Automated Amino Acid Analysis Using an Agilent Poroshell HPH-C18 Column

Application Note

Food Testing, Agriculture, Small Molecule Pharmaceuticals

Author

William Long
Agilent Technologies, Inc.

Abstract

In this application note, an automated precolumn OPA/FMOC amino acid method, previously developed on 3.5 and 1.8 μm Agilent ZORBAX Eclipse Plus C18 columns, is expanded to include 2.7 μm Agilent Poroshell HPH-C18 superficially porous columns. This column exhibits good lifetime and transferability to different column dimensions, both of which are shown in this work. Applications of the column to fermentation products are also shown.

Agilent Technologies

Introduction

Superficially porous particle (SPP) technology is based on particles with a solid core and a superficially porous shell. These particles consist of a 1.7 µm solid core with a 0.5 µm porous shell. In total, the particle size is about 2.7 µm. The 2.7 µm superficially porous particles provide 40 to 50% lower backpressure and 80 to 90% of the efficiency of sub-2 µm totally porous particles. The superficially porous particles have a narrower particle size distribution than totally porous particles. This results in a more homogeneous column bed, and reduces dispersion in the column. At the same time, the thin porous shell provides lower resistance to mass transfer. The result is minimal loss of efficiency at higher flow rates [1]. Additionally, since the columns incorporate a 2 µm frit, they are as resistant to clogging as 3.5 and 5 µm columns. Until recently, all silica-based SPP materials possessed limited lifetime in higher pH buffers, including phosphate buffers. To achieve these longer lifetimes, it is necessary to protect the base particle by either surface modification or special bonding modification. The surface of Agilent Poroshell HPH-C18 particles are chemically modified to form an organic layer, resistant to silica dissolution at high pH conditions, using a proprietary process.

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 method developed on HP 1090 Series HPLC systems, and later updated for the Agilent 1100 Series, has now evolved further taking advantage of the Agilent 1260 Infinity Binary LC and superficially porous Agilent Poroshell HPH-C18 columns [2-8].

Experimental

Preparation of HPLC mobile phase

Mobile phase A contained 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and 5 mM NaN_3. For 1 L, weigh 1.4 g anhydrous Na_2HPO_4 plus 3.8 g $Na_2B_4O_7 \cdot 10H_2O$ in 1 L water plus 32 mg NaN_3. Adjust to about pH 8.4 with 1.2 mL concentrated HCl, then add small drops of acid to pH 8.2. Allow stirring time for complete dissolution of borate crystals before adjusting pH. Filter through 0.45 µm regenerated cellulose membranes (p/n 3150-0576). Mobile phase B contains acetonitrile:methanol:water (45:45:10, v:v:v). All mobile-phase solvents were 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.

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. Some precautions include:

- OPA is shipped in ampoules under inert gas to prevent oxidation. Once opened, the OPA is potent for about 7 to 10 days. We recommend transferring 100 µL aliquots of OPA to microvial inserts. Label with name and date, cap, and refrigerate. Replace the OPA autosampler microvial daily. Each ampoule lasts 10 days.

- FMOC is stable in dry air but deteriorates in moisture. It should also be transferred in 100 µL aliquots to microvial inserts. Label with name and date, cap tightly, and refrigerate. Like the OPA, an open FMOC ampoule transferred to 10 microvial inserts should last 10 days (one vial/day).

- Borate buffer can be transferred to a 1.5-mL autosampler vial without a vial insert. Replace every three days.

Preparation of amino acid standards

Solutions of 17 amino acids in five concentrations are available from Agilent (10 pmol/µL to 1 nmol/µL) 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.

To make the 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 the 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.

For primary amino acid ISTD stock solutions, weigh 58.58 mg norvaline into a 50 mL volumetric flask. For secondary amino acids, weigh 44.54 mg sarcosine into the same 50 mL flask. Fill half way 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 are 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.

Pump parameters

Pump parameters for all methods include compressibility ($\times 10^{-6}$ bar) A: 35, B: 80, with minimal stroke A, B of 20 µL.

Online derivatization

Depending on the autosampler model, the automated online derivatization program differs slightly. For the Agilent G1376C well plate automatic liquid sampler (WPALS), with injection program:

1. Draw 2.5 µL from borate vial (p/n 5061-3339).

2. Draw 1.0 µL from sample vial.

3. Mix 3.5 µL in wash port five times.

4. Wait 0.2 minutes.

5. Draw 0.5 µL from OPA vial (p/n 5061-3335).

6. Mix 4.0 µL in wash port 10 times default speed.

7. Draw 0.4 µL from FMOC vial (p/n 5061-3337).

8. Mix 4.4 µL in wash port 10 times default speed.

9. Draw 32 µL from injection diluent vial.

10. Mix 20 µL in wash port eight times.

11. Inject.

12. Wait 0.1 minutes.

13. Valve bypass

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

- Vial 1: Borate buffer

- Vial 2: OPA

- Vial 3: FMOC

- Vial 4: Injection diluent

- P1-A-1: Sample

Thermostatted column compartment (TCC)

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

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.

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.

Results and Discussion

As can be seen in Figure 1, using the same chromatographic conditions, the separation was very similar. The elution order of the mixture on both columns was the same, and as shown in Figure 2, the relationship of retention times of the amino acid samples was highly correlated between an Eclipse Plus C18 and a Poroshell HPH-C18, with a correlation co-efficient of 0.997. As can be seen in the chromatograms, the retention times were slightly less on the Poroshell HPH-C18 column. Some chromatographic differences are notable. Thus, separation of leucine and lysine looks better on Poroshell HPH-C18, while the separation between lysine and hydroxyproline and the sarcosine/proline pair looks worse. As suggested in previous application notes, the chromatography can be altered to enhance resolution of desired peak pairs.
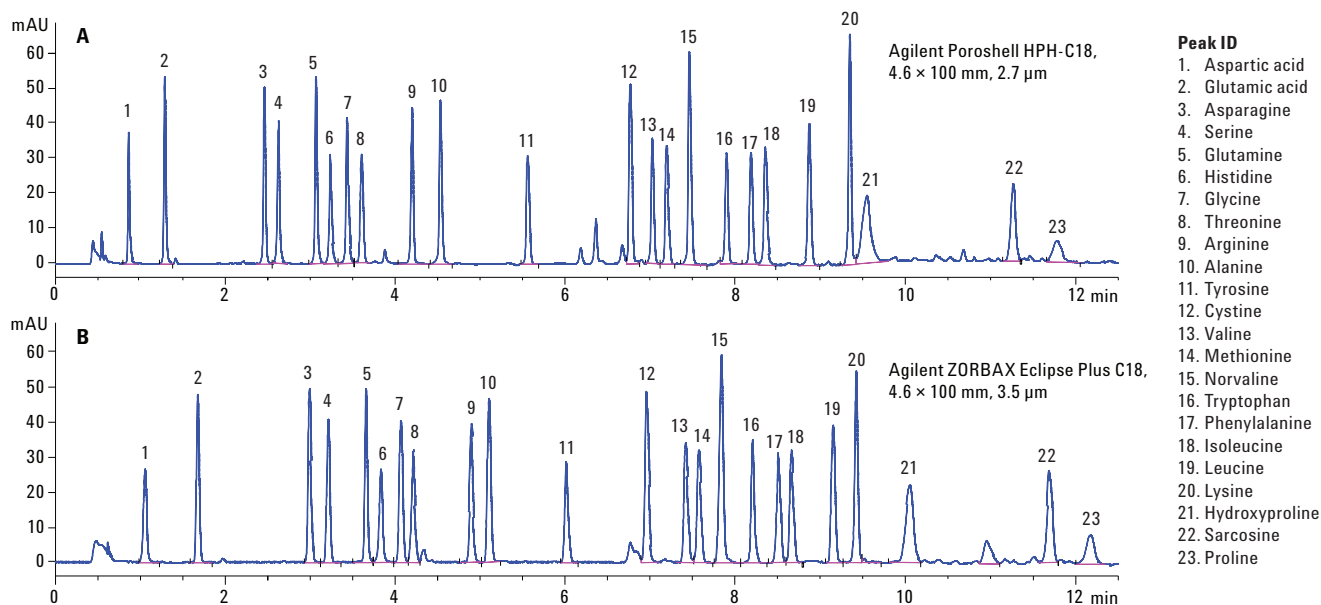

Peak ID
1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Serine
5. Glutamine
6. Histidine
7. Glycine
8. Threonine
9. Arginine
10. Alanine
11. Tyrosine
12. Cystine
13. Valine
14. Methionine
15. Norvaline
16. Tryptophan
17. Phenylalanine
18. Isoleucine
19. Leucine
20. Lysine
21. Hydroxyproline
22. Sarcosine
23. Proline

Figure 1. Comparison of an Agilent Poroshell HPH C18 to an Agilent ZORBAX Eclipse Plus C18 column using the Amino Acid Method.

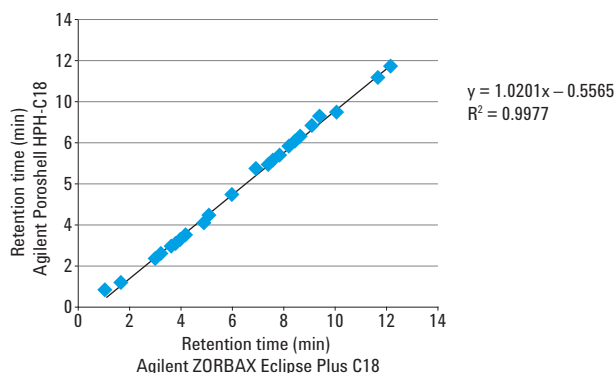$$y = 1.0201x - 0.5565$$
$$R^2 = 0.9977$$

Figure 2. Correlation of retention times using Agilent Poroshell HPH-C18 and Agilent ZORBAX Eclipse Plus C18 columns.

Table 1. Conditions for Figure 1.

Column: Agilent Poroshell HPH C18, 4.6 × 100 mm, 2.7 µm
(p/n 695975-702) or
Agilent Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm
(p/n 959961-902)

Flow rate: 1.5 mL/min

Gradient:	Time (min)	%B
	0	2
	0.35	2
	13.4	57
	13.5	100
	15.7	100
	15.8	2
	18	end

Column dimensions

The method can easily be scaled to different column dimensions. In this work, three column dimensions were studied. All columns were 100 mm in length with 4.6, 3.0, or 2.1 mm internal diameter, as shown in Figure 3. In this case, the only changes to the method were made by altering the flow rate. Table 1 lists the gradient program used throughout. Flow rates are changed geometrically with the diameter of the column. The flow rate used with the 4.6 × 100 mm column was 1.5 mL/minute. The flow rates for the 3 and 2.1 mm columns were 0.62 and 0.21 mL/min, respectively. In all cases, the low-volume heat exchanger was used with short red tubing to minimize extra column volume. Using the Agilent 1260 Infinity Binary LC with low dispersion heating and tubing, the column pressure was approximately 175 bar.

We observed that retention time of all analytes increased slightly (without changing selectivity) as columns were changed from larger to smaller internal diameter. This is due to the increase in gradient delay time. As the flow rates are scaled and consequently reduced from larger to smaller column ids, the gradient delay volume remains constant, thereby increasing the time it takes for the gradient to reach the column. The difference in retention between various column ids could potentially be reduced or eliminated by scaling the gradient delay volume on the LC system (adding or removing capillary length/diameter/volume between the pump and column inlet).
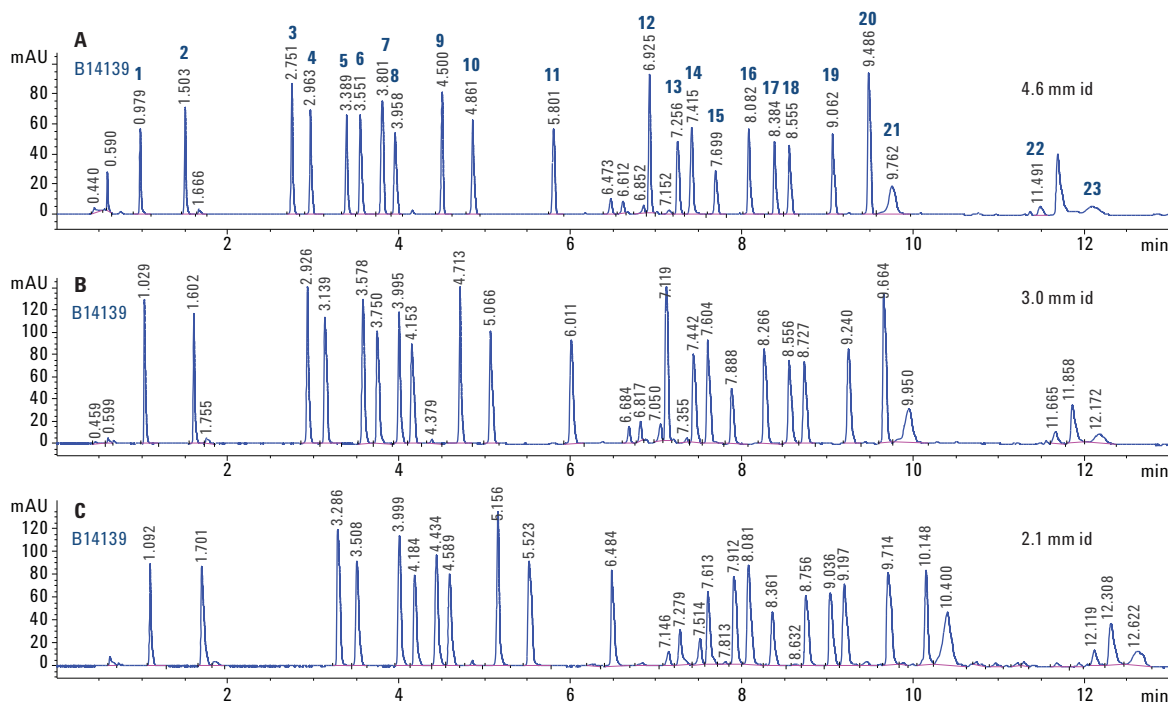

Figure 3. Agilent Poroshell HPH-C18 100 mm columns of different inside dimension using the amino acid method.

Lot-to-lot variability

Batch-to-batch or lot-to lot reproducibility is also an import factor in method development. It is recommended that, before a method is adopted, one of the earliest validation steps is to examine the method performance on at least three columns made from different lots.

Following good validation practice, three columns loaded with particles from different production batches were examined for 4.6, 3.0, and 2.1 × 100 mm columns. The overlays of these three sets are shown in Figures 4A-C. As can be seen in Figure 4A, the amino acid separation on the 4.6 × 100 mm column achieved good peak as well as baseline separation shape for all compounds. No change in elution order was noted, and lot-to-lot reproducibility looked good. A slight change in retention time can be seen in Figure 4A though the k' remained constant. However, a slight change in the wavelength switch time is required as it is tied to the elution times of lucine and hydroxyproline. Similar reproducibility is evident in Figures 4B and 4C for the smaller id columns.
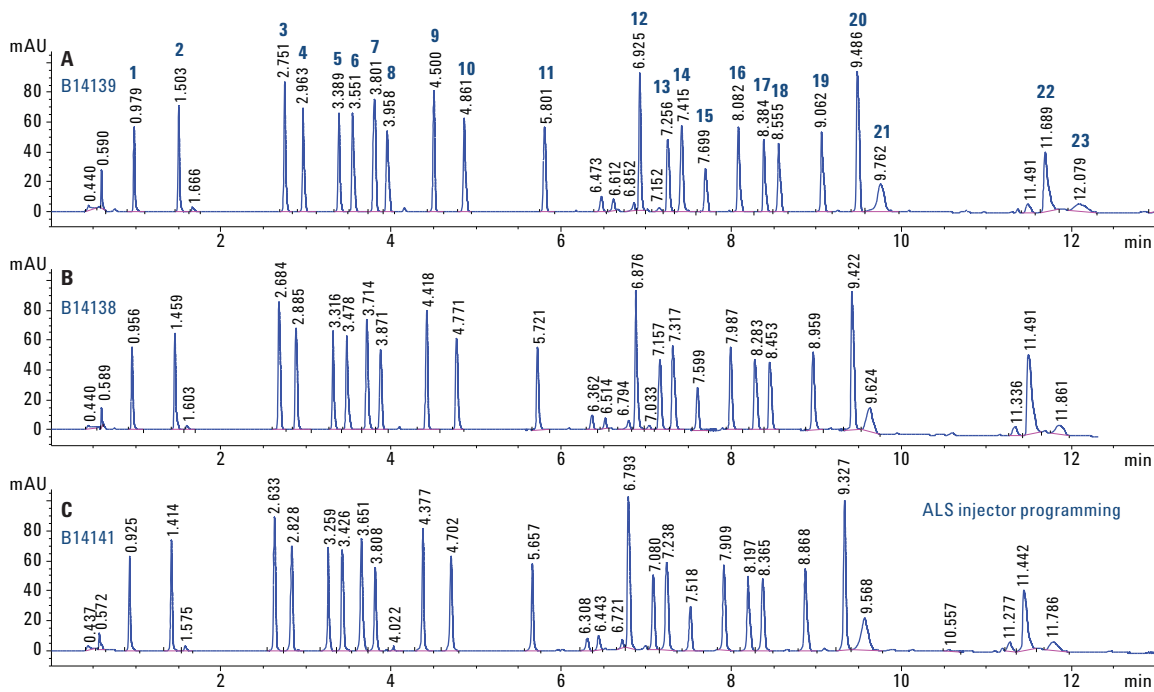

Figure 4A. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-702).
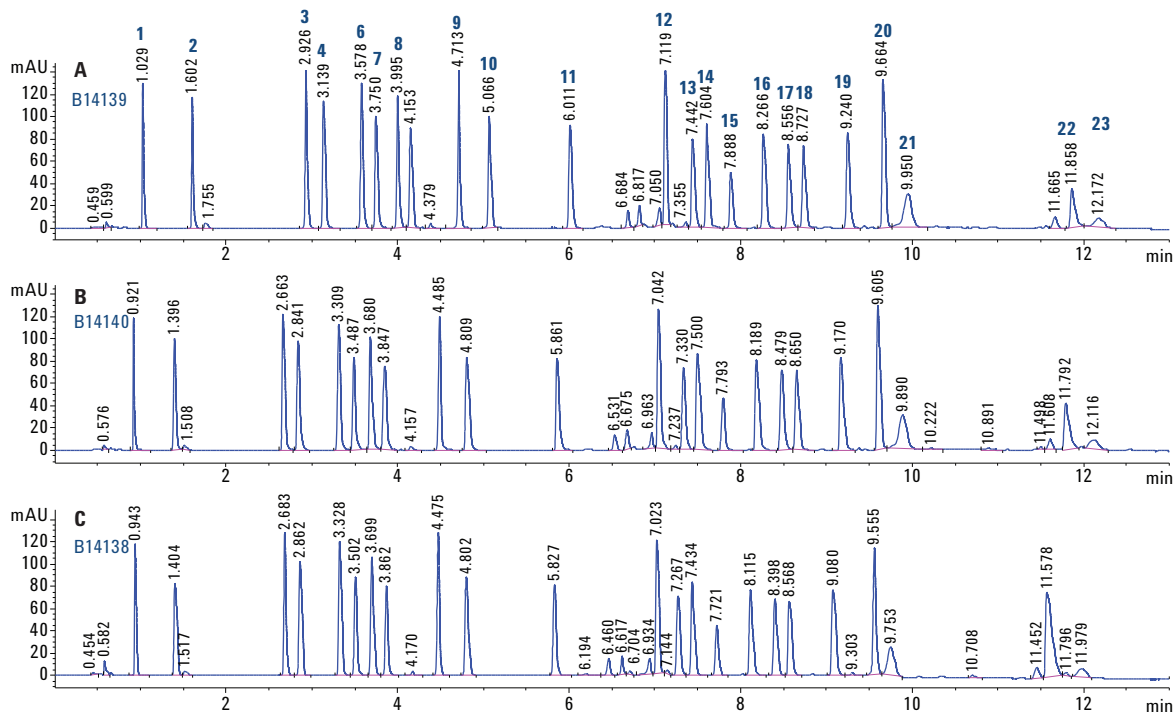
Figure 4B. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 3 × 100 mm, 2.7 µm (p/n 695975-502).
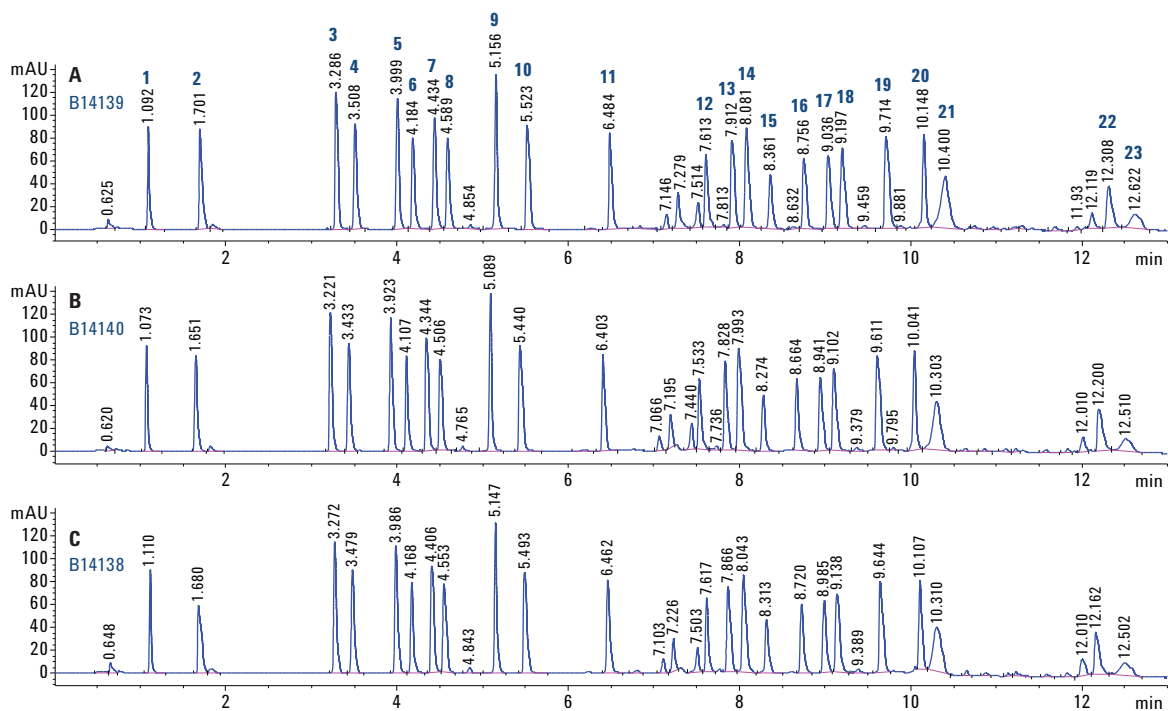

Figure 4C. Separation of amino acid and internal standards on three lots of Agilent Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-702).

Lifetime

Column lifetime is an important consideration for chromatographers analyzing amino acid samples. Most silica columns lose efficiency after prolonged exposure to these conditions. Kirkland *et al.* [9] and Tindall and Perry [10] discussed possible reasons for the reduced lifetime of silica columns in phosphate buffer, but both agree that columns do not last as long.

There are two approaches to achieving high pH stability in silica HPLC columns. One way is to employ special bonding chemistry, as in the Agilent ZORBAX Extend C18 column. This column uses bidentate bonding to protect the silica from dissolution at high pH. Another way to achieve high pH stability is to modify the silica itself, making it less soluble. The surface of Poroshell HPH particles are chemically modified to form an organic layer, resistant to silica dissolution at high pH conditions, using a proprietary process [11].

Figure 5 is an overlay of four chromatograms. Single 4 L bottles of mobile phase A and B were prepared. A single 2.1 × 100 mm column was used for lifetime testing from a series of 500 analyses over a period of four weeks. In this series, approximately 102 injections were made each week using freshly opened amino acid standard mix and reagents. At the end of the sequence, the column was flushed with 100% B mobile phase for 40 minutes and the instrument was shut down. In this manner, the method was run for 3.5 days and the column was stored with no analysis for 3.5 days. This simulated typical practice in a lab where samples are run for an extended time, and then a column is washed and stored. Storing a column in 100% mobile phase B was recommended in the original amino quant methods, and is common practice in many successful laboratories that frequently run amino acids. A realistic lifetime study was carried out, showing excellent lifetime of the column over one month of use, with over 500 standard injections, shutting down and storing after each sequence. As can be seen in Figure 5, the 17 amino acid sample lost no resolution and only a slight retention time shift was seen.
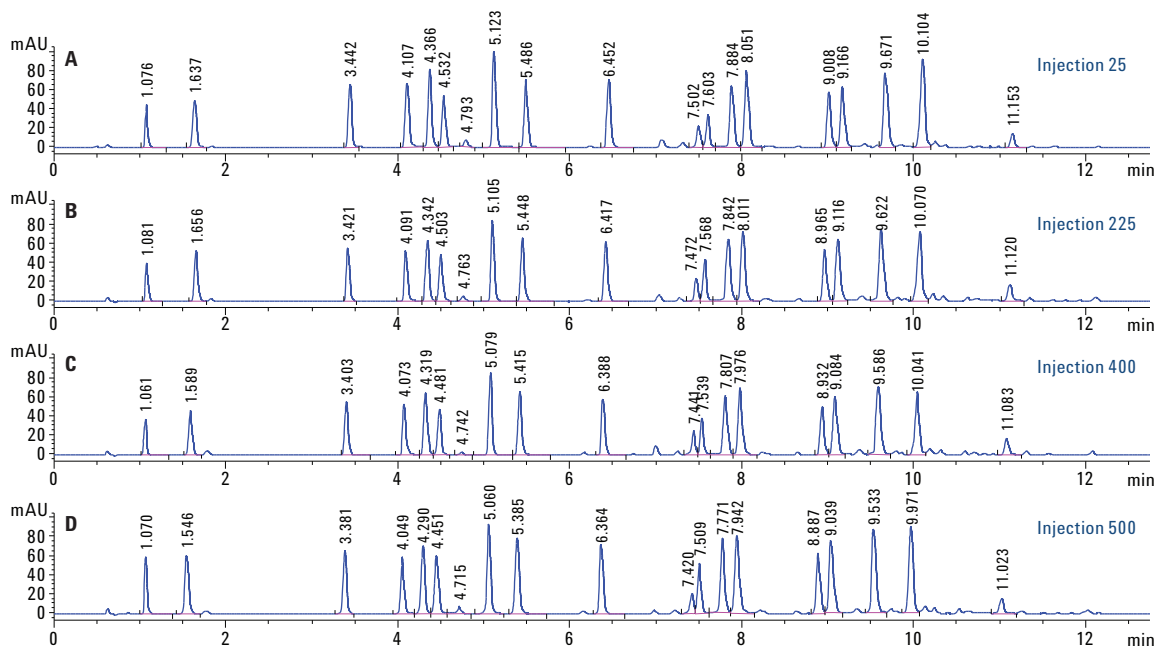

Figure 5. Column lifetime test using an Agilent Poroshell HPH-C18, 2.1 × 100 mm column running an amino acid method.

Conclusions

Agilent Poroshell HPH-C18 has selectivity similar to totally porous Agilent ZORBAX Eclipse Plus C18. This allows easy transfer of existing methods such as the amino acid method. In this work, no changes to the chromatographic conditions were made although changes in the gradient could be done to improve resolution on selected amino acids. In most cases, Poroshell HPH-C18 was slightly less retentive than totally porous Eclipse Plus C18. The method was investigated with 4.6, 3.0, and 2.1 mm × 100 mm columns. Use of the low volume column heater is recommended. In total, four particle lots were investigated, requiring only slight changes to the wavelength switch time. A realistic lifetime study was carried out, showing excellent lifetime of the column over one month of use, with over 500 standard injections, shutting down and storing after each sequence.

References

1. Wang, X.; Barber, W. E.; Long, W. J. Applications of superficially porous particles: High Speed, high efficiency or both? *J. Chromatogr. A*. **2012**, *1228*, 72-88.

2. Schuster, R.; Apfel, A. A new technique for the analysis of primary and secondary amino acids; Application note, Hewlett-Packard Publication number 5954-6257, **1986**.

3. Schuster, R. Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and high-performance liquid chromatography. *J. Chromatogr. B*. **1988**, *431*, 271-284.

4. Henderson, Jr., J. W.; Ricker, R. D.; Bidlingmeyer, B. A.; Woodward, C. Rapid, Accurate, Sensitive and Reproducible HPLC Analysis of Amino Acids; Application note, Agilent Technologies, Inc. Publication number 5980-1193E, **2000**.

5. Woodward, C.; Henderson, Jr., J. W.; Todd Wielgos, T. High-Speed Amino Acid Analysis (AAA) on Sub-Two Micron Reversed-phase (RP) Columns; Application note, Agilent Technologies, Inc. Publication number 5989-6297EN, **2007**.

6. Gratzfeld-Huesgen, A. Sensitive and Reliable Amino Acid Analysis in Protein Hydrolysates using the Agilent 1100 Series HPLC; Application note, Agilent Technologies, Inc. Publication number 5968-5658EN, **1999**.

7. Greene, J.; Henderson, Jr., J. W.; Wikswo, J. P. Rapid and Precise Determination of Cellular Amino Acid Flux Rates Using HPLC with Automated Derivatization with Absorbance Detection; Application note, Agilent Technologies, Inc. Publication number 5990-3283EN, **2009**.

8. Henderson, Jr., J. W.; Brooks, A. Improved Amino Acid Methods using Agilent ZORBAX Eclipse Plus C18 Columns for a Variety of Agilent LC Instrumentation and Separation Goals; Application note, Agilent Technologies, Inc. Publication number 5990-4547EN, **2010**.

9. Kirkland, J. J.; van Straten, M. A.; Claessens, H. A. Reversed-phase high-performance liquid chromatography of basic compounds at pH 11 with silica-based column packings. *J. Chromatogr. A*. **1998**, *797*, 111-120.

10. Tindall, G. W.; Perry, R. L. Explanation for the enhanced dissolution of silica column packing in high pH phosphate and carbonate buffers. *J. Chromatogr. A*. **2003**, *988*, 309-312.

11. Anon. Extending Column Lifetime in Pharmaceutical Methods with High pH-Stable Poroshell HPH Chemistries; Technical overview, Agilent Technologies, Inc. Publication number 5991-5022EN, **2014**.

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Agilent Technologies