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EXTRACTION AND PURIFICATION OF LUTEIN FATTY ACID ESTERS FROM MARIGOLD FLOWER

Miss Janya Vechpanich

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
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งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อหาสภาวะที่เหมาะสมของกระบวนทำสารฟรีลูทีนให้บริสุทธิ์จากดอกดาวเรือง ซึ่งประกอบด้วย 2 กระบวนการ คือ ขั้นตอนแรกเป็นการเปลี่ยนรูปลูทีนเอสเทอร์เป็นฟรีลูทีนโดยสะปอนนิฟิเคชันด้วยสารละลายโพแทสเซียมไฮดรอกไซด์ และขั้นตอนที่สองสารเมื่อผ่านสะปอนนิฟิเคชันแล้วจะผ่านกระบวนการทำให้บริสุทธิ์เพื่อให้ได้ความบริสุทธิ์สูงที่สุด สำหรับขั้นตอนสะปอนนิฟิเคชันได้ศึกษาผลของเวลาในการทำปฏิกิริยาในช่วง 0-7 ชั่วโมง, ปริมาณสารละลายด่าง(สารละลายโพแทสเซียมไฮดรอกไซด์)ต่อ 1 กรัมโอลิโอเลซินในช่วง 0.3-1 มิลลิลิตรและความเข้มข้นของสารละลายโพแทสเซียมไฮดรอกไซด์ที่ใช้ 25-45% โดยปริมาตร โดยทำการทดลองที่อุณหภูมิ 75 องศาเซลเซียส พบว่าความเข้มข้นของฟรีลูทีนเพิ่มมากขึ้นเมื่อเวลาเพิ่มจนถึง 4 ชั่วโมง และหลังจากนั้นจะเกิดการสลายตัว สำหรับปริมาณและความเข้มข้นของสารละลายโพแทสเซียมไฮดรอกไซด์เมื่อทำปฏิกิริยาที่ 4 ชั่วโมง พบว่าความเข้มข้นของฟรีลูทีนจะมีค่ามากที่สุดที่ 40% และ 0.5 มิลิลิตรของโพแทสเซียมไฮดรอกไซด์ หลังจากกระบวนการสปอนนิฟิเคชั่นแล้วฟรีลูทีนที่ได้ยังคงมีสิ่งเจือปนอยู่จึงต้องมีการทำสารให้บริสุทธิ์โดยผ่านกระบวนการตกผลึกโดยใช้วิธีการเติมศึกษาน้ำกับเอทานอลที่อัตราส่วน 2:0.5 เป็นเวลา 0.5 ชั่วโมง จะได้ฟรีลูทีนที่มีร้อยละผลได้ 99.12% และความบริสุทธิ์ 85.53% ซึ่งเป็นสภาวะที่ดีที่สุด

ภาควิชา.....วิศวกรรมเคมี.....ลายมือชื่อนิสิต.....

สาขาวิชา.....วิศวกรรมเคมี.....ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2551.....

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KEY WORD : MARIGOLD / LUTEIN / LUTEIN ESTER / EXTRACTION / SAPONIFICATION / CRYSTALLIZATION

JANYA VECHPANICH: EXTRACTION AND PURIFICATION OF LUTEIN FATTY ACID ESTERS FROM MARIGOLD FLOWER.
ADVISOR: ASST. PROF. ARTIWAN SHOTIPRUK, Ph.D., 61 pp.

This study aims optimize process for purification of free lutein obtained from marigold flowers. First, saponification was carried out to convert lutein fatty acid esters in the marigold oleoresin to free lutein . Then, further purification of free lutein could be achieved by crystallization. At a fixed temperature of 75 ºC, we determined the effects of saponification conditions such as time (0-7 hrs), volume to weight ratio of KOH solution to oleoresin (0.3ml: 1g-1ml: 1g), and the concentration of KOH solution (25%-45%) on the concentration of free lutein. The results showed that the concentration of free lutein increased with an increase in time up to 4 hrs, where the maximum concentration was obtained. Extended saponification above 4hrs resulted in observable product degradation. For saponfication of 1 g of oleoresin at 75 C for 4 hr, the suitable volume of KOH solution and concentration of KOH solution were found to be0.5ml and 40% v/v, respectively The volume and concentration higher than 0.5ml and 40% caused product degradation as free lutein is unstable at extreme pH. After saponification, the purity of free lutein was improved by crystallization. The effect of the system of crystallization solvent (water to ethanol and water to isopropanol), the volume ratio of crystallization solvent (2:0.3-2:1 v/v), and crystallization time (0.25-2 hrs) on the yield and purity of free lutein were studied. The suitable crystallization process was found to be that employing 2:0.5 (water to ethanol) and the crystallization time was 0.5 hrs. The purity and yield of free lutein as analyzed by HPLC were found to be 85.53% and 99.12%, respectively.

Department : Chemical Engineering……Student’s Signature……
Field of study: Chemical Engineering…...Advisor’s Signature……
Academic Year…….2008……

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CHAPTER I

INTRODUCTION

1.1 Motivation

Marigold is an herbaceous and ornametal plant in daisy family, cultivated in tropical areas in Southwest of the United States, South America, and Asia (Sowbhagya et al., 2004).Marigold flowers are of various shades from yellow, red, orange, dark orange, to orange brown depending on their varieties. Those mostly culviated in Thailand is of *Tagetes erecta* species. Recently, the use of marigold flower extract as nutritional and medicinal supplements has also been explored and the potential benefits of the marigold extract have been reported including the prevention of cancer and cardiovascular disease, enhanced immune function, inhibition of auto-oxidation of cellular lipids, and protection against oxidant-induced cell damage (Soon et al., 1998).

The most important group of bioactive compounds extracted from marigold is xanthophylls, and of these, lutein is the main component and its health-benefiting functions are increasingly being recognized. In marigold flowers, lutein occurs in a more stable against heat and UV-light, as acylated form or lutein fatty esters.The lutein fatty acid esters compared with their non-esterified (free lutein) form (Subagio et al., 2003). However, it is free lutein that are responsible for protective activity against the two common eye diseases of aging: cataracts and age-related macular degeneration (Olmedilla et al., 2003), which are the main causes of irreversible loss of vision. In addition, a high intake of free lutein may also have other beneficial effects on human health, including protection against cardiovascular disease (Dwyer et al., 2001), stroke, and UV-radiation-induced skin damage.

For these reasons, the development of lutein esters isolation processes as well as the purification process to obtain high purity free lutein from marigold flowers have recently become of considerable interest. Philip et al. (1977) isolated lutein fatty acid esters by dissolution of oleoresin in hot alkanol followed by precipitation of lutein fatty acid ester by cooling the solution. The resulting lutein fatty acid ester contained 51% lutein. At such purity, the product was still not suitable for use as human food. To improve the quality of the extracted lutein, saponification of lutein

fatty acid esters to free lutein is required, followed by crystallization to obtained purified lutein crystals. The process for isolation and purification of lutein from marigold oleoresin is shown in Figure 1.1. The free lutein obtained by this process was found to have 97 % purity (Khachik et al., 1995, 2001). In the work conductd by Khachik et al., 1995, petroleum ether was used for extraction of marigold oleoresin. The authors later proposed the use of more benign solvent such as tetrahydroflouran (THF), which can be easily removed by washing with water or alcohol (Khachik et al., 2001). The purified carotenoids can be safely used as nutritional supplements or food coloring additives.

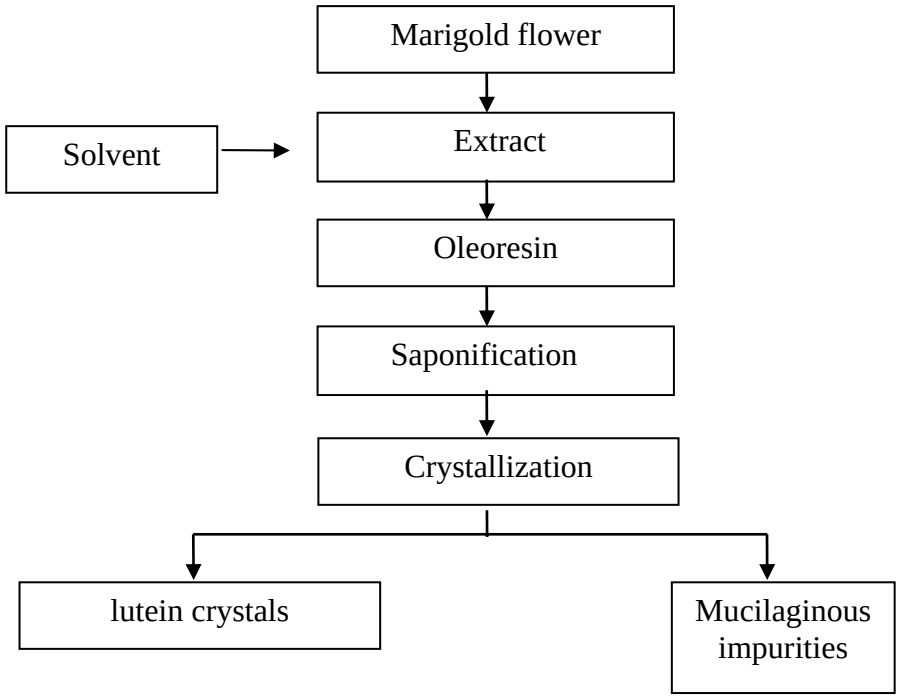


Figure 1.1 Procedures for isolation and purification of free lutein from marigold flower

Although several processes have been proposed to extract and purify lutein from marigold flowers (Philip et al., 1977, Khachik et al., 1995, Sadano et al., 2003, Xu et al., 2006, Madhavi et al., 2007), most of these studies emphasized only on the purification process to obtained free lutein. However, to determine the technical and economical feasibility of the entire processes starting from extraction, saponfication, and purification, the experimental data for the entire process are required. It is therefore the objective of this study to determine the experimental data for extraction

of xanthophylls from marigold flowers, the saponification process, as well as the purification process to obtain free lutein. First, the total amounts of xanthophylls in the starting marigold flowers were determined. Then, the detailed investigation was carried out to determine the suitable saponification conditions such as the amount and concentration of saponification agent and saponification time. In addition, the crystallization conditions (the type, amount, and composition of crystallization solvents and crystallization time) used to obtain high purity free lutien was examined.

1.2 Objectives

1. To determine the suitable conditions for the saponification of lutein fatty acid ester to free lutein.
2. To determine the suitable conditions for the crystallization of lutein crytstals.

1.3 Working scopes

1. Determine the total amount of xanthophylls in marigold flower.
2. Determine the suitable saponification time (0 hr, 3hr, 4hr, 5hr, and 7hr), the v/w ratio of KOH solution to marigold oleoresin (0.3:1, 0.5:1, 0.8:1, and 1:1), and concentration of KOH solution (25%, 35%, 40%, and 45% in MeOH) for concentration of free lutein at 75 °C.
3. Determine the suitable type of binary solvent mixture for crystallization such as water: ethanol and water: isopropanol on the yield and purity of free lutein.
4. Determine the effect of the v/v ratio of crystallization solvent (2: 0.3, 2: 0.5, 2: 0.8, and 2: 1) on the yield and purity of free lutein.
5. Determine the suitable crystallization time (15min, 0.5 hr, 1hr, 1.5hr, and 2 hours) on the yield and purity of free lutein.

1.4 Expected benefits

1. To propose the suitable procedure and conditions for the saponification of lutein fatty acid esters and the purification of free lutien from marigold flower.

CHAPTER II

BACKGROUNDS & LITERATURE REVIEWS

Background

2.1 Introduction of marigold

Marigold is a branching herbaceous plants in the daisy family. The plants are normally about 60-90 cm tall (Sowbhagya et al., 2004), and are extensively cultivated in temperate climate in tropical parts of the world. The vast quantities of marigold flowers are used in garlands and decoration for weddings, festivals, and religious events. Different varieties and flowers are available in various shades of yellow, red, orange, dark-orange, and orange-brown. Three common species of marigold are distinguished as *Tagetes erecta*, *Tagetes patula*, and *Tagetes tenuifolia*. The species grown mostly in Thailand is *T. erecta* (Figure 2.1).

Figure 2.1 Marigold (*Tagetes erecta*)[1]

[1]Source: www.seedsofindia.com/shop/?shop=1&cat=15

T. erecta have pinnate green leaves, and white, golden, orange, yellow, to an almost red floral heads typically 5-18 inch diameter, generally with both ray florets

and disc florets. The foliage has a musky/pungent scent, though some later varieties have been bred to be scentless. They have been used as a source of essential oil, food colorants, and as herbal medicine for skin complaints, wounds and burns, conjunctivitis and poor eyesight. The flowers may be used as fresh or dried, and can be made into tea, tincture, and creams.

2.2 Carotenoids of marigold

Carotenoids are the most widespread group of natural pigments, with over 600 of carotenoids being characterized structurally. They are present in all photosynthetic organisms and are components of the fruit, vegetables, and flowers having yellow to red colors.

Carotenoids are isoprenoids generally consisting of eight isoprene units joined together with covalent bond (Figure 2.2). The molecule of carotenoids could be presented in the form of straight chain such as lycopene, or may contain the ring at the tail of the chain like β-carotene. In general, carotenoids are classified into 2 groups, hydrogenated and oxygenated carotenoids. Hydrogenated carotenoid derivatives or carotenes are the molecules that composed of hydrocarbon chain, which lead themselves to be characterized as non-polar molecules which can be easily dissolved in oil. Examples of carotenes are β-carotene and lycopene (Figure 2.3). The second group, called oxygenated carotenoid derivatives or xanthophylls is the group of carotenoids whose molecules are composed of oxygen atoms. Thus, they are more polar and dissolve less in oil than the carotenoids in the first group. The examples of xanthophylls are such as lutein, zeaxanthin, astaxanthin (Figure 2.4).

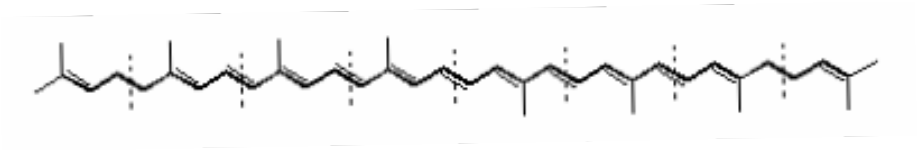


Figure 2.2 General formula of carotenoids,
broken lines indicate formal division into isoprenoid units[2]

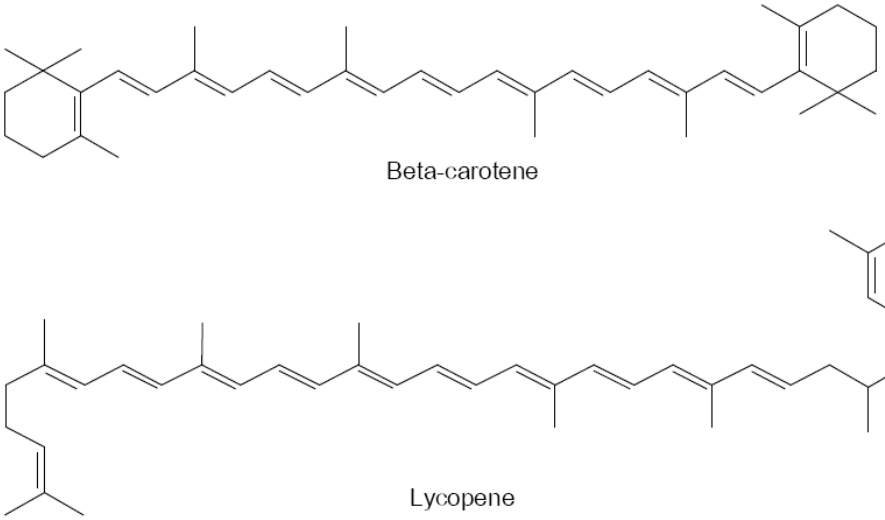


Figure 2.3 Chemical structure of hydrogenated carotenoids deriavatives[3]

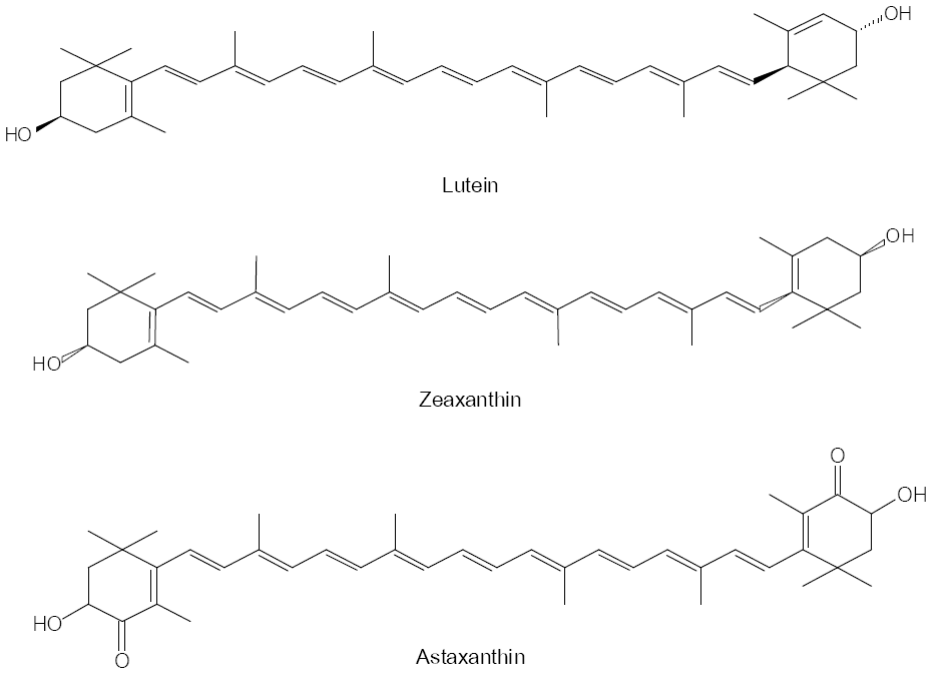


Figure 2.4 Chemical structure of oxygenated carotenoids derivatives[3]

Carotenoids in marigold flower consist mostly of xanthophylls, among which, the three main components are lutein, 70% to 90%; zeaxanthin, 10% to 25%; and a

small proportion of β-cryptoxanthin (Table2.1). Lutein is the main coloring component in the marigold flower.

Table 2.1 Carotenoids in marigold flower[4]

Carotenoids	Distribution (%)	Carotenoids	Distribution (%)
Phytoene	2.4	α-Cryptoxanthin	0.8
Phytofluene	2.6	β-Cryptoxanthin	0.5
α-Carotene	0.1	Lutein	72.3
β-Carotene	0.5	Antheraxanthin	0.1
Zeacarotene	0.5	Zeaxanthin	16.4
		Neoxanthin	0.8

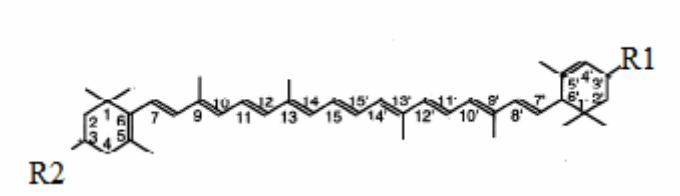
[4]Sowbhagya et al., 2004

2.3 Lutein

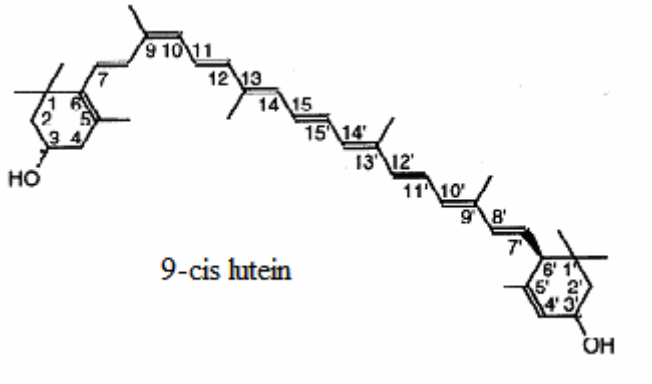
Lutein is one of the major constituents of green vegetable, orange fruits, and egg yolk, and is the main antioxidant identified within the eye retina It plays an important role in the prevention of cataracts and the maintenance of optimum eye health, whilst preventing macular degeneration (AMD) (Xin-yu et al., 2005). The antioxidant qualities of the compound also help promote healthy skin during sun exposure. Currently lutein is widely consumed as in the diet or as supplement, or is applied to the skin through a growing number of personal care products containing supplemental Lutein (Sowbhagya et al., 2004; Liu et al., 2007; Schalch et al., 2007).

Lutein exists in different isomeric forms and in nature they occur as a mixture of *trans* (60-90%) and *cis* forms (10-40%). The different isomers of lutein present in marigold flower are 9-cis lutein, 13-cis lutein, 15-cis lutein and all-trans lutein (Figure 2.5). In marigold flowers, lutein exists in the esterified form of fatty acids such as myristate, myristate palmitate, palmitate stearate, and distearate lutein esters (Table 2.2). These lutein fatty acid esters must first be converted to free lutein upon ingestion before it can be absorbed and metabolized by the body. Thus, to obtain lutein in the

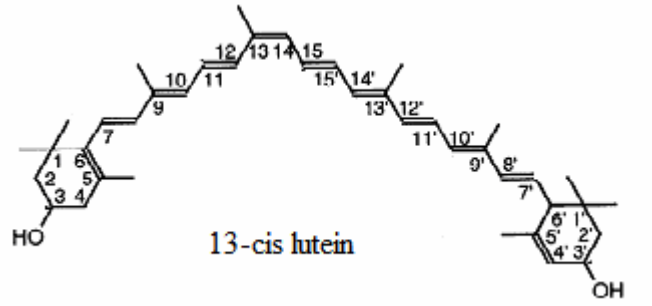
free form that can be readily absorbed by human, processes for isolation of lutein esters and conversion of these esters to free lutein have been proposed (Khachik et al., 2001 and Sowbhagya et al., 2004).



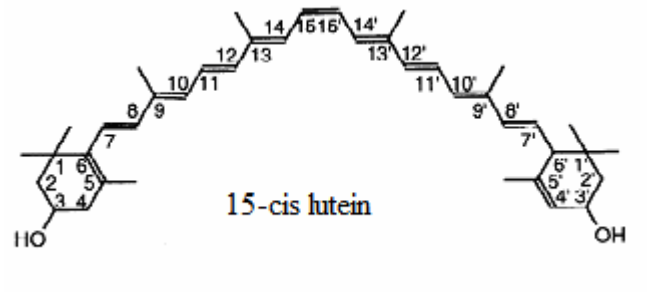
All-trans lutein



9-cis lutein



13-cis lutein



15-cis lutein

Figure 2.5 Structure of isomers lutein of marigold flower [4]

[4]Grazyna et al., 1998

Table 2.2 Composition of lutein fatty acid ester in marigold flower

Lutein fatty acid ester	Structure of lutein fatty acid ester	
	R_1	R_2
Free lutein	OH	OH
Monomyristate	OH	$OCO(CH_2)_{12}CH_3$
Monopalmitate	OH	$OCO(CH_2)_{14}CH_3$
Monostearate	OH	$OCO(CH_2)_{16}CH_3$
Dimyristate	$OCO(CH_2)_{12}CH_3$	$OCO(CH_2)_{12}CH_3$
Myristate palmitate	$OCO(CH_2)_{12}CH_3$	$OCO(CH_2)_{14}CH_3$
Dipalmitate	$OCO(CH_2)_{14}CH_3$	$OCO(CH_2)_{14}CH_3$
Palmitate stearate	$OCO(CH_2)_{14}CH_3$	$OCO(CH_2)_{16}CH_3$
Distearat	$OCO(CH_2)_{16}CH_3$	$OCO(CH_2)_{16}CH_3$

2.4 Isolation of Lutein from Marigold Flowers

A process for isolation and purification of xanthophylls crystal from marigold oleoresin includes extraction the marigold with solvent, saponification the lutein fatty acid ester to free lutein with potassium hydroxide, and crystallization by antisolvent.

2.4.1 Extraction of marigold oleoresin

As lutein is used in nutritional supplements and food colorants, the techniques for extracting, concentrating lutein to produce high purity product are often necessary. The first step involves extraction of marigold flower and the selection of appropriate solvent plays a very important role. The key criterion for solvent selection corresponds to the solubility of solute in the solvent. The solubility is a measure of solute concentration that is in equilibrium with the solvent at a given temperature. Therefore, the most appropriate extraction solvents or mixtures of solvents should have nearly the same polarities as those of the solutes. Due to the non-polar activity of

lutein, more efficient solvents for its extractions are non-polar solvents such as hexane, petrolether and tetrahydrofurane. More polar solvents such as ethanol or acetonitrile have lower chemical potentials against lutein and consequently have lower solubility properties. Lutein, a rich source of Vitamin A (necessary for normal eyesight, growth, and emryonic development), from pumpkin has been extracted with hexane Seo et al., 2005). In some study, acetone was employed for extraction of lutein and zeaxanthin contents such as the extraction of three local leafy vegetables such as Chekup manis, pea tree leaves, and drumstick tree leaves (Liu et al., 2007), and carotenoids from apricots (Kurz et al., 2008). In addition, the solvent mixture of acetone and hexane has also been used for extraction of pumpkin fruit.

Furthermore, extraction of lutein has also been carried out using supercritical fluid extraction. Seo et al., 2005 extracted carotenoid from pumpkin by liquid-liquid extraction which resulted in the oleoresin containing 2% lutein contents and compared it with supercritical fluid extraction which gave the extract with 3% lutein contents. Supercritical fluid extraction is developed as a simpler, faster, and less toxic than liquid-liquid extraction but its disadvantage is high condition and cost. Organic solvent is more economically feasible for extraction of marigold flower especially for the extraction of crude marigold oleoresin.

2.4.2 Isolation and purification of lutein from marigold oleoresin

The isolation of free lutein from marigold oleoresin involves two main steps: saponification of lutein fatty acid esters to free lutein and the crystallization to purify the product.

Saponification

Marigold flower petals are an excellent source of lutein. However, lutein in marigold flowers does not exist as free lutein but in esterified form with fatty acids such as lauric, myristic, and palmitic acids (Khachik et al., 2001). Upon saponification of the marigold extract, the lutein fatty acid esters (consist of R1 and R2 function) are converted to free lutein which can be more readily absorbed by haman (Figure 2.6). In this process, a fatty acid salt or soap is produced as a by-product.

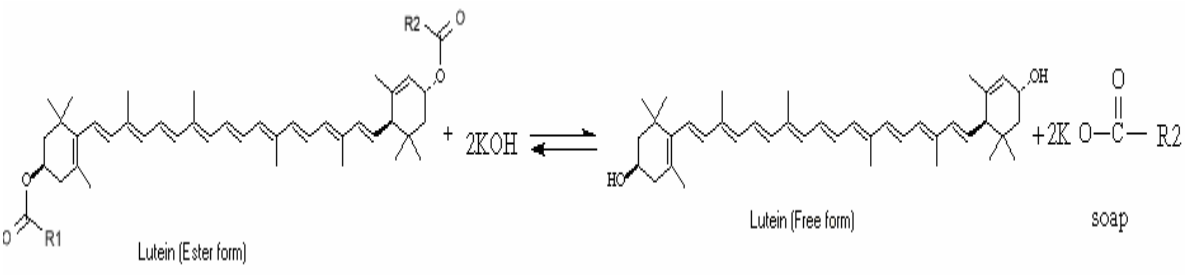


Figure 2.6 Saponification of a lutein fatty acid ester

Crystallization

After saponification, free lutein is resulted. However it is still required to remove other impurities to improve the purity of lutein, and this could be achieved by crystallization. Crystallization is the process of formation of solid crystals precipitating from an identical solution or melt which mass transfer of a solute from the liquid solution to a pure solid crystalline phase occurs. The crystallization process consists of two major steps: nucleation and crystal growth. Nucleation is the step where the solute molecules dispersed in the solvent to gather into clusters, which become stable under the current operating conditions. These stable clusters constitute the nuclei. However when the clusters are not stable, they redissolve. Therefore, the clusters need to reach a critical size in order to become stable nuclei. Such critical size is dictated by the operating conditions (temperature, supersaturation, etc.). It is at the stage of nucleation that the atoms arrange in a defined and periodic manner that defines the crystal structure. The crystal growth is the subsequent growth of the nuclei that succeed in achieving the critical cluster size. Nucleation and growth continue to occur simultaneously while the supersaturation exists. Supersaturation is the driving force of the crystallization; hence the rate of nucleation and growth is driven by the existing supersaturation in the solution. Depending upon the conditions, either nucleation or growth may be predominant over the other, and as a result, crystals with different sizes and shapes are obtained (control of crystal size and shape constitutes one of the main challenges in industrial manufacturing, such as for pharmaceuticals). Once the supersaturation is exhausted, the solid-liquid system reaches equilibrium and the crystallization is complete, unless the operating conditions are modified from equilibrium so as to supersaturate the solution again. In order for crystallization to occur from a solution it must be supersaturated. This means that the solution has to

contain solute much more than it can be dissolved (saturated solution). There are various methods to achieve supersation: 1) solution cooling, 2) addition of a second solvent to reduce the solubility of the solute (technique known as antisolvent or drown-out), 3) chemical reaction, and 4) change in pH. Other methods, such as solvent evaporation, can also be used. The most common among these methods are solution cooling and using antisolvent.

In antisolvent crystallization, the supersaturation is generated by the addition of another solvent that decreases the solubility of solutes. The solvents used for this purpose are referred to as antisolvents. This method is attractive since it can lead to significant savings in energy consumption and operation costs in comparison to the solution cooling. Furthermore it can be seen as an alternative methodology for the limited temperature stability of the product, as in the case of pharmaceuticals and biochemical.

2.5 Literature reviews

Several studies have been conducted on extraction and purification of lutein from marigold flowers. Philip et al. (1977) extracted marigold oleoresin with petroleum ether. Upon the dissolution of oleoresin in hot alkanol, and followed by cooling, and evaporating of solvent, precipitation of lutein fatty acid ester took place as shown in Figure 2.7.

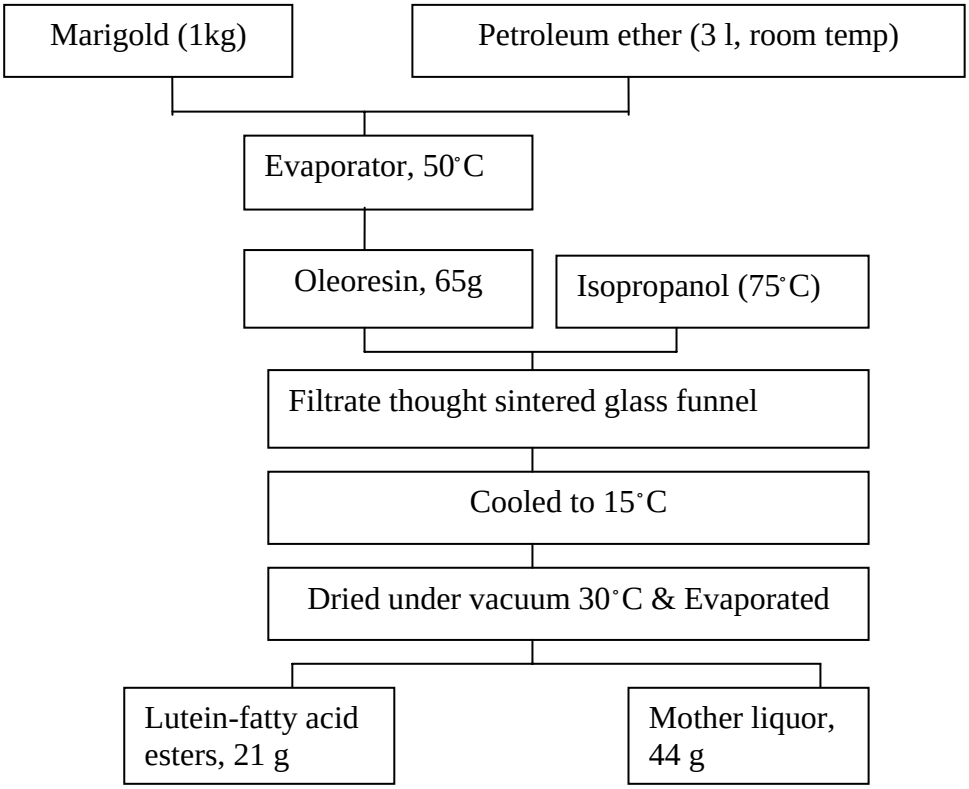


Figure 2.7 Extraction and isolation of lutein fatty acid esters from marigold flower by Philip et al., 1977.

Madhavi et al. (2007) later proposed a procedure for extraction of lutein esters from crude marigold oleoresin, instead of the marigold flower using the process shown in Figure 2.8. The extraction solvents used are alcohols such as isopropanol, n-propanol, ethanol, or co-solvents such as mixtures of isopropanol or n-propanol with ethanol. In this process, alcohols are used which are less toxic than other previously used solvents.

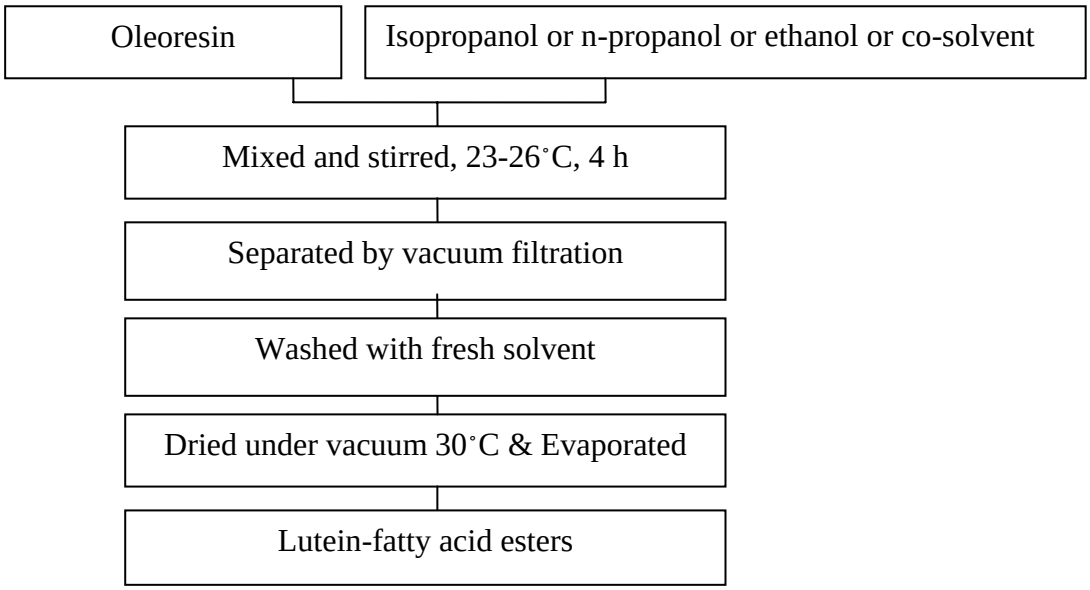


Figure 2.8 The process for the purification used as a solvent modifier or co-solvent by Madhavi et al. 2007

From the extraction processes described above, the lutien was extracted from marigold flower or oleoresin in the form of lutien fatty acid esters. Since human body could absorb lutein that are in a free form more easily than the luetien esters, Khachik et al. (1995) therefore proposed a saponification step in which lutein fatty acid esters in ethanol was reacted with KOH to form free lutein as shown in Figure 2.9. In this step, soap is the by-product of the reaction.

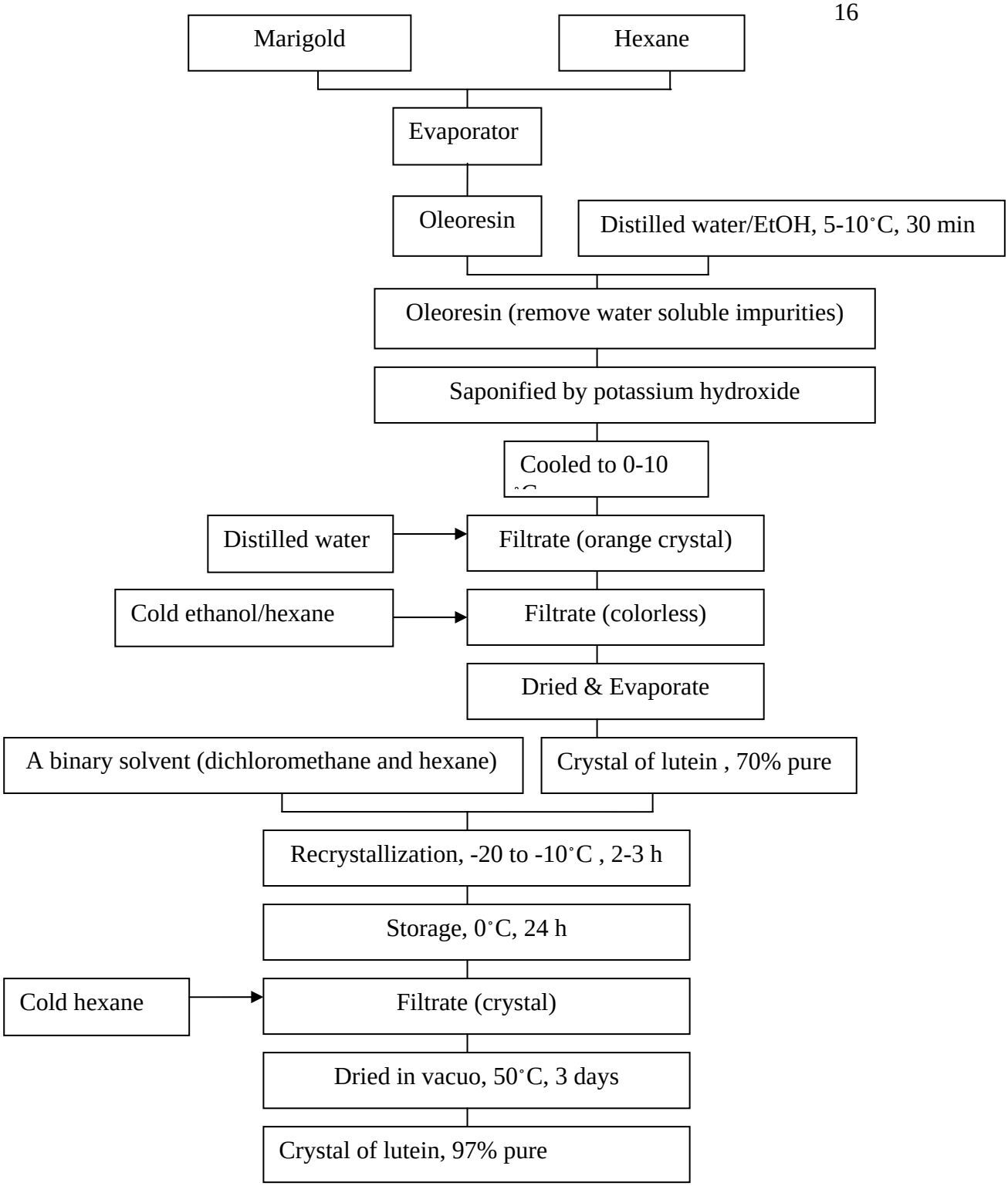


Figure 2.9 Process for isolation, purification, and recrystallization of lutein fatty acid ester from marigold oleoresin by Khachik et al., 1995.

This method used rather harmful organic solvents (i.e., hexane and dichloromethane), which make it unsuitable for human use. The same author later (Khachik et al., 2001) proposed to extract, saponify, and purify lutein and zeaxanthin, and a mixture of several rare carotenoid from marigold without the use of these harmful organic solvents. In their study, tetrahydrofuran (THF) was used instead,

which can be easily removed by washing with water or alcohol. Moreover, in the recrystallization steps, THF and distillate water were used instead dichloromethane and hexane from patent of Khachik et al. (1995). The purified carotenoids obtained by this method could be safely used as nutritional supplements or food coloring additives.

To further process the extract from marigold more easily into soft capsules for dietary supplement, Sadano et al. (2003) developed a process to obtain marigold oleoresin that has low viscosity and high lutein content. This was carried out by dissolving marigold oleoresin with a vegetable oil, which was then extracted with supercritical fluid extraction. Viscous impurities such as wax and resin would dissolve in oil while the lutein content in the oleoresin would dissolve in CO_2. Then, oleoresin obtained from $SCCO_2$ extract was then redissolved in acetone, and the solution was allowed to crystallize upon cooling. This process is shown in Figure 2.10.

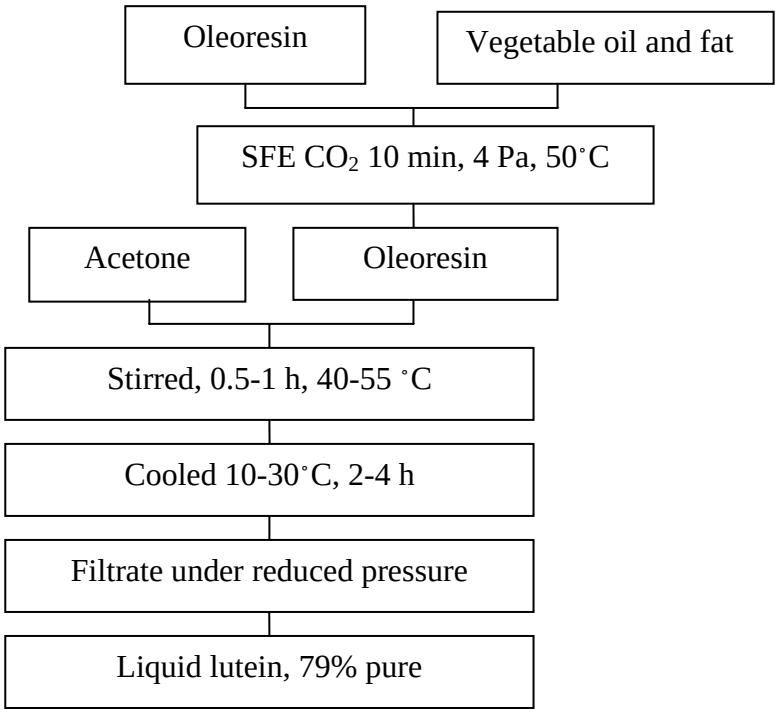


Figure 2.10 Purification of marigold oleoresin by Sadano et al. (2003).

Xu et al. (2006) isolated and purified lutein and zeaxanthin from crude marigold oleoresin with minimal level of organic solvents. This process involves hydrolyzing carotenoids esters in plant oleoresin using ethanol, water and alkali for 3-5 hours, followed by crystallization in a warm mixture of water and alcohol. This process employed the use of antisolvent to achieve supersaturation instead of the system cooling. Xanthophylls crystals obtained by this technique contained at least 80% total xanthophylls, of which at least 90% was trans-lutein and zeaxanthin, and trace amount of cis-lutein and other carotenoids were obtained.

Reviews of processes for extraction purification of lutein fatty acid esters from marigold flower are summarized in Table 2.3.

Table 2.3: Reviews on the studies of process for purification lutein fatty acid ester from marigold flower.

Author	***Solvent for extraction***	***Solvent for purification***	***Yield & Purity***	***Application***	***Objective***
1.Philip et al.,1977	Petroleum ether	Isopropanol	21 g/Kg 51% pure	To use for coloring icecream by direct addition to mixing	To Purifying luten-fatty acid ester
2. Khachik et al., 1995	Hexane	Distilled water/EtOH	97% pure	To test for herbicides, pesticides, and oral supplementation	To isolation and purification the free form of lutein from chemical impurities.
3. Khachik et al., 2001	THF	Distilled water/THF	97% pure	To use for food coloring additives	To safety source of nutritional supplement for human consumption as well as providing a suitable and effective color additive for human food

Author	*Solvent for extraction*	*Solvent for purification*	*Yield & Purity*	*Application*	*Objective*
4. Sadano et el., 2003	Hexane	Acetone	79% pure	To use soft capsules	To study method for decrease viscosity by supercritical fluid extraction
5. Xu et al., 2006	Hexane	EtOH	150.4 g /Kg 91.4% pure	To use as a nutritional supplement and as an additive in food	To purity and useed as little as possible toxic organic solvent and operation steps, and with high yield rate
6. Madhavi et al. 2007	Hexane	Isopropanol , N-propanol , Ethanol , or Co-solvent	Isopropanol 62 % (w/w), 61% pure N-propanol 64 % (w/w), 62% pure Ethanol 95 % (w/w), 35% pure Isopropanol+ethanol 78% (w/w), 57% pure N-propanol+ethanol 73% (w/w), 62% pure	To use for food coloring additives	To improvement of a lower-grade oleoresin for more purity and yield.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and chemicals

The dried marigold flowers were obtained from Marigold Production Group, Nakhonratchasima, Thailand. Solvents, hexane, used for extraction of pigments were purchased from Merck, USA. All chemicals; ethanol, potassium hydroxide, hydrochlolic acid, used for the determination of total carotenoids and lutein are analytical grade, and were purchased from Sigma-Aldrich, Germany.

3.2 Sample preparation

Samples of marigold were prepared from petals and calyces, which were dried, ground into fine powder, and stored at 4 °C in a domestic refrigerator until use. In order to determine the total amount of xanthophylls in marigold flower, 100 grams of fine ground sample was extracted with 500 ml of hexane at 40 ^{o} C first for 4 hrs. The remaining sample residue was separated and re-extracted 3 times, each with 500 ml of hexane at 30 ^{o} C, for 1 day, 3 days, and 10 days, respectively. The amount of the xanthophylls in each extract was determined by first evaporating the solvent from the sample solution under vacuum at 40 ^{o}C for 15 minutes. After the vacuum evaporation, the oleoresin was continued to dry for 2 hours in vacuum oven at 30 ^{o}C. The dried oleoresin was then dissolved in ethanol and the amount of total xanthophylls extracted was then measured using a spectrophotometer. The total xanthophylls contents in the marigold oleoresin were measured.

3.3 Separation and purification of lutein

To isolate free lutein from marigold flowers, several steps are necessary. The procedures for the separation and purification to obtain free lutein from marigold consisted of three main steps: extraction, saponification and crystallization. The different steps and variables investigated in this study are summarized in Figure 3.1

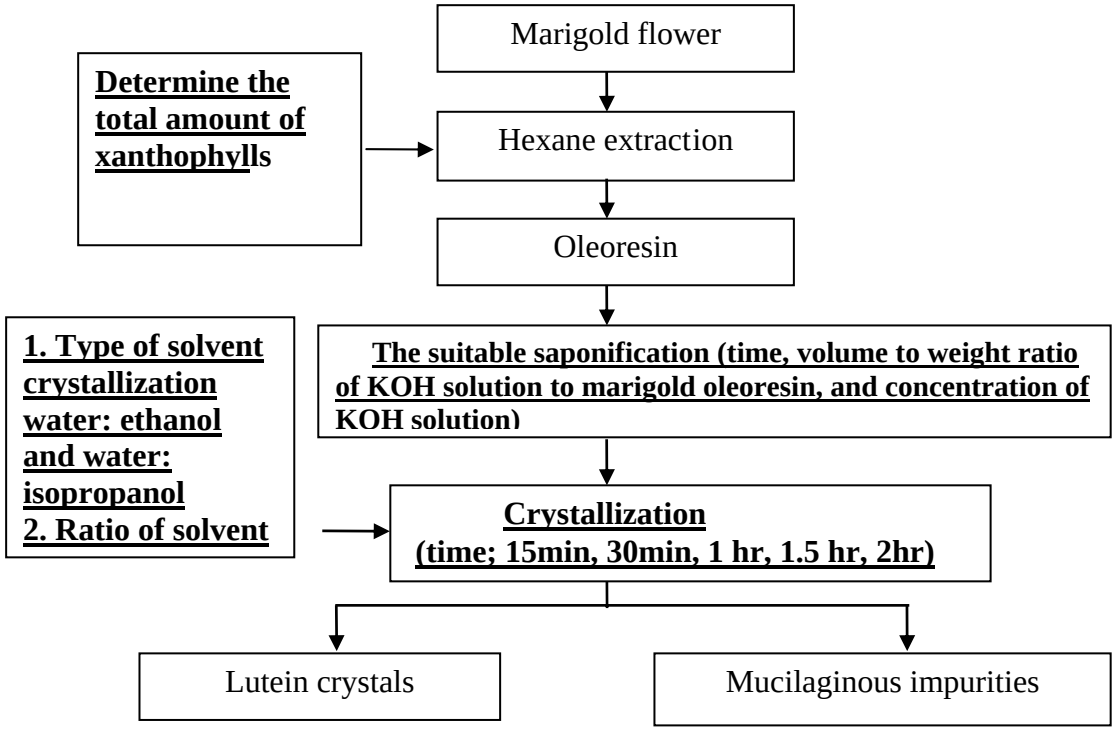


Figure 3.1 Procedures for separation lutein from marigold flower and variables studied (in bold face).

3.3.1 Extraction of marigold oleoresin

100 grams of fine ground sample were extracted with 500 ml of hexane at 40 ^{o}C for 4 hrs. The extract was evaporated by using a rotary vacuum evaporator (EYELA rotary evaporator N-100) at 40 ^{o}C for 15 minutes. To prepare sufficient amount of the oleoresin for this study, 10 batches of extraction were carried out as described above, that is the total of 1 kilogram of fine ground sample was extracted with the total amount of 5 L of hexane. After the vacuum evaporation, drying of the oleoresin was continued for 2 hours in a vacuum oven at 30 ^{o}C. For each 100 grams of fine ground sample, approximately 10 grams of dried oleoresin was obtained. The oleoresin prepared will be used for further investigation on lutein separation and purification process.

3.3.2 Saponification of lutien fatty acid esters

In the first sets of experiment saponfication was carried out to determine the suitable process conditions such as saponification time, the volume to weight ratio of KOH solution to marigold oleoresin, and the concentration of KOH solution. Initially, the suitable saponification time was determined. Here, 1 grams of marigold oleoresin was dissolved in 2 ml of ethanol at the temperature 75 °C. In to the mixture, 1 ml of 45 % potassium hydroxide solution was added slowly. Saponification of marigold oleoresin was allowed to take place for 0 hr, 3 hrs, 4 hrs, 5 hrs, and 7 hrs to determine the suitable saponification times. After a specified saponification time was reached, the mixture was cooled to 65 °C, and the pH was adjusted to about 7.0 with aqueous solution of hydrochloric acid. This step is considered as a completion step for saponification. The resulted mixture can generally be subjected to crystallization to further purify the free lutein product (Section 3.3.3). For the anlaysis of resulted free lutein however, the following steps are required. That is, in to the saponified mixture, 50 ml of ethanol was added, and the mixture was sonicated at 30^{+}/-5 °C for 15 minutes. After sonication, the mixture was allowed to settle and the two phases were separated. The top phase was a clear yellow ethanol rich phase that contained the desired free lutein product, while the bottom phase contained mostly soap, water, and salt that was resulted from nuetralization. This was then centriguted and the two phases were separated. To be sure to recover all the free lutein, bottom phase separated from the previous step was extracted again in 50 ml of ethanol under ultrasonication at the same condition. The mixture was centrifuged, and the clear solution was again separted and combined with the solution from the first step, which was then analyzed for the amount of free lutein by high performance liquid chromatography (HPLC). The suitable saponification times was selected such that the maximum concentration of free lutein per a gram of oleoresin was obtained. This saponification time was then used in further study for the determination of a suitable volume to weight ratio of KOH solution to marigold oloresin. Once the suitable ratio was determined, experiments were carried out at this condition to determine the suitable concentration of KOH solution for saponifcation process. Table 3.1 summarizes all the variables and their ranges employed in this study summarized. All experiments were conducted in duplicate.

Table 3.1 Ranges of variables saponification

Variables	Fix variables	Ranges
Saponification step		
• Saponification time	75 °C, 45 % KOH solution ,1ml	0, 3, 4, 5, and 7 hr
• The volume of KOH solution	75 °C, 45 % KOH solution , 4hrs	0.3ml, 0.5ml, 0.8ml, and 1ml
• Concentration of KOH solution	75 °C, 0.5ml of KOH solution , 4hrs	25%, 35%, 40%, and 45%

3.3.3 Crystallization of lutein by antisolvent

In this study, the effects of key factors for antisolvent crystallization that affect the yield and purity of free lutein crystals was determined. The variables investigated included the type of binary crystallization solvent mixtures, volume ratio of binary solvent mixture, and the crystallization time. First, binary solvent mixture systems were examined: water: ethanol and water: isopropanol at various ratios to determine the most suitable crystallization solvent system. The selected system was then employed to determine the suitable crystallization time. The ranges of experimental variables for the crystallization experiments are summarized in Table 3.2. To carry out the crystallization experiments, with the saponified oleoresin mixture was first prepared at the optimal saponification condition following the procedure described in section 3.3.2. The saponified oleoresin mixture after neutralization was then dissolved in a specified crystallization solvent mixture at 65 °C. The mixture was then kept at 65 °C for 30 min, after which the yellow precipitate was obtained. The precipitate obtained was then filtered by Whatman filter paper No.5 under suction until dried. The dried precipitate was weighed and analyzed for the amount of free lutein to determine the yield (amount of free lutein in precipitateper the total free lutein) and the purity (amount of free lutein per weight of dryprecipitate) of the resulted product. In order to analyze the lutein content in the final product (precipitate), the precipitate was dissolved in 50 ml of ethanol under sonication at 30^{+}/-5 °C for 15 minutes. A small amount of insoluble impurity suspended in the solution was separated from the

soluble portion by centrifugation. The solid impurity was extracted again with 50 ml of ethanol to recover all the lutein, and the extract solutions were then combined and analyzed for the concentration of free lutein with HPLC.

Table 3.2 Ranges of variables crystallization

Variables	Fix variables	Ranges
Crystallization step		
• The type of binary solvent mixtures for crystallization	65 °C , 30 minutes	water: ethanol and water: isopropanol
• The v/v ratio of crystallization solvent		2ml: 0.3ml, 2ml: 0.5ml, 2ml: 0.8ml, and 2ml: 1ml
• Crystallization time	65 °C , water: ethanol (2:0.5)	15min, 30min, 1hr, 1.5hr, and 2hr

3.5Analytical method

3.5.1 Analysis of total xanthophylls

The oleoresin was dissolved in ethanol and the amounts of total xanthophylls contents in the oleoresin were determined by measuring the absorbance at 478 nm with a spectrophotometer using lutein as a standard (Prommuak et al. 2008).

3.5.2 Analysis of free lutein and lutein fatty acid esters

The free luteins and lutein fatty acid esters from saponification and crystallization steps were analyzed by HPLC following the method described by Piccaglia et al. The sample solution was injected to Lichrocart C-18 column, a Diode Array Detector Module 335 and an automatic injector. A 5 μm reversed-phase was used. Chromatographic separation was obtained with a gradient solvent system of acetonitrile:methanol (9:1, v:v) (A) and ethylacetate (B), from 0% to 100% of B using a linear gradient over 30 min, at a flow rate of 1 ml/min and detection at 450 nm.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Total amount of xanthophylls in marigold flower

In order to determine the total xanthophylls contents in marigold flower, the repeated extractions of dried ground marigold flower were conducted using hexane as extracting solvent. The initial extraction time was 4 hours (at 40 ^{o} C), followed by extraction with hexane at 30 ^{o} C for 1 day, 3 days, and 10 days respectively. The content of total xanthophylls in each extract was quantified and was summarized in Table 4.1. The results of Table 4.1 show that the total xanthophylls contents the dried marigold flower sample was 25.77 mg/g of dry weight (257.7 mg/g oleoresin). Of this amount, more than 80% (20.94 mg/g of dry weight or 209.4 mg/g oleoresin) was extracted after the initial extraction with hexane at 40 ^{o} C for 4 hours, and only small percentages, 12.29%, 6.18%, and 0.26%, were extracted after the subsequent repeated extraction with hexane at 30 ^{o} C after 1, 2, and 7 days, respectively. It is noted that for the preparation of the marigold oleoresin for subsequent study, extraction was carried out only one time with hexane for 4 hr at 40 ^{o} C, after which the extract was dried to obtained marigold oleoresin.

Table 4.1 Total xanthophylls content in marigold flower

Run	Time/ Temperature (C)	Total xanthophylls (ug/ml)				Total xanthophylls (mg/g oleoresin)	Total xanthophylls (mg/g marigold)	% Total xanthophyll
		Exp.1	Exp.2	Average	SD			
1	4hr-40C	0.625	0.612	0.619	0.009	209.4	20.9	81.27
2	1day-30C	0.095	0.092	0.094	0.002	31.66	3.166	12.29
3	2day-30C	0.051	0.043	0.047	0.006	15.91	1.591	6.18
4	7day-30C	0.003	0.001	0.002	0.001	0.68	0.068	0.26
SUM						257.7	25.77	100

4.2 Determination of sutiable saponification conditions

Because human body could more easily absorb free lutein than the lutien esters, saponification of lutein fatty acid esters to free lutein is a necessary step. In the present study, experiments were conducted to determine the effects of times, volume of KOH solution, and concentration of KOH solution on saponicification process, and from these results, the most suitable saponification condition that gave the maximum yield of free lutein would be obtained.

4.2.1 Effects of saponification time

In an initial experiment, saponification was performed in which 1 g of marigold oleoresin and 1ml of 45 % potassium hydroxide solution was reacted for various times at 75°C. After the specified saponification time, the reaction was stopped by neutralizing the system with HCl solution. Into the saponified product, ethanol was added and the mixture was sonicated, after which the mixture was separated into two phases. The top phase was a clear solution containing free lutein, and the bottom phase was white paste composed mostly of insoluble soap and the salt obtained during nuetralization. The clear portion was separated and analyzed for the amount of free lutein by high performance liquid chromatography (HPLC). Typical chromatograms those of free lutein standard solution, solution of marigold oleoresin in ethanol, and the ethanol soluble portion of the sample obtained after saponification are shown in Figure 4.1, 4.2, and 4.3 respectively. As seen in the figures, the retention time of free lutein was 16 minutes, while the more nonpolar lutein esters eluted at later time with the reversed phase chromatography employed in this study. These figures clearly indicated that the conversion of lutein esters into free lutein occurred as a result of saponfication. The conversion however was found to depend on saponification time as seen in Figure 4.4, which shows the free lutein content per unit weight of oleoresin obtained at different saponification times between 0-7 hours. The results demonstrated the increase in free lutein content with an increase in saponification time up to 4 hours, after which the free lutein content decreased. The increase in free lutein during the first 4 hours was possibly due to the fact that different times are required for different types of lutein fatty acid esters. During the

initial period, sapnoification of monoesters of lutein containing an alkyl group, such as lutein esters of lauric, myristic, or palmitic acids could more readily occur. While the lutein diesters such as lutein dimyristate, dipalmitate, or distearate, having two alkyls groups took longer time to convert into free lutein. The decrease in free lutein concentration at 5 and 7 hours however could be due to the degradation of the products after extended period of exposure to alkali condition. From this experiment, the content of free lutein as high as 126 mg/g oleoresin was achieved in 4 hours of saponification. The saponification time of 4 hour will be used in the subsequent experiment to determine the suitable volume to weight ratio of KOH to oleoresin and the suitable concentration of KOH solution.

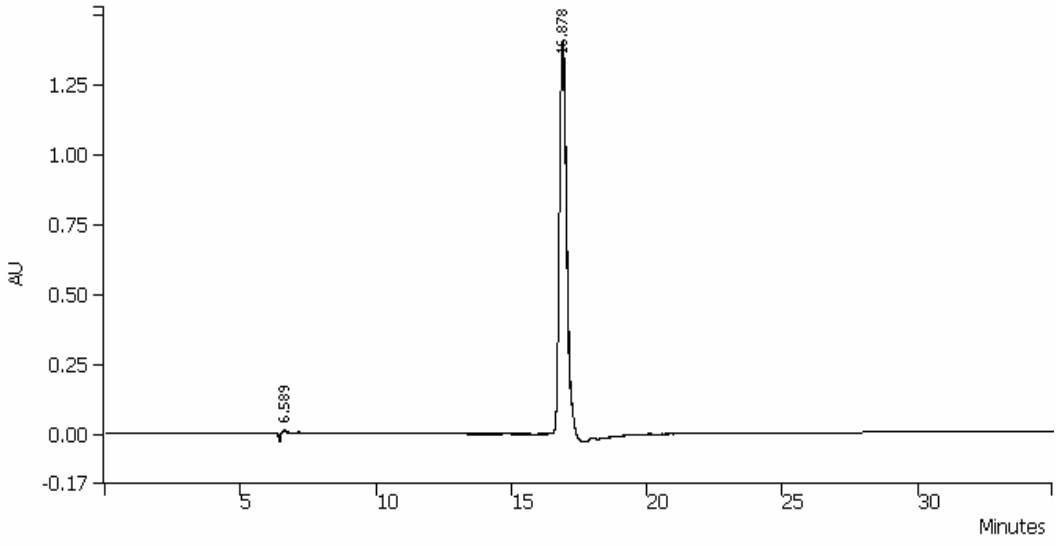


Figure 4.1 HPLC chromatogram of standard free lutein

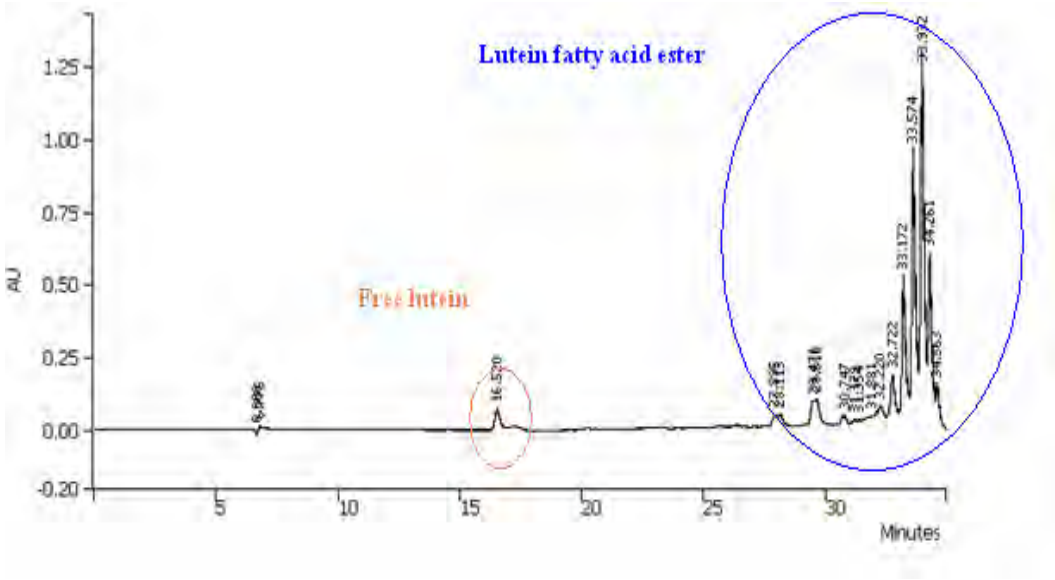


Figure 4.2 HPLC analyses of free lutein and lutein fatty acid esters in marigold flower before saponification.

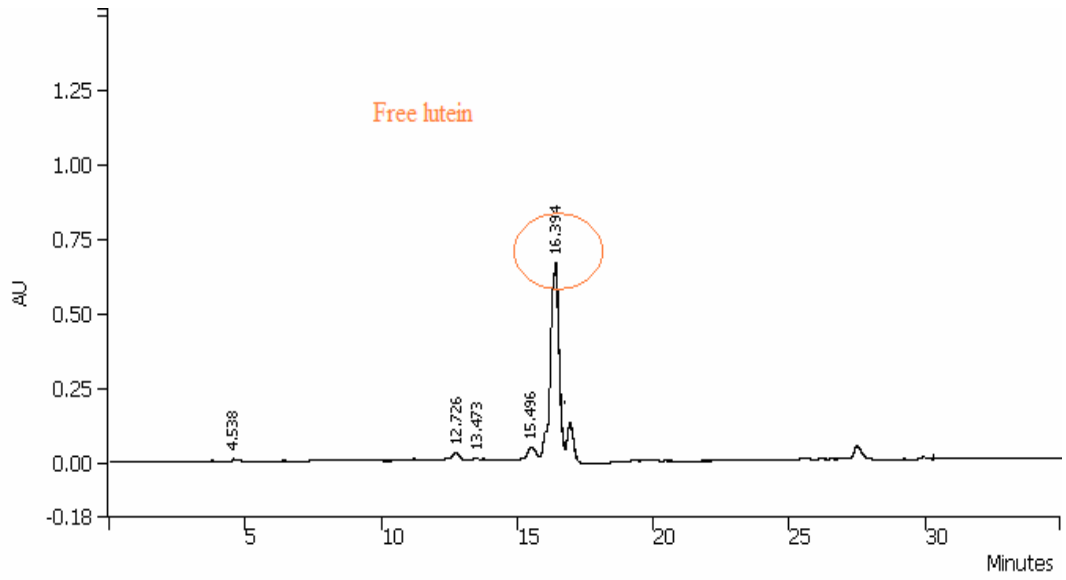


Figure 4.3 HPLC analyses of free lutein and lutein fatty acid esters in marigold flower after saponification in 4 hours.

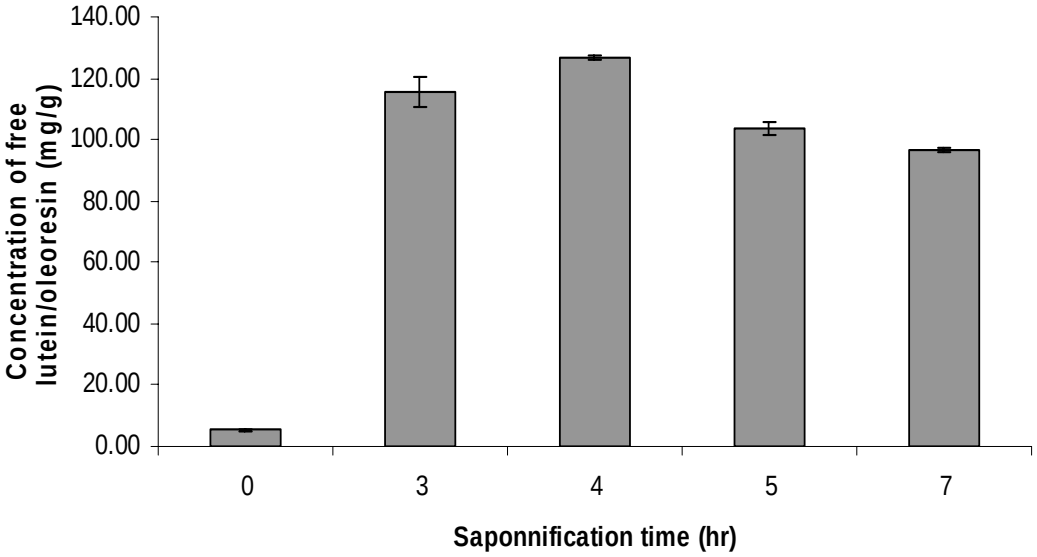


Figure 4.4 Effects of time on saponification to obtain free lutein

4.2.2 Effects of volume to weight ratio of KOH solution to marigold oleoresin for saponification

For the complete saponification to occur, it is generally requires that the sufficient amount of alkali be used. This required amount could possibly be prepared at various concentrations alkali solution, given that corresponding volumes were used. It would be desirable to use smallest volume of KOH at rather high concentration to minimize the reactor size, thus making the process more economically feasible. However, the concentrated KOH could cause degradation of product, and thus the suitable KOH concentration and thus volume would be those that yield the maximum results. In this investigation, the concentration of KOH solution of 45% was initially chosen as a starting point, based on preliminary study and that reported by other investigators (Xu et al., 2007). What remained to be determined however was the suitable volume of this KOH solution. Here, various volumes, 0.3, 0.5, 0.8, and 1 ml, of 45% KOH solution were reacted with 1 g of marigold oleoresin at 75 °C for 4 hours. The results in Figure 4.5 show that the highest amount of free lutein (179 mg/g oleoresin) was obtained with 0.5 ml of 45% KOH solution. The lower volume of KOH solution probably could not provide sufficient amount of reactant for

saponification, while on the other hand, larger volume of KOH solution (0.8 or 1 ml) caused degradation, and thus lowered the lutein content. This result agree with the study by Sowbhagya et al. (2004) which reported that long exposure of lutein at extreme pH conditions (lower than pH3 or greter than pH9) results in isomerization and degradation.

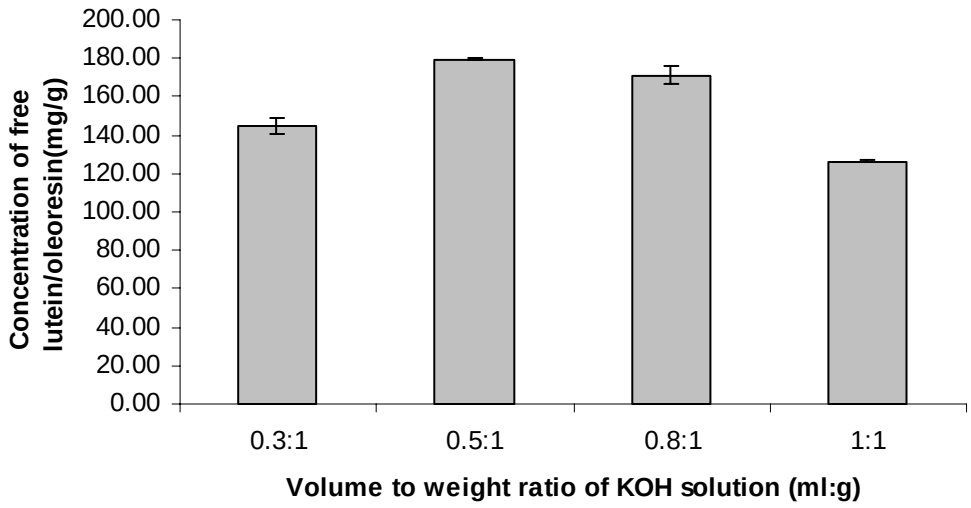


Figure 4.5 Effects of volume to weight ratio of KOH solution to marigold oleoresin on saponification to obtain free lutein

4.2.3 Effects of KOH concentration

The results obtained previously indicated that the highest free lutein obtained was 179 mg/g oleoresin after 4 hours of saponification process at 75 C with 45% w/v KOH at the 0.5 ml to 1 g marigold oleoresin volume to weight ratio. The most suitable conditions found were based on the fixed concentration of 45% w/v KOH taken from literature. This set of experiment was therefore carried out to fine tune the process once again so as to determine the most suitable concentration of KOH that yeiled the maximum results. Here, saponification of 1 g of oleoresin was carried out for 4 hours at 75°C in 0.5 ml of KOH solution at various KOH concentrations (25, 35,

40, and 45 % w/v). The results of free lutein obtained are shown in Figure 4.6 which reveals that the highest amount of free lutein (194 mg/g oleoresin) was obtained with 0.5 ml of 40% KOH solution. This condition was therefore the most suitable condition for saponification, and was therefore employed to prepare the saponified products used for further crystallization experiments.

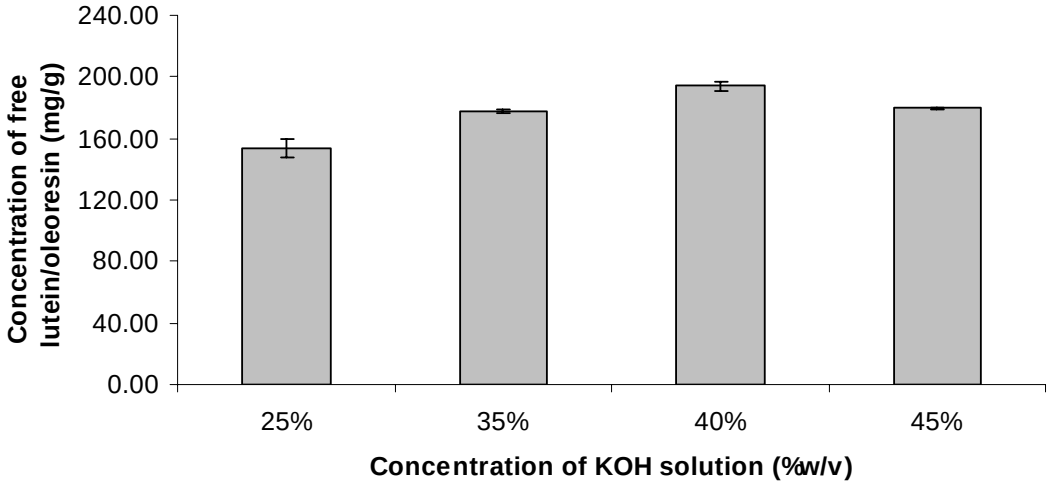


Figure 4.6 Effects of concentration of KOH solution on saponification to obtain free lutein

4.3 Determination of suitable crystallization conditions

After saponification steps, free lutein was resulted. However, further step would still be required to obtain the purified free lutein product, and this could be achieved by crystallization. In this case, anti solvent crystallization was employed in which an anti solvent (or solvent mixture) was added to adjust the solubility of mixture to achieve supersaturation, and thus the desired crystallization. Thus, optimization of process conditions, such as the ratio of crystallization solvent (antisolvent) used, and crystallization time, is of great importance.

4.3.1 Effects of volume ratio of crystallization solvent (H_2O: EtOH and H_2O: iso-propanol)

In the present work, the effects of solvent mixtures such as water: EtOH and water: isopropanol at various volume ratio of crystallization solvent in the range of 2ml: 0.3ml, 2ml: 0.5ml , 2ml: 0.8ml , and 2ml: 1ml were determined on the yield of free lutein (Figure 4.7) and the purity of free lutein crystals obtained (Figure 4.8) Here, the process was examined at a fixed temperature at 65 °C and for the crystallization time of 30 min.

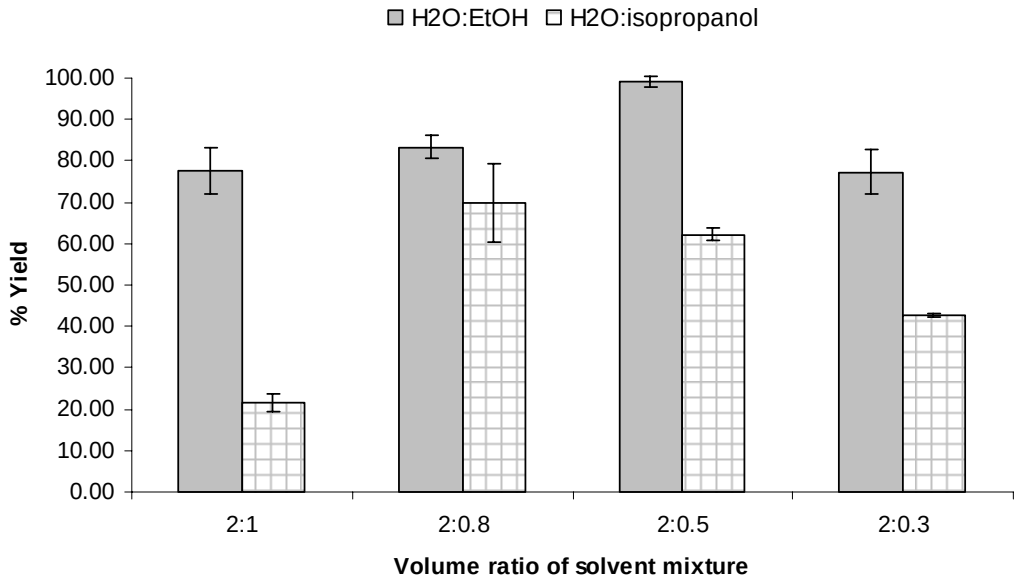


Figure 4.7 Effects of volume ratio of crystallization solvent (H_2O: EtOH and H_2O: iso-propanol) on yield of free lutein.

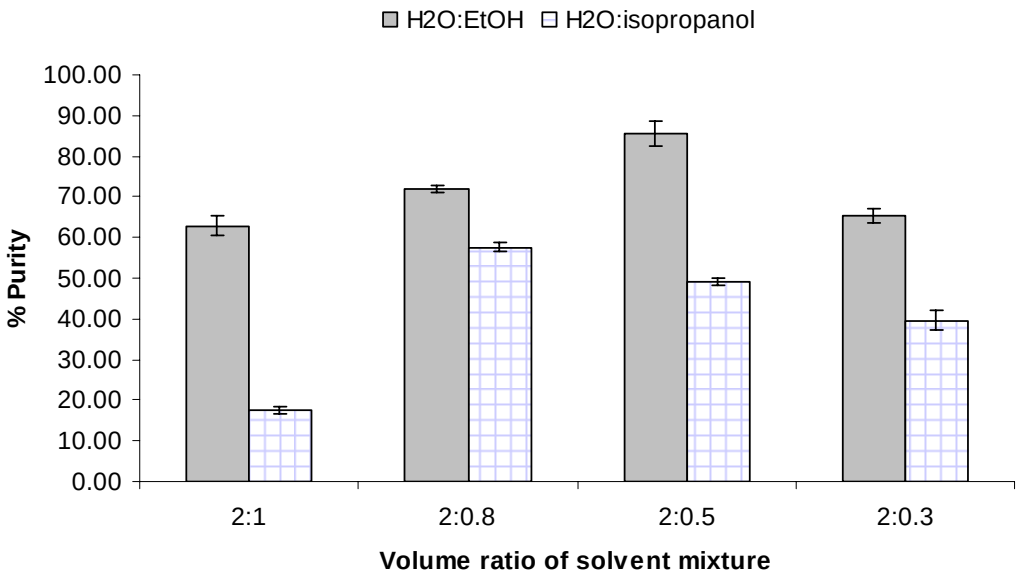


Figure 4.8 Effects volume ratio of crystallization solvent (H_2O: EtOH and H_2O: isopropanol) on purity of free lutein.

The yield and purity of free lutein obtained in water:EtOH and water:isopropanol solvent systems at various ratios are shown in Figure 4.7 and Figure 4.8.From these figures, it can be seen that the yield and purity were found to be higher when water:EtOH system was used compared with water:isopropanol system. Moreover, the yield of free lutein in oleoresin and the purity of free lutein in the resulted crystals increased as the content of water in water: EtOH mixture increased (from 2:1.0 up to 2:0.5) and the water: isopropanol ratio increased to (from 2:1.0 to 2:0.8). At the higher content of water, the polarity of the antisolvent was favorable for the precipitation free lutein. Solvent with high water would increase the polarity of the solvent system, thus making unfavorable for the dissolution of non-polar compound such as free lutein. As a result, the precipitation of free lutein would occur. When the content of water in both solvent systems further increased, the yield decreased, indicating the system polarity of the antisolvent might be unsuitable for crystallization. At this condition, the purity of free lutein also decreased, possibly because at such conditions, precipitatation of of other impurities increased.

At the ratio of 2:0.5 (water: EtOH), the yield of free lutein were the highest, 99.12% (or 192 mg free lutein in crystal/194 mg free lutein in oleoresin). For the

system of water:isopropanol, the highest free lutein yield of about 69.71% (135 mg free lutein/194 mg free lutein in oleoresin) was obtained at 2:0.8 (water: isopropanol). The purities of lutein crystals obtained after crystallization with the two solvent systems were 85.53% (192 mg free lutein/ 225 mg crystals) and 57.61% (135 mg of free lutein/235 mg crystals), respectively.

4.3.2 Effects of crystallization time

In this work, effects of time for crystallization on 0.25, 0.5, 1, 1.5, and 2 hours on yield of free lutein and purity of free lutein (Figure 4.9) were investigated at a fixed temperature at 65 °C for crystallization in water: EtOH solvent at the oleoresin to solvents ratios of 1g oleoresin: 2 ml water: 0.5 ml ethanol.

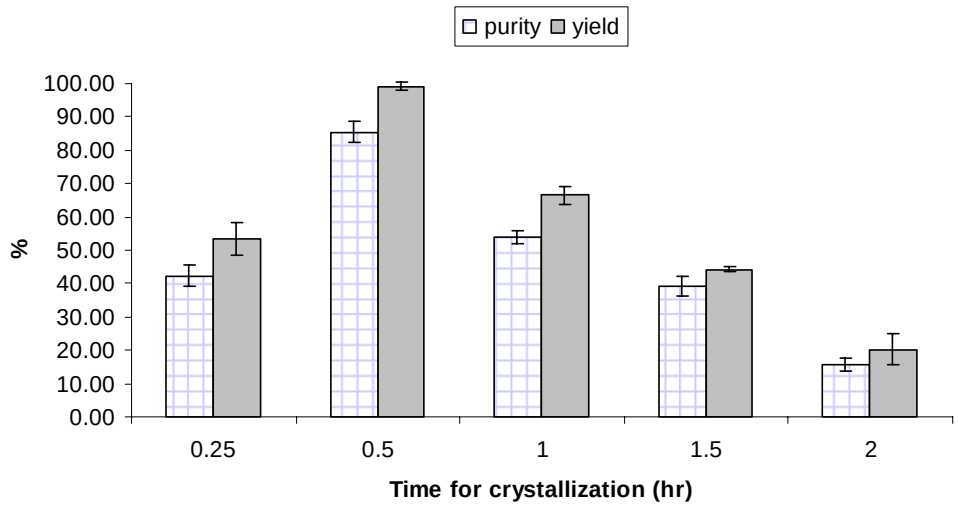


Figure 4.9 Effects of time for crystallization on yield of free lutein and purity of free lutein.

The yield and purity of free lutein obtained at various crystallization times are shown in Figure 4.9, which demonstrated that the yield and purity of free lutein increased with an increase in crystallization time up to 0.5 hours, where the highest yield of 99.12% (85.53% purity) was obtained, and then decreased as the crystallization time was longer. It is generally known that in typical crystallization process, nucleation and growth to size require adequate period of time. However, it was found in this case that too long period of crystallization time, the free lutein are

likely to be converted back to lutein esters as can be seen in the chromatograms of crystallization products obtained after a 1 hour crystallization period (Figure 4.11), compared with after a 30 minute crystallization period (Figure 4.10).

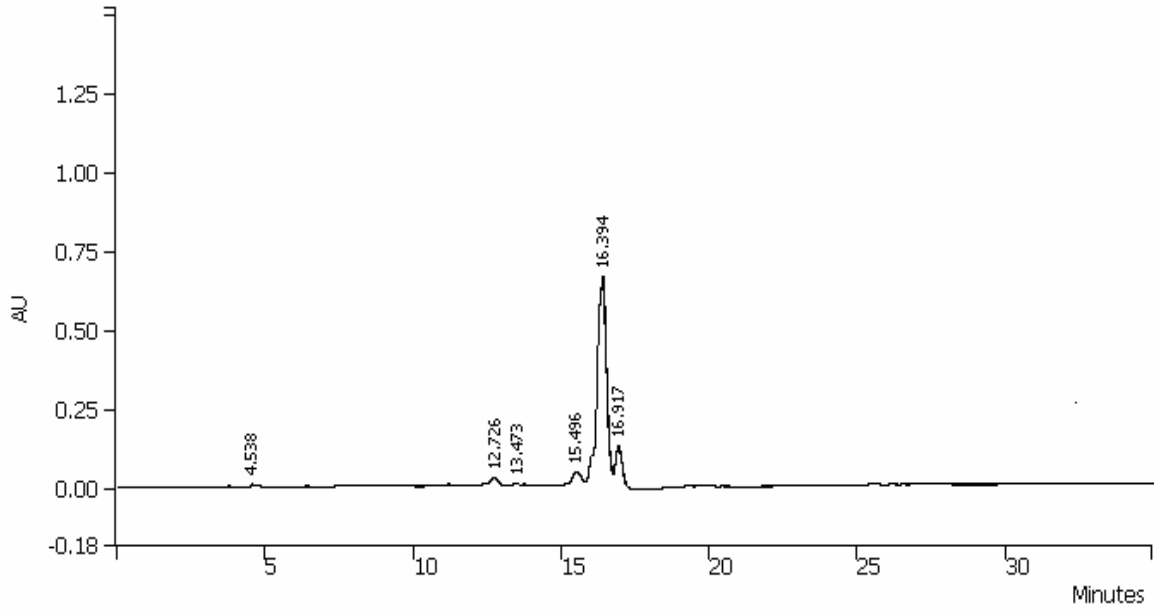


Figure 4.10 HPLC analyses of free lutein and lutein fatty acid esters in marigold flower after 30 minutes of crystallization times.

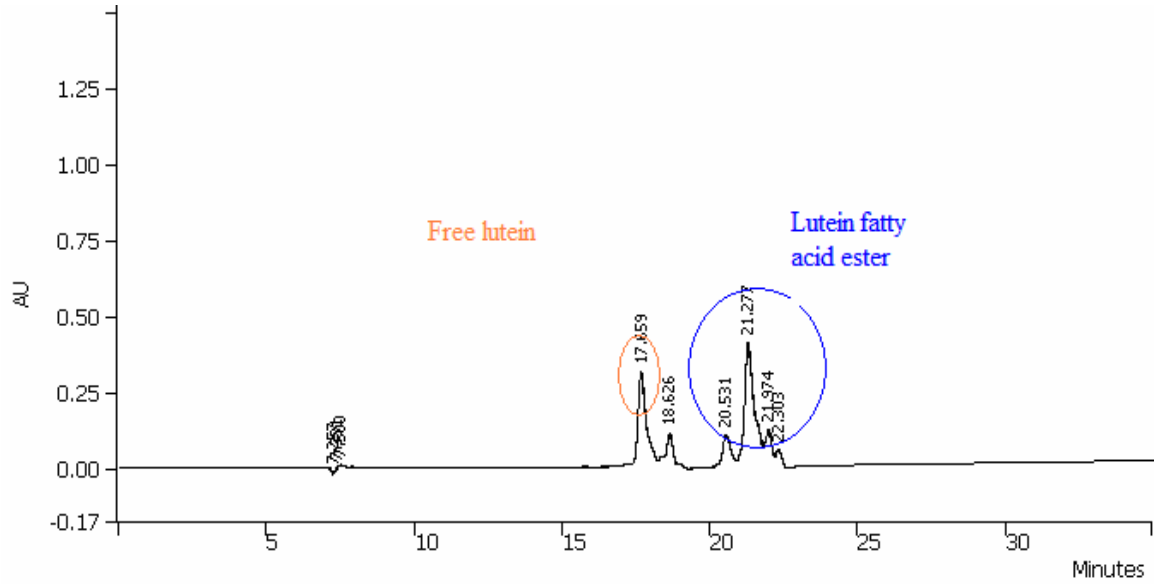


Figure 4.11 HPLC analyses of free lutein and lutein fatty acid esters in marigold flower after various crystallization times.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The total amount of xanthophylls in marigold flower was approximately 25.77 mg xanthophylls/g dry weight of marigold flowers.
2. The suitable condition for saponification of free lutein from Marigold oleoresin was found. For 1 g of oleoresin, 0.5ml of KOH at 40% w/v provided the suitable amount of KOH to complete saponification. The suitable reaction was found to be 4 hours, and at such conditions, approximately 194 mg free lutein /g oleoresin was obtained.
3. For crystallization of free lutein from Marigold oleoresin, water:EtOH was found to be more preferable crystallization solvent compared with water:isopropanol. The suitable solvent ratio of 2:0.5 (water:EtOH) and the crystalizatin time for 30 minutes are found to be suitable conditions, at which approximately 99.12% yield and 85.53% purity were resulted.

5.2 Recommendations

1. The solubility of lutein in solvent mixtures of interest should be examined at various temperatures in order to better predict the crystallization behavior.
2. As evidence by the HPLC chromatograms which show that lutein could be easily converted back to lutein esters at longer crystallization. This indicated that low stability of the free lutein obtained. For the commercial production of the compound, the product stability is a key issue that should be further investigated in the future study.
3. Higher purity of free lutein could be obtained by recrystallization. In addition, the use of cooling crystallization is another possibility that could be worth investigating.

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APPENDICES

APPENDIX A

EXPERIMENTAL DATA FOR ANALYSIS

A-1 Standard calibration curve of xanthophylls from spectrophotometer analysis

Table A-1 Standard calibration curve data of xanthophylls from spectrophotometer analysis

Concentration of xanthophylls (ug/ml)	Absorbance at 478 nm
0	0
1	0.186
2	0.336
3	0.534
4	0.622
5	0.932
6	1.061
7	1.262

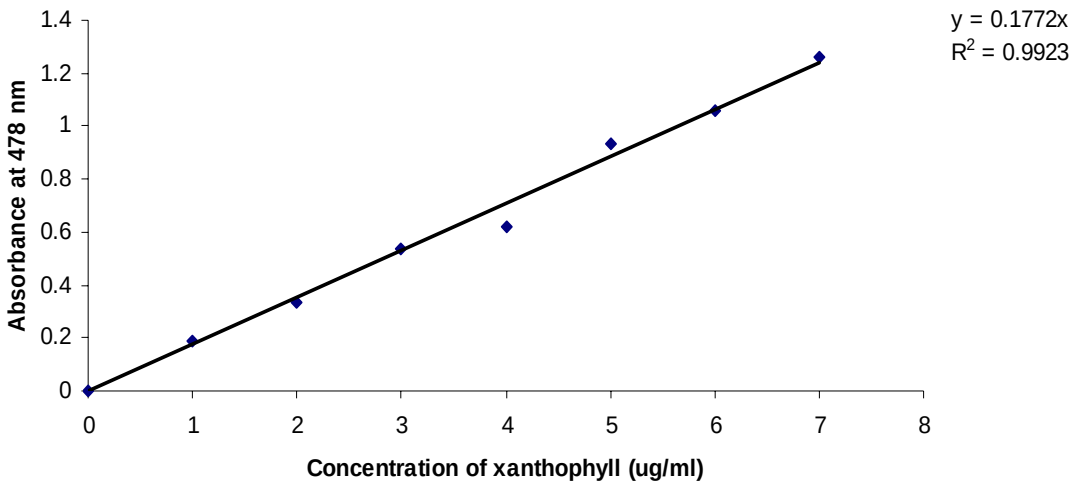

Figure A-1 Standard calibration curve data of xanthophylls from spectrophotometer analysis

A-2 Standard calibration curve of xanthophylls from HPLC analysis

Table A-2 Standard calibration curve data of xanthophylls from HPLC analysis

Concentration of lutein (mg/ml)	Peak Area
0	0
0.005	4097947
0.0625	54401204
0.125	65841064
0.25	220985872
0.5	405019296

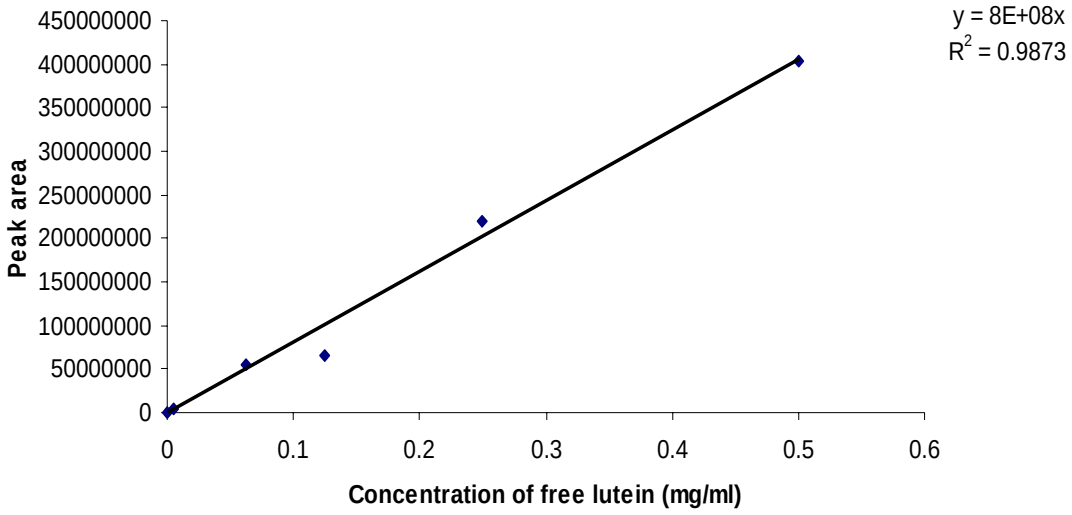

Figure A-2.1 Standard calibration curve data of xanthophylls from HPLC analysis

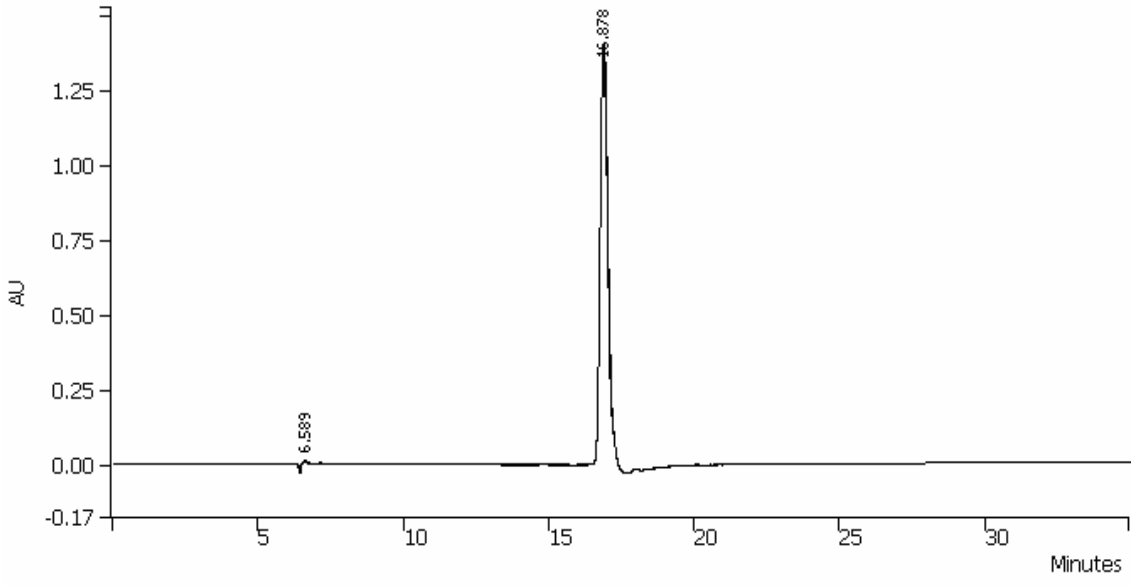

Figure A-2.2 HPLC chromatogram of free lutein standard

APPENDIX B

EXPERIMENTAL DATA

B-1 Experimental data of total xanthophylls in marigold flower

Table B-1 Total xanthophylls content in marigold flower

Run	Time/Temperature (C)	Total xanthophylls (ug/ml)				Total xanthophylls (mg/g oleoresin)	Total xanthophylls (mg/g marigold)	% Total xanthophyll
		Exp.1	Exp.2	Average	SD			
1	4hr-40C	0.625	0.612	0.619	0.009	209.42	20.9	81.27
2	1day-30C	0.095	0.092	0.094	0.002	31.66	3.166	12.29
3	2day-30C	0.051	0.043	0.047	0.006	15.91	1.591	6.18
4	7day-30C	0.003	0.001	0.002	0.001	0.68	0.068	0.26
SUM						257.67	25.767	100

Marigold flower 100 g were converted to 10 g oleoresin

B-2 Experimental data for the study of suitable saponification

Table B-2.1 Effect of time for saponification

Saponnification time (hr)	Area		Concentration of free lutein (mg/ml)		Concentration of free lutein (mg/g oleoresin)		Concentration of free lutein (mg/g oleoresin)	Sd
	Exp 1	Exp 2	Exp1	Exp 2	Exp 1	Exp 2		
0	6530279	7716713	0.01	0.01	4.83	5.71	5.27	0.62
3	151732720	160626880	0.19	0.20	112.21	118.79	115.50	4.65
4	171610480	170515792	0.21	0.21	126.91	126.10	126.51	0.57
5	141709536	137842448	0.17	0.17	104.80	101.94	103.37	2.02
7	130233048	131281600	0.16	0.16	96.31	97.09	96.70	0.55

Table B-2.2 Effect of volume of KOH solution

Volume of KOH solution (ml)	Area		Concentration of free lutein (mg/ml)		Concentration of free lutein (mg/g oleoresin)		Concentration of free lutein (mg/g oleoresin)	Sd
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2		
0.3	191827872	200082736	0.24	0.25	141.86	147.97	144.91	4.32
0.5	243154912	242029088	0.30	0.30	179.82	178.99	179.40	0.59
0.8	227030624	236301872	0.28	0.29	167.90	174.75	171.32	4.85
1	171610480	170515792	0.21	0.21	126.91	126.10	126.51	0.57

Table B-2.3 Effect of concentration of KOH solution

Concentration of KOH solution (ml)	Area		Concentration of free lutein (mg/ml)		Concentration of free lutein (mg/g oleoresin)		Concentration of free lutein (mg/g oleoresin)	Sd
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2		
25%	213890288	201745056	0.26	0.25	158.18	149.20	153.69	6.35
35%	239664928	241257952	0.30	0.30	177.24	178.42	177.83	0.83
40%	265024336	259750320	0.33	0.32	195.99	192.09	194.04	2.76
45%	243154912	242029088	0.30	0.30	179.82	178.99	179.40	0.59

B-3 The study of suitable crystallization

B-3.1 Calculation of yield and purity of free lutein

$$\% \text{ yield of free lutein} = \frac{\text{Amount of free lutein in sample}}{\text{Amount of total free lutein}} * 100$$

$$\% \text{ purity of free lutein} = \frac{\text{Amount of free lutein in sample}}{\text{Weigh of sample}} * 100$$

B-3.2 Experimental data

Table B-3.1 Effect of the v/w ratio of crystallization solvent (H2O:EtOH and H2O:iso-propanol) to oleoresin on yield of free lutein

The v/w ratio of crystallization solvent to oleoresin (H2O:solvent:oleoresin)	Type of crystallization solvent	Area		Concentration of free lutein (mg/ml)		Concentration of free lutein (mg/g oleoresin)			%Yield (free lutein/ xanthophyll)	Sd
		Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Ave		
2:0.3:1	H2O:EtOH	207909296	197848688	0.26	0.24	153.75	146.31	150.03	77.32	5.2609
	H2O:iso-propanol	111590544	112458040	0.14	0.14	82.52	83.166	82.845	42.69	0.4536
2:0.5:1	H2O:EtOH	258938480	261215623	0.32	0.32	191.49	193.18	192.33	99.12	1.1908
	H2O:iso-propanol	164404704	161501792	0.20	0.20	121.58	119.44	120.51	62.10	1.518
2:0.8:1	H2O:EtOH	221545264	215915072	0.27	0.27	163.84	159.68	161.76	83.36	2.9442
	H2O:iso-propanol	192039968	173749584	0.24	0.21	142.02	128.49	135.26	69.71	9.5645
2:1:1	H2O:EtOH	209375920	198609184	0.26	0.24	154.84	146.88	150.86	77.75	5.6302
	H2O:iso-propanol	58670820	54682664	0.07	0.07	43.39	40.439	41.914	21.60	2.0855

Table B-3.2 Effect of the v/w ratio of crystallization solvent (H2O:EtOH and H2O:iso-propanol) to oleoresin on purity of free lutein

The v/w ratio of crystallization solvent to oleoresin (H2O:solvent:oleoresin)	Type of crystallization solvent	Concentration of free lutein (mg/g oleoresin)		Crystal (mg)		%Purity			Sd
		Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Ave	
2:0.3:1	H2O:EtOH	153.75	146.31	240	220	64.06	66.51	65.29	1.73
	H2O:iso-propanol	82.52	83.166	200	220	41.26	37.80	39.53	2.45
2:0.5:1	H2O:EtOH	191.49	193.18	230	220	83.26	87.81	85.53	3.22
	H2O:iso-propanol	121.58	119.44	250	240	48.63	49.76	49.20	0.80
2:0.8:1	H2O:EtOH	163.84	159.68	230	220	71.23	72.58	71.91	0.95
	H2O:iso-propanol	142.02	128.49	250	220	56.81	58.41	57.61	1.13
2:1:1	H2O:EtOH	154.84	146.88	240	240	64.52	61.20	62.86	2.35
	H2O:iso-propanol	43.39	40.439	240	240	18.08	16.85	17.46	0.87

Table B-3.3 Effect of time for crystallization on yield of free lutein

Time for crystallization (hr)	Area		Concentration of free lutein (mg/ml)		Concentration of free lutein (mg/g oleoresin)			%Yield (free lutein/ xanthophyll)	Sd
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Ave		
0.25	135322000	144526336	0.17	0.18	100.07	106.88	103.48	53.33	4.81
0.5	258938480	261215623	0.32	0.32	191.49	193.18	192.33	99.12	1.19
1	177132352	171816736	0.22	0.21	130.99	127.06	129.03	66.50	2.78
1.5	115351704	116808192	0.14	0.14	85.31	86.38	85.84	44.24	0.76
2	57716952	48938876	0.07	0.06	42.68	36.19	39.44	20.32	4.59

Table B-3.4 Effect of time for crystallization on purity of free lutein

Time for crystallization (hr)	Concentration of free lutein (mg/g oleoresin)		Crystal (mg)		%Purity			Sd
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Ave	
0.25	100.07	106.88	250	240	40.03	44.53	42.28	3.18
0.5	191.49	193.18	230	220	83.26	87.81	85.53	3.22
1	130.99	127.06	250	230	52.40	55.24	53.82	2.01
1.5	85.31	86.38	230	210	37.09	41.13	39.11	2.86
2	42.68	36.19	250	250	17.07	14.48	15.77	1.84

APPENDIX C

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Extraction and Purification of Marigold Lutein Fatty Acid Esters

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Abstract

Marigold flowers contain various xanthophylls such as lutein, zeaxanthin, and cryptoxanthin. Of the xanthohyll produced and accumulated in marigold, lutein is found in the highest amout. The compound has been proven scientifically to reduce the risk of age related macular degeneration and cataracts, however in marigold, lutein is present in the form of lutien fatty acid esters, which in comparison with the free form, is medicinally inactive. Therefore, this study aims to investigate the extraction of total xanthophylls from marigold flower as well as the conversion of extracted lutein fatty acid esters into free lutein by saponification. The results showed that hexane was able to extract approximately 41.52 mg xanthophylls/g dry weight of marigold flowers, and the extracted lutein esters could be converted into free luteins by saponification of the extract, in which an increase of more than 2 folds of free lutein could be obtained.

Keywords **:** extraction, purification, lutein fatty acid esters, marigold flower

1. Introduction

Marigold (*Tagetes erecta* L.) is a common ornamental plant which is available in various shades from yellow, red, orange, dark orange, to orange brown. The bioactive compounds, mainly xanthophylls extracted from marigold are well known for the antimicrobial, antiseptic properties, and have a long history of being used as herbal remedies. The use of marigold extract as nutritional and medicinal supplements has also been explored and the potential benefits of the marigold extract including the prevention of cancer and cardiovascular disease, enhanced immune function, inhibition of auto-oxidation of cellular lipids, and protection against oxidant-induced cell damage have been reported. [1]

One of the major xanthophylls found in marigold is lutein whose health-benefiting functions are increasingly being recognized. In marigold flowers, lutein occurs in the acylated form as lutein esters, which are more stable than their non-esterified form against heat and UV-light [2], however it the free lutein that has been identified as a major constituent of the macular pigment of the human retina and has the ability to absorb high-energy blue light from sunlight. Research has strongly suggested that free lutein has protective activity against the two common eye diseases of aging, cataracts and age-related macular degeneration [3], which are the main causes of irreversible loss of vision. In addition, a higher intake of free lutein may also have other beneficial effects on human health, including protection against cardiovascular disease [4], stroke, and UV-radiation-induced skin damage.

It is therefore the objective of this study to obtain free lutein from marigold flowers. Here, the study was divided into two parts. Part I involved the study of extraction of lutein fatty acid esters to determine the effect of the types of extraction solvents on the total amount of xanthophylls extracted. Part II involved the saponification of lutein fatty acid esters to obtain free luteins.

2. Materials and methods

2.1 Materials and chemicals

The dried marigold flowers were obtained from Marigold Production Group, Nakhonratchasima, Thailand. Solvents used as a solvent for extraction of pigments was purchased from Mreck, USA. All chemicals used for the determination of total carotenoids and lutein are analytical grade, and were purchased from Sigma-Aldrich, Germany.

2.2 Sample preparation

Samples of marigold were prepared from petals and calyces, which were dried, ground into fine powder, and stored at 4 °C in a domestic refrigerator until use.

2.3 Extraction of xanthophylls

Two grams of the fine ground sample was weighed into a thimble and was extracted with 200 ml of various solvents (hexane, EtOH, and acetone) for 4 hours. The extract was evaporated under vacuum. The dried extracts were redissolved in EtOH and the amounts of total xanthophylls extracted by different solvents were measured using a spectrophotometer.

2.4 Saponification

Marigold oleoresin was dissolved in 50 ml of ethanol at the temperature 40-70 °C. A 45 % potassium hydroxide solution (2ml) [5] is added slowly to the dissolved oleoresin, and the mixture was incubated at 40-70 °C under agitation between 3 and 5 hours to form a saponified products. After that the mixture was cooled, and pH adjusted to about 7.0 with aqueous solution of hydrochoric acid. Then potassium salts are removed from the product mixture, and the solution was analyzed for the amount of free lutein by high performance liquid chromatography (HPLC).

2.6 Analytical method

1) Analysis of total xanthopylls

The total xanthopylls contents in the extracts were determined by measuring the absorbance at 478 nm with a spectrophotometer using lutein as a standard [6].

2) Analysis of free luteins and lutein fatty acid esters

The free luteins and lutein fatty acid esters were analyzed by HPLC following the method described by Piccaglia et al. [7]. The sample extract was injected to Lichrocart C-18 column, a Diode Array Detector Module 335 and an automatic injector. A 5 μm reversed-phase was used. Chromatographic separation was obtained with a gradient solvent system of acetonitrile: methanol (9:1,v:v) (A) and ethylacetate (B), from 0% to 100% of B using a linear gradient over 30 min, at a flow rate of 1 ml/min and detection at 450 nm.

3. Results and Discussion

Part I: Extraction of lutein fatty acid esters

3.1 Types of solvent

The selection of the solvent plays very important role in increasing the efficiency of extraction due to the corresponding solubility properties. Figure 1 presents the total xanthopyll contents from marigold flower obtained by soxhlet extraction with various organic solvents such as hexane (Hex), acetone, and ethanol (EtOH).

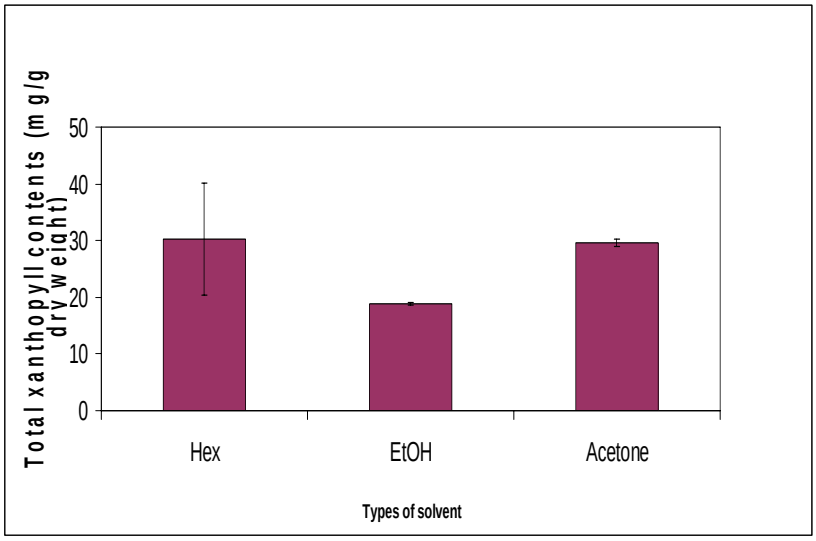

Fig.1 Total xanthopyll contents (mg/g dry weight) by soxhlet extraction with 200 ml of solvent for 4 hr at difference solvent.

Hexane and acetone could extract higher amount of xanthophylls compared with EtOH. This was due to the solubility properties of solvents as a result of different polarities of the solvents used. Non-polar solvents such as hexane and acetone are therefore more efficient for extraction of non-polar lutein compound, while the more polar solvents such as EtOH have lower solubility for lutein.

3.2 Total amount of xanthopylls in marigold flower

In order to determine the total xanthopyll contents in marigold flower, the repeated extraction of marigold flower was conducted 5 times with hexane in soxhlet extractor. The first extraction was carried out for 4 hours, followed by the subsequent 2 hour extractions. The total xanthopylls contents were quantified for all samples were determined and the results are summarized in Table 1. Approximately 92% of xanthophylls in the marigold flower (38.39 mg/g of dry weight out of 41.52 mg/g of dry weight) could be extracted by the first 4-h extraction. The xanthopyll extracted at 6, 8, 10, and 12 h in the subsequent extractions accounted for 4.43%, 1.73%, 1.08%, and 0.31% of the total amount in the sample, respectively.

No. of repeated soxhlet	Content of total xanthopylls (mg/g dry weight)[C]	% of total xanthopylls
1[a]	38.39	92.46
2[b]	1.84	4.43
3[b]	0.72	1.73
4[b]	0.45	1.08
5[b]	0.13	0.31
SUM	41.52	100

Table 1 The content of total xanthopylls in Marigold flowers by soxhlet extraction with hexane.

[a] Extraction by soxhlet 95% n-hexane, 80 ºC , 4 hours

[b] Soxhlet extraction by 95% n-hexane, 80 ºC , 2 hours

[C] Marigold sample: solvent ratio=2:200 w/v

Part II: purification of lutein fatty acid esters by sanponification

3.3 Total amount of free luteins after saponification

3.3.1 Chromatogram of standard free lutein

Figure 2 shows the chromatogram of free lutein standard. The retention time of the compound was found to be 10 minutes.

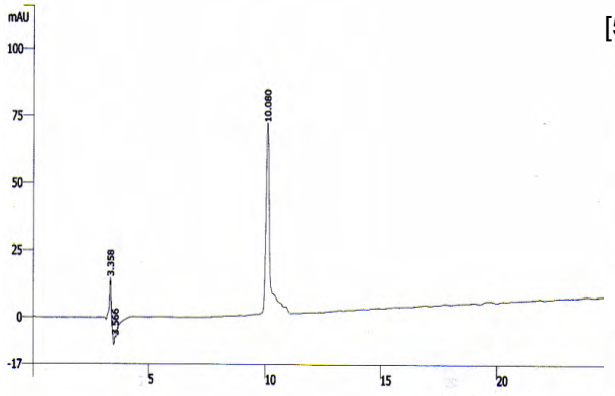

Fig.2 Chromatogram of free lutein standard

3.3.2 Chromatogram of sample

Figure 3 presents the chromatogram of free lutein and lutein fatty acid esters before saponification. The peak at 10 min was identified as a lutein according to the retention of the lutein standard. Others peaks at later retion times were the lutein fatty acid esters, which for example include dimyristate, myristate-palmitate, dipalmitate and palmitate-stearate. The chromatogram of the saponified samples is shown in Figure 4. The peak area of the free lutein in this sample was increased compared with the unsaponified sample (Figure 3), thus indicating that the amount of free lutein increased after saponification, while the contents of lutein fatty acid esters decreased. It can be seen from this result that saponification of marigold oleoresin caused the lutein fatty acid esters to be converted into free luteins. Other than the free lutein, zeaxanthin and other xanthophylls are also obtained along with the potassium salts of fatty acids like myristic acid, palmitic acid and stearic acid [5].

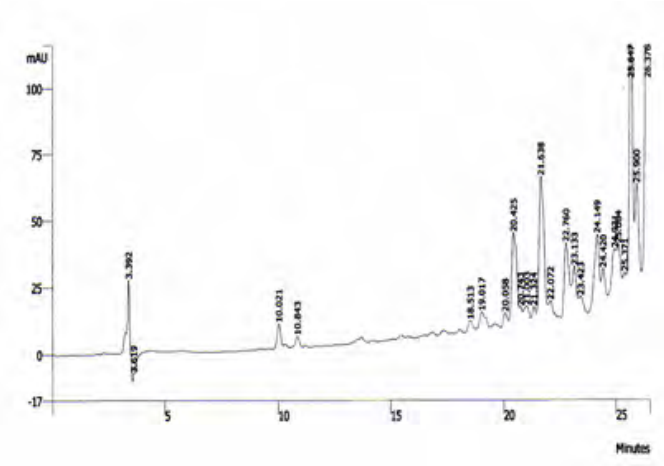

Fig.3 Chromatograme of free lutein and lutein fatty acid esters before saponification.

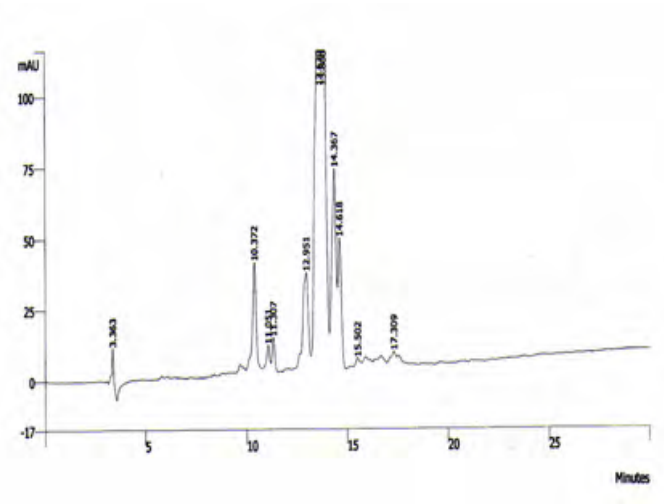

Fig.4 Chromatograme of free lutein and lutein fatty acid esters after saponification.

4. Conclusions

Hexane was found the appropriate for extraction of total xanthophylls from marigold flower and the majority (92%) of the xanthopylls pigment could be removed within the first 4 hours of extraction. Furthermore, saponification of marigold oleoresin was shown to cause the conversion of lutein fatty acid esters into more active free lutein.

5. Acknowledgements

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