Application Note

Food Testing & Agriculture

Agilent

Trusted Answers

Automation of Sample Derivatization Using the Agilent 1260 Infinity II Prime LC System for Amino Acid Analysis

Suitable for Agilent 1260 Infinity III LC

Author

Lucas Willmann
Agilent Technologies, Inc.

Abstract

Amino acid analysis plays an important role in research, industrial processes, and the assessment of food quality. This application note describes the use of an Agilent 1260 Infinity II Prime LC System for amino acid analysis in different beverages. Using the Agilent 1260 Infinity II Multisampler, amino acids were automatically derivatized by an injector program enabling a fast reaction time and high reproducibility. By application of the Agilent 1260 Infinity II Flexible Pump combined with an Agilent AdvanceBio Amino Acid Analysis (AAA) column, 23 analytes could be separated in a run time of 9 minutes showing a retention time precision of less than 0.1% RSD for all analytes. The multi-emission feature of the Agilent 1260 Infinity II Fluorescence Detector enabled sensitive detection of all amino acid derivatives in a single run showing LODs down to 0.225 pmol/µL. To show its potential for several application areas, the developed method is used for analysis of amino acids in a soft drink and red wine sample.

Introduction

Amino acids are small organic molecules containing an amino and carboxyl group that are relevant for formation of peptides and building of proteins. They are involved in several other biological functions as key precursors for a variety of nitrogenous compounds and hormones playing a role in chemical messaging and energy metabolism. As many amino acids are essential nutrients and present in a variety of food and beverages, reliable determination of amino acids for assessment of food quality is indispensable.

Amino acid analysis can be performed using a variety of analytical methods (e.g., CE/MS, GC/MS, or LC/MS). Automated derivatization of amino acids before analysis via reversed-phase chromatography in combination with fluorescence or diode array detection has proven value and eliminates the need of MS detection. In-loop derivatization with *o*-phthalaldehyde (OPA) and 3-mercaptopropionic acid for primary as well as 9-fluorenylmethylchloroformate (FMOC) for secondary amino acids provides a rapid and easy approach to overcome the insufficient analyte retention on reversed-phase columns as well as weak fluorescence and ultraviolet absorbance.[1]

This application note demonstrates the use of a 1260 Infinity II Prime LC System with a 1260 Infinity II Fluorescence Detector for sensitive and precise analysis of amino acids in different beverages. Thereby, the 1260 Infinity II Multisampler is used for automated, precolumn derivatization of amino acids, enabling fast and reproducible masking of amine functionalities with protective groups without any manual labor.

Experimental

Instruments

- Agilent 1260 Infinity II Flexible Pump (G7104C), no mixer equipped
- Agilent 1260 Infinity II Multisampler (G7167A), 100 µL loop (G4267-60311), 0.12 mm seat assembly (G4267-87012)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A), standard heat exchanger (G7116-60015)
- Agilent 1260 Infinity II Fluorescence Detector Spectra (G7121B), 8 µL FLD cell (G1321-60005)
- 0.12 mm id system capillaries

Software

Agilent OpenLab CDS (Version 2.4)

Analytical standards/samples

- Amino Acid Supplement (part number 5062-2478) containing: L-asparagine, L-glutamine, L-tryptophan, L-4-hydroxyproline, L-norvaline, and sarcosine (1 g each)
- AA standard, 1 nmol/µL (part number 5061-3330)
- AA standard, 250 pmol/µL (part number 5061-3331)
- AA standard, 100 pmol/µL (part number 5061-3332)
- AA standard, 25 pmol/µL (part number 5061-3333)
- AA standard, 10 nmol/µL (part number 5061-3334)

Extended amino acid (EAA) stock solution (1.8 nmol/µL) and internal standard (IS) stock solution (1 nmol/µL) were prepared in 0.1 M HCl in water. EAA stock solution includes L-asparagine, L-glutamine, L-tryptophan, and L-4-hydroxyproline. IS stock solution consists of L-norvaline and sarcosine.

To avoid freeze-thaw cycles, it is recommended to distribute stock solutions (e.g., in 1 mL aliquots) and store them at –20 °C. The EAA and IS stock solutions were mixed 1:1 to get the desired volume of the EAA-IS stock solution. Afterwards, the EAA-IS stock solution can be diluted 1:10 with, for example, 100 pmol/µL AA standard solution to reach the final concentration of 90 pmol/µL for each amino acid and 50 pmol/µL for internal standards.

Samples (soft drink and red wine) were obtained from a local store and were filtered using 15 mm Agilent Captiva premium syringe filters with 0.2 µm regenerated cellulose membrane (part number 5190-5108)

All samples and standard mixtures were transferred into amber vials (part number 5182-0716) with glass inserts with polymer feet (part number 5181-1270) and screw caps (part number 5190-7024)

Columns

- Agilent AdvanceBio AAA LC column, 3.0 × 100 mm, 2.7 µm (part number 695975-322)
- Agilent AdvanceBio AAA guard columns, 3.0 × 5 mm, 2.7 µm, 3/pk (part number 823750-946)

Solvents

- **Mobile phase A:** Weigh in 2.8 g of sodium phosphate dibasic (Na_2HPO_4) and 7.6 g of disodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$), add 1.9 L of water and 3.5 mL of fuming hydrochloric acid (37%), mix until homogeneous, fill up to the total volume of 2 L with water. It is recommended to use an amber 2 L solvent bottle (part number 9301-6341) to avoid algae growth.

- **Mobile phase B:** acetonitrile/methanol/water 45/45/10 (v/v/v)
- Fresh, ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point of use cartridge (Millipak).
- Other mobile phase ingredients were obtained from Merck, Germany.

Reagents

- **Borate buffer:** 0.4 M in water, pH 10.2, 100 mL (part number 5061-3339)
- **FMOC reagent:** 2.5 mg/mL in ACN, 10 × 1 mL ampoules (part number 5061-3337)
- **OPA reagent:** 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid, 6 × 1 mL ampoules (part number 5061-3335)
- **Injection diluent:** 5 mL mobile phase A + 100 µL *ortho*-phosphoric acid (85%) from Merck, Germany

After opening an OPA or FMOC ampoule, the reagents should be distributed to amber vials (part number 5182-0716) with inserts (part number 5181-1270) and screw caps (part number 5190-7024) and stored for no longer than a week. Borate buffer and injection diluent can be transferred to vials without inserts. All reagents should be stored at 4 °C and should be exchanged daily.

Injector program

1. Draw 5.00 µL from location 1 (borate buffer) with the default speed using the default offset.
2. Wash the needle as defined in the method.
3. Draw 1.00 µL from the sample with the default speed using the default offset.
4. Wash the needle as defined in the method.
5. Draw 1.00 µL from location 2 (OPA reagent) with the default speed using the default offset.
6. Wash the needle as defined in the method.
7. Mix 7.00 µL from air with the default speed 10 times.
8. Draw 0.40 µL from location 3 (FMOC reagent) with the default speed using the default offset.
9. Wash the needle as defined in the method.
10. Mix 7.40 µL from air with the default speed 10 times.
11. Draw 32.00 µL from location 4 (injection diluent) with the maximum speed using the default offset.
12. Wash the needle as defined in the method.
13. Mix 20.00 µL from air with the maximum speed 5 times.
14. Inject.

Calibration/limit of detection

Calibration was conducted using 0.5 to 90 pmol/µL of analytical standards diluted in 0.1 M HCl in water. Limit of detection (LOD) values were determined using a signal-to-noise ratio (S/N) of at least 3.

Method parameters

Parameter	Value
Flow	1.2 mL/min
Timetable	0 min: 2% B, 0.2 to 6.8 min: 2 to 57% B, 7.0 to 7.4 min: 100% B, 7.5 min: 2% B
Stop Time	9 min
Needle Wash	5 s in flush port, wash solvent: 0.1 M HCl in water/acetonitrile 1/1 (v/v)
Column Compartment Temperature	40 °C
Advanced Multisampler Parameters	
Draw Speed	100 µL/min
Eject Speed	400 µL/min
Wait Time After Draw	1.2 s
Offset	0 mm
Vial/Well Bottom Sensing	Off
Advanced Pump Parameters	
Minimum Stroke/Primary Channel	Automatic
Flow Ramp Up/Down	50 mL/min²

FLD parameters

Parameter	Value
Multi-Emission	A) 455 nm B) 315 nm
Excitation	0 min: 345 nm 5.68 min: 265 nm
PMT Gain	10
Peak Width	>0.013 min (0.25 s resp. time) (37.04 Hz)

Results and discussion

To enable chromatographic separation by a reversed-phase column and detection via FLD, derivatization of the primary and secondary amine functionalities of amino acids was conducted using the 1260 Infinity II Multisampler. Therefore, in-loop derivatization with OPA and FMOC was executed by an injector program resulting in high reproducibility without any manual work. During the derivatization program, samples were alkalized with borate buffer, derivatized with OPA/FMOC, and quenched with injection diluent in around 3.5 minutes. After each draw of the sample or reagent, a 5-second needle wash step using a 1:1 mixture of sample solvent and acetonitrile was included to minimize carryover. By automation of these processes, a peak area precision (n = 10) of less than 1% relative standard deviation (RSD) could be achieved for the majority of the compounds (Table 1).

Due to the pH sensitivity of the reaction, derivatization of samples with an overly acidic pH value can result in a strongly decreased yield and consequently lower signal intensity. Thus, samples with a pH value below the pH value of the sample solvent (0.1 M HCl has a pH value of 1) might be neutralized before derivatization. For example, after a classical hydrolysis method for cleavage of proteins into amino acids using 6 M hydrochloric acid,[2] sample neutralization might be a necessary step before derivatization. For samples containing too many matrix components, a more selective method might be considered to avoid coelution of amino acids with matrix components. Application of hydrophilic interaction chromatography with low-pH solvents and positive ion mode in MS detection using multiple reaction monitoring has shown to be a suitable approach for amino acid analysis.[3]

Table 1. Method validation showing calibration linearity, detector sensitivity, and repeatability (RSD calculations are based on 10 consecutive injections using the standard mixture with a final concentration of 4.5 pmol/µL for amino acids and 2.5 pmol/µL for internal standards).

Peak No.	Compound	Calibration Range (pmol/µL)	LOD (pmol/µL)	R^2	RSD RT (%)	RSD Area (%)
1	L-Aspartic acid	0.9 to 90	0.225	0.9999	0.08	0.95
2	L-Glutamic acid	0.9 to 90	0.225	1.0000	0.10	0.90
3	L-Asparagine	0.9 to 90	0.225	0.9996	0.04	0.90
4	L-Serine	0.9 to 90	0.225	0.9997	0.04	0.92
5	L-Glutamine	0.5 to 45	0.225	0.9985	0.03	0.83
6	L-Histidine	0.9 to 90	0.225	0.9998	0.02	1.62
7	Glycine	0.9 to 90	0.225	0.9997	0.02	0.56
8	L-Threonine	0.9 to 90	0.225	0.9998	0.02	0.80
9	L-Arginine	0.9 to 90	0.225	0.9997	0.04	0.90
10	L-Alanine	0.9 to 90	0.225	0.9998	0.02	0.95
11	L-Tyrosine	0.9 to 90	0.225	0.9998	0.01	0.95
12	L-Cystine	5 to 90	2.25	0.9990	0.05	2.61
13	L-Valine	0.9 to 90	0.225	0.9997	0.04	0.90
14	L-Methionine	0.9 to 90	0.225	0.9998	0.04	0.74
15	L-Norvaline	1.25 to 50	0.25	0.9998	0.03	0.80
16	L-Tryptophan	0.9 to 90	0.225	0.9998	0.03	0.84
17	L-Phenylalanine	0.9 to 90	0.225	0.9998	0.03	0.91
18	L-Isoleucine	0.9 to 90	0.225	0.9999	0.03	0.97
19	L-Leucine	0.9 to 90	0.225	0.9998	0.03	0.81
20	L-Lysine	4.5 to 90	0.9	0.9990	0.05	1.26
21	L-4-Hydroxyproline	4.5 to 90	0.9	0.9996	0.03	1.84
22	Sarcosine	5 to 50	1.25	0.9995	0.01	1.88
23	L-Proline	4.5 to 90	0.9	0.9995	0.02	4.15

An AdvanceBio AAA LC column and the corresponding guard column enabled separation of 23 target substances in a run time of 9 minutes (Figure 1). The superficially porous particle technology of the Agilent InfinityLab Poroshell column resulted in good chromatographic separation at a moderate backpressure of up to 510 bar.

OPA- and FMOC-derivatized amino acids can be detected via FLD using an emission wavelength of 455 and 315 nm, respectively. To detect both derivatives in a single run, the multi-emission functionality of the 1260 Infinity II Infinity Fluorescence Detector was used. Additionally, the excitation wavelength needs to be switched from 345 to 265 nm after the elution of L-leucine (Peak 19) to detect the FMOC-derivatized L-4-hydroxyproline (Peak 21), L-proline (Peak 22), and sarcosine (Peak 23). If FMOC-derivatized amino acids are not of interest, it is recommended to keep the excitation and emission wavelength at 345 and 455 nm, respectively, over the entire run time.

Calibration was performed using individual concentrations from 0.5 to 90 pmol/µL and showed an excellent linearity with R^2 values of around 0.99 for all amino acids (Table 1). LODs showed an S/N of at least 3 and ranged from 0.225 to 2.25 pmol/µL, showing the high sensitivity of the 1260 Infinity II Infinity Fluorescence Detector. The use of the 1260 Infinity II Prime LC system resulted in excellent retention time precision (n = 10), showing values lower than 0.1% RSD for all compounds (Table 1).

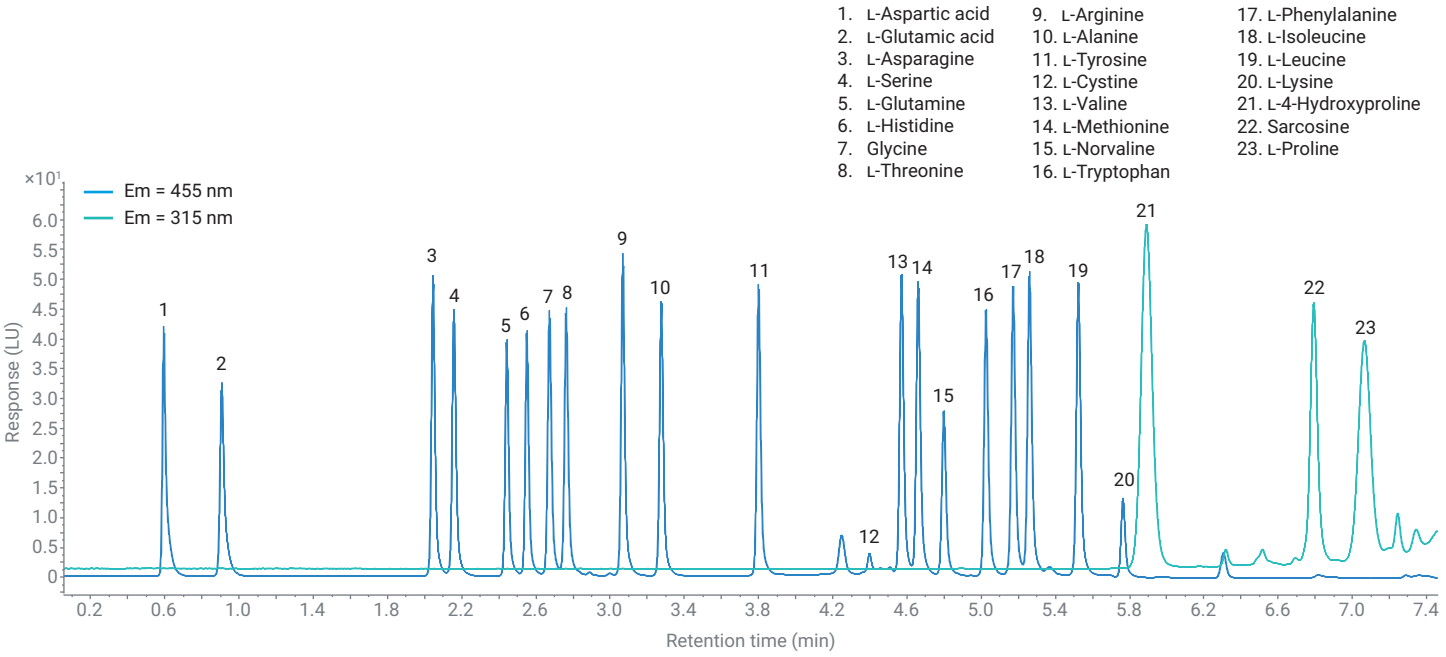

Figure 1. Analysis of 22.5 pmol/µL amino acid standard mixture showing an overlay of FLD signals at an emission wavelength of 455 and 315 nm.

To demonstrate the utility of this method, a commercially available soft drink and a red wine sample were analyzed showing different amino acid profiles (Figure 2). Samples were filtered using a 0.2 µm regenerated cellulose membrane, and the red wine sample was diluted 1:10 with water before injection. Due to high concentrations of certain amino acids, the photomultiplier tube (PMT) gain was adapted to the samples' individual concentration range to avoid oversaturated signals. Each step of the PMT gain approximately doubles the signal; signal-to-noise ratio is decreased with lower values for PMT gain. For the soft drink sample, the PMT gain was decreased from 10 to 9 over the entire run, and for the diluted red wine sample, the PMT gain was switched from 10 to 8 after 6.5 minutes to get a sufficient peak height for proline, which is usually the most abundant amino acid in red wine.[4] Consequently, recalibration should be performed daily and in accordance with the required PMT gain settings.

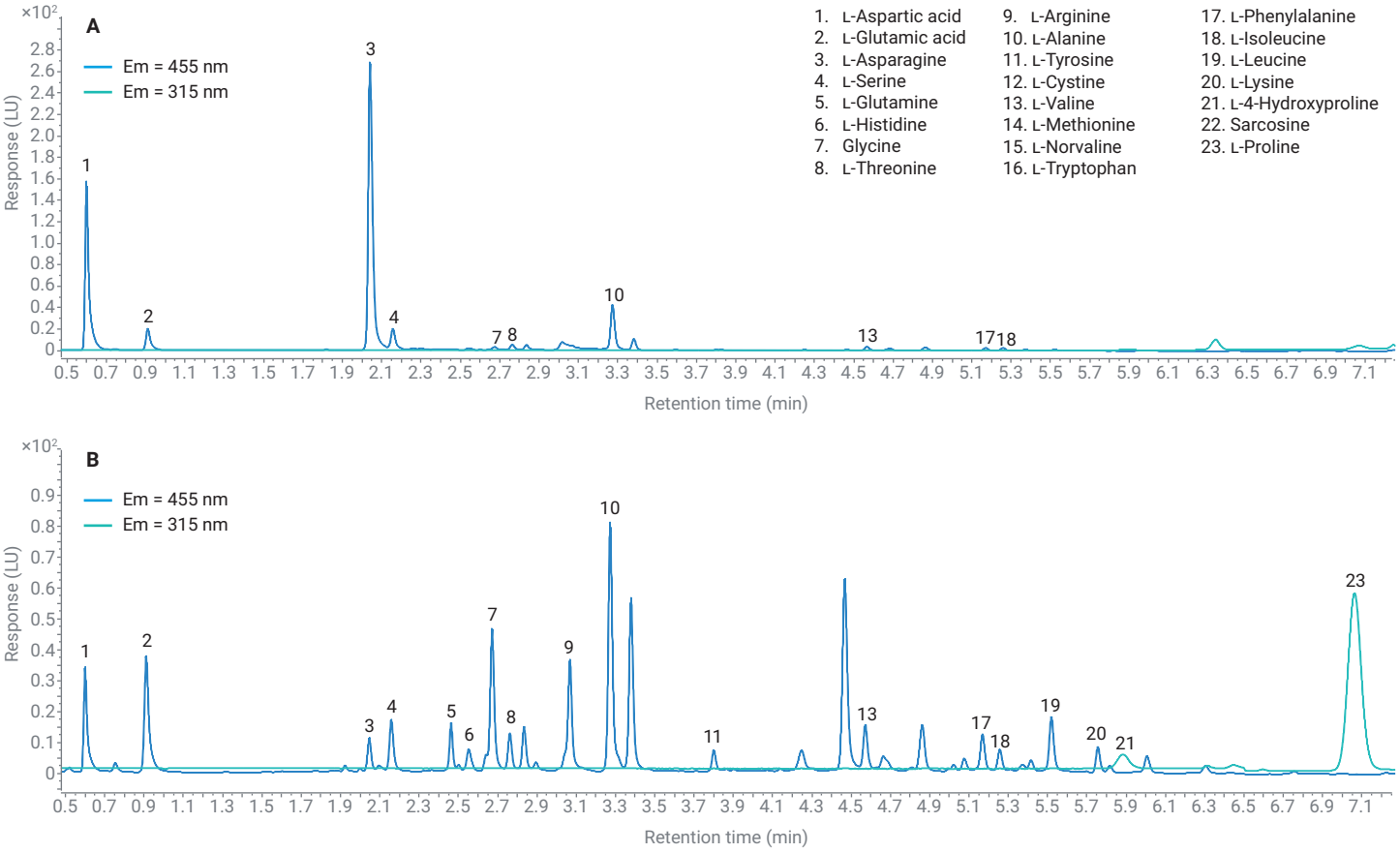

Figure 2. Analysis of amino acids in (A) soft drink and (B) red wine showing an overlay of FLD signals at an emission wavelength of 445 and 315 nm.

Conclusion

This application note demonstrates the use of a 1260 Infinity II Prime LC System for efficient and reliable analysis of amino acids. The 1260 Infinity II Multisampler was used for automated in-loop derivatization of amino acids with OPA and FMOC without the need of any manual work. Derivatization could be achieved in approximately 3.5 minutes and showed high reproducibility, with a peak area precision of less than 1% RSD for most of the compounds. Using the 1260 Infinity II Flexible Pump resulted in excellent chromatographic separation of 23 analytes in a run time of 9 minutes, showing a retention time precision of less than 0.1% RSD. Application of the 1260 Infinity II Infinity Fluorescence Detector enabled simultaneous detection of OPA and FMOC derivatives and showed a high sensitivity with LODs down to 0.225 pmol/µL for most of the compounds. Application of the method for the analysis of a red wine and soft drink sample demonstrates its potential for use in several application areas.

References

1. 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. Biomed. Appl.* **1988**, *431*, 271–284.
2. Davidson, I. Hydrolysis of Samples for Amino Acid Analysis. Methods in Molecular Biology - Protein Sequencing Protocols, Vol. 211, 2nd ed. Humana Press, Totowa, New Jersey; 2003.
3. Hitchcock, J. *et al.* Analysis of Amino Acids in Animal Feed Matrices Using the Ultivo Triple Quadrupole LC/MS System. *Agilent Technologies application note*, publication number 5994-0586EN, **2019**.
4. Long, D. *et al.* Rapid Method for Proline Determination in Grape Juice and Wine. J. *Agric. Food Chem.* **2012**, *60(17)*, 4259–4264.

www.agilent.com

DE.2627430556

Printed in the USA, October 15, 2024
5994-2189EN

Agilent

Trusted Answers