should be followed if an internal standard or other amino acids (for example, the extended amino acids) are added. Table 2 describes "standard sensitivity" concentrations typically used in UV analysis. Table 3 is typically used for "high sensitivity" fluorescence analysis.

Table 2. Standard Sensitivity Calibration Standards

	Concentration of final AA solutions (pmol/µL)		
	900	**225**	**90**
Take 5 mL of 18 nmol EAA	5 mL	5 mL	5 mL
Dilute with water	—	15 mL	45 mL
Diluted EAA mix	**5 mL**	**20 mL**	**50 mL**
Take 5 mL of diluted EAA mix	5 mL	5 mL	5 mL
Add 10 nmol ISTD solution	5 mL	5 mL	5 mL
EAA-ISTD mix	**10 mL**	**10 mL**	**10 mL**
Take 100 µL of EAA-ISTD mix	100 µL	100 µL	100 µL
For 1 nmol AA, add	900 µL	—	—
For 250 pmol AA, add	—	900 µL	—
For 100 pmol AA, add	—	—	900 µL
Final AA solution with EAA and 500 pmol/µL ISTD	**1 mL**	**1 mL**	**1 mL**

Table 3. High Sensitivity Calibration Standards

	Concentration of final AA solutions (pmol/µL)		
	90	**22.5**	**9**
Take 5 mL of 1.8 nmol EAA	5 mL	5 mL	5 mL
Dilute with water	—	15 mL	45 mL
Dilute EAA mix	**5 mL**	**20 mL**	**50 mL**
Take 5 mL diluted EAA mix	5 mL	5 mL	5 mL
Add 1 nmol ISTD solution	5 mL	5 mL	5 mL
EAA-ISTD mix	**10 mL**	**10 mL**	**10 mL**
Take 100 µL EAA-ISTD mix	100 µL	100 µL	100 µL
For 100 pmol AA, add	900 µL	—	—
For 25 pmol AA, add	—	900 µL	—
For 10 pmol AA, add	—	—	900 µL
Final AA solution with EAA and 50 pmol/µL ISTD	**1 mL**	**1 mL**	**1 mL**

Distinct Method Parameters

The LC pump

See Table 4. Other pump parameters for all methods include:

- Compressibility (×10^{-6} bar) A: 35, B: 80
- Minimal Stroke A, B: 20 µL

The automated liquid sampler (ALS): online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly:

G1376C well plate automatic liquid sampler (WPALS), with injection program:

1. Draw 2.5 µL from borate vial (Agilent p/n 5061-3339).
2. Draw 1.0 µL from sample vial.
3. Mix 3.5 µL in washport 5 times.
4. Wait 0.2 min.
5. Draw 0.5 µL from OPA vial (Agilent p/n 5061-3335).
6. Mix 4.0 µL in washport 10 times default speed.
7. Draw 0.4 µL from FMOC vial (Agilent p/n 5061-3337).
8. Mix 4.4 µL in washport 10 times default speed.
9. Draw 32 µL from injection diluent vial.
10. Mix 20 µL in washport 8 times.
11. Inject.
12. Wait 0.1 min.
13. Valve bypass.

Table 4. Mobile Phase Gradient Programs

Traditional high resolution method gradients, 5 µm

	4.6 × 250 mm p/n 959990-902	3.0 × 250 mm p/n custom
time (min.)	**%B**	**%B**
0	2	2
0.84	2	2
33.4	57	57
33.5	100	100
39.3	100	100
39.4	2	2
40	end	end
flow (mL/min.)	1.5	0.64

Rapid Resulution method gradients, 3.5 µm

	4.6 × 150 mm p/n 959963-902	3.0 × 150 mm p/n 959963-302	2.1 × 150 mm p/n 959763-902
time (min.)	**%B**	**%B**	**%B**
0	2	2	2
0.5	2	2	2
20	57	57	57
20.1	100	100	100
23.5	100	100	100
23.6	2	2	2
25	end	end	end
flow (mL/min.)	1.5	0.64	0.42

Rapid Resolution High Throughput method gradients 1.8 µm, 100 mm

	4.6 × 100 mm p/n 959963-902	2.1 × 100 mm p/n 959763-902
time (min.)	**%B**	**%B**
0	2	2
0.35	2	2
13.4	57	57
13.5	100	100
15.7	100	100
15.8	2	2
16	end	end
flow (mL/min.)	1.5	0.42

Rapid Resolution High Throughput method gradients 1.8 µm, 50 mm

	4.6 × 50 mm p/n 959941-902	3.0 × 50 mm p/n 959941-302	2.1 × 50 mm p/n 959741-902
time (min.)	**%B**	**%B**	**%B**
0	2	2	2
0.2	2	2	2
7.67	57	57	57
7.77	100	100	100
8.3	100	100	100
8.4	2	2	2
9	end	end	end
flow (mL/min.)	2.0	0.85	0.42

G1329A automatic liquid sampler (ALS), with injection program:

1. Draw 2.5 µL from borate vial (Agilent p/n 5061-3339).
2. Draw 1.0 µL from sample vial.
3. Mix 3.5 µL in air default speed 5 times.
4. Wait 0.2 min.
5. Draw 0.5 µL from OPA vial (Agilent p/n 5061-3335).
6. Mix 4.0 µL in air, 10 times default speed.
7. Draw 0.4 µL from FMOC vial (Agilent p/n 5061-3337).
8. Mix 4.4 µL, 10 times default speed.
9. Draw 32 µL from injection diluent vial
10. Mix 20 µL in air default speed 8 times.
11. Inject.
12. Wait 0.1 min.
13. Valve bypass.

The location of the derivatization reagents and samples is up to the analyst, and ALS tray configuration. Using the G1367C with a 2 × 56 wellplate tray (G2258-44502), the locations were:

- Vial 1: Borate buffer
- Vial 2: OPA
- Vial 3: FMOC
- Vial 4: Injection Diluent
- P1-A-1: Sample

The Thermostatted Column Compartment (TCC)

Left and right temperatures are set at 40 °C. Enable analysis when temperature is within ± 0.8 °C. See Table 5 for which heat sink to use.

The diode array detector (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. 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 is determined by examining signal A and B timeframes between peaks 20 and 21, then choosing a suitable point in time to switch wavelengths. Once switch time is established and programmed into the method, signal A and B are optional.

Peak width settings were:

- >0.01 min for the Rapid Resolution High Throughput (RRHT) 1.8-µm column methods
- >0.03 min for the Rapid Resolution (RR) 3.5-µm column methods
- >0.03 min for the Traditional High Resolution 5-µm column methods

Table 5. Flow Path Specifics

	Traditional High Resolution Methods (5 µm)		Rapid Resolution Methods (3.5 µm)		
Method name	**4.6 × 250 mm**	**3.0 × 250 mm**	**4.6 × 150 mm**	**3.0 × 150 mm**	**2.1 × 150 mm**
LC Model	1100	1200	1200 SL	1200 SL	1200 SL
Pump	G1312A	G1311A quat	G1312B	G1312B	G1312B
Dampener	yes	n/a	yes	yes	bypassed
Static mixer	G1312-87330	n/a	G1312-87330	G1312-87330	bypassed
Purge valve to ALS	G1328-87600 (green 500 mm)	G1328-87600 (green 500 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)
ALS	G1367A	G1329A	G1367C	G1367C	G1367C
Needle seat	G1367-87101 (green)	G1313-87201 (green)	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)
ALS to heat exchanger	G1313-87305 (green 180 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)
Heat exchanger	G1316 A 3 µL	G1316 A 3 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL
Heat exch. to column or guard	5021-1817 (green 150 mm)	5021-1816 (green 105 mm)	5021-1820 (red 105 mm)	5021-1820 (red 105 mm)	5021-1820 (red 105 mm)
Optional guard cartridge	820950-936-4 pk, 4.6 id	821125-936-4pk, 2.1 id	820950-936-4 pk, 4.6 id	821125-936-4pk, 2.1 id	821125-936-4pk, 2.1 id

Table 5. Flow Path Specifics

	Traditional High Resolution Methods (5 µm)		Rapid Resolution Methods (3.5 µm)		
Method name	**4.6 × 250 mm**	**3.0 × 250 mm**	**4.6 × 150 mm**	**3.0 × 150 mm**	**2.1 × 150 mm**
Column	959990-902	custom	959963-902	959963-302	959763-902
Post column to union	5065-9931 (200 mm green)	5065-9931 (200 mm green)	n/a	n/a	n/a
ZDV union to flow cell	5022-2184	5022-2185	n/a	n/a	n/a
Detector	G1315B	G1315D	G1315C	G1315C	G1315C
Flow cell	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024
	Rapid Resolution High Throughput Methods (1.8 µm 100 mm)		**Rapid Resolution High Throughput Methods (1.8 µm 50 mm)**		
Method name	**4.6 × 100 mm, 1.8 µm**	**2.1 × 100 mm, 1.8 µm**	**4.6 × 50 mm, 1.8 µm**	**3.0 × 50 mm, 1.8 µm**	**2.1 × 50 mm, 1.8 µm**
LC Model	1200 SL	1200 SL	1200 SL	1200 SL	1200 SL
Pump	G1312 B	G1312 B	G1312 B	G1312 B	G1312 B
Dampener	yes	bypassed	yes	bypassed	bypassed
Static Mixer	G1312-87330	bypassed	G1312-87330	bypassed	bypassed
Purge valve to ALS	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)	5021-1823 (red 400 mm)
ALS	G1367C	G1367C	G1367C	G1367C	G1367C
Needle seat	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)	G1367-87201 (red)
ALS to heat exchanger	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)	01090-87611 (red 105 mm)
Heat exchanger	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL	G1316-80003 1.6 µL
Optional guard cartridge	none	none	none	none	none
Column PN	959964-902	959764-902	959941-902	959941-302	959741-902
Column to flow cell	directly connected	directly connected	directly connected	directly connected	directly connected
Detector	G1315C	G1315C	G1315C	G1315C	G1315C
Flow cell	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024	2 µL G1315-60024

Columns and Guard Cartridges

See Table 5 for Agilent ZORBAX Eclipse Plus C18 columns and recommended guard cartridges. A Cartridge Hardware Kit (p/n 820888-901) is needed to house the guard cartridge. Cartridges should be flushed with a few milliliters of mobile phase B before connecting to column for equilibration.

The 3.0 × 250 mm, 5 µm is a custom column configuration as of this printing, but can be ordered from the LC Custom Column Shop:
http://www.chem.agilent.com/cag/lccustom_1page.asp?&rcat=LCColumns.

Results and Discussion

The scalability of Agilent ZORBAX Eclipse Plus C18 stationary phase columns between three particle sizes (5, 3.5, 1.8 µm) allows for many fine tuned methods. As summarized in Table 1, the analyst can choose a column or method based on analysis time, resolution factors, solvent usage, or instrument model.

Each of the three categories has its own method parameters. Of particular importance is the mobile phase flow path configuration for the smaller volume columns. Truly scaling a gradient method between different column diameters and lengths is somewhat complicated, especially because different flow rates produce different gradient delay times, which is the time it takes the gradient to travel from its formation point to the column head.

Besides flow rate, the delay time is determined by the flow path volume between the gradient formation point and the head of the column. The delay volume can vary from instrument to instrument. The specific flow paths used to produce all the chromatograms in this application note are listed in Table 5. Instead of trying to exactly scale the methods, a simpler approach was used with the flow path volumes listed in Table 5 and flow rates that produced a good separation. For example, the 4.6 × 50 mm, 1.8 um separation has a flow rate of 2 mL/min instead of 1. 5 mL /min, which is the equivalent flow rate used for the longer 100, 150 and 250 mm, 4.6 mm id columns.

Traditional 5 µm High Resolution methods

The first group is "Traditional 5 µm High Resolution" options. These two options are ideal for large sample volumes or high resolution analyses. All amino acids have a resolution factor greater than 2.0 and pressures under 400 bar. The 3.0-mm id Solvent Saver option uses 57% less solvent than the 4.6-mm id method (See Figure 1). The 250-mm column length means they have the longest analysis times compared to the "Rapid Resolution 3.5-µm" and the "Rapid Resolution High Throughput 1.8-µm" options.

The first 20 amino acids in Figure 1 are derivatized with OPA. The last three, hydroxyproline, sarcosine and proline, are derivatized with FMOC. A programmable wavelength switch from 338 to 265 nm takes place after lysine (peak 20) elutes and before hydroxyproline (peak 21) elutes.

Rapid Resolution Methods

The second group, "Rapid Resolution" methods also has resolution factors > 2, but analysis times are reduced from 40 to 25 minutes, and solvent use is reduced as well. The 3.5-µm columns have higher efficiency than 5-µm columns, so similarly high resolution can be achieved in a shorter length column with shorter analysis time. Figure 2 compares the three column dimensions.

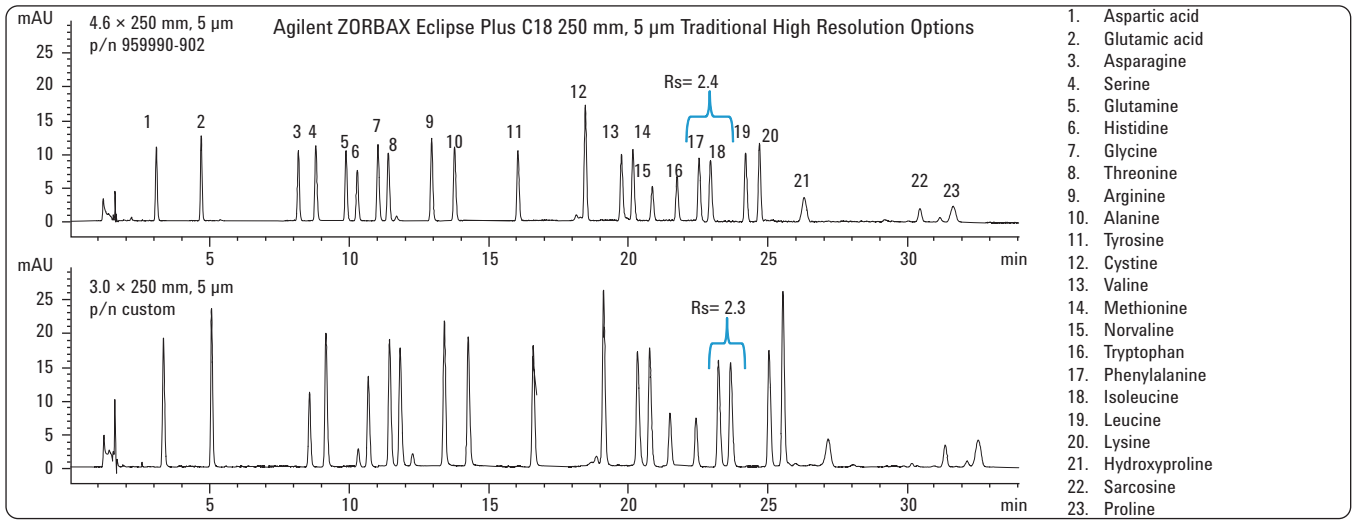


Figure 1. Amino acid analysis using 250 mm, Agilent ZORBAX Eclipse Plus C18, 5 µm columns.

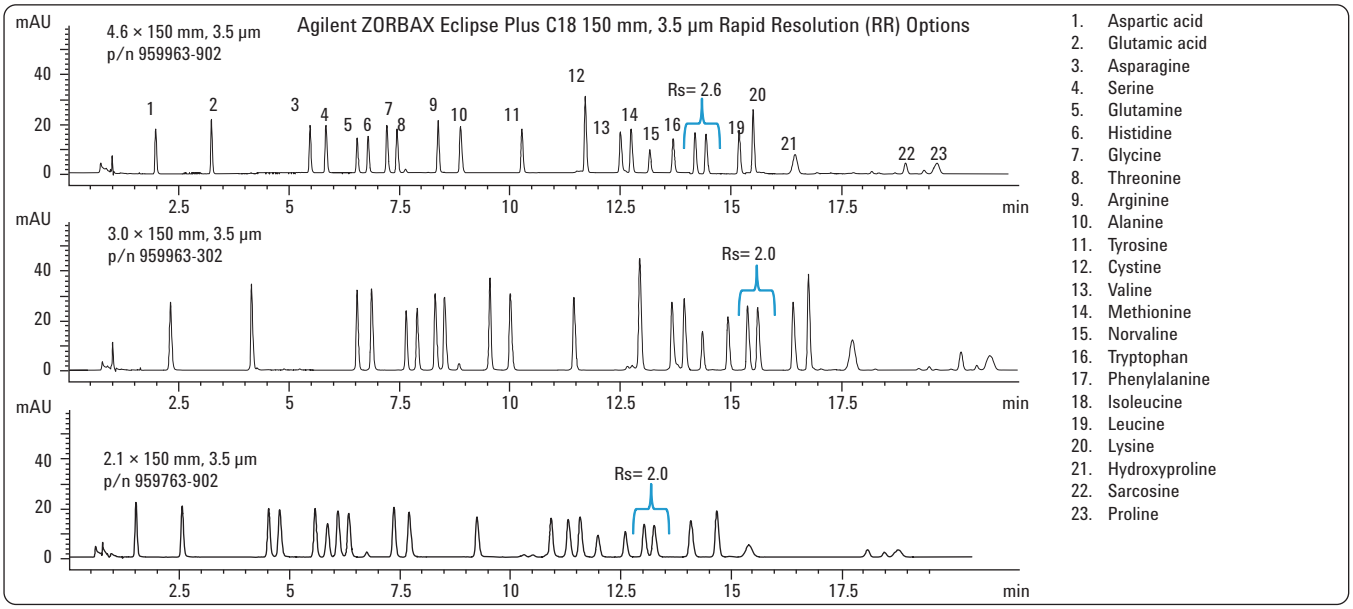


Figure 2. Amino acid analysis using 150 mm, Agilent ZORBAX Eclipse Plus C18 3.5 µm columns.

Upgrading from the original AminoQuant method to an Agilent ZORBAX Eclipse Plus C18 AA method

The Rapid Resolution methods include the 2.1 × 150 mm, 3.5 µm column which has the same inner diameter and similar efficiency of the AminoQuant column (2.1 × 200 mm, 5 µm). Consequently, the column produces a chromatogram with a similar analysis time and peak widths.

AminoQuant users that upgrade to an Eclipse Plus C18 amino acid method and want a comparable chromatogram to their AminoQuant results should use the 2.1 × 150 mm, 3.5 µm method with an Agilent 1200 SL Series HPLC as listed in Table 5.

Peak elution order of the standard amino acid mixture including the supplemental amino acids (gln, asn, trp, nva, hyp, sar) is the same for the two methods except for arginine. In the AminoQuant method, arginine elutes immediately after alanine. In the Eclipse Plus C18 method (and all previous ZORBAX amino acid methods), arginine elutes immediately before alanine.

AminoQuant users that upgrade to an Eclipse Plus amino acid method can choose one of the other nine methods if they are not constrained to a chromatogram similar in analysis time to their original AminoQuant chromatogram. These users should choose the method that best suits their needs regarding analysis time, resolution, solvent consumption, or instrumentation.

Rapid Resolution High Throughput Methods

These methods use 100 or 50-mm column dimensions and 1.8-µm particles. Resolution factors of the 100-mm RRHT columns shown in Figure 3 are greater than the 3.5-µm options in Figure 2 and are about the same value as the "Traditional High Resolution" columns in Figure 1.

This demonstrates that a 100 mm, 1.8 µm column has about the same efficiency as a 250-mm, 5-µm column. These high-speed, high-resolution methods require an Agilent 1200 SL system, as system pressure is about 400 bar for the analyses.

When the column length is halved, the analysis time (and pressure) is halved, but resolution is reduced. The benefit of

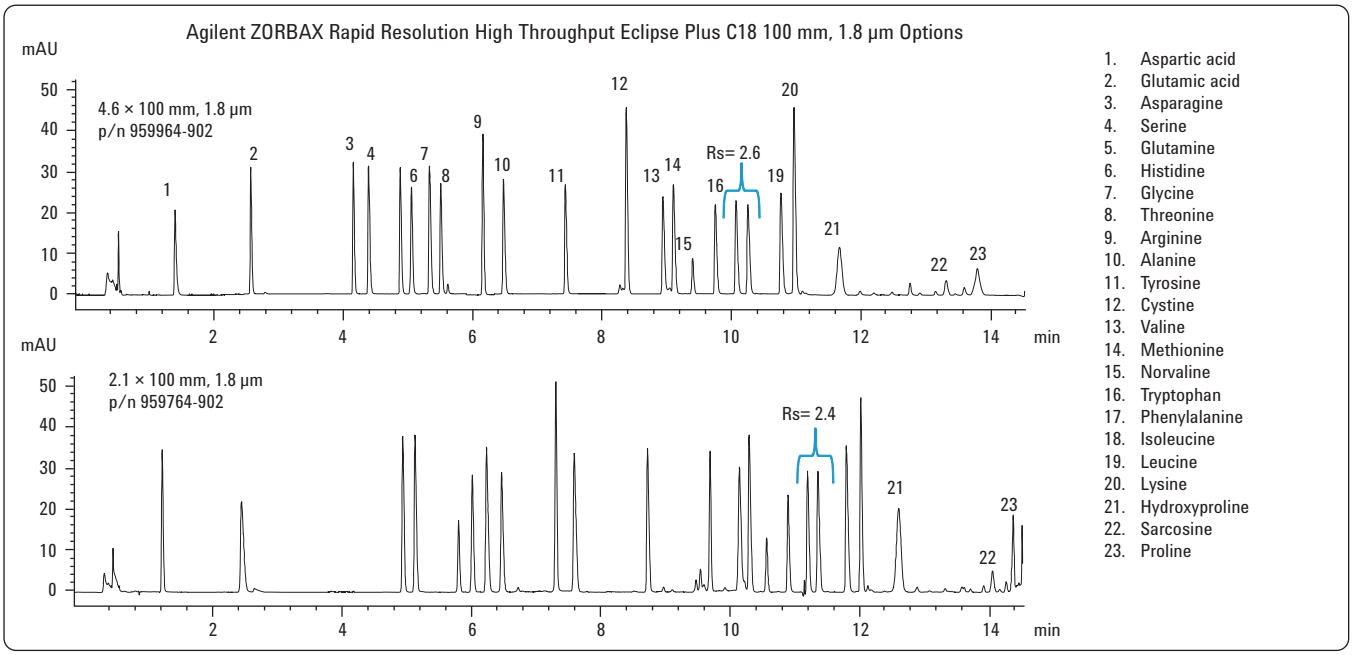


Figure 3. Amino acid analysis using 100 mm, Agilent ZORBAX Eclipse Plus C18, 1.8 µm columns.

using the short RRHT columns is a nine-minute analysis time including re-equilibration with critical pair Rs factors of less than two (Figure 4). Again, the 1200 SL is needed for these 1.8-µm methods for its higher DAD detector sampling rate to accurately detect and quantify the narrow peaks, and flexible pump flow path configurations to minimize gradient delay volume.

Batch-to-Batch Reproducibility

Batch-to-batch or lot-to lot reproducibility was measured by performing the analysis on three different columns made from different lots of packing material. Separation factor (α), or selectivity, was then measured for the closely spaced peaks.

Selectivity is a good measure of batch-to-batch reproducibility because it has a direct effect on the analyte equilibrium distribution between the stationary phase and the mobile phase. The equilibrium distribution is influenced by the mobile phase composition, the nature of the stationary phase, and the temperature. Since mobile phase composition and temperature are constant, any differences in separation factors are due to differences in stationary phase.

Notice the selectivity factors of closely spaced peaks in Figures 5, 6, and 7 are the same.

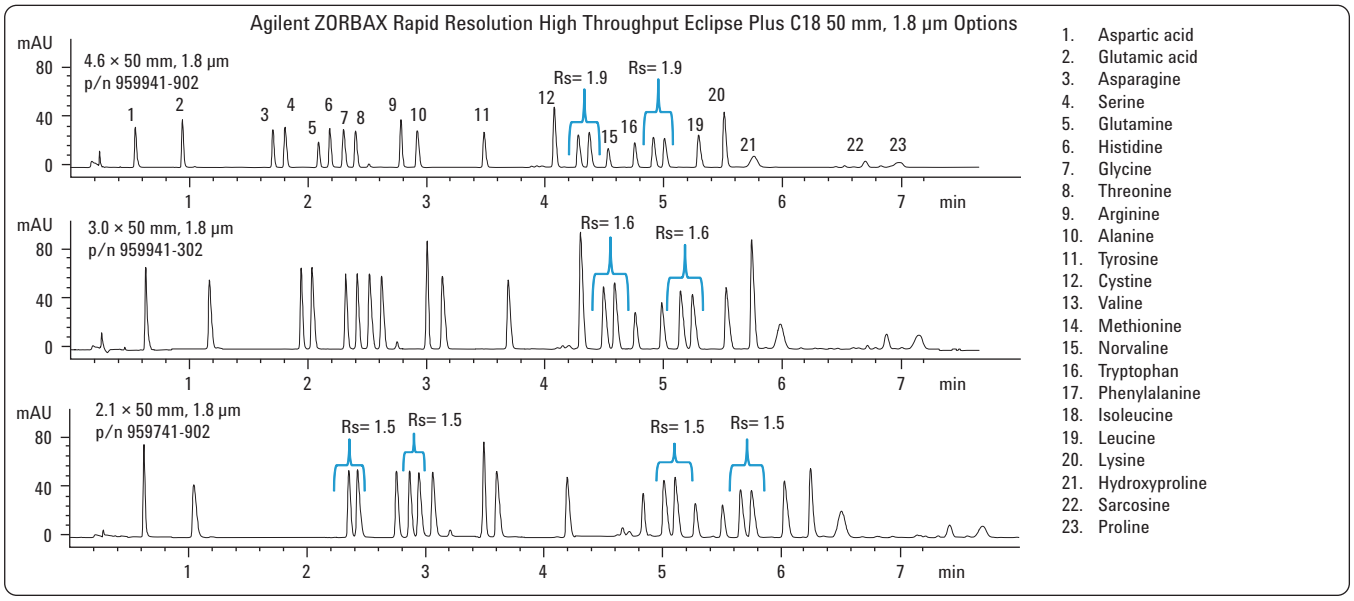


Figure 4. Amino acid analysis using 50 mm Agilent ZORBAX Eclipse Plus C18, 1.8 µm columns.

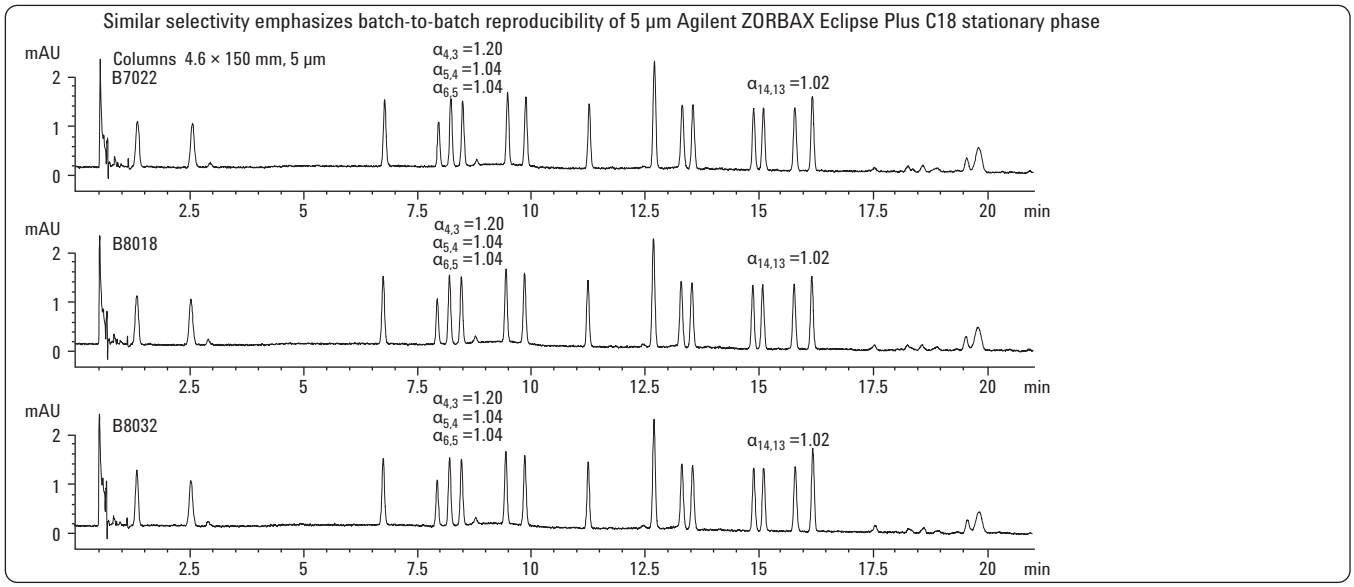


Figure 5. Batch-to-Batch Reproducibility of Agilent ZORBAX Eclipse Plus C18, 5 µm

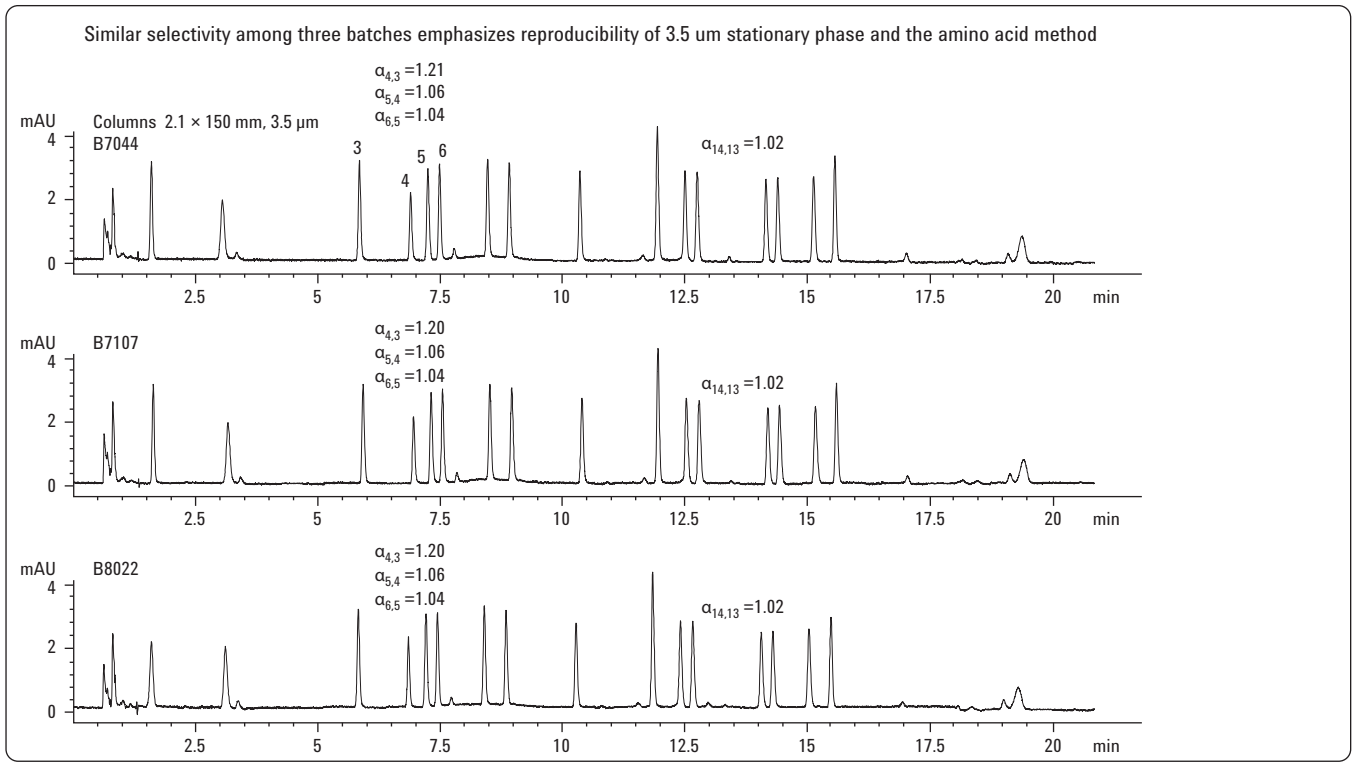


Figure 6. Batch-to-batch reproducibility of Agilent ZORBAX Eclipse Plus C18, 3.5 µm

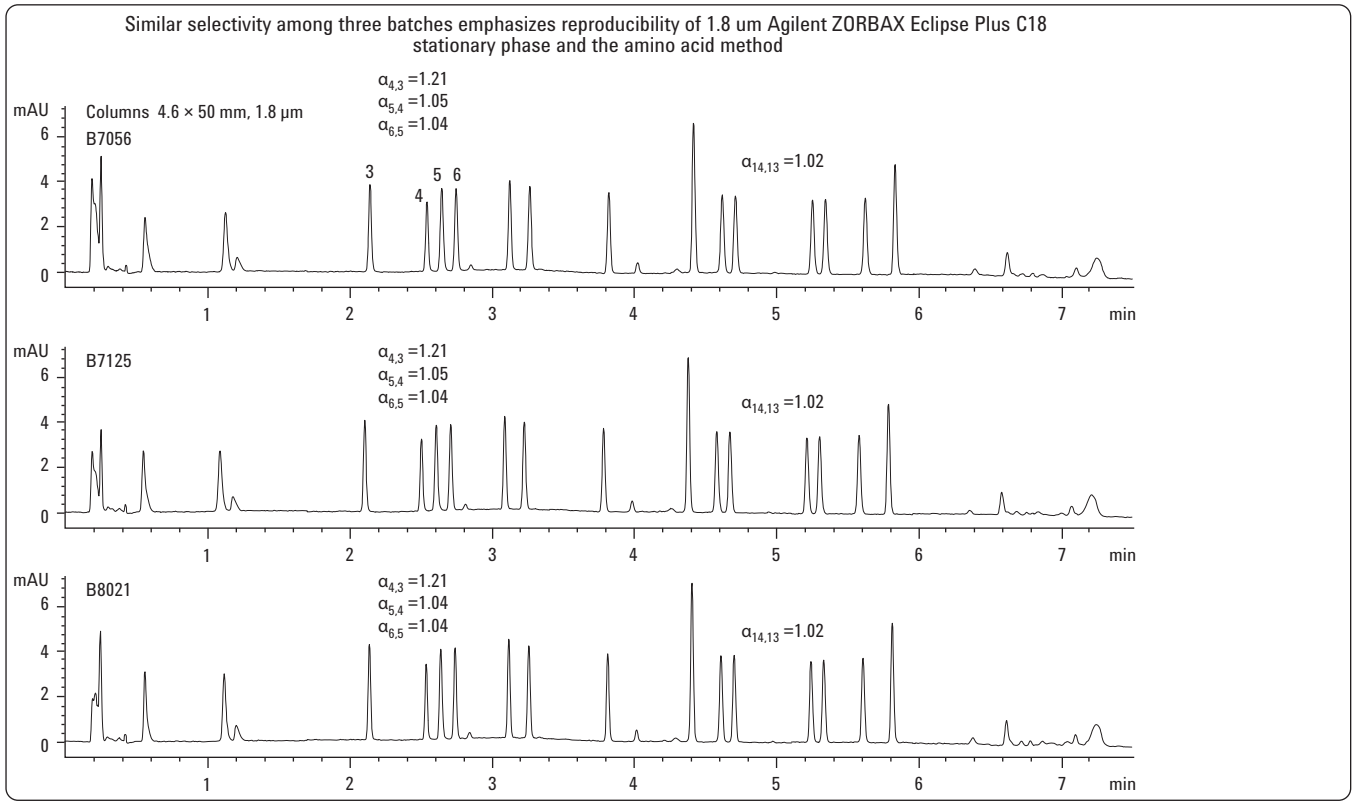


Figure 7. Batch-to-batch reproducibility of Agilent ZORBAX Eclipse Plus C18, 1.8 µm

In only one instance, the selectivity factor differs by 1%. Batch-to-batch reproducibility is consistent and reliable for the three particle sizes.

Different column dimensions were used for batch-to-batch comparisons:

- 4.6 × 50 mm for 1.8 µm particle comparisons
- 2.1 × 150 mm for 3.5 µm particle comparisons
- 4.6 × 150 mm for µm particle comparisons

Different sizes are suitable because selectivity is independent of column dimensions. Also, they further exemplify method robustness. In fact, the 4.6 × 150 mm dimension (See Figure 5) showing batch-to-batch reproducibility is a configuration not suggested in this application note. This is because although the 5-µm column separates the amino acids with similar selectivity as a 3.5-µm column, the 4.6 × 150-mm, 3.5-µm column has higher efficiency, and therefore better resolution. That makes it a better option than the 5-µm column.

Lifetime/ Longevity

Figure 8 shows a ChemStation sequence that repeated the amino acid method continuously for ten calendar days to obtain 500 injections. An additional longevity test (See Figure 9) was done using the G1311A quaternary pump and a 3.0 × 250 mm column. The longer column took an additional four days, for a total of 14 days of nonstop operation to complete 500 analyses. Resolution gradually lessened over the two week periods in both cases, but still produced R_s factors greater than 1.5.

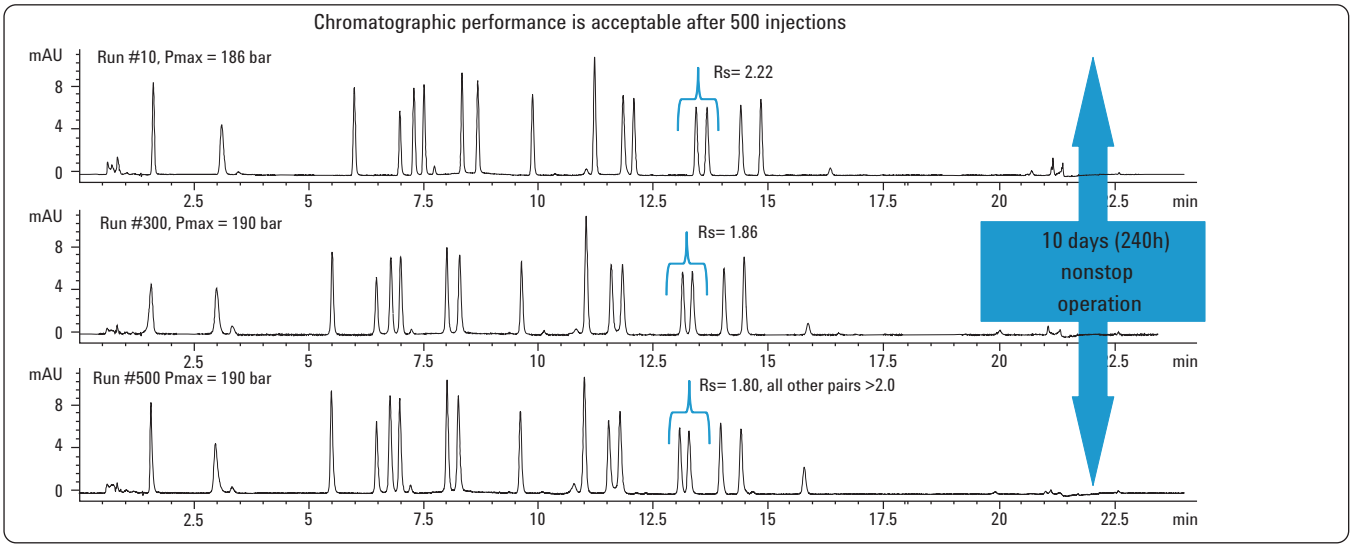


Figure 8. Longevity of Agilent ZORBAX Eclipse Plus C18, 2.1 × 150 mm, 3.5 µm

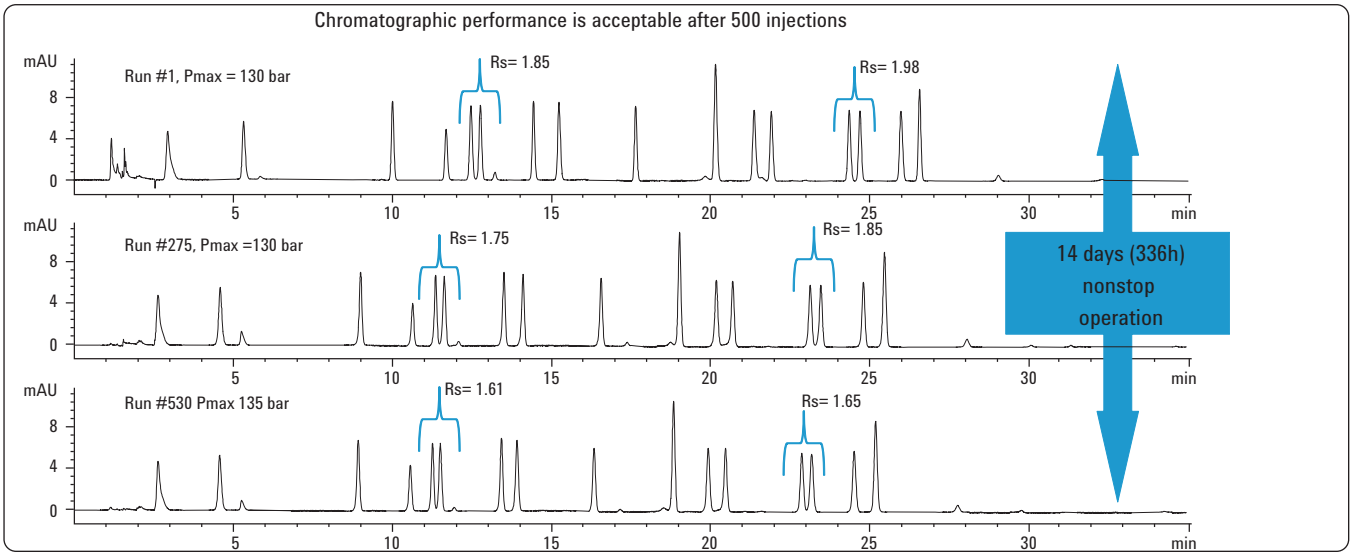


Figure 9. Longevity of Agilent ZORBAX Eclipse Plus C18 3.0 × 250 mm, 5 µm on a Quaternary 1200 system

Injection-to-injection reproducibility

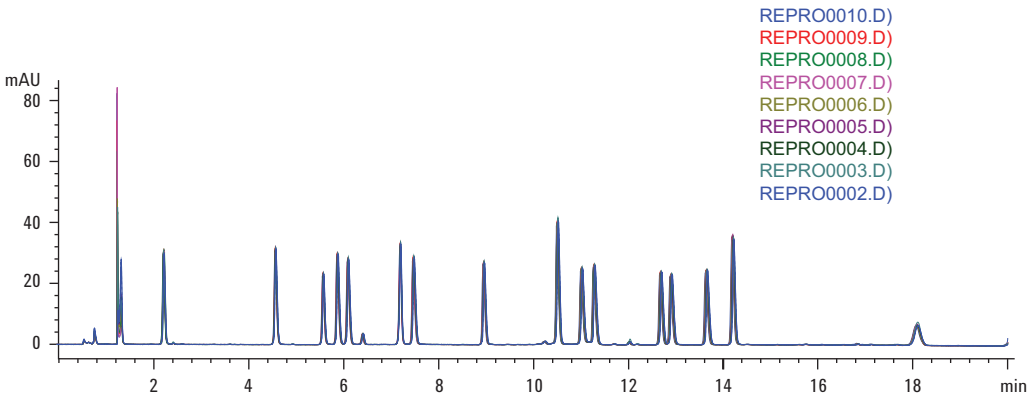
From the longevity data, injection reproducibility was recognized also. Figure 10 is an overlay of nine repeated injections. It allows retention time reproducibility and peak area to be easily compared.

The percent relative standard deviation (%RSD) for peak area of early, middle, and late eluting amino acids is calculated below the chromatogram. Similar results have been reported using different column dimensions [5, 7].

Linearity

Two columns, the 2.1 × 150 mm, 3.5 µm and the 4.6 × 100 mm, 1.8 µm, were chosen to demonstrate linearity. The three-point "standard sensitivity" calibration curve from the earlier amino acid protocols was extended to a five-point calibration curve using the "standard sensitivity" amino acid mixture standards of 100, 250, and 1000 pmol/µL and the additional 10 and 25 pmol/µL "high sensitivity" standards (for fluorescence detection).

Consistent peak area and retention time indicate method ruggedness, including online derivitization and column re-equilibration



	Peak Area											
amino acid	Injection 1	Injection 2	Injection 3	Injection 4	Injection 5	Injection 6	Injection 7	Injection 8	Injection 9	StDEV	Mean	%RSD
glu	90.7	93.7	92.4	93.1	93.8	94.7	92.5	95.7	93.2	1.43	93.3	1.5
ala	109.9	113.2	111.8	112.1	113.3	114	111.6	116	113.3	1.72	112.8	1.5
cys	153.2	157	155	157	157.8	159	154.4	162.6	158	2.79	157.1	1.8
lys	142.3	142.5	137.1	144.4	141.9	143.5	137.9	140.7	138.9	2.55	141.0	1.8
pro	60.8	62.9	61.8	65.8	60.9	63.2	61.2	70.2	61	3.11	63.1	4.9

Figure 10. Overlay of back-to-back injections, Agilent ZORBAX Eclipse Plus C18, 2.1 × 150 mm, 3.5 µm, 1 nmol/µl sample

Figures 11 and 12 plot curves for early, middle and late eluting amino acids, including an FMOC derivatized amino acid, for the two column configurations. The curves are representative of the other amino acids in the standard. Table 6 summarizes all the R^2 values for both columns using the five point curve, and a three point curve with the higher concentrations as described in earlier iterations of the amino acid method. Linearity is as robust from 10 to 1000 pmol/µL as it is from 100 to 1000 pmol/µL.

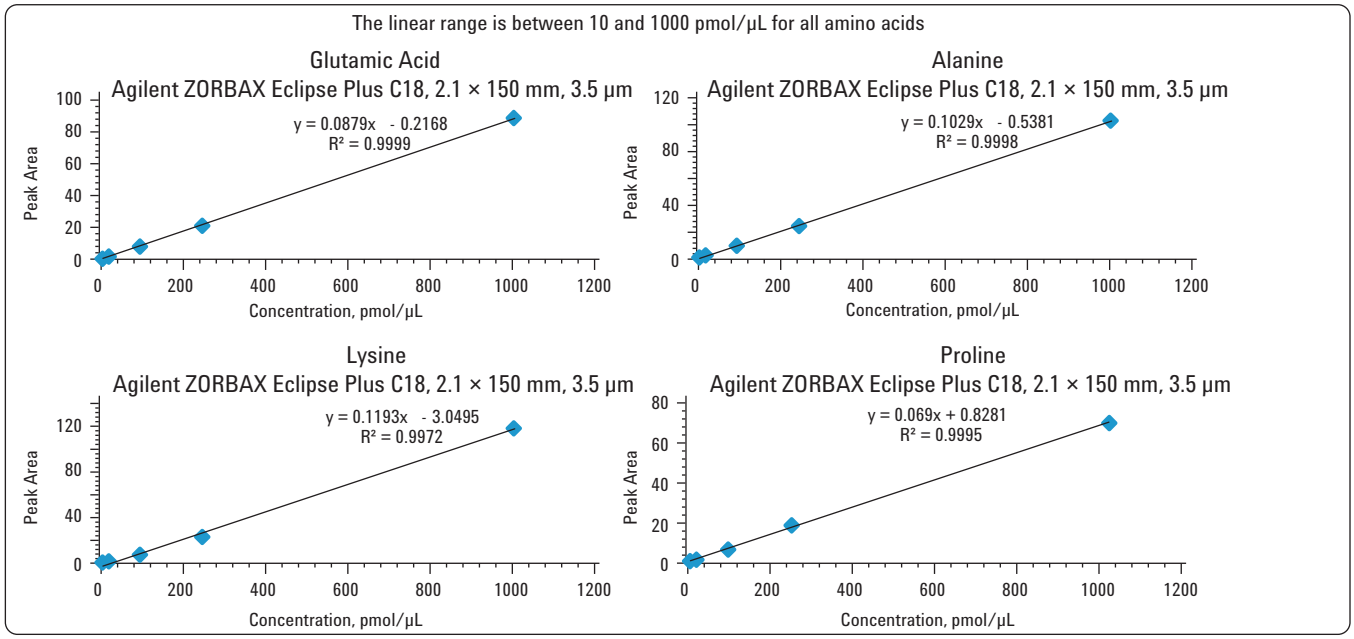


Figure 11. Five point calibrations of early, middle, and late eluting amino acids on the Agilent ZORBAX Eclipse Plus C18 2.1 × 150 mm, 3.5 µm column

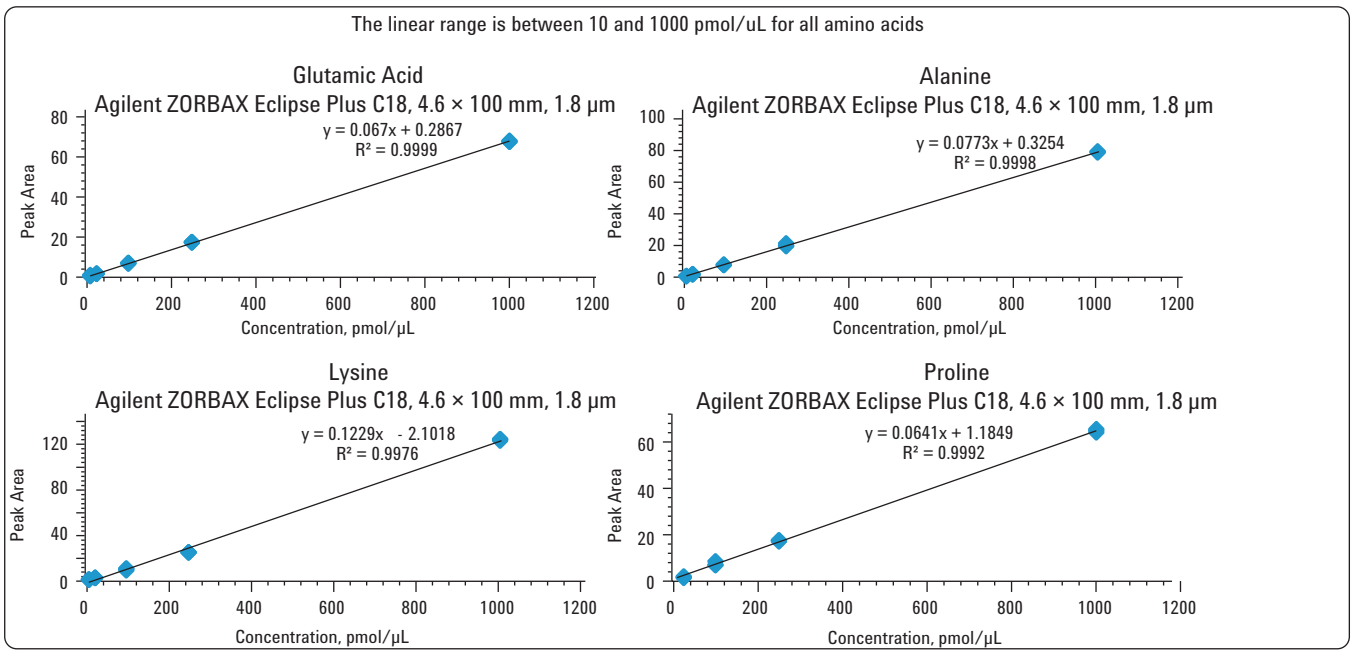


Figure 12. Five point calibrations of early, middle, and late eluting amino acids on the Agilent ZORBAX Eclipse Plus C18 4.6 × 100 mm, 1.8 µm column

Table 6 Five and Three Point Calibration Curve R^2 Values

Five point calibration curve R^2 values (10-1000 pmol/µL)

Amino Acid	2.1 mm × 150 mm, 3.5 µm Calibration Curve	R^2	4.6 mm × 100 mm, 1.8 µm Calibration Curve	R^2
ASP	y=0.0952x-0.2994	0.9999	y=0.0723x+1.1861	0.9976
GLU	y=0.0879x-0.2168	0.9999	y=0.0670x+0.2867	0.9999
SER	y=0.0983x-0.2968	0.9996	y=0.0746x+0.1128	1.0000
HIS	y=0.0716x-0.4109	0.9997	y=0.0555x+0.1748	1.0000
GLY	y=0.0956x-0.8918	0.9993	y=0.0758x-0.1374	0.9999
THR	y=0.0990x-0.3423	0.9999	y=0.0744x+0.2991	0.9999
ARG	y=0.1001x-0.5964	0.9997	y=0.0769x+0.1386	0.9999
ALA	y=0.1029x-0.5381	0.9998	y=0.0773x+0.3254	0.9998
TYR	y=0.0934x-0.4675	0.9998	y=0.0716x+0.1888	1.0000
CYS	y=0.1472x-2.0912	0.9994	y=0.1202x-0.5441	0.9998
VAL	y=0.1029x-0.3804	0.9998	y=0.0794x+0.2804	0.9999
MET	y=0.1008x-0.4284	0.9998	y=0.0780x+0.0927	1.0000
PHE	y=0.0945x-0.5314	0.9998	y=0.0730x+0.2714	0.9999
ILE	y=0.0982x-0.3882	0.9998	y=0.0739x+0.3198	0.9999
LEU	y=0.1001x-0.5408	0.9998	y=0.0767x+0.2263	0.9999
LYS	y=0.1193x-3.0495	0.9972	y=0.1229x-2.1018	0.9976
PRO	y=0.0690x+0.8281	0.9995	y=0.0641x+1.1849	0.9992

Three point calibration curve R^2 values (100-1000 pmol/µL)

Amino Acid	2.1 mm × 150 mm, 3.5 µm Calibration Curve	R^2	4.6 mm × 100 mm, 1.8 µm Calibration Curve	R^2
ASP	y=0.0958x-0.7365	0.9999	y=0.0710x+2.2162	0.9982
GLU	y=0.0884x-0.6010	1.0000	y=0.0668x+0.4858	0.9999
SER	y=0.0995x-1.2270	0.9999	y=0.0745x+0.2204	1.0000
HIS	y=0.0723x-0.9605	0.9998	y=0.0554x+0.2005	0.9999
GLY	y=0.0971x-2.1402	0.9997	y=0.0762x-0.4390	1.0000
THR	y=0.0996x-0.7722	1.0000	y=0.0742x+0.4565	0.9999
ARG	y=0.1011x-1.4018	0.9998	y=0.0768x+0.1926	0.9999
ALA	y=0.1037x-1.1823	0.9999	y=0.0771x+0.5060	0.9997
TYR	y=0.0941x-1.0366	0.9999	y=0.0714x+0.3964	1.0000
CYS	y=0.1489x-3.4631	0.9997	y=0.1201x-0.4196	0.9999
VAL	y=0.1038x-1.1299	0.9999	y=0.0792x+0.4669	0.9998
MET	y=0.1016x-1.1133	0.9999	y=0.0779x+0.1347	1.0000
PHE	y=0.0954x-1.2188	0.9999	y=0.0728x+0.4522	1.0000
ILE	y=0.0990x-1.0161	0.9999	y=0.0737x+0.5095	0.9999
LEU	y=0.1009x-1.1797	0.9999	y=0.0765x+0.3618	0.9999
LYS	y=0.1233x-6.2802	0.9993	y=0.1261x-4.6725	0.9986
PRO	y=0.0686x+1.0919	0.9994	y=0.0635x+1.6958	0.9995

Customization

The flow path configuration for each method is listed in Table 5. The mobile phase gradient is crucial to resolve the many amino acids. The known flow path assures that the gradient delay volume is similar between the LC systems used for this application note and the LC system in your laboratory. The robustness of the columns, mobile phase, and LC systems, however, does leave room for some variability or customization. For example, a longer length flow cell can be substituted to increase sensitivity (See Figure 13).

Another way of customizing the method is to eliminate the FMOC derivatization step in the injector program and modify the gradient if secondary amino acids are not important. [7]

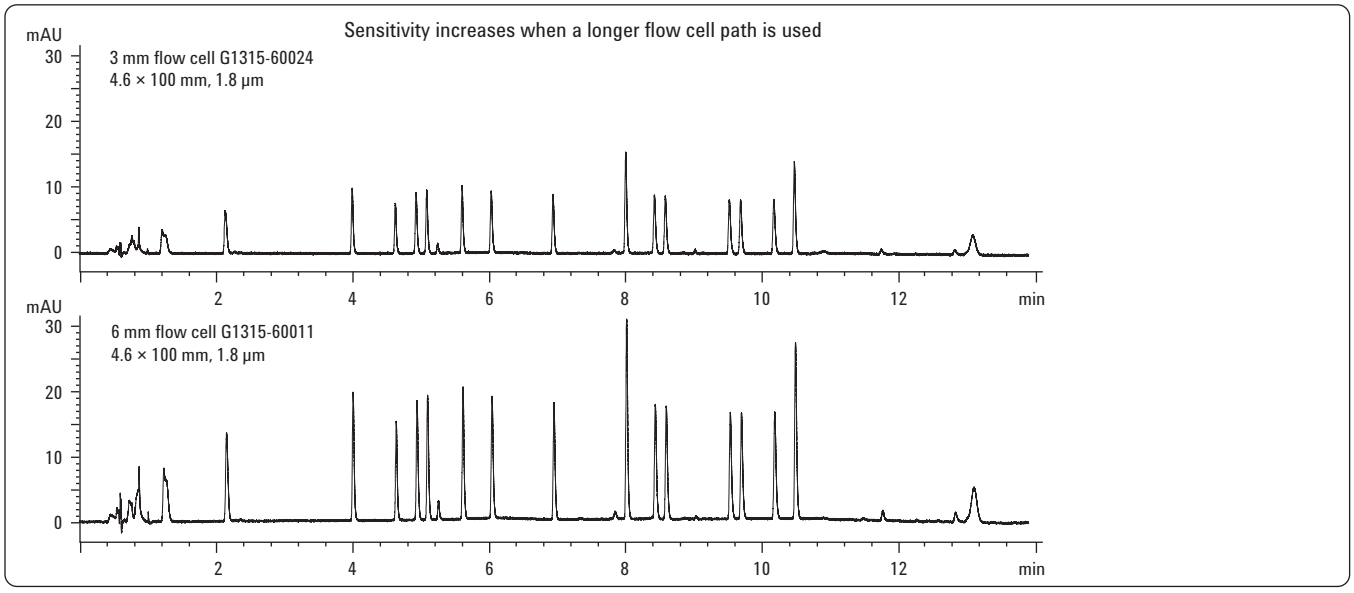


Figure 13. RRHT amino acid analysis using 100 mm Agilent ZORBAX Eclipse Plus C18 columns and different flow cells.

Conclusion

An automated online derivatization method for amino acids was enhanced using Agilent ZORBAX Eclipse Plus C18 columns in ten column dimensions including length, column id, and three particle sizes. The variety of column choices offers the analyst high resolution, high speed, reduced solvent consumption, or a combination that bests suits his needs.

The 2.1 × 150 mm, 3.5 µm column produces a chromatogram most similar to the AminoQuant method. Another benefit of this protocol over previous iterations is the flexibility to transfer the method between one type of LC system to another, such as a quaternary pump LC to a binary pump LC, or a 400-bar LC (Agilent 1100) to a 600-bar LC (Agilent 1200 SL). This protocol also gives better retention of the first two eluting amino acids, aspartic and glutamic acid. Method ruggedness was demonstrated by longevity, batch-to-batch and linearity testing, and ability for customization. The ZORBAX Eclipse Plus C18 columns coupled with reliable Agilent LC instrumentation and proven automated online derivatization advanced the analysis of free amino acids.

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Table 7. Ordering Information

Derivatization Reagents Description	Agilent Part No.
Borate Buffer: 0.4 M in water, pH 10.2, 100 mL	5061-3339
FMOC Reagent, 2.5 mg/mL in ACN, 10 × 1 mL ampoules	5061-3337
OPA Reagent, 10mg/mL in 0.4M borate buffer and 3-mercaptoproprionic acid, 6 × 1mL ampoules	5061-3335
DTDPA Reagent for analysis of cysteine, 5 g	5062-2479

Mobile Phase and Injection Diluent Components Description	Manufacturer	Mfgr.'s Part No.
Na_2HPO_4, Sodium Phosphate, Dibasic	Sigma	S 7907
$Na_2B4O_7{\cdot}10H_2O$, Sodium Tetrborate Decahydrate	Sigma	S 9640
NaN_3, Sodium Azide	Sigma	S 2002
H_3PO_4, ortho Phosphoric Acid	Sigma	79617

Vials Description	Agilent Part No.
100 µL Conical insert with polymer feet, 100/pk	5181-1270
Amber, wide-opening, write-on, screw-top vial, 2 mL,100/pk	5182-0716
Blue polypropylene cap, PTFE/silicone septum, 100/pk	5182-0721
Clear glass screw cap vial, 6 mL,16 mm cap size, 100/pk	9301-1377
Screw caps, 16 mm, 100/pk	9301-1379
PTFE/silicone septa, 16 mm, 100/pk	9301-1378

Standards Description	Agilent Part No.
Amino Acid Standards in 0.1 M HCl, 10 × 1mL ampoules	
1 nmol / µL	5061-3330
250 pmol / µL	5061-3331
100 pmol / µL	5061-3332
25 pmol / µl	5061-3333
10 pmol / µL	5061-3334

Supplemental Amino Acids:	
Nva, Sar, Asn, Gln, Trp, Hyp, 1 g each	5062-2478

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