Improved OPA /FMOC Derivatized Amino Acid Methods using Many Column Configurations for a Range of Speed and Resolution Options

Henderson J.W. Jr., Brooks, A., Joseph, M.
Agilent Technologies 2850 Centerville Rd. Wilmington, Del. USA 19808

FBA32-Tu
HPLC2009
Dresden, Germany

Agilent Technologies

Introduction

A well established online automated OPA/FMOC derivitization method for amino acids has been improved and updated. The updated method can be used with a variety of ZORBAX Eclipse Plus C18 column configurations including 5, 3.5, and 1.8 µm particle sizes, column lengths from 250 to 50 mm, and column inner diameters of 4.6, 3.0 and 2.1 mm in order to provide the sensitivity and flexibility for fast analyses and a variety of sample types and sizes. Scalability, lot-to-lot reproducibility, linearity, and data will be presented. Ten column options will be shown, ranging from rapid nine minute analyses of 23 amino acids including re-equilibration using short (50 mm) ZORBAX Rapid Resolution High Throughput columns (1.8 µm), to 40 minute analyses using 250 mm traditional 5 µm columns.

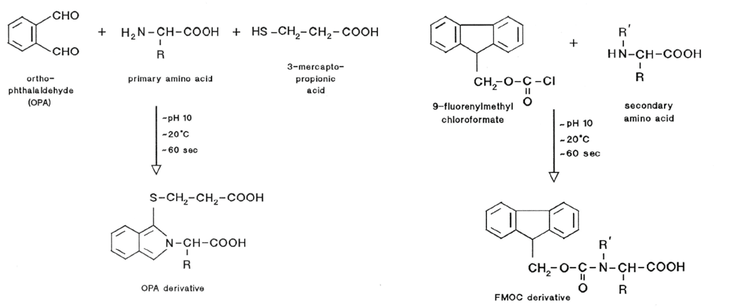
The Ten ZORBAX Eclipse Plus C18 Options

#	Method Category	Column (mm)	Analysis Time with Re-equilibration	Typical Minimum Resolution Factor	Approx. mL Solvent/ Analysis*	Agilent HPLC
1	Traditional High Resolution	4.6 x 250, 5 µm	40 min	2.4	64	1200 or 1200 SL
2		3.0 x 250, 5 µm	40 min	2.4	28	
3	Rapid Resolution	4.6 x 150, 3.5 µm	25 min	2	42	1200 or 1200 SL
4		3.0 x 150, 3.5 µm	25 min	2	18	
5		2.1 x 150, 3.5 µm	25 min	2	12	
6	Rapid Resolution High Throughput	4.6 x 100, 1.8 µm	16 min	2.4	28	1200SL
7		2.1 x 100, 1.8 µm	16 min	2.4	8	
8		4.6 x 50, 1.8 µm	9 min	1.5	23	
9		3.0 x 50, 1.8 µm	9 min	1.5	10	
10		2.1 x 50, 1.8 µm	9 min	1.5	5	

* includes injector program and pre run DAD autobalancing (2.42 min), and re-equilibration time.

The Online Pre-Column Derivatizations

The primary amino groups react with ortho-phthalaldehyde (OPA) in the presence of 3-mercaptopropionic acid (3-MPA) at about pH 10 to form an isoindole derivative. Secondary amino groups do not react. The OPA derivatized amino acid is then detected by UV at 338 nm.



The secondary amino groups react with 9-fluorenylmethyl chloroformate (FMOC) at about pH 10 to form a secondary amide. Secondary amino groups do not react. The FMOC derivatized amino acid is then detected by UV at 262 nm.

Method Parameters

Amino Acid Identification and Detection

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

Primary Amino Acids 1-20 are detected at UV wavelength 338nm (DAD1, Sig=338,10 Ref=390,20 TT)

Secondary Amino Acids 21-23 are detected at UV wavelength 262nm (DAD1, Sig=262,4 Ref=390,20)

A programmed signal wavelength change from 338 nm to 262 nm is determined by choosing a switching time after lysine elutes and before hydroxyproline elutes

The Automated Derivatization

G1376C well plate automatic liquid sampler (WPALS):

1) Draw 2.5 µL from Borate vial (Agilent PN 5061-3339)
2) Draw 1.0 µL from Sample vial
3) Mix 3.5 µL in washport 5X
4) Wait 0.2 min
5) Draw 0.5 µL from OPA vial (Agilent PN 5061-3335)
6) Mix 4.0 µL in washport 10X max speed
7) Draw 0.4 µL from FMOC vial (Agilent PN 5061-3337)
8) Mix 4.4 µL in washport 10X max speed
9) Draw 32 µL from Injection Diluent vial
10) Mix 20 µL in washport 8X
11) Inject
12) Wait 0.1 min
13) Valve bypass

G1329A automatic liquid sampler (ALS):

1) Draw 2.5 µL from Borate vial
2) Draw 1.0 µL from Sample vial
3) Mix 3.5 µL in air, max speed 5X
4) Wait 0.2 min
5) Draw 0.5 µL from OPA vial
6) Mix 4.0 µL in air, max speed 10X max speed
7) Draw 0.4 µL from FMOC vial
8) Mix 4.4 µL in air, max speed, 10X max speed
9) Draw 32 µL from Injection Diluent vial
10) Mix 20 µL in air, max speed 8X
11) Inject
12) Wait 0.1 min
13) Valve bypass

The Agilent LC Flow Paths

The flow path is recorded carefully because it can have a significant effect on resolution, especially for gradients (delay volume) and 2.1 i.d. columns (extra column volume).

	Traditional (5 µm) High Resolution Methods		Rapid Resolution (3.5 µm) Methods		
Method name	**4.6 x 250, 5 µm**	**3.0x 250, 5 µm**	**4.6x 150, 3.5 µm**	**3.0x 150, 3.5 µm**	**2.1x 150, 3.5 µm**
LC Model	1100	1200	1200 SL	1200 SL	1200 SL
Pump	G1312A	G1311A quat	G1312B	G1312B	G1312B
Dampener/static mixer	yes	n/a	yes	yes	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
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
	RRHT 1.8 µm Methods (100 mm)		**RRHT 1.8 µm Methods (50 mm)**		
Method name	**4.6 x 100, 1.8 µm**	**2.1 x 100, 1.8 µm**	**4.6 x 50, 1.8 µm**	**3.0 x 50, 1.8 µm**	**2.1 x 50, 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/static mixer	yes	bypassed	yes	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)

The Linear Gradients

The gradient profile (%B) is identical for all columns. The different gradient delay times are mitigated by reducing delay volume and the isocratic hold in the beginning of the gradient program.

Traditional high resolution method gradients, 5 µm

	4.6 x 250, 5 µm PN 959990-902	3.0 x 250, 5 µm PN 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 Resolution method gradients, 3.5 µm

	4.6 x 150, 3.5 µm PN959963-902	3.0 x 150, 3.5 µm PN959963-302	2.1x 150, 3.5 µm PN959763-902
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 x 100, 1.8 µm PN959964-902	2.1 x 100, 1.8 µm PN959764-902
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

	4.6x50, 1.8 µm PN959941-902	3.0x50, 1.8 µm PN959941-302	2.1x50, 1.8 µm PN959741-902
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

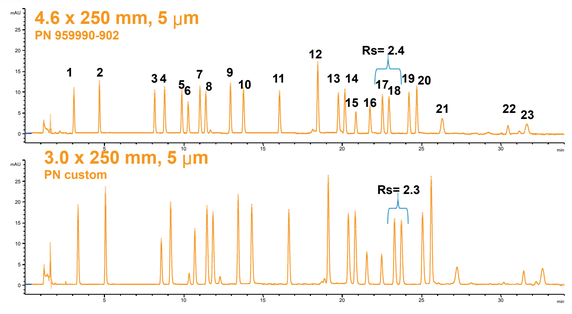
The Mobile and Stationary Phase

Stationary Phase: ZORBAX Eclipse Plus C18
Column Temperature: 40 °C
Mobile Phase A: 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 5 mM NaN_3
Mobile Phase B: Acetonitrile: Methanol: Water (45:45:10, v: v: v)
Injection Diluent: (0.25 mL H_3PO_4 + 100 mL H_2O)

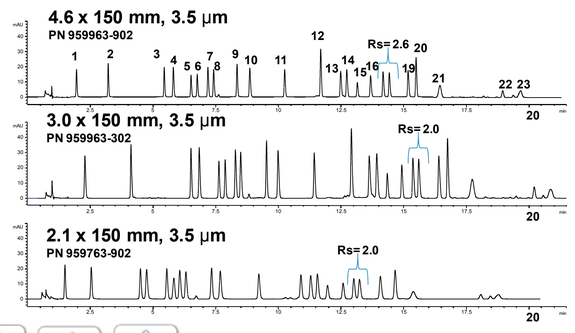
Results

Assuring Reproducibility | Improved Peak Shape | Improved Resolution | Saving Time/money

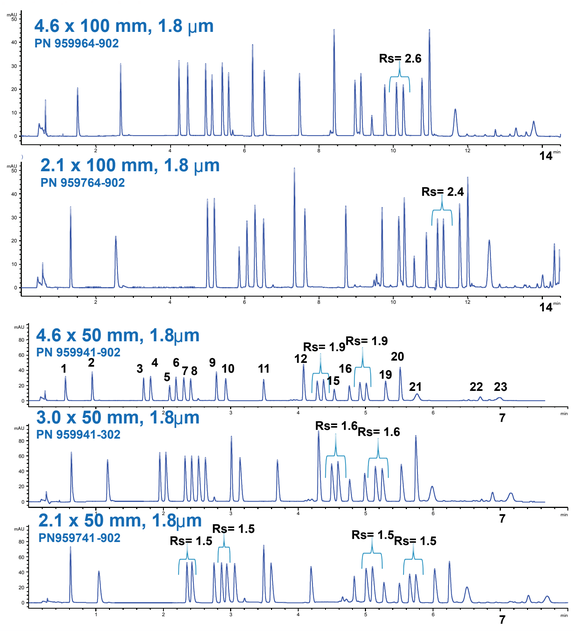
The Eclipse Plus C18 5 µm Options



Rapid Resolution 3.5 µm Options



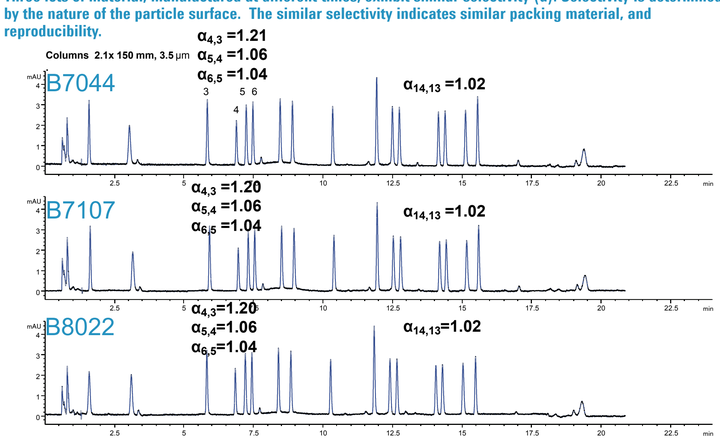
RRHT 1.8 µm Options



Method Ruggedness

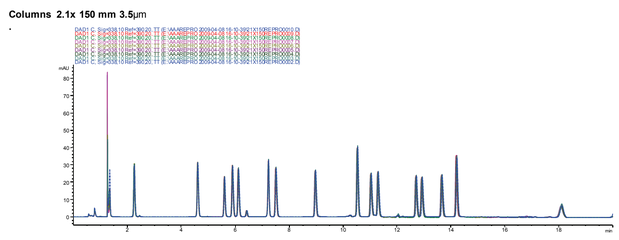
Lot-to-Lot Reproducibility

Three lots of material, manufactured at different times, exhibit similar selectivity (α). Selectivity is determined by the nature of the particle surface. The similar selectivity indicates similar packing material, and reproducibility.



Injection-to-Injection Reproducibility

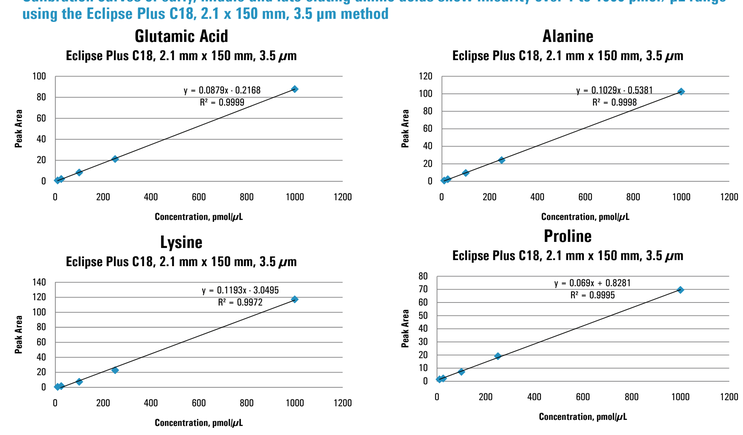
Overlay of eight sequential injections showing reproducibility of the online derivatization and gradient programs. Peak area of early, middle and late eluting amino acids are statistically tabulated below. The other amino acids had similar statistics.



amino acid	Peak Area 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**
cy2	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**

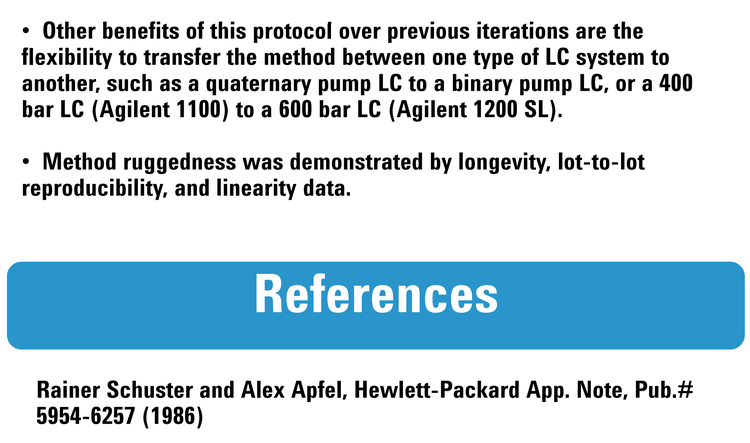
Linearity

Calibration curves of early, middle and late eluting amino acids show linearity over 1 to 1000 pmol/µL range using the Eclipse Plus C18, 2.1 x 150 mm, 3.5 µm method



Lifetime

Overlay of early middle and late chromatograms of a 500 injection sequence



Conclusions

- **An automated online derivitization method for amino acids was updated using ZORBAX Eclipse Plus C18 columns in ten column dimensions, with varied length, column ID, and three particles sizes.**
- **The variety of Eclipse Plus C18 column choices offers the analyst high resolution, high speed, reduced solvent consumption, in a combination that bests suits one's needs.**
- **Other benefits of this protocol over previous iterations are 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).**
- **Method ruggedness was demonstrated by longevity, lot-to-lot reproducibility, and linearity data.**

References

Rainer Schuster and Alex Apfel, Hewlett-Packard App. Note, Pub.# 5954-6257 (1986)

Rainer Schuster, J. Chromatogr., 431, 271-284 (1988)
Herbert Godel, Petra Seitz, and Martin Verhoef, LC-GC International, 5(2), 44-49 (1992)

Angelika Gratzfeld-Huesgen, "Sensitive and Reliable Amino Acid Analysis in Protein Hydrolysates using the Agilent 1100 Series HPLC" Agilent Pub.# 5968-5658EN (1999)

John W. Henderson Jr, Robert D. Ricker, Brian A. Bidlingmeyer, and Cliff Woodward, "Rapid, Accurate, Sensitive and Reproducible HPLC Analysis of Amino Acids" Agilent Pub.# 5980-1193E (2000)

Cliff Woodward, John W Henderson Jr. and Todd Wielgos, "High-Speed Amino Acid Analysis (AAA) on Sub-Two Micron Reversed-phase (RP) Columns" Agilent Pub.# 5989-6297EN (2007)

To download a PDF of this poster go to:

www.zorbaxmethod.com/productivityposter