High-Speed Amino Acid Analysis (AAA) on 1.8 µm Reversed-Phase (RP) Columns

Application

Pharmaceuticals and Foods

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Abstract

Amino acid analysis (AAA) is commonly used in proteomics and food quality testing as a tool to discover the precise amino acid (AA) makeup of samples. In order to do this in a timely fashion a short turnaround time is needed; to do it with limited sample amounts, a highly sensitive method is needed; and to maximize laboratory productivity, an automated method is desirable. This paper describes a new methodology using recently developed columns with an engineered particle size of 1.8 µm. These patented particles are specifically designed to create less backpressure than other sub-two-micron particles on the market; therefore, they can be used on 400 bar HPLC systems as well as higher pressure limit instruments. We take advantage of the OPA/FMOC chemistry first introduced by Hewlett-Packard/Agilent in 1988 and improve on the precision, run time, column longevity, and ruggedness of the previous methods [1—4].

Introduction

The detection and quantitation of amino acids has been a large part of protein and food analysis since Moore and Stein developed the first analyzer in 1951 utilizing ion exchange chromatography to separate underivatized amino acids (AAs) followed by post-column derivatization with ninhydrin and detection in the visible region [5]. They were able to develop a completely automated analyzer, introduced commercially by Beckman in 1958. This was a revolution in the analysis of proteins and strongly contributed to the winning of the 1972 Nobel Prize in Chemistry for their work on ribonuclease. A single analysis had previously taken weeks to complete but was now accomplished in less than a day. Subsequent work reduced the analysis time to approximately 110 minutes on the last revision of the Beckman Amino Acid Analyzer produced, but the sensitivity was still a problem using ninhydrin.

Post-column reaction with o-phthalaldehyde (OPA) and mercaptoethanol offered some promise, but only primary AAs were detected (see Figure 1a). In 1971 the first precolumn derivatization of AAs with OPA was published [6]. The derivatives were rather unstable but the process could be automated. Sensitivity was enhanced, particularly when fluorescence detection (FLD) was used. While the OPA is nonfluorescent, the derivatives are highly fluorescent; however, the secondary AAs are still not detected. Another derivatizing agent, 9-fluorenylmethyl chloroformate (FMOC), formed stable derivatives and derivatized both pri-

mary and secondary AAs but was itself strongly UV absorbing and highly fluorescent (see Figure 1b) [7]. An extraction step or reaction with very hydrophobic amine at the end was needed to remove the excess FMOC and its reaction byproducts [8]. This latter methodology was also automated as a precolumn method [9] but had problems, in commercial versions, of having higher relative standard deviations (RSDs) than systems using no back extractions.

By combining these two chemistries in a sequential way, in 1986 Hewlett-Packard/Agilent was able to fully automate the derivatization, chromatography, detection, and reporting of AAs from protein hydrolysates without back extraction [1]. A commercial analyzer was developed and sold by 1988 that had a total turnaround time of 36 minutes with femtomole sensitivity. This was definitely a step in the right direction for biotech companies and university researchers, who were sample limited, particularly in the early R&D phases of drug discovery.

A few chemical innovations were needed in order to make this commercially viable: OPA derivatives were made more stable by changing the source of incorporated thiol to 3-mercaptopropionic acid (MPA); all primary AAs were reacted first with OPA to quantitatively remove them from further reaction; then FMOC was introduced to react only with the secondary AAs. Since FMOC, its derivatives, and reaction byproducts were all more hydrophobic than any of the OPA derivatives, they did not interfere with any primary AA detection. Because there were only a few FMOC derivatives, it was possible, using a simple two-segment gradient, to adequately separate those AAs from the reaction byproducts and FMOC [1]. This is summarized in Figures 1 and 2. The names corresponding to the peak numbers are given in Table 1.

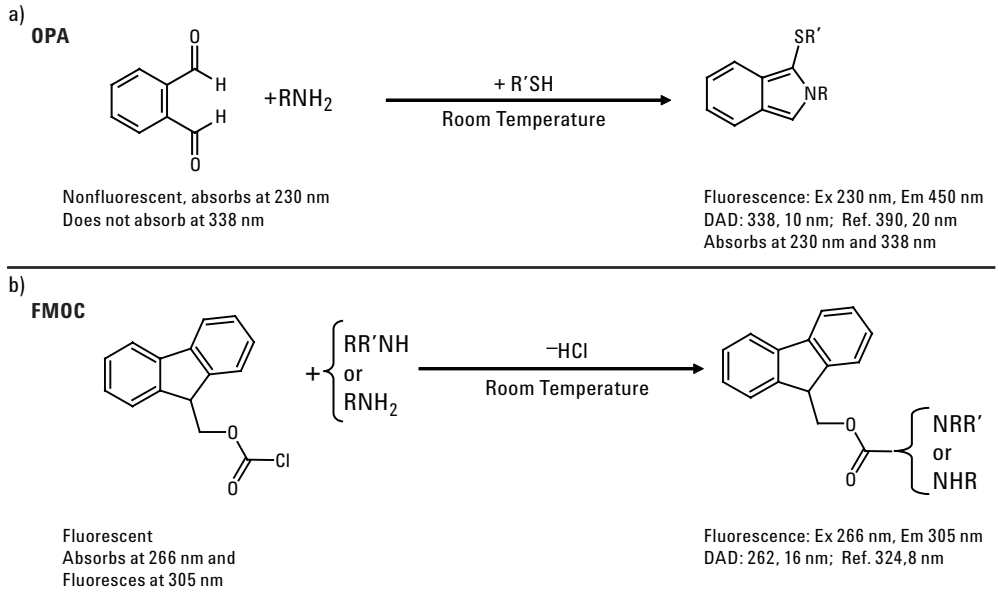

Figure 1. o-Phthalaldehyde (OPA) and 9-Fluorenylmethyl chloroformate (FMOC) reactions with amines.

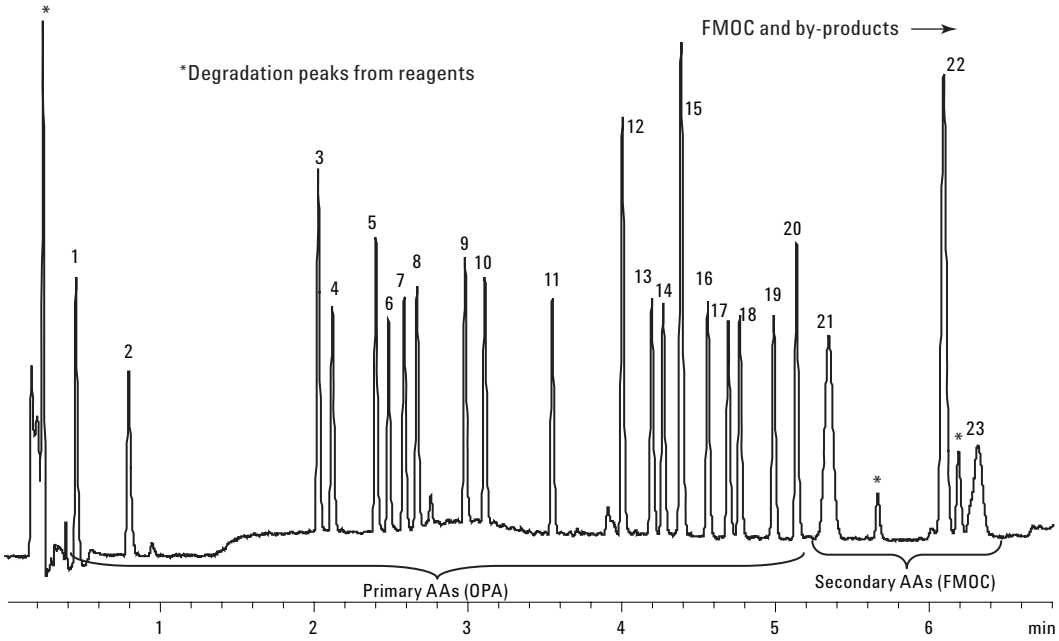

Figure 2. Amino acid analysis on Rapid Resolution HT Eclipse Plus C18, 4.6 x 50 mm, 1.8-µm column: DAD 125 pMoles on column.

Table 1. Names and Order of Elution for OPA and FMOC Derivatives of Amino Acids

Peak #	AA name	AA abbreviation	Derivative type
1	Aspartic Acid	ASP	OPA
2	Glutamic Acid	GLU	OPA
3	Asparagine	ASN	OPA
4	Serine	SER	OPA
5	Glutamine	GLN	OPA
6	Histidine	HIS	OPA
7	Glycine	GLY	OPA
8	Threonine	THR	OPA
9	Arginine	ARG	OPA
10	Alanine	ALA	OPA
11	Tyrosine	TYR	OPA
12	Cystine	CYS-CYS	OPA
13	Valine	VAL	OPA
14	Methionine	MET	OPA
15	Norvaline†	NVA	OPA
16	Tryptophan	TRP	OPA
17	Phenylalanine	PHE	OPA
18	Isoleucine	ILE	OPA
19	Leucine	LEU	OPA
20	Lysine	LYS	OPA
21	Hydroxyproline	HYP	FMOC
22	Sarcosine†	SAR	FMOC
23	Proline	PRO	FMOC

† Internal Standard

Experimental

Instrument

The results obtained in this application were all performed on an Agilent 1200SL HPLC system consisting of the following components:

G1312B, binary pump SL
G1379B, micro degasser
G1367C, high-performance well plate autosampler (WPS) configured with 54 x 2 mL sample tray in front and 15 x 6 mL tray in back
G1316B, thermostatted column compartment SL (TCC) with low dispersion kit installed
G1315C, diode array detector SL (DAD) with semi-micro flow cell
G1321A, fluorescence detector (FLD)

All tubing used throughout the instrument is 0.12 mm id (0.005 inch).

It is likely that older instruments, such as 1100 binary systems, and possibly 1100 and 1200 quaternary pump-based systems can run this analysis on 4.6 mm id columns, though this has not been tested. The use of 3.0 mm id and 2.1 mm id is not recommended on those systems without extensive testing due to the larger delay volume encountered using those systems; this may cause anomalies in some of the earlier eluting peaks due to dwell volume effects.

Instrument Configuration

Pump Parameters: mixer and pulse dampener bypassed with 100 mm long tubing (0.12 mm id), bypass programmed (at 0.1 min after inject command; see WPS parameters below), compressibility settings used: A = 35, B = 80

Flow: 0.42 mL/min for 2.1 mm id; 0.85 mL/min for 3.0 mm id; 2.00 mL/min for 4.6 mm id

Gradient Timetable:	Time (min)	%B
	0.0	2.0
	1.0	2.0
	7.0	57.0
	7.1	100.0
	8.4	100.0
	8.6	2.0
	Stop time 8.7	

DAD: PW 0.01 min; slit 4 nM; Stop time 7 min (adjust as needed); Cell volume = 5 µl, 6 mm flow path (Agilent P/N G1315-60025)
Signal A: 338, 10 nm; Ref 390, 20 nm
Signal B: 262, 16 nm; Ref 324, 8 nm
Signal C: 338, 10 nm; Ref 390, 20 nm
Signal D: 230, 16 nm; Ref 360, 100 nmM
Timetable Signal C): 0.00 min 338, 10 nm; Ref 390, 20 nm; 5.53 min 262, 16 nm; Ref 324, 8 nm (adjust as needed; 3.0 mm id transition at approximately 5.45 min, 4.6 mm id transition at approximately 5.4 min)

Table 2. 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 (pMoles/µL)		
	900	**225**	**90**
Take 5 mL 18 nMoles EAA	5 mL	5 mL	5 mL
Dilute with 0.1 N HCl		15 mL	45 mL
Diluted EAA mix	**5 mL**	**20 mL**	**50 mL**
Take 5 mL diluted EAA mix	5 mL	5 mL	5 mL
Add 10 nMoles ISs solution	5 mL	5 mL	5 mL
EAA-ISs mix	**10 mL**	**10 mL**	**10 mL**
Take 100 µL EAA-ISs mix	100 µL	100 µL	100 µL
Add 1000 pMoles AA standard	900 µL	—	—
Add 250 pMoles AA standard	—	900 µL	—
Add 100 pMoles AA standard	—	—	900 µL
Final AA solution with EAA and 500 pMoles/µL ISs	**1 mL**	**1 mL**	**1 mL**

FLD: PW 0.01 min; Stop time 7 min (adjust as needed), never use this detector in series before another due to fragility of flow cell Ex 230 nm; Em 450 nm; Filter 295 nm (Default filter)
Timetable Signal: 0.00 min Ex 230 nm, Em 450 nm; PMT Gain 9 (as needed) 5.53 min Ex 266 nm, Em 305 nm; PMT Gain 9 (as needed; wave length transition times as for DAD + approximately 0.03 min)

TCC: used with low dispersion kit installed; T = 40 °C for column side, 30 °C for exit side. Low dispersion kit used on both sides.

WPS: Default volume set to 0.5 µl, default speed used throughout injector program is 200 µl/min

Injector program
1) Draw 2.5 µl from Borate vial (Agilent P/N 5061-3339)
2) Draw 0.5 µl from Sample vial
3) Mix 3 µl in washport 5X
4) Wait 0.2 min
5) Draw 0.5 µl from OPA vial (Agilent P/N 5061-3335)
6) Mix 3.5 µl in washport 6X
7) Draw 0.4 µl from FMOC vial (Agilent P/N 5061-3337)
8) Mix in 3.9 µl in washport 10X
9) Draw 32 µl from Injection Diluent vial
10) Mix 20 µl in washport 8X
11) Inject
12) Wait 0.10 min
13) Valve bypass

Amino Acid Mix for Calibration Curves

For the construction of calibration tables and curves, 17 amino acids, plus the 4 extended amino acids, are combined at various concentrations with fixed amounts of internal standards. The internal standards (ISs) (norvaline and sarcosine) are part of the supplemental amino acid kit (P/N 5062-2478). The remaining amino acids in this kit (glutamine [GLN], asparagine [ASN], tryptophan [TRP], and hydroxyproline [HYP]) form the extended amino acids (EAA). To make the appropriate solutions, refer to Tables 2 and 3 for low- and high-sensitivity standards, respectively.

Table 3. 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 (pMoles/µL)		
	90	**22.5**	**9**
Take 5 mL 1.8 nMoles EAA	5 mL	5 mL	5 mL
Dilute with 0.1 N HCl	—	15 mL	45 mL
Diluted EAA mix	**5 mL**	**20 mL**	**50 mL**
Take 5 mL diluted EAA mix	5 mL	5 mL	5 mL
Add 1.0 nMoles ISs solution	5 mL	5 mL	5 mL
EAA-ISs mix	**10 mL**	**10 mL**	**10 mL**
Take 100 µL EAA-ISs mix	100 µL	100 µL	100 µL
Add 100 pMoles AA standard	900 µL	—	—
Add 25 pMoles AA standard	—	900 µL	—
Add 10 pMoles AA standard	—	—	900 µL
Final AA solution with EAA and 50 pMoles/µL ISs	**1 mL**	**1 mL**	**1 mL**

Amino Acid Standards (10 pMoles/µL to 1 nMoles/µL): 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. Calibration curves may be made using from two to five 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 (P/N 5062-2478). For use with low-sensitivity standards (Table 2), make a 25-mL solution containing 18 nMoles/µL of glutamine, asparagine, tryptophan, and 4-hydroxy-proline in deionized water. Sonicate the solution until dissolved. Store the solution at 4 °C. For use with high-sensitivity standards (Table 3), make a 1.8 nMoles/µL solution by diluting 5 mL of the 18 nMoles/µL standard with 45 mL deionized H_2O.

ISs Stock Solution: These solutions are made using two of the six amino acids in the supplemental amino acid kit (P/N 5062-2478). For use with low-sensitivity standards (Table 2), make a 25-mL solution containing 10 nMoles/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 3), make a 1 nMoles/µL solution by diluting 5 mL of the 10 nMoles/µL standard with 45 mL deionized H_2O. Store at 4 °C.

HPLC Columns

ZORBAX Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm, P/N 959741-902
ZORBAX Rapid Resolution HT Eclipse Plus C18, 3.0 x 50 mm, 1.8 µm, P/N 959941-302
ZORBAX Rapid Resolution HT Eclipse Plus C18, 4.6 x 50 mm, 1.8 µm, P/N 959941-902

Mobile Phase and Injection Diluent

Mobile phase A: 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 0.5 mM NaN_3 (5.6 gm anhydrous Na_2HPO_4 + 15.2 gm $Na_2B_4O_7 \cdot 10H_2O$ in 4 L water + 32 mg NaN_3). Adjust to approximately pH 9 with 6 mL concentrated HCl and then small drops until pH 8.2. ***Be cautious with strong acids***. Filter through 0.45 µm regenerated cellulose membranes (Agilent P/N 3150-0576). Stable for approximately 1.5 weeks at room temperature.

Mobile phase B: Acetonitrile methanol: water (45:45:10 by volume). All mobile-phase solvents must be HPLC grade.

Injection Diluent: 100 ml mobile phase A + 1,500 µL concentrated H_3PO_4 in a 100-mL bottle. Keep at 4 °C. Dispense into 6-mL vials before use. ***Be cautious with strong acids.***

See *Ordering Information* for descriptions and part numbers of all supplies available for AAA.

Derivatization Reagents

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

FMOC Reagent: Agilent P/N 5061-3337
Pipette 200-µL aliquots of the 1-mL FMOC reagent into conical inserts, cap immediately, and refrigerate (4 °C); solution is useable for 7 to 10 days, maximum, after dispensing if kept at 4 °C; useable for 1 day if kept at room temperature.

OPA Reagent: Agilent P/N 5061-3335
Pipette 200-µL aliquots of the 1-mL OPA reagent into conical inserts, cap immediately, and refrigerate (4 °C); solution is useable for 7 to 10 days, maximum, after dispensing, if kept at 4 °C; useable for 1 day if kept at room temperature.

Water: Deionized water, HPLC Grade

See *Ordering Information* for descriptions and part numbers of all supplies available for AAA.

Sample Preparation

The various bottled beer samples analyzed in this paper were obtained locally. The only preparation needed was degassing, which was accomplished quite simply by sonication. Caution must be used because sonication causes instantaneous, explosive degassing of carbonated beverages. It is recommended that the beer be poured into a wide-mouth beaker of greater volume than the beer, to allow for the large amount of foam generated.

Results and Discussion

Separation Options

In this application we demonstrate that the new method allows AAA to be done on a wider variety of columns than in previous versions. We report the results run on Rapid Resolution HT Eclipse Plus C18 columns, all of which were made with 1.8-µm particles in 4.6-, 3.0-, or 2.1-mm id and 50-mm length. The only differences in each method are the flow rate, so that a constant column flow velocity is maintained, and detector wavelength switching time, so that secondary AAs can be included on the same chromatogram.

We have also run this separation on Rapid Resolution HT Eclipse Plus C8 columns and on Rapid Resolution HT Eclipse XDB-C18 and -C8 columns, packed with 1.8-µm particles and the same variety of dimensions (data not shown). All columns gave very similar results to those obtained on the Rapid Resolution HT Eclipse Plus C18 (data not shown). The comparative separations obtained on different diameter Rapid Resolution HT Eclipse Plus C18 columns are shown in Figures 3 and 4 for DAD and FLD, respectively. As may be seen, there are only slight shifts in retention times for all chromatograms no matter what diameter is used. The shifts are caused by the slight changes in delay times among the three different diameter columns owing to the fact that the delay volume is constant; thus, the delay times are less for higher flow rates.

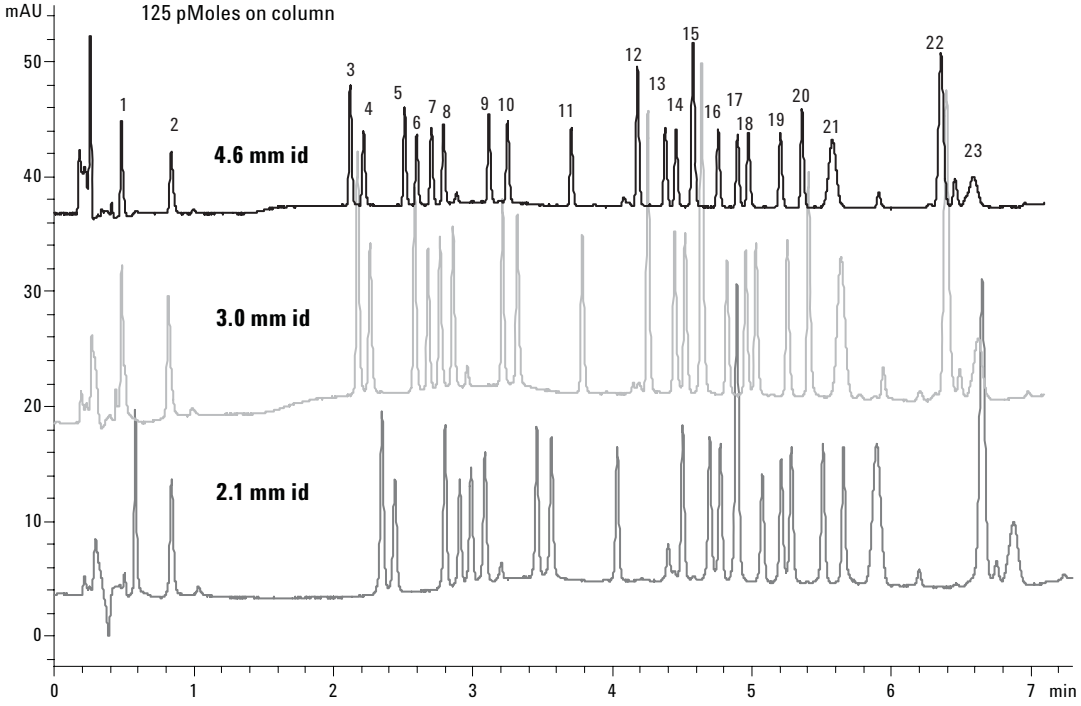

Figure 3. Scalability of AAA on various Rapid Resolution HT Eclipse Plus C18, 50 mm long, 1.8-µm columns, DAD.

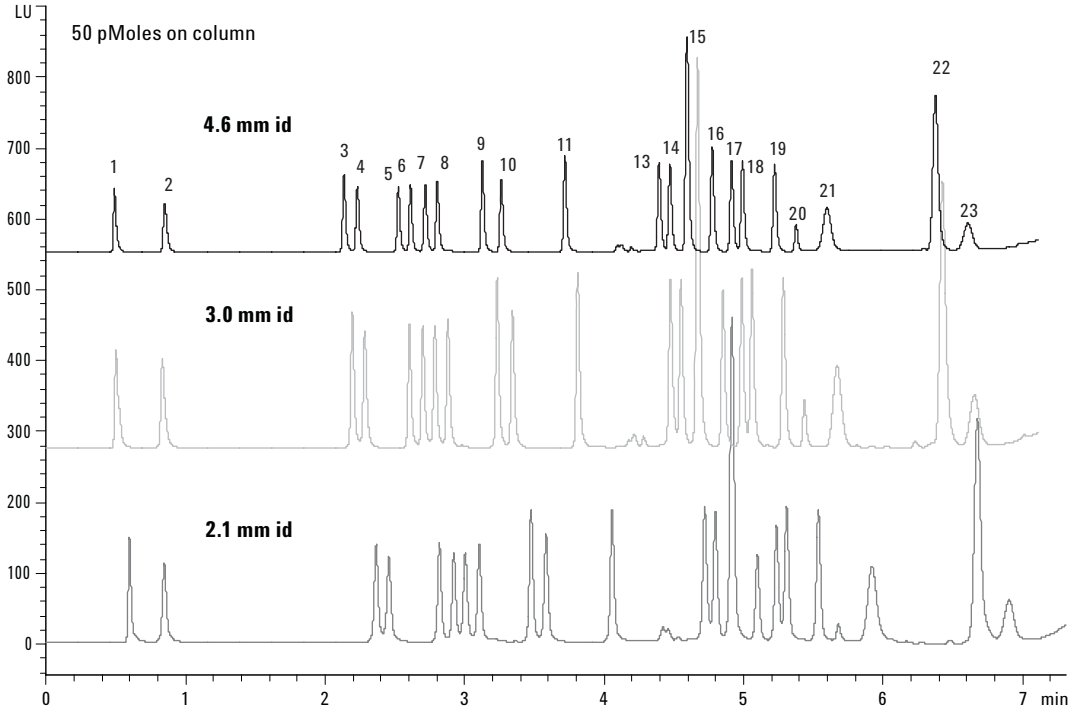

Figure 4. Scalability of AAA on various Rapid Resolution HT Eclipse Plus C18, 50 mm long, 1.8-µm columns, FLD.

Reproducibility and Linearity

The reproducibility of the secondary AAs in previous versions of this analysis was always less than that of the primaries [1−4]. With the current methodology we have improved the secondary AAs' reproducibility to equality with the primary AAs'. This data is shown in Tables 4 and 5 for ZORBAX Eclipse Plus-C18, 2.1-mm id columns, without ISs and in Table 6 for 4.6-mm id columns with ISs. The absolute peak area reproducibilities in Table 4 are superior. The average RSD is below 2% and only two are above 3% for all the AAs. Note that the linearities shown in Tables 5 and 6 are also excellent for all the AAs, being very close to 1.00 for all with a small edge to the linearities obtained with ISs. The results without ISs may be seen graphically in Figures 5 and 6 for Eclipse Plus-C18, 3.0-mm id columns. The chromatographic reproducibility of low-level AAA on the Eclipse Plus-C18, 2.1-mm id column may be seen in Figure 7 for 5.0 pMoles on column. The staggered overlay allows the retention time reproducibility and peak sizes to be easily compared. This is raw data with no corrections and no ISs.

Table 4. Absolute Peak Area Reproducibility on Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8-µm Columns Using an Agilent 1200SL HPLC System, no ISs

Detector reproducibility
50 pMoles FLD; 125 pMoles DAD; raw data areas

AA name	Abbreviation	FLD RSD (n = 10)	DAD RSD (n = 12)
Aspartic acid	ASP	1.1	0.8
Glutamic acid	GLU	0.8	2.3
Asparagine	ASN†	1†	1.6†
Serine	SER	0.9	1
Glutamine	GLN*	2.9*	2.1*
Histidine	HIS	0.8	2.1
Glycine	GLY	1	1.4
Threonine	THR	1.2	0.9
Arginine	ARG	1	1.9
Alanine	ALA	0.9	.3
Tyrosine	TYR	1	1
Cystine	CYS-CYS	NA	1.1
Valine	VAL	1.5	0.9
Methionine	MET	2.7	0.8
Norvaline	NVA	3.3	2.3
Tryptophan	TRP	1.3	1.1
Phenylalanine	PHE	1	0.9
Isoleucine	ILE	0.9	1.5
Leucine	LEU	2.1	0.7
Lysine	LYS	4.4	0.8
Hydroxyproline	HPA	1.8	1.7
Sarcosine	SAR	1.9	2.3
Proline	PRO	3.0	2.2
Average RSD =		1.7	1.4

† Asparagine is somewhat unstable in solution
* Glutamine is very unstable in solution

Table 5. Linearity of AAA on Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8-µm Columns Using an Agilent 1200SL HPLC System, no ISs

	Using raw data areas	
Amino acid	DAD coefficients of linearity (r^2)	FLD coefficients of linearity (r^2)
ASP	0.9949	0.9992
GLU	0.9976	0.9995
ASN†	0.9928†	1.0000†
SER	0.9933	0.9997
GLN*	0.9966*	0.9937*
HIS	0.9951	0.9996
GLY	0.9931	0.9996
THR	0.9942	0.9996
ARG	0.9960	0.9998
ALA	0.9965	0.9996
TYR	0.9936	0.9997
CYS-CYS	0.9818	NA
VAL	0.9952	0.9998
MET	0.9944	0.9997
NVA	0.9989	0.9991
TRP	0.9923	0.9987
PHE	0.9925	0.9996
ILE	0.9934	0.9993
LEU	0.9944	0.9994
LYS	0.9857	0.9957
HYP	0.9980	0.9924
SAR	0.9975	0.9960
PRO	0.9981	0.9969

† Asparagine is somewhat unstable in solution.
* Glutamine is very unstable in solution.

Table 6. Linearity of AAA on Rapid Resolution HT Eclipse Plus C18, 4.6 x 50 mm, 1.8-µm Columns Using an Agilent 1200SL HPLC System with ISs

Amino acid	DAD coefficients of linearity (r^2)	FLD coefficients of linearity (r^2)
ASP	0.99995	0.99887
GLU	0.99916	0.99879
ASN†	1.00000†	0.99581†
SER	0.99982	0.99880
GLN*	0.99996*	0.99549*
HIS	0.99978	0.99993
GLY	0.99982	0.99681
THR	0.99996	0.99941
ARG	0.99993	0.99946
ALA	0.99984	0.99924
TYR	0.99991	0.99946
CYS-CYS	0.99992	NA
VAL	0.99992	0.99886
MET	0.99992	0.99996
NVA	IS+	IS+
TRP	0.99997	0.99687
PHE	0.99988	0.99943
ILE	0.99973	0.99910
LEU	0.99986	0.99932
LYS	0.99956	0.99979
HYP	0.99991	0.99751
SAR	IS+	IS+
PRO	0.99998	0.99979

† Asparagine is somewhat unstable in solution.
* Glutamine is very unstable in solution.
+ IS at 250 pMoles on column for DAD and 25 pMoles on column for FLD

Extended Amino Acids (EAAs) and Internal Standards (ISs)

There are several amino acids that are destroyed during acid hydrolysis of proteins (asparagine, glutamine and tryptophan) and one that is rarely seen anywhere but in structural proteins (hydroxyproline). Additionally, many analysts prefer to use ISs in their sample in an effort to improve accuracy and reproducibility. It is necessary to choose the ISs carefully in order to obtain the best results. There are two common ways these are used:

1. Addition to the sample before any sample manipulations at all (i.e., before acid hydrolysis). This corrects for all variations, providing, of course, that the ISs are stable to hydrolysis and have the same derivatization reactivity as the other components in the sample. In order to properly use this mode the standards should also undergo the hydrolysis process; but this usually leads to more variation due to the different stabilities of various amino acids. An example is serine, which is destroyed progressively, dependent on hydrolysis time. This brings us to the most useful way of running with ISs.
2. Addition just before analysis, which corrects for volumetric pipetting errors and autosampler variations and also assumes that the reactivities of ISs and sample components are the same. This is the method used in this application.

For the OPA/FMOC analysis demonstrated here, there are two ISs available: norvaline for primary amino acids and sarcosine for secondary amino acids. These AAs may be used for both methods of using ISs (unpublished data); but method 2 is definitely the preferred way. When the four amino acids listed above (EAAs) are included in the standards using the Agilent Amino Acids Supplement

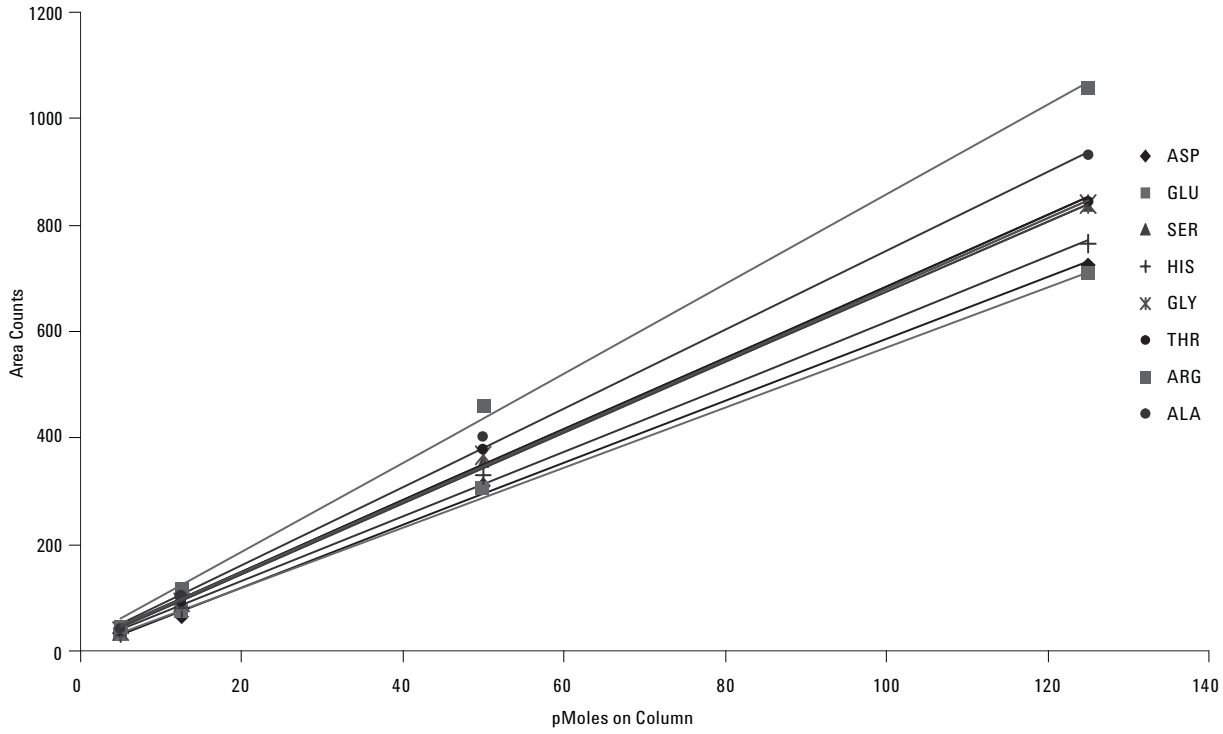

Figure 5. Amino acid linearities by FLD on Rapid Resolution HT Eclipse Plus C18, 3.0 x 50 mm, 1.8 µm.

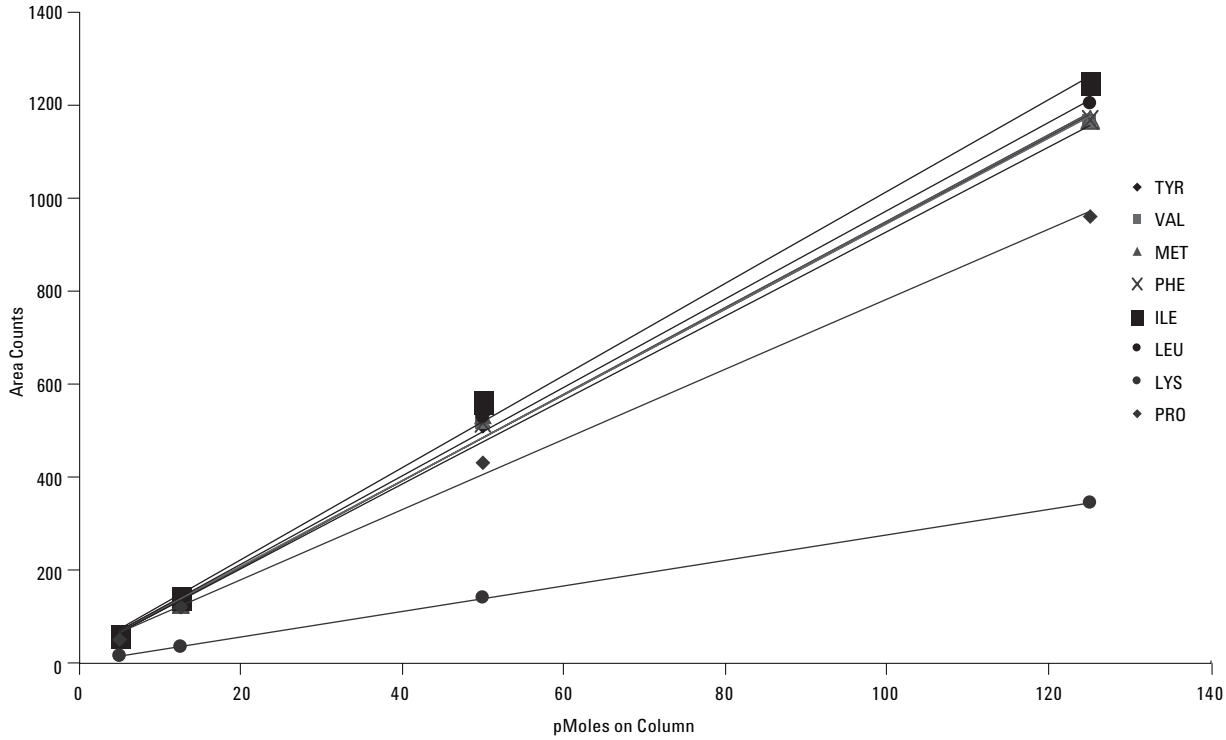

Figure 6. Amino acid linearities by FLD on Rapid Resolution HT Eclipse Plus C18, 3.0 x 50 mm, 1.8 µm.

kit (P/N 5062-2478), all are well separated in this new version of AAA. This has been shown in Figures 3 and 4 already on a Rapid Resolution HT Eclipse Plus C18, 2.1, 3.0, or 4.6 x 50 mm, 1.8-µm columns. In these two figures the ISs, norvaline and sarcosine, are added at 100 pMoles on column for FLD chromatograms and at 250 pMoles on column for DAD simply to demonstrate where they appear in the chromatogram. The original process for the addition of the EAAs and ISs has been described previously [4]. This procedure is used to produce an IS-based calibration table for the quantitation needed for real-world samples. The process is described in the Experimental section and is shown in Tables 2 and 3.

Real samples

The result of a comparison of a variety of lager beers is shown in Figure 8. Each beer has been spiked with ISs at a level of 500 pMoles/µL each so that a comparison of the relative AA content

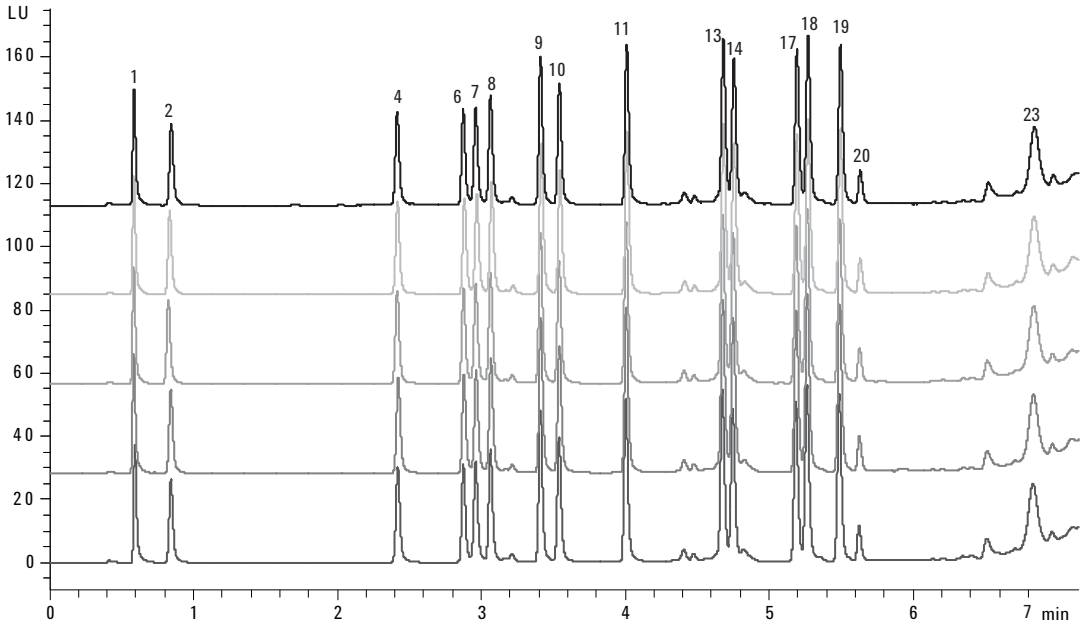

Figure 7. Reproducible AAA of 5.0 pMoles on column using a Rapid Resolution HT Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm, FLD, no ISs.

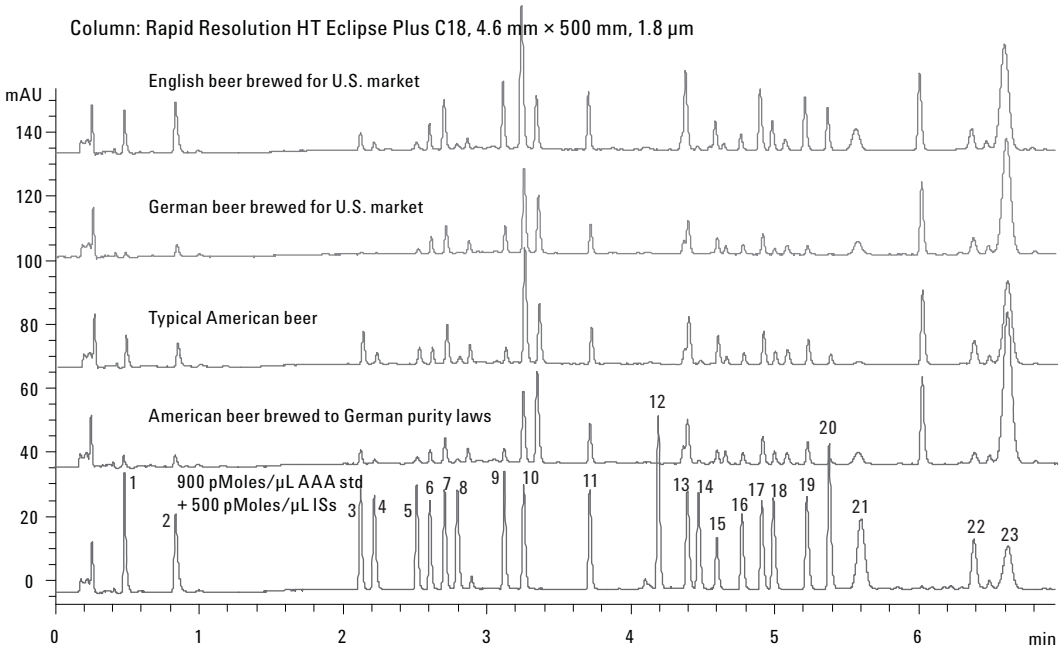

Figure 8. Comparison of a variety of beers available in the USA using the 1200SL, DAD and ISs.

could be calculated. This comparison shows that the relative AA content of these beers is not consistent with many peoples' perception—that "typical" American beers have lower AA content than do traditional German beers. The results indicate that at least some German and English beer manufacturers now brew their beers sold in the U.S. for American taste. It may be that the micro-brew from the U.S. is more consistent with a German beer brewed for German distribution, since it is brewed in compliance with German purity laws and therefore, more representative of "traditional" German brewing. The results are tabulated in Table 7.

Acid hydrolysis and subsequent AAA are commonly used in the analysis of proteins. Cell culture media are also monitored, both in the laboratory and in the new Process Analytical Technology (PAT) initiative now being implemented throughout biotechnology and pharmaceutical companies. AAA on these subjects will be presented in subsequent papers.

Table 7. Comparison of the Amino Acid Content of Various Beers

Amino acid	Typical American beer µMoles /mL	American beer brewed to German purity laws µMoles /mL	German beer brewed for U.S. market µMoles /mL	English beer brewed for U.S. market µMoles /mL
ASP	1.06	0.73	0.26	1.15
GLU	1.07	0.93	0.80	1.98
ASN	1.19	0.98	0.14	0.54
SER	0.45	0.40	0.12	0.26
GLN	0.67	0.76	0.38	0.33
HIS	0.68	1.00	1.07	0.89
GLY	1.54	2.00	1.61	1.57
THR	0.30	0.34	0.74	0.20
ARG	0.48	0.94	1.29	1.83
ALA	4.28	5.14	4.56	4.60
TYR	1.36	2.86	1.65	1.77
CYS-CYS	0.08	0.10	0.08	0.05
VAL	1.92	3.35	1.93	2.83
MET	0.17	0.27	0.11	0.16
NVA	IS	IS	IS	IS
TRP	0.58	1.07	0.80	0.67
PHE	1.33	2.21	1.23	2.07
ILE	0.54	0.97	0.34	0.94
LEU	1.02	1.71	0.55	1.75
LYS	0.30	0.32	0.08	0.90
HYP	0.20	1.74	1.17	1.26
SAR	IS	IS	IS	IS
PRO	8.65	34.84	15.88	10.29
Total =	27.9	62.7	34.8	36.0

Conclusions

All aspects of the AAA have been improved by this work. The use of 1.8-µm sized particle columns has enabled the cycle time of the analysis to be more than cut in half, from approximately 35 minutes to approximately 13.5 minutes. The peak shapes of the early eluting aspartic acid and glutamic acid have been improved. The reproducibility of the secondary AAs has been improved, as has that of the slowest AA to react, lysine. The linearity is excellent (nearly 1); and the average peak area reproducibility is below 2%. Use of the new Rapid Resolution HT Eclipse Plus C18, 1.8-µm columns of any diameter from 2.1 to 4.6 mm has been demonstrated. The new mobile phase and injector conditions have made it very simple to convert from 2.1- to 3.0- or 4.6-mm id columns by simply changing flow rate. It is also possible to use Rapid Resolution HT Eclipse Plus C8 or Rapid Resolution HT Eclipse XDB-C18 or -C8 columns as well (data not shown).

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ZORBAX Rapid Resolution HT Eclipse Plus C18 HPLC Columns

Description	Size (mm)	Particle Size (µm)	Agilent Part No.
Eclipse Plus C18	4.6 x 50 mm	1.8 µm	959941-902
Eclipse Plus C18	3.0 x 50 mm	1.8 µm	959941-302
Eclipse Plus C18	2.1 x 50 mm	1.8 µm	959741-902

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 x 1 mL ampoules	5061-3337
OPA Reagent, 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 x 1 mL ampoules	5061-3335
DTDPA Reagent for analysis of cysteine, 5 g	5062-2479

Mobile Phase and Injection Diluent Components

Description	Manufacturer	Manufacturer Part No.
Na_2HPO_4, Sodium Phosphate, Dibasic, Anhydrous	Sigma	71639
$Na_2B_4O_7.10H_2O$, Sodium Tetraborate 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-cap 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 x 1 mL ampoules	
1 nMoles/µL	5061-3330
250 pMoles/µL	5061-3331
100 pMoles/µL	5061-3332
25 pMoles/µL	5061-3333
10 pMoles/µL	5061-3334
Supplemental Amino Acids: Nva, Sar, Asn, Gln, Trp, Hyp, 1 g each	5062-2478

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