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Research Article

Determination of theanine in green tea, green tea-based beverages, and dietary supplements by high performance liquid chromatography using ortho-phthalaldehyd (OPA) derivation

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Abstract

Theanine is a natural amino acid abundant in green tea and is added to green tea-based beverages and health supplements containing green tea extract. In this study, a method for analyzing theanine using HPLC with o-phthalaldehyde (OPA) derivatization has been developed and validated. Theanine was extracted from the sample using water and then derivatized with OPA reagent, then chromatographically separated on a Xbridge C18 column (250 mm × 4.6 mm; 5 µm), with a mobile phase consisting of 20 mM ammonium acetate and methanol in a gradient program. Fluorescence detector was used with an excitation wavelength of 345 nm and an emission wavelength of 455 nm. The method demonstrated good specificity, recovery and accuracy required by AOAC. The limits of detection and quantification were 3.0 µg/g and 10.0 µg/g, respectively. This method has been applied to analyze the theanine content in tea samples, beverages, and health supplements containing green tea extract at the National Institute for Food Control.

Keywords: *Theanine, green tea, green tea beverage products, OPA.*

1. INTRODUCTION

Tea is one of the most popular beverages around the world, most tea is made from the leaves and buds of tea trees (*Camellia sinnesis*) [1]. Nowadays, based on fermentation rate during processing, tea is classified into 6 main types ranging from the lowest to highest rate of fermentation: white tea, green tea, yellow tea, black tea, and Pu'er tea. Tea supplements

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cardiovascular health due to its ability to reduce blood pressure and cholesterol LDL, improves blood circulation and decreases risks of chest pain and cardiac arrest. Also, tea also increases immunity, enhances brain function, assists digestion and reduces stress and anxiety [2]. However, these benefits can change depending on how they are prepared and consumed. The benefits are due to the presence of caffeine, catechin, flavonoids, amino acids, carbohydrates, etc. in tea. Theanine has the highest concentration, usually accounting for 50% the total amount of free amino acids in tea [3]. The chemical structure of theanine is provided in Figure 1.

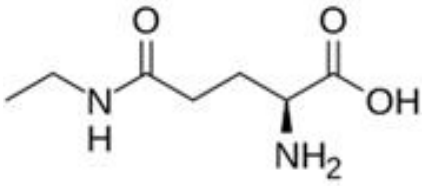

Figure 1. *Theanine's chemical structure*

Theanine is usually found in tea leaves in structural isomer form of L-theanine. It is the only secondary metabolite found in tea, resulting in the sweet umami taste in tea. Theanine can also be used as one of the important components in measuring the freshness of tea [4]. Also, it has similar structure to glutamic acid, allowing it to affect glutamate receptors [5]. This is supposed to increase serotonin and dopamine amount in the brain. Theanine can also interact with Kainate and NMDA receptors due to having similar structure to glutamic acid. Therefore, theanine has many health benefits such as anti-oxidants, anti-inflammatory, anti-cancer, anxiety, metabolism regulations, protecting the heart as well as the liver and kidneys. Theanine-supplementing has been proven to help relax and improve stress endurance [5]. Because of its various health benefits, theanine has a wide range of applications, one of them being among the ingredients to beverages or supplements.

Health supplements and theanine-containing beverages are usually available ready-to-be-consumed in the form of tablets and capsules with concentrations from 50 to 200 mg and, in liquid form, 1 to 3 mg/100mL. Although theanine is present in most green tea, the amount of L-theanine changes according to the harvest location and time. Normally, a cup of tea contains an average of 15 to 30 mg theanine [3], depending on the kind of tea used and the mixing method. However, tea products and beverages made from tea do not reveal the specific amount of theanine on their label. Some supplements containing L-theanine are ubiquitous on the market, many not having clear origins and may affect consumers' health.

There are several theanine quantifying methods devised all over the world with different techniques such as high performance liquid chromatography (HPLC) with fluorescent dectector (FLD) [6]; LC-MS/MS with ESI ionization source [7], high performance thin layer chromatography (HPTLC-UV) [8] and capillary electrophoresis [9]. Recently, AOAC has announced an SMPR draft for theanine in tea sample in 2016 [10]. This method utilizes pH 2.2 citrate buffer solution as an extraction solvent without needing to decontaminate. This is also a study using high performance liquid chromatography combined with UV-Vis detector and post column derivatization by ninhydrin agent.

Theanine's molecular structure lacks conjugate double-bonds, resulting in its poor UV-Vis absorption, therefore a derivatizing agent is used to improve detection. However, ninhydrin is only suitable for high temperature sample processing conditions, reactions' colors can be affected by different environmental factors such as light and moisture due to ninhydrin being easily degraded over long periods of exposure to light. Due to the fact, in this study, OPA is the derivatizing agent used due to its relatively easy process, its reactions happening under room temperature and in basic condition. OPA has a high sensitivity suitable for samples with low concentrations.

In 1996, Dorrestejin *et al.* [11] has devised a method for determining 13 amino acids by HPLC with pre-column derivatization. Theanine contains an amino group (NH_2) and a carboxyl group (COOH) similar to other amino acids, but there is an ethylamine group instead of an amino group directly attached to the carbon chain, unlike traditional amino acids. OPA reacts to amino groups of amino acids with a thiol group (usually 2-mercaptoethanol or N-acetylcystein), forming fluorescent isoindole complexes. Therefore, the study begins investigating sample preparation conditions and theanine and OPA combined with 2-mercaptoethanol (MCE) reaction conditions using the high performance liquid chromatography technique. The reaction mechanism of theanine with OPA-MCE derivative is shown in Figure 2.

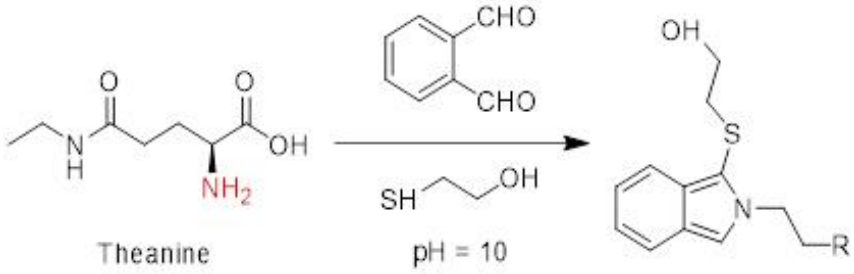

Figure 2. *Reaction mechanism between theanine and OPA-MCE derivatizing agent*

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

2.1.1. Chemicals

L-theanine standard (97.6% pure, Biopurify) with lot number: PRF223030748. Stock standard solution (1000 µg/mL) is used to prepare working standard solution. The concentration of the working standard solution is in the range of 0.80 – 40.0 µg/mL.

Chemicals used in this study includes distilled water for chromatography and substances, including: 2-mercaptoethanol (Sigma), boric acid (Merck), hydrochloric acid (Merck), ammonium acetate (Merck), o-phthalaldehyd (Sigma), sodium hydroxids (Merck), methanol (Merck).

Stock OPA derivatizing agent (10 mg/g) was prepared by weighing o- phthalaldehyde and dilute to volume with methanol and shake well. Transfer into a dark-colored bottle or cover with aluminum foil and store at 2-8ºC for 2 months.

OPA-MCE derivatizing agent (2 mg/g) was prepared by pipetting 2 mL stock OPA agent with MCE into a 10 mL volumetric flask, add pH 9.2 borate buffer to volume to create a basic environment for the reaction between OPA and theanine, shake well and avoid light. Working derivatizing agent are freshly made with every analytical batch.

The blank sample chosen to be investigated and confirm the method's functioning value is a chamomile tea not containing L-theanine and a herbal tea containing honeysuckle, daisy, monkfruit, woundwort, desmondium, cotton tree flower, *Microcos panicutula L.* plant, *Grewia paniculata plant and licorice.*

Blank sample with standard: Add 0.2 mL of 1000 µg/mL theanine standard solution to 1g blank sample like above (making 20 µg/mL) to begin the investigation.

Real sample consists of 5 tea samples, 5 tea beverages, 8 health supplements containing L-theanine and 2 ingredient samples bought in Vietnam.

2.1.2. Instrumental Analysis

This study utilizes Acquity UPLC H-Class ultra-high performance liquid chromatography system connected Waters brand fluorescent detector. Some other devices include: XS105 analytical balance (0.0001 g accuracy) (Metler Toledo); pH meter (Metler Toledo); horizontal shaker (IKA); ultrasound water bath (Elma); centrifuge (Hermle); Waters XBridge C18 colum (250 mm × 4.6 mm, 5 µm).

2.2. Methods

2.2.1. Sample processing method

Tea samples usually contain components such as catechin, caffeine, polyphenols, flavonoids, etc. For tea beverage samples, this study uses tea samples mainly containing EGCG, theanine, water, sugar and other flavoring components. Health supplement samples usually contain similar components to tea with the addition of some excipients. These compounds mostly do not affect theanine when using OPA derivatizing agent. Therefore, the sample preparation step needing to decontaminate as well as optimizing theanine extracting conditions are investigated on tea and beverage containing tea extract samples. The method, after being established, is validated on health supplement samples to confirm its usage value.

Some parameters investigated include:

(1) OPA/theanine ratio

(2) OPA/theanine reaction time

(3) Investigation of extraction method: ultrasound-assisted and shaking;

(4) Investigation of lipid removal conditions

(5) Investigation and selection of extracting solvents: water, 0.1 M HCl, pH 9.2 borate buffer;

(6) Investigation and selection of extraction time: 10, 20 and 30 minutes

(7) Investigation and selection of extraction temperature: 20°C, 40°C, 60°C and 80°C.

The investigations are performed on tea, tea beverages and health supplements samples, then validated on all three samples above and 100% theanine ingredient. The sample preparation and derivatization processes are shown in Figure 3.

2.2.2. Analytical method

The study utilizing the HPLC method with fluorescent detector (FLR) is investigated for optimized conditions like the mobile phase program [12]. Based on [11, 12] and readily available conditions at the laboratory, Waters XBridge C18 (250 mm × 4,6 mm, 5 μm) chromatography column with two channels: A: CH_3COONH_4 20 mM in water and B: methanol is used to determine theanine. The excitation wavelength is set to 345 nm and the radiation wavelength to 455 nm.

2.2.3. Method validation

Validation of method is carried as specified in AOAC's Appendix F by measurements of: Specificity, standard range, limit of quantification, and accuracy – precision.

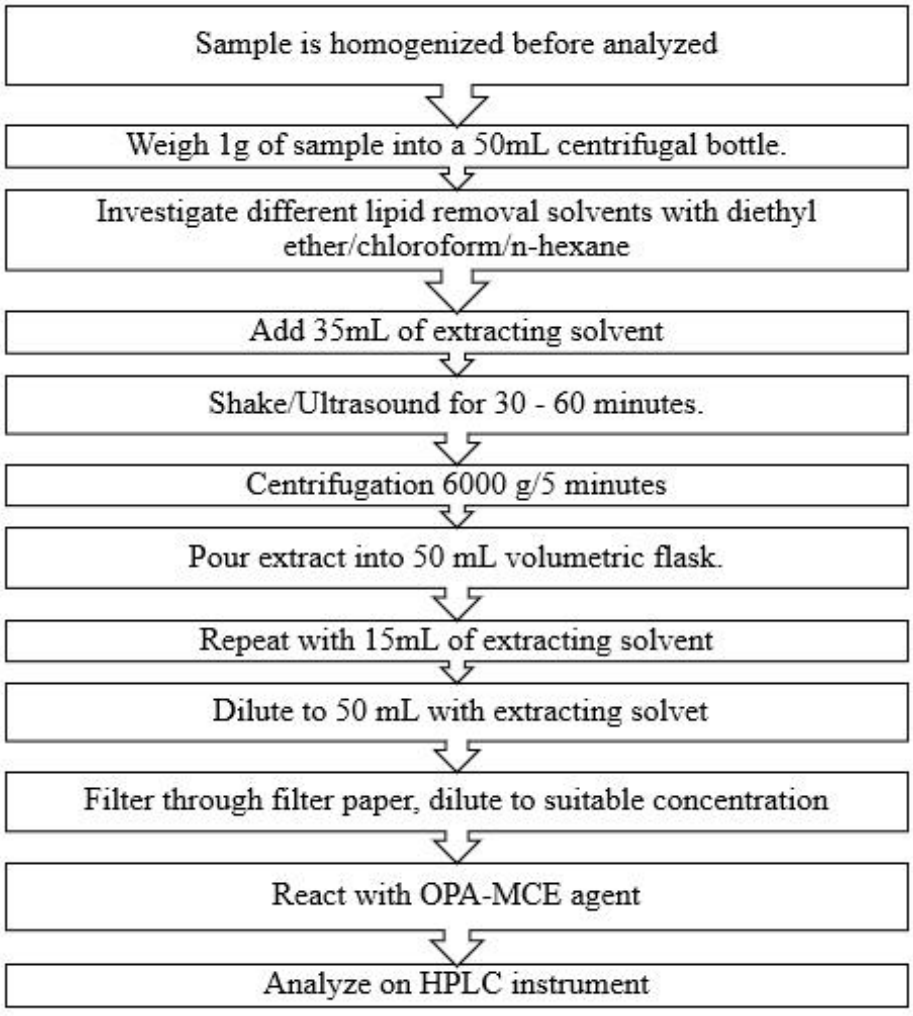

Figure 3. *Expected analytical process*

2.2.4. Applied analysis of real samples

Analysis of real samples (5 tea samples, 5 beverages made from tea samples, 8 health supplement pill samples and 2 ingredient samples) containing theanine bought randomly in the Hanoi area.

3. RESULTS AND DISCUSSION

3.1. Chromatographic conditions

After referencing [11, 12] and some preliminary investigations at the laboratory, the process of optimizing the mobile phase, a program has been constructed as displayed in Table 1.

Table 1. *Mobile phase program for theanine analysis*

Time (mins)	CH_3COONH_4 20mM	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

A chromatogram, provided in Figure 4, is obtained after analyzing the theanine standard solution at 20 µg/mL, along with data regarding the suitability of the system calculated according to USP (US Pharmacopeia): number of theoretical plates: 24801; tailing factor: 1.38, resolution of theanine with OPA: 7.40.

The results are up to standard due to compliance with the following requirements: number of theoretical plates N ≥ 4000 plates, peak tailing factor 0.9 ≤ 1.38 ≤ 2.0 [1].

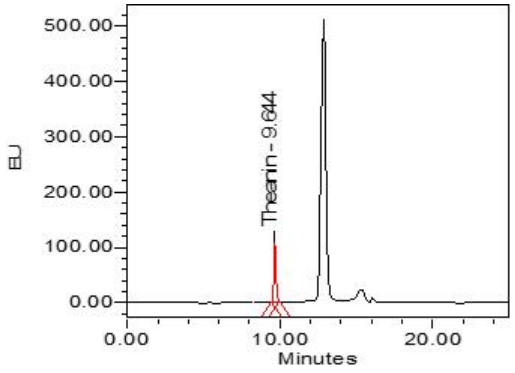

Figure 4. *Chromatogram of standard at 20 µg/mL*

3.2. Theanine and OPA derivatizing agent reaction conditions

3.2.1. OPA/theanine ratio

Theanine solution (8 µg/mL) is derivatized with OPA according to the following theanine to OPA ratios, respectively: 2:1; 1:1; 1:2 at 1-minute reaction time. Results after analysis of these solutions were obtained and shown in Figure 5.

From the results above, it can be observed that higher OPA compositions yielded larger signals. The results also show that, with the 1:1 and 1:2 ratios of OPA agent reacted, the peaks are not considerably differentiated, however, these two ratios' peak size are larger than that of the 1:2 ratio. On the other hand, large amounts of derivatizing agent entering the column will dirty it quicker, leading to the degradation of the analytical column. So to optimize the amount of OPA, derivatization signal and the longevity of the column, this study has chosen the OPA/theanine ratio to be 1:1. This result is also backed by past researches [14].

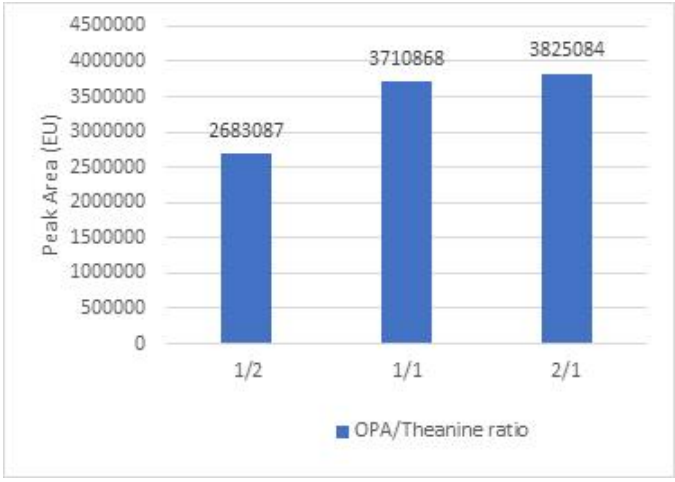

Figure 5. *Results on different theanine to OPA ratios*

3.2.2. OPA/theanine reaction time

The derivatization is carried out with the following reaction times, in order, 0.5, 1, 2, 3 and 4 minutes, and analyzed with the HPLC system. The time of reaction results are shown in Figure 6.

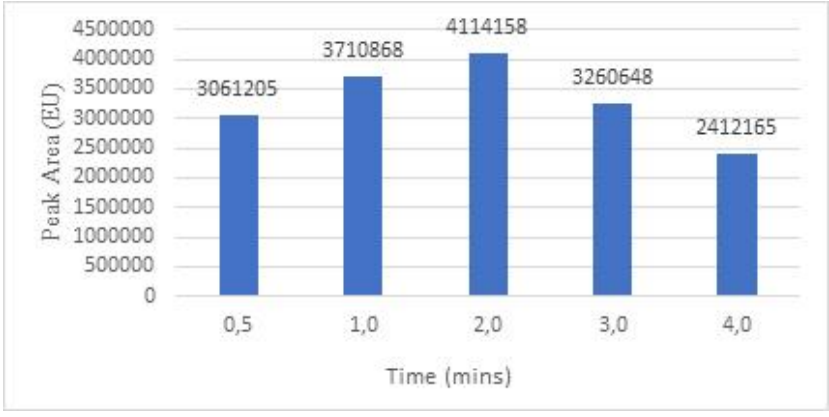

Figure 6. *Results on different reaction times*

The results in Figure 6 shows that the 2-minute time has the highest peak area. Because the derivatizing product of amino acid and OPA is an unstable one, too much time spent to react will lead to the product degrading, and too little time will be insufficient for them to completely react with one another. Therefore, the derivatization time has been chosen to be 2 minutes. This result also corresponds to a past study [14].

3.3. Sample processing conditions investigation

3.3.1. Investigation of extraction methods

The sample is extracted with water, shaken and ultrasonicated. Afterwards, the samples are analyzed on HPLC. The results are displayed on Figure 7.

From the results above, the sample extracted with ultrasonication offers a higher extraction yield compared to the shaking method. Due to theanine not being easily degraded by high heat (100°C), the ultrasound-assisted method will be able to eliminate some unstable impurities and extract theanine better.

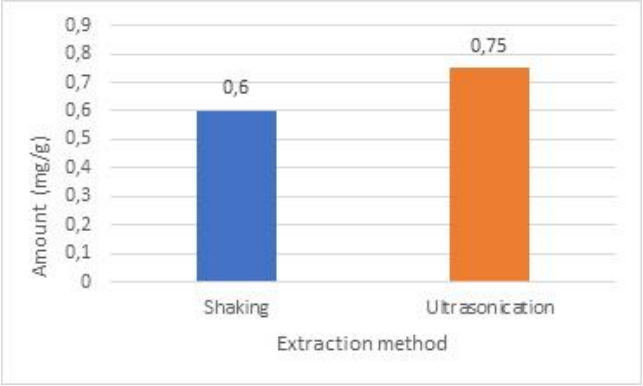

Figure 7. *Results on different extraction methods*

3.3.2. Investigation of lipid removal conditions

The samples are prepared with aliphatic solvents, in order: diethyl ether, chloroform, n-hexane. Three available solvents which theanine does not dissolve in are selected at laboratory conditions. At the same time, excipients used in soft capsule sample: soy bean oil and white beeswax, usually dissolve well in nonpolar solvents. The results of the lipid removal conditions investigation are displayed on Figure 8.

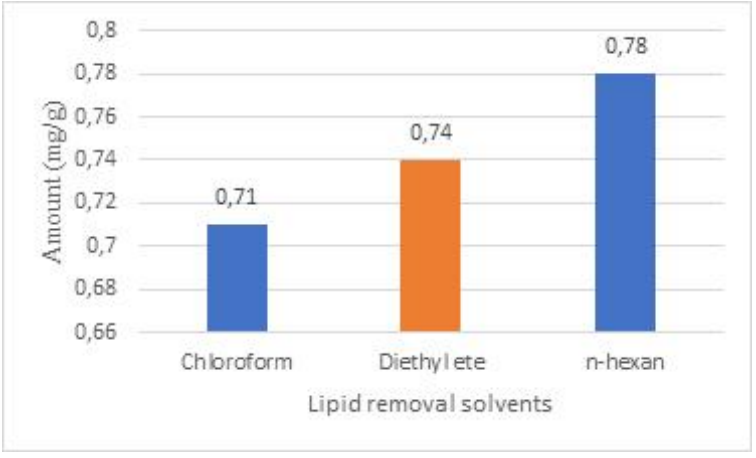

Figure 8. *Results of lipid removal solvents*

The results in Figure 8 shows that defatting with n-hexane yields the highest amount of theanine, while defatting with chloroform yields the lowest. These results are also supported by other studies as theanine dissolves well in polar solvents, therefore, when the polarity of the solvent is increased, the theanine amount will decrease.

3.3.3. Investigation of extraction solvents

The sample is extracted with solvents, respectively, H_2O, 0.1 M HCl và pH 9.2 borate buffer. These three solvents are selected due to L-theanine dissolving well in H_2O and 0.1 M HCl creating the environment to form theanine's saline form. The pH 9.2 borate buffer is used in this investigation due to its ability to create a basic environment for the OPA and theanine derivatizing process. The extraction solvents investigation results is given in Figure 9.

From the Figure 9, the water (H_2O) solvent gave the highest extraction yield. The fact that pH 9.2 borate buffer had a lower yield may be due to theanine not being able to dissolve

as well in a saline environment. The lowest yield by 0.1 M HCl might be a result of the analyte being prone to degrading in an acidic environment.

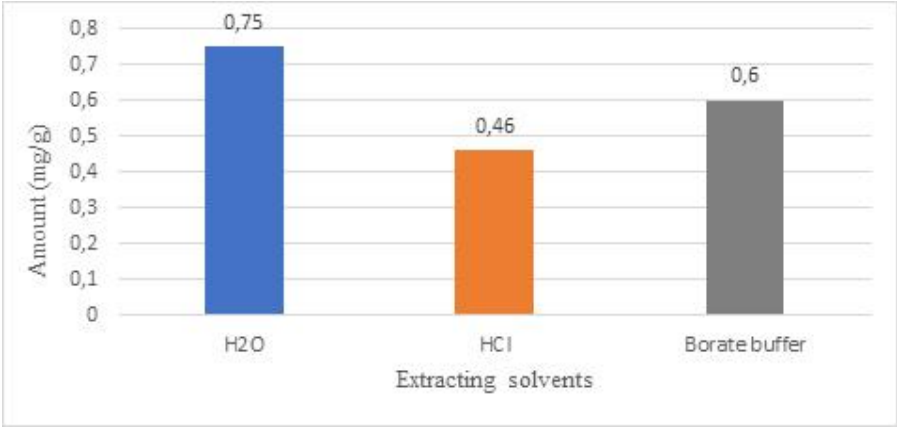

Figure 9. *Results on different extracting solvents*

3.3.4. Investigations of extracting time

The samples are extracted with water and sonicated for the following durations: 10, 20 and 30 minutes, then analyzed through HPLC to yield the results in Figure 10 below.

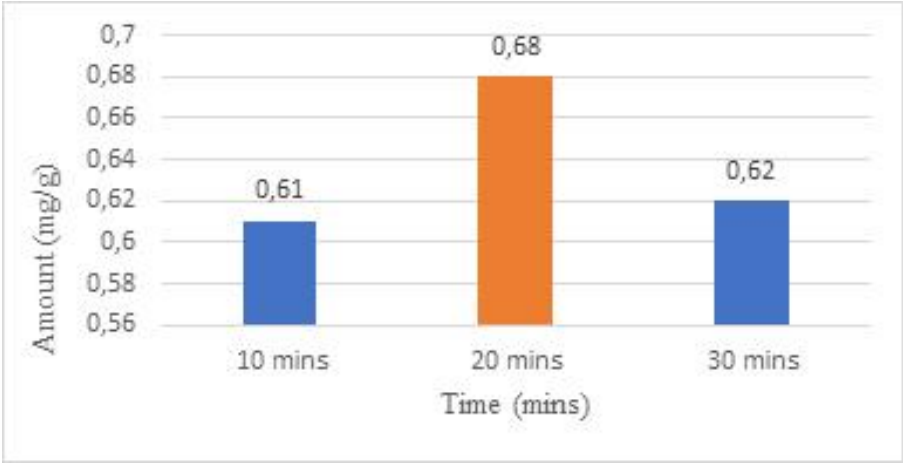

Figure 10. *Results on different extraction time*

From the results above, there were no discernible differences between 10 and 30-minute extraction time but the results are both lower than that of the 20-minute extraction time. To ensure that the extraction time is optimized, the study has decided to extract the samples for 20 minutes. These results have been more optimal than that of past studies [14].

3.3.5. Investigation of extraction temperatures

The samples are ultrasound-assisted extracted with H_2O at temperatures of 20°C, 40°C, 60°C, 80°C, then analyzed by HPLC, giving results like shown in Figure 11.

Based on the results above, the higher the extraction temperature, the larger the theanine signals. This makes sense due to theanine's being easily-dissolved in water and stable under heat. Because of this, 80°C has been chosen to be the optimal extraction temperature. This result is also supported by past researches [15].

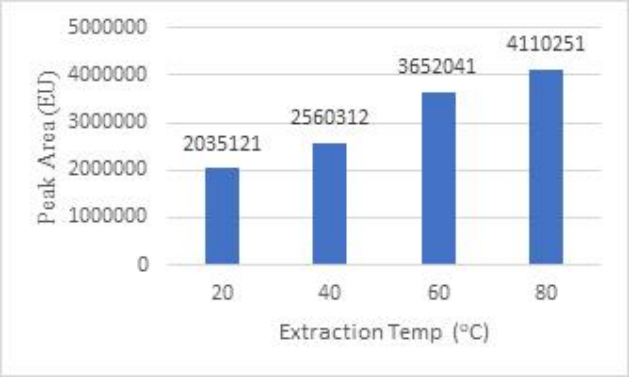

Figure 11. *Results on different extraction temperatures*

3.3.6. Optimized analytical method after investigations

After sample preparation conditions have been investigated, the process of analyzing theanine in tea, tea beverages and health supplements is utilized to validate the method as follows:

Sample Preparation: Begin with homogenizing the sample, weigh 1g of homogenized sample into a centrifugal bottle. For samples with high concentrations of oils and fats, add 30 mL of n-hexane, vortex to completely homogenize (be careful as not to bursting the cap open or blowing up the bottle by lightly shake and let the gas exhaust). Add about 35 mL of distilled water into the centrifugal bottle, lid, sonicate at 80°C for 20 minutes. Centrifuge at 6000 rpm for 5 minutes and transfer the extracts to a 50 mL volumetric flask. Repeat the process for the undissolved solids one more time. Dilute to volume with distilled water. Shake well, filter through 0.45 µm filter paper, transfer the filtrate into vials and analyze on the HPLC system.

Derivatization: Auto-derivatize on the HPLC system with 1:1 OPA/theanine ratio.

Chromatography Conditions: analytical column: C18 (250 mm × 4,6 mm, 5 µm); flowrate 0,8 mL/min; column temperature: 30°C, analysis time: 20 phút, mobile phase: channel A: 20 mM CH_3COONH_4 and channel B: Methanol according to the gradient program in Table 1.

3.4. Method validation

3.4.1. System stability

The 20 µg/mL standard are run for 6 times, yielding %RSD results of 1.60 ≤ 2%, therefore corresponding with the stability of the system (Table 2).

Table 2. *System stability results*

No.	Peak Area	SD%	RSD%
1	10623372	0.33	1.60
2	10759828		
3	10196632		
4	10664555		
5	10521974		
6	10769432		

3.4.2. Specificity of method

The selectivity of the method is determined as follows:

Standard: 40 µg/mL theanine standard solution

Blank: Herbal (amin) tea is used to be the representative blank

Standard with blank in tea sample: Weigh 1.0g of the blank sample from above, and add 0.5 mL of 1000 µg/mL standard solution

The results are shown in Figure 12.

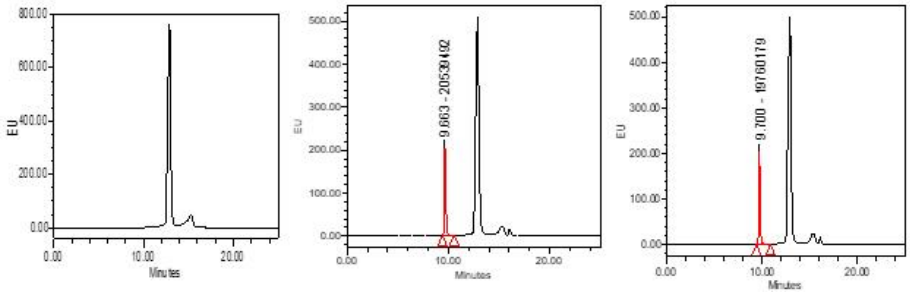

Figure 12. *Chromatogram of the specificity of (a) Blank, (b) Standard and (c) Blank with Standard*

Figure 12 has shown that there are no peaks on the blank sample's chromatogram at the analyte's retention time, the difference between the standard's and blank + sample's retention time is 0.38% < 2,0% which corresponds with [16].

3.4.3. Standard curve of L-theanine

L-theanine standard solutions are prepared at 0.8; 2; 4; 8; 20; 40 µg/mL, in this specific order. The solutions are derivatized with OPA and analyzed with HPLC. The curve is established to demonstrate the correlation between the peak area and concentration of the corresponding analyte. The standard curve of L-theanine is displayed in Figure 13.

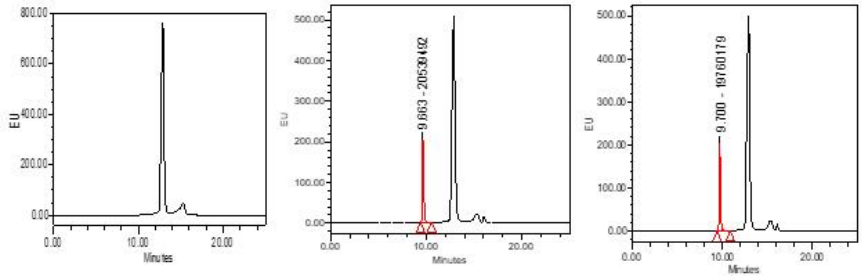

Figure 13. *L-theanine's standard curve*

The results shown above shows that the standard curve has a coefficient of determination $R^2 \geq 0{,}995$, and the bias of all data points are under 15%, fulfilling the requirements of AOAC [16].

3.4.4. Limit of detection, limit of quantification (LOD, LOQ)

The standards are added to the blank sample to reach a concentration of 0.8 µg/mL. The analysis is performed as discussed above. The signal to noise ratio (S/N) calculated with the system's Empower software given an S/N value of ≥ 10. Therefore, the chosen LOQ is 10 µg/g and LOD, 3 µg/g. These LOQ and LOD values are calculated based on the R-value of 10 repeated runs at low concentration (0.8 µg/mL). The R-value with the estimated LOD and LOQ value satisfies the requirement at 4<R<10.

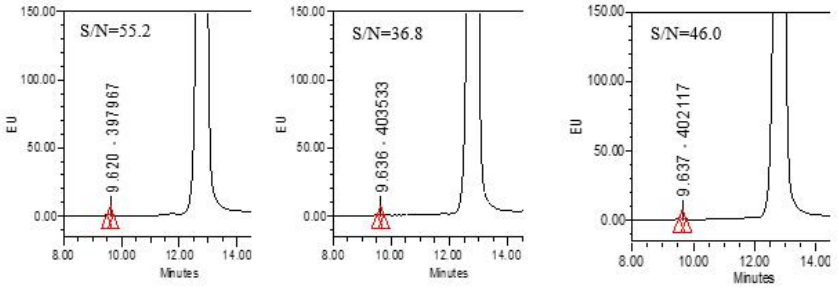

Figure 14. *Chromatogram of theanine at LOQ*

3.4.5. Accuracy and precision

The accuracy and precision of the method is determined through the recovery rate, repeatability and internal reproducibility. Theanine standard is added to blank samples at 3 concentrations: 0.8 µg/mL; 20 µg/mL and 40 µg/mL and analyzed as specified above. Each concentration is repeated for 6 times. The internal reproducibility is performed similarly on different days. The validation results show that the method has met the requirements for accuracy and precision for all three samples (Table 3).

Table 3. *Results on the accuracy and precision of theanine*

Sample	Amount	Accuracy (% Recovery)	Precision	
			Repeatability (RSD_r %)	Reproducibility (RSD_R %)
Teas	20 µg/g	95.9 - 102	3.26	5.52
	2 mg/g	97.8 - 104	2.34	5.17
	4 mg/g	97.8 - 104	3.02	4.11
Beverages made from Tea	20 µg/g	96.8 - 104	3.49	5.26
	2 mg/g	97.8 - 105	2.93	4.28
	4 mg/g	98.1 – 104	2.70	3.65
Health Supplements	20 µg/g	97.0 – 104	3.19	5.33
	2 mg/g	98.7 – 104	2.86	4.12
	4 mg/g	99.2 – 103	1.17	1.29
Ingredients	100%	98.6 – 101	1.05	1.87

The results obtained from Table 3 shows that the method has satisfied AOAC on recovery rate (80-110%), repeatability (≤ 7%) and internal reproducibility (≤ 10%) of tea, tea-based beverages and health supplements bases. In ingredient samples, the results have also achieved the recovery rate (98-102%), repeatability (≤ 1.2%) and internal reproducibility (≤ 2%). The validation results shows that the method meets the requirements for methodological efficiency to be performed and applied in analyzing real samples.

3.5. Application of real samples analysis

After the validation has fulfilled the requirements of AOAC, the process was applied to analyze the amount of theanine in actual samples bought randomly over the Hanoian area, including: 5 tea samples, 8 health supplements, 5 beverages made from tea and 2 ingredient samples. The results are shown in Figure 15.

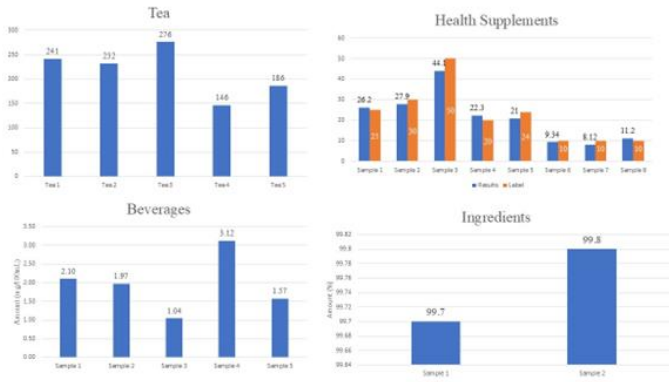

Figure 15. *Results on analyzing real samples*

From the results above, theanine amounts in tea samples falls in the range of 146 – 276 mg/100g. In health supplements, there is a discrepancy between the amounts listed on the labels and all the analytical results of 20% lower concentration in the labels. Moreover, the theanine amount in tea-based beverages fluctuates between 1.04 and 3.12 mg/100mL. Lastly, the ingredients sample yielded results from 99.7 to 99.8 g/100g, displaying high accuracy in the methodology.

The utilization of OPA derivatization to quantify theanine approach shows potential in analyzing theanine in tea sample, tea beverages and health supplements. However, other theanine-supplemented samples need to continuous investigations and improvements. In the upcoming time, the research group will continue to develop this method to be more suitable for applying to many more sample bases.

4. CONCLUSION

This study had succeeded in quantifying theanine in tea, beverages and health supplements containing green tea extract via high performance liquid chromatography method using OPA derivatization. The process has been optimized for both samples. This method has also been validated for specificity, standard curve, precision, accuracy and limit of detection and limit of quantification. The validation data has all satisfied AOAC's requirements. It has been applied to analyze tea, beverages made from tea and health supplements containing theanine. This method can be further extended to quantify theanine's amount in many other sample types.

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