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USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE QUANTITATIVE ANALYSIS OF AMINO ACIDS IN VIETNAM EDIBLE BIRD'S NEST

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ABSTRACT: This paper describes a method utilizing high-performance liquid chromatography with fluorescence detection (HPLC-FLD) developed for the simultaneous quantitation analysis of 15 acidic amino acids in Edible Bird's Nest. Samples underwent hydrolysis in acidic media (1:1 HCl) at 100 °C for 16.0 hours, followed by neutralization with NaOH (30 %). The mean recoveries of the 15 analytes ranged from 80.1 % to 110.8 %, with MDL and MQL values of 2.0 ppm and 6.0 ppm, respectively. The method demonstrated accuracy, repeatability, and recovery unaffected by matrix effects. Its application enabled the determination of total amino acids content in Vietnam bird's nest samples, yielding results of 43.30 to 45.08 g/100 g for refined bird's

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nest, 36.69 to 41.40 g/100 g for raw bird's nest, and 1221 to 1723 mg/kg for distilled bird's nest.

Keywords: amino acids, bird'nest, pre-column, HPLC-FLD

Introduction

Edible Bird's Nest (EBN) holds a revered status among Vietnamese, Korean, and Chinese communities for its culinary excellence. This esteemed delicacy is crafted from the solidified saliva of insectivorous swiftlets, which form intricate nests. The processed EBN commands prices ranging from 3.0 to 6.00 million Vietnamese dong (VND)/100 g, while the unprocessed variant ranges from 1.00 to 2.00 million VND/100 g. This substantial price tag reflects both its esteemed value and the widespread recognition of its nutritional advantages, which are brimming with essential trace elements that promote health and well-being. EBN is rich in glycoproteins, primarily comprising proteins (62.0 – 63.0 %) and carbohydrates (25.6 – 27.3 %). Among these carbohydrates, 9% consist of sialic acid, 7.2 % galactosamine, 5.3 % glucosamine, 16.9 % galactose, and 0.7 % fructose, contributing to its esteemed nutritional profile.[1,2] Meanwhile, EBN contains all 9 essential amino acids, and 9 non-essential amino acids, totaling 30.17 g/100 g of EBN samples. These amino acids are crucial for supporting various vital in vivo processes.[3] EBN has been reported to prevent influenza virus infection by neutralizing the virus and inhibiting the agglutination of red blood cells caused by the virus.[4] Matsukawa et al. showed that EBN exhibited the ability to reduce bone density loss by improving calcium concentration and slowing down the aging of the skin.[5] EBN was also found to synergistically induce cell proliferation which is important in cell regeneration and wound healing.[6] The extract of EBN was found to demonstrate a neuroprotective effect, enhancing memory and suppressing neuroinflammation in Wistar rats. It's suggested that the presence of sialic acid from EBN could hold potential benefits in combating neurodegenerative diseases.[7] All these studies have highlighted the significant interest in Edible Bird's Nest (EBN) for enhancing consumer health, thereby benefiting the development and improvement of the immune system. Chemical methods commonly employed for EBN characterization primarily rely on chromatographic techniques, including gas chromatography (GC) and liquid chromatography (LC), coupled with various types of detectors. Research reports have extensively documented the applications of GC and LC analytical techniques in determining the structure of glycans and a diverse range of compounds such as amino acids, monosaccharides, fatty acids, and hormones in EBN, food, and pet food.[8-12]

This study presents a straightforward method for the simultaneous quantification of 15 acidic amino acids in Edible Bird's Nest (EBN) within a short timeframe, utilizing o-Phthalaldehyde (OPA) as a derivatizing agent. OPA reacts with primary amines in the presence of 2-Mercaptoethanol to produce highly fluorescent products. The pre-column reaction of amino acids with o-Phthalaldehyde, facilitated by 2-Mercaptoethanol (MCE), occurs rapidly at room temperature, resulting in the formation of isoindolic derivatives in a straightforward manner. This method was effectively applied to assess the amino acid content following the hydrolysis of EBN using HPLC-FLD in the samples collected in the Vietnam market.

Materials and method

Chemicals and Reagents

All reagents utilized in the experiment were of analytical grade. Reference standards for Aspartic acid (Asp), Glutamic acid (Glu), Serine (Ser), Histidine (His), Arginine (Arg), Glycine (Gly), Threonine (Thr), Tyrosine (Tyr), Alanine (Ala), Methionine (Met), Valine (Val), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu), and Lysine (Lys) were procured from TRC (Canada). Methanol and Acetonitrile, both of HPLC grade, were obtained from J. T. Baker (Phillipsburg, USA). O-phthalaldehyde, 2-mercaptoethanol, and formic acid (FA) were sourced from Sigma-Aldrich (Germany).

Instrumentation

The method development and optimization experiments were conducted on a high-performance liquid chromatography (HPLC) system including the column oven and thermostat autosampler (HPLC 1260, Agilent Technologies) with the column (Eclipse plus C18 250 x 4.6 mm, 5 µm column) in combination with fluorescence detector. The fluorescence was followed using an excitation wavelength of 340 nm and an emission wavelength of 450 nm.

Standard solutions

Individual stock standard solutions of each amino acid at a concentration of 1.0 mg/mL each were prepared in deionized water (DI) and hydrochloric acid (HCl). A working solution was then prepared before use by diluting 0.1 mL of the stock standard solution with 10 mL of DI water, resulting in a known concentration of 0.01 mg/mL. All stock solutions were refrigerated at 2 – 8 °C when not in use.

Online Pre-column derivatization

The derivatization reaction of amino acids with OPA was conducted online in the HPLC autosampler, following a procedure adapted from a referenced document and adjusted to suit real conditions.[13] Briefly, the derivatization reaction was achieved by thoroughly mixing borate buffer (0.4 M $Na_2B_4O_7 \cdot 10H_2O$ buffer at pH 10.6), derivatization reagent (prepared as 5 mM of OPA, 225 mM of 3-mercapto-propionic acid in 0.4 M borate buffer), diluent (1.0 M acetic acid), and samples solution at room temperature. After derivatization, the solution was injected into the column. The entire procedure, including needle washing, took approximately 5.0 minutes. Importantly, the derivatizing reagents were frozen and stored in the dark until use, and the borate buffer was freshly prepared.

Mobile phase A: A mixture containing 3.8 g Sodium tetraborate decahydrate ($Na_2[B_4O_5(OH)_4] \cdot 8H_2O$), 1.4g Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), and 0.032g extra pure Sodium azide (NaN_3) was dissolved in a 1 L glass container filled with 600 mL of distilled water. The pH was adjusted to 7 using 1:1 HCl and the volume was adjusted to 1 L with distilled water. Finally, the solution was filtered through a 0.45 µm filter and degassed for 15 minutes.

Mobile phase B: This mobile phase was prepared by mixing acetonitrile, methanol, and deionized distilled water in a ratio of 4.5:4.5:1.0. Analogously, this solution was degassed for 15 minutes.

Sample preparation

0.5 g of the bird's nest sample was homogenized in a 100 mL Schott-Duran flask. Subsequently, a mixture consisting of 25 mL of HCl solution in distilled water (1:1) containing 0.1 % phenol was added to the flask, which was then covered and shaken thoroughly. The mixture was then subjected to hydrolysis at 100 °C for 16 hours in a drying oven. After hydrolysis, the sample was allowed to cool down, and the pH was adjusted to a neutral value. The solution was then transferred into a 100 mL flask and further diluted with distilled water. Following this, the sample was filtered using filter paper and supplementary centrifuged at 3000 rpm. Finally, the supernatant was filtered through a 0.45 µm membrane filter transferred into a 1.5 mL vial, and analyzed using an HPLC-FLD system.

Chromatographic analysis

For liquid chromatographic separation, the Eclipse Plus C18 column (250 x 4.6 mm, 5µm) coupled with a fluorescence detector (1260 Agilent Technologies) was employed to separate target analytes at 40 °C. The fluorescent was monitored at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The binary mobile phases (A) and (B), along with the mobile phase program for the loading pumps, are detailed in Table 1, while the flow rate remained constant at 1.2 mL/min throughout the entire chromatographic analysis process. Samples and standard solutions were stored at 10 °C in the sample tray. A 2.0 µL aliquot of the standard or samples was injected into the HPLC-FLD system via an autosampler.

Table 1. The mobile phase gradient program.

Time (min)	A (%)	B (%)	Flow rate (mL/min)
0.0	95.0	5.0	1.2
0.84	95.0	5.0	1.2
40.00	57.0	43.0	1.2
40.10	0.0	100.0	1.2
44.30	0.0	100.0	1.2
44.40	95.0	5.0	1.2
46.00	95.0	5.0	1.2

Method validation

Linearity

Linearity was evaluated by analyzing triplicate reference standard solutions of Asp, Glu, Ser, His, Arg, Gly, Thr, Tyr, Ala, Met, Val, Phe, Ile, Leu, and Lys at concentrations of 1, 2, 5, 10, 20, 50, and 100 µg/mL. Standard solutions at each concentration level were measured in triplicate. The standard curve equation was constructed based on the graph representing peak area and standard addition concentration.

MDL and MQL

In this study, the Method Detection Limit (MDL) and Method Quantification Limit (MQL) of amino acids were determined using a minimum concentration (Cmin) of 1 mg/L. The test was repeated 11 times to determine the standard deviation (SD). The MDL was calculated using the formula MDL = (t × SD) × F, and the MQL was determined as MQL = 10 × MDL, where SD represents the standard deviation and t is the Student's t-value at the 95 % confidence level. Additionally, F represents the degrees of freedom, which are calculated as n – 1.[14-16]

Recovery

For the recovery assessment, 0.5 g of each sample was weighed into a 50 mL centrifuge tube, followed by the addition of 0.5 mL of a standard solution containing the above-mentioned amino acids at a concentration of 1.0 mg/mL. The procedure was conducted like that outlined in the previous section. The resulting solution was diluted with acetonitrile to achieve final analyte concentrations of 10 mg/L. The experiment was repeated seven times to calculate the standard deviation (SD), recovery percentage (R%), and relative standard deviation (RSD%) at each level.[17,18]

Precision

The method precision was evaluated using a reference standard solution containing fifteen amino acids at concentrations of 20, 40, and 100 mg/L. This solution was analyzed six times on the same day, and the relative standard deviation (RSD) of peak areas was calculated.

The repeatability (intra-day precision) and intermediate precision (inter-day precision) were assessed by analyzing ADE and COR sample solutions at concentrations of 20, 40, and 100 mg/L. All samples were prepared daily and analyzed over the next three days. The RSD of peak areas (n = 11) was evaluated for both repeatability and intermediate precision assessments.

Results and discussion

Chromatographic analysis

The mobile phase gradient program for HPLC is outlined in Table 1. The chromatogram of standard solutions containing Asp, Glu, Ser, His, Arg, Gly, Thr, Tyr, Ala, Met, Val, Phe, Ile, Leu, and Lys under selected conditions is depicted in Fig. 1, showing the respective retention times. Successful separation and quantification of the 15 amino acids within a 40-minute chromatogram have been achieved. The distinct retention times of these substances are attributable to their differing structures and polar properties. For instance, glutamic acid, with its two carboxylic acid groups, exhibits a preference for interaction with the polar mobile phase compared to phenylalanine, which possesses an aromatic ring. Furthermore, the benzene ring of phenylalanine interacts strongly with the stationary phase

C18, leading to a longer retention time than glutamic acid. Detailed information regarding the retention times of the 15 amino acids is provided in Table 2.

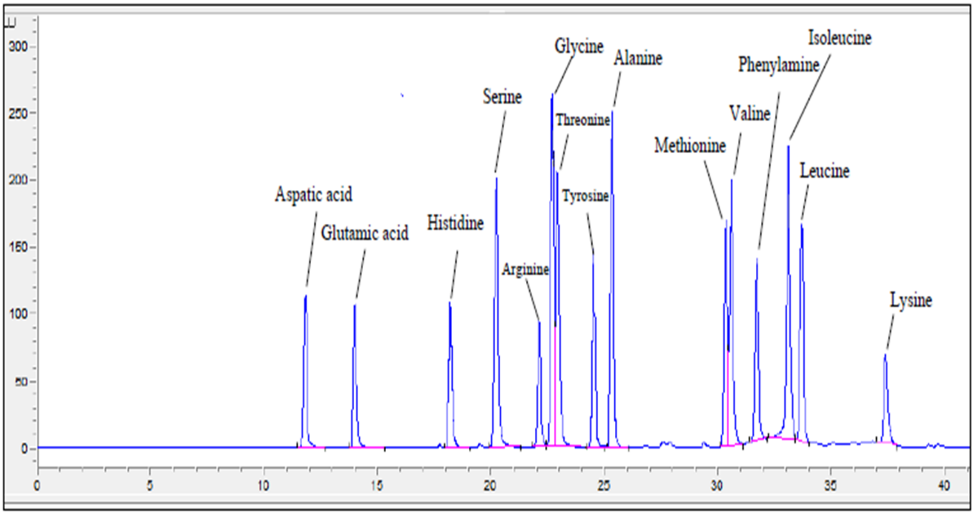

Figure 1. Representative HPLC-FLD chromatogram of the 15 amino acids in standard solution containing 10 μg/L of each amino acid.

Method validation

The calibration curve data for the 15 amino acids are provided in Table 2, demonstrating linearity over the range of 1.0 to 100 mg/L. The correlation coefficients exceeded 0.999, indicating excellent linearity. The method detection limit (MDL) and method quantitation limit (MQL) values presented in Table 2 signify that this method was suitable for the analysis of Asp, Glu, Ser, His, Arg, Gly, Thr, Tyr, Ala, Met, Val, Phe, Ile, Leu, and Lys in real samples, as per the standards set by the European Commission and USFDA.[19]

The recovery percentages (R%) for the 15 acidic amino acids ranged from 79.2 % to 116.5 %, which aligns with the specifications of the AOAC[20] and are consistent with findings from previous studies. System precision, repeatability (intra-day), and intermediate precision (inter-day) were evaluated by determining the relative standard deviation (RSD) across a series of assessments. The observed RSD values for precision studies were all below 5.0 %, indicating that this method is sufficiently precise for routine analysis. The recovery and precision achieved in this study are comparable to or better than those reported in several previous studies, indicating satisfactory repeatability and reproducibility of the proposed method.

Table 2. Parameters of method validation.

						Repeatability, RSD (%)	
Analytes	**Retention time, min**	**The correlation coefficient, R^2**	**Recovery, R(%)**	**MDL, ppm**	**MQL, ppm**	**intra-day**	**inter-day**
Asp	11.9	0.9992	83.2÷96.1	0.6	6.0	1.8	3.5
Glu	14.0	0.9991	89.3÷98.5	0.5	5.0	1.7	2.9
Ser	18.3	0.9993	85.8÷97.6	0.3	3.0	0.9	4.2
His	20.4	0.9990	80.2÷101.2	0.4	4.0	2.5	3.8
Arg	22.1	0.9992	81.3÷104.6	0.4	4.0	3.1	5.7
Gly	22.9	0.9992	89.6÷114.3	0.7	7.0	1.8	2.1
Thr	23.1	0.9993	82.9÷99.2	0.3	3.0	2.5	3.2
Tyr	24.6	0.9991	84.4÷99.8	0.6	6.0	2.9	3.9
Ala	25.3	0.9991	79.9÷95.5	0.4	4.0	1.5	2.5
Met	30.3	0.9994	87.8÷110.1	0.3	3.0	3.2	4.6
Val	30.6	0.9991	81.9÷99.5	0.2	2.0	2.5	4.2
Phe	31.8	0.9993	88.2÷96.9	0.5	5.0	2.1	2.9
ILe	33.1	0.9992	85.7÷95.9	0.3	3.0	3.4	4.9
Leu	33.7	0.9990	81.8÷116.5	0.6	6.0	2.2	5.2
Lys	37.3	0.9992	84.8÷106.1	0.4	4.0	1.9	2.5

Effect of the temperature and time hydrolysis on amino acids content

Figure 2 illustrates that the amino acid content remains low at temperatures below 100 °C. This phenomenon could be attributed to incomplete hydrolysis of the bird's nest samples, resulting in peptide bonds not being fully cleaved, or amino acids not being effectively separated from impurities. For instance, at 60 °C, the highest observed Threonine content is only 1.13 g/100 g. The temperature exerts a significant influence on the hydrolysis of the peptide chain. Consequently, samples hydrolyzed at 100 °C exhibit a notably higher amino acid content. For example, the yield of Aspartic acid at 100 °C is 4.77 g/100 g, compared to 4.25 g at 80 °C and 0.56 g at 60 °C. As depicted in Figure 2, the content of these acidic amino acids at 100 °C and 120 °C is nearly identical. Therefore, a hydrolysis temperature of 100 °C was selected for further examination.

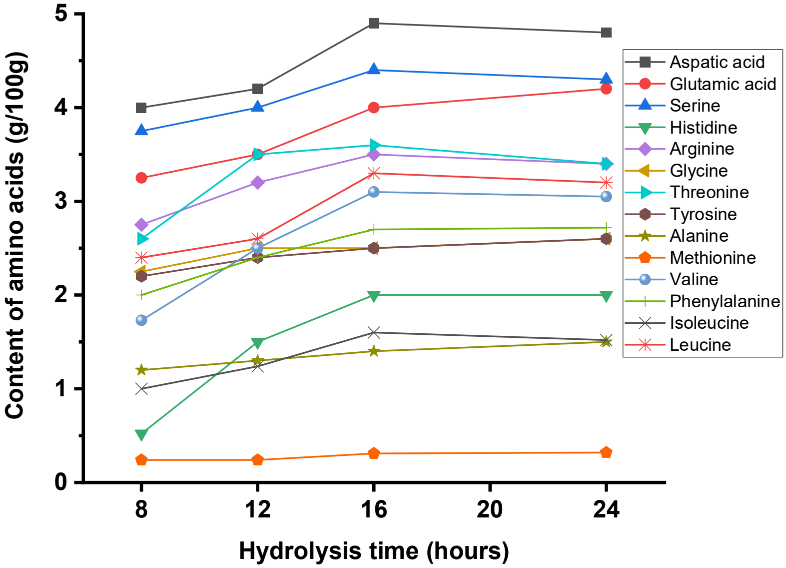

Figure 2. Effect of hydrolysis temperature on the content of amino acids.

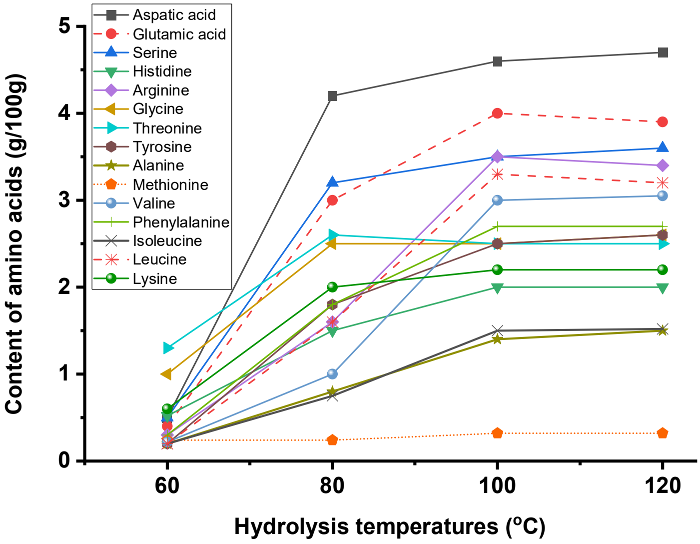

Figure 3. Effect of hydrolysis time on the content of amino acids.

The data depicted in Fig. 3 reveals that after 16 hours of incubation, the samples were not fully hydrolyzed. However, extending the hydrolysis time from 12 hours to 16 hours resulted in a substantial increase in content. Based on these findings, a hydrolysis time of 16 hours is deemed appropriate for the analysis of amino acids.

Application

The HPLC-FLD method was employed to determine the contents of amino acids in the real samples. Table 3 reveals that all 15 amino acids present in both raw bird's nest and refined bird's nest samples exhibit very high content levels. Notably, Aspartic acid emerges as the amino acid with the highest content, ranging from 4.76 to 5.64 g/100 g for the raw bird's nest and 4.96 to 5.92 g/100 g for the refined bird's nest. Conversely, Met demonstrates the lowest acid amine content, with values ranging from 0.29 to 0.34 g/100 g for the raw bird's nest and 0.39 to 0.46 g/100 g for the refined bird's nest. Additionally, the total content of acid amines was calculated, revealing ranges of 43.30 to 45.08 g/100g for refined bird's nest, 36.69 to 41.40 g/100 g for raw bird's nest, and 0.122 to 0.172 g/100 g for distilled bird's nest. These analysis results are consistent with previous studies on the composition and content of amino acids in bird's nests conducted in Vietnam and other countries in the ASEAN region.[21]

Table 3. The content of amino acids in real samples (g/100 g).

Amino acid	Raw EBN, (n = 10) g/100 g	Refined EBN, (n = 12) g/100 g
Asp	4.76 ÷ 5.63	4.96 ÷ 5.92
Glu	4.41 ÷ 5.12	4.92 ÷ 5.72
Ser	4.44 ÷ 4.78	4.81 ÷ 5.21
His	1.98 ÷ 2.67	2.05 ÷ 2.92
Arg	2.96 ÷ 3.63	3.01 ÷ 4.05
Gly	1.92 ÷ 2.56	2.67 ÷ 3.51
Thr	3.03 ÷ 3.62	4.20 ÷ 4.62
Tyr	2.71 ÷ 3.12	2.94 ÷ 3.64
Ala	1.24 ÷ 1.87	1.82 ÷ 2.19
Met	0.29 ÷ 0.34	0.39 ÷ 0.46
Val	2.95 ÷ 3.12	3.01 ÷ 3.95
Phe	2.76 ÷ 3.68	2.93 ÷ 3.74
ILe	1.14 ÷ 1.52	1.68÷ 1.94
Leu	3.27 ÷ 3.47	3.72 ÷ 3.90
Lys	1.73 ÷ 1.98	1.82 ÷ 2.08

Conclusion

The developed HPLC-FLD method enables the simultaneous quantitation of 15 amino acids in Edible Bird's Nests (EBNs) through online pre-column derivatization with OPA. This method has demonstrated excellent performance in terms of linearity, selectivity, repeatability, intermediate precision, and minimal matrix effects during method validation. Employing the validated method, the content of these compounds was successfully quantified in real samples. The analytical outcomes affirm the suitability and efficacy of the proposed method for accurately quantifying amino acids in various real samples.

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