การสกัดกรดไขมันลูทีนเอสเตอร์จากดอกดาวเรืองโดยใช้ไดเมทิลอีเทอร์เหลวเป็นสารสกัด

นางสาวเปมิกา ตุลยสิทธิกุล

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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Extraction of Lutein Fatty Acid Esters from Marigold Flower using Liquefied Dimethyl Ether (DME) as extractant

Miss Pemika Tunyasitikun

จุฬาลงกรณ์มหาวิทยาลัย
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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
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เปมิกา ตุลยสิทธิกุล : การสกัดกรดไขมันลูทีนเอสเตอร์จากดอกดาวเรืองโดยใช้ไดเมทิลอีเทอร์เหลวเป็นสารสกัด (Extraction of Lutein Fatty Acid Esters from Marigold Flower using Liquefied Dimethyl Ether (DME) as extractant) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อาทิวรรณ โชติพฤกษ์, 54 หน้า.

งานวิจัยนี้มุ่งศึกษาการสกัดกรดไขมันลูทีนเอสเตอร์จากดอกดาวเรืองโดยใช้ไดเมทิลอีเทอร์เหลวเป็นสารสกัด ซึ่งเป็นตัวทำละลายที่ไม่เป็นพิษ โดยที่อุณหภูมิและความดันปกติจะอยู่ในสถานะแก๊ส ทำให้ตัวทำละลายแยกออกจากผลิตภัณฑ์ได้ง่ายโดยการลดความดัน งานวิจัยนี้ได้แบ่งการศึกษาเป็น 3 ส่วน ส่วนที่ 1 เป็นการหาสภาวะที่เหมาะสมของกระบวนการสกัดด้วยไดเมทิลอีเทอร์เหลว โดยทำการศึกษาปัจจัยของอัตราส่วนของตัวทำละลายต่อดาวเรืองแห้ง และอุณหภูมิในการสกัดที่มีผลต่อปริมาณลูทีนเอสเตอร์ที่สกัดได้ โดยทำการทดลองที่ความเร็วรอบในการปั่นกวน 400 รอบต่อนาที เป็นเวลา 30 นาที สภาวะที่เหมาะสมที่สุดในการสกัดคือ อัตราส่วนของตัวทำละลายต่อดาวเรืองแห้ง 33:0.5 และ 35 องศาเซลเซียส ซึ่งจะได้ปริมาณลูทีนเอสเตอร์ 20.65 มิลลิกรัมต่อ 1 กรัมดาวเรืองแห้ง จากนั้นเปรียบเทียบกับกระบวนการสกัดด้วยตัวทำละลายที่เป็นเฮกเซน และกระบวนการสกัดด้วยคาร์บอนไดออกไซด์วิกฤตยิ่งยวด รวมทั้งเปรียบเทียบกับกระบวนการสกัดด้วยไดเมทิลอีเทอร์เหลวโดยใช้ดาวเรืองเปียกที่ปริมาณความชื้น 80% และ 70% ส่วนที่ 2 เป็นการหาสภาวะที่เหมาะสมของกระบวนการสะปอนนิฟิเคชั่นเพื่อเปลี่ยนลูทีนเอสเตอร์ให้อยู่ในรูปลูทีนอิสระ พบว่าที่อัตราส่วนของเอทานอลต่อโอลีโอเรซิน 20:1, ความเข้มข้นของสารละลายโพแทสเซียมไฮดรอกไซด์ 2.5%, 35 องศาเซลเซียส เป็นเวลา 4 ชั่วโมง เป็นสภาวะที่เหมาะสมที่สุดในการทำปฏิกิริยา ซึ่งจะได้ปริมาณลูทีนอิสระ 150 มิลลิกรัมต่อ 1 กรัมโอลีโอเรซิน และส่วนที่ 3 เป็นการหาสภาวะที่เหมาะสมของกระบวนการสกัดด้วยไดเมทิลอีเทอร์เหลวพร้อมกับการทำปฏิกิริยาสะปอนนิฟิเคชั่น พบว่าสภาวะที่เหมาะสมที่สุดคือ อัตราส่วนของเอทานอลต่อดาวเรืองแห้ง 10:0.5, ความเข้มข้นของสารละลายโพแทสเซียมไฮดรอกไซด์ 5%, 35 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ซึ่งจะได้ปริมาณลูทีนอิสระ 20.71 มิลลิกรัมต่อ 1 กรัมดาวเรืองแห้ง และเปรียบเทียบกับกระบวนการที่สกัดด้วยไดเมทิลอีเทอร์เหลวก่อนแล้วนำไปทำปฏิกิริยาสะปอนนิฟิเคชั่นต่อ ซึ่งได้ปริมาณลูทีนอิสระ 16.65 มิลลิกรัมต่อ 1 กรัมดาวเรืองแห้ง อีกทั้งเปรียบเทียบกับกระบวนการสกัดด้วยไดเมทิลอีเทอร์เหลวพร้อมกับการทำปฏิกิริยาสะปอนนิฟิเคชั่นโดยใช้ดาวเรืองเปียก พบว่าได้ปริมาณลูทีนอิสระ 19.22 มิลลิกรัมต่อ 1 กรัมดาวเรืองแห้ง

ภาควิชา วิศวกรรมเคมี ลายมือชื่อนิสิต .....

สาขาวิชา วิศวกรรมเคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5770234021 : MAJOR CHEMICAL ENGINEERING
KEYWORDS: DIMETHYL ETHER, LUTEIN, MARIGOLD FLOWERS

PEMIKA TUNYASITIKUN: Extraction of Lutein Fatty Acid Esters from Marigold Flower using Liquefied Dimethyl Ether (DME) as extractant. ADVISOR: PROF. ARTIWAN SHOTIPRUK, 54 pp.

This study will focus on the investigation of extraction of lutein fatty acid esters from marigold flowers using liquefied dimethyl ether (DME) which is nontoxic solvent as extractant. Since it is gaseous at normal temperature and pressure, DME can be easily removed from the final product by pressurizing. In this study, was divided into 3 parts. Part I involves the determination of suitable DME extraction. Effect of extraction conditions including solvent to sample ratio (w/w) and extraction temperature at 400 rpm for 30 min on extracted yield of lutein esters were investigated. The highest amount of lutein esters was found to be 20.65 mg/g dried marigold at the condition of 33:0.5 solvent to sample ratio and 35^{o}C. The results were compared with solvent extractions using hexane as solvent and supercritical carbon dioxide extraction. Then, compared with DME extraction using wet sample with 80% and 70% moisture content. Part II deals with the determination of suitable saponification condition in order to convert the lutein esters to free lutein that was ratio of ethanol to oleoresin 20:1, 2.5% KOH concentration, 35 °C for 4 h. at such conditions, approximately 150 mg free lutein /g oleoresin was obtained. In part III, the evaluation of simultaneous DME extraction and saponification was carried out. The suitable condition for simultaneous DME extraction and saponification of lutein esters to free lutein was ratio of ethanol to dried marigold 10:0.5, 5% KOH concentration, 35 °C for 1 h. at such conditions, approximately 20.71 mg free lutein /g dried marigold was obtained. Then compared with those obtained with sequential DME extraction followed by saponification for free lutein that is 16.65 mg free lutein /g dried marigold. And also compared with simultaneous DME extraction and saponification using wet sample, at this study the amount of free lutein was 19.22 mg free lutein /g dried marigold.

Department: Chemical Engineering Student's Signature .....
Field of Study: Chemical Engineering Advisor's Signature .....
Academic Year: 2016

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CHAPTER I
INTRODUCTION

1.1 Motivation

Nowadays, since it is not easy to receive sufficient amount nutrients from modern-day diets, food supplements such as vitamin C, vitamin E, vitamin A, phytochemical (polyphenols, β-carotene, chlorophyll, lutein, etc.) are gaining increased attention. Those derived from natural sources are of particular importance; lutein being a good example of a dietary supplement that not only does it offer several health benefits, it can also be used for medicinal purposes (Siriamornpun et al., 2012). Lutein is known to have the ability to prevent age-related macular degeneration (Tian et al., 2015), cancer and enhance immune function (Soon et al., 1998). It can be found in various natural sources including vegetables (spinach, kale and yellow carrots), fruits (peaches, olives, avocados), micro-algae (*chlorella*) and marigold flowers (Chen et al., 2016). Among those sources, lutein is found in large quantity in the flowers of marigold (*Tagetes erecta*), grown widely in Asia and South East Asia (Sowbhagya et al., 2004). The extraction of lutein from marigold flowers used for nutraceutical application is therefore very attractive and the worldwide market for extracted lutein is expected to grow to US$308 million by 2018 (Lin et al., 2015). Lutein from marigold is generally in the form of lutein esters, mainly lutein dipalmitate (50%), lutein dimyristate (30%), and lutein monoester (6%) (Attokaran 2011). It should be noted that lutein esters must be converted into free lutein by a reaction in a potassium hydroxide solution (usually in an alcohol such as ethanol), the process known as saponification, to exert the bio-availability to human (Piccaglia et al., 1998).

Extraction of lutein esters from marigold flowers can be achieved by using conventional organic solvents. Of these, the most commonly used is hexane. However, the process is time-consuming as it requires separation of the toxic organic solvent. Alternatively, supercritical carbon dioxide (SC-CO_2) extraction is more environmentally friendly as it does not require an additional step of solvent removal, which may result in loss of product yeild. Moreover, the high operating pressure leads to high equipment cost (Gao et al., 2009, Palumpitag et al., 2011).

Alternatively, liquefied dimethyl ether (DME) is an interesting solvent and is regarded as a safe extractant for food or daily ingredients. Data on inhalation exposure of animals and humans to dimethyl ether indicates a very low degree of toxicity. Because of its low boiling point (-24.8^{o}C), the separation of solvent residue from the final product after extraction can be easily achieved upon depressurization of the extraction system. The separated DME can also be recovered and reused easily. In addition, the operating pressure (normally less than 1 MPa) of liquefied DME is lower compared with that of CO_2, which means the lower equipment and operating costs. In addition, compared with CO_2, DME is slightly polar, and it has been shown that liquefied DME was suitably be used to simultaneously remove both organic compounds such as polychlorinated biphenyls (PCBs) and water from river sediment (Oshita et al., 2010). Goto et al., 2015 have developed a wet extraction process using liquefied DME as a solvent for extraction of fucoxanthin from algae. Provided with the possibility to eliminate the drying, cell disruption, and solvent evaporation processes, the simple and low-energy DME based system can be realized. Catchpole et al., 2010 carried out liquefied DME extraction of lipid from fermentation biomass using as DME and found that it was able to effectively extract both neutral and complex lipids thus yielding substantially higher amount of lipid than that obtained with the non-polar SC-CO_2.

In this paper, we propose to investigate DME extraction of lutein esters from marigold flowers as well as the suitable conditions for converting lutein fatty acid esters into free lutein. The processes: extraction and saponification are generally conducted in sequence; extraction followed by saponification. Nevertheless, it has been shown in a recent study by Wang et al., 2015 that simultaneous extraction and saponification to obtain free lutein, not only is possible, but it has economic potential. In their study, the process was carried out in an aqueous two-phase system using isopropanol-KOH aqueous solution, with isopropanol being both an extractant and a saponification medium. The concentrations of isopropanol and KOH, the mass of marigold flower, extraction temperature and time were investigated. As high as 86.73% extraction rate, 84.64% leaching efficiency and 93.98% saponification rate were achieved with use of 38% of isopropanol, 18% of KOH, 0.05 g added mass of

marigold flower powder, and the process was carried out at 50^{o}C and 1.5 h mixing time.

In the first part of this study, we proposed to determine the suitable conditions for extraction of lutein esters from marigold flowers using liquefied DME. The results will be compared with extractions that achieved by employing conventional organic solvents such as hexane, SC-CO_2, and wet extraction (that concerned with the study of moisture content). The second part was the determination of suitable saponification conditions to convert lutein esters in the marigold oleoresin to be free lutein. After obtained suitable conditions for DME extraction and saponification, we proposed to determine amount of free lutein of sequential DME extraction and saponification. In the last part, the possibility of simultaneous DME extraction and saponification of lutein esters to obtain free lutein will be investigated. The results will be compared with that obtained sequential DME extraction and saponification, employing the most suitable conditions for both case (simultaneous and sequential processes).

1.2 Objectives

1.2.1 To study the effect of extraction conditions including solvent to sample ratio (w/w) and operating temperature and moisture content, on extraction of lutein esters from marigold flower using liquefied DME.

1.2.2 To determine the suitable condition including concentration of KOH solution (in ethanol), extraction temperature and time for the simultaneous extraction and saponification of lutein fatty acid esters to free lutein.

1.3 Working scopes

1.3.1 Determine the suitable extraction variables on DME extraction of lutein fatty acid esters from marigold.

Variables	Ranges			
Solvent to sample ratio (w/w)	20:0.5	26:0.5	33:0.5	40:0.5
Temperature (°C)	25	30	35	40

; Fixed variables: agitation rate at 400 rpm. and time 30 min.

- Comparisons of extraction yields with solvent extraction and SC-CO_2 extraction at the best condition based on the results from (Palumpitag et al., 2011) at 40 °C, 4 h for solvent extraction and 60 °C, 4 h, 40 MPa for SC-CO_2 extraction.
- Comparisons of lutein esters content of marigold with wet extraction and dry extraction base on optimal condition of DME extraction.
  ; Control variable: dry marigold powder at 0 %moisture content.

Variables	Ranges		
Moisture content (%)	0	70	80

; Fixed variables: agitation rate at 400 rpm. and time 30 min.

1.3.2 Determine the suitable saponification variables.

Variables	Ranges		
Ratio of ethanol to oleoresin (ml/g)	10:1	20:1	30:1
Concentration of KOH in ethanol (%w/v)	1.5	2.5	3.5
Temperature (°C)	30	35	40
Time (h)	0.5	2	4

; Fixed variables: agitation rate at 150 rpm.

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- Sequential DME extraction and saponification: using optimal condition of DME extraction and optimal condition of saponification to compare with simultaneous extraction and saponification process.

1.3.3 Determine the suitable condition for simultaneous extraction and saponification of lutein fatty acid esters to free lutein.

Variables	Ranges		
Ratio of ethanol to sample (ml/g)	5:0.5	10:0.5	15:0.5
Concentration of KOH in ethanol (%w/v)	3	5	7
Time (h)	0.5	1	2

- Comparisons of simultaneous DME extraction and saponification with sequential DME extraction and saponification, employing the most suitable conditions.

1.4 Expected benefits

This study investigates the use of alternative green solvent such as DME to obtain high value compound, lutein esters, from marigold flowers. In addition, the proposed simultaneous process of extraction and saponification to obtain free lutein does not only require fewer steps (as shown in figure 1.1), but will improve the overall rate of extraction. Since free lutein is more easily soluble in DME, putting the two processes together, having the esters convert to free lutein will enhance the overall extraction rate. The process should reveal shorter process time as well as milder process conditions.

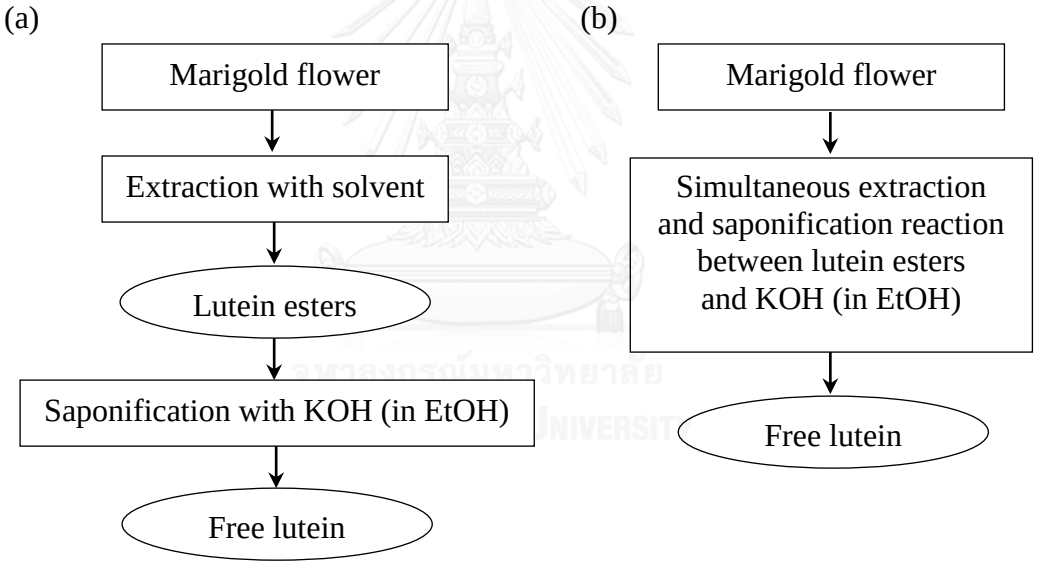


Figure 1.1 Procedures for (a) conventional method and (b) simultaneous extraction and saponification of free lutein from marigold flowers

CHAPTER II
BACKGROUND & LITERATURE REVIEWS

Background

2.1 Marigold flowers

Marigolds are wildly cultivated all over the world, also in Thailand, particularly the species T.erecta, T.patula and T.tenuifolia. They grow well in almost any sort of soil especially in soil with good drainage, also need full sun. Marigold is a medicinal and ornamental plant in the composite family, has plenty of natural antioxidant lutein. They are used in decoration for festivals and religious purposes.

Marigold can be in size from 0.1 to 2.2 m. tall. Most species have pinnate green leaves, floral heads are 4-6 cm diameter with occur in gold, orange and yellow (Figure 2.1).

Figure 2.1 Marigold Flower[1]

http://www.infojardin.com/foro/showthread.php?t=259725

Applications of marigold flowers

Marigold flower heads is the only part that are used for medicinally, as they can be made into infusion, tinctures and ointments that are good for skin wound, birthing scars, sore and all kind of skin problems. Marigold flower helps to prevent DNA damage, cancer, strokes, slowing the ageing process. Marigolds have high content of antioxidants which is protect people from the damage that may be caused by environmental factor such as pollution, cigarette smoke etc. Antioxidants present in marigold are also good for eye degeneration and cataracts. Marigold can be made for yellow food coloring and a natural fabric dye. They are plenty of natural compounds and volatile essential oils (Vasudevan et al., 1997).

2.2 The pigment in marigold

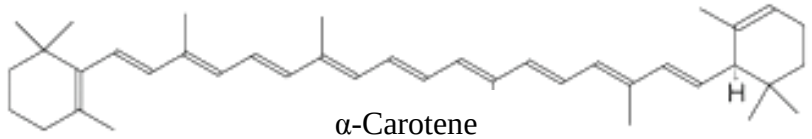
Marigold flower pigment is the interesting component as used as colorants in food. It is one of the richest sources of xanthophylls which are a group of carotenoids that molecules contain oxygen (Vasudevan et al., 1997). An enzymatic pretreatment method was developed for improved extraction of pigments from marigold flowers. Lutein is the major pigment present in the marigold flower. About 95% of lutein present in the flowers is in the form of esters out of which lutein palmitate is the major pigment (Gau et al., 1983). Marigold flower pigment is used in poultry feeds to obtain the desirable skin color and fatty tissues as the poultry pigmentation combines good health and premium quality (Hencken 1992).

Carotenoids

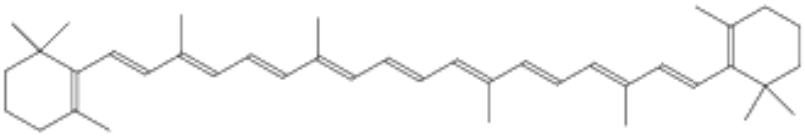
Carotenoids are carotenes and xanthophylls consisting of eight isoprenoid units joined in a head to tail pattern. Most of carotenoids have 40 carbon atoms (Figure 2.2).

Figure 2.2 General formulas of carotenoids

Carotenes characters are non-polar molecules as containing only carbon and hydrogen. Examples of carotenes chemical structures are shown in figure 2.3



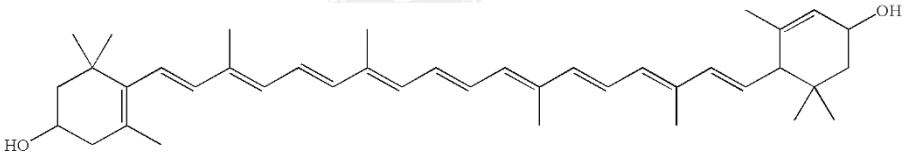
α-Carotene



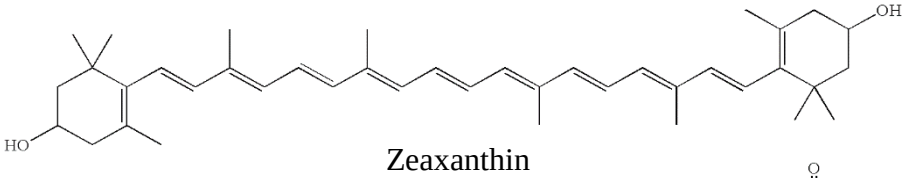
β-Carotene

Figure 2.3 Chemical structures of carotenes

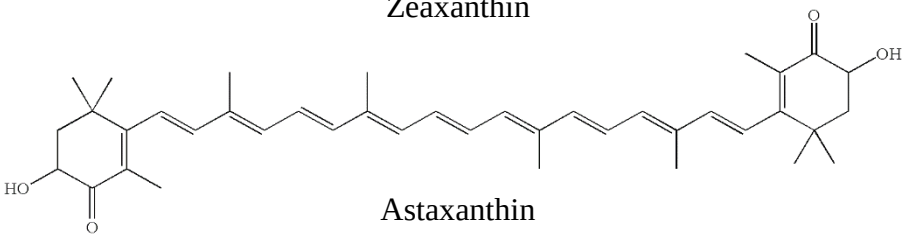
Xanthophylls contain oxygen as have more polar than carotenes. Chemical structures of xanthophylls are lutein, zeaxanthin and astaxanthin as shown in figure 2.4.



Lutein



Zeaxanthin



Astaxanthin

Figure 2.4 Chemical structures of xanthophylls

Carotenoid in marigold flowers are 70% to 90% lutein, 10% to 25% zeaxanthin and beta-carotene proportion are shown in Table 2.1 (Sowbhagya et al., 2004)

Table 2.1 Carotenoids of marigold

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

Carotenoid structure

Carotenoid structure molecules consist of eight isoprene units joined as covalent bond and extensive conjugated double bond. Conjugation system causing carotene to absorb energy of ultraviolet and white light and making carotenoid as pigments, suitable for molecule oxidation resistant of carotenoid. That might be straight line form found in lycopene or ring form at the end of molecule found in beta-carotene. Carotenoid can divide into two groups. Those are hydrogenated and oxygenated carotenoids. Hydrogenated carotenoid derivatives (carotene) are hydrocarbon molecule, non-polar, soluble such as beta-carotene and lycopene. Oxygenated carotenoid derivatives (xanthophyll) have more polar and less soluble than carotenoid as oxygen atoms exist in molecules such as lutein, zeaxanthin and astaxanthin (Simpson et al., 1989).

Application of carotenoids

Carotenoids are group of naturally pigments, usually red, orange or yellow in color. They are used as natural colorants for food, feed and cosmetics. They are a main dietary source of vitamin A in humans with reduced risk of several chronic health disorders such as cancer, heart disease and eye degeneration. The benefits of carotenoid pigments are abundance as can slow the growth of skin tumors, dermatological disease, and cancer in human (Mathews et al., 1982).

Lutein

Lutein ($C_{40}H_{56}O_2$) is one of carotenoid and is major xanthophylls that are in the marigold flowers. Lutein is a carotenoid in yellow pigment. It was also found in many kinds of fruits and vegetables, also in egg yolk. (Sivel et al., 2013) The capacity of lutein in many sources depends on their kind, maturation, processing temperature, preservation or storage. Lutein is an antioxidant that helps protect eye damage as it neutralizes free radical caused by ultraviolet radiation and prevent macular degeneration (AMD). Humans can only consume lutein from fruits, vegetables and food supplements (Calvo 2005). Marigold flowers materials contain all-trans-isomer of lutein; nevertheless cis-isomers of lutein also contained, varies by light and temperature during extraction and sample analysis. Lutein from marigold flower was found in form of lutein fatty acid esters and must be converted to free lutein before people can consume, the process for conversion is saponification (Khachik et al., 1995).

Free luteins are in leafy vegetables such as spinach, broccoli and cabbage. Esters with fatty acids are in fruits and vegetables such as mango, orange, papaya, red or green pepper, yellow corn etc.(Khachik et al., 1986). The quantitative extraction with organic polar or non-polar solvents must be done prior to and HPLC analysis of lutein. Using instrumental method also depend on the native of the sample, performing sole extraction or proceeding by sample saponification (Calvo 2005). The structure of free lutein as shown in figure 2.5

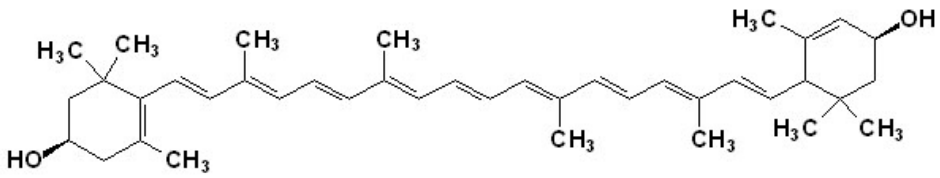
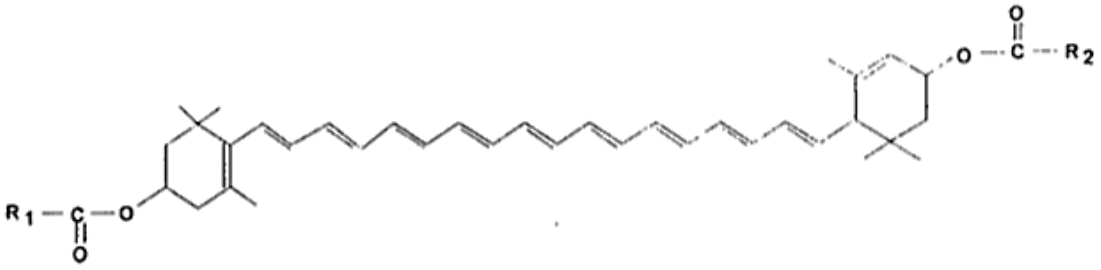


Figure 2.5 Chemical structure of free lutein

Lutein esters in marigold flowers range are 3.8 to 791 ug/g. Most esters in this flowers is lutein palmitate and the rest are dimyristate, myristate-palmitate, palmitate-sterate and distearate (Table 2.2) (Sowbhagya et al., 2004). The structures of lutein fatty acid esters in marigold flower as shown in figure 2.6

Table 2.2 Composition of lutein fatty acid esters (%)

Xanthophyll esters	Gau et al.	Helrich et al.
Dipalmitate	35.5	37.57
Dimyristate	12.6	11.57
Myristate - palmitate	24.7	24.23
Palmitate-stearate	14.4	15.55
Di-stearate	2.4	3.63



Xanthophyll esters	R_1	R_2
Dipalmitate	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{12}$
Dimyristate	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{12}$
Myristate - palmitate	$H_3C(CH_2)_{14}$	$H_3C(CH_2)_{12}$
Palmitate-stearate	$H_3C(CH_2)_{16}$	$H_3C(CH_2)_{14}$
Di-stearate	$H_3C(CH_2)_{16}$	$H_3C(CH_2)_{16}$

Figure 2.6 Structures of lutein fatty acid esters in marigold flower

2.3 Extraction of lutein fatty acid esters from marigold flowers

2.3.1 Solvent extraction

Solvent extraction is a technique that is useful for separation of compound from either a solid or liquid based on their relative solubility of solute compound in solvent. Solvent is a substance that dissolves a solute in form of liquid, solid and gas. Solvent is mostly liquid but can also be solid or gas. Dissolution of solvents varies with temperature. Water is polar solvent for people used. Solvent extraction is the first commercial method to use for chemical and biochemical industries because of its simplicity. Suitable solvents for extraction are non-polar such as hexane, benzene, toluene, diethyl ether etc., hexane is the most effective solvent (Verghese 1998) for extraction compared to ethanol and acetonitrile that are polar solvents. Solvent extraction use to extract vegetable oil from its seed with hexane as solvent, extract colored pigments out of vegetables, extract medicine from herbal plants and extract essential oil from plants. Despite the simplicity of this technique, solvent extraction method involves multiple extraction steps that use time-consuming, use large quantities of solvents that cost more expensive than other. Also, hexane is toxic solvent that is unsuitable for food products. The methods are used to separate a compound consist of

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1) Solid/Liquid Extraction

Solid-liquid extraction allows soluble components to be removed from solids using a solvent. Applications of this unit operation include obtaining oil from oil seeds or leaching of metal salts from ores.

Solvent is used to remove the transition component from extraction material (consisting of solid carrier phase and transition component). Ideally, this results in solvent with dissolved transition component, with the completely depleted solid carrier phase. In reality, the solid carrier phase will still contain some transition component after completion of the extraction. In addition, some of the solvent will still be adsorptive bonded to the solid carrier phase.

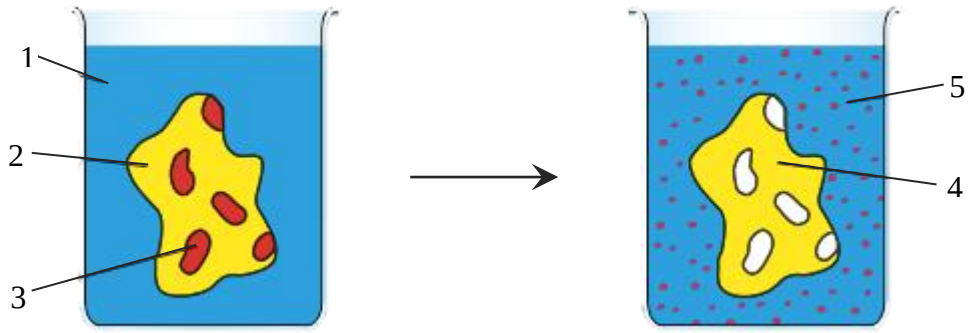

Figure 2.7 Schematic of solid-liquid extraction – before extraction (left) and after extraction (right): 1 solvent, 2 extraction material (solid carrier phase with transition component), 3 transition component, 4 depleted solid carrier phase, 5 solvent with dissolved transition component

To achieve the fastest and most complete solid extraction possible, the solvent must be provided with large exchange surfaces and short diffusion paths. This can be done by pulverizing the solid to be extracted. However, an excessively small grain size can cause agglutination and make it more difficult for the solvent to permeate.

2.) Liquid/Liquid Extraction

Liquid-liquid extraction involves using a liquid solvent to remove a liquid component from a liquid mixture. The component dissolves preferably in the solvent. Applications of this process include removal of vitamins from aqueous solutions and aromatic compounds from crude oil fractions.

In the simplest case, three components are involved: Transition component (A), solvent (B) and carrier liquid (C). The transition component A is combined with the carrier liquid C as the initial mixture (feed). If the initial mixture and the solvent B are mixed together, the transition component A is transferred into the solvent B. The requirement for this is that the solubility of the transition component A in the solvent B is higher than in the carrier liquid C. In turn, the carrier liquid C should be almost insoluble in the solvent B.

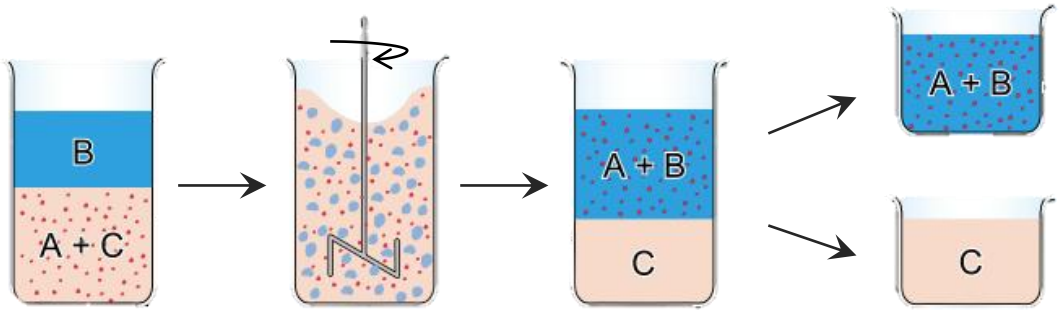

Figure 2.8 Schematic of liquid-liquid extraction – Transition component (A), solvent (B) and carrier liquid (C)

2.3.2 Supercritical fluid extraction

Supercritical fluid extraction (SFE) is technique of using any substance at temperature and pressure above its critical point to permeate solid as same as gas, and dissolve as same as liquid such as supercritical CO_2 (critical point pressure 73.8 bar, critical point temperature 31.1^{o}C) that has state of matter mixed between gas and liquid, dissolved well in non-polar compound. SFE is appropriate technique for extraction of samples that are easily degraded by light, oxygen and high temperature such as vegetable and essential oil (Brunner et al., 2005), Feasible control of fluid property by changing the temperature or pressure to increase the solvent power. In the food and cosmetic industries, SFE is used to extract agent such as flavoring agent, coloring agent, essential oil, caffeine, vitamin etc. SFE is safe for environment and consumer much more than organic solvents extraction which cannot totally separate a solute and still remained solvent residues.

Supercritical fluids (SCF) such as carbon dioxide have their properties similar to liquid organic solvents, but with higher dispersion, low viscosity and lower surface tension. Since SCF properties can be adjusted by changing the pressure or temperature, separation of solvent compound then is fast and easy.

2.3.3 DME extraction

Dimethyl Ether (DME) has chemical formula as CH_3OCH_3 or C_2H_6O contact with liquid will cause frostbite. It can be either liquid or vapor when leaked. DME has solubility of water between 10 and 20 mole%. Then, pre-drying of sample before extraction is not necessary. DME is also easily separated from lipids and other residues as its low boiling point (-24.8^{o}C) and is not remained in the final products at normal temperatures. DME has been authorized as save solvents to be used in foodstuff and food ingredients by the European Food Safety Authority (EFSA), by the Food Standards Australia, New Zealand and by the United States. DME extraction when used for removing fat from animal protein raw materials must be vacuum which assures that most of the volatile dimethyl ether is not present in the final animal protein products.

Generally, organic solvents and supercritical fluids are used as extraction techniques but unfavorable as high cost and toxicity. Recently, a new extraction technique of liquefied dimethyl ether have been extensively studied and used as a green solvent, extracting solvents for the separation of lipid from various natural sources without drying, cell disruption and solvent heating, obtained economically efficient and environmentally friendly. DME becomes method practicable in several industrial fields, such as food, pharmacy and bio-fuels as has such a low toxicity and has been examined as a prospective solvent.

Physical and chemical properties of dimethyl ether

DME is basically inert chemically. It’s a powerful solvent that has a partially soluble in water. It is soluble in ether, acetone, ethyl alcohol also soluble in organic solvents except for polyalcohols. DME becomes a new extraction in several industrial as it is a gas at room temperature and atmospheric pressure that cause residues could easily be removed from the final product.

Dimethyl ether is a colorless, with a faint ethereal odor at room temperature and atmospheric pressure. As liquid density is a function of temperature and pressure.

Basic physical and chemical properties from Flick, 1985 and Food Standards Australia New Zealand (FSANZ), 2011 are given in Table 2.3

Table 2.3 Physical and chemical properties of dimethyl ether

Characteristic	Property
Boiling point	-24.8°C at 1 atmosphere
Freezing point (melting point)	-141.5°C at 1 atmosphere
Flash point	-41°C
Critical pressure	52.5 atm
Critical Temperature	129°C
Heat of melting	25.6 cal/g
Heat of vaporization	111.6 cal/g
Density of liquid	0.665 g/cm^3 at 25°C, when liquefied
Density of gas	1.92 g/L at 1 atmosphere and 25°C
Vapour pressure	4450 mmHg (593 kPa) at 25°C
Solubility in water	7% by weight at 18°C and 1 atmosphere

2.4 Saponification

Lutein fatty acid esters cannot be absorbed by human (Roberta et al., 1997). As lutein in marigold flowers is esterified form with fatty acid such as layric, myristic and palmitic acids (Khachik et al., 2001). The sponified process can converted lutein fatty acid ester to free lutein and obtained fatty acid salt or soap as by-product.

Saponification is the lipid reaction (such as triglyceride, phosphor lipid, wax) and the hydrolysis reaction of esters with metallic alkali such as sodium hydroxide (NaOH) and potassium hydroxide (KOH). Saponification used to enhance the pigmentation value of the extract during the manufacture of commercial preparations.

Figure 2.9 as shown the saponification process. The lutein fatty acid esters consist of R1 and R2 function are converted to free lutein and by-product is fatty acid salt (soap).

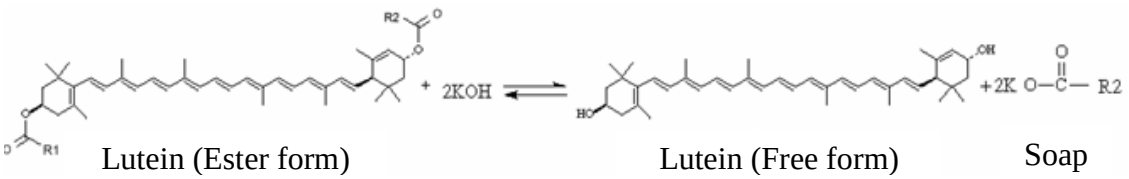

Figure 2.9 Saponification of lutein fatty acid esters

Figure 2.10 as shown mechanism of saponification. There is an irreversible ester hydrolysis, called saponification, which is a process involving a base (i.e. NaOH). The ester bond is cleaved and the hydroxide anion (–OH) is attached to the carbonyl carbon. This manipulation momentarily results in a carboxylic acid and a strong base (the alkoxide ion with a negatively charged oxygen atom that is associated with the positive sodium ion). The formed carboxylic acid is quickly deprotonated (its proton is taken) by the strong base (alkoxide ion) to give the final products of saponification, which are the carboxylate salt and the alcohol.

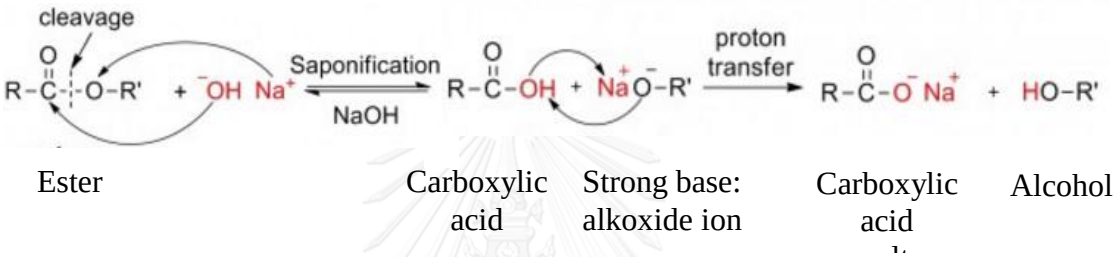

Figure 2.10 Mechanism of saponification

The studies on saponification of lutein fatty acid ester from marigold flowers have been investigated. (Hao Lin et al. 2015) determined the contents of free lutein and lutein ester of marigold flowers. The hydroxyl groups at both ends of free lutein can react with fatty acids to form a lutein mono-ester or di-ester. Conversely, lutein ester can be converted to free lutein via saponification with alkali. The sample was extracted with acetone and the saponification of the oleoresin (dissolved in methanol) was carried out with KOH to form free lutein. The resulting of the crude extract has low quantity of free lutein (0.15 g/kg). After saponification, free lutein contents were very high (19.4 g/kg). (Hojnik et al., 2008) studied the extraction kinetics behavior of lutein from marigold flower petals and simultaneous alkali hydrolysis (saponification). The process of lutein extraction depends on the reaction rate of saponification of lutein esters into free lutein. The data obtained in the present study show that marigold flower petals are a very rich of lutein esters and that the isolation of free lutein can be performed in a single-step procedure, which is composed of extraction and simultaneous saponification. It was found that the main operating parameters influencing the process kinetics are type of solvent, temperature, amount

of solvent and alkali solution, and concentration of the alkali solution. Experimentally determined optimal operating parameters are hexane as organic solvent, temperature 40 °C, solvent/material = 5 L/kg, alkali solution/material = 3.75 L/kg and concentration of KOH in ethanol of 5% (w/v). It was shown that, at optimal conditions, up to 80% of total lutein initially.

2.5 Literature reviews

In marigold flowers, lutein is present in form of lutein fatty acid esters. Conventional method for marigold lutein fatty acid esters extraction is achieved by solvent extraction generally using hexane (Navarrete-Bolanos et al. 2005). Non-polar solvent like hexane was the most efficiency for lutein extraction (Verghese et. al.1998). However, hexane is a toxic solvent and not suitable for food products. Khachik et al., 2001 proposed to extract lutein from marigold with tetrahydrofuran (THF) instead of hexane which is relative to nontoxic solvent. THF is a more polar solvent which cause the obtained yield was lower than hexane extraction. However, several method have been discussed to solve this problem and also other such as long extraction time, the process lead to thermal degradation, etc. Reviews processes for extraction of lutein fatty acid esters from marigold flower are summarized in Table 2.4

For these reason, Supercritical fluid technology has been studied for the application in food industry. Supercritical carbon dioxide (SC-CO_2) is the environment friendly and non-toxic extraction solvent. Supercritical fluid extraction (SFE) of lutein esters from marigold studies were found to be much effective than conventional solvent extraction (Palumpitug et al.2009, Peter Amala Sujith et al. 2012). SFE is suitable for extracting compounds that are easily degraded by light, oxygen and high temperature, easily controlling of the fluid property by change temperature or pressure in order to increase the solvent power. SC-CO_2 extraction is however required high operating pressure that might cause higher equipment cost.

Alternatively, liquefied dimethyl ether (DME) is a new extraction technique and is green solvent that has attracted tremendous attention as it is economically efficient and environmentally friendly. DME can also be used so as to provide milder

extraction conditions. DME is a strong solvent for lipids and is non-toxic that has widely used in food, pharmaceutical and cosmetic applications. DME is non-reactive, does not cause a pH change in aqueous solution and has a sufficiently high vapor pressure at room temperature that complete solvent removal can be carried out easily and at moderate temperatures (Catchpole et al., 2008). DME can extract wet sample without pre-drying step which is required for high energy consuming, leading to the higher total yields compare to SC-CO_2. Catchpole et al., 2008 studied the extraction of lipids from a specialist dairy stream and fermentation biomass using near critical DME extraction (Catchpole et al., 2010). Goto et al., 2015 studied wet extraction of fucoxanthin from raw microalgae by liquefied dimethyl ether. Reviews processes of liquefied dimethyl ether (DME) extraction are summarized in Table 2.5

However, the extracted lutein by those methods is still in esters form which must be saponified to obtain free lutein that requires several steps. Han et al., 2013 studied simultaneous aqueous two-phase extraction and saponification reaction of chlorophyll from silkworm excrement. The aim of their study was to develop a simple and convenient method for the preparation of sodium copper chlorophyllin which synthesized by the saponification reaction between chlorophyll and sodium hydroxide in the ethanol. The concentrations of NaOH, saponification temperature and time were investigated. Wang et al., 2015 studied on combining extraction and saponification of lutein from dry marigold flower by isopropanol-KOH, which is aqueous two-phase extraction. The concentrations of isopropanol and KOH, the added mass of marigold flower powder, extraction temperature and time were investigated. When compare with the conventional method, the method of simultaneous process is not only required less steps in the process, but also avoid the use of large quantities of organic solvent. Reviews processes of simultaneous extraction and saponification reaction are summarized in Table 2.6.

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Table 2.4 Reviews processes for extraction of lutein fatty acid esters from marigold flower

Author	Title	Method /model	Raw material	Compound studied	Condition	Yield	Analysis
Palumpitug et al., 2009	Extraction of lutein fatty acid ester from marigold flower with supercritical carbon dioxide using palm oil as co-solvent	Supercritical carbon dioxide extraction	Marigold flower	Lutein fatty acid esters	- T = 60 ˚C - P = 40 MPa. - time = 4 h. - co-solvent (palm oil) = 10%(w/w)	<u>Without co-solvent</u> % recovery of lutein esters = 74.4% (Total lutein esters yield increased with increasing temperature and pressure) <u>With Co-solvent</u> % recovery of lutein esters = 87.2%	Lutein ester by spectro-photometer at 474 nm. (AOAC method)

Table 2.4 Reviews processes for extraction of lutein fatty acid esters from marigold flower

Author	Title	Method /model	Raw material	Compound studied	Condition	Yield	Analysis
Gao et al., 2009	Supercritical CO_2 extraction of lutein esters from marigold (*Tagetes erecta* L.) enhanced by ultrasound	Supercritical carbon dioxide extraction /Sovova model	Marigold flower	Lutein fatty acid esters	- T = 55 ˚C - P = 32.5 MPa. -particle size = 0.245-0.350 mm -CO_2 flow rate = 10 kg/h -ultrasonic power =400W -ultrasonic frequency = 25 kHz	Without ultrasound -lutein esters = 4.98 mg/g With ultrasound -lutein esters = 6.9 mg/g	Lutein ester by spectro-photometer (WHO/ FAO method)

Table 2.5 Reviews processes of liquefied dimethyl ether (DME) extraction

Author	Title	Method	Raw material	Compound studied	Condition	Yield	Analysis
Catchpole et al., 2010	Extraction of lipids from fermentation biomass using near-critical dimethyl ether	Dimethyl ether extraction, Supercritical carbon dioxide extraction	Microorganisms *(Mortierella alpina, Phaffia rhodozyma and Agrobacterium tumefaciens)* ,algal biomass	Lipids	- T = 313-333 K - P = 40 bar	-The lipid extracts produced were rich in arachidonic acid, astaxanthin, co-enzyme Q10 and EPA - DME is able to extract polar lipids that cannot be extracted with sc-co_2 and that are poorly soluble if at all in sc-co_2 + ethanol. - fucoxanthin and astaxanthin are also more effectively extracted with DME.	GC, HPLC

Table 2.5 Reviews processes of liquefied dimethyl ether (DME) extraction

Author	Title	Method	Raw material	Compound studied	condition	Yield	Analysis
Oshita et al., 2010	Extraction of PCBs and water from river sediment using liquefied dimethyl ether as an extractant	Dimethyl ether extraction	River sediment	Polychlorinated biphenyls (PCBs) and water	- T = 25 ˚C - P = 0.6–0.8 MPa. - time = 4320 s. - liquefied DME /sediment ratio = 60 mL/g.	- The maximum extraction efficiencies of liquefied DME for PCBs = 99% and water = 97%. - A large amount of PCBs and water could be extracted by increasing the extraction time and the liquefied DME/sediment ratio.	GC-MS

Author	Title	Method	Raw material	Compound studied	Condition	Yield	Analysis
Catchpole et al., 2008	Extraction of lipids from a specialist dairy stream	Dimethyl ether extraction, Supercritical carbon dioxide extraction	Beta-serum	Lipids	DME -T = 333 K -P = 40 bar CO_2 -T = 313 K -P = 300 bar	-DME is used as a solvent for neutral and complex lipids -Good yields of phospholipids were obtained in this process, particularly at low feed to solvent flow ratios and with low solids concentrations.	P NMR
Goto et al., 2015	Extraction of carotenoids and lipids from algae by supercritical CO_2 and subcritical dimethyl ether	Dimethyl ether extraction, Supercritical carbon dioxide extraction	Wet brown seaweed (*Undaria pinnatifida*)	Fuco-xanthin	-T = 25 ◦C -P =0.59 MPa -time = 43 min	-Amount of fucoxanthin = 390 μg/g dry of wet *U.pinnatifida*	HPLC

Table 2.6 Reviews processes of simultaneous extraction and saponification reaction

Author	Title	Method	Raw material	Compound studied	condition	Yield	Analysis
Han et al., 2013	Simultaneous aqueous two-phase extraction and saponification reaction of chlorophyll from silkworm excrement	Aqueous two-phase extraction (ATPE) and saponification reaction (using ethanol–NaOH)	Silkworm excrement	Chlorophyll (Sodium copper chlorophyllin)	- NaOH concentration = 0.4 g/ml. - Saponification temperature = 333.15 K - Saponification time = 2 h	- The simultaneous aqueous two-phase extraction and saponification reaction process shows a better performance. - The amount of sodium chlorophyllin in the top system reached its maximum in the investigated range.	UV–Vis spectro-photometer

Table 2.6 Reviews processes of simultaneous extraction and saponification reaction

Author	Title	Method	Raw material	Compound studied	condition	Yield	Analysis
Wang et al., 2015	Combined process of reaction, extraction and purification of lutein in marigold flower by isopropanol-KOH aqueous two- phase system	Aqueous two-phase extraction (ATPE) and saponification reaction (using isopropanol–KOH)	Marigold flower	Lutein	- Isopropanol concentration = 38%(w/w) - KOH concentration = 18%(w/w) - Marigold flower powder = 0.05 g. - Temperature = 50 ˚C - Mixing time = 1.5 h	- The extraction rate of total lutein = 86.73% - The leaching efficiency of total lutein = 84.64% - The partition coefficients of total lutein = 5.115 - The saponification rate of lutein = 93.98%	UV–Vis spectro-photometer, TLC

CHAPTER III
MATERIALS AND METHODS

This chapter describes the materials and methodologies used for carrying out this study, which is divided into 3 parts. Part I involves the determination of suitable DME extraction. Part II deals with the determination of suitable saponification condition. In part III, the evaluation of simultaneous DME extraction and saponification was carried out and the results, in terms of the efficiency of the recovery of free lutein, are compared with those obtained with sequential DME extraction followed by saponification.

3.1 Materials and chemicals

Pulverized dried marigold flowers sample was obtained from PTT Chemical (Rayong, Thailand). Liquefied dimethyl ether (DME) (Spray-work air can 420D) used for extraction was purchased from Siam Tamiya Co., Ltd., Thailand. Hexane (purity>99.5%) used for extraction of lutein esters was supplied by Sigma-Aldrich. High purity carbon dioxide used for SC-CO_2 extraction was obtained from Thonburiwattana Co. (Bangkok, Thailand). Ethanol (purity>99.5%) and potassium hydroxide used for saponification were purchased from Merck, USA. Diethyl ether and sodium sulfate was supplied by Merck, Thailand. Lutein standards (analytical grade) were purchased from Sigma-Aldrich, Germany.

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3.2 Methodology for extraction

3.2.1 Determination of total amount of lutein esters in marigold flowers

The extraction of lutein esters from pulverized dried marigold flowers can be achieved by various methods such as DME extraction, solvent extraction and SC-CO_2 extraction, which would yield different amounts of lutein esters. To evaluate the efficiency of each extraction method, it is necessary to determine the total amount of lutein esters in marigold flowers sample by repeated extraction with hexane.

First, 10 gram of dried marigold flowers was extracted by 50 ml of hexane at 40^{o}C for 4 h in a digital incubator shaker (New Brunswick Scientific Co., Inc., innova 4000, USA), after which, the system was left to stand for 5 min for the residue to settle. Then the extract was separated from the solid sample residue by filtration using filter paper (Grade. No.1: 11 μm). The remaining sample residue was re-extracted 3 times, each with 50 ml of hexane at 30^{o}C for 1 day, 3 days and 10 days, respectively. The extract was diluted with hexane and the prior to the analysis for the amount of the lutein esters by a spectrophotometer (Thermo Spectronic model 4001/4, USA).

In Part I of this study, powder of marigold flowers was extracted with DME and the extraction yields of lutein esters were compared with those obtained with hexane, supercritical carbon dioxide extraction. In addition, the DME extraction of wet marigold sample was evaluated. The experimental procedures for carrying out the above investigation are described as follows:

3.2.2 DME extraction

DME extraction of pulverized dried marigold flowers was carried out in an apparatus schematically shown in Fig.3.1, which consists of a stainless steel extractor, connected to a DME storage vessel thru a needle valve and to a separation unit thru a ball valve. The separation unit is composed of an 80 ml hyper-glass vessel in polycarbonate housing. The temperature of the extractor was controlled by heating jacket connecting with control box. Agitation of the extraction system was provided using a magnetic stirrer. An extraction run begins with charging 0.5 gram of pulverized dried marigold flowers into the extractor, along with an 8 mm diameter magnetic bar. The needle valve was open to allow liquefied DME from the DME storage vessel into the extractor at a specified solvent to sample ratio. Extraction was carried out for 30 minutes at a fixed agitation rate of 400 rpm at a controlled extraction temperature. The operating variables for the DME extraction experiments were solvent (DME) to sample ratio and extraction temperature, whose ranges are summarized in Table 3.1. After each extraction was complete, the ball valve is open to allow the extract to flow out of the reactor to the separation unit through a filter

paper (Grade. No.1: 11 µm) placed at the exit of the extractor and then through a stainless steel filter. By depressurizing the separation unit, DME was evaporated. The remaining sample, namely *marigold oleoresin*, was diluted with hexane prior to further analysis for the amount of lutein esters by a spectrophotometer.

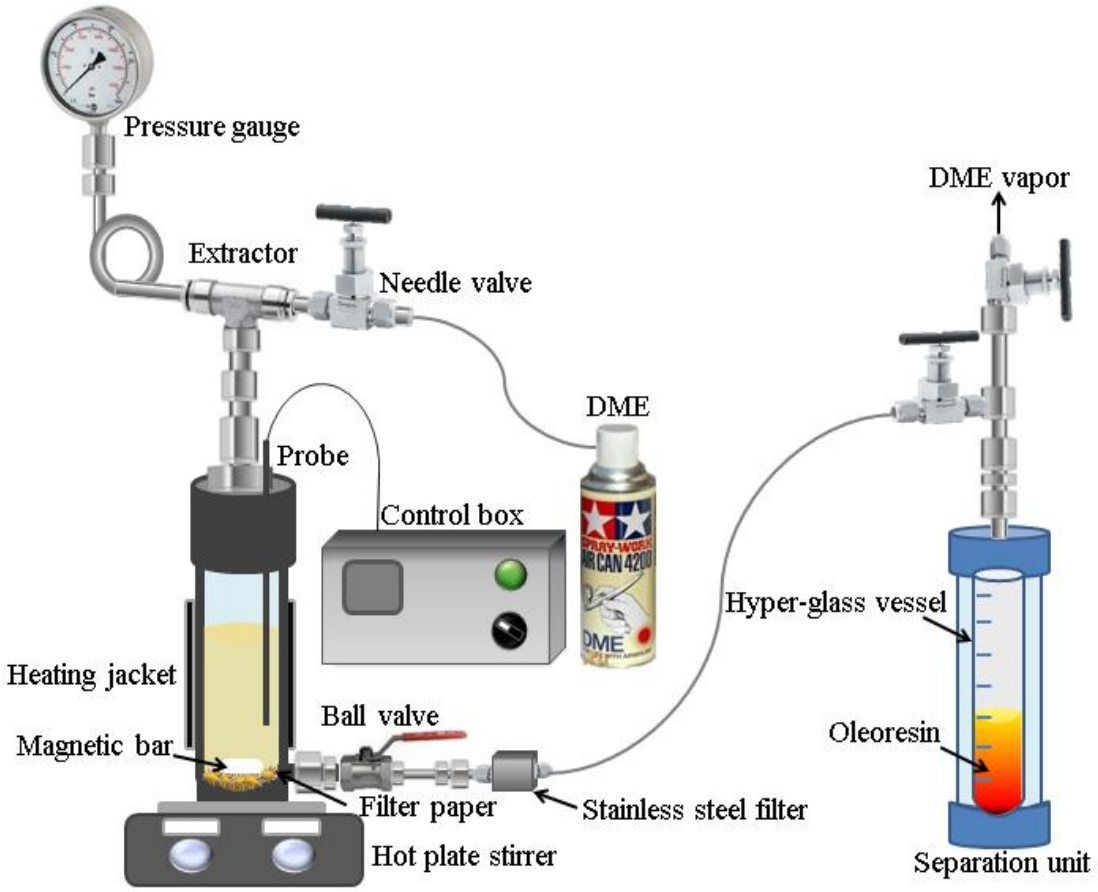

Figure 3.1 DME extraction apparatus

Table 3.1 Ranges of variables for DME extraction

Variables	Ranges	Fix variables
Ratio of solvent to sample (w/w)	20:0.5, 26:0.5, 33:0.5, 40:0.5	35^{o}C, 30 min, 400 rpm.
Temperature (^{o}C)	25, 30, 35, 40	30 min, 400 rpm.

3.2.3 Solvent extraction

10 grams of dried marigold was extracted by 50 ml of hexane at 40^{o}C for 4 h in digital incubator shaker. After the extraction was complete, the system was left to stand for 5 min. Then the extract was filtered to separate the filtrate from the solid marigold residue and diluted with hexane prior to the quantification of the amount of lutein esters by a spectrophotometer.

3.2.4 Supercritical carbon dioxide extraction

0.5 gram of dried marigold sample was placed into a 10 ml extraction vessel, filled with silica sand to distribute the sample. The vessel was tightened, and assembled in a supercritical carbon dioxide extractor (model SFX-220, ISCO.). The extraction was carried out at 60^{o}C, 40 MPa for 4 h, the most suitable condition suggested by our previous study (Palumpitag et al., 2011). The extract was collected in a sample vial containing hexane trap (wrapped with aluminum foil), and was diluted with hexane and analyzed by spectrophotometer for the total amount of lutein fatty acid esters.

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3.2.5 DME extraction of wet sample of marigold flowers

With the most suitable condition for DME extraction of dried marigold flowers determined as described in section 3.2.2, the efficiency of DME for extraction of wet sample of marigold flowers was investigated and compared with that of DME extraction of dried sample. The predetermined amounts (using equation A-2) of distilled water were added to 0.5 gram of dried marigold flower sample to prepare the wet marigold flower sample of 70% and 80% moisture contents (the approximate water contents of fresh marigold flowers). The sample of specified moisture content was then extracted with DME following the method described in section 3.2.2.

3.3 Determination of suitable saponification condition

In Part II of this study, experiments were carried out to determine the most suitable saponification conditions to convert lutein esters in the marigold oleoresin to be free lutein. The procedure is described as follows:

3.3.1 Preparation of marigold oleoresin

To prepare marigold oleoresin for the investigation of suitable saponification conditions, 100 grams powder of dried marigold flowers was extracted with 500 ml of hexane at 40^{o}C for 4 h. The extract was filtered to remove the solid marigold residue. The filtrate was evaporated to remove hexane by using a rotary vacuum evaporator (EYELA rotary evaporator N-100) at 40^{o}C. The resulted oleoresin was further dried for 2 hours in a vacuum oven at 35^{o}C. For each 100 grams of dried marigold flowers, approximately 10 grams of dried oleoresin was obtained.

3.3.2 Saponification of lutein fatty acid esters

The saponification experiment carried out to determine the suitable condition involves the reaction of 1 gram of marigold oleoresin with the KOH solution in ethanol in a 35 ml glass vial at a controlled extraction temperature and time, at a constant agitation rate 150 rpm using a magnetic bar. The operating variables to be studied are the ratio of ethanol to oleoresin, concentration of KOH solution, saponification time and temperature, whose ranges are summarized in Table 3.2. After each saponification run, 50 ml of ethanol was added to the saponified mixture. The mixture was subsequently transferred to a separation funnel, into which 100 ml of 5% Na_2SO_4 solution (in distilled water) and 80 ml of diethyl ether were loaded. The liquids were mixed and were then allowed to separate into two phases. The upper phase containing free lutein was collected, while the lower phase which was the water-soluble impurities was discarded. The free lutein rich organic phase was washed repeatedly with 5% Na_2SO_4 aqueous solution until the aqueous phase became colorless (Shibata et al., 2004). Diethyl ether in the washed free lutein rich phase was then evaporated by purging with nitrogen. The dried sample was re-dissolved with

50 ml of ethanol and stored at 4^{o}C until analysis by high pressure liquid chromatography (HPLC, Varian Inc., model 410, USA).

Table 3.2 Experimental ranges of variables for saponification of lutein esters

Variables	Ranges	Fixed conditions
Ratio of ethanol to oleoresin (ml/g)	10:1, 20:1, 30:1	KOH 0.5 g, 35^{o}C, 4 h
Concentration of KOH in ethanol (%w/v)	1.5, 2.5, 3.5	35^{o}C, 4 h
Temperature (^{o}C)	30, 35, 40	4 h
Time (h)	0.5, 2, 4	-

As mentioned, to obtain free lutein from marigold flowers, extraction and saponification processes are required. Nevertheless, they may be carried out either sequentially or simultaneously. In Part III, the simultaneous DME extraction and saponification of lutein esters to obtain free lutein will be investigated to determine the most suitable conditions for such process. The results will be compared with that obtained by sequential DME extraction, followed by saponification carried out at the most suitable DME extraction and saponification conditions as suggested by the results of Part I and Part II, , respectively. The experimental procedures are detailed as follows:

3.4 Simultaneous DME extraction and saponification

Simultaneous DME extraction and saponification of pulverized dried marigold flowers was carried out in the same apparatus used for DME extraction (Fig.3.1). For this part of experiment, 0.5 gram of dried marigold powder and KOH solution (in ethanol) at specified concentrations (%w/v) were charged into extractor. Then, liquefied DME was then charged into the extractor at optimal DME to sample ratio

determined from the previous experiment. The extraction was carried out at 35^{o}C, agitation rate 400 rpm and at a specified mixing time as shown in Table 3.3. After the extraction was complete, the extract was then transferred to the separation unit, after which DME was then evaporated by depressurizing the separation unit. Into the remaining sample mixture (oleoresin, KOH and ethanol), 50 ml of ethanol was added, and the mixture was subsequently transferred to a separation funnel. The free lutein was extracted from the saponified product, and subsequently washed following the same method described in section 3.3.2. The washed free lutein solution was then stored in a 4^{o}C until analysis by HPLC.

Table 3.3 Ranges of variables for simultaneous DME extraction and saponification

Variable	Ranges	Fix variables
Ratio of ethanol to sample (ml/g)	5:0.5, 10:0.5, 15:0.5	KOH 0.5 g, 35^{o}C, 0.5 h
Concentration of KOH in ethanol (%w/v)	3, 5, 7	35^{o}C,0.5 h
Time (h)	0.5, 1, 2	35^{o}C

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3.5 Sequential DME extraction and saponification

The sequential or 2-step process began with DME extraction, followed by saponification of the oleoresin, in each step, the most suitable condition as suggested by the results in the previous sections was employed. 0.5 gram powder of dried marigold flowers was extracted by DME following the procedure described in section 3.2.2. Once, the extraction was complete, DME was evaporated. Marigold oleoresin was diluted with hexane and then transferred to 35 ml. vial. Then, hexane was evaporated by purging with nitrogen. The oleoresin was reacted with KOH in ethanol for the further saponification as already mentioned in section 3.3.2. At the end, the washed free lutein solution was then stored in a 4^{o}C until analysis by HPLC.

3.6 Analytical method**s**

3.6.1 Analysis of lutein fatty acid esters

The amount of lutein esters in marigold flowers extract was determined by measuring spectrophotometric absorbance at 428 nm using the WHO/FAO method (JECFA, 2014) by adding about 80 ml hexane and 5 ml 2-propanol to oleoresin and pour into a 100 ml volumetric flask. Place the volumetric flask into a shaker to achieve complete dissolution. Adjust to the 100 ml volume mark with hexane. Mixed well, make serial dilutions with hexane and using hexane as blank. The equation to determine the lutein esters was calculated by equation A-1 as shown in Appendix A.

3.6.2 HPLC analysis

The saponified solutions obtained from Part II and Part III was analyzed by using HPLC to identify the components of free lutein and lutein fatty acid esters. The reversed phase HPLC analysis was carried out using Lichrocart C-18 column, a Diode Array Detector Module 335 and an automatic injector. The mobile phase was a gradient solvent system of acetonitrile:methanol (9:1,v:v) (A) and ethyl acetate (B), from 0% to 100% of B using a linear gradient injected to over 30 min, at a flow rate of 1 ml/min. The sample injection volume was 20 µl and the detection wavelength was at 450 nm (Palumpitag et al., 2011).

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CHAPTER IV
RESULTS AND DISCUSSION

4.1 Total amount of lutein esters in dried marigold flowers

Dried marigold flowers were repeatedly extracted with hexane in order to determine the total amount of lutein esters in marigold flowers. The initial extraction was at 40^{o}C for 4 h., followed by extraction at 30^{o}C for 1 day, 2 days, and 7 days respectively. The yield of marigold oleoresin was 10 g/100 g of dried flowers. The total lutein esters content in marigold flowers in each extract was quantified and summarized in Table 4.1, from which, it can be seen that the total amount of lutein esters was 23.31 mg/g dried marigold flowers. Approximately 75.39% of this amount was extracted in the initial extraction with hexane at 40^{o}C for 4 h., and only small percentages, 18.42%, 5.01% and 1.19% were extracted in subsequent extractions with hexane at 30^{o}C for 1, 2 and 7 days respectively. The extraction yield of lutein esters in this research is comparable to those reported by Vechpanich et al. (2008) and Palumpitug et al. (2011), which were 25.77 mg/g dried marigold and 27.26 mg/g dried marigold respectively.

Table 4.1 Total lutein esters content in dried marigold flowers

Run	Time/Temperature	Total lutein esters (mg/g dried marigold flowers)				% Total lutein esters
		Exp.1	Exp.2	Average	SD	
1	4 h./ 40^{o}C	17.39	17.76	17.58	0.26	75.39
2	1 day/ 30^{o}C	4.07	4.52	4.29	0.32	18.42
3	2 day/ 30^{o}C	1.16	1.18	1.17	0.01	5.01
4	7 day/ 30^{o}C	0.30	0.25	0.28	0.03	1.19
Sum		22.92	23.71	23.32	0.63	100

4.2 Determination of suitable DME extraction conditions

In Part I, DME extraction was investigated in order to find the suitable solvent to sample ratio and temperature that gave the maximum yield of total lutein esters. The yield obtained by DME extraction was then compared with those resulted from solvent extraction using hexane and SC-CO_2 extraction. In addition, the DME extraction yields of dried and wet marigold flowers were compared. The results are presented as follows.

4.2.1 Effect of solvent to sample (w/w) ratio

Initially, DME extraction experiment was performed with 0.5 g of dried marigold. The DME to dried marigold sample ratio was varied 20:0.5, 26:0.5, 33:0.5 and 40:0.5 that determined at 35^{o}C, 400 rpm for 30 min. All the extracts were determined for the amount of total lutein esters by measuring the absorbance at 428 nm with a spectrophotometer, whose results are shown in Fig. 4.1. The total lutein esters content was found to increase with increasing solvent to sample ratio from 20:0.5 to 33:0.5 and stayed rather constant above this ratio. This is possibly due to the fact that, as the solvent increases, solute–solvent interactions are also enhanced, and this then enhances the total amount of lutein esters extracted and solubilized into the solvent. The highest lutein esters yield of 20.65 mg/g dried marigold in this experiment was obtained at the solvent to sample ratio of 33:0.5. This ratio was then used in subsequent experiment to determine the suitable extraction temperature.

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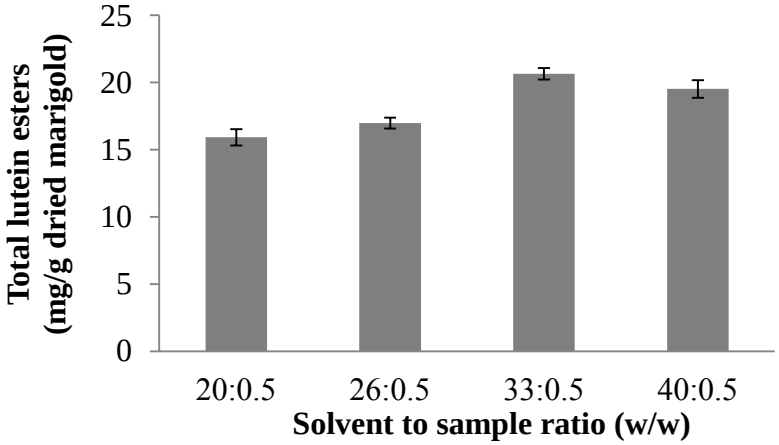

Figure 4.1 Effect of solvent to sample ratio on amount of lutein esters obtained by DME extraction (35^{o}C, 400 rpm and 30 min)

4.2.2 Effect of extraction temperature

The dried marigold flowers was extracted at four temperatures: 25°C, 30°C, 35°C and 40°C, with a solvent to sample ratio of 33:0.5, which is the ratio yielding the highest amount of lutein esters as determined in section 4.2.1. As shown in Fig.4.2, the lutein esters yield increases only gradually with increasing temperature up to 35°C and decreased slightly at 40°C, probably due to the high sensitivity of the compounds to high temperature conditions. Since no significant differences are seen among various temperatures up to 35°C, the subsequent experiment was carried out at 35°C, the closest to the ambient temperature.

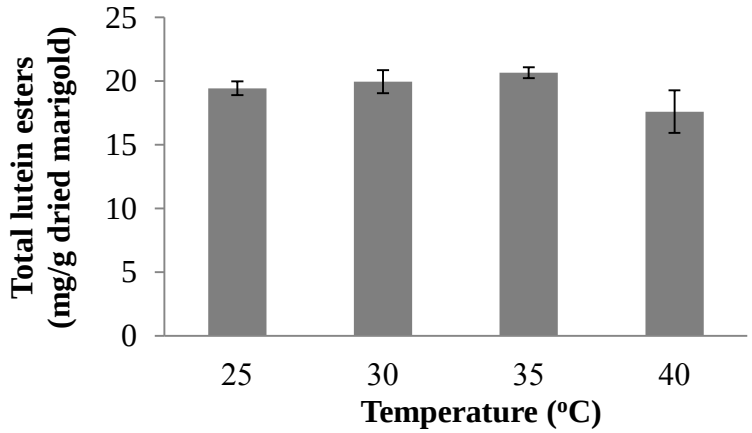

Figure 4.2 Effect of temperature on amount of lutein esters obtained by DME extraction (solvent to sample ratio 33:0.5 400 rpm, and 30 min).

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4.2.3 Comparison of yields obtained from extraction with DME, SC-CO_2 extraction and hexane

Three extraction methods using different solvents were evaluated to compare the yield of marigold lutein esters, and the results are shown in Table 4.2. For DME extraction, the highest lutein esters content was found to be 20.65 mg/g dried marigold at the most suitable conditions (33:0.5 ratio of DME to dried marigold, 35°C for 30 min). The yield is higher than that obtained by extraction with hexane (17.58 mg/g dried marigold obtained at the 5:1 ratio of hexane to dried marigold, 40°C for 4 h) Although these numbers could not be directly compared due to use of different solvent to sample ratio, it can be clearly seen from these results that among the three

solvents evaluated, SC-CO_2 extraction yielded the lowest amount of lutein esters (15.91 mg/g dried marigold, at 60^{o}C, 40 MPa for 4 h), despite the highest solvent to sample ratio of 428 ml : 0.5 g and the most suitable conditions employed, suggested by previous study (Palumpitug et al., 2011). This result suggested that although SC-CO_2 extraction leaves no residue solvents in the extract, and is thus safe to use for human consumption, it's small molecule is not favorable to solubilize lutein esters, high molecular weight (although rather non-polar) compounds. Based on high extraction yield and the fact that the solvent can be easily separated from the extract, DME is considered a favorable solvent for extraction of marigold lutein esters.

Table 4.2 Comparing amount of lutein esters from each extraction method

Method	Solvent to sample ratio	Conditions	Total lutein esters (mg/g dried marigold)
DME extraction / batch process	DME : dried marigold = 33 g : 0.5 g.	35^{o}C, 400 rpm and 30 min.	20.65
Solvent extraction using hexane / batch process	Hexane : dried marigold = 50 ml : 10 g.	40^{o}C, 150 rpm and 4 h.	17.58
SC-CO_2 extraction / continuous flow process	CO_2 : dried marigold = 428ml : 0.5 g.	60^{o}C, 40 MPa and 4 h.	15.91

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4.2.4 Comparison of lutein esters yields by DME extraction of dried and wet marigold flowers

The feasibility of DME extraction of wet marigold flowers is evaluated here and the results are compared with that obtained with DME extraction of dried marigold sample (0% water content) from previous section. Wet marigold flowers were prepared by adding pre-calculated amount of water to the dried marigold flowers to 70 and 80% moisture content, approximate water content of fresh marigold flowers

and that of fresh marigold flowers having undergone a dewatering process by physical compression, respectively. As shown in Figure 4.3, lutein esters yields of 17.89 mg/g dried marigold and 17.71 were obtained with DME extraction of wet marigold samples of 80% and 70% moisture content, respectively, slightly lower than that obtained with dried marigold flowers. The presence of water might have increased the overall solvent polarity of water, lowering the solvent's ability to extract rather non-polar compounds like lutein esters. Nevertheless, if the lower extraction yields could be compensated by the amount of time and energy saved from not needing to dry the biomaterials (marigold flowers), DME extraction of wet sample would be favorable.

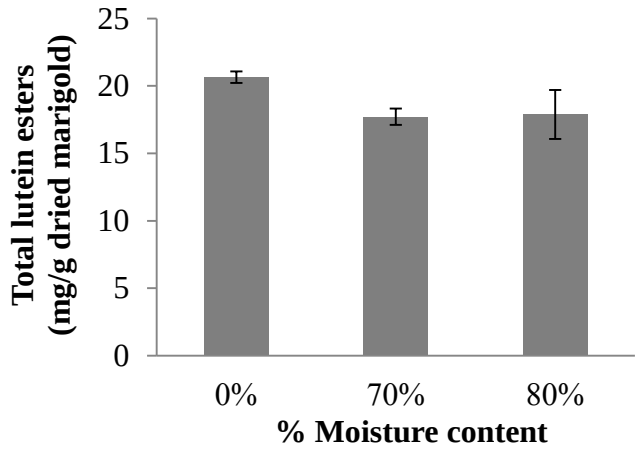


Figure 4.3 Comparison of lutein esters yields obtained by DME extraction of wet and dry marigold flowers.
(solvent to sample ratio 33:0.5, 35^{o}C, 400 rpm and 30 min.)

4.3 Determination of suitable saponification conditions

To suggest the suitable saponification condition, in Part II of this study, the effect of volume of ethanol to weight of marigold oleoresin ratio, KOH concentration, saponification temperature and time were determined on the amount of free lutein from the process.

For the analysis of saponified solution, 50 ml ethanol was added to the saponified product. The mixture was then filtered to isolate insoluble impurities. The resulting clear solution was then analyzed for the amount of free lutein by high performance liquid chromatography (HPLC). Fig.4.4, 4.5, and 4.6 show the

chromatograms of lutein standard, oleoresin before saponification and saponified mixture after saponification respectively. The peak at 9.885 min. in Fig. 4.4 was identified as free lutein, and the other peaks at later retention times were lutein fatty acid esters. Fig.4.6 indicated that conversion of lutein esters into free lutein occurred as a result of saponification.

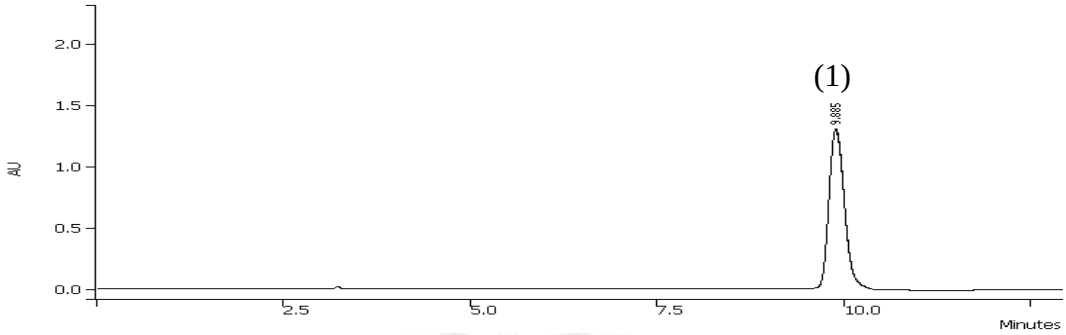


Figure 4.4 Chromatogram of lutein standard, Peaks: (1) lutein

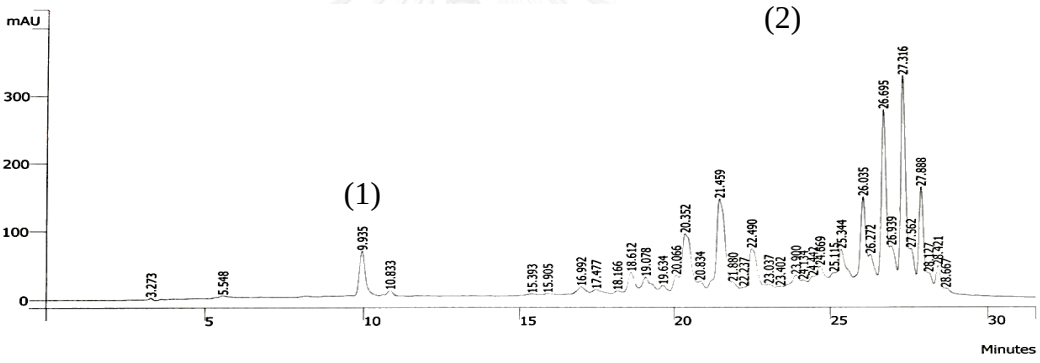


Figure 4.5 Chromatogram of oleoresin before saponification
Peaks: (1) lutein, (2) lutein fatty acid ester

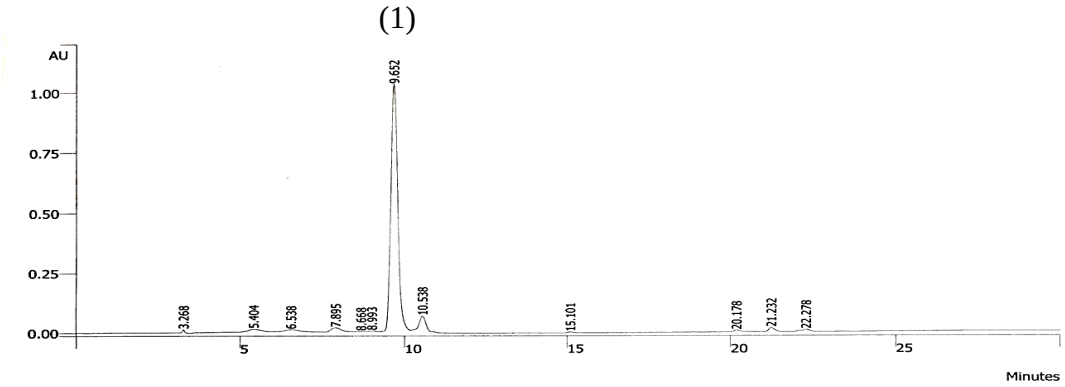


Figure 4.6 Chromatogram of saponified mixture after saponification.
Peaks: (1) lutein

4.3.1 Effect of volume of ethanol to weight of marigold oleoresin ratio

In practice, the use of the smallest volume of ethanol at rather high concentration is preferable as this would minimize the reactor size, and thus make the process more economical. In this set of experiment, The effect of the ratio of volume of ethanol to weight of marigold oleoresin (10:1, 20:1 and 30:1 ml of ethanol to 1 g oleoresin) on saponification efficiency were investigated at the fixed amount of KOH of 0.5 g used in the reaction at 35 °C for 4 h. The results shown in Fig. 4.7 revealed that the highest amount of free lutein (150.79 mg/g oleoresin) was obtained with the ratio of 20:1 ml of ethanol to 1 g oleoresin. The amount of free lutein increased when the ratio of ethanol volume to weight of oleoresin increased from 10:1 to 20:1, but then decreased when the ratio was increased to 30:1. The increase of ethanol volume initially helped improve mass transfer, thus the contact between KOH and oleoresin, which in turn promoted saponification. Nevertheless, the increase of ethanol volume to 30 ml on the other hand, could lower KOH concentration, which thus lowered the rate of reaction. This resulted in incomplete reaction, as evidenced by the presence of lutein esters peaks in the chromatogram shown in Fig. 4.8 (c).

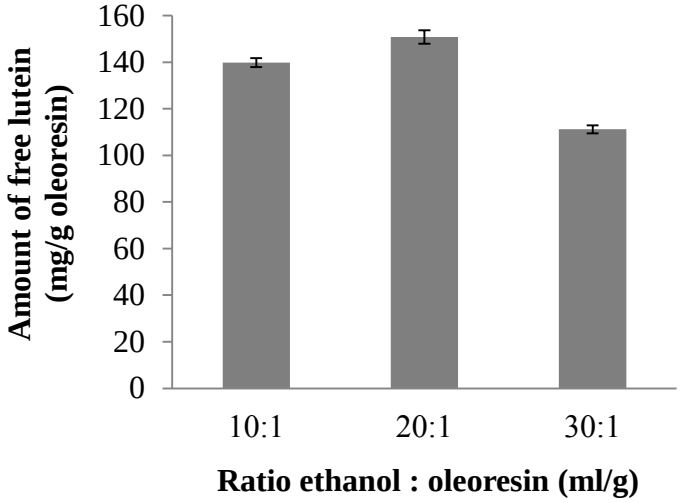


Figure 4.7 Effect of ethanol to oleoresin ratio on amount of free lutein obtained by saponification (KOH 0.5 g, 35^{o}C, 150 rpm and 4 h)

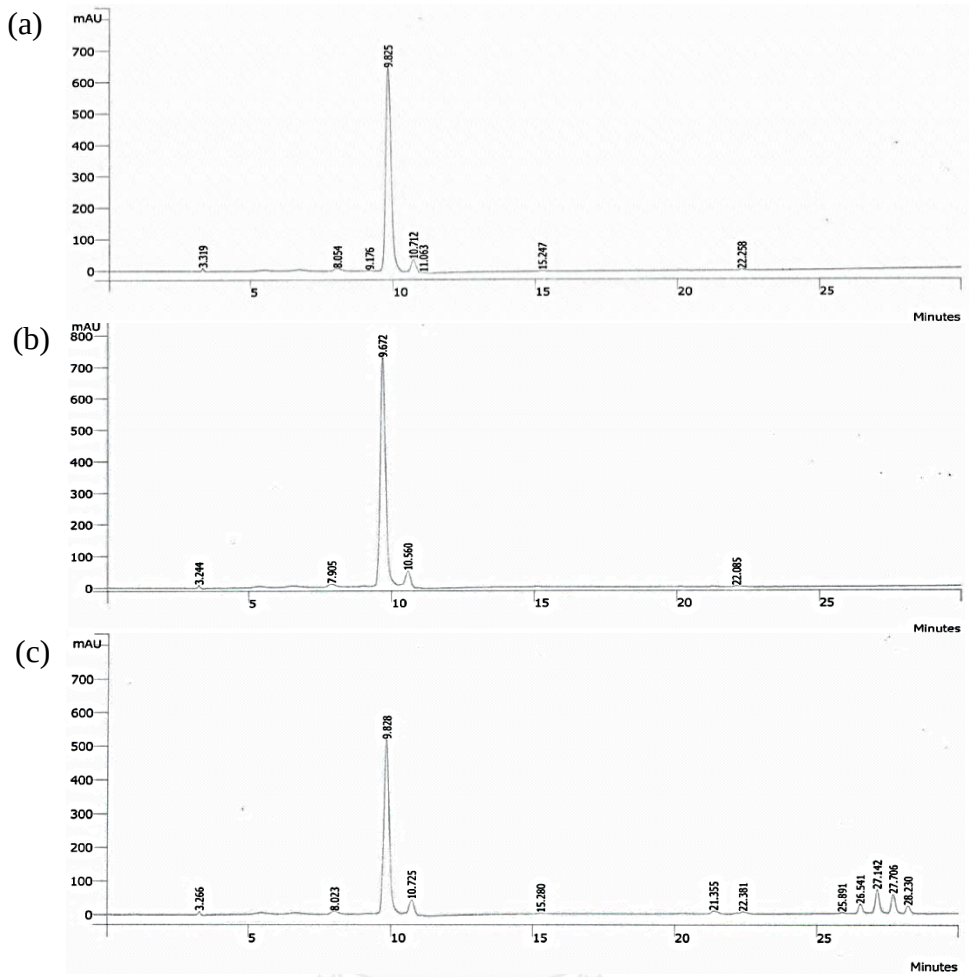


Figure 4.8 Chromatogram of saponified mixture at ratio of ethanol:oleoresin (a) 10:1, (b) 20:1, (c) 30:1 (KOH 0.5 g, 35^{o}C, 150 rpm and 4 h)

4.3.2 Effect of KOH concentration

To complete the saponification, it is generally required that the sufficient amount of alkali be used. Given the suitable ethanol volume to weight of oleoresin ratio of 20:1 determined from previous section, the most suitable concentrations of alkali solution that would yield the highest amount of free lutein was determined. Saponification was conducted in which 1 g of marigold oleoresin was dissolved in 20 ml of KOH solutions in ethanol at various concentrations (1.5, 2.5, 3.5% (w/v)). The reaction was further allowed to take place at 35^{o}C for 4 h. The results indicated that at 2.5%w/v of KOH concentration, the highest amount of free lutein of 150.79

mg/g oleoresin was obtained. As shown in Fig.4.9, the amount of free lutein increased with increasing KOH concentration, nevertheless, at higher concentration of KOH the conversion to free lutein decreased. This was possibly due to the degradation of lutein esters at in strong alkaline environment (high pH).

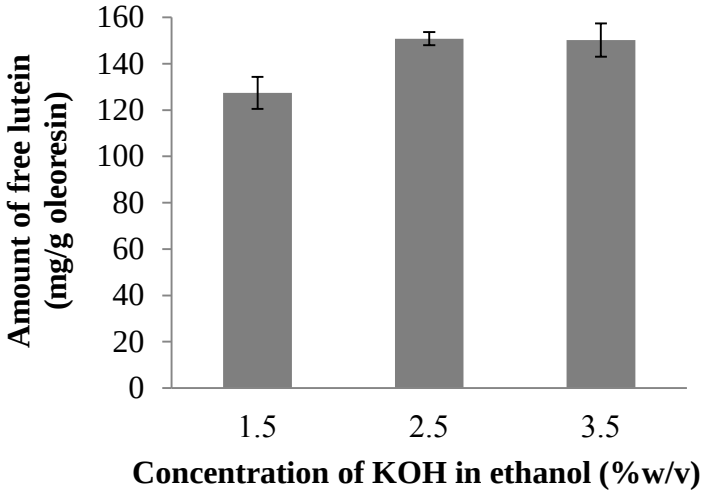


Figure 4.9 Effect of KOH concentration on amount of free lutein obtained by saponification (ethanol to oleoresin ratio 20:1,35^{o}C, 150 rpm and 4 h).

4.3.3 Effect of saponification temperature

Suitable temperature was determined for saponification of marigold oleoresin in 2.5% KOH in ethanol 20 ml. The reaction was allowed to take place at various temperatures 30°C, 35°C and 40°C for 4 h. Fig. 4.10 shows the amount of the free lutein obtained at different saponification temperature. At 30°C, the oleoresin seemed to have not been dissolved completely (Fig.4.11 (a)). However, at 35°C, the oleoresin could completely dissolve (Fig.4.11 (b)), thus increased the KOH and ethanol interaction with the oleoresin. At 40°C, the amount of free lutein obtained only slightly increased, therefore, the most suitable temperature was 35°C.

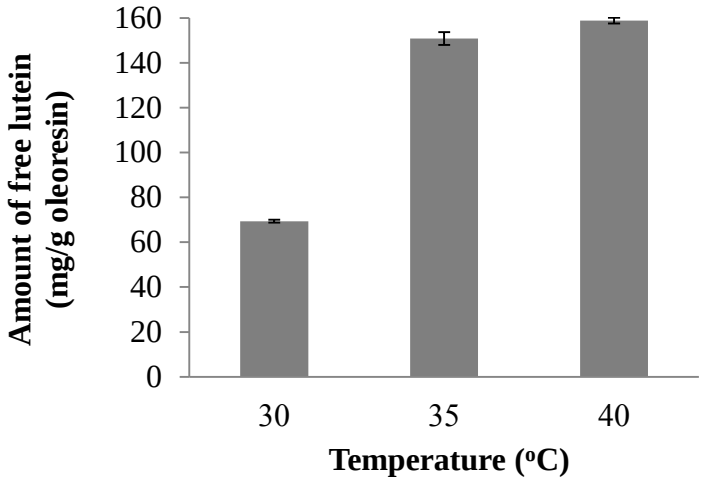


Figure 4.10 Effect of temperature on amount of free lutein obtained by saponification (ethanol to oleoresin ratio 20:1, 2.5% KOH, 150 rpm and 4 h)

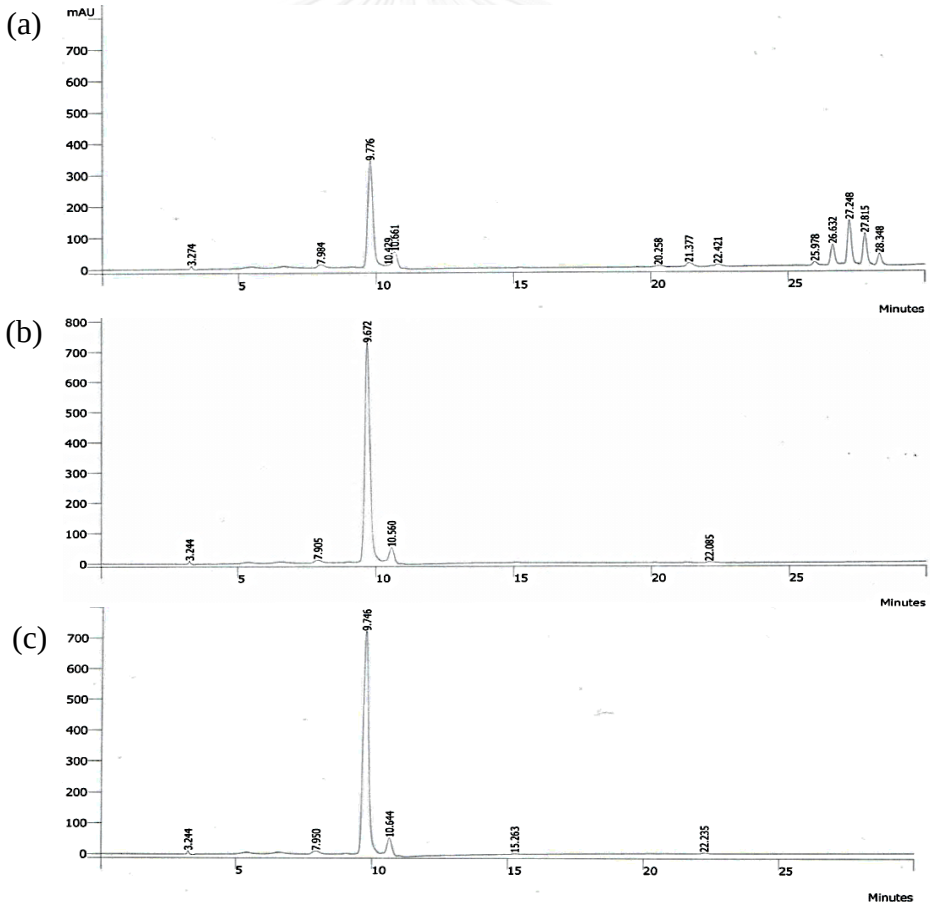


Figure 4.11 Chromatogram of saponified mixture at saponification temperature (a) 30°C, (b) 35°C, (c) 40°C (ethanol to oleoresin ratio 20:1, 2.5% KOH, 150 rpm and 4 h)

4.3.4 Effect of saponification time

The experiment was conducted to determine the effect of reaction time (0.5 h, 2 h and 4 h) on saponification of 1 g of marigold oleoresin in 2.5%KOH in ethanol 20 ml at 35°C. The content of free lutein increased with increasing saponification time with the highest (150.79 mg/g oleoresin) obtained after 4 h of saponification was achieved after (Fig 4.12). This result was supported by the chromatograms shown in Fig 4.13, revealing that the conversions of lutein esters to free lutein were not complete after 0.5 and 2 h, but was so after 4 h.

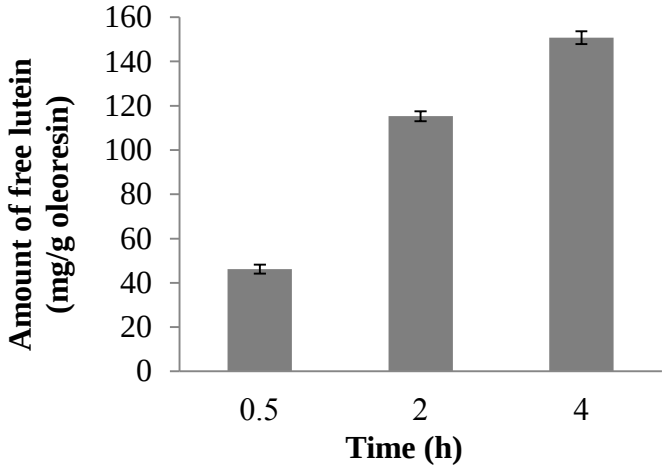
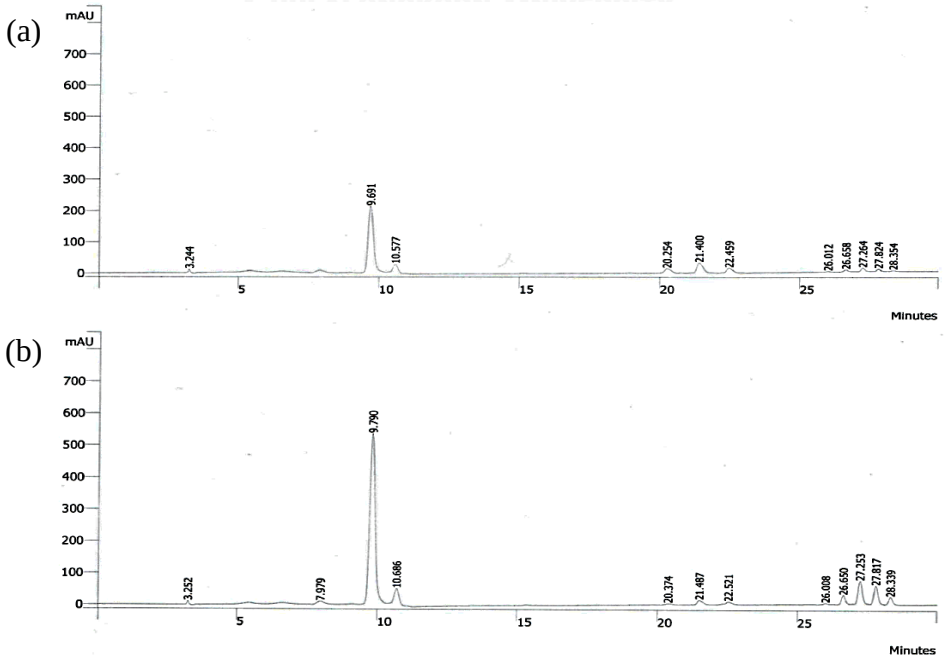


Figure 4.12 Effect of time on amount of free lutein obtained by saponification (ethanol to oleoresin ratio 20:1, 2.5% KOH, 35^{o}C and 150 rpm)



(c)

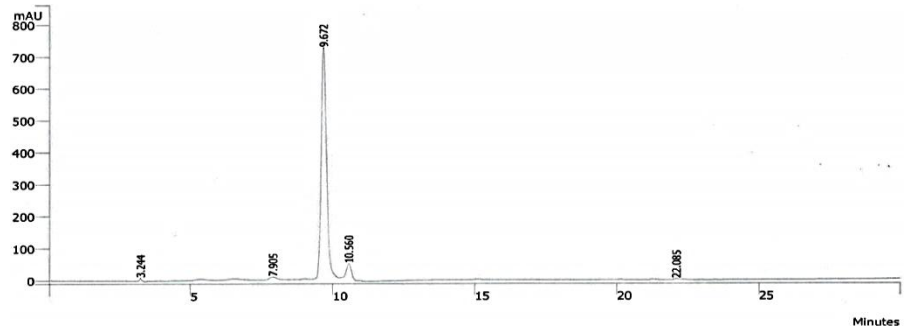


Figure 4.13 Chromatogram of saponified mixture at saponification time (a) 0.5 h., (b) 2 h., (c) 4 h (ethanol to oleoresin ratio 20:1, 2.5% KOH, 35^{o}C and 150 rpm)

4.4 Simultaneous DME extraction and saponification of lutein fatty acid esters to free lutein

Suitable conditions for DME extraction and saponification to convert lutein esters in marigold oleoresin to free lutein were determined in Part I and Part II. In this part (Part III), the possibility of simultaneous DME extraction and saponification of lutein esters to obtain free lutein will be investigated. The effects of the following factors: volume of ethanol to weight of dried marigold ratio (5:0.5-20:0.5 ml/g), KOH concentration (3%-7%w/v), and treatment time (0.5-2 h.) on free lutein yield were determined. The results will be compared with that obtained with sequential process of DME extraction, followed by saponification of oleoresin.

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4.4.1 Effect of volume of ethanol to weight of dried marigold ratio

The results in Fig. 4.14 showed that the effects of volume of ethanol to weight of dried marigold ratio affect the extraction and reaction for the production of free lutein. The experiments begin with using dried marigold 0.5 g and fixed amount of KOH 0.5 g at 35^{o}C, 400 rpm for 30 min. and the solvent to sample ratio 33:0.5. At the ethanol to dried marigold ratio of 5:0.5, only a small amount of free lutein was obtained, due to the incomplete saponification reaction as shown in the chromatogram in Fig. 4.15 (a). At ratio 10:0.5 and 15:0.5 (Fig 4.15 (b), (c)) higher amounts of free lutein were achieved as a result of complete saponification reaction (18.96 mg/g dried marigold), as ethanol acted as a carrier for solvent-solute interaction. The ratio of

10:0.5 was found to be most suitable ratio since it was able to achieve comparable amount of free lutein to that obtained a the ratio 15:0.5.

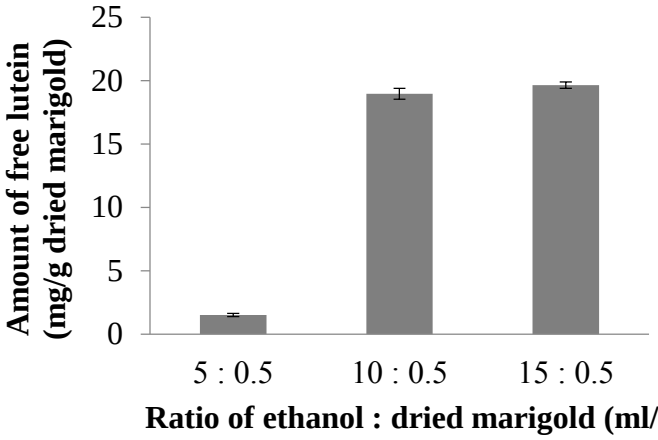

Figure 4.14 Effect of ethanol to dried marigold ratio on amount of free lutein obtained by simultaneous process (KOH 0.5 g, 35^{o}C, 400 rpm and 30 min)

(a)

(b)

(c)

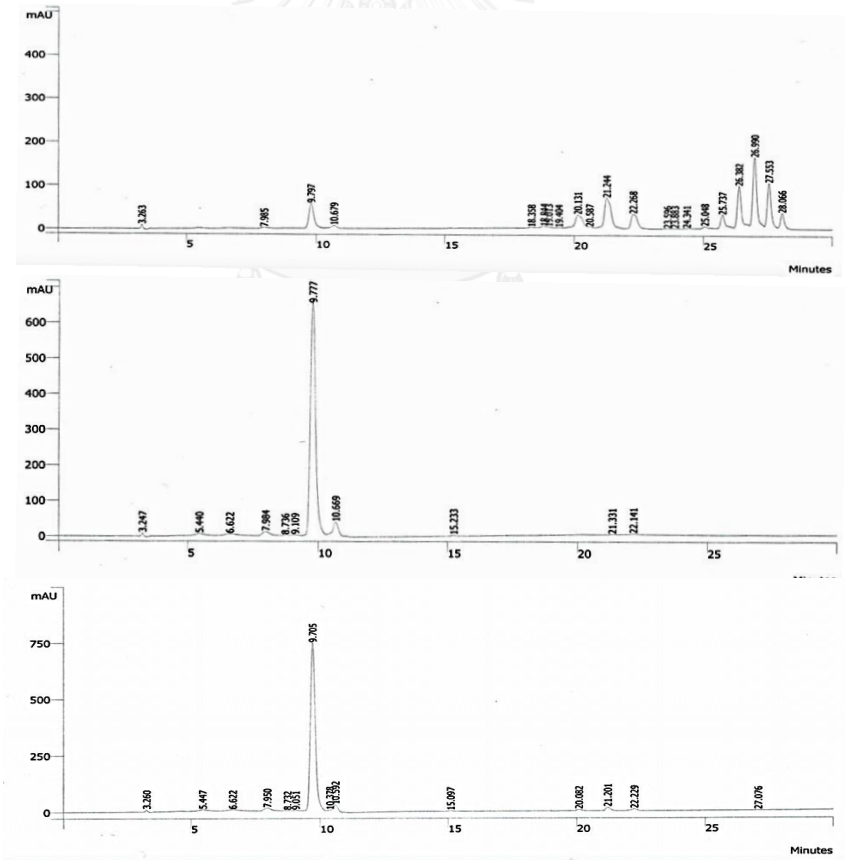

Figure 4.15 Chromatogram of simultaneous DME extraction and saponification of lutein esters to free lutein at ratio of ethanol:dried marigold
(a) 5 : 0.5, (b) 10 : 0.5, (c) 15 : 0.5 (KOH 0.5 g, 35^{o}C, 400 rpm and 30 min.)

4.4.2 Effect of KOH concentration

The effect of concentration of KOH in ethanol (3%, 5% and 7%w/v) at 35^{o}C, 400 rpm for 30 min is shown in Fig. 4.16. With the increase in KOH concentration from 3% to 5% but at 7%, the concentration of KOH decline. Although, the increase of KOH concentration was desirable for higher free lutein would be produced. Nevertheless, lutein was unstable in strong alkaline environment, so amount of free lutein decreased at high concentration of KOH. The results showed that the maximum amount of free lutein was obtained at the KOH concentration of 5%w/v (18.96 mg/g dried marigold), and it was chosen for further study.

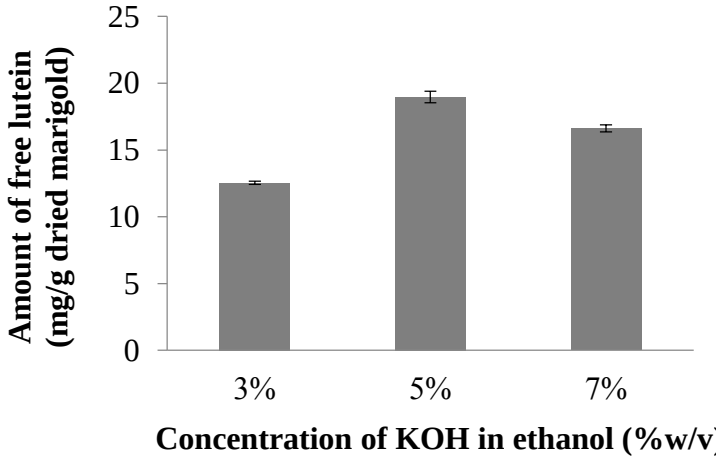

Figure 4.16 Effect of KOH concentration on amount of free lutein obtained by simultaneous process
(ethanol to dried marigold ratio 10:0.5, 35^{o}C, 400 rpm and 30 min)

4.4.3 Effect of mixing time

From previous experiment, we obtained the optimal KOH concentration at 5% KOH in ethanol 10 ml. Here, in this section, the effect of mixing time was determined with a fixed temperature at 35^{o}C and agitation rate of 400 rpm. The results in Fig. 4.17 indicated a slight increase in free lutein yield when the mixing time increased from 0.5 h. to 1 h, but stayed relatively constant with further increase of mixing time from 1 h to 2 h. Therefore, 1 h would be the most suitable time for the simultaneous process, yielding approximately 20.71 mg free lutein /g dried marigold.

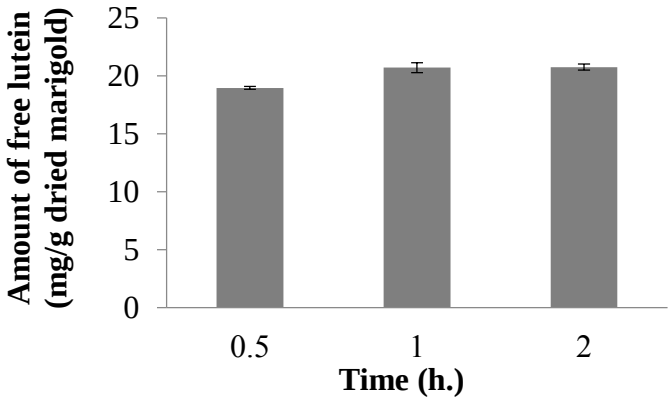

Figure 4.17 Effect of time on amount of free lutein obtained by simultaneous process (ethanol to dried marigold ratio 10:0.5, 5% KOH, 35^{o}C and 400 rpm)

4.4.4 Comparison of simultaneous DME extraction and saponification of dried sample with simultaneous DME extraction and saponification of wet sample

Table 4.3 summarizes the yields of free lutein from dried and wet marigold flower by simultaneous DME extraction and saponification. The results indicated that both the dried and the wet sample gave comparable yields (20.71 mg/g dried marigold and 19.22 mg/g dried marigold respectively). The slightly lower yield from wet sample was probably a result of the high polarity of water in the wet sample, which reduces the overall polarity solvent and thus its ability to extract the sample. The small difference however makes the wet DME extraction process rather attractive since cost of drying marigold flowers can be considerably saved.

Table 4.3 Comparison of free lutein yield obtained by simultaneous DME extraction and saponification of dried and wet marigold flowers

Method	Conditions	Amount of free lutein (mg/g dried marigold)
Simultaneous process / using dried sample	DME to dried marigold ratio 33:0.5, ethanol to dried marigold ratio 10:0.5, 5% KOH, 35^{o}C, 400 rpm and 1 h.	20.71
Simultaneous process / using wet sample		19.22

4.5 Comparison of simultaneous and sequential DME extraction and saponification process

The yield of free lutein from simultaneous and sequential DME extraction and saponification processes, each carried out at the most suitable conditions determined from the previous section were compared. For the simultaneous process is carried out in a DME extraction apparatus with 0.5 g of dried marigold flowers with the solvent to sample ratio 33:0.5, ethanol to sample ratio 10:0.5, 5% KOH concentration, at 35^{o}C, 400 rpm for 1 h. The sequential process consisted of DME extraction carried out with using solvent to sample ratio 33:0.5, 35^{o}C, 400 rpm for 30 min, followed by saponification of the resulting oleoresin at the ratio of ethanol to oleoresin of 20:1, 2.5% KOH concentration, 35^{o}C for 4 h, the most suitable conditions previously determined (Section 4.3) that begin with using an oleoresin 1 g but the yield of oleoresin derived from DME extraction was approximately 0.2 g/0.5 g of dried marigold flowers. Therefore, the conditions of saponification process must be scale down from the ratio of ethanol to oleoresin of 20:1 to 4:0.2. The results are summarized in Table 4.4, which showed that the content of free lutein in the simultaneous process was higher than that of the sequential process (20.71 versus 16.72 mg/g dried marigold). As from chromatogram Fig. 4.18 showed that both of simultaneous process and sequential process completed saponification reaction occur to convert to free lutein. Also, simultaneous process does not only require fewer steps, but will improve the overall rate of extraction. Since free lutein is more easily soluble in DME, putting the two processes together, having the esters convert to free lutein will enhance the overall extraction rate that lead to obtain free lutein more than sequential process 20% due to some of the lutein esters that still remain and can be re-extracted again which obtained lutein esters 2.20 mg/g dried marigold (about 10%more) and the rest were loss during the process as per several steps. In simultaneous process, for re-extraction obtained lutein esters 0.91 mg/g dried marigold. This showed that simultaneous process, lutein could be extracted from the samples almost completely. Moreover, the simultaneous process should reveal the need for shorter process time.

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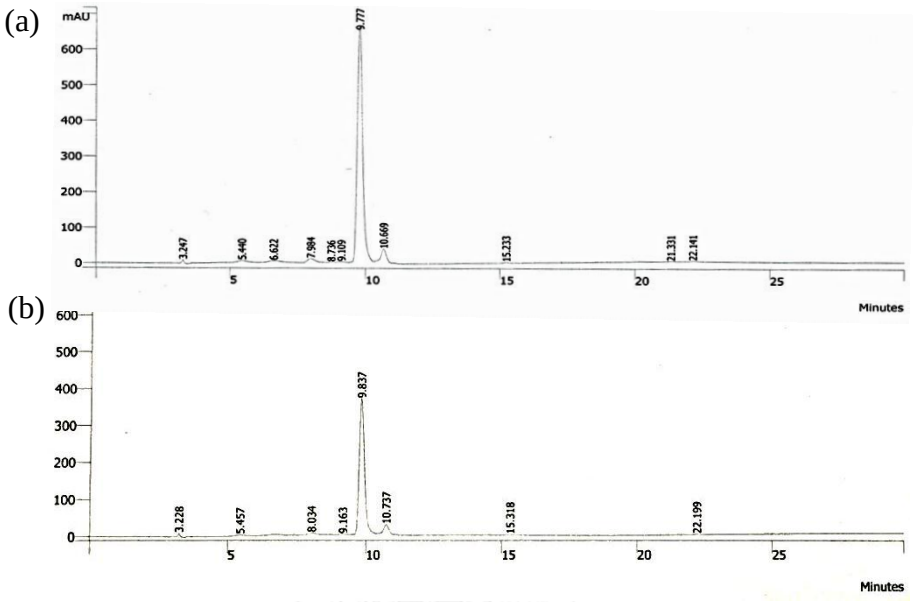

Figure 4.18 Chromatogram of (a) simultaneous process (ethanol to dried marigold ratio 10:0.5, 5% KOH, 35^{o}C and 1 h), (b) sequential process (ethanol to oleoresin ratio 4:0.2, 2.5% KOH, 35^{o}C and 4 h)

Table 4.4 Comparing amount of free lutein obtained by simultaneous process and sequential process

Method	Conditions	Amount of free lutein (mg/g dried marigold)
Simultaneous process / using dried sample	DME to dried marigold ratio 33:0.5, ethanol to dried marigold ratio 10:0.5, 5% KOH, 35^{o}C, 400 rpm and 1 h.	20.71
Sequential process	DME extraction; DME to dried marigold ratio 33:0.5, 35^{o}C, 400 rpm, 30 min. Saponification; ethanol to oleoresin ratio 4:0.2, 2.5% KOH, 35^{o}C, 150 rpm, 4 h.	16.72

CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The total amount of lutein esters in dried marigold flowers, reported in terms of total lutein esters, was approximately 23.32 mg/g dried marigold from solvent extraction using hexane at 40°C for 4 h and repeated at 30°C for 10 days.

2. Part I, The suitable condition for DME extraction was solvent to sample ratio 33:0.5, 35°C, 400 rpm for 30 min. The highest lutein esters content is 20.65 mg/g dried marigold. Therefore, the lutein esters content obtained by DME extraction under the optimal condition studied was found to be higher than that obtained by solvent extraction using hexane and by SC-CO_2 extraction under each of the optimal conditions. Solvent extraction obtained lutein esters 17.58 mg/g dried marigold when extracted with using 50 ml hexane at 40^{o}C for 4 h. SC-CO_2 extraction obtained lutein esters 15.91 mg lutein esters /g dried marigold, at 60^{o}C, 40 MPa for 4 h. Then, compared with DME extraction using wet sample with 80% moisture content obtained lutein esters 17.89 mg/g dried marigold and with 70% moisture content obtained lutein esters 17.71 mg/g dried marigold, which found less than the DME extraction using dried sample (0% moisture content).

3. Part II, The suitable condition for saponification of free lutein from marigold oleoresin was found. For 1 g of oleoresin, at ratio of ethanol to oleoresin 20:1, 2.5% KOH concentration, 35 °C for 4 h. at such conditions, approximately 150 mg free lutein /g oleoresin was obtained.

4. Part III, The suitable condition for simultaneous DME extraction and saponification of lutein esters to free lutein was found. For 0.5 g of dried

marigold flowers, at ratio of ethanol to dried marigold 10:0.5, 5% KOH concentration, 35 °C for 1 h. at such conditions, approximately 20.71 mg free lutein /g dried marigold was obtained. Moreover, comparison of simultaneous DME extraction and saponification with sequential DME extraction and saponification for free lutein is 16.65 mg free lutein /g dried marigold. Also compared with simultaneous DME extraction and saponification using wet sample obtained the amount of free lutein is 19.22 mg free lutein /g dried marigold.

5.2 Recommendations

1. The optimizing DME extraction process, as this study was batch process that should be developed to continuous process to ease the process of feed DME into reactor and through the separation unit. In order to obtain economic value in the future, it is feasible to use scale up process to further develop pilot plant.
2. The free lutein could be obtained by simultaneous DME extraction and saponification. Investigating the purification of free lutein should be studied for the commercial production.
3. The application of lutein as medical products should be essentially studied for information about the compound structures, activities and also toxicity.
4. The determination of lutein solubility of DME should be study more from calculation or experiment to be as basic data.

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APPENDICES

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APPENDIX A
EXPERIMENTAL AND DATA ANALYSIS

A-1 JECFA ; Lutein esters from Tagetes Erecta

Equation A-1 Determine the total content of carotenoid esters

Calculation: Total carotenoid ester content (% w/w) = $\frac{Abs \ x \ d \ x \ 100}{A^{1\%}_{isobestic} \ x \ W}$

Where: Measure absorbance of the sample at 428 nm

Abs = measured absorbance, d = dilution factor

$A^{1\%}_{isobestic}$ (specific absorbance of lutein ester at the wavelength of the isobestic point) = 898 , W = weight of sample (g)

A-2 Method of determination of moisture content

Equation A-2

Calculation: $\%W = \frac{A-B}{B} x \ 100$

Where: %W = percentage of moisture in the sample,

A = weight of wet sample (g)

B = weight of dried sample (g)

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A-3 HPLC analysis of standard lutein

Table A-3 Standard calibration data of lutein

Concentration of lutein (mg/ml)	Peak area
0	0
0.005	19514630
0.0625	54401204
0.125	165841064
0.25	316505248
0.5	515303680

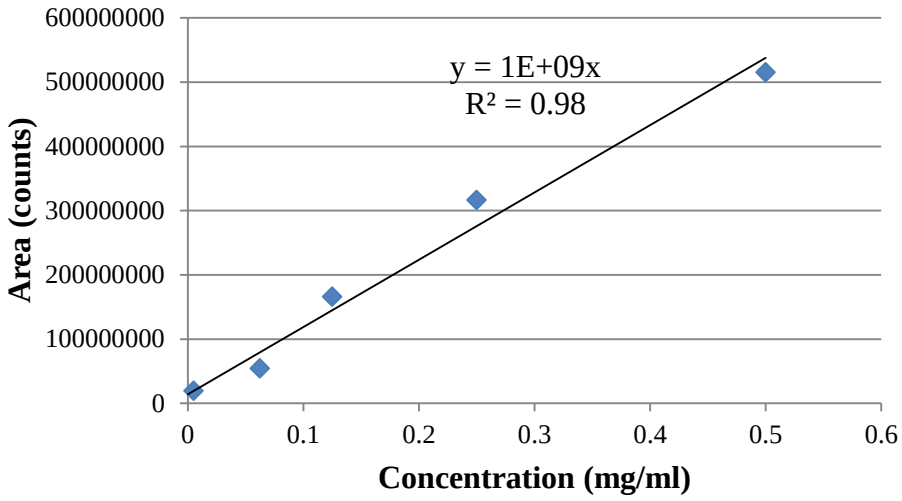

Figure A-3 Standard calibration curve for HPLC analysis of lutein

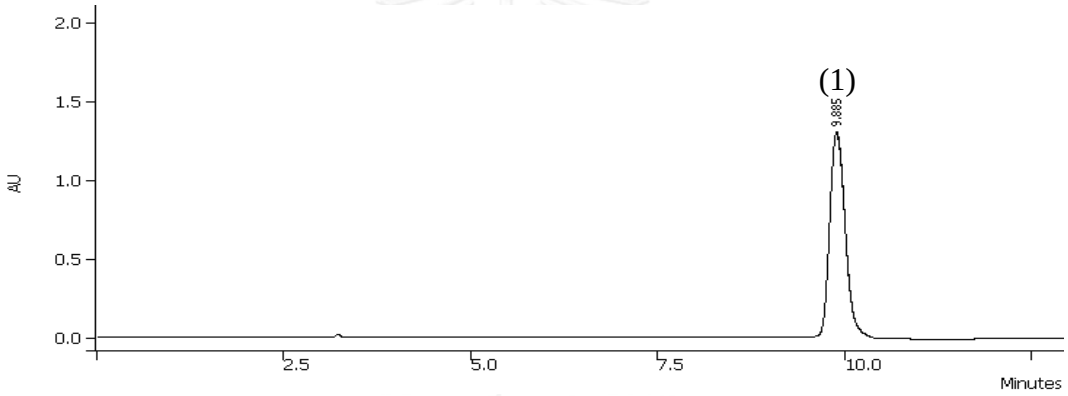

Figure A-4 Chromatogram of lutein standard, Peak: (1) lutein

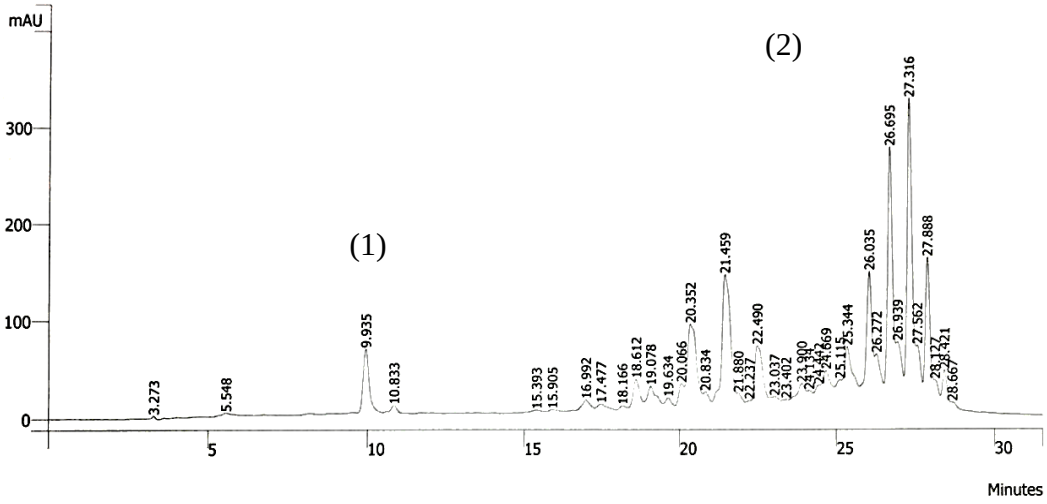

Figure A-5 Chromatogram of marigold extract before saponification

Peaks: (1) lutein, (2) lutein fatty acid ester

(1)

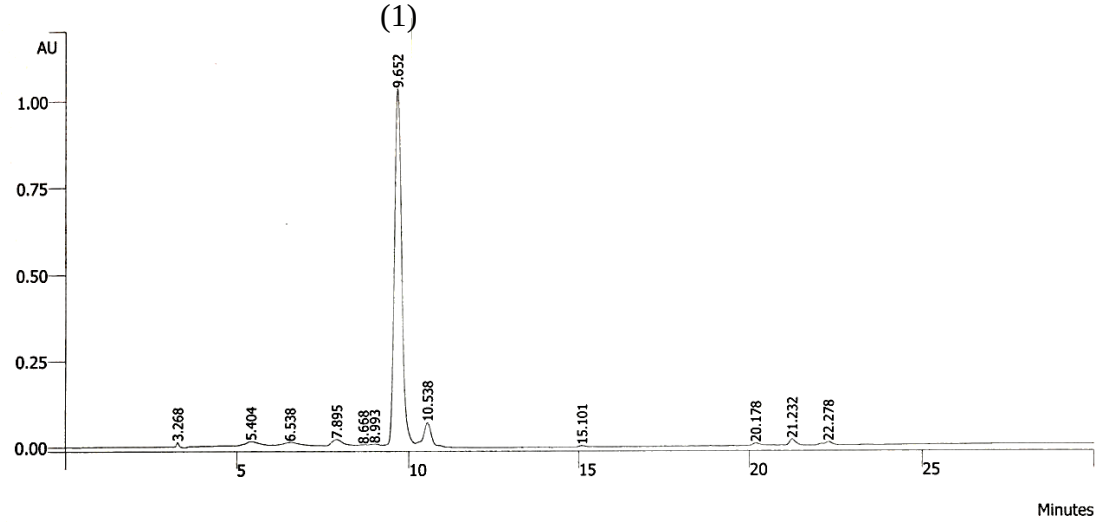

Figure A-6 Chromatogram of marigold extract after saponification
Peaks: (1) lutein

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APPENDIX B
EXPERIMENTAL DATA

B-1 Experimental data of total lutein esters in marigold flowers

Table B-1 Total lutein esters content in marigold flowers

Run	Time/Temperature	Total lutein esters (mg/g dried marigold)				% Total lutein esters
		Exp.1	Exp.2	Average	SD	
1	4 h./ 40^{o}C	17.39	17.76	17.58	0.26	75.39
2	1 day/ 30^{o}C	4.07	4.52	4.29	0.32	18.42
3	2 day/ 30^{o}C	1.16	1.18	1.17	0.01	5.01
4	7 day/ 30^{o}C	0.30	0.25	0.28	0.03	1.19
sum		22.92	23.71	23.32	0.63	100

B-2 Experimental data for the study of suitable DME extraction

Table B-2.1 Effects of solvent to sample ratio (w/w) on DME extraction

Solvent to sample ratio (w/w)	Total lutein esters (mg/g dried marigold)			
	Exp 1	Exp 2	Average	SD
20 : 0.5	16.36	15.50	15.93	0.61
26 : 0.5	17.25	16.68	16.96	0.40
33 : 0.5	20.35	20.95	20.65	0.43
40 : 0.5	19.99	19.06	19.53	0.66

Table B-2.2 Effects of extraction temperature on DME extraction

Temperature (^{o}C)	Total lutein esters (mg/g dried marigold)			
	Exp 1	Exp 2	Average	SD
25	19.81	19.03	19.42	0.55
30	19.31	20.60	19.96	0.91
35	20.35	20.95	20.65	0.43
40	16.43	18.78	17.60	1.66

Table B-2.3 Comparisons of DME extraction with supercritical carbon dioxide ($SC\text{-}CO_2$) extraction and solvent extraction with hexane

Solvent	Total lutein esters (mg/g dried marigold)			
	Exp 1	Exp 2	Average	SD
DME	20.35	20.95	20.65	0.43
Hexane	17.39	17.76	17.58	0.26
$SC\text{-}CO_2$	16.64	15.18	15.91	1.03

Table B-2.4 Comparisons of lutein esters content of marigold with wet extraction and dry extraction base on optimal condition of DME extraction

% Moisture content	Total lutein esters (mg/g dried marigold)			
	Exp 1	Exp 2	Average	SD
0%	20.35	20.95	20.65	0.43
70%	17.28	18.14	17.71	0.61
80%	19.17	16.61	17.89	1.81

B-3 Experimental data for the study of suitable saponification

Table B-3.1 Effects of volume of ethanol to weight of marigold oleoresin ratio

ethanol : oleoresin Ratio (ml/g)	Free lutein (mg/ml)		Free lutein (mg/g oleoresin)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
10 : 1	0.087	0.088	138.51	141.19	139.85	1.90
20 : 1	0.093	0.096	148.76	152.84	150.80	2.89
30 : 1	0.069	0.070	109.99	112.37	111.18	1.68

Table B-3.2 Effects of KOH concentration

Concentration of KOH in ethanol (%w/v)	Free lutein (mg/ml)		Free lutein (mg/g oleoresin)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
1.5	0.083	0.070	132.29	112.48	122.38	14.00
2.5	0.093	0.096	148.76	152.84	150.80	2.89
3.5	0.097	0.091	155.30	145.10	150.20	7.21

Table B-3.3 Effects of saponification temperature

Temperature (^{o}C)	Free lutein (mg/ml)		Free lutein (mg/g oleoresin)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
30	0.043	0.044	68.95	69.80	69.37	0.60
35	0.093	0.096	148.76	152.84	150.80	2.89
40	0.100	0.099	159.69	157.87	158.78	1.28

Table B-3.4 Effects of saponification time

Time (h)	Free lutein (mg/ml)		Free lutein (mg/g oleoresin)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
0.5	0.028	0.030	44.70	47.62	46.16	0.339
2	0.071	0.073	113.71	116.85	115.28	0.175
4	0.093	0.096	148.76	152.84	150.80	2.89

B-4 Experimental data for the study of suitable condition for simultaneous extraction and saponification

Table B-4.1 Effects of volume of ethanol to weight of dried marigold ratio

ethanol:dried marigold Ratio (ml/g)	Free lutein (mg/ml)		Free lutein (mg/g dried marigold)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
5 : 0.5	0.008	0.007	1.54	1.49	1.51	0.04
10 : 0.5	0.093	0.096	18.66	19.27	18.96	0.43
15 : 0.5	0.097	0.099	19.47	19.83	19.65	0.26

Table B-4.2 Effects of KOH concentration

Concentration of KOH in ethanol (%w/v)	Free lutein (mg/ml)		Free lutein (mg/g dried marigold)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
3	0.068	0.057	13.64	11.46	12.55	1.54
5	0.093	0.096	18.66	19.27	18.96	0.43
7	0.088	0.079	17.50	15.73	16.61	1.26

Table B-4.3 Effects of saponification temperature

Time (h)	Free lutein (mg/ml)		Free lutein (mg/g dried marigold)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
0.5	0.093	0.096	18.66	19.27	18.96	0.43
1	0.103	0.104	20.63	20.80	20.71	0.12
2	0.103	0.105	20.58	20.93	20.76	0.25

Table B-4.4 Comparisons of simultaneous DME extraction and saponification with sequential DME extraction and saponification

Process	Free lutein (mg/ml)		Free lutein (mg/g dried marigold)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
Simultaneous	0.103	0.104	20.63	20.80	20.71	0.12
Sequential	0.084	0.083	16.72	16.57	16.65	0.11

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Table B-4.5 Comparisons of simultaneous DME extraction and saponification of dry sample with simultaneous DME extraction and saponification of wet sample

Process	Free lutein (mg/ml)		Free lutein (mg/g dried marigold)			
	Exp 1	Exp 2	Exp 1	Exp 2	Average	SD
Dry process	0.103	0.104	20.63	20.80	20.71	0.12
Wet process	0.097	0.095	19.35	19.09	19.22	0.18

APPENDIX C

The Pure and Applied Chemistry International Conference 2016

(PACCON 2016)

February 9-11, 2016

at Bangkok International Trade and Exhibition Centre (BITEC)

Bangkok, Thailand

Extraction of Lutein Fatty Acid Esters from Marigold Flower using Liquefied Dimethyl Ether (DME) as extractant

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Abstract:

Marigold flower is a rich source of antioxidant compound called lutein. It shows high antioxidant activity and has many beneficial effects to human such as reducing the failure of the eyesight, coronary heart disease and cancer. In this study, extraction of lutein fatty acid esters from marigold flower was carried out by using a green solvent, namely liquefied dimethyl ether (DME). Since it is gaseous at normal temperature and pressure, DME can be easily removed from the final product by depressurization. The solvent-free product therefore can be obtained without additional energy intensive solvent evaporation step. This extraction method is suitable for heat sensitive compounds including lutein owing to the mild operating conditions (moderate pressure and low temperature). The main objective of this study is to determine the effects of extraction conditions including solvent to sample ratio (w/w) and extraction temperature on extracted yield of lutein fatty acid esters. The highest amount of extracted lutein fatty acid esters was found to be 20.65 mg/g of dry sample at the condition of 33:0.5 solvent to sample ratio, 35^{o}C at fixed stirred rate and time at 400 rpm and 30 min. The results were compared with solvent extraction with hexane and with supercritical carbon dioxide extraction.

1. Introduction

Marigold flower (*Tagetes erecta*) which is used to make garlands for Buddhist ceremonies has become a very interesting source of lutein. Lutein is an important compound used as a food colorant in livestocks or for medicinal purposes[1]. In human, it is an antioxidant known to prevent aged-related macular degeneration[2]. Lutein can be found in various sources such as vegetables, fruits and micro-algae[3]. However, marigold is one of the most interesting sources of lutein due to the high lutein content and its low price.

Lutein extracted from natural sources including marigold is widely used in pharmaceutical and nutraceutical applications. However, lutein is present in nature as lutein esters form, which must be further converted into free lutein by saponification[4]. The extraction of lutein esters from marigold can be achieved by solvent extraction which is generally hexane or supercritical carbon dioxide (SC-CO_2) extraction[5]. However, the toxicity of organic solvents is concerned and SC-CO_2 extraction requires high operating pressure.

Alternatively, liquefied dimethyl ether (DME) is a safe extractant for food or daily ingredients. The animal and human data on

inhalation exposure to dimethyl ether indicates a very low degree of toxicity. DME exists as a gas at temperatures above -24^{o}C[6] but DME is prior compressed to produce liquefied DME to use as extractant and for the easy transportation. Because of its low boiling point, the separation of solvent residue from final product can be easily achieved by depressurization. The lower vapor pressure of DME compared with CO_2 allows the lower operating pressure which might cause less equipment cost. The extraction of lutein esters from marigold using liquefied DME as extractant is examined in this study. The effects of extraction conditions including solvent to sample ratio (w/w) and extraction temperature on the extracted yield of lutein fatty acid esters were investigated. The total amount of lutein esters obtained by the most suitable DME extraction condition was compared to those obtained by hexane and SC-CO_2 extractions. The extracted lutein esters from marigold were further converted to free lutein by saponification.

2. Materials and Methods

2.1 Materials and chemicals

Dried marigold flowers in the powder form were obtained from PTT Chemical (Rayong, Thailand). Liquefied dimethyl ether (DME) used for extraction was obtained from Siam Tamiya Co., Ltd., Thailand. For solvent extraction of lutein esters, hexane (purity>99.5%) was used and was supplied by Sigma-Aldrich. Ethanol (purity>99.5%), potassium hydroxide and sodium sulfate used for saponification were purchased from Merck, USA. Diethyl ether was supplied by Merck, Thailand. Lutein standards used in HPLC experiment (analytical grade) were purchased from Sigma-Aldrich, Germany.

2.2 DME extraction

The schematic of DME extraction is shown in Fig.1. 0.5 Grams of dry marigold was put into a 80 ml hyper-glass along with a 8 mm diameter magnetic bar. The hyper-glass was then assembled with a pressure-resistant polycarbonate extractor. Liquefied DME was filled into the extractor at required solvent to sample ratio. The extraction was carried out for 30 minutes at controlled extraction temperature. After extraction, the extractor was connected to the separation unit which is the same apparatus with the extractor. The extracts were allowed to flow through membrane filter (0.65 µm pore diameter) to separate the solid marigold residue. The DME was then evaporated by depressurizing the separation unit. The remained sample called oleoresin was then dilute with hexane and analyzed the amount of lutein esters by spectrophotometer (Thermo Spectronic model 4001/4, USA).

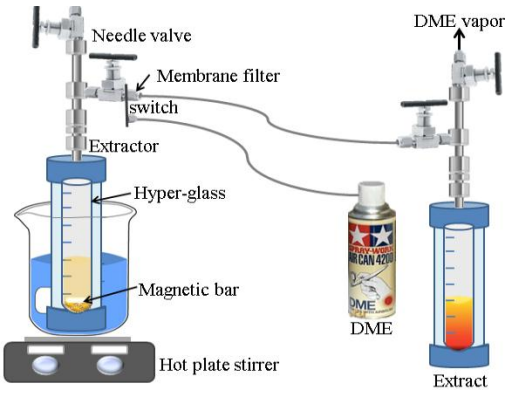

Figure1. DME extraction apparatus

2.3 Solvent Extraction

10 grams of dried marigold was extracted by 50 ml of hexane at 40^{o}C

for 4 h in Digital Incubator Shaker (New Brunswick Scientific Co., Inc., innova 4000, USA). After extraction, the extract was filtered and evaporated to remove the solid marigold residue and hexane, respectively. The obtained oleoresin was then diluted by hexane prior to the quantification of the amount of lutein esters by a spectrophotometer.

2.4 SC-CO_2 Extraction

Dried marigold 0.5 gram was placed into a 10 ml extraction vessel. To distribute the sample throughout the extraction chamber, the vessel was filled with silica sand. The extraction was carried out at 60^{o}C, 40 MPa for 4 h which is the best condition based on the results from our previous study [5]. The extract was collected in a sample vial containing hexane trap (wrapped with aluminum foil). After removal of hexane and re-dilution with the exact amount of hexane, the extract was analyzed by spectrophotometer

2.5 Saponification

10 ml of 60% w/v KOH solution (in ethanol) was added into 125 ml flask which contained 1 gram of marigold oleoresin collected from DME extraction. The reaction was performed at 150 rpm and 50^{o}C for 4 h. After the reaction was completed, additional 50 ml of ethanol was added to the saponified mixture. Then, mixture was transferred to a separation funnel where 100 ml of 5%Na_2SO_4 solution (in distilled water) and 80 ml of diethyl ether were loaded. The liquids were mixed and then separated into two phases. The upper phase containing free lutein was collected, while the lower phase which was the water-soluble impurities was discarded. The free lutein rich solution was remained and washed repeatedly with 5%Na_2SO_4 solution until the water phase became colorless[7]. The washed free lutein solution was then stored in a -20^{o}C refrigerator until analysis by High Pressure Liquid Chromatography (Varian Inc., model 410, USA).

2.6 Analytical methods

2.6.1 Analysis of lutein fatty acid esters

The amount of lutein esters in marigold flower extract was determined by measuring spectrophotometric absorbance at 428 nm using the WHO/FAO method[8].

2.6.2 HPLC analysis

The extracted and saponified solutions were analyzed by using HPLC to identify the components of free lutein and lutein fatty acid esters. The reversed phase HPLC analysis was carried out using Lichrocart C-18 column, a Diode Array Detector Module 335 and an automatic injector. The mobile phase was a gradient solvent system of acetonitrile:methanol (9:1,v:v) (A) and ethyl acetate (B), from 0% to 100% of B using a linear gradient injected to over 30 min, at a flow rate of 1 ml/min. The sample injection volume was 20 µl and the detection wavelength was at 450 nm[5].

3. Results & Discussion

3.1 DME extraction

3.1.1 Effect of solvent to sample ratio (w/w) on DME extraction

The effects of DME to dried marigold sample ratio (20:0.5, 26:0.5, 33:0.5 and 40:0.5), were determined at 35^{o}C, 400 rpm for 30 min. The results are shown in Fig.2. The total lutein esters content was found to increase with increasing of solvent to sample

ratio from 20:0.5 to 33:0.5 and slightly decrease at 40:0.5 of solvent to sample ratio. The highest lutein ester observed in this experiment was 20.65 mg/g dry marigold. The solvent to sample ratio at 33:0.5 was therefore selected as the optimum condition and used for the next experiment.

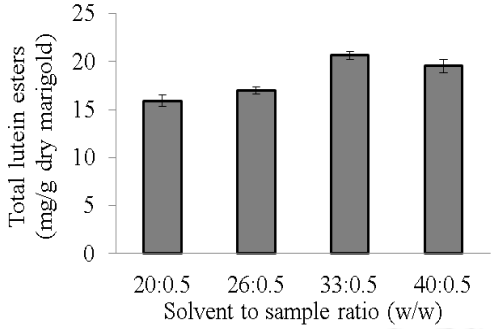

Figure2. Effect of solvent to sample ratio (w/w) on lutein esters extracted at 35°C, 400 rpm for 30 min.

3.1.2 Effect of extraction temperature on DME extraction

The dried marigold flowers were extracted at four temperatures 25°C, 30°C, 35°C and 40°C, with solvent to sample ratio 33:0.5 for 30 min as shown in Fig.3. The lutein esters yield was found to increase with increasing temperature and became highest at 35°C (20.65 mg/g dry marigold).

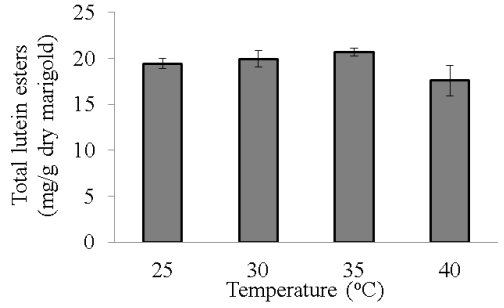

Figure3. Effect of temperature (°C) on lutein esters extracted at 400 rpm for 30 min and solvent to sample ratio 33:0.5

3.2 Comparisons of extraction yields

After suitable conditions for DME extraction were obtained, the highest lutein esters content at 20.65 mg/g dry marigold was compared with solvent extraction using hexane and also with SC-CO_2 extraction. Solvent extraction obtained lutein esters at 17.58 mg/g dry marigold when extracted with 50 ml hexane at 40°C for 4 h. SC-CO_2 extraction obtained lutein esters at 15.91 mg/g dry marigold when extracted at 60°C, 40 MPa for 4 h. as shown in Fig.4.

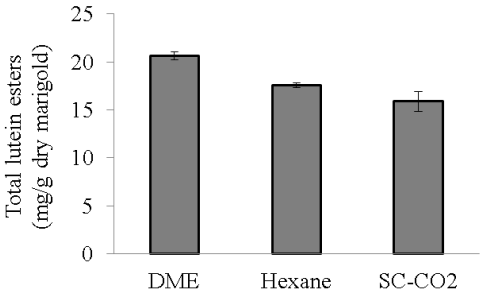

Figure4. Comparing amount of lutein esters obtained with DME extraction, solvent extraction using hexane and SC-CO_2 extraction.

3.3 Saponification of lutein esters extract

The HPLC analysis of extract obtained at the optimum condition (solvent to sample at ratio 33:0.5, 35°C and 400 rpm for 30 min) is shown in Fig.5, which indicates the presence of free lutein and lutein fatty acid esters prior to saponification. The peak at 9 min was identified as free lutein and the other peaks at later retention times were lutein fatty acid esters. Fig.6 presents the chromatogram of the saponified extract, which shows the increase of free lutein peak area, while those of lutein fatty acid esters were decreased, as they were converted to free lutein.

4. Conclusions

The total lutein esters extracted increased with increasing solvent to sample ratio and temperature but results indicated that there existed a limit of extraction conditions as the optimum condition for extraction was obtained at solvent to sample 33:0.5, 35^{o}C at fixed stirred rate and time at 400 rpm and 30 min. At this condition the amount of total lutein esters obtained with DME (21.86 mg/g dry marigold) was higher than that obtained with solvent extraction with 25 ml hexane (17.58 mg/g dry marigold), and with SC-CO_2 extraction at 60^{o}C, 40 MPa for 4 h.(15.91 mg/g dry marigold).

Acknowledgements

The authors greatly appreciate PTT Global Chemical Public Company limited (Rayong, Thailand) for providing the marigold flower sample.

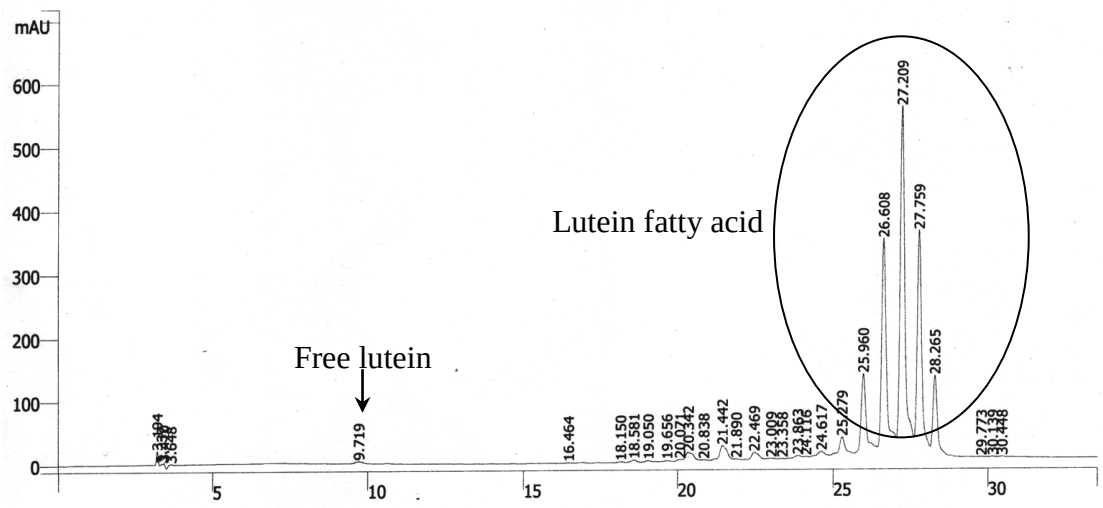

Figure5. Chromatogram of free lutein and lutein fatty acid esters before saponification

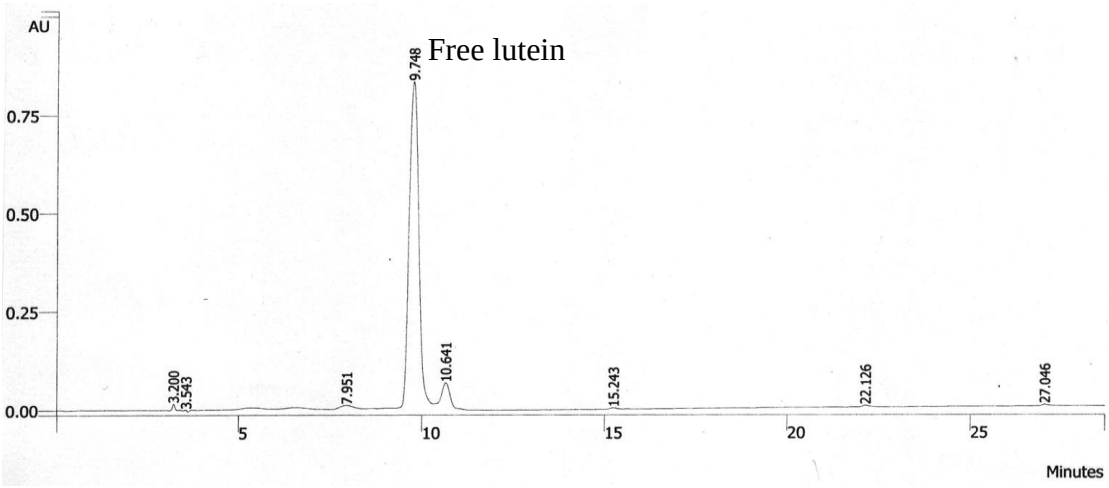

Figure6. Chromatogram of free lutein and lutein fatty acid esters after saponification

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