Analysis of Amino Acids and Biogenic Aminesby High Performance Liquid Chromatography

การวิเคราะห์กรดอะมิโน และไบโอเจนิกเอมีนโดยโครมาโทกราฟีของเหลวสมรรถนะสูง

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ABSTRACT

The simultaneous analysis of 10 amino acids (arginine, aspartic acid, asparagine, glutamic acid, histidine, leucine, lysine, phenylalanine, tryptophan and γ-aminobutyric acid) and 6 biogenic amines (cadaverine, putrescine, histamine, spermidine, tryptamine and tyramine) as their o-phthalaldehyde-ethanethiol (OPA-ET) derivatives prior to reversed phase high performance liquid chromatography (RP-HPLC) with fluorescence detection has been studied. Parameters affect the analysis such as derivatization conditions and optimum HPLC condition were investigated. Gradient elution with a mixture of methanol and water was used, and complete separation was achieved within 30 min. The proposed method is easy and was applied to determine amino acids and biogenic amines in beer samples.

บทคัดย่อ

งานวิจัยนี้ได้ศึกษาการวิเคราะห์ปริมาณกรดอะมิโน 10 ชนิด (อาร์จินิน กรดแอสพาร์ติก แอสพาราจิน กรดกลูตามิก ฮิสทิดีน ลิวซีน ไลซีน ฟีนิลอะลานีน ทริปโทแฟน และกรดแกรมมาอะมิโนบิวไทริก) และไบโอเจนิกเอมีน 6 ชนิด (คาดาเวอรีน พูเทรสซีน ฮิสทามีน สเปอร์มิดีน ทริปทามีน และไทรามีน) พร้อมกันโดยการเตรียมอนุพันธ์กับ ออร์โท-พาทาลอัลดีไฮด์-อีเทนไทออล ก่อนการแยกด้วยโครมาโทกราฟีของเหลวสมรรถนะสูงแบบรีเวิร์สเฟส และตรวจวัดแบบฟลูออเรสเซนต์ ได้ศึกษาปัจจัยที่มีอิทธิพลต่อผลการวิเคราะห์ เช่น สภาวะการเตรียมอนุพันธ์ และสภาวะที่เหมาะสมในการแยกด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง การวิเคราะห์สามารถเสร็จสิ้นภายใน 30 นาที โดยการชะแบบเกรเดียนท์ด้วยเมทานอลและน้ำ วิธีการวิเคราะห์นี้ง่าย และได้นำไปประยุกต์ใช้ในการวิเคราะห์ปริมาณกรดอะมิโน และไบโอเจนิกเอมีนในตัวอย่างเบียร์

Key Words : Amino acids, Biogenic amines, O-phthalaldehyde
คำสำคัญ : กรดอะมิโน ไบโอเจนิกเอมีน ออร์โท-พาทาลอัลดีไฮด์

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Introduction

Amino acids are small biomolecules that consist of basic amino group and acidic carboxyl group. They play central roles both as building block of protein and as intermediates in metabolism. The decarboxylation of amino acids or their metabolites give rise to amine, known as biogenic amines. Biogenic amines are nitrogen compounds of biological matters that formed during normal metabolic processes in living organisms and are present in food products especially fermented products. Histamine, putrescine, cadaverine, tyramine, tryptamine, and spermidine are considered to be the most important biogenic amines occurring in foods, they induce toxicological and health risks (Karovičová and Kohajdová, 2005; Ertan Anli et al., 2006). Analysis of biogenic amines is important because of their toxicity and their usage as indicators of the degree of freshness or spoilage of foods (Bauza et al., 1995; Herraez-Hernandez et al., 2006). Several modern analytical methods mainly based on liquid chromatography have been developed for determination of amino acids and biogenic amines in foods. UV detection can not be used to determine most of them due to the lack of chromophores. The detection of amino acids and biogenic amines is widely achieved via fluorimetry with derivatization (Nedjeljko, 1996; González et al., 2006; Peris-Vicente et. al., 2006).

Beer is the alcoholic beverage that consumed all over the world (Hughes and Baxter, 2001). Consumption of beer may pose chemical risks to human health due to the occurrence of biogenic amines during its fermentation process.

This research is aim to develop reversed phase HPLC for simultaneous determination of biogenic amines and their amino acid precursors using OPA-ET as derivatization reagent and apply the proposed method to determine amino acids and biogenic amines in beer samples.

Materials and methods

Standards and reagents

Biogenic amine standards (cadaverine, putrescine, histamine, spermidine, tryptamine and tyramine), some amino acids standards (asparagine, glutamic acid, and γ-aminobuthyric acid), sodium tetraborate, ethanethiol (ET) and *o*-phthalaldehyde (OPA) were from Fluka; Switzerland, others amino acids (arginine, aspartic acid, histidine, leucine, lysine, phenylalanine and tryptophan) were from Acros; Belgium, methanol for HPLC from Lab-Scan; Thailand, boric acid from Merck; Germany, sodium hydroxide from Carlo Erba; France, ultrapure water with resistivity 18.2 MΩ cm was obtained from a Milli-Q system (Millipore).

Preparation of standard and reagent solutions

Standard solutions of amino acids and biogenic amines (10 mmol/L each) were prepared in de-ionized water. All of standard solutions were stored at 4 + 1 OC and used within a month.

The HPLC grade methanol was used as a solvent to prepared 100 mmol/L OPA and 1,000 mmol/L ET solutions. These reagents were stabled at 4 ± 1°C for a month.

Chromatographic conditions

The HPLC system was a Waters HPLC instrument equipped by a Waters515 controller

binary pump, with Waters in-line degasser AF and a 20 μL Rheodyne injector, detected with Jasco FP920 fluorescence detector and operated with Millenium32® software. The column was a Symmetry C8 column, Waters (3.9 x 150 mm, 5 μm).

Chromatography was operated under ambient temperature with a flow rate of 0.5 mL/min. Detection has been carried out by fluorescence detection at the excitation wavelength of 340 nm and emission wavelength of 457 nm. Mobile phase composition was investigated.

Preparation of derivatization reagent

Three milliliters of 100 mmol/L OPA solution and 2 mL of 100 mmol/L Borate buffer pH 9 were transferred into a 10 mL volumetric flask and mixed for 1 min, then 3 mL of 1,000 mmol/L ET solution was added and mixed for 1 min, finally the mixing solution was diluted with MeOH.

Derivatization procedure

An appropriate volume of standard AAs and BAs and/or extract from samples was transfered into 10 mL vial and mix for 1 min with appropriate volume of OPA-ET reagent then filtered through 0.22 mm nylon filter before injecting into HPLC system.

Study on parameters affect derivatization

1) *pH*

pH of 100 mmol/L borate buffer solution was studied at pH 8, 9 and 10.

2) *Methanol content in OPA-ET reagent*

Content of methanol in OPA-ET reagent was studied using three difference reagents A, B and C contained methanol 40, 60 and 70%, respectively (**Table 1**). An appropriate content of methanol was selected by considering peak area and precipitation of the derivatives.

Table 1 Composition of reagent A, B and C

Reagent	OPA 50 mmol/L	ET 50 mmol/L	Buffer 100 mmol/L	MeOH
A	2.5*	7.5	15	0
B	2.5	7.5	10	5
C	2.5	7.5	7.5	7.5

*volume in mL

3) *Molar ratio of OPA:ET*

Molar ratio of OPA:ET reagent was investigated using three various ratios 1:3, 1:10 and 1:20, respectively.

Sample preparation

Beer sample was sonicated with ultrasonic bath for 15 min, filtered through 0.22 mm nylon filter before derivatized with OPA-ET and finally injected into HPLC.

Results and discussion

Chromatographic conditions

The optimum compositions of mobile phase were achieved by gradient elution of methanol and water as shown in **Figure 1.** Complete separation was obtained without any buffer eluent application. The separation of 10 amino acids and biogenic amines was achieved within 30 min, as the chromatogram shown in **Figure 2A.**

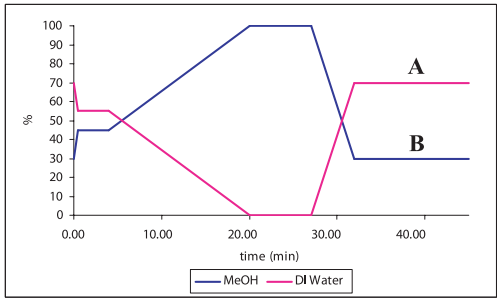

Figure 1 Gradient elution profile of (A) MeOH and (B) water.

Derivatization of amino acids and biogenic amines

The reaction was simply occurred in mild condition at room temperature and basic condition. Factors affect efficiency of derivatization was investigated such as buffer pH, methanol content and molar ratio of OPA to ET.

1) *pH of buffer solution*

Borate buffer pH 8, 9 and 10 produced OPA-ET reagent pH 9, 11 and 14, respectively. Although derivatizes could occurred at all the studied pH. However, buffer pH 9 was chosen for further studies because the reaction is completed and to prevent the damage of HPLC column from high pH.

2) *Methanol content in OPA-ET reagent*

To study effect of MeOH content, 3 difference reagents (A, B and C) were used for demonstration. Reagent A, B and C contained MeOH 40, 60 and 70%, respectively (Table 1). These reagents could produce amines-OPA-ET derivatives for all the studied amino acids and biogenic amines. All of the final solutions were clear, except the solutions of cadaverine and putrescine with reagents A and B (Table 2). However, these solutions were clear when reagent C was used. It can be concluded that methanol content in the reagent affect solubility of the derivatives. Thus, reagent C contained 70% methanol was used throughout.

3) *Molar ratio of OPA:ET*

Three various molar ratio of OPA:ET = 1:3, 1:10 and 1:20 were investigated. Peak area acquired from these reagents was not significant difference. However, reagent contained OPA:ET at 1:3 was unstable after 15 hours, while the other ratios (1:10 and 1:20) can be used for 24 hours. The reagent contained OPA:ET at 1:10 was chosen for further studies, due to high toxic to respiratory system of ET.

Table 2 Derivatization of 10 amino acids and 6 biogenic amines with reagents A, B and C

AAs or BAs	code	A	B	C
Asparagic acid	Asp	✓	✓	✓
Glutamic acid	Glu	✓	✓	✓
Asparagine	Asn	✓	✓	✓
Histidine	His	✓	✓	✓
γ-aminobutyric acid	GABA	✓	✓	✓
Tryptophan	Trp	✓	✓	✓
Phenylalanine	Phe	✓	✓	✓
Leucine	Leu	✓	✓	✓
Arginine	Arg	✓	✓	✓
Lysine	Lys	✓	✓	✓
Histamine	Htm	✓	✓	✓
Tryptamine	Tpm	✓	✓	✓
Putresscine	Put	✓P	✓P	✓
Cadaverine	Cad	✓P	✓P	✓
Tyramine	Trm	✓	✓	✓
Spermidine	Spd	✓	✓	✓

Note : ✓ = obtained peak, P = cloudy solution

Application

Figure 2B shows a typical chromatogram obtained from beer sample. Identification of peaks

was performed using retention time and also confirmed by spiking test, seven amino acids were found in beer samples.

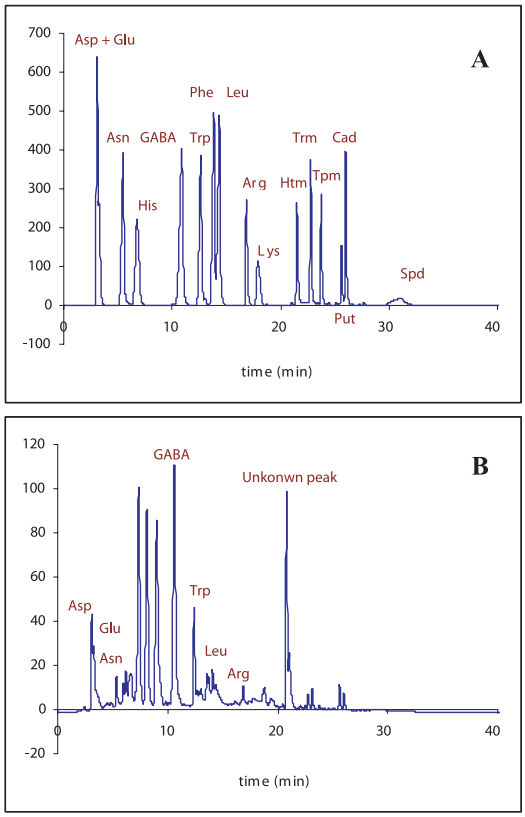

Figure 2 Chromatograms of standard amino acids and biogenic amines (A) and beer (B).

Conclusion

The method for the simultaneously analysis of biogenic amines and their amino acids precursors was developed. The method based on precolumn derivatization with OPA-ET and reversed phase HPLC analysis of the derivatives. The proposed method can analyze 10 amino acids and 6 biogenic amines in one analysis and shows the ability to analyze amino acids and biogenic amines in beer samples which the quantitative study is carry on.

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