DETERMINATION OF THE TOTAL TRYPTOPHAN IN FOOD BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COMBINED WITH ENZYME HYDROLYSIS

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Abstract

High performance liquid chromatography was applied for the determination of tryptophan in food. The sample was hydrolyzed in a duration from 16 to 24 hour by protease enzyme at 50°C. The extract was separated on the C8 reversed phase column with mobile phase of NaH_2PO_4 50mM pH 2.3: Methanol (82:18; v/v). The linearity of the method was kept in the range of 0.5 - 50 mg/L. Limit of detection was found to be 4.6 mg/100g. Recovery was determined by standard addition method, giving values of recovery in the range of 97 - 103% and RSD (n = 6) in the range of 0.077 - 2.27%. Internal standard was used to reduce the errors in analysis process and good reproducibility.

Keywords: *HPLC, Tryptophan, food, amino acid*

1. INTRODUCTION

Tryptophan (Tryp) is an essential amino acid that the human body cannot synthesize itself, but must be supplemented through diet. It has two important functions that are (1) to be converted into niacin (vitamin B3) by the liver and (2) to provide the precursor of serotonin - a neurotransmitter that helps the body regulate appetite and good sleep [5,7]. Tryp is found in natural food containing protein, nutritional products and pharmaceuticals. Heating in food processing can lead to disruption of protein bonds. Development of analytical methods to determine Tryp content is necessary to assess food quality.

Currently, there are numerous methods for determination of Tryp in food. Among them, HPLC method was the most common [4, 6, 8, 9]. In order to separate and quantify the Tryp on the HPLC system, the sample preparation involves hydrolysis of protein to form free amino acids, including the Tryp. The published studies focused on optimizing the protein hydrolysis conditions, using reagents such as acid, alkaline and enzymatic. Hydrolysis using acid or alkaline may result in low performance due to oxydation of Tryp. Protease enzyme can improve this problem because enzymes were specific to substrate and the hydrolysis was not affected by high temperature. Therefore, this study focused on optimization of the enzyme hydrolysis combined with a high performance liquid chromatographic method to determine the total tryptophan content in food.

2. MATERIALS AND METHOD

Standards and reagents: Tryptophan and 5-Methyl-DL-Tryptophan were from Sigma-Aldrich. Trizma base and Protease from *Streptomyces griseus* were from Sigma Aldrich; other chemicals including NaH_2PO_4, HCl, Methanol, Acetonitril were from Merck.

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Instrument: HPLC (Waters) with fluorescence detector, column YMC PAC C8 (3 mm x 50 mm x 3 µm)

3. RESULTS AND DISCUSSION

3.1. HPLC conditions

Mobile phase is the decisive factor for chromatographic separation efficiency. In general, the mobile phase can affect selectivity, solubility, resolution, the width of peak and so on. Referred to former studies [8,9] and based on the current procedure of the laboratory, columns C8 and C18 combined with some mobile phases such as: NaH_2PO_4 - Methanol, H_2O - Acetonitril at the ratio of 82: 18 were applied. The standard chromatogram using column C8 was shown in Fig. 1:

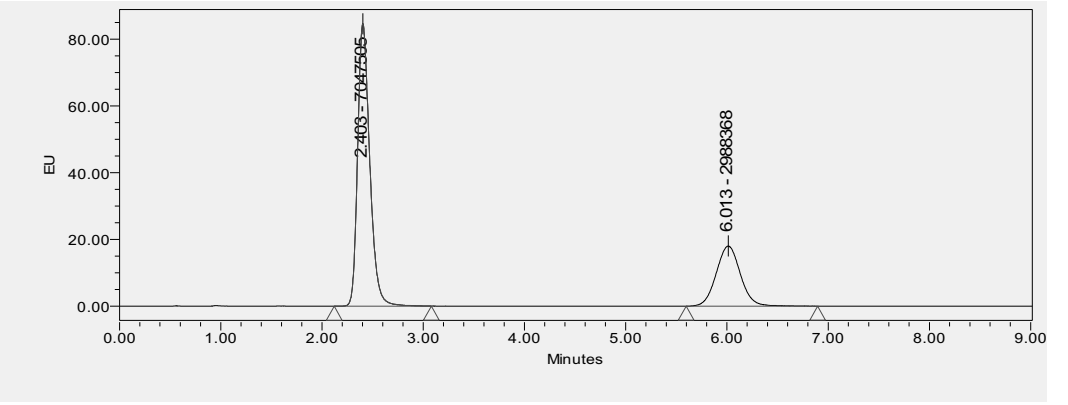

Figure 1. *Chromatogram of Tryp and -Methyl-DL-Tryptophan (C8 column, 3 mm x 50 mm x 3 µm, mobile phase NaH_2PO_4 – Methanol)*

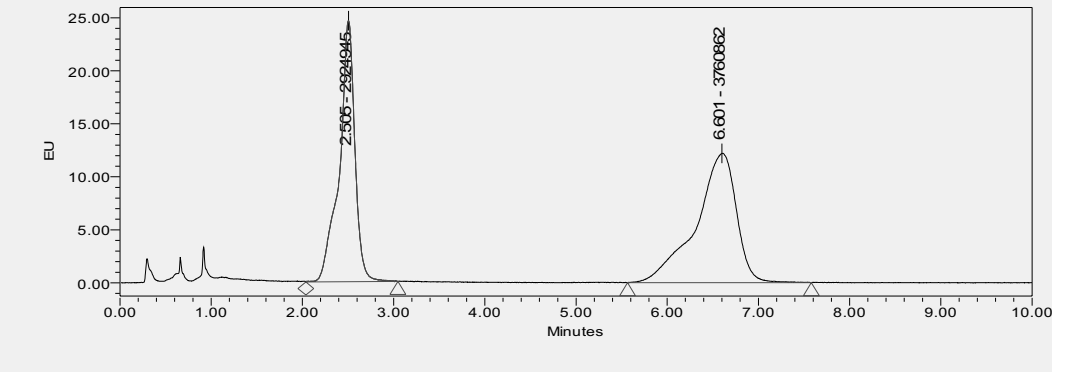

Figure 2. *Chromatogram of Tryp and -Methyl-DL-Tryptophan (C8 column, 3 mm x 50 mm x 3 µm, mobile phase H_2O - Acetonitril)*

The results showed that for C8 column combined with mobile phase of water - ACN the spreaded peaks were achieved. For C8 column combined with mobile phase of NaH_2PO_4 50 mM pH 2.3 and MeOH very sharp and smooth peaks were achived. The results of combination of C18 column with mobile phase of water - ACN showed that peaks were spread and large. While using the mobile phase of NaH_2PO_4 50 mM, pH = 2.3 and MeOH gave very sharp, smooth peaks. However, the biggest disadvantage of C18 column was the long analysis time required.

As a result, in this study, C8 column was selected with mobile phase of NaH_2PO_4 50 mM, pH 2.3 and MeOH

The optimal conditions for determination of Tryp by HPLC: C8 column (3 mm x 50 mm x 3 µm), fluorescence detector with Excitation wavelength: 295 nm, Emission wavelength: 345 nm; flow rate of 0.5 mL/min, sample injection volume: 10 µL; and mobile phase: NaH_2PO_4 50 mM, pH

= 2.3: Methanol (82:18, v/v).

3.2. Sample preparation

The sample preparation involves hydrolysis of protein to form free amino acids, including Tryp. The amount of enzyme depends on protein content. In order to completely hydrolyze the protein and avoid waste of enzymes, it is necessary to investigate the ratio of enzyme and sample amount. In addition, hydrolysis time and hydrolysis temperature also greatly affect the performance of hydrolysis. Therefore, optimizing parameters of sample preparation include ratio of enzyme amount and sample amount, hydrolysis duration, and hydrolysis temperature.

- During sample treatment, 0.5 mL of 5-Methyl-DL-Tryptophan internal standard was added to all samples to control hydrolysis.

- The protease enzyme also was hydrolyzed to Tryp because it is a protein. Therefore, a blank analysis should be carried out in all analysis batches.

- For milk powder samples, it is necessary to reconstitute the sample before the analysis. Reconstitution was as the following: 25 g milk powder was weighted accurately in a 500 mL glass bottle, distilled water was added to 225 g, sample was shaken well and stir on the stirrer (about 30 minutes) to ensure homogenization. Reconstitution would help increase uniformity and representativeness of the sample.

3.1.2.1. Hydrolysis temperature

Hydrolysis temperature greatly affects enzyme activity. High temperatures inactivate the enzyme, while low temperatures prolong the analysis process and reduce the hydrolysis efficiency. Affection of hydrolysis temperature was shown in Fig. 3.

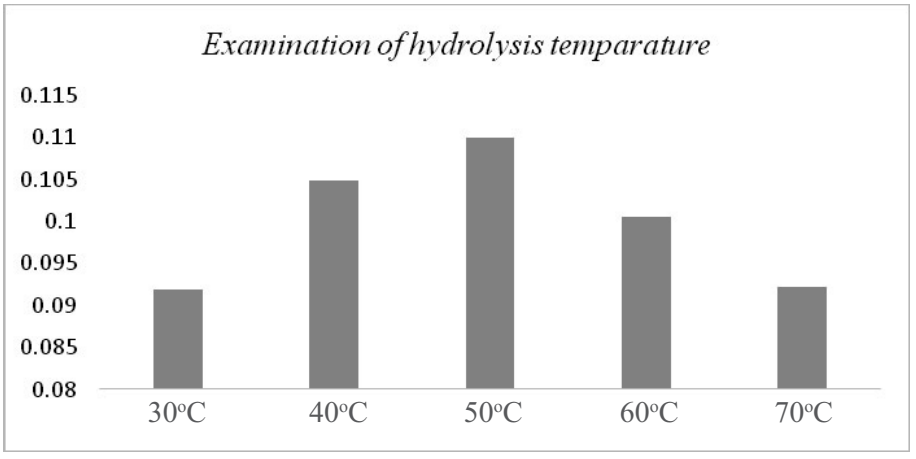

Figure 3. *Tryp content obtained at different temperatures*

The highest Tryp content was obtained when hydrolysis was fixed at 50°C. At the temperature higher than 50°C, inhibited enzyme caused low content of Tryp. Turbid sample solutions obtained at low temperatures (30, 40°C) and high temperatures (60, 70°C) may be due to the incomplete hydrolysis. At the temperature of 50°C, clean sample solutions with the highest Tryp content were obtained. Therefore, the temperature of 50°C was selected for the optimization.

3.1.2.2. Hydrolysis duration

Reconstituted milk samples were used in order to investigate the optimum hydrolysis duration. The results were shown in Fig. 4:

There was a change in Tryp content obtained at different hydrolysis durations. The Tryp content increased as the hydolysis duration rose from 10 to 16 hours. This can be explained that lower time (<14 hours) was not enough to completely hydolyze the protein. In order to save time, the hydrolysis duration of 16 hours was selected. In the case that the next steps could not be immediately proceeded,

samples could be allowed to take to 30 hours.

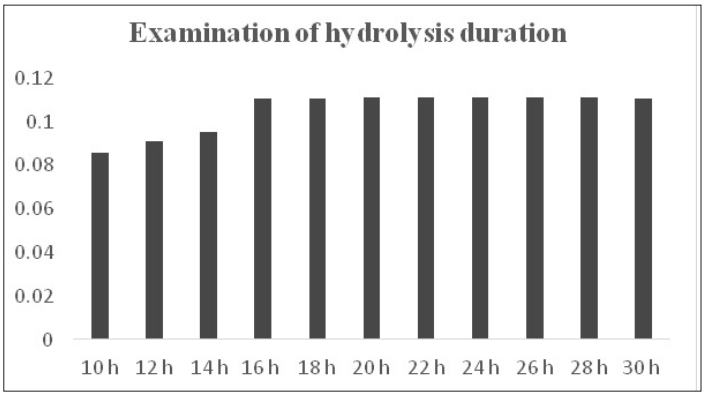

Figure 4. Tryp content obtained at different hydrolysis duration

3.1.2.3. Ratio of enzyme amount and sample amount

Samples containing different protein contents such as: Milk powder (15 - 21%), liquid milk (2.8 - 3.2%), soybean (35 - 40%), beef (15 - 20%), and egg (11 - 14%) were used to investigate optimal amount of enzyme. The amount of 0.5 mL of protease enzyme was added to all samples while changing the volume of sample. Solution obtained from reconstitution of milk powder (15 - 21% protein) with a proportion of about 10% was similar to liquid milk. Tryp contents were insignificantly changed during the process. For the sample amount of 3.5 - 4g, the Tryp content decreased due to the fact that the amount of enzyme was not enough to hydrolyze the sample. Therefore, for these liquid milk and powdered milk samples, the amount of 2.5 g sample would be hydrolyzed completely by 0.5 mL of protease enzyme.

Eggs are high protein products (11 - 14%), therefore, the amount of sample for hydrolysis should be small. With the sample amount of 0.05g to 2g, results showed that Tryp content was not significantly different, however, with the sample amount of 0.5g to 2g, Tryp content decreased gradually. Therefore, for egg samples, it was necessary to weigh about 0.1 g so that the protein in the sample could be completely hydrolyzed.

A beef sample is about 18% in content of protein. With the sample amount of 0.05 g to 0.15 g, the Tryp was not significantly different, indicating that the amount of protease enzyme could hydrolyze thoroughly the protein content in the sample. With the sample amount of 0.2 g to 2 g, enzyme was not enough to hydrolyze the protein and resulted in reducing the Tryp content.

Soybean has very high protein content (41%). With the sample amount of 0.025g to 0.05g the Tryp content was not significantly different, indicating that the amount of protease enzyem was 0.5 mL of hydrolyzed protein completely. With the sample amount of 0.1 g to 1 g, the enzyme amount was not enough to hydrolyze the protein, so the Tryp content decreased.

The optimal conditions for sample prepartion were: 0.1g of meat; 2.5g of reconstituted milk of liquid milk; 0.05g of soybean; 0.1 g of egg; add the amount of 0.5 mL of internal standard and protease enzyme, and then 3mL of Tris. Sample was hydrolyzed overnight within 16 - 24h at the temperature of 50^{o}C.

3.2. Method validation

3.2.1. Limit of detection (LOD) and limit of quantitation (LOQ)

Based on the standard deviation of 10 blank samples, the corresponding LOD and LOQ were determined to be 4.6 mg/100g and 5.7 mg/100g.

3.2.2. Calibration curve

The stock standard solution was diluted and added a fixed amount of internal standard to different standard concentrations, achieving a working range. Solutions were injected into the HPLC

system with optimal conditions. The correlation between the ratio of Tryp concentration and internal standard with ratio of Tryp peak area and internal standard area was linear in the range of 0.5 - 5 ppm with correlation coefficient $R^2 = 0.9998$.

3.2.3. Repeatability

Repeatability was carried out on samples such as milk powder, liquid milk, meat and eggs. The analysis was repeated six times to evaluate the repeatability. The analytical results showed good repeatability as required by AOAC. The RSD% of milk powder, liquid milk, meat, eggs were 0.77; 1.56; 2.05 and 2.27%, respectively.

3.2.4. Intermediate reproducibility

In order to evaluate the internal reprodutibility of the method, samples including powdered milk, liquid milk, meat, eggs were analyzed by two analysts on two different days The analytical results showed good repeatability as required by AOAC. The RSD% of milk powder, liquid milk, meat, eggs, and beans were 1.16; 0.78; 1.69; 2.17 and 1.92%, respectively.

3.2.5. Recovery

Liquid milk, milk powder, meat, eggs and soybeans, were spiked with three different concentration levels (low, medium and high). Repeat analysis three times in order to evaluate the recovery rate. The recovery rate was in the range of 97% - 103%, showing that the method meet the requirements of AOAC.

3.3. Analysis of food samples

Results of analysis of Tryp content on 23 food samples collected in Hanoi were shown in Table 1:

Table 1. *Tryp content on 23 food samples*

No.	***Sample***	***Tryp content (g/100g)***
1	Beef	0.321
2	Pork	0.297
3	Chicken	0.217
4	Cashew nut 1	0.388
5	Cashew nut 2	0.394
6	Walnut	0.228
7	Peanut	0.307
8	Bean	0.452
9	Seaweed	0.839
11	Egg 1	0.201
12	Egg 2	0.215
13	Dietary supplement 1	2.45
14	Dietary supplement 2	0.084
15	Dietary supplement 3	0.183
16	Milk powder 1	0.201

17	Milk powder 2	0.259
18	Milk powder 3	0.184
19	Milk powder 4	0.233
20	Liquid milk 1	0.024
21	Liquid milk 2	0.044
22	Liquid milk 3	0.056
23	Liquid milk 4	0.078

The table showed that, Tryp content was in ranges from 0.217 to 0.321 g/100g in meat products. In particular, Tryp content in beef was the highest (0.321 g/100g). Tryp content in bean samples ranged from 0.228 to 0.452 g/100g. The Tryp content of soybeans was the highest (0.452 g/100g). Two samples of chicken eggs had the same Tryp content. The seaweed samples had very high Tryp content of 0.839 g/100g. In the four samples of milk powder, Tryp content were in the range from 0.184 to 0.259 g/100g. In four samples of liquid milk, Tryp content were ranges from 0.024 to 0.078 g/100g. Tryp content ranges from 0.084 to 2.45 g/100g in the three functional food products.

4. CONCLUSIONS

The study was successful in determining the Tryp content in food samples by high-performance liquid chromatography combined with fluorescent detector. The analytical procedure was quite simple, the method was assessed for specificity, calibration, repeatability, accuracy and detection limits. The method validation results showed that the process was appropriate to determine the Tryp content in food. The method was applied to analyze 23 food samples for different Tryp content depending on the matrices.

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Tóm tắt

XÁC ĐỊNH HÀM LƯỢNG TỔNG TRYPTOPHAN TRONG THỰC PHẨM BẰNG PHƯƠNG PHÁP SẮC KÝ LỎNG HIỆU NĂNG CAO KẾT HỢP THỦY PHÂN MẪU BẰNG ENZYME

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Phương pháp sắc ký lỏng hiệu năng cao được áp dụng để xác định hàm lượng tryptophan trong thực phẩm. Mẫu được thủy phân 16-24 giờ bằng protease enzyme ở 50°C. Dịch chiết được tách trên cột C8 với pha động là đệm NaH_2PO_4 50mM pH 2,3 : Methanol (82:18;v/v). Khoảng tuyến tính của Tryptophan 0,5 - 50 mg/L. Giới hạn hạn phát hiện của phương pháp là 4,6 mg/100g. Độ thu hồi được thực hiện bằng phương pháp thêm chuẩn, kết quả thu được trong khoảng 97% -103% và RSD (n = 6) trong khoảng 0,77% - 2,27% cho thấy phương pháp có độ đúng và độ chính xác tốt. Sử dụng nội chuẩn giúp quá trình phân tích giảm sai sót và cho kết quả lặp lại tốt.

Từ khóa: *HPLC, Tryptophan, thực phẩm*