Rapid, Accurate, Sensitive, and Reproducible HPLC Analysis of Amino Acids

Amino Acid Analysis Using Zorbax Eclipse-AAA Columns and the Agilent 1100 HPLC

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An ideal, quantitative amino acid analysis combines speed and sensitivity with reliability of both the derivatization reaction and the analytical technique. These goals are achieved with automated, online derivatization using o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids; the automated derivatization is then integrated with rugged HPLC analysis. The complete procedure is rapid, accurate, sensitive, and reproducible using the Agilent 1100 HPLC.

Combining OPA and FMOC chemistries enables fast pre-column derivatization of amino acids (AA) for chromatographic analysis. The reaction mixture is buffered at a pH of 10.2, which allows direct derivatization of acid hydrolyzed protein/peptide samples. The primary AA's are reacted first with OPA using 3-mercaptopropionic acid (3-MPA). The secondary AA's do not react with the OPA; but are then derivatized using FMOC. The incorporation of 3-MPA into the indoles decreases their hydrophobicity, and as a result, the OPA-derivatives elute chromatographically before the FMOC derivatives. Excess FMOC and its degradation products elute after the last of the secondary AA's and do not interfere with the analysis.
The derivatization process is fast and is easily automated using the Agilent 1313A autosampler. Because of the advantageous reaction speeds, both derivatizations are complete at room temperature. The automated procedure provides a high degree of reproducibility. Total analysis from injection to injection can be achieved in as little as 14 min (10-min analysis time) on the 75-mm column. On the 150-cm column total run time is 26 min (16-min analysis time). Both analyses provide high sample throughput.

SEPARATION OPTIONS

The Zorbax Eclipse-AAA column contains batch-qualified reversed-phase material. When used according to the protocol described in this *Technical Note*, the column enables the user to separate the amino acids commonly found in protein/peptide hydrolysates.

The A and B mobile-phase components are easy to prepare, and the gradient consists of linear segments (refer to *Experimental Conditions*, *Mobile Phase* section, for details). This combination contributes to a rugged protocol that can be accomplished on the Agilent 1100 HPLC using either binary or quaternary solvent-delivery systems.
The chromatogram in Figure 1 illustrates typical routine sensitivity in high-throughput applications that can be obtained on the Agilent 1100 HPLC binary system using the 1100 Diode Array Detector (DAD). A single run can be completed in 14 minutes (including re-equilibration) with adequate resolution. Separation conditions are listed in

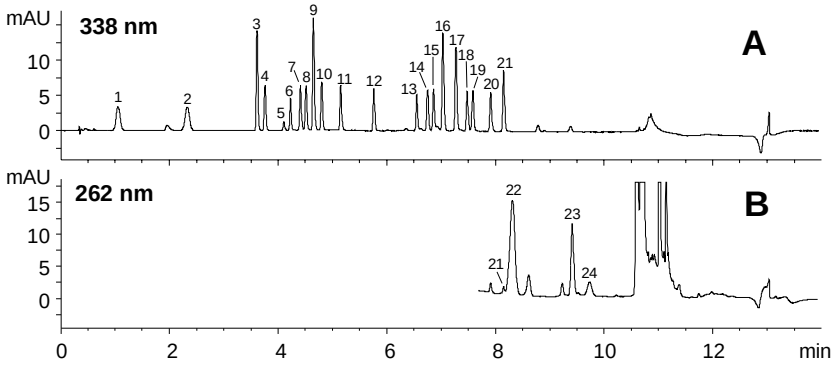

Figure 1: Routine Analysis, High-Throughput Separation of 24 Amino Acids Using the Eclipse-AAA Protocol. The column dimensions are 4.6 x 75 mm, 3.5 µm. See Table 1 for peak identification. Detection: A. 338 nm (OPA amino acids), B. 262 nm (FMOC-amino acids).

the *Experimental Conditions* section. The primary amino acids (OPA-derivatized) shown in Figure 1A, are monitored at 338 nm while the secondary amino acids (FMOC-derivatized) shown in Figure 1B, are monitored at 262 nm. The amount injected was 125 pmoles of each amino acid, in 0.5 µL.

Table 1. Amino Acid Elution Order Using Eclipse-AAA Protocol

Peak No.	Amino Acid	3-Letter Code
1	Aspartate	ASP
2	Glutamate	GLU
3	Asparagine	ASN
4	Serine	SER
5	Glutamine	GLN
6	Histidine	HIS
7	Glycine	GLY
8	Threonine	THR
9	Citrulline	CIT
10	Arginine	ARG
11	Alanine	ALA
12	Tyrosine	TYR
13	Cystine	CY2
14	Valine	VAL
15	Methionine	MET
16	Norvaline	NVA
17	Tryptophan	TRP
18	Phenylalanine	PHE
19	Isoleucine	ILE
20	Leucine	LEU
21	Lysine	LYS
22	Hydroxyproline	HYP
23	Sarcosine	SAR
24	Proline	PRO

When more resolution is desired than is available from the high-throughput separation on a 75-mm column (Fig. 1), a 150-mm column length should be used. Figure 2 shows a typical routine sensitivity, high-resolution separation obtained from two different Zorbax Eclipse-AAA 150-mm length columns – one with 3.5µm particles and the other with 5µm particles. The chromatograms show the 338 nm UV signal that detects the OPA-derivatized primary amino acids. These separations are quite similar in appearance, but the chromatogram for the 3.5-µm column demon-

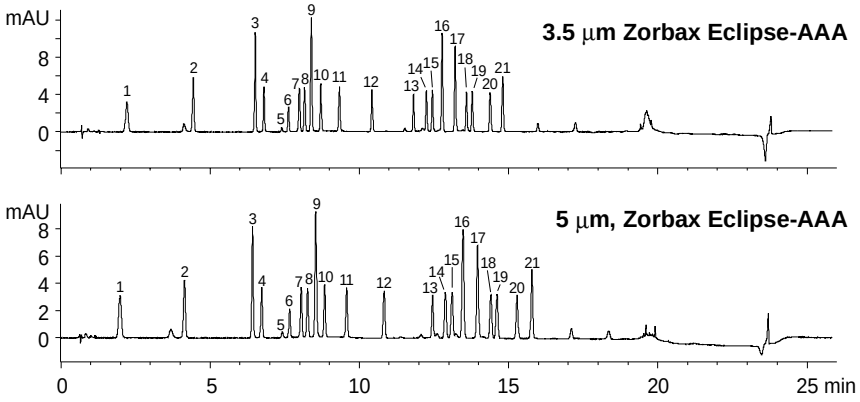

Figure 2: High-Resolution Analysis of 21 Amino Acids: on the 5µm and 3.5µm Zorbax Eclipse-AAA Column. Column dimensions are 4.6 x 150 mm. See Table 1 for peak identification. Detection: 338 nm (OPA amino acids).

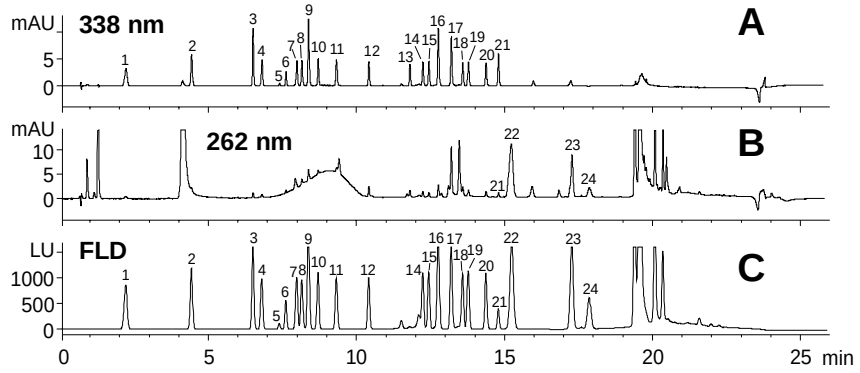

Figure 3: High Sensitivity, High-Resolution Analysis of Amino Acids Using Different Detection Modes and the Zorbax Eclipse-AAA Protocol. The column dimensions are 4.6 x 150 mm, 3.5 µm. See Table 1 for peak identification. Detection: A. UV 338 nm (OPA amino acids), B. UV 262 nm (FMOC-amino acids), C. fluorescence (see *Experimental Conditions*).

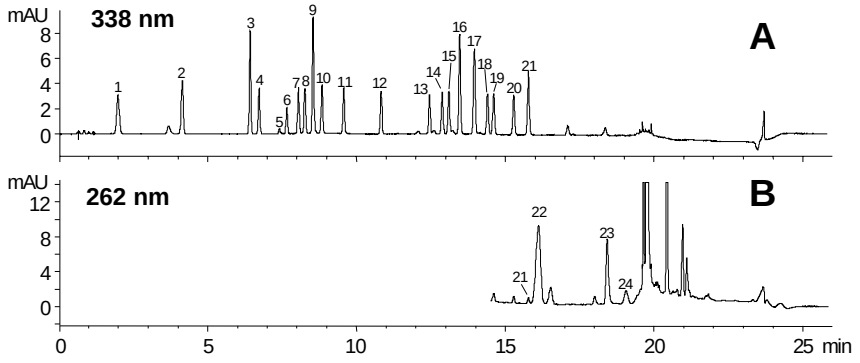

Figure 4: Routine Analysis, High Resolution of 24 Amino Acids Using the Eclipse-AAA Protocol. The column dimensions are 4.6 x 150 mm, 5 µm. See Table 1 for peak identification. Detection: A. UV 338 nm (OPA amino acids), B. UV 262 nm (FMOC-amino acids).

strates higher resolution resulting from higher efficiency. Back pressure on the column containing the smaller, 3.5 µm, particles is 240-300 bar (3530-4410 psi), while the back pressure of the column having 5 µm particles is 160-210 bar (2350-3090 psi). Thus, if only primary amino acids are of interest and the 15-cm column length is used, the larger 5-µm packing is a better choice because of less system backpressure.

The value of the 15-cm column containing 3.5-µm particles, over its 5-µm counterpart, is clearly illustrated by comparing Figures 3 and 4. Figure 3A shows separation of the primary amino acids, monitored at 338 nm; Figure 3B shows the separation monitored at 262 nm; and Figure 3C shows the analysis using fluorescence detection. Note the resolution between peaks #21 (lysine) and #22 (hydroxyproline) in Figures 3 and 4. The increase in resolution between these peaks, when using the longer column with smaller particles (150mm, 3.5 µm), provides a longer time-window that facilitates wavelength switching of the DAD or FLD between peaks #21 and #22.

When monitoring at 262 nm (Fig. 3B), a small baseline "hump" elutes between 7 and 10 minutes due to derivatization byproducts. Since only the primary AA's are monitored (338 nm) during this time, the "hump" has no impact on their detection or resolution. It is best to monitor at two wavelengths for detection of secondary amino acids such as hydroxyproline. If this is not desirable, wavelength switching can be used.

The specific time to switch fluorescence (or UV) wavelengths may differ due to minor variations in temperature, mobile phase, etc. In Figure 3C the FLD signal monitored at 450 nm (Ex = 340 nm), is programmed to change to 305 nm (Ex = 266 nm) after peak #21 (lysine) elutes but before peak #22 (hydroxyproline) elutes. In this case, the switch was programmed to occur at exactly 15 minutes. For specific details, see the *Experimental Conditions* Section, *Detection Settings*. After Peak #24 (proline) elutes, the gradient increases to 100% channel B to elute reaction byproducts from the column. After the step gradient up to 100% B for 3.7 minutes, a programmed return to the starting conditions equilibrates the column for the next injection. Also note in the fluorescence chromatogram, that Peak #13 (cystine) does not fluoresce under these conditions and is not detected.

Lysine–Hydroxyproline Separation and Wavelength Switching

Analysis of lysine and hydroxyproline has a major impact on the choice of detection parameters and column configuration, as well as the resulting runtime. Amino acids eluting prior to hydroxyproline (up to and including lysine) are derivatized with OPA and are detected at 338 nm. Hydroxyproline elutes immediately after lysine and is the first FMOC-derivatized amino acid to elute; detection must be at 262nm. The simplest solution is continuous collection of 338 nm and 262 nm data in two separate signals using the Agilent 1100 DAD or MWD (multiwavelength detector).

If the DAD and MWD are not available, the wavelength collected in a single channel can be switched under carefully chosen conditions, for detection of both OPA and FMOC-derivatized amino acids. Collection of data in a single channel may be necessary, for instance, when using the Agilent 1100 VWD (variable wavelength detector). Increased resolution between lysine and hydroxyproline is possible when using more-complex gradient-profiles. Check on the web for additional information at www.agilent.com/chem in the "Technical Support" / "User Contributed Software".

When hydroxyproline is not of interest in the sample (e.g., in analysis of protein hydrolysates), it is possible to use any of the column configurations and switch wavelength at a time between lysine elution and elution of the first FMOC-amino acid (sarcosine or proline). In this scenario, the 4.6 x 75 mm column size is adequate and has the advantage of half the analysis time.

COMPARISON WITH AMINOQUANT METHOD ON THE HP1090 HPLC

Figure 5A shows a chromatogram

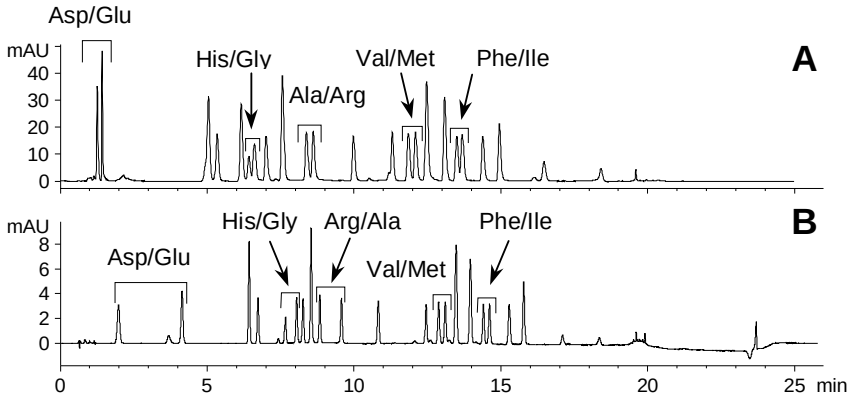

Figure 5: Comparison of Amino-Acid Analyses. A) AminoQuant Method on the HP 1090 HPLC with a Hypersil AA Column. B) Zorbax Eclipse-AAA Column (4.6 x 150 mm, 5 µm) on the Agilent 1100 HPLC.

from the original AminoQuant method (Application Notes, HP Pub. No. 5954-6257) on the HP1090 HPLC using the specified mobile phase, column and flow rate. There are five critical pairs (asp/glu, his/gly, ala/arg, val/met and phe/ile) in this separation. The asp/glu pair elutes very closely to the void volume.

Figure 5B shows a chromatogram obtained using the Zorbax Eclipse-AAA column. The resolution of all of the critical pairs is improved, especially the first pair, asp/glu, which now has increased retention and is moved significantly away from the column's void volume. Note also, that arginine elutes before alanine when using the Eclipse-AAA column, compared to the original AminoQuant method on the same HP 1090 instrument.

REPRODUCIBILITY

Table 2 shows the results from replicate injections (n=6) of the amino acid reaction mixture separated using a Zorbax Eclipse-AAA column (4.6 x 150 mm, 3.5 µm). Each run represents an individual derivatization and its chromatographic separation. Retention time reproducibility is quite good with an average % relative standard deviation (%rsd) of 0.18%. The reproducibility of the derivatization, as represented by the peak area has an average %rsd of 2.0. These data are comparable to those published for the original AminoQuant method on the HP 1090 instrument (0.23 % and 2.3 % respectively (*LC/GC International*, Volume 5, Number 2, Feb 1992, pp. 44-49).

LINEARITY AND SENSITIVITY

Linearity for the Eclipse-AAA protocol is demonstrated for the range of 4.5 pmoles to 450 pmoles, using amino acid standards (0.5µl sample). Figure 6 shows calibration curves for several amino acids using DAD or FLD detection. The correlation coefficient for all 24 amino acids is between 0.99900 and 1.00000 using either the DAD or FLD detector for calibration.

Detection of derivatized amino acids at two low levels, 5 pmoles and 50 pmoles, is shown in Figures 7 and 8 for the DAD and FLD, respectively. Using the DAD (Fig. 7), each amino acid in the standard mix can be resolved at a level of approximately 10 pmoles. The FLD (Fig. 8) shows higher sensitivity than the DAD.

Amino Acid	Retention % rsd	Peak Area % rsd
ASP	0.58	0.8
GLU	0.33	3.0
ASN	0.16	2.2
SER	0.12	2.8
GLN	0.12	2.4
HIS	0.11	2.7
GLY	0.15	2.5
THR	0.12	1.1
CIT	0.10	3.5
ARG	0.36	2.3
ALA	0.11	0.9
TYR	0.12	0.7
CY2	0.17	0.6
VAL	0.16	0.5
MET	0.17	1.1
NVA	0.15	0.7
TRP	0.18	0.8
PHE	0.14	0.8
ILE	0.14	1.1
LEU	0.18	1.0
LYS	0.19	3.2
HYP	0.13	4.2
SAR	0.14	6.8
PRO	0.12	2.7
Mean	**0.18**	**2.0**

Table 2: Reproducibility of the Zorbax Eclipse-AAA Protocol for the Analysis of Amino Acids. An Agilent 1100 System with quaternary pump was used. Values represent six replicate analyses.

CONCLUSION

Using OPA and FMOC chemistries, amino acid analysis of protein and peptide hydrolysates can be performed in ten minutes using the Zorbax Eclipse-AAA column and the Agilent 1100 HPLC. This separation approach offers improved retention of five critical pairs compared to the original AminoQuant method on the HP1090 HPLC system. The reproducibility of the analysis of amino acids on the Eclipse-AAA column is comparable to that of the original AminoQuant method, and the mobile phase is more straightforward to prepare with only a pH adjustment of the buffer (solvent A) needed.

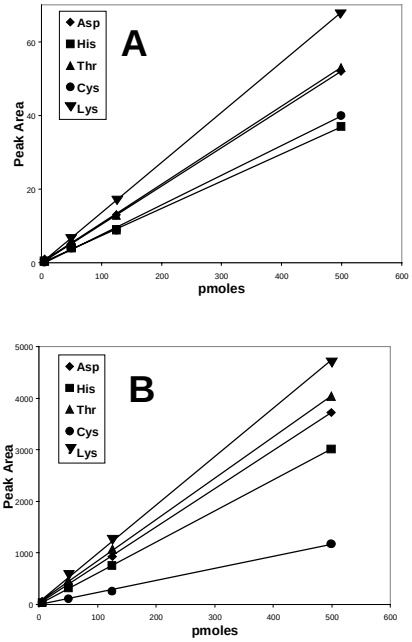

Figure 6: Calibration curves for analysis of amino acid derivatives by UV (A) or FLD (B) detection. Linearity is demonstrated for the concentration range of 4.5 to 450 pmoles in 0.5 µl of sample.

The choice of column depends upon the analysis speed and resolution desired:

ZORBAX Eclipse-AAA
4.6 x 75 mm (3.5 µm) for routine sensitivity, high-throughput work using the DAD.

ZORBAX Eclipse-AAA
4.6 x 150 mm (5 µm), for routine sensitivity, high-resolution work at lower back pressures, using the DAD

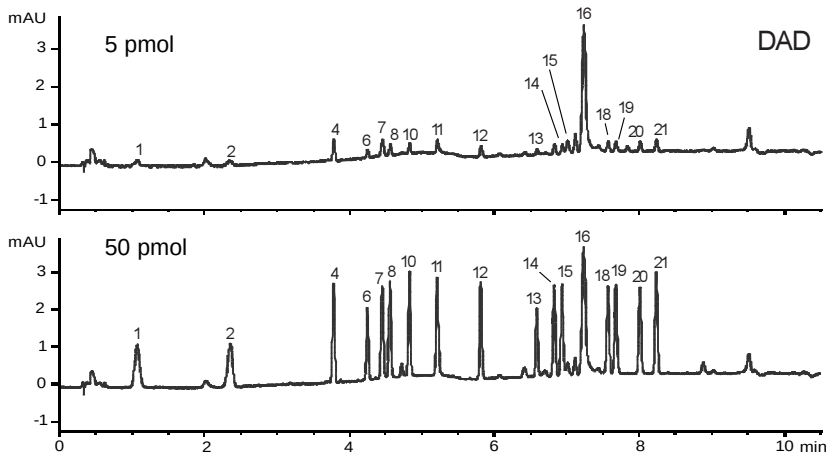

Figure 7: UV-Detector Response 338 nm (OPA-derivatives) Using Different Concentrations of Amino Acids.

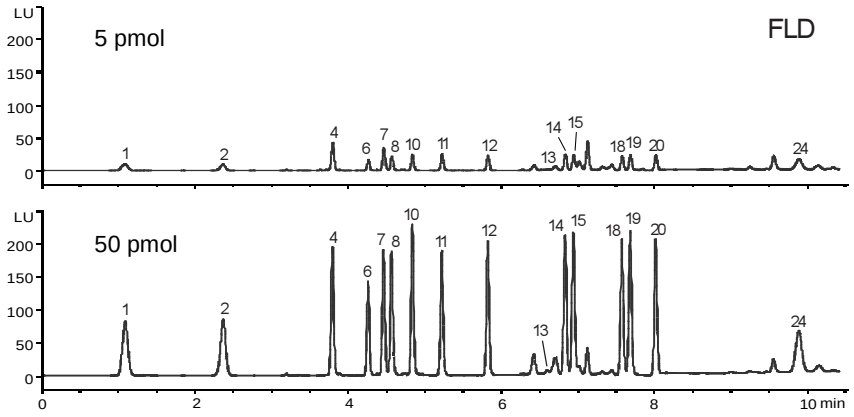

Figure 8: Fluorescence-Detector Response Using Different Concentrations Amino Acids. Gain for the Photomultiplier Tube (PMT) = 10.

ZORBAX Eclipse-AAA
4.6 x 150 mm (3.5 µm), for high sensitivity, high-resolution work using the FLD.

ZORBAX Eclipse-AAA
3.0 x 150 mm, (3.5 µm), available in Fall of 2000, is for high sensitivity, high-resolution work with less solvent and sample consumption.

Using the Agilent 1313A auto-sampler to automate the pre-column derivatization procedure results in a speedy and reproducible reaction with minimal operator intervention. This protocol can be used for routine analysis of both primary and secondary amino acids using the DAD and collecting two wavelengths, or with programmed wavelength switching.

For high-sensitivity work, the fluorescence detector is required.

EXPERIMENTAL CONDITIONS

Chromatograms shown in Figures 1 – 8 were obtained using the following experimental conditions:

Instrument
The recommended chromatographic system is the Agilent 1100 HPLC: G1312A Binary pump with G1315A Diode Array Detector (DAD), 6-mm or 10-mm flow cell, and/or G1315A Fluorescence Detector (FLD). While the results shown here were obtained the binary pump, this procedure has also been used with the Agilent 1100 quaternary pump (G1311A).

HPLC Columns
ZORBAX Eclipse-AAA
4.6 x 75 mm, 3.5 µm
PN 966400-902

ZORBAX Eclipse-AAA
4.6 x 150 mm, 3.5 µm
PN 963400-902

ZORBAX Eclipse-AAA
4.6 x 150 mm, 5 µm
PN 993400-902
Optional guard column*
ZORBAX Eclipse–AAA
4.6 x 12.5 mm, 5 µm, 4/PK
PN 820950-931

ZORBAX Eclipse-AAA
3.0 x 150 mm, 3.5 µm
Available in Fall of 2000.
* The optional guard column should be installed directly preceding the analytical column, using a low dead-volume connector.

Mobile Phase

A: 40 mM Na_2HPO_4 pH 7.8 [5.5 g NaH_2PO_4, monohydrate + 1 liter water, adjust to pH 7.8 with NaOH solution (10 N)]
B: ACN: MeOH: water (45:45:10, v/v/v)
It is convenient to make Mobile Phase A as a 10X stock solution with no pH adjustment. The solution can be kept for several weeks and can be diluted and titrated to pH 7.8, as needed.
All mobile-phase solvents should be HPLC grade.

Pump Settings

Flow: 2 mL/min
Stoptime: 14 min (75-mm column) or 26 min (150-mm column)
Post time: off

Auxiliary Pump Settings:
Max. flow ramp: 100 mL/min^2
Compressibility A: 50 x 10^{-6}
Minimal Stroke A: 20 µL
Compressibility B: 115 x 10^{-6}
Minimal Stroke B: Auto

Gradients:

For 75 mm column length

Time (min)	% B
0	0
1	0
9.8	57
10	100
12	100
12.5	0
14	0

For 150 mm column length

Time (min)	% B
0	0
1.9	0
18.1	57
18.6	100
22.3	100
23.2	0
26	0

Note: To extend column life, flush column with 10 column volumes of 100% B when column will not be used for periods of overnight or longer.

Detector Settings

DAD:

Required Lamps:
UV lamp: yes
Vis. lamp: no
UV: 338 nm, 10 nm bandwidth (bw), reference: 390 nm, 20 nm bw (for OPA-amino acids)
262 nm, 16 nm bw, reference: 324 nm, 8 nm bw (for FMOC-amino acids)
Peakwidth: >0.03 min (0.5 s)
Slit: 4 nm

FLD:

For 75 mm column

Time (min)	Ex/Em (nm)	PMT Gain
0	340/450	10
8.5*	266/305	9

For 150 mm column

Time (min)	Ex/Em (nm)	PMT Gain
0	340/450	10
15*	266/305	9

*The specific time to switch fluorescence wavelengths may differ due to variations in temperature, mobile phase, etc.

Peakwidth: >0.5 min

Autosampler:

See vial positioning (Fig. 9)

Injector program:
Draw 2.5 µL from vial 1 (borate buffer)
Draw 0.5 µL from sample (e.g., choose vial position #11 for amino acid sample)
Mix 3 µL "in air", max speed, 2x
Wait 0.5 min
Draw 0 µL from vial 2 (needle wash using water in uncapped vial)
Draw 0.5 µL from vial 3 (OPA)
Mix 3.5 µL "in air", max speed, 6x
Draw 0 µL from vial 2 (needle wash using water in uncapped vial)
Draw 0.5 µL from vial 4 (FMOC)
Mix 4 µL "in air", max speed, 6x
[Optional needle rinse for high sensitivity use: Draw 0.0 µL from vial 6 (ACN, acetonitrile)]
Draw 32 µL from vial 5 (water)
Mix 18 µL "in air", max speed, 2x
Inject

Auxiliary:
Drawspeed: 200 µL/min
Ejectspeed: 600 µL/min
Draw position: 0.0 mm

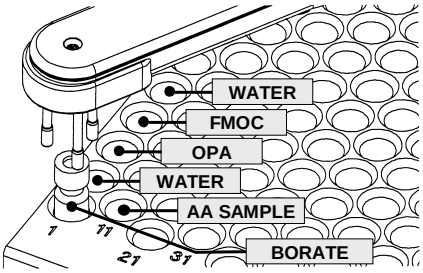

Figure 9: Position of reagent vials in the Agilent 1313A autosampler. This positioning of vials is designed for the listed injector program.

Vials:

Conical vial inserts with polymer feet (Fig. 10A) are required to hold the OPA and FMOC reagents because of the limited volumes involved. The inserts are compatible with wide-opening screw-top (Fig. 10B-C) or crimp-top vials. For this procedure snap-cap vials should not be used because an airtight seal is needed for both FMOC, because it is highly volatile, and OPA, because it slowly degrades in the presence of oxygen. Be careful not to use vials or caps designed for other instruments, as these may damage the Agilent G1313A autosampler.

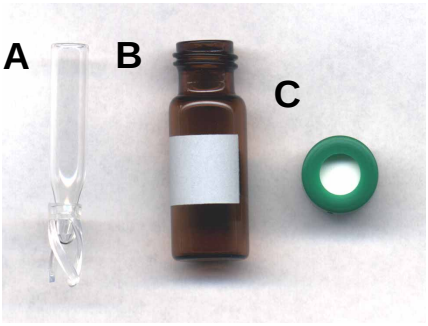

Figure 10: Insert, Vial, and Cap. Photo of conical insert **A** (Agilent PN 5181-1270), amber wide-opening vial **B** (Agilent PN 5182-0716), and screw cap **C** (Agilent PN 5182-0721), for amino acid analysis using the Agilent 1100 autosampler.

Column Compartment:

Temperature: 40°C (left and right side)
Enable analysis: When temperature is within setpoint +/- 0.8°C

Derivatization Reagents

Borate Buffer:

Agilent PN 5061-3339
Solution is 0.4 N in water, pH 10.2. Keep refrigerated (4°C). Dispense as necessary.

FMOC Reagent:

Agilent PN 5061-3337
Pipette 100-µL aliquots of the 1-mL FMOC reagent into conical inserts, cap immediately and refrigerate (4°C); solution is useable for 7 - 10 days, maximum, after dispensing.

OPA Reagent:
Agilent PN 5061-3335
Pipette 100-µL aliquots of the 1-mL OPA reagent into conical inserts, cap immediately and refrigerate (4°C); solution is useable for 7 - 10 days, maximum, after dispensing.

Water: Deionized, HPLC grade

See *Ordering Information* for descriptions and part numbers.

SAMPLE PREPARATION
Note: Each reagent vial should be replaced every day. Each 1 mL ampoule of reagent contains sufficient solution to last about ten days (1000 µL/100 µL = 10 days).

Amino Acid Mix for Chromatographic Comparisons
For chromatographic analyses, 17 amino acids from the 250 pmol/µL standard mix (PN: 5061-3331), plus citrulline and the 6 supplemental amino acids, were combined at a concentration of approximately 250 pmol/µL. The mixture was prepared by combining the two stock solutions described below. Add 1 µL of the supplemental amino acid stock solution to a fresh aliquot of the 250-pmole standard (100µL) in the conical vial insert. Mix using a vortex mixer to complete the 24-component standard ready for injection (250 pmol/L).

250 pmole standard:
Divide 1 mL ampoule of 250pmol/µL amino acids (PN 5061-3331) into 100 µL portions in conical vial inserts, cap and refrigerate aliquots at 4°C.

Supplemental amino acid stock solution:
Weigh about 0.25 mmoles of each auxiliary amino acid (gln, asn, trp, nva, hyp, sar) from kit (PN 5062-2478) into a 20-mL vial. Add 5 mL deionized water and sonicate in a hot water bath until dissolved. Add another 5 mL water to complete dilution. Store in refrigerator (4°C). Citrulline (Sigma-Aldrich Co., St. Louis, MO) was added in this mix at the same concentration.

For storage, do **not** combine supplemental amino acids with amino acid standards. Some of these supplemental amino acids degrade in HCl (especially glutamine, and to a lesser extent, asparagine).

Amino Acid Mix for Calibration Curves
For the construction of calibration curves, 17 amino acids, plus the 4 extended amino acids, are combined at various concentrations with fixed amounts of internal standards. The internal standards (ISTD) (norvaline and sarcosine) are part of the supplemental amino acid kit (PN: 5062-2478). The remaining amino acids in this kit (gln, asn, trp, hyp) form the extended amino acids (EAA). To make the appropriate solutions, refer to Tables 3 and 4 for low and high sensitivity standards, respectively.

Amino acid standards (10 pmol/µL to 1nmol/µL):
Divide each 1 mL ampoule of standards PN 5061-3330 through 5061-3334) into 100 µL portions in conical vial inserts, cap and refrigerate aliquots at 4°C. Calibration curves may be made using from 2 to 5 standards, depending on experimental need.

Extended amino acid (EAA) stock solution:
This solution is made using four of the six amino acids in the supplemental amino acid kit (PN: 5062-2478). For use with low-sensitivity standards (Table 3), make a 25-mL solution containing 18 nmol/µL of glutamine, asparagine, tryptophan, and 4-hydroxy-proline in deionized water. Sonicate the solution until dissolved. Store the solution refrigerated at 4°C. For use with high-sensitivity standards (Table 4), make a 1.8 nmol/µL solution by diluting 5 mL of the 18 nmol/µL standard with 45 mL deionized H_2O.

Internal standards (ISTD) stock solution:
These solutions are made using two of the six amino acids in the supplemental amino acid kit (PN: 5062-2478). For use with low-sensitivity standards (Table 3), make a 25-mL solution containing 10 nmol/µL of norvaline and sarcosine in deionized water. Sonicate the solution until dissolved. Store in refrigerator (4°C). For use with high-sensitivity standards (Table 4), make a 1 nmol/µL solution by diluting 5 mL of the 10 nmol/µL standard with 45 mL deionized H_2O. Store in refrigerator (4°C).

ADDITIONAL SUPPORT

User-contributed Chemstation Method files for each column type, written documentation, as well as an amino acid report and macro, are available by download via the Agilent web site at www.agilent.com/chem, under "Technical Support" / "User Contributed Software".

Table 3: Preparation of Low-Sensitivity Amino Acid Standard Solutions. Prepare the three low-sensitivity standards by mixing together stock solutions in the volumes shown.

	Concentration of Final AA Solutions (pmol/µL)		
	900	**225**	**90**
Take 5 mL 18 nmol EAA	5mL	5mL	5mL
Dilute with 0.1NHCl	—	15mL	45mL
Diluted EAA mix	**5mL**	**20mL**	**50mL**
Take 5 mL diluted EAA mix	5mL	5mL	5mL
Add 10 nmol ISTD solution	5mL	5mL	5mL
EAA-ISTD mix	**10mL**	**10mL**	**10mL**
Take 100 µL EAA-ISTD mix	100µL	100µL	100µL
Add 1000 pmol AA standard	900µL	—	—
Add 250 pmol AA standard	—	900µL	—
Add 100 pmol AA standard	—	—	900µL
Final AA Solution with EAA and 500 pmol/µL ISTD	**1 mL**	**1 mL**	**1 mL**

Table 4: Preparation of High-Sensitivity Amino Acid Standard Solutions. Prepare the three high-sensitivity standards by mixing together stock solutions in the volumes shown.

	Concentration of Final AA Solutions (pmol/µL)		
	90	**22.5**	**9**
Take 5 mL 1.8 nmol EAA	5mL	5mL	5mL
Dilute with 0.1NHCl	—	15mL	45mL
Diluted EAA mix	**5mL**	**20mL**	**50mL**
Take 5 mL diluted EAA mix	5mL	5mL	5mL
Add 1 nmol ISTD solution	5mL	5mL	5mL
EAA-ISTD mix	**10mL**	**10 mL**	**10 mL**
Take 100µL EAA-ISTD mix	100µL	100µL	100µL
Add 100 pmol AA standard	900µL	—	—
Add 25 pmol AA standard	—	900µL	—
Add 10 pmol AA standard	—	—	900µL
Final AA Solution with EAA and 50 pmol/µL ISTD	**1mL**	**1mL**	**1mL**

ORDERING INFORMATION

Eclipse-AAA HPLC Columns			
Description	Size (mm)	Particle Size (µm)	Agilent Part No.
Analytical routine sensitivity, high-resolution	4.6 x 150	5µm	993400-902
Analytical high sensitivity, high resolution work using the FLD	4.6 x 150	3.5µm	963400-902
Analytical, routine sensitivity, high-throughput	4.6 x 75	3.5µm	966400-902
Analytical high sensitivity, high resolution work–DAD or FLD	3.0 x 150	3.5µm	961400-302
Guard (4/pk)	4.6 x 12.5	5µm	820950-931
Guard Hardware Kit	—	—	820777-901

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

Vials	
Description	Agilent Part No.
100 µL Conical insert with polymer feet, 100/pk	5181-1270
Amber, wide-opening, write-on, screw-top vial, 2mL,100/pk	5182-0716
Green Screw Cap, PTFE/silicone septum, 100/pk	5182-0721

Standards	
Description	Agilent Part No.
Amino Acid Standard in 0.1 M HCl, 10 x 1mL ampoules	
1 nmol /ml	5061-3330
250 pmol /ml	5061-3331
100 pmol /ml	5061-3332
25 pmol /ml	5061-3333
10 pmol /ml	5061-3334
Supplemental Amino Acids: Nva, Sar, Asn, Gln, Trp, Hyp, 1g each	5062-2478

Additional Support
Description
User-contributed Chemstation method files, custom amino-acid report and macro, and any additional documentation are available through the web at www.agilent.com/chem under “Technical Support” / “User Contributed Software”.

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