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Research Article

Quantification of L-ornithine L-aspartate in health supplement by HPLC in combination with o-phthalaldehyde derivative technique

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Abstract

Dietary supplements containing L-ornithine L-aspartate (LOLA) are on the rise. Thus, a simple, reliable method of analysis for determining LOLA has been developed, which helps to control product quality. In this study, a method for determining LOLA in dietary supplements by HPLC in combination with o-phtalaldehyde derivatives (OPA) was developed and validated. The study optimized conditions for extraction of LOLA from the matrix, then derived with OPA and chromatographic separation on column C18. The detection limits and quantitative limits of the method were conducted on low-content real samples, with LOD and LOQ values achieved at 6.0 and 20.0 µg/g, respectively. The method has good recovery (98 – 106%) and repeatability (1.32 – 3.50%) that meet the method performance requirements according to AOAC. The validated method has been applied to analyze LOLA content in dietary supplements at the National Institute for Food Control.

Keywords: *L-ornithine L-aspartate, health food supplement, HPLC, amino acids, pre-column derivatization, o-phthalaldehyde, OPA.*

1. INTRODUCTION

LOLA (L-ornithine L-aspartate) is a peptide consisted of the amino acids ornithine and aspartic acid bonded by an amide bond; its molecular formula is indicated in the Figure 1 [1]. Ornithine is an amino acid that is not incorporated into proteins in the body although it is involved in the urea cycle. Ornithine is accumulated in the body under conditions of ornithine transcarbamylase deficiency [5]. Aspartic acid also known as aspartate has the abbreviated form as Asp and has the chemical structure which is $HOOCCH(NH_2)CH_2COOH$. Aspartate is

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an ion, salt, or ester of aspartic acid often containing the carboxylate anion. Aspartic acid is an amino acid that forms component of proteins and it is one of the 20 amino acids [3].

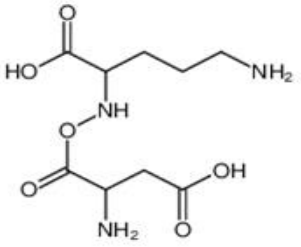


Figure 1. *LOLA molecular structure*

Effect of LOLA is resembled to decrease the levels of ammonia and produce urea in cirrhotic patients. There is a decrease in enzyme activities including carbamoyl phosphate synthetase and arginase in animals with cirrhosis as suggested by the data from prior investigations on the issue. It is worthwhile to note that the employment of LOLA amplifies the activity of such enzymes by a certain degree [4]. Thus, the addition of LOLA in health food products especially in the products that are developed for supporting those who have liver disorders or bad liver functioning are gaining even more interest.

Hence, the purpose of the following work is to propose an analytical method to assess and control the quality of LOLA, practically and theoretically. At the current moment, several methods have been elaborated by means of different techniques including capillary zone electrophoresis [5], high performance liquid chromatography [6], as well as the high performance liquid chromatography combined with pre-column derivatization [7] for analysis of amino acids. The most used technique of protein analysis is called capillary electrophoresis; nevertheless, its parameters are capillary and voltage. HPLC with UV detection can be applied for quantification of LOLA, but the sensitivity of this method is not very high mainly because of the presence of α-amino acid molecular that does not have π-bonds and very low detection wavelength is easily influenced by the matrix. As the method mentioned above, to elevate the sensitivity and specificity of the method, the derivatives of amino acids contain ninhydrin, o-phthalaldehyde (OPA), phenylisothiocyanat, 9-fluorenylmethyl chloroformat (FMOC), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamat (AQC), … reagents which can be used together with HPLC improve the sensitivity of analysis of amino acids [8]. Among these derivatives, OPA is the most frequently used because this process is quite simple, the reaction takes place at the room temperature, and in water and borate buffer as solvents. Thus, the OPA derivatives are generally less polar than the starting amino acids, and a good separation on reverse-phase HPLC [7, 8]. In 1996, Dorresteijn and colleagues developed a method for the identification of 13 amino acids by HPLC combined with pre-column derivation, but the method has not yet been developed for the analysis of L-ornithine l-aspartate. Therefore, to meet the demand, a method for analyzing LOLA is needed. The study is expected to conduct a survey of the sample processing conditions and reaction conditions of LOLA and OPA followed by chromatographic analysis using HPLC.

2. MATERIALS AND METHODS

2.1 Chemicals and equipment

2.1.1. Chemicals

The LOLA reference material (99%, Sigma-Aldrich, USA), batch number: SLBW4848. The working stock standard solution at a concentration of 1000 µg/mL was applied. The concentrations of the working standard solutions were from 0.20 to 40.0 µg/mL. The chemicals used in this study included: Methanol (MeOH), ammonium acetate (CH_3COONH_4), boric acid, sodium hydroxide, hydrochloric acid, 2-mercaptoethanol, o-phthalaldehyde (OPA) and ultrapure water for chromatography that was of HPLC grade. Merck brand chemicals or equivalent were used throughout the study and all chemicals used were of chromatographic purity.

The blank samples selected for the study and validation of the method's applicability were health supplement products aimed at liver function enhancement, in the form of hard capsules, soft capsules, and syrups containing the following ingredients: Silymarin 40% extract, Solanum procumbens extract, Gymnema sylvestre extract and other excipients : all these components of the products none of which contained LOLA.

Spiked samples: 5 mL of the LOLA standard solution prepared at concentration of 1000 µg/mL was used in 1g of test sample (0.5 mg/g).

The sample consists of ten health supplement products bought in Hanoi.

2.1.2. Instrument

The instrument used for the analysis was the Waters Acquity UPLC H-Class system accompanied with an Acquity FLD fluorescence detector purchased from Waters Corporation, USA. Other accessories used in the analysis were an analytical balance- XS105 (Mettler Toledo) with an accuracy of 0.0001 g; pH meter (Mettler Toledo); horizontal shaker (IKA); ultrasonic bath (Elma); and centrifuge (Hermle).

2.2. Research methodology

2.2.1. Sample preparation method

The analysis based on the health supplement samples was carried out given that such systems include additional components such as excipients, herbs, vitamins as well as amino acids. Thus, prior to analyzing the extraction of LOLA, sample preparation is required to remove impurities and determine the best extraction condition.

Several investigations were conducted to optimize the analysis process, improve the extraction efficiency of LOLA, and enhance the reaction between LOLA and OPA in the health supplement matrix, including: Several investigations were conducted to optimize the analysis process, improve the extraction efficiency of LOLA, and enhance the reaction between LOLA and OPA in the health supplement matrix, including: (1) Examining and selection of extraction method, (2) Examining and selection of the extraction solvent, (3) Examining and selection of extraction time, (4) Comparative analysis of the relationships between the outcomes of the LOLA and OPA, (5) Studying the time-dependency of the reaction of LOLA with OPA.

The given scheme in Figure 2 shows the sample processing and derivatization. The method was evaluated on hard capsule matrices and validated on all three matrices: These packages include hard capsules, soft capsules, and solutions.

2.2.2. Analytical method

This study included the use of High-Performance Liquid Chromatography with fluorescence detector as its method, and the conditions that were adjusted include the solvent program. Based on previous studies [7-9] and the available laboratory conditions, the Waters XBridge C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase consisting of two channels—Channel A: The concentration of 20 mM ammonium acetate in water as well as methanol—was used in Channel B which was used to determine LOLA. The excitation wavelength was 345 nm and the emission wavelength that was used was 455 nm.

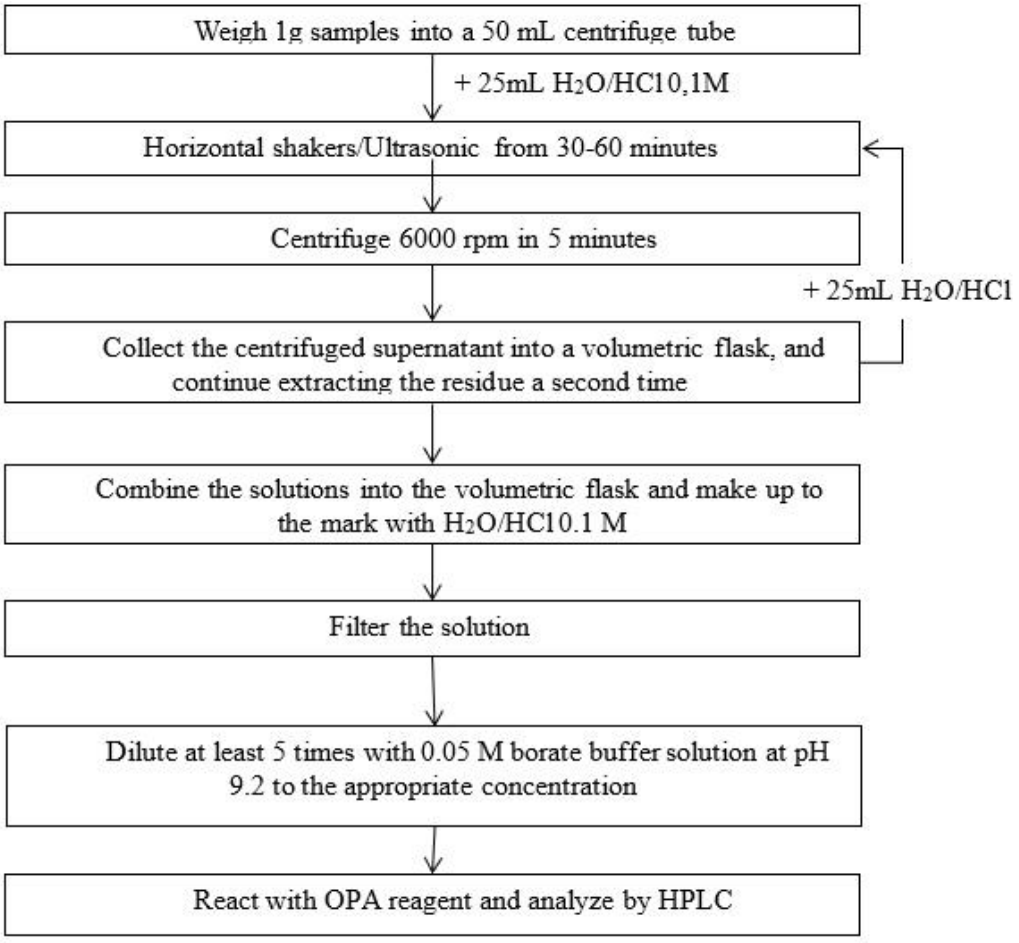


Figure 2. *Proposed analytical process*

2.2.3. Method validation

The optimized method was validated according to AOAC Appendix F parameters including: Specificity, calibration range, limit of quantitation, accuracy, and precision.

2.2.4. Application to real sample analysis

Ten functional food samples containing L-ornithine L-aspartate were collected from the Hanoi area for analysis.

3. RESULTS AND DISCUSSION

3.1. Chromatographic condition survey

Based on the literature [7-9] and preliminary surveys in the laboratory, the standard LOLA solution at a concentration of 10 µg/mL was analyzed under the following mobile phase programs:

- Program 1: 20 mM ammonium acetate: Methanol in a ratio of 70:30 (v/v)
- Program 2: 20 mM ammonium acetate: Methanol in a ratio of 50:50 (v/v)
- Program 3: Gradient program with two channels of 20 mM ammonium acetate and Methanol in the ratios shown in Table 1.

Table 1. *LOLA analytical mobile phase program*

Time (min)	CH_3COONH_4 20 mM	Methanol
0.00	70	30
8.00	70	30
15.0	20	80
18.0	20	80
18.5	70	30
25.0	70	30

The results were obtained as shown below

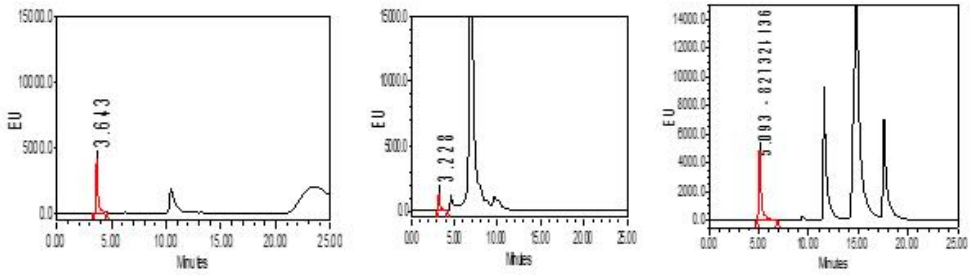


Figure 3. *Chromatogram of LOLA standard solution with concentration 10 µg/mL*

In the chromatogram illustrated in Figure 3, the peak corresponding to the compound of interest, LOLA, elutes at 5.093 minutes is distinctly separated from other derivatives of OPA reaction which has three high peaks that elute at a retention time of 12, 15 and 18 minutes. Also, the analysis time is short in the case as compared to the gradient program run under isocratic conditions.

3.2. Investigation of the reaction conditions between LOLA and OPA reagent

3.2.1. Investigation of reaction time

The reaction time with the LOLA standard solution and OPA was conducted at 0.5, 1, 2, 3 minutes. Identification was done on the HPLC system, with the results as displayed in Figure 4.

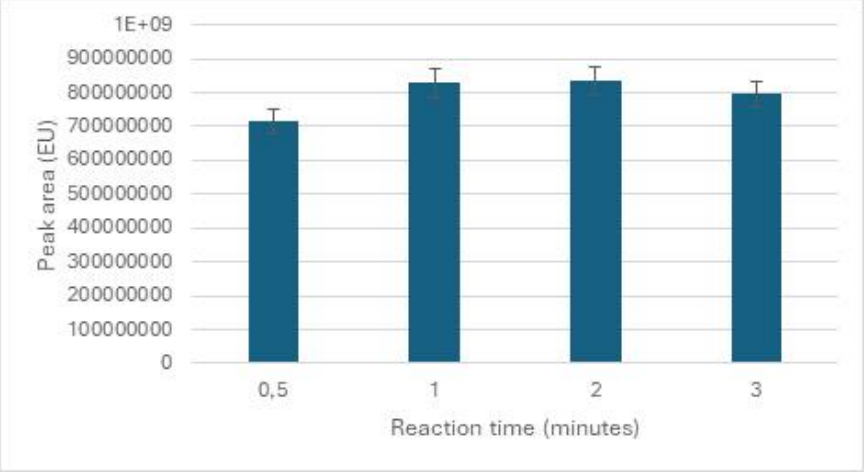


Figure 4. *Results of reaction time*

By analyzing the results illustrated in the Figure 3, it is evident that an increase in the level of derivatization efficiency is achieved at 1 minute. The low signal is produced when the number of bead displacements is small after 0.5 minute. The obtained signal values for the derivatization times of 1 and 2 min are the highest, and the increase of time up to 3 min leads to the significant signal decrease. This is inline with previous findings PEA reagent, the OPA derivative product reveals that the product is unstable and slowly degrades once it has been derivatized [10]. Having compared the signal at derivatization time of 1 minute and 2 minutes, it can be concluded that these two values are not significantly different. Thus, to maximize the time and efficacy for derivatization, the study chose 1 minute as the reaction time for the procedure.

3.2.2. Investigation of the ratio of LOLA and OPA

For derivatization of LOLA solution at a concentration of 8.0 µg/mL with OPA, the procedure was done at the following LOLA concentration ratios: 1/2; 1/1; 2/1; 4/1, reaction time is 1 minute. The results achieved from the solutions obtained after derivatization are illustrated in the Figure 5.

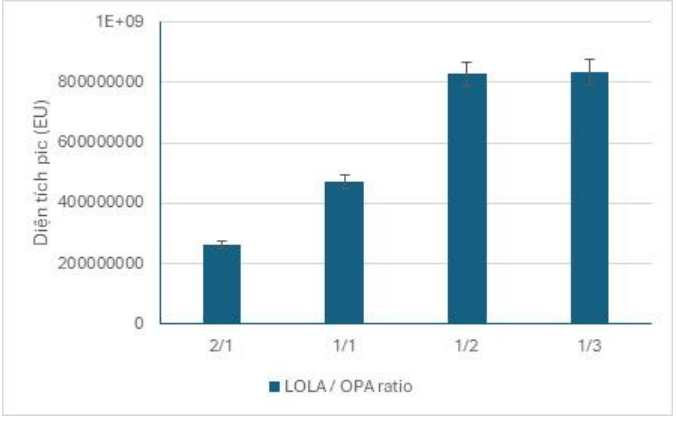


Figure 5. *LOLA/OPA ratio results*

It can also be seen by the results indicated in the graph in Figure 5 that as the ratio of OPA increases, the signal similarly rises though at a very slow gradation. This paper finds

out that the average signal response for the chosen ratios of 1/2 and 1/3 are almost equal and offer the highest signals. If the content of OPA derivative rises, the signal rises as well; however, there is also more derivative entry to the column, which can easily deplete column and decrease its lifetime. Thus, to get maximum extraction yield of OPA, maximum signal in the derivation step and the durability of the analytical column, the study settled for a LOLA/OPA ratio of 1/2.

3.3. Investigation of sample preparation conditions

3.3.1. Investigation of the extraction method

The sample was treated based on the discussed flowchart (Figure 2) and was extracted with water, shaken in the horizontal plane, and using ultrasounds. The sample undergoing this research was analyzed through HPLC and the outcomes illustrated in Figure 6.

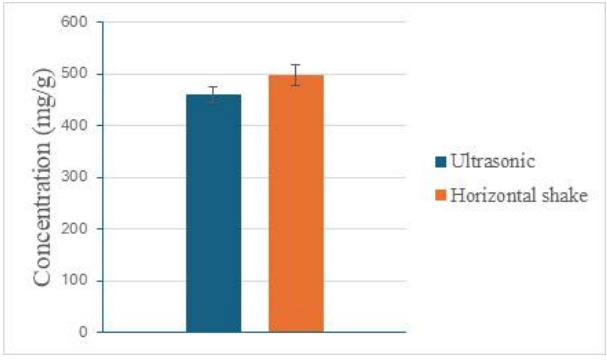


***Figure* 6.** *Extraction method results*

Therefore, considering the results in Figure 6, it can be noted that the extraction efficiency obtained using the horizontal shaking method was higher than that of the ultrasonic method. LOLA in the solution degrades when it is subjected to ultrasonication at high temperatures. According to current literature [11], this finding is consistent.

3.3.2. Investigation and selection of the extraction solvent

According to the analytical method described in the Figure 2 in the research, Sample B was processed with H_2O and 0.1M HCl, respectively. The sample was analysed on the HPLC system and the same was plotted as a graph which is shown in Figure 7.

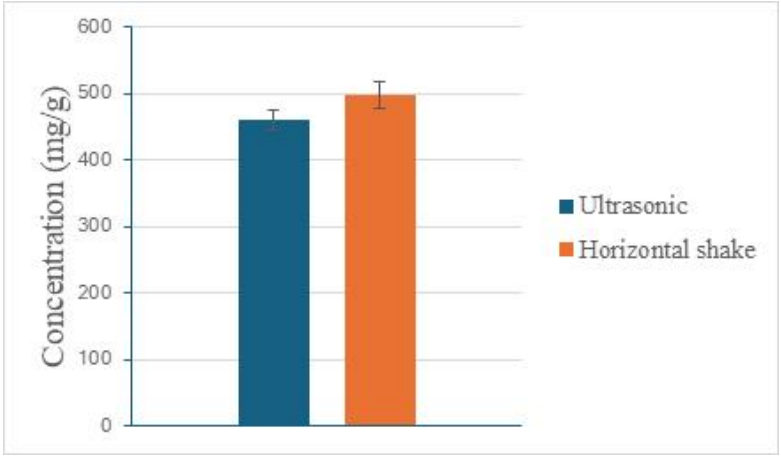


Figure 7. *Extraction solvent method results*

Analyzing the results shown in Figure 7, it can be noted that the highest extraction efficiency is achieved for water (H_2O) as the solvent higher than 0.1M HCl solution. This is in concordance with other evaluations revealing that solubility of LOLA is low in an acidic solution than in a neutral solution [1].

3.3.3. Investigation of extraction time

The sample was extracted in accordance with the outlined analysis plan illustrated in Figure 2 under extraction time of 15 minutes, 30 minutes, and 45 minutes respectively. The samples were then subjected to the HPLC system's analysis with the following data shown in Figure 8.

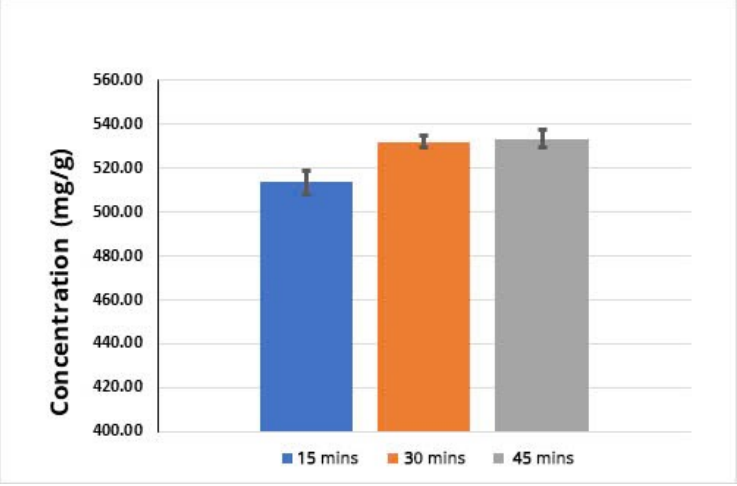


Figure 8. *Extraction time results*

From the results in Figure 8, it can be observed that there is no significant difference between the extraction times of 30 and 45 minutes. The 15-minute extraction time yielded lower results, possibly because LOLA was not completely extracted within 15 minutes. Therefore, to optimize both extraction efficiency and analysis time, the study selected a 30-minute extraction time.

3.3.4. Optimized analytical method after investigation

After investigating the sample preparation conditions, the LOLA analysis procedure for health supplements used to validate the method as described below.

- Sample Preparation: Homogenize the sample, weigh 1g of the homogenized sample into a centrifuge tube. Transfer 25 mL of distilled water to the centrifuge tube, shake thoroughly using a horizontal shaker for 30 minutes. Centrifuge at 6000 rpm for 5 minutes, decant the supernatant into a 50 mL volumetric flask. Re-extract the residue. Make up to the mark with distilled water. Shake thoroughly, filter through a 0.45 µm membrane filter, transfer the filtrate into a vial, and analyze using the HPLC system.

- Derivatization Procedure: The derivatization is carried out automatically on the HPLC system following the sample injection procedure as follows: (1) Pipetted 2 µL from the sample/LOLA standard vial; (2) Pipetted 4 µL from the OPA derivatization reagent vial; (3) Mix in the syringe for 1 minute; (4) Inject the mixed solution into the chromatographic system.

- Chromatographic Conditions: Analytical column: C18 (250 × 4.6 mm, 5 µm); Mobile phase: Channel A: 20 mM ammonium acetate and Channel B: Methanol according to the following gradient program: from 0 to 8 minutes at 70% A, from 8 to 15 minutes gradient to 20% A and maintain at 20% A in 3 minutes then return to 70% A and equilibration for next analysis. The flow rate was 0.8 mL/minute; Column temperature was 30°C. Total analysis time was 25 minutes.

3.4. Method validation

3.4.1. Specificity of the method

The specificity of the method was determined as follows:

- Standard Sample: Use the LOLA standard solution at a concentration of 10 µg/mL.
- Blank Sample: Weigh 1.0 g of health supplement in the form of hard capsules, soft capsules, or syrup that does not contain LOLA.

Spiked Blank Sample: Weigh 1.0 g of the above blank sample, add 0.5 mL of 1000 µg/mL standard solution. The results are shown in Figure 9.

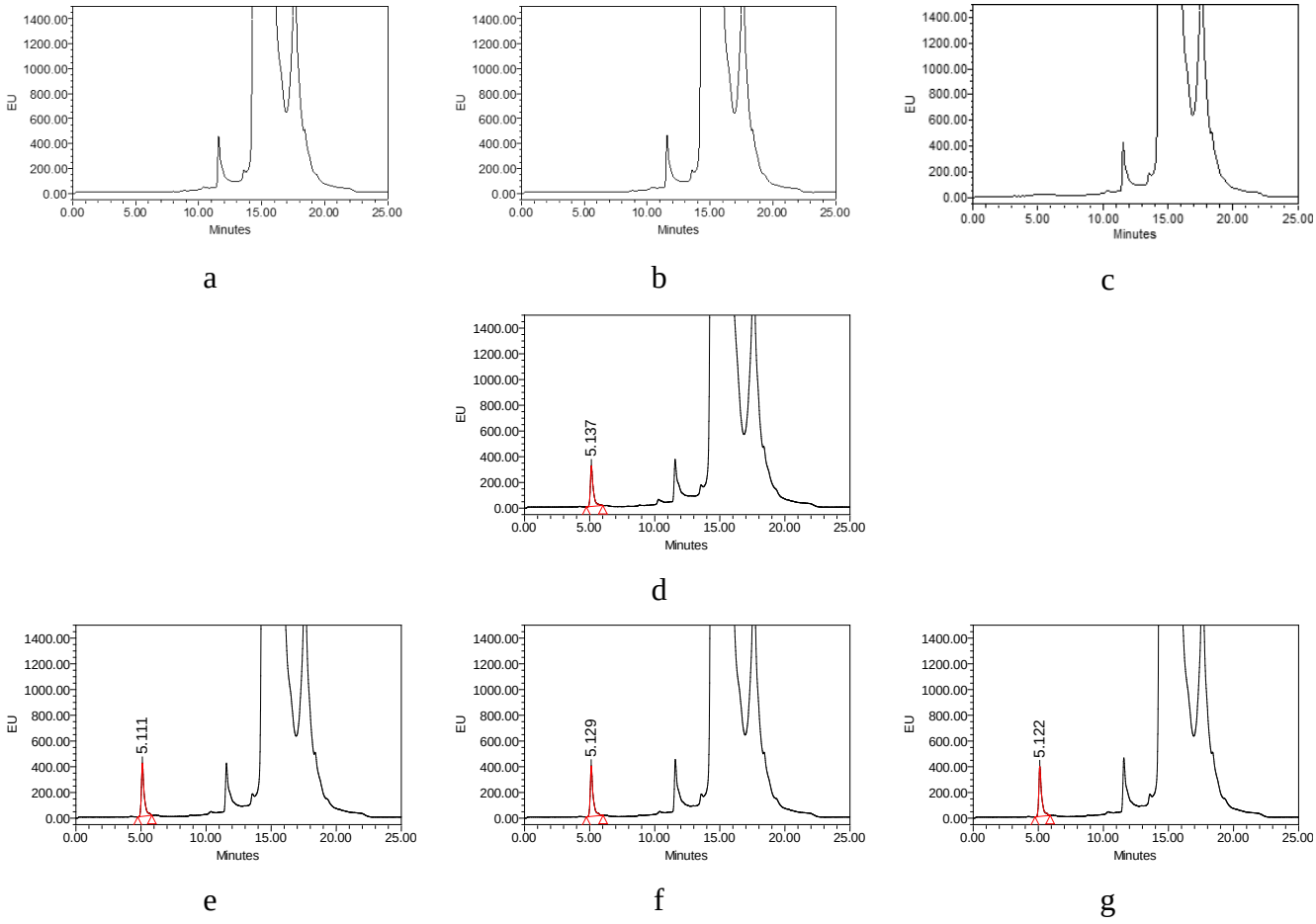


Figure 9. *Specificity Chromatograms: a: Blank sample with hard capsule matrix, b: Blank sample with soft capsule matrix, c: Blank sample with liquid matrix, d: LOLA standard sample, e: Spiked sample with hard capsule matrix, f: Spiked sample with soft capsule matrix, g: Spiked sample with liquid matrix.*

3.4.2. Standard curve

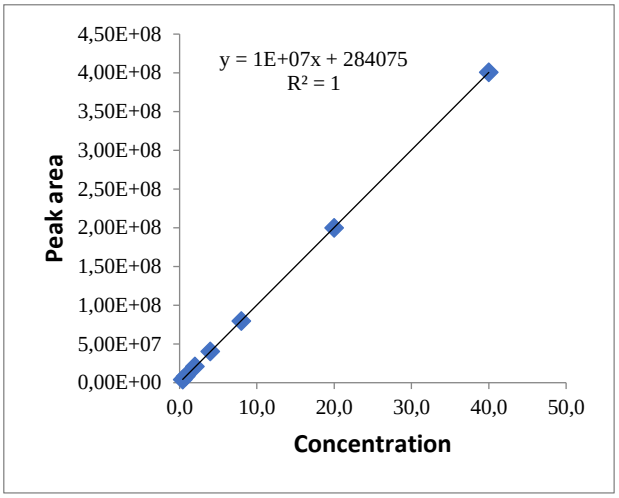
Prepare LOLA standard solutions with concentrations from 0.2 to 40 µg/mL. The calibration curve consists of 7 points with the following concentrations: 0.2 µg/mL, 0.4 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL, 20 µg/mL, 40 µg/mL. Perform derivatization with the OPA reagent and analyze using HPLC instrument. A calibration curve was constructed showing the relevance between the peak area and the analyte concentration, with the results as shown in Table 2.

Table 2. *Results of standard curve analysis*

LOLA standard

Concentration (µg/mL)	**Peak area**	**Ctt** (µg/mL)	**Bias (%)**
40.0	4.01E+08	40.0	0.08
20.0	2.00E+08	19.9	-0.26
8.00	7.97E+07	7.9	-0.76
4.00	4.05E+07	4.01	0.37
2.00	2.09E+07	2.06	3.11
0.80	8.42E+06	0.81	1.65
0.40	4.18E+06	0.39	-2.62

LOLA calibration curve



From the results in Table 2, it can be seen that the calibration curve has a correlation coefficient of $R^2 = 1 > 0{,}995$, và with the deviation of the calibration points less than 15%, meeting the AOAC requirements [12].

3.4.3. Limit of detection and limit of quantification (LOD, LOQ)

The standard was spiked into blank samples of hard capsules, soft capsules, and liquids at a concentration level of 20 µg/g. The signal-to-noise ratio (S/N), calculated using the Empower software of the instrument, showed that S/N ≥ 10 (Figure 10). According to market surveys, health supplement products contain LOLA at levels of 1 mg/g or higher. Therefore, an LOQ of 20 µg/g and an LOD of 6.0 µg/g are viable for analyzing LOLA in health supplements.

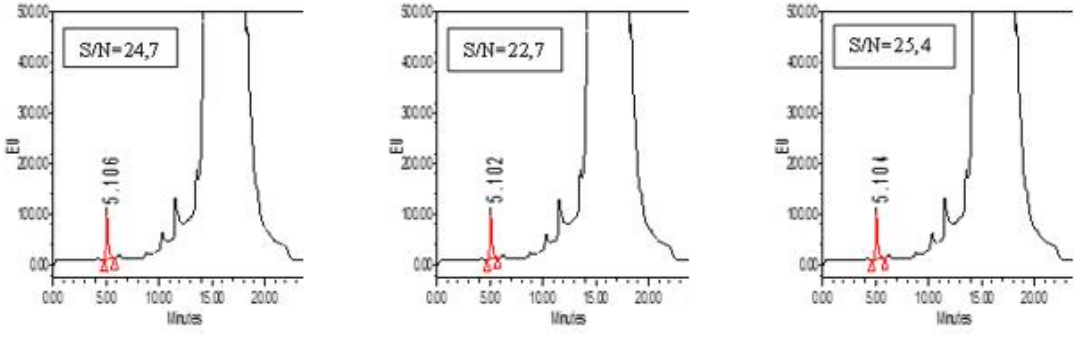


Figure 10. *LOLA Chromatogram at LOQ: a: hard capsule; b: soft capsule; c: liquid sample*

3.4.4. Accuracy and precision

The accuracy and precision of the analytical method were examined through repeatability, recovery and internal reproducibility. The LOLA standard was added into the blank samples at three concentration: 20, 100, and 1000 µg/g (equivalent to LOQ, 5LOQ, 50LOQ) and analyzed as the procedure described. Repeat six times with each concentration. Internal reproducibility was performed similarly on different days. The results showed that the analytical method met the accuracy and precision value across all three sample matrices which is shown in Table 3.

***Table 3**. Accuracy and precision of LOLA in health supplements*

Sample type	Concentration (µg/g)	Accuracy (% recovery)	Precision	
			Repeatability (RSD_r %)	Within-lab reproducibility (RSD_R %)
Tablets	20	98-103	2.02	3.43
	100	100-106	2.89	2.67
	1000	98-106	3.24	3.49
	20	99-106	3.30	3.50
Soft cyst	100	100-106	2.93	2.47
	1000	99-106	2.60	2.61
Liquid	20	99-104	1.65	2.48
	100	99-105	1.71	1.32
	1000	101-104	1.09	1.82

The results obtained from Table 3 show that the method meets AOAC requirements for recovery (90 – 107%), repeatability (3.7%), and internal reproducibility (6%). The validation results indicate that the method meets the performance criteria required for implementation and application in real sample analysis.

3.4. Application to real sample analysis

The method was applied to analyze LOLA in 10 health supplement samples purchased in the Hanoi area. The results of the LOLA content analysis in 10 health supplement samples purchased on the market are shown in Table 4.

***Table 4.** Analytical results of some health supplement in the market*

Sample name	Test results	Label content	RSD (%)
Tablets 1	79.6 mg/capsule	80 mg/ capsule	1.53
Tablets 2	263 mg/capsule	300 mg/ capsule	1.02
Tablets 3	162 mg/capsule	200 mg/ capsule	1.14
Hard capsules 1	87.9 mg/capsule	100 mg/ capsule	1.54
Hard capsules 2	46.9 mg/capsule	100 mg/capsule	0.65
Hard capsules 3	105 mg/capsule	200 mg/capsule	1.04
Hard capsules 4	49.3 mg/capsule	50 mg/capsule	1.07
Soft capsules 1	44.8 mg/capsule	50 mg/capsule	1.15
Soft capsules 2	47.3 mg/capsule	50 mg/capsule	1.33
Tablets 4	866 mg/capsule	900 mg/ apsule	1.54

From the results in Table 4, in 10 of analyzed health supplement samples, the lowest LOLA content recorded at 44.8 mg/capsule and the highest was 866 mg/capsule. All analysis results showed a deviation from the labeled content less than 20%. The method has an appropriate LOQ and linear range for determining LOLA content in health supplement matrices.

The method using OPA to determine LOLA content shows consistency for analyzing LOLA in complex health supplement matrices. On the other hand, the study has only been dealt with health supplement matrices., not other samples in the market. There are other supplements that contain LOLA, such as nutritional food for special diets, dietary supplements, etc., which need further analysis. The research team will continue to develop this method for application to other matrices.

4. CONCLUSION

In conclusion, it is possible to confirm that the proposed method of pre-column OPA derivatization and HPLC-FLR analysis has successfully established and tested an analytical method for the determination of the contents of LOLA in health supplements. The LOD and LOQ of the method are 6 and 20 μg/g for the method. Based on the results obtained, the method had high recovery rates (98 – 106%) and precision (1.32 – 3.49%) to the AOAC requirements. This analytical method is currently applied to analyze LOLA at the National Institute for Food Control (NIFC).

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