Separation of two sulfurated amino acids with other seventeen amino acids by HPLC with pre-column derivatization

Application

Abstract

An analytical method using an Agilent 1200 RRLC system was developed for the separation of 19 amino acids which contains two sulfurated amino acids and transferred to a Agilent 1100 HPLC system successfully. Good separation of this method was achieved by using Agilent ZORBAX Eclipse Plus C18 column on both two system. The results demonstrated that this analytical method was simple, reliable for analyzing these 19 amino acids, and the method is useful in comprehensively evaluating the quality of feed, food and pharmaceutical products which containing sulfurated amino acids.

Introduction

The detection and quantitation of amino acids have played an important role in feed, food and pharmaceutical Industry. Pre-column derivatization method with OPA and FMOC reagents for amino acids analysis has become a very reliable method. A wide variety of column dimensions could be successfully used for this application with almost all Agilent LC configurations[1]. However, all those applications only focused on standard amino acids or 17 hydrolyzed amino acids, and there are very few reports on separation of sulfurated amino acids together with all other amino acids.

Sulfurated amino acids are the common ingredient in feedstuff, and they could be destroyed by common hydrolysis method. this kind of amino acids were not as usual as other amino acids, and the analysis of the samples containing these amino acids by using the reverse phase LC method for separation of familiar amino acids[2] would be more difficult because their retention and separation characteristic. So an improved method was developed to meet the requirement of these kinds of amino acids, the method was firstly developed by using Agilent 1200SL RRLC system, and then transferred to a Agilent 1100 HPLC quaternary gradient system.

Experimental

Equipment:

Agilent 1100 Series LC system

Agilent 1100 Series LC system was used. It includes:

- Agilent 1100 Series quaternary Pump (G1311A)
- Agilent 1100 Series Degasser (G1379A)
- Agilent 1100 Series Autosampler (G1313A)
- Agilent 1100 Series Thermostatted Column Compartment (G1316A)
- Agilent 1100 Series Variable Wavelength Detector (G1314A)

Agilent 1200 SL Rapid Resolution LC system (RRLC)

For fast method development, an Agilent 1200 SL RRLC system was used. The system consisted of:

- Agilent 1200 SL RRLC System
- Agilent 1200 Series Binary Pump SL (Low Delay Volume Configuration) (G1312B)
- Agilent 1200 Series Degasser (G1379B)
- Agilent 1200 Series High Performance Autosampler SL (G1367D)
- Agilent 1200 Series Thermostatted Column Compartment SL (G1316B)
- Agilent 1200 Series Diode Array Detector SL (G1315C)

Chemicals and materials

HPLC-grade acetonitrile, methanol were purchased from Dimark Chemical LTD. Pure water was obtained from a Millipore pure water system. In the experiment the 100 pMoles/µL Amino Acid Standards (Agilent P/N 5061-3332),Borate Buffer (Agilent P/N 5061-3339), FMOC Reagent (Agilent P/N 5061-3337) and OPA Reagent (Agilent P/N 5061-3335) were used as the standard and the Derivatization Reagents, Methionine Sulfone and Cysteic Acid Standards were kindly provided by the customer.

Preparation of Mobile Phase and Injection Diluent

Mobile phase A: 10 mM Na2HPO4: 10 mM Na2B4O7, pH 8.2 (1.4 gm anhydrous Na2HPO4 +3.8 gm Na2B4O7 · 10H2O in 1 L water). Adjust the pH 8.2 with concentrated HCl.

Mobile phase B: Acetonitrile : methanol: water (45:45:10 by volume).

Injection Diluent: 100 ml mobile phase A + 1,500 µL concentrated H3PO4.

Pre-column derivatization Program (Autosampler settings)

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
2) Draw 0.5 µl from Sample vial
3) Mix 3 µl in washport 5 times
4) Wait 0.2 min
5) Draw 0.5 µl from OPA vial
6) Mix 3.5 µl in washport 6 times
7) Draw 0.4 µl from FMOC vial
8) Mix in 3.9 µl in washport 10 times
9) Draw 32 µl from Injection Diluent vial
10) Mix 20 µl in washport 8 times
11) Inject

Chromatographic conditions

	1100 Series LC system		1200 SL RRLC system	
Column (Agilent Zorbax Eclipse Plus C18)	4.6 × 150 mm, 5 µm		4.6 × 50 mm, 1.8 µm	
Flow Rate	2 mL/min		2 mL/min	
Time Table	Time (min)	B%	Time (min)	B%
	0	0	0	0
	3	0	1	0
	10.4	18.5	3.8	18.8
	23	57	8	57
	23.1	100	8.1	100
	27	100	9.4	100
	27.5	0	9.6	0
Stop Time(min)	27.5		9.6	
Post Time(min)	5		4.5	
Column Temp.	40°C		40°C	
Detector Settings	0~19 min: 338 nm; 19~27.5 min: 262 nm		338/10 nm Ref.=390/20 nm 262/16 nm Ref.=324/8	

Results and discussion

Figure 1 shows the separation of the same amino acids using the Agilent 1200SL RRLC method. A Zorbax Eclipse C18 column (4.6 × 50 mm, 1.8 µm) was used. The analytes were separated in approximately 10 minutes. Considering the re-equilibration time of the column, the method had a total run time of 14.1 minutes.

Figure 2 shows the separation of 19 amino acids using the Agilent 1100 HPLC method which was transferred from the 1200SL RRLC system. A same stationary phase with a dimension of 4.6 × 150 mm, 5 µm column was used. The analytes were separated within approximately 28 minutes. However, the runtime of this method was approximately 32.5 min to re-equilibrate the column, to ensure the reproducibility of the retention time of Aspartic acid which would be influenced distinctly by the re-equilibration status of the column.

A comparison of Figures 1 and 2 shows that the Agilent 1200SL RRLC system separates the analytes approximately 2.5 times faster than the Agilent 1100 HPLC system. Enhance the analysis speed is one of advantages of the STM (Sub two micro) technology and the UHPLC system.

The sensitivity of two methods was also different, for the same concentration and injection volume of the sample, the peak height of the RRLC method was more higher than the HPLC method, this because of less peak expanding taken by the shorter column and analysis time.

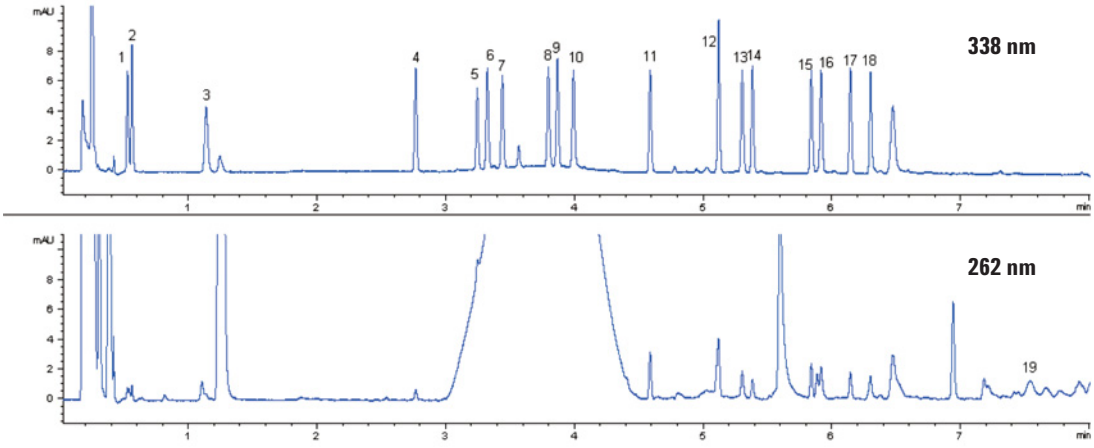

Figure 1. 19 Amino acids analysis on 1200SL RRLC system with Zorbax Eclipse Plus C18, 4.6 x 50 mm, 1.8-µm column, DAD.

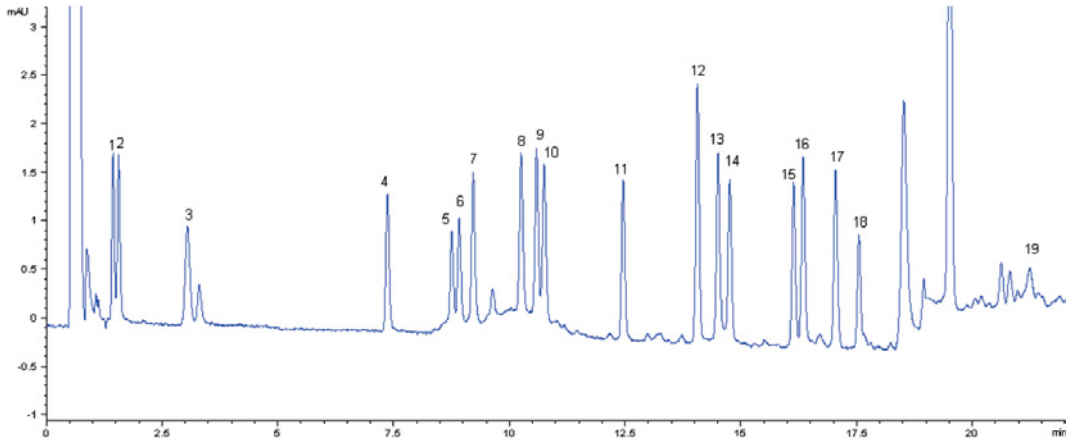

Figure 2. 19 Amino acids analysis on 1100 HPLC system with Zorbax Eclipse Plus C18, 4.6 x 150 mm, 5-µm column,VWD.

Table 1. Names and Order of Elution for OPA and FMOC

Peak #	AA name	AA abbreviation	Derivative Type
1	Methionine sulfone		OPA
2	Aspartic Acid	ASP	OPA
3	Glutamic Acid	GLU	OPA
4	Serine	SER	OPA
5	Histidine	HIS	OPA
6	Glycine	GLY	OPA
7	Threonine	THR	OPA
8	Cysteic Acid		OPA
9	Arginine	ARG	OPA
10	Alanine	ALA	OPA
11	Tyrosine	TYR	OPA
12	Cystine	CYS	OPA
13	Valine	VAL	OPA
14	Methionine	MET	OPA
15	Phenylalanine	PHE	OPA
16	Isoleucine	ILE	OPA
17	Leucine	LEU	OPA
18	Lysine	LYS	OPA
19	Proline	PRO	FMOC

Both Methionine sulfone and aspartic acid are very strong polar compounds, so it is very hard to be retained by the reversed phase column. The method use a 100% aqueous phase as the starting of the mobile phase gradient to make them retain and separate. For this reason, the re-equilibration time needs to be longer than usual to ensure the column totally re-equilibrated, otherwise the retention time of Methionine sulfone would be unstable, and the resolution with the Aspartic acid peak would also reduce.

Comparing to the result by using standard method transfer calculator, the gradient times in for 1100 were adjusted due to the different delay volume of 1100 LC and RRLC systems (with low delay volume configuration). The separation of Cysteic Acid, Arginine(ARG) and Alanine (ALA) was sensitive to this difference. when the method was transferred to 1100 HPLC system from 1200 RRLC, the separation changed. The gradient time was adjusted to compensate the difference of delay volumes.

Conclusions

All the 19 amino acid got a satisfactory separation by both the RRLC and HPLC method. The transferring of the two methods was easy, Because of the different delay volume of the two system, the gradient time table was adjusted. The use of 1.8 µm sized particle columns has enabled the cycle time of the analysis to be cut in half, from 32.5 minutes to 14.5 minutes. The retention and resolution of the early eluting Methionine sulfone and aspartic acid could meet the requirements of quantitative analysis. The peak shapes and resolution of other peaks were also good.

References

1. John W Henderson Jr and Anne Brooks, Agilent App. Note, 5990-4547EN (2009)
2. Cliff Woodward and John W. Henderson Jr. Agilent App. Note, 5989-6297EN (2007)

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