MINISTRY OF EDUCATION AND TRAINING VIETNAM NATIONAL CHEMICAL GROUP

VIETNAM INSTITUTE OF INDUSTRY CHEMISTRY

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RESEARCH ON THE EXTRACTION, PURIFICATION OF LUTEIN AND ZEAXANTHIN, AND FORMULATION OF NANOSIZED EMULSION PRODUCTS FROM MARIGOLD PETALS (*TAGETES ERECTA* L.)

Major: Organic Chemistry

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SUMMARY OF THE DOCTORAL THESIS IN CHEMISTRY

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The thesis will be defended before the State-level Thesis Evaluation Council at the at the Vietnam Institute of Industry Chemistry

The thesis can be studied at the library: Vietnam Institute of Industrial Chemistry

A. INTRODUCTION OF THESIS

1. The urgency of the thesis

Lutein and zeaxanthin are two isomers belonging to the carotenoid group, and during the process of extraction, separation, and purification, these two compounds are always found together. The active ingredient content of the product is considered as the combined amount of these two compounds. In fact, this mixture can be named after the predominant active ingredient, lutein, or referred to as lutein and zeaxanthin.

Lutein is an active ingredient that provides protection to the eyes and skin against the harmful effects of high-energy visible radiation. It helps prevent certain eye diseases in the elderly, such as macular degeneration and cataracts. Additionally, it reduces the risk of atherosclerosis and certain types of cancers, while also improving cognitive abilities, language skills, and memory in older individuals. The human body is incapable of synthesizing lutein and zeaxanthin naturally, hence the necessity of incorporating products containing these compounds.

The majority of commercial lutein products worldwide are obtained through extraction from marigold petals (*Tagetes erecta* L.). Global trends, including those in Vietnam, involve the exploration and utilization of bioactive natural compounds for disease prevention and treatment. Lutein extracted from marigold petals is a natural compound possessing significant biological and pharmacological activities.

Despite their considerable medicinal properties, lutein and zeaxanthin have large molecular sizes and are challenging to dissolve in water, which makes their absorption within the body difficult. To overcome this limitation, reducing the size of the active ingredients to the nanoscale has been proposed as a solution to enhance their absorption capacity and biological activity.

However, research on the extraction and purification of lutein and zeaxanthin in Vietnam for pharmaceutical purposes remains unexplored.

Based on this foundation, the research direction of extracting, purifying lutein and zeaxanthin, and formulating nanosized emulsion products from Marigold petals has been selected to be implemented as the thesis topic.

2. Research objectives of the thesis

Within the framework of the research directions of the Key Laboratory of Refining and Petrochemical Technology (RDDD), the main objectives of the thesis are as follows:

- Conduct systematic research on the extraction and purification of lutein and zeaxanthin from marigold plants in order to obtain a product that meets the standards specified by the United States Pharmacopeia (USP), thereby enabling its commercialization.
- Develop a highly stable nanodispersion system of lutein and zeaxanthin to provide guidance for their application in the pharmaceutical industry.

3. The main contents of the thesis

• Selection of raw materials and methods for the preliminary processing and preservation of marigold materials.

• Extraction, separation, and purification of lutein and zeaxanthin to meet the standards set by the United States Pharmacopeia (USP 40).

• Isolation of high-purity lutein and zeaxanthin for use as active analyte standards in laboratory experiments.

• Conducting stability and toxicity testing of lutein and zeaxanthin.

• Preparation of highly stable lutein nano-emulsions to enhance the oral bioavailability of lutein.

4. Scientific and practical significance of the thesis

The findings of this thesis hold significant scientific and practical value in the field. The successful purification of high-purity lutein and the development of lutein nano-emulsions using a simple method have potential implications for commercialization. These advancements pave the way for a proactive domestic supply of lutein and zeaxanthin, thereby contributing to the enhancement of ocular health among the Vietnamese population.

5. New contributions of the thesis

The process of extracting and purifying lutein and zeaxanthin from marigold petals was systematically studied, starting from the preliminary processing of raw materials to the preservation of the final product. The obtained lutein, after hydrolysis of the extract, was recrystallized at 50 ºC using an ethanol/water solvent system (1/1 v/v ratio) to obtain high-purity lutein that met the standards specified in the United States Pharmacopeia (USP 40). This recrystallization method offers high purity, short crystallization time, low solvent volume, and does not involve the use of any toxic solvents, making it suitable for industrial-scale deployment.

Lutein and zeaxanthin were isolated from the product mixture using silica gel column chromatography. From a mixture containing 96% total lutein, 260 mg of lutein standard (with a content of over 98%) and 6 mg of zeaxanthin standard (with a content of over 95%) were successfully isolated. These isolated compounds met the quality criteria as analytical standards for high-performance liquid chromatography (HPLC).

A lutein-containing nano-emulsion was successfully prepared with an optimized formula consisting of lutein (0.5% in soybean oil), soybean oil (1%), Tween 80 (18%), Span 60 (4%), pectin (0.06%), and double-distilled water (100 ml). The resulting nanoparticles had an average size of

approximately 56 nm, which remained stable in size and shape for 43-44 days. After 24 months of storage, under specified conditions, the emulsion system did not undergo phase separation, and the particle size was measured at 97 nm.

6. The structure of the thesis

The thesis comprises 152 pages (excluding appendices) and includes 63 tables and 57 figures. It is structured into the following sections: Introduction (2 pages), Theoretical Overview (32 pages), Experimental Methods (19 pages), Results and Discussion (80 pages), Conclusion (2 pages), New Contributions of the Thesis (1 page), List of Published Scientific Works (1 page), and References (15 pages) containing a total of 162 references.

B. MAIN CONTENT OF THE THESIS

Chapter 1: OVERVIEW

Marigold flowers (*Tagetes erecta* L.) are widely recognized as the primary natural source of lutein. Lutein exhibits notable protective effects on the eyes, including defense against age-related macular degeneration, retinal nerve damage, and cataracts. Additionally, lutein possesses antioxidant and anti-inflammatory properties, as well as anti-cancer and cardiovascular protective activities.

Numerous publications worldwide have focused on the extraction and purification of lutein and zeaxanthin from marigold flowers. However, obtaining lutein in a high-purity form remains challenging.

In Vietnam, several research projects have recently emerged concerning the extraction of lutein and zeaxanthin-rich extracts from marigold flowers for the development of functional foods. However, there is a lack of studies specifically addressing the production of lutein and zeaxanthin preparations

that meet the standards outlined in the United States Pharmacopeia (USP) using marigold flowers.

Chapter 2: EXPERIMENTS

2.1. Chemicals, tools and equipment

Chemicals and supplies were sourced from reputable suppliers such as Sigma Aldrich, Merck (USA), as well as suppliers from China and Vietnam. The thesis utilized standard laboratory tools including beakers, glass extraction columns, Pasteur pipettes, micro pipettes, and analytical devices such as UV-Vis spectrophotometers, HPLC (High-Performance Liquid Chromatography), LC-MS/MS (Liquid Chromatography-Mass Spectrometry), as well as other equipment including drying ovens, rotary vacuum evaporators, stirrers, ultrasonic machines, homogenizers, etc.

The raw materials used in the study were fresh marigold flowers sourced from Nam Dinh, Vietnam. The flowers were harvested from December of the previous year to May of the following year.

2.2. Research Methods.

2.2.1. Methods for preliminary processing and preservation of raw materials.

Fresh marigold flowers were carefully separated from stalks, sepals, and pistils, and only the yellow-orange petals were collected. The flowers underwent treatment and drying processes according to the research protocols. Subsequently, the dried petals were finely ground and stored in sealed bags at -10 °C.

To determine the total lutein content, 1000 g of dried samples prepared using different drying methods were finely ground and extracted with ethyl acetate for 24 hours. The extracted solution was then evaporated to obtain

the extract, which was analyzed using UV-Vis spectroscopy. Based on the results, the appropriate drying and preservation methods were selected.

2.2.2. Preprocessing Method for Dried Marigold Flower Powder

The dried powder of marigold petals underwent pretreatment using NaOH, sulfuric acid, or citric acid, followed by extraction to obtain lutein esters. The resulting extract was analyzed for total lutein content using a UV-Vis spectrophotometer. The influencing factors such as temperature, time, and concentration of the treatment agents were investigated. Based on these studies, the optimal method for preprocessing the raw materials was determined.

2.2.3. Extraction of Lutein Ester-Rich Extracts from Marigold Flowers

A 100 g sample of marigold petal powder was soaked in an extraction solvent for 2-24 hours with stirring at temperatures ranging from 30°C to 70°C. After extraction, the filtrate was collected using vacuum filtration with Whatman filter paper No. 1. The solvent was then distilled from the filtrate using a rotary vacuum evaporator with a heating device set at 50-60°C. The resulting extract was stored at -10°C under vacuum and protected from light. The total lutein content in the extract was determined using a UV-Vis spectrophotometer.

2.2.4. Enrichment of the Extract

A 20-gram portion of the extract was dissolved in 99.7% ethanol and heated at 30-70°C until complete dissolution. Partially distilled water was added to the solution while maintaining a temperature of 70°C for 20 minutes, followed by continued stirring to dissolve the extract. The temperature of the system was gradually reduced to 60°C. A less polar solvent (such as n-hexane or ethyl acetate) was added to the ethanol-water system. The mixture was stirred for 10 minutes and then left to settle. The upper layer containing the lutein ester was collected, while the bottom water

phase was transferred to another container. The extraction process was repeated twice using the lower layer. The collected extracts were then combined and subjected to rotary evaporation.

2.2.5. Extraction, Hydrolysis, and Crystallization of Free Lutein

The extract was hydrolyzed using a KOH solution in 96% ethanol (EtOH) to obtain crude lutein.

The crude lutein was dissolved in EtOH, and then water (H2O) was added at 50°C. The resulting mixture was hot filtered to collect the crystals, which were subsequently washed multiple times with hot water. The crystals were dried in a vacuum oven at 50°C for 8 hours.

The purified lutein was evaluated for storage conditions, stability, acute toxicity, and long-term toxicity.

2.2.6. Preparation of Lutein and Zeaxanthin Standards for HPLC Analysis

Lutein and zeaxanthin were isolated using silica gel column chromatography.

2.3. Methods for Preparing Lutein Nanoemulsions

Preparation Method:

Oil Phase Preparation: Lutein and a lipophilic surfactant were dissolved in soybean oil and heated to 60°C.

Aqueous Phase Preparation: Pectin, a hydrophilic surfactant, was dissolved in water and heated to 70°C.

The two phases were combined and subjected to dispersion using a homogenizer at 9,000 rpm to form a crude oil/water (O/W) emulsion.

Nanoparticle preparation was achieved by subjecting the emulsion to sonication at 40 Hz for 3-15 minutes. The emulsion was further homogenized

to reduce the particle size using a D-160 homogenizer at 10,000 rpm for 2 minutes at 25°C.

The nanoemulsion was stored in a dark glass jar with a tight-fitting lid at room temperature. Ultrasound working and resting times were set to 5 seconds and 7 seconds, respectively, to prevent overheating. Circulating cold water was used in the storage tank to maintain the sample temperature at 30-40°C for stabilization.

Experimental Design: The experimental design followed the central composite design method with the assistance of Modde software.

Evaluation: The lutein nanoemulsion was evaluated for shape, particle size, and stability.

2.4. Methods for Analyzing Lutein and Zeaxanthin Content

The content of lutein and zeaxanthin was analyzed using UV-Vis spectrophotometry, high-performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) instruments.

Thin-layer chromatography and column chromatography were employed to detect the presence of lutein and zeaxanthin in raw materials and to isolate them.

One-dimensional (1D) nuclear magnetic resonance (NMR) spectroscopy, including H1 NMR and 13C-NMR, as well as two-dimensional (2D) NMR techniques such as heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum coherence (HSQC), were utilized to identify lutein and zeaxanthin in $CDCl_3$ and CD_3OD solvents.

Chapter 3: RESULTS AND DISCUSSION

3.1. Results of Marigold Material Selection and Initial Processing Methods

3.1.1. Results of Surveying the Shade Drying Method

The optimal drying time for fresh marigold petals was determined to be 36 hours. After drying, the marigold petals with a moisture content of 16.6% yielded a total lutein content of 8.5 mg/g in the dried flowers.

3.1.2. Results of Surveying the Drying Method with a Blower

The suitable drying time for the blower oven drying method was found to be 24 hours at a temperature of 50°C. After drying, the dried marigold petals with a moisture content of 5.0% exhibited a total lutein content of 12.6 mg/g in the dried flowers.

3.1.3. Results of Surveying the Vacuum Drying Method

The optimal vacuum drying temperature was determined to be 50°C for 16 hours. After drying, the dried marigold petals with a moisture content of 1% yielded a total lutein content of 12.9 mg/g in the dried flowers.

3.1.4. Comparison and Selection of the Appropriate Drying Method

In the laboratory setting, the vacuum drying method at 50°C for 16 hours resulted in the highest lutein content in the dried marigold petals.

On a large scale, the oven drying method with a blower for 24 hours at 50°C was chosen. This method offered high drying efficiency, comparable to vacuum drying, while significantly reducing investment and operating costs compared to vacuum drying.

3.2. Results of Lutein and Zeaxanthin Extraction and Purification Meeting Pharmacopoeial Standards

3.2.1. Results of Pretreatment of Dried Marigold Flower Powder

Pretreating the CVT powder resulted in improved extraction efficiency and required fewer extraction cycles compared to untreated CVT powder. Citric acid pretreatment was found to be particularly effective. Therefore, the CVT powder was pretreated with 0.6% citric acid at 50°C for 2 hours, using a material-to-agent ratio of 1:10 g/mL. The citric acid-pretreated CVT powder, when subjected to extraction twice, yielded a lutein ester extraction efficiency of 92.95%, which was higher than that of the untreated CVT powder extracted four times (92.33% yield).

3.2.2. Extraction Results of Lutein Ester-Rich Extracts from Marigold Flowers

Lutein ester was extracted from dried marigold flower powder using ethyl acetate as the solvent. The extraction process involved a 6-hour extraction at 60°C, using a solvent-to-material ratio of 10:1 (v/w) and a stirring speed of 200 rpm. Two extractions were performed, resulting in an extraction efficiency of 92.95%, which is significantly high.

3.2.3. Results of Enrichment in the Extraction Process Using the Liquid-Liquid Extraction Method

n-Hexane was employed as the solvent for the liquid-liquid extraction to enrich lutein in the extract.

Enrichment process conditions: The extract was mixed with ethanol and water in a ratio of 7:3 (v/v). Lutein was enriched using the liquid-liquid extraction method with n-hexane solvent. The ratio of total extract/ethanol-water/n-hexane solvent was 1:10:15. Each extraction cycle lasted for 10 minutes, and two extraction cycles were conducted at a temperature of 60°C. The recovery efficiency of the active ingredients after enrichment reached 96.9%.

3.2.4. Results of Hydrolysis of Lutein and Zeaxanthin-Rich Extracts

The optimal conditions for hydrolyzing the lutein-rich extract from marigold flowers were as follows: The post-enriched extract was dissolved in ethanol at a ratio of extract to ethanol of 0.8 g/mL. The hydrolysis process was carried out using KOH with a KOH-to-extract ratio of 0.18 (w/w) at 70°C for 80 minutes. The hydrolysis efficiency of lutein ester was 82.94%.

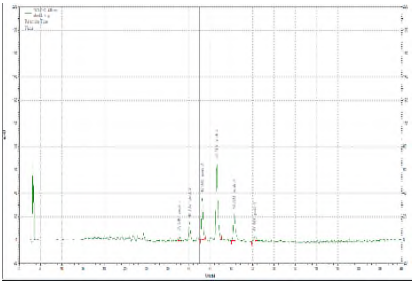


Figure 3.1. HPLC chromatogram of unhydrolyzed extract (lutein ester)

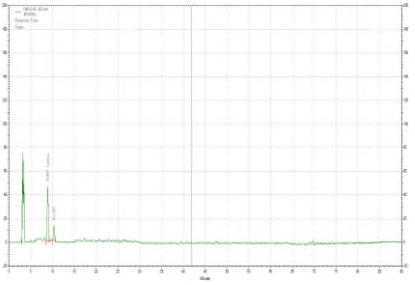
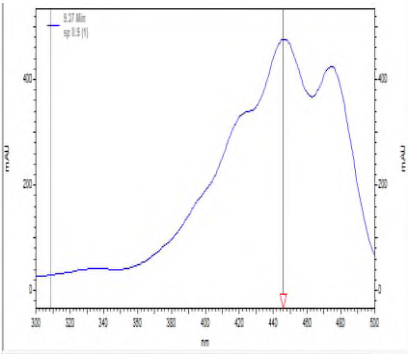
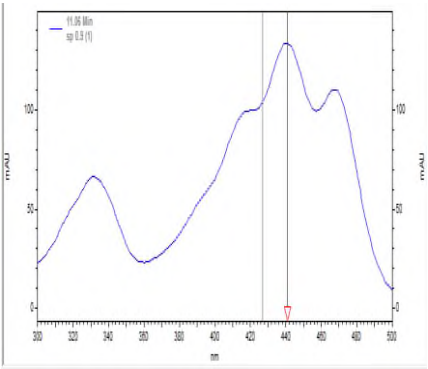


Figure 3.2. HPLC chromatogram of the extract after hydrolysis (free lutein)



trans-lutein:

λ_{max} = 424; 446; 474 (nm)

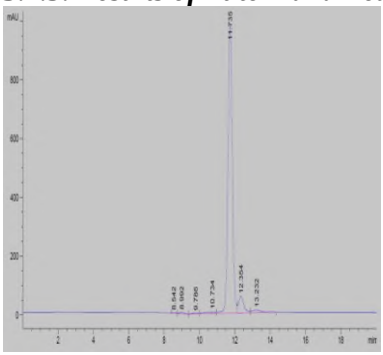


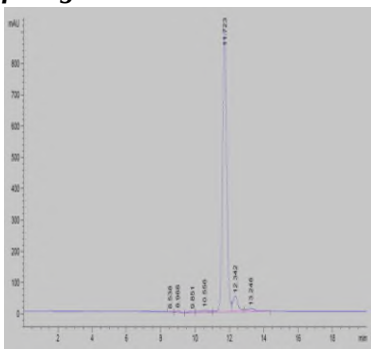
cis-lutein:

λ_{max} = 328; 418; 442; 468 nm

Figure 3.3. UV-Vis absorption spectrum of sample lutein

3.2.5. Results of Lutein and Zeaxanthin Refining Process





Sample of initial crystallized lutein *Sample of double crystallized lutein*

Figure 3.4. HPLC results of lutein crystal samples after crystallization

The hydrolyzed extract underwent purification using an ethanol/water solvent system with a ratio of 1:1 (v/v) at 50°C. Water was used as the crystallizing agent, with a solvent ratio of 100:1 (v/w). The crystallization process achieved an efficiency of 76.3%. The refined product had a total lutein content of 96%, including 88.9% lutein and 7.1% zeaxanthin.

3.2.6. Results of Lutein and Zeaxanthin Isolation as Analytical Standards

The purified lutein was used to isolate lutein and zeaxanthin using silica gel column chromatography.

From a mixture of products containing 96% total lutein, 260 mg of lutein standard (with a content of over 98%) and 6 mg of zeaxanthin standard (with a content of over 95%) were isolated. These standards met the quality criteria for analytical standards in HPLC. The structure of the isolated lutein standard was also determined.

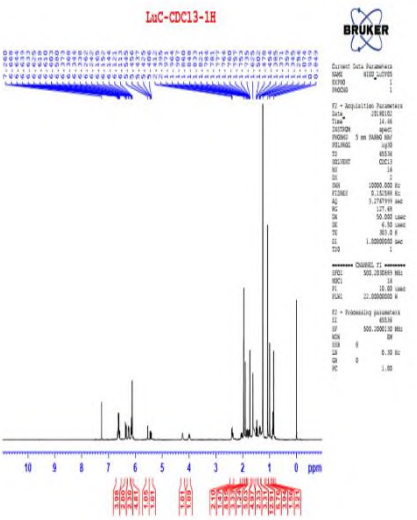


Figure 3.5. 1H-NMR spectrum of the standard lutein

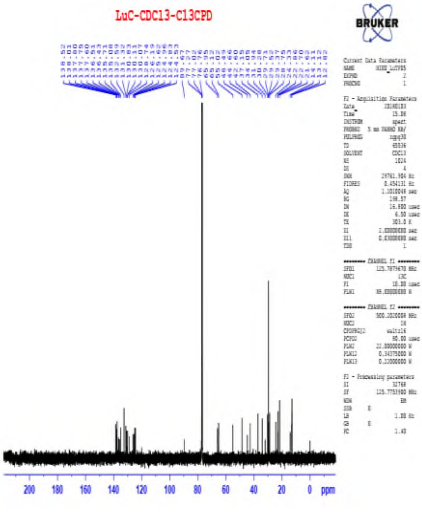
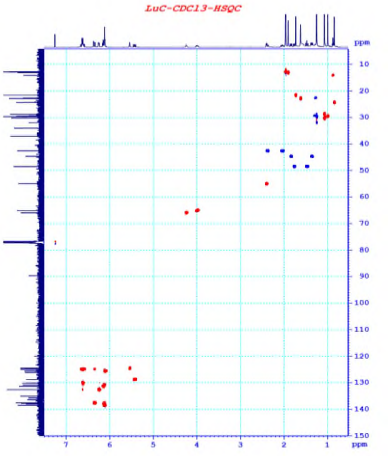
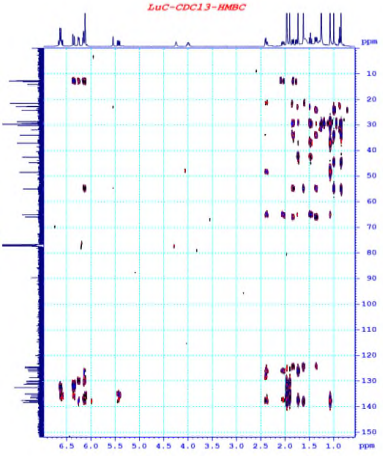


Figure 3.6. 13C-NMR spectrum of the standard lutein



HSQC spectrum



HMBC spectrum

Figure 3.7. HSQC and HMBC spectra of the standard lutein

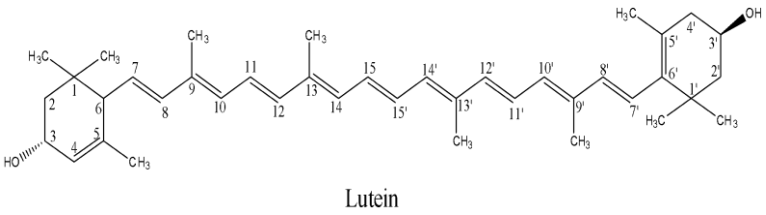


Figure 3.8. Chemical structure of lutein

Lutein, after isolation, was analyzed using UV-Vis spectroscopy, HPLC, and LC-MS/MS, comparing it with the Sigma Aldrich standard.

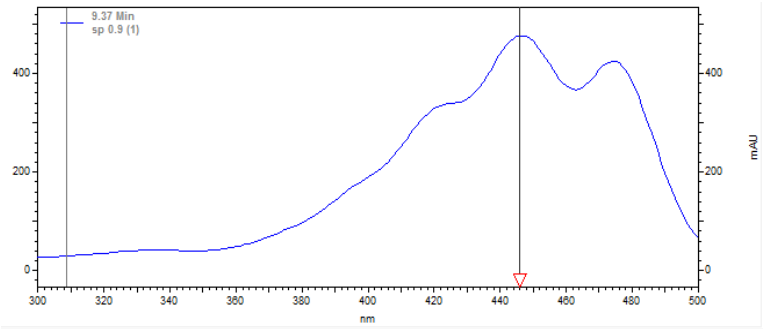


Figure 3.9. UV-Vis spectroscopy of lutein

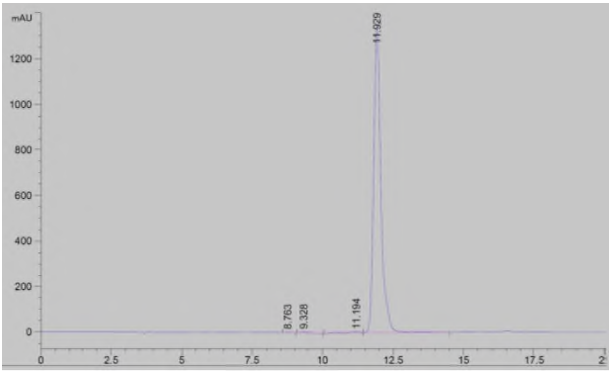


Figure 3.10. HPLC chromatogram of the reference standard lutein

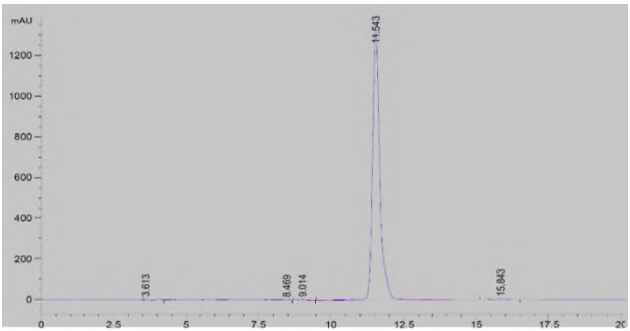


Figure 3.11. HPLC chromatogram of the purified lutein standard

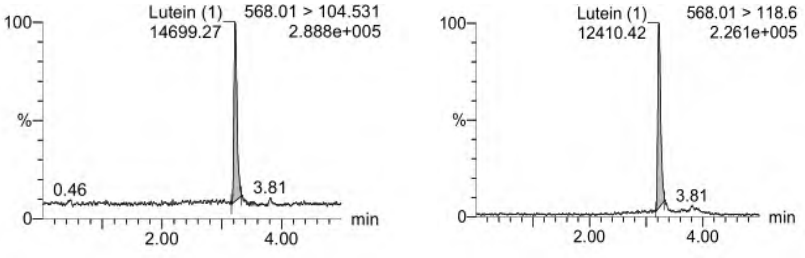


Figure 3.12. LC-MS/MS chromatograms of purified lutein standards

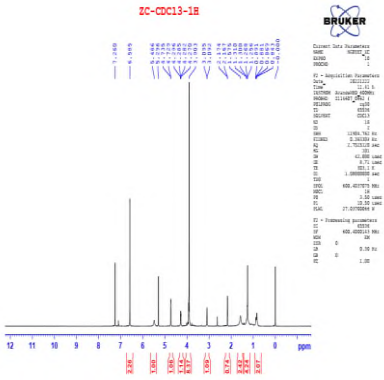


Figure 3.13. 1H-NMR spectrum of the zeaxanthin standard

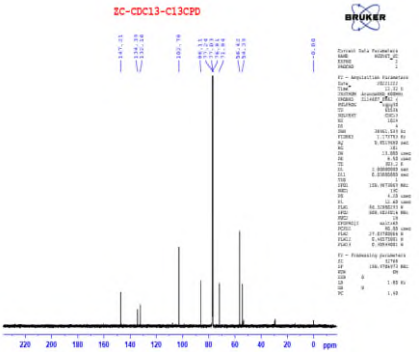
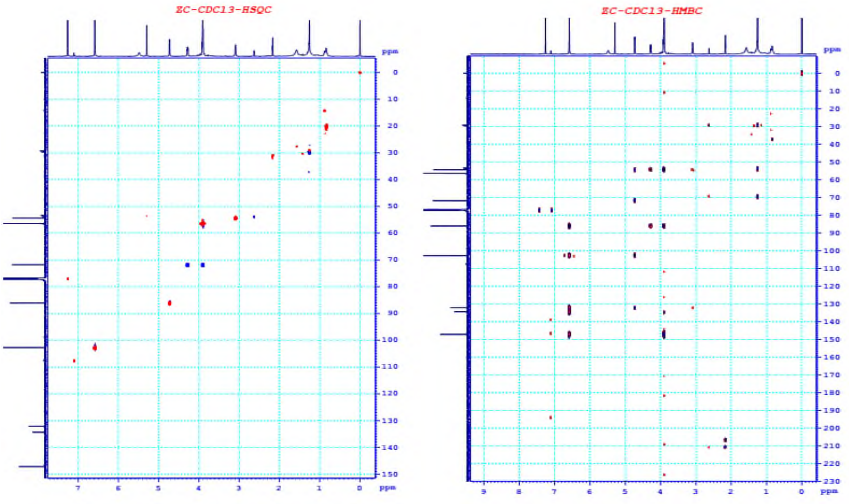


Figure 3.14. 13C-NMR spectrum of the zeaxanthin standard



HSQC *HMBC*

Figure 3.15. HSQC and HMBC spectra of the zeaxanthin standards

Figure 3.16. Chemical structure of zeaxanthin

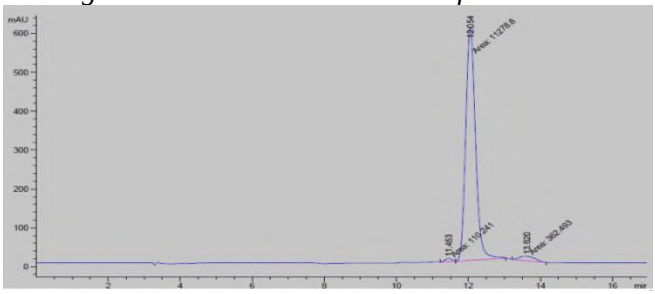


Figure 3.17. HPLC chromatogram of purified zeaxanthin standard

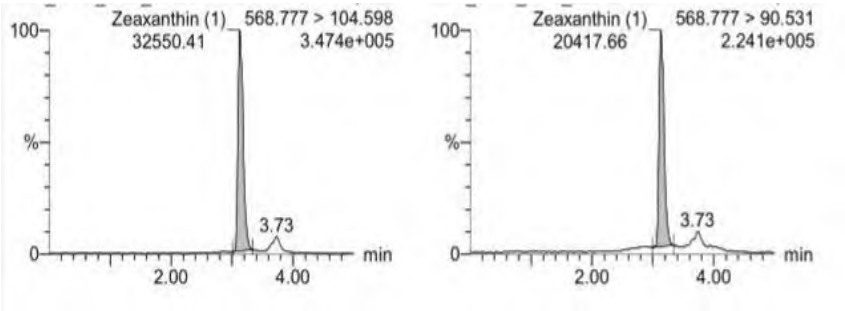


Figure 3.18. LC-MS/MS chromatograms of purified zeaxanthin standards

3.2.7. Study on the Preservation Method of Lutein and Zeaxanthin Products

Lutein is highly susceptible to light degradation, and consistent exposure to light can lead to rapid metabolism and reduced quality. Storage under natural light also results in decreased lutein quality. After 1 week, the lutein content decreased from 96.3% to 87.9%; after 2 weeks, it further decreased to 84.2%; after 3 weeks, it decreased to 75.7%; and after 4 weeks, it decreased to 74.1%. The results demonstrate that lutein is well-preserved when stored in a light-protected environment or a sealed brown bottle. However, under environmental exposure conditions, lutein tends to undergo oxidation, gradually reducing its quality. Lutein remains nearly unchanged in quality for 4 weeks when stored away from light and refrigerated at -10 ºC.

3.3. Evaluation of the Stability of the Extraction and Purification Method of Lutein from Marigold Flowers

The stability assessment results of the extraction and purification method of lutein from a large quantity of dried marigold powder are presented in Table 3.1.

The extraction and purification method of lutein from marigold flowers exhibits high stability. The resulting product, with a purity of 92.9%, meets the USP 40 standard of the US Pharmacopeia. The results have been independently tested at the Institute of Functional Foods.

Table 3.1: Results of Lutein Product Quality

No,	Attributes	Unit	Quality	
			Required	Actual result
1	Total lutein and zeaxanthin content	%	≥ 80	92,9
2	Lutein	%	≥74	85,85
3	Zeaxanthin	%	≤ 8,4	7,05
4	Appearance	-	Yellow-orange	Red-orange
5	Sulfate ash	%	≤ 2,0	1,0
6	Moisture	%	≤ 1,0	Negligible

3.4. Lutein Stability and Toxicity Testing

3.4.1. Lutein Stability Study

The purity of lutein was determined to be 96.3%. The study results indicate that when stored in a vacuum environment at the tested temperatures, the total lutein content remains above 90%, demonstrating stability for over 9 months. However, it was observed that lutein content changes less when samples are stored at lower temperatures, such as -10 ºC, compared to higher temperature ranges.

Therefore, lutein products can be stored at room temperature or at cooler temperatures in a vacuum-sealed sample storage cabinet, protected from light.

3.4.2. Results of Acute and Subchronic Toxicity Testing of Lutein

- Acute oral toxicity (LD50) in white mice: No LD50 value could be determined for a mixture of lutein and zeaxanthin when administered orally to white mice at the highest possible dose of 3000 mg/kg body weight.
- Subchronic toxicity in white rats: Batches of rats were administered a mixture of lutein and zeaxanthin at doses of 1.68 mg/kg body weight/day and 8.40 mg/kg body weight/day for 90 consecutive days. The results showed:
  - Rats remained healthy and exhibited consistent weight gain;
  - Hematological parameters (red blood cells, hemoglobin, hematocrit, mean volume of red blood cells, white blood cells, platelets) remained unchanged;
  - Blood biochemical parameters evaluating liver and kidney function (AST, ALT enzymes, plasma albumin, total cholesterol, creatinine) showed no significant changes;
  - No histopathological damage was observed in the liver, spleen, or kidney.

3.5. Results of Lutein Nanoemulsion Preparation

3.5.1. Selection of Experimental Parameters

Tween 80 and span 60 were chosen as emulsifiers for the formulation of lutein nanoemulsions. Pectin was used as a gelling agent and stabilizer due to its ability to increase emulsion viscosity and reduce the interfacial tension between oil and water, resulting in desirable emulsion textures.

3.5.2. Experimental Design

The formulation of lutein nanoemulsion was designed according to Table 3.2.

Table 3.2. Nanoemulsion Formulation

No.	Ingredients	Content
1	Lutein	5 %
2	Soybean oil	1 %
3	Tween 80 (X_1)	Not fixed
4	Span 60 (X_2)	Not fixed
5	Pectin (X_3)	Not fixed
6	Double distilled water	100 ml

Objective function:

- Average nanoemulsion size (Y_1): $Y_1 \leq 100$ nm.

- Emulsion stability (Y_2): Y_2 –> max (day).

An experimental matrix consisting of 17 experiments was designed using the Modde software.

3.5.3. Impact Analysis

The Modde software was used to process experimental data and analyze the impact of input variables on the objective function, which was then optimized experimentally.

The regression equations are as follows:

$Y_1 = 82.2328 – 13,519\ X_1 – 8,06399\ X_2 + 0.234379\ X_1X_2 – 0.23438\ X_1X_3 – 0.23438\ X_2X_3$.

$Y_2 = 33.4708 + 5.06543\ X_2 + 3.1659\ X_3 – 0.469286\ X_2X_3$.

The analysis of variance (ANOVA) resulted in regression coefficients (R_2) of 0.999 and 0.994 for the functions Y_1 and Y_2, respectively. This

indicates a strong correlation between the input variables and the objective functions, demonstrating the high accuracy, reliability, and existence of an optimal point in the experimental model.

The Modde software provides the following formula for creating lutein-containing nanoemulsions: Lutein 5% (in soybean oil), soybean oil 1%, Tween 80 18%, Span 60 4%, pectin 0.06%, distilled water 2 times 100 ml.

3.5.4. Some Physicochemical Properties of the Lutein Microemulsion System

The lutein nanoemulsion exhibits a dark orange color, with a light refraction of 1.35 ± 0.02 and a density of 1.02 ± 0.01 g/ml. It is completely dispersed in water.

3.6. Researching and Addressing Environmental Issues

During the trial production, citric acid used in the pretreatment of raw materials was recovered and reused multiple times.

The extraction residue, after being completely separated from the solvent through the drying method, is used as a microbial organic fertilizer.

CONCLUSION

1. A stable laboratory process has been established for extracting the active ingredients lutein and zeaxanthin from marigold flowers, consisting of the following consecutive steps:

- Vacuum drying of marigold petals at 50 ºC for 16 hours.
- Pretreatment of dried marigold powder with 0.6% citric acid at 50 ºC for 2 hours, with an agent/material ratio of 10/1 (v/v). The recovery efficiency reached 92.95%.
- Extraction of lutein and zeaxanthin esters from the dried marigold powder using ethyl acetate for 6 hours at 60 ºC, with a solvent ratio

of 10/1 (v/w), agitation at 200 rpm, and two extraction cycles. The extraction efficiency reached 92.95%.

- Dissolution of the extract in ethanol/water (7/3, v/v) and enrichment; extract/ethanol-water/n-hexane = 1/10/15, with two extraction cycles of 10 minutes each at 60 ºC. High extraction enrichment efficiency of 96.9% was achieved.
- Extraction hydrolysis using KOH(C_2H_5OH)/extract = 0.18 (w/w), with a concentration of KOH in C_2H_5OH of 0.144 (g/ml), extract concentration of 0.8 g/mL, at 70 ºC for 80 minutes. The hydrolysis efficiency reached 82.94%.
- Refining of lutein using ethanol/water = 1/1 (v/v) at 50 ºC, with a solvent/lutein ratio of 100/1 (v/w). The refining efficiency was 76.3%. The refined product had a total lutein content of 96%. This utility solution has been recognized and granted a utility solution patent number 2730 (2021) in Vietnam.

2. The process of extracting the active ingredients lutein and zeaxanthin from marigold flowers has been scaled up to produce a lutein and zeaxanthin mixture with high efficiency in lutein ester extraction, extraction enrichment, and hydrolysis. The extraction and refining efficiencies of lutein are 88.92% and 95.4%, respectively. The obtained results yielded 10 kg of product with a total lutein content of 92.9%, consisting of 85.85% lutein and 7.05% zeaxanthin (according to independent test results from the Testing Center - Vietnam Institute of Dietary Supplements). The product meets the USP 40 standard of the US Pharmacopeia, enabling its commercialization for the domestic pharmaceutical industry.

3. Lutein and zeaxanthin were isolated from the product mixture using silica gel column chromatography. From a product mixture containing 96%

total lutein, 260 mg of lutein standard (with a content of over 98%) and 6 mg of zeaxanthin standard (with a content of over 95%) were successfully isolated, meeting the quality criteria as analytical standards for HPLC. This achievement marks the first time that lutein and zeaxanthin achieved standard quality in Vietnam.

4. The quality of the extracted lutein mixture has been tested. The product demonstrates high stability for over 9 months when stored in a vacuum, protected from light at -10 ºC. Although the product can be stored at room temperature, its quality slightly decreases after 9 months. The product shows no acute or semi-permanent toxicity when used at the appropriate dosage and timing.

5. A total of 1500 mL of lutein nanoemulsion has been prepared using the optimized formula: lutein (5% in soybean oil), soybean oil (1%), Tween 80 (18%), Span 60 (4% in 4%), pectin (0.06%), and double distilled water (100 ml). The lutein nanoemulsion product exhibits a nanoparticle size of approximately 56 nm, which remains unchanged in size and shape after 43-44 days. The lutein nanoemulsion is highly soluble in water, exhibits excellent stability, and represents a pioneering development in this field. After 24 months of storage under specified conditions, the emulsion did not separate, and the particle size remained at 97 nm.

6. Research has been conducted on the reuse of waste materials generated during the extraction and refining process of lutein. This includes the recovery and reuse of pretreatment agents, extraction solvents, and composted residues as organic fertilizers. This closed-loop, zero-waste technology promotes sustainability and environmental consciousness.

C. LIST OF SCIENTIFIC REPORTS

● **Scientific reports:**

1. Nguyen Thi Minh Nguyet, Tran Van Hieu, Hoang Thi Hue An, Nguyen Thi Bay, Tran Minh Thu, Nguyen Thanh Binh, Vu Thi Thu Ha (**2018**). Influence of solvents and extraction conditions on lutein extraction from marigold flowers. *Journal of Chemistry*, 56(6E1): 90–93.

2. Nguyen Thi Minh Nguyet, Vu Thi Thu Ha, Nguyen Thanh Binh, Nguyen Minh Dang, Nguyen Thi Bay (2019). Quantification of lutein from Marigold flower (*Tagetes erecta* L.) petals by liquid chromatography – tandem mass spectrometry method, *Vietnam Journal of Chemistry*, 57(2): 240–244.

DOI: 10.1002/vjch.201900020

3. Nguyen Thi Minh Nguyet, Vu Thi Thu Ha, Nguyen Thi Bay, Nguyen Phuong Hoa, Nguyen Hoang Ngan, Nguyen Thanh Binh (**2021**). Acute and subchronic toxicity studies of lutein and zeaxanthin extracted from marigold flowers (*Tagetes erecta* L.). *Journal of Analytical Chemistry, Physics and Biology*, 26(2): 208–213.

4. Nguyen Thi Minh Nguyet, Nguyen Thi Bay, Bach Thi Tam, Nguyen Thanh Binh, Vu Thi Thu Ha (**2021**). Effects of pectin and emulsifiers on the size and stability of lutein nanoemulsions. *Journal of Analytical Chemistry, Physics and Biology*, 20(special issue): 65-69.

● **Intellectual Property:**

1. Vu Thi Thu Ha, Nguyen Thi Minh Nguyet, Nguyen Minh Dang, Nguyen Thi Phuong Hoa, Nguyen Thi Bay (**2021**). Method for the purification of lutein obtained from the saponification of marigold flower extract (oleoresin), *Utility Solutions Patent*, No. 2730.