รายงานวิจัยฉบับสมบูรณ์

โครงการ การสกัดน้ำมันจากเมล็ดต้นยางพาราสำหรับการผลิตไบโอดีเซลโดยใช้ไดเมธิลอีเทอร์เหลว

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เดือน พฤษภาคม ปี 2561

สัญญาเลขที่ MRG5980085

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
มหาวิทยาลัยนเรศวร

Abstract (บทคัดย่อ)

Project Code :
(รหัสโครงการ) MRG5980085

Project Title :
(ชื่อโครงการ) การสกัดน้ำมันจากเมล็ดต้นยางพาราสำหรับการผลิตไบโอดีเซลโดยใช้ไดเมธิลอีเทอร์เหลว

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Project Period :
(ระยะเวลาโครงการ) 2 ปี

Abstract

The objective of this study was to find the optimal conditions for liquefied dimethyl ether (DME) extraction of rubber seed oil (RSO). Response surface methodology (RSM) with the spherical central composite design (CCD) model was employed to determine optimal extraction conditions, including seed moisture content (%wt), solvent to solid weight ratio (g/g), and extraction temperature (^{o}C). The quadratic regression equation suggested the optimal extraction condition to be at a moisture content of 56.4%wt, a solvent to solid ratio of 6.7 (g/g), and a temperature of 33.3^{o}C. At this condition, the RSO yield predicted by the statistical model gave a slight deviation of 1.8% from the experimentally validated result (41.48 versus 42.24%). Having a kinematic viscosity of 36.8 cSt, an acid value of 10.7 KOH/g oil, a fatty acid content of 5.1%, an unsaturated fatty acids content of 80%, the resulting RSO has potential in the production of biodiesel, biolubricants, and biodegradable plastic.

Keywords : rubber seed oil; extraction; dimethyl ether; response surface; optimization

บทคัดย่อ

วัตถุประสงค์ของงานวิจัยนี้คือหาสภาวะที่เหมาะสมของการสกัดน้ำมันเมล็ดยางพาราโดยใช้ไดมิธิลอีเทอร์หลว วิธีการพื้นที่ผิวตอบสนองโดยการใช้แบบจำลองการออกแบบ spherical central composite design (CCD) ถูกนำมาใช้เพื่อหาสภาวะที่เหมาะสม คือ ความชื้นของเมล็ด (ร้อยละโดยน้ำหนัก) อัตราส่วนของตัวทำละลายต่อเมล็ดยางพารา (กรัมต่อกรัม) และ อุณหภูมิในการสกัด (องศาเซลเซียส) สมการที่ได้แนะนำสภาวะการสกัดที่ดีที่สุดคือ ความชื้นเมล็ดยางพาราร้อยละ 56.4 โดยน้ำหนัก อัตราส่วนของตัวทำละลายต่อเมล็ดยางพารา 6.7 (กรัมต่อกรัม) และ อุณหภูมิในการ 33.3 องศาเซลเซียส ที่สภาวะดังกล่าวน้ำมันยางพาราที่ถูกคาดการณ์มีความเบี่ยงเบนไปจากค่าจากการทดลองเล็กน้อยเพียงร้อยละ 1.8 (ร้อยละ 41.48 ต่อ ร้อยละ 42.24). น้ำมันยางพาราที่ได้มีความหนืด 36.8 cSt ค่าความเป็นกรด 10.7 KOH/g oil กรดไขมัน of 5.1% มีกรดไขมันอิ่มตัว 80% ซึ่งบ่งชี้ว่าน้ำมันที่ได้มีความเป็นไปได้ในการนำไปใช้ในการผลิตไบโอดีเซล น้ำมันหล่อลื่น และ ไบโอพลาสติก

(คำหลัก) น้ำมันเมล็ดยางพารา, การสกัด, ไดเมธิลอีเทอร์, พื้นผิวตอบสนอง, การหาค่าเหมาะสมที่สุด

Executive Summary

สรุปงานวิจัยอย่างย่อ

งานวิจัยนี้ได้ศึกษาการสกัดน้ำมันจากเมล็ดยางพาราโดยการใช้ไดเมธิลอีเทอร์เหลวเป็นสารสกัด จากงานวิจัยพบว่าเมล็ดยางพาราเป็นวัตถุดิบที่มีความชื้นสูง หากต้องการสกัดน้ำมันซึ่งมีสภาพความมีขั้วต่ำจะต้องทำการระเหยแห้งเพื่อนำน้ำออกก่อนจะสกัดด้วยตัวทำละลายที่ไม่มีขั้วเช่น เฮกเซน เป็นต้น เนื่องจากเฮกเซนไม่สาสามรถละลายเข้ากันกับน้ำได้จึงมีประสิทธิภาพการสกัดที่ลดลง อย่างไรก็ตาม ไดเมธิลอีเทอร์เหลวสามารถละลายเข้ากันกับน้ำได้บางส่วนจึงเป็นตัวทำละลายที่มีประสิทธิภาพสามารถสกัดเมล็ดยางพาราได้โดยไม่ต้องระเหยน้ำออกทั้งหมด ซึ่งถูกพจารณาว่าเป็นกระบวนการที่ประหยัดพลังงาน คณะผู้วิจัยจึงได้ศึกษาหาสภาวะในการสกัดที่เหมาะสมเพื่อให้ได้ปริมาณน้ำมันที่มีคุณภาพสูงสุด ทั้งนี้ได้ใช้การวิเคราะห์แบบพื้นผิว (response surface methodology) เพื่อหาสภาวะที่เหมาะสมดังกล่าว และเมื่อได้สภาวะที่เหมาะสมจึงการวิเคราะห์ทางสถิติแล้ว จึงทำการทดลองเพื่อตรวจสอบความแม่นยำของการคาดการณ์ของข้อมูล และนำน้ำมันที่สกัดได้ทดสอบคุณสมบัติ พบว่าน้ำมันเมล็ดยางพาราที่สกัดโดยการใช้ไดเมธิลอีเทอร์เหลวเป็นสารสกัดมีความเป็นไปได้ในการนำไปใช้ผลิตไบโอดีเซล ไบโอพลาสติก และ น้ำมันหล่อลื่น โดยรายละเอียดของเนื้อหาของงานวิจัยได้ถูกนำเสนอในรายงานฉบับนี้อย่างครบถ้วน

1. Introduction

Rubber trees (*Hevea brasiliensis*), despite their Amazon rainforest origin, are now grown extensively for natural rubber production in many Asian countries, including Thailand, Indonesia, Malaysia, India, and China. In rainy seasons, each rubber tree produces about 5 kg of fresh rubber seeds, each weighing 3 to 6 grams, and consisting of 42-51% shell and 49-58% kernel. Fresh rubber seeds, especially the kernels, are known to have very high moisture content of approximately 29-50%wt (40-100% in dry basis) [1]. When dried, the rubber seeds have been reported to contain as much as 40-60% oil [2-5]. However, due to the presence of poisonous hydrogen cyanide, rubber seed oil (RSO) is non-edible [6], the seeds are therefore regarded as agricultural waste. Being non-edible oil, nevertheless, makes the RSO a more interesting and economical feedstock for various industrial applications than other edible oils such as palm and coconut oils. Owing to its chemical composition and physico-chemical properties such as kinematic viscosity, flash point and cloud point, RSO has particularly been suggested to be suitable for the production of biodiesel, biolubricants and biodegradable plastic [2-5, 7-9].

To recover oil from rubber seeds, various extraction techniques can possibly be applied. The simplest is the oil extraction by a mechanical press but it is reported to give low RSO yield [10]. Extraction using a non-polar organic solvent such as hexane or petroleum ether gives higher oil yield [11], however it requires energy intensive steps for the removal of toxic solvent (i.e. by evaporation). Alternatively, extraction with supercritical carbon dioxide (SC-CO_2) allows the solvent to be separated readily, by depressurizing the system. Unfortunately, this process utilizes high pressures, leading to high equipment and operating costs [12-14], which makes the process uneconomical in many cases. Moreover, extraction of oil from fresh rubber seeds with non-polar organic solvents including SC-CO_2 gives low yield due to accessibility of the solvents into the relatively wet seeds. The oil yield can generally be improved by drying the raw seeds prior to extraction, nevertheless, the drying process can be extremely energy intensive.

Recently, liquefied dimethyl ether (DME), a chemical widely used as fuel, aerosol propellant, assistant solvent, vesicant and refrigerant [15], has been reported to be an effective solvent for extraction of lipid and active compounds from various natural materials [16-21]. Compared with other organic solvents, DME is a non-toxic

solvent for extraction. In addition, DME is considered a green solvent since it has zero ozone depletion potential (ODP) and low global warming potential (GDP) [22]. DME can also be easily removed from the final product in a similar manner as in SC-CO_2 extraction, by de-pressurizing the system. However, extraction with DME has an advantage over SC-CO_2 extraction in that it requires lower extraction pressure, approximately at 1 MPa (or 20-60 times lower pressure) [13, 16-17]. In addition, DME is partially miscible with water [23-24], and so has been successfully applied for extraction of high moisture content materials without requiring prior drying [20-24].

This study therefore focused on the investigation of RSO extraction using liquefied DME. Response surface methodology (RSM) with a spherical central composite design (CCD) was employed for the optimization of process conditions giving the maximum RSO yield, including seed moisture content (%wt in dry basis), solvent to solid weight ratio (g/g), and extraction temperature (^{o}C). The resulting RSO was then characterized chemical characteristics and physico-chemical properties, including kinematic viscosity, acid value, and % fatty acid.

2. Materials and Methods

2.1. Materials and Chemicals

Fresh rubber seeds were obtained from a local farm in Phitsanulok Province, Thailand. The rubber seeds were manually peeled to collect only the inner white kernel. The kernels were weighed and then cut into rough pieces and dried in a hot air oven at 60°C for 72 h. The dried kernels were then weighed again, and the moisture content of the fresh kernels was calculated on a dry basis and found to be approximately 86%. The dried sample was flaked to obtain approximately 40-60 mesh kernel flakes and then stored in a desiccator until extraction to prevent the flakes from absorbing moisture. Liquefied DME (Spray Work Air Can 420D) to use for extraction was purchased from Siam Tamiya Co., Ltd., Thailand. Hexane (purity>99.5%) was purchased by Sigma-Aldrich.

2.2. Rubber seed oil extraction with liquefied DME

For DME extraction, approximately 10 g of the dried kernel flakes were first mixed with an exact amount of distilled water to achieve the desired moisture content in dry basis. Each sample was then loaded into a cellulose thimble (30x100

mm) along with an 8-mm-diameter magnetic bar, which was then placed into an extractor (100-ml stainless-steel). To achieve the required solvent to solid weight ratio (g/g), liquefied DME was added through a needle valve into the pre-weighed extractor. The extraction was then conducted at a controlled temperature, under constant stirring at 500 rpm for 30 min. After extraction, the liquid containing DME and the extracts was transferred through a stainless-steel filter (7 μm pore diameter, Swagelok, Thailand) to a 100-ml beaker. This liquid was then dried by a hot plate stirrer at 120°C for 15 min. The resulting oil was weighed to determine the oil yield based on dried kernel weight.

In order to compare DME extraction with hexane extraction, 10 grams of dried kernel flakes were extracted with 200 ml of n-hexane in a Soxhlet apparatus for 18 hours. The total oil content determined by this method was found to be 4.45 grams (44.5% yield).

2.3. Design of experiment and optimization of extraction conditions by response surface methodology

Response surface methodology with the spherical central composite design model was used to determine the optimal extraction conditions. Three extraction parameters were defined as seed moisture content = X_1 (%wt in dry basis), solvent to solid weight ratio = X_2 (g/g) and extraction temperature = X_3 (°C). The value of α for the CCD was defined as $\alpha = \sqrt{K}$ where K is the number of parameters (K = 3), so the α values used in this study were -1.73 and +1.73. The low, middle and high levels of each parameter were therefore designated as -1, 0, and +1, respectively. The corresponding actual values for each parameter are shown in Table 1. The correlation between the parameters and the response is described by Equation 1, as follows:

$$Yield = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X^2{}_i + \sum_{i=1}^{3}\sum_{i=1}^{3}\beta_{ij} X_i X_j \qquad (1)$$

Where X_i = actual values of parameters, β_0 = interception, β_{ii} = regression coefficient and β_{ij} = cross product coefficient.

The analysis of the spherical CCD experimental design, analysis of variance (ANOVA) and optimization of the conditions were carried out using Minitab Statistical Software (version 17).

Table 1. Levels of actual and coded factors

X_i	**-1.73**	**-1**	**0**	**1**	**1.73**
Moisture content (%wt)	5.5	20	40	60	75
Solvent to solid weight ratio (g/g)	3.3	4	5	6	6.7
Temperature (^{o}C)	31	35	40	45	49

2.4 Characterization of rubber seed oil

To determine its chemical characteristics, RSO obtained at the optimal condition was characterized by FT-IR and ^{1}H NMR analysis. FT-IR was conducted by using an FT-IR instrument (Perkin Elmer Frontier) following the analysis method described by Ali et al., 2015 [11]. ^{1}H NMR spectra of the RSO were obtained by a 600 MHz NMR spectrometer (Avance-II Ultrashield-400 MHz, Bruker) using a 5-mm-diameter NMR tube according to the procedure described by Aigbodion et al., 2005 [25]. Physico-chemical properties including kinematic viscosity, acid value and % fatty acid of RSO were determined following standard methods of ASTM D445, AOCS (Te 1a-64), and AOCS (Te 1a-64), respectively. The fatty acid composition was determined according to ISO 5508 [26] using a Gas Chromatography (GC-FID detector, Shimadzu 2010 model).

3. Results and discussion

3.1 Design of experiment (DOE) and optimization of extraction conditions

3.1.1 Quadratic regression model and variance analysis

The 17 sets of operating conditions with their corresponding experimental RSO yields as well as their model-predicted yields are shown in Table 2. The analysis of variance (ANOVA) values for the quadratic regression equation and the significance of regression coefficients from the spherical CCD are shown in Table 3. From the results,

the predicted data from the quadratic regression equation were fitted to the experimental data, which showed an R^2 value of 0.9135 and an adjusted R^2 value of 0.802. RSM suggested a quadratic equation with an F-value of 8.21 and a P-value of 0.006. The significantly lower P-value compared to the F-value indicates that the model is acceptable [11]. The ANOVA revealed that the P-values of seed moisture content (X_1) and solvent to solid weight ratio (X_2) were 0.005 and less than 0.0001, respectively. These two values indicate that seed moisture content and solvent to solid weight ratio affected the RSO yield significantly. The quadratic polynomial equation substituted by the actual parameter values is shown in Equation 2, as follows:

$$\text{Yield} = -268.0 + 1.399X_1 + 43.2X_2 + 7.57X_3 - 0.00541X_1^2 - 2.128\,X_2^2 - 0.0658X_3^2 - 0.0490X_1X_2 - 0.0139X_1X_3 - 0.361X_2X_3 \quad (2)$$

where: X_1, X_2 and X_3 are parameters in actual values.

Table 2. Spherical CCD experimental parameters, experimental and predicted data.

Run	Moisture content (%wt) (X_1)	Solvent to solid weight ratio (g/g) (X_2)	Temperature (^oC) (X_3)	Experimental %Yield (g oil/g dried seed)	Predicted %Yield (g oil/g dried seed)
1	20	4	35	15.57	16.90
2	60	4	35	29.90	28.21

3	20	6	35	32.99	33.43
4	60	6	35	37.63	40.82
5	20	4	45	23.40	22.86
6	60	4	45	26.39	28.61
7	20	6	45	27.84	32.17
8	60	6	45	32.68	34.00
9	5.5	5	40	24.85	23.23
10	75	5	40	35.98	34.61
11	40	3.3	40	18.66	19.55
12	40	6.7	40	42.37	38.51
13	40	5	31	31.34	30.98
14	40	5	49	32.58	30.21
15	40	5	40	35.88	35.41
16	40	5	40	33.81	35.41
17	40	5	40	36.39	35.41

Table 3. Analysis of variance (ANOVA) for the quadratic regression equation.

Source	Sum of Square	df	Mean Square	F-value	P-value
Model	714.515	9	79.931	8.21	0.006*
X_1	151.730	1	151.730	15.69	0.005*
X_2	422.786	1	422.786	43.72	<0.0001*
X_3	0.944	1	0.944	0.10	0.764

X_1X_2	7.675	1	7.675	0.79	0.403
X_1X_3	15.496	1	15.496	1.60	0.246
X_2X_3	26.043	1	26.043	2.69	0.145
X_1X_1	56.818	1	56.818	5.88	0.046*
X_2X_2	55.015	1	55.015	5.69	0.049*
X_3X_3	32.906	1	32.906	3.40	0.108
Error	67.698	7	9.671		
Total	782.213	16			
R^2	0.9135				
Adjusted R^2	0.802				

** The parameters significantly affect RSO yield (P-value less than 0.05)*

3.1.2 Effect of extraction parameters

The effect of extraction conditions including moisture content, solvent to solid weight ratio, and extraction temperature on RSO yield were observed from the 3D surface and contour plots generated by the quadratic equation as shown in Fig. 1-3. Figs. 1 and 2 show that a higher seed moisture content resulted in a higher RSO yield. One possible explanation of the results is that the presence of water could act as a co-solvent for oil extraction leading to the increase in RSO yield. Similar results were also observed in previous research [18], which indicated that the presence of water in the seed is necessary for oil extraction using DME. However, it is worth noting that too high the amount of water might increase the overall solvent polarity which could potentially cause a lower oil yield.

The effect of the solvent to solid weight ratio on RSO yield is shown in Figs. 1 and 3. The results revealed that an increase in solvent to solid weight ratio significantly increased RSO yield. The results might be explained by the fact that solute-solvent interactions are known to be enhanced by an increase in solvent. It follows that increased DME would thus enhance the RSO extraction and solubilization into the DME.

The effect of extraction temperature on RSO yield is shown in Figs. 2 and 3. The RSO yield initially increased with increasing temperature, but then decreased at the extraction temperature higher than 38°C. Increasing the temperature of an

extraction generally increases the solubility of oils into the solvents, however the density of DME is also known to decrease at high temperatures [27] and that decreased density of DME results in a lower solvent power. At high extraction temperature, the decrease in solvent power might outpace the increase in solubility of the RSO into the DME, resulting in a lower RSO yield.

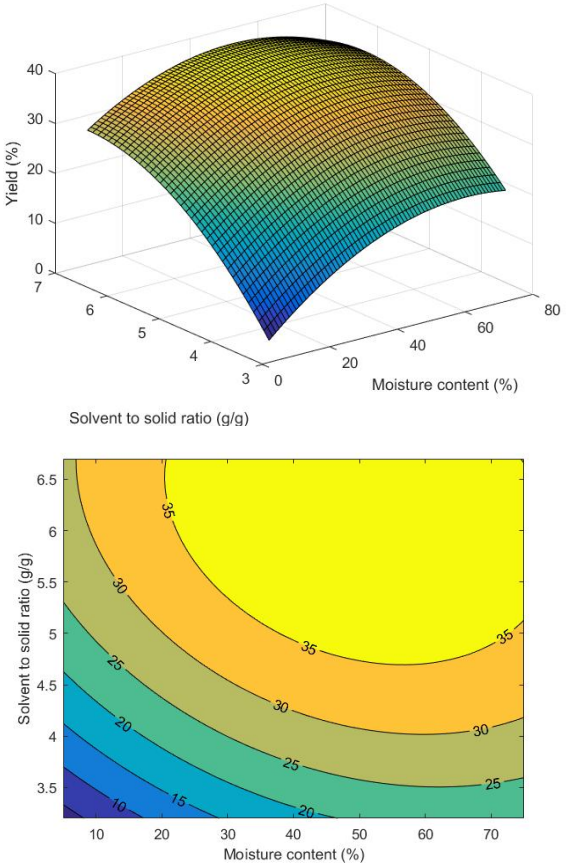

Fig. 1. 3D surface and contour plots explaining the interaction effects of moisture content (X_1) and solvent to solid ratio (X_2).

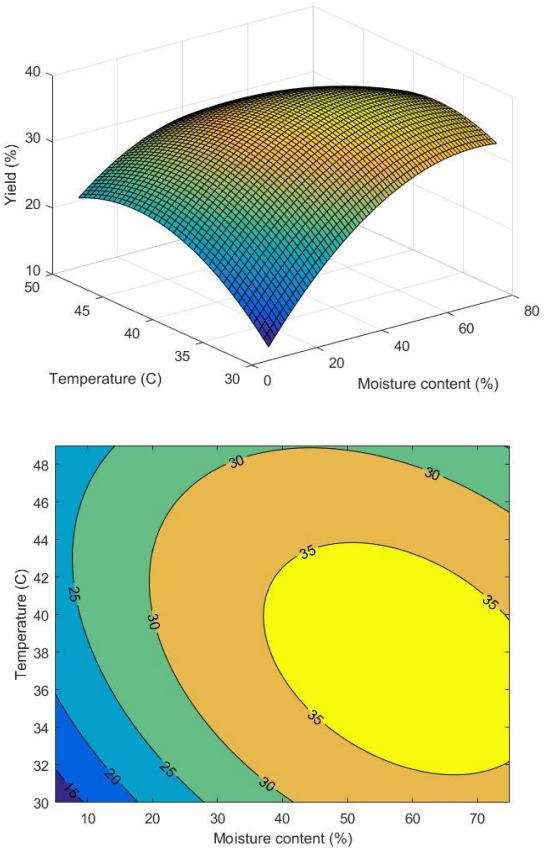

Fig. 2. 3D surface and contour plots explaining the interaction effects of moisture content (X_1) and temperature (X_3).

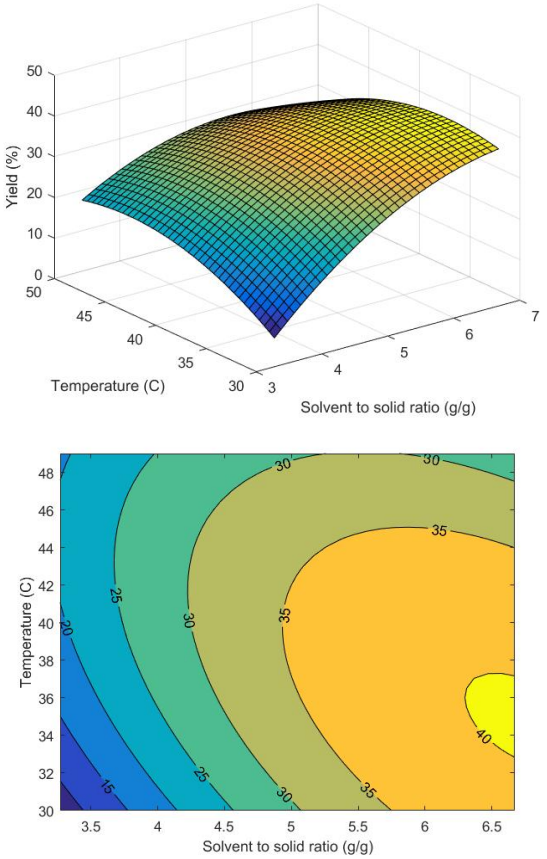

Fig. 3. 3D surface and contour plots explaining the interaction effects of solvent to solid ratio (X_2) and temperature (X_3).

3.1.3 Process validation

The regression model's suggested optimal extraction conditions to obtain the maximum RSO yield of 41.48% were a seed moisture content of 56.4%wt, a solvent to solid ratio of 6.7 (g/g), and a temperature of 33.3°C. These figures were rounded to 56%wt, 6.7, and 33°C, respectively, and the experiment was conducted. The actual RSO yield obtained was 42.24%, which showed that the regression equation's RSO yield prediction was reasonable (1.8% error).

Compared with Soxhlet extraction using hexane as solvent, DME extraction gave a slightly lower RSO yield (42.24% versus 44.5%). However, the extraction process using liquefied DME theoretically requires lower energy consumption. DME extraction does not require completely dried rubber seeds to optimize oil yield but the suitable moisture content of 56% should be maintained. Moreover, the differences in RSO yield that resulted from varying extraction temperature in the range of 30-50°C were not statistically significant, since the P-value for temperature (X_3) was greater than 0.05 (as seen in Table 3). Therefore DME extraction, at least at the local room temperature (approximately 30-45°C), requires no additional energy input to optimize the RSO yield. It can be drawn from the results that liquefied DME has potential to be used for recovery of RSO.

3.2 Characterization of rubber seed oil

RSO obtained at the optimum extraction conditions was characterized to determine its chemical characteristics by FT-IR and ^{1}H NMR analysis. The FT-IR spectra of RSO are shown in Fig. 4. The main peaks and their corresponding functional groups are given in Table 4. FT-IR analysis revealed that the main component in RSO was triglyceride (TG). The strong absorption band of the ester carbonyl functional group of TG was observed around 1742 cm^{-1}. In addition, the fingerprint region of RSO can be seen at the FT-IR spectra from 1458 to 585 cm^{-1}[28].

The signal assignments of RSO obtained from ^{1}H NMR analysis are shown in Table 5 and Fig. 5. RSO's structure consists of the terminal methyl of the FA chain (0.859-0.979 ppm), the methylene group next to the terminal methyl (1.597 ppm),

methylene groups next to one double bond (1.989-2.061 ppm), and methylene groups next to two double bonds (2.739-2.79 ppm). The other groups are carbon-carbon double bond (C=C) and methylene of glyceryl (α: 4.137 – 4.152 ppm and β: 5.309-5.369 ppm).

The physico-chemical properties of DME extracted RSO are reported in Table 6. The results show that the RSO had a golden yellow color, a kinematic viscosity of 36.8 cSt, an acid value of 10.7 KOH/g oil, and fatty acid content of 5.1%. The fatty acid content of RSO obtained by liquefied DME extraction was lower than the extraction using other organic solvents reported in previous work of Wuttichai et al, 2017 [29] who found that fatty acid contents of RSO using hexane, acetone, dichloromethane, and ethyl acetate were 5.2%, 10.1%, 11.8%, and 8.1%, respectively. Despite the lower fatty acid content of RSO in this work, which might be caused by the difference in growth conditions, it can be implied that the low fatty acid content of the RSO suggests its potential use for biodiesel production. Through alkali-catalyzed transesterification, fatty acid content must be minimized to avoid the side saponification reaction. Compared with *Jatropha* oil, RSO has a slightly higher fatty acid content (3.3% versus 5.02%) [30]. However, rubber seed is more attractive source than *Jatropha* because it contains higher oil content (40-69% versus 17-36%) [30]. In addition, RSO obtained in this work also shows possibility to be used in the production of biolubricants corespending to its kinematic viscosity, which is in the range of standard lubricants (viscosity grade: 22 (at 40°C = 19.2-24.2 cSt, at 100°C $\leq$ 4 cSt)) [8].

Moreover, RSO's fatty acid profile as determined by GC analysis is shown in Table 7. The total unsaturated fatty acid content was 80.01% which was identified as 41.21% linoleic, 15.25% linolenic, and 23.10% oleic acids. The total saturated fatty acid content of RSO was 15.59%, which was mainly palmitic acid (8.80%). The results suggested that RSO is also suitable for use as a feedstock in biosynthesis of polyhydroxyalkanoates (PHA), a starting material of biodegradable plastic production, because the oil containing high amount of unsaturated fatty acid is favorably utilized by the bacteria [9].

Table 4. The main peaks in the FT-IR spectra of RSO

Peak (cm^{-1})	Functional group
3009	=C-H stretching of nonconjugated (methylene group)
2921- 2852	C-H stretching vibration (methyl group)
1742	-C=O stretching vibration (carbonyl)
Fingerprint region (1435-585 cm^{-1})	
1458	C-H bending frequency (alkene)
1377	C-H bending vibration (alkene)
1236	C-O stretching vibration (ester)
1159	C-O stretching vibration (ester)
1097	=C-O-C stretching vibration (ester)
721	C-H bending vibration (aromatic)
587	C-H vibration (alkane)

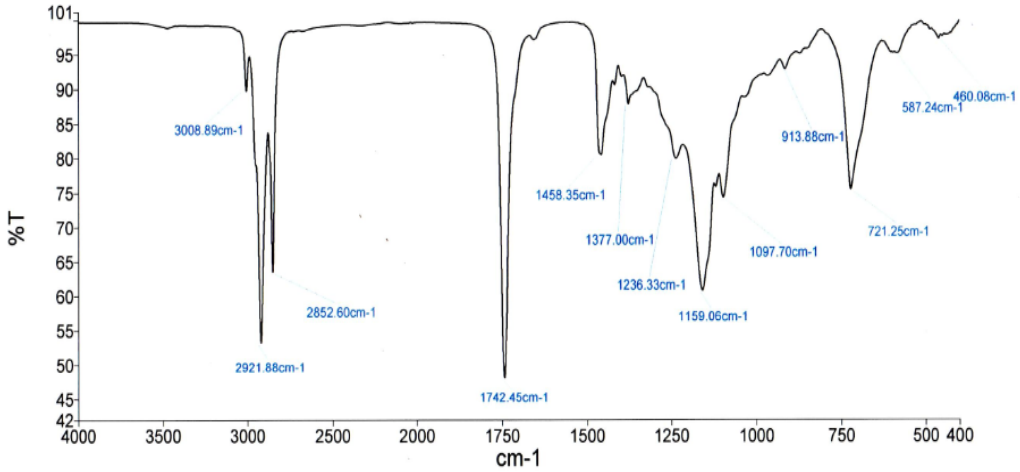

Fig. 4. FT-IR spectra of RSO obtained at optimum condition.

Table 5. The signals present in ^{1}H NMR spectra of RSO and their assignment.

δ (ppm)	Assignment
0.859-0.979	-CH_3 terminal methyl
1.244-1.358	-CH_2 saturated aliphatic chain
1.597	-CH_2-C methylene α to terminal methyl
1.989-2.061	-CH_2-C=C allylic methylene
2.275-2.319	-CH_2-O-C=O acyl methylene
2.739-2.79	-C=C-CH_2-C=C- diallylic methylene
4.137-4.152	-CH_2-O-CO- in α position in glyceryl
4.265-4.275	-CH=CH- olefinic (FA chain)
5.309-5.369	-CH-O-CO- in β position in glyceryl

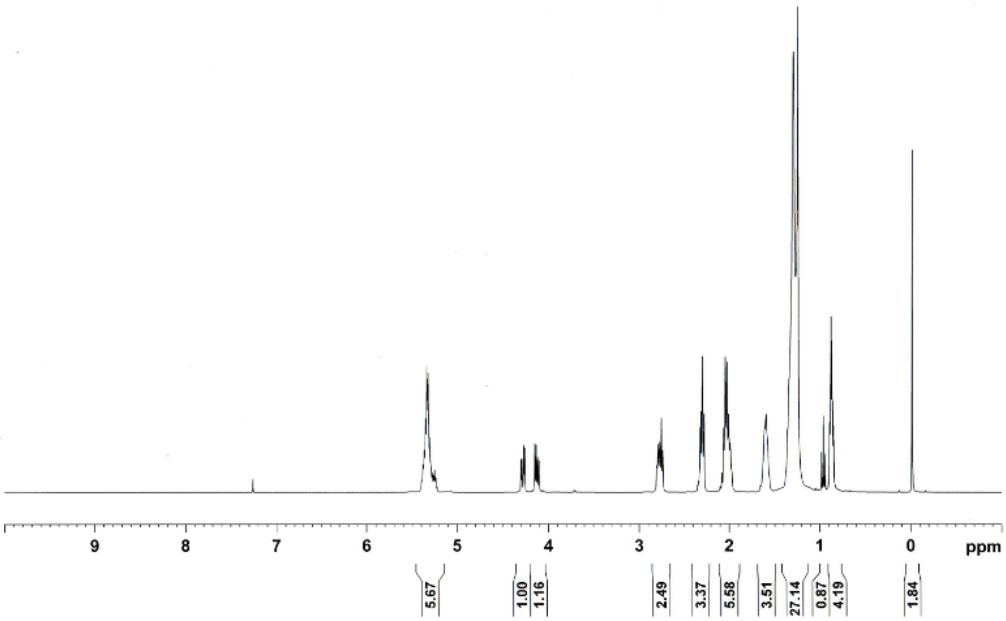

Fig. S2. The ^{1}HNMR analysis of RSO obtained at optimum condition.

Table 6. Physico-chemical properties of rubber seed oil.

Properties	Rubber seed oil	Testing methods
Color	Golden yellow	

Kinematic viscosity (Cst at 25 °C)	36.8	ASTM (D445)
Acid value (mg of KOH/g oil)	10.7	AOCS (Te 1a-64)
Free fatty acid (%)	5.06	AOCS (Te 1a-64)

Table 7. Fatty acid composition of RSO.

Fatty acid	Percentage
Unsaturated fatty acid	80.01
Linoleic acid	41.21
Linolenic acid	15.25
Oleic acid	23.10
Palmitoleic acid	0.29
Elcosenoic acid	0.16
Saturated fatty acid	15.59
Myristic acid	0.12
Stearic acid	6.39
Palmitic acid	8.80
Arachidic acid	0.28

4. Conclusions

Liquefied DME was found to be an effective solvent for rubber seed oil extraction. The maximum RSO yield of 42.24% can be achieved at 56%wt of seed moisture content, 6.7 of solvent to solid ratio and 33°C of extraction temperature which are the reasonable predicted extraction conditions by response surface methodology (RSM). The physico-chemical properties of the RSO were found to be kinematic viscosity of 36.8 cSt, acid value of 10.7 KOH/g oil, fatty acid content of 5.1%, and unsaturated fatty acids content of 80%. This current research provides a conservation energy consumption process of DME extraction for recovery oil from high moisture content rubber seeds. As the results, using DME as solvent at local

room temperature with wet extraction achieved high yield of RSO, which could be considered a potential feedstock for the industrial applications including production of biodiesel, biolubricants, and biodegradable plastic.

References

[1] Widyarani, Enny, R., Johan, P.M.S., Marieke, E.B., 2014. Biorefinery methods for separation of protein and oil fractions from rubber seed kernel. Ind. Crops. Prod. 62, 323-332.

[2] Ramadhas, A.S., Jayaraj, S., Muraleedharan, C., 2005. Characterization and effect of using rubber seed oil as fuel in the compression ignition engines. Renew. Energy. 30(5), 795-803.

[3] Ramadhas, A.S., Jayaraj, S., Muraleedharan, C., 2005. Biodiesel production from high FFA rubber seed oil. Fuel. 84(4), 335-340.

[4] Ramadhas, A.S., Muraleedharan, C., Jayaraj, S., 2005. Performance and emission evaluation of a diesel engine fueled with methyl esters of rubber seed oil. Renew. Energy. 30(12), 1789-800.
[5] Ikwuagwu, O.E., Ononogbu, I.C., Njoku, O.U., 2000. Production of biodiesel using rubber [Hevea brasiliensis (Kunth. Muell.)] seed oil. Ind. Crops. Prod. 12(1), 57-62.
[6] Salimon, J., Abdullah, M.B., Nadia, S., 2012. Rubber (Hevea brasiliensis) seed oil toxicity effect and Linamarin compound analysis. Lipids Health Dis. 11, 74.
[7] Khazaai, S.N.M., Maniam, G.P., Rahim, M.H.A, Yusoff, M.M., Matsumurada, Y., 2017. Review on methyl ester production from inedible rubber seed oil under various catalysts. Ind. Crops. Prod. 97, 191-195.
[8] Kamalakar, K., Rajak, A.K., Prasad, R.B.N., Karuna, M.S.L., 2013. Rubber seed oil-based biolubricant base stocks: A potential source for hydraulic oils. Ind. Crops. Prod. 51, 249-257.
[9] Kynadi, A.S., Suchithra, T.V., 2017. Formulation and optimization of a novel media comprising rubber seed oil for PHA production. Ind. Crops. Prod. 105, 156-163.
[10] Herry, S., Iryanto, Maria, I., 2014. Effects of temperature, pressure, preheating time and pressing time on rubber seed oil extraction using hydraulic press. Procedia Chem. 9, 248-256.
[11] Ali, S.R., Pankaj, T., Vaibhav, V.G., 2015. Extraction of oil from rubber seeds for biodiesel application: Optimization of parameters. Fuel. 150, 636-644.
[12] Xiang X., Jie D., Xuefeng M., Liping S., 2011. Supercritical CO2 extraction of oil, carotenoids, squalene and sterols from lotus (*Nelumbo nucifera* Gaertn) bee pollen. Food bioprod. process. 89, 47-52.
[13] Lee, N.Y., Setapar, S.H.M., Sharif, N.S.M., Ahmad, A., Khatoon, A., Azizi, C.Y.M., Idayu, M.I., 2013. Extraction of rubber (Hevea brasiliensis) seed oil using supercritical carbon dioxide and soxhlet extraction. Res. J. Chem. Environ. 17 (10), 46-52.
[14] Fiori, L., 2010 Supercritical extraction of grape seed oil at industrial-scale: Plant and process design, modeling, economic feasibility. Chem. Eng. Process. 49, 866-872.
[15] Jianguo, Y., Jiangtao, W., Xianyang, M., Ilmutdin, A., 2011. Compressed liquid density measurements of dimethyl ether with a vibrating tube densimeter. J. Chem. Thermodynamics. 43, 1371-1374.

[16] Kanda, H., Li, P., Ikehara ,T., Hirose, M.Y., 2012. Lipids extracted from several species of natural blue–green microalgae by dimethyl ether: Extraction yield and properties. Fuel. 95, 88-92.
[17] Li, P., Kanda, H., Makino, H., 2014. Simultaneous production of bio-solid fuel and bio-crude from vegetal biomass using liquefied dimethyl ether. Fuel. 116, 370-376.
[18] Tallon, S., Catchpole, O., Grey, J., Fletcher, K., Fletcher, A., 2008. Extraction of lipids from a specialist dairy stream. J. Supercrit. Fluids. 45, 314-321.
[19] Catchpole, O., Ryan, J., Zhu, Y., Fenton, K., Grey, J., Vyssotski, M., MacKenzie, A., Nekrasov, E., Mitchell, K., 2010. Extraction of lipids from fermentation biomass using near-critical dimethylether. J. Supercrit. Fluids. 53, 34-41.
[20] Tallon, S., Catchpole, O., Grey, J., Fenton, K., Fletcher, K., Fletcher, A., 2007. Extraction of lipids from aqueous protein-rich streams using near-critical dimethyl ether, Chem. Eng. Technol. 30, 501-510.
[21] Boonnoun, P., Tunyasitikun, P., Clowutimon, W., Shotipruk, A., 2017. Production of free lutein by simultaneous extraction and de-esterification of marigold flowers in liquefied dimethyl ether (DME)-KOH-EtOH Mixture. Food bioprod. process. 106, 193-200.
[22] Azizi, Z., Rezaeimanesh, M., Tohidian, T., Rahimpour, M.R., 2014. Dimethyl ether: A review of technologies and production challenges. Chem. Eng. Process. 82, 150-172.
[23] Pozo, M.E., Streett, W.B., 1984. fluid phase equilibria in the system dimethyl ether/water from 50 to 200 C and pressures to 500 bar J. Chem. Eng. Data. 29, 324-329.
[24] Holldorff, H., Knapp, H., 1988. Binary vapor–liquid–liquid equilibrium of dimethyl ether-water and mutual solubilities of methyl chloride and water: experimental results and data reduction. Fluid Phase Equilib. 44, 195-209.
[25] Aigbodion, A.I., Bakare, I.O., 2005. Rubber seed oil quality assessment and authentication. JAOCS. 82 (7), 465-469.
[26] International Organization for Standardization (ISO). Animal and vegetable fats and oils - Analysis by gas chromatography of methyl esters of fatty acids; 1990.
[27] Ihmels, E. C., Lemmon, E. W., 2007. Experimental densities, vapor pressures, and critical point, and a fundamental equation of state for dimethyl ether. Fluid Phase Equilib. 260, 36-48.

[28] Onoji, S.E., Iyuke, S.E., Igbafe, A.I., 2016. Hevea brasiliensis (Rubber Seed) oil: extraction, characterization, and kinetics of thermo-oxidative degradation using classical chemical methods. Energy Fuels. 30, 10555-10567.
[29] Wuttichai, R., Theeranun, S., Boonyawan, Y., Taweesak, S., Vinich, P., 2017. Rubber seed oil as potential non-edible feedstock for biodiesel production using heterogeneous catalyst in Thailand. Renew. Energy. 101, 937-944.
[30] Talita, F.S.B., Nair, H.C.A., Messias, F.Q., Pedro D.F., Simone, M., José, A.A.R., Everaldo, P.M., 2015. Fatty acid profiles of species of *Jatropha curcas* L., *Jatropha mollissima* (Pohl) Baill. and *Jatropha gossypiifolia* L. Ind. Crops. Prod. 73, 106-108.

ภาคผนวก

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

- Boonnoun P, Tunyasitikun P, Clowutimon W, Shotipruk A. Production of free lutein by simultaneous extraction and de-esterification of marigold flowers in liquefied dimethyl ether (DME)-KOH-EtOH mixture. Food and Bioproducts Processing. 2017; 106: 193-200 (ISI)
- Boonnoun P, Shotipruk A, Kanda H, Goto M. Response surface optimization of liquefied dimethyl ether extraction conditions of rubber seed oil. Chemical Engineering Communications. Submitted (under review updated on 14th May 2018)

2. การนำผลงานวิจัยไปใช้ประโยชน์

   -

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
   - Thungoen A, Shotipruk A, Boonnoun P. Extraction of phenolic compounds from fenugreek seeds using liquefied dimethyl ether (DME). The 2018 Pure and Applied Chemistry International Conference (PACCON 2018). 7-9 Feb 2018. Prince of Songkla University, Thailand. (Proceeding)

Contents lists available at ScienceDirect

Food and Bioproducts Processing

journal homepage: www.elsevier.com/locate/fbp

Production of free lutein by simultaneous extraction and de-esterification of marigold flowers in liquefied dimethyl ether (DME)–KOH–EtOH mixture

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ARTICLE INFO

Article history:
Received 25 July 2017
Received in revised form 4 October 2017
Accepted 6 October 2017
Available online 14 October 2017

Keywords:
Lutein
Liquefied dimethyl ether
Solvent extraction
De-esterification
Marigold flowers
Simultaneous process

ABSTRACT

Extraction of xanthophylls from marigold flowers using liquefied dimethyl ether (DME) was investigated. The most suitable DME extraction condition was found to be 33:0.5 (w/w) DME to dried marigold flowers ratio and at 35 °C, giving 20.65 mg of total xanthophylls/g of dried marigold flowers. Following extraction, the suitable de-esterification conditions to convert the extracted lutein fatty acid ester (the major compound in xanthophylls) to free lutein, a more bio-available form, were determined to be 2.5%w/v KOH–EtOH, at 35 °C for 4 h. While employing the most suitable conditions in each of the two steps gave favorable free lutein yield (16.65 mg free lutein/g dried marigold flowers), a one-step process in which simultaneous extraction and de-esterification carried out at the most suitable condition: the DME to dried marigold flowers ratio 33:0.5 (w/w), EtOH to dried marigold flowers ratio 10:0.5, 5% KOH–EtOH concentration, at 35 °C for 1 h, could lead to about 20% improvement (20.71 mg free lutein/g dried marigold flowers). Furthermore, the results from this study suggested that wet marigold samples could possibly be used both in the DME extraction for xanthophylls and in the simultaneous process to obtain free lutein.

1. Introduction

Marigold (*Tagetes erecta*) is an ornamental plant originated in Mexico, but nowadays has been grown widely in tropical and subtropical regions of the world including South-East Asia (Lin et al., 2015a). The plant has been around Thailand for many years and is grown commercially all over the country for cut flowers used in Buddhist ceremonies and for use in poultry industry to provide yellow color to the skin of broilers and egg yolks. The key constituents in the marigold flowers that are responsible for the natural yellow color are xanthophylls, oxygenated carotenoids, and of which, approximately 60–70% is lutein (Gong et al., 2012).

Lutein ($C_{40}H_{56}O_2$) is a dihydroxy carotenoid with two ionone rings at each end of the molecule. Known not only as an important food colorant, lutein has also been well documented for its several health benefits including prevention and remedy of age-related macular degeneration (Prommuak et al., 2008; Luengo et al., 2014; Tian et al., 2015) and cancers, as well as enhancement of immune functions (Park et al., 1998; Siriamornpun et al., 2012). Given its relatively high amount of lutein in marigold flowers and its large market size ($150 million in the US) (Ho et al., 2015), extraction of marigold lutein for nutraceutical application is therefore becoming very attractive. Indeed, the worldwide market for lutein extracted from marigold flowers is expected to grow to US$308 million by 2018 (Lin et al., 2015b).

Lutein naturally occurs in the acylated form, in which it is chemically bound to various types for fatty acids, with the majority being lutein fatty acid diesters, due to the two hydroxy groups at the ionone rings. In marigold flowers, the main lutein fatty acid esters are lutein

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https://doi.org/10.1016/j.fbp.2017.10.002

dipalmitate (50%) and lutein dimyristate (30%) (Attokaran, 2011). Unfortunately, the high lipophilicity of long chain carbon skeleton of lutein fatty acid esters leads to low solubility in digestive fluids and subsequently poor bio-availability (Hojnik et al., 2008). To improve the bio-availability and the bio-activity, lutein fatty acid esters are generally de-esterified to free lutein by reacting with an alkali solution (i.e. KOH), usually in an alcohol such as ethanol (EtOH) or isopropanol (IPA) (Attokaran, 2011).

Conventionally, extraction of lutein fatty acid esters (or xanthophylls) from marigold flowers can be carried out by using organic solvents such as hexane and acetone (Hojnik et al., 2008). A major concern with this process is mainly the requirement for complicated separation steps to remove the toxic solvent. Provided with the low temperature operation and the ease of solvent separation, simply by depressurizing the extraction system, supercritical carbon dioxide (SC-CO_2) has been viewed as a potential green alternative to toxic organic solvent for extraction of lutein esters from marigold flowers (Gao et al., 2009, 2010; Palumpitag et al., 2011). Nevertheless, SC-CO_2 extraction has some disadvantages, such as high operating pressures (Espinosa-Pardo et al., 2017), which leads to high equipment and operating costs. Alternatively, owing to its low degree of toxicity, liquefied dimethyl ether (DME) has recently gained increasing interest and has been regarded as a safe extractant for food ingredients or dairy products (Noriyasu et al., 2015). In addition, its low boiling point (−24.8 °C) makes it easy to be separated from the final product upon depressurization of the extraction system. Nevertheless, compared with SC-CO_2, the requirement on extraction pressure is considerably lower (normally less than 1 MPa vs. 20–60 MPa (Calderone and Tallon, 2008; Skerget et al., 2010)). Furthermore, DME is slightly polar, making extraction of relatively non-polar compounds from wet samples possible. Specifically, liquefied DME has been shown to successfully extract lipids and a number of anti-oxidative compounds such as fucoxanthin from wet samples including fermentation biomass and algae (Goto et al., 2015; Catchpole et al., 2010).

In a typical marigold lutein manufacturing process, extraction of lutein fatty acid esters from marigold flowers is carried out first, and is subsequently followed by the de-esterification process to obtain free lutein. This sequential process can be time and energy consuming and causes high loss of product. As suggested by a number of studies (Han et al., 2013; Wang et al., 2016), when the processes of extraction and de-esterification are combined into one step, higher product yields could be achieved in fewer overall steps and shorter period of time.

To the best of the authors' knowledge, liquefied DME extraction has never been applied to extraction of marigold lutein esters. This paper therefore aims to provide the results of thorough investigation on this subject. Specifically, the effects of extraction conditions were determined and the extraction yields were compared with those achieved by employing hexane and SC-CO_2. In addition, the possibility of further improving the free lutein productivity by a combined process was evaluated, in which DME extraction and de-esterification of marigold flowers were carried out simultaneously. The results were compared with those carried out with sequential liquefied DME extraction, followed by de-esterification. In this study, the effects of using wet versus dried marigold flowers as starting raw materials were also determined.

2. Materials and methods

2.1. Materials and chemicals

Pulverized dried marigold flowers sample was obtained from PTT Global Chemical Company Ltd. (Rayong, Thailand). Liquefied 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 fatty acid esters was supplied by Sigma–Aldrich, Germany. High purity carbon dioxide used for SC-CO_2 extraction was obtained from Thonburiwattana Co. (Bangkok, Thailand). EtOH (purity >99.5%) and KOH anhydrous (purity >85%) used for de-esterification 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.

2.2. Methodology for extraction

2.2.1. DME extraction

DME extraction of dried marigold flowers was carried out to determine the effects of solvent to sample weight ratio (20:0.5–40:0.5 g/g) and temperature (25–40 °C) on total xanthophylls yield. As schematically shown in Fig. 1, the apparatus used consists of a 120-ml stainless steel batch extractor and a separation unit. The separation unit is composed of an 80-ml-hyper-glass vessel in a polycarbonate housing. The extractor

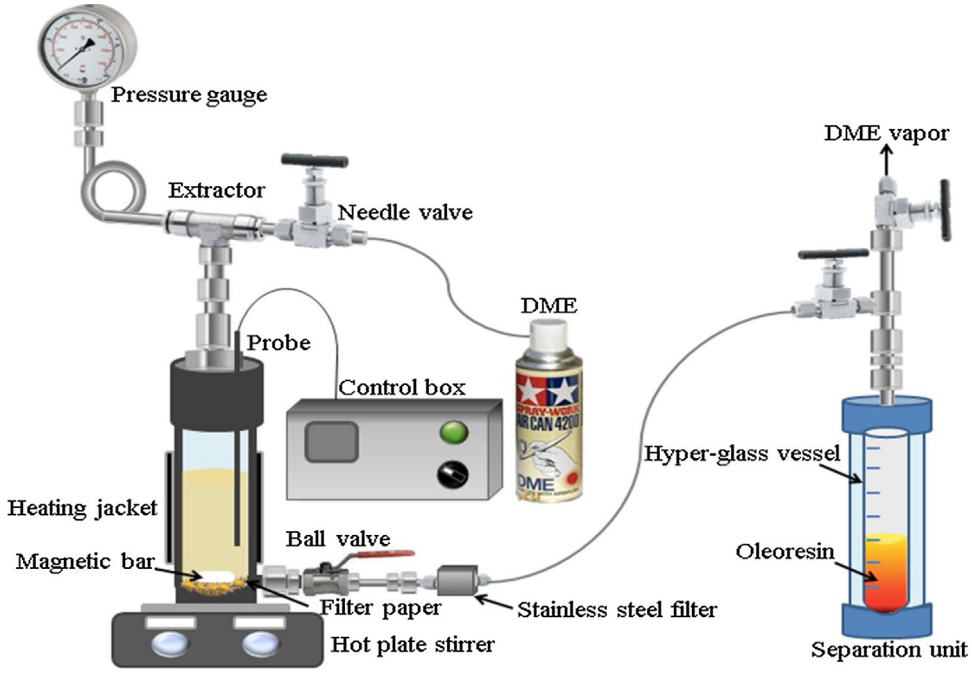

Fig. 1 – DME extraction apparatus.

was heated by a heating jacket connected to a temperature control box. Agitation of the extraction system was provided using a magnetic stirrer. In a typical run, extraction run begins with charging 0.5 g of dried marigold flowers into the extractor, along with an 8-mm-diameter magnetic bar. To achieve a specified solvent to sample ratio (g/g), the needle valve was then opened to allow a specified amount of liquefied DME from the DME storage vessel into the pre-weighed extractor. The extraction was carried out for 30 min at a fixed agitation rate of 400 rpm at a controlled extraction temperature. After the completion of each extraction, the ball valve was opened to allow the extract to flow out of the extractor to the separation unit through a Whatman No.1 filter paper, placed at the exit of the extractor, and then through a stainless steel in-line filter (7 μm of pore size diameter) behind the ball valve. 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 total xanthophylls by a spectrophotometer. For each 0.5 g of dried marigold flowers, approximately 0.1–0.2 g (20–40%) of marigold oleoresin was obtained.

To determine the possibility of wet extraction of xanthophylls from marigold, wet marigold flower was extracted using the most suitable DME extraction condition obtained from dried marigold extraction experiment. The predetermined amounts of distilled water were added to 0.5 g of dried marigold flower sample to prepare the wet sample of 80% and 70% moisture contents. These, respectively, are approximate water contents of pretreated (by fermentation) marigold flowers and that of fresh marigold flowers having undergone a dewatering process by physical compression (Lin et al., 2015b). The sample of specified moisture content was then extracted with DME following the method described above.

2.2.2. *Solvent extraction*

For solvent extraction, the procedure and conditions were slightly modified from the previous work of Vechpanich and Shotipruk (2010). Here, 10 g of dried marigold flowers was extracted by 50 ml of hexane at 40 °C for 4 h in a digital incubator shaker (Innova 4000) at 150 rpm. 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 was diluted with hexane prior to the quantification of the amount of total xanthophylls by a spectrophotometer.

2.2.3. *Supercritical carbon dioxide extraction*

0.5 g of dried marigold flowers 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 °C, 40 MPa for 4 h, which is 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 total xanthophylls.

2.3. *Determination of suitable de-esterification condition*

2.3.1. *Preparation of marigold oleoresin*

To prepare marigold oleoresin for the investigation of suitable de-esterification conditions, 100-g powder of dried marigold flowers was extracted with 500 ml of hexane at 40 °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 °C. The resulting marigold oleoresin was further dried for 2 h in a vacuum oven (Model VD23, Binder) at 35 °C. For each 100 g of dried marigold flowers, approximately 10 g of marigold oleoresin was obtained.

2.3.2. *De-esterification of lutein fatty acid esters*

Experiments were carried out to determine the effect of de-esterification conditions: EtOH to oleoresin ratio (10:1 ml/g–30:1 ml/g), KOH concentration (1.5–3.5%w/v), temperature (30–40 °C) and time (0.5–4 h) on free lutein yield. A typical de-esterification run involved the reaction of 1 g of marigold oleoresin with the KOH–EtOH solution in a 35-ml glass vial at a controlled temperature and time, at a constant agitation rate of 150 rpm using a magnetic stirrer. Upon the completion of the reaction, free lutein was removed from the de-esterified mixture according to the method of Shibata et al. (2004). Briefly, 50 ml of EtOH was added to the de-esterified mixture, which 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 added. After rigorous mixing, followed by adequate settling time, two phases: the EtOH–water bottom phase and the diethyl ether top phase (free lutein rich phase) were formed. Upon evaporation of the diethyl ether by purging with nitrogen, the dried residue was obtained, and was subsequently re-dissolved with EtOH and stored at 4 °C until analysis by HPLC.

2.4. *Simultaneous DME extraction and de-esterification*

Simultaneous DME extraction and de-esterification of dried marigold flowers was carried out in the same apparatus used for DME extraction (Fig. 1) to determine the operating conditions: the effects of EtOH to dried marigold flower ratio (5:0.5–15:0.5 ml/g), KOH–EtOH concentration (3–7%w/v) and mixing time (0.5–2 h) on free lutein yield. For this experiment, 0.5 g of dried marigold flowers and KOH–EtOH solution at specified concentrations (%w/v) were charged into the extractor. Liquefied DME at the most suitable DME to sample weight ratio suggested by the previous experiment was then charged into the extractor. Simultaneous DME extraction and de-esterification was carried out at 35 °C, agitation rate of 400 rpm and at a specified mixing time. The extract consisting of DME and de-esterified mixture was then transferred to the separation unit, after which DME was then evaporated by depressurizing the separation unit and the de-esterified mixture was obtained. Into the de-esterified mixture, 50 ml of EtOH was added, and the solution was subsequently transferred to a separation funnel. The free lutein was extracted from the solution, and subsequently washed following the same method described in Section 2.3.2. The washed free lutein solution was then stored at 4 °C until analysis by HPLC.

The possibility of simultaneous DME extraction and de-esterification of wet marigold samples was also investigated using the suitable condition determined from simultaneous DME extraction and de-esterification of dried marigold sample. For this experiment, the predetermined amounts of distilled water were added to 0.5 g of dried marigold flower sample to prepare the wet sample of 80% moisture contents.

The wet sample was then simultaneously extracted and de-esterified following the method described above.

2.5. Sequential DME extraction and de-esterification

The sequential or 2-step process began with DME extraction, followed by de-esterification of the oleoresin. In each step, the most suitable extraction condition and de-esterification condition as suggested by the results in the previous sections were employed. First, 0.5-g powder of dried marigold flowers was extracted by DME following the procedure described in Section 2.2.1. Once the extraction was complete, DME was evaporated. Approximately 0.2 g of marigold oleoresin was diluted with hexane and was then transferred to a 35-ml vial. Then, hexane was evaporated by purging the solution with nitrogen. As already mentioned in Section 2.3.2, the oleoresin was reacted with KOH–EtOH solution at the suitable de-esterification condition determined from the previous experiment. The resulting de-esterified product was washed and extracted according to the method of Wang et al. (2016) as described in Section 2.3.2. The washed free lutein solution was then stored at 4 °C until analysis by HPLC.

2.6. Analytical methods

2.6.1. Analysis of total xanthophylls

The amount of total xanthophylls in marigold flowers extract was determined by measuring spectrophotometric absorbance at 428 nm using the WHO/FAO method (JECFA, 2014). Briefly, 80 ml of hexane and 5 ml of 2-propanol were added to the extract, and the mixture was then poured into a volumetric flask in which its volume was adjusted to 100 ml, prior to the spectroscopic measurements of total xanthophylls.

2.6.2. HPLC analysis

The de-esterified solutions were analyzed using HPLC to determine the amount of free lutein. 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).

3. Results and discussion

3.1. Determination of suitable DME extraction conditions

The effect of DME extraction condition including DME to dried marigold flowers weight ratio and extraction temperature on total xanthophylls yield are shown in Fig. 2. It is noted that the error bars in Fig. 2 and other results were determined based on the duplicate experiments. The total xanthophylls content was found to increase with increasing DME to dried marigold flowers ratio from 20:0.5 to 33:0.5 (g/g) and stayed rather constant above this ratio as shown in Fig. 2(a). The highest total xanthophylls yield was found to be 20.65 mg/g dried marigold flowers at the solvent to sample ratio of 33:0.5 (g/g). The results might be explained by the fact that, as the solvent increases, solute-solvent interactions are also enhanced, and

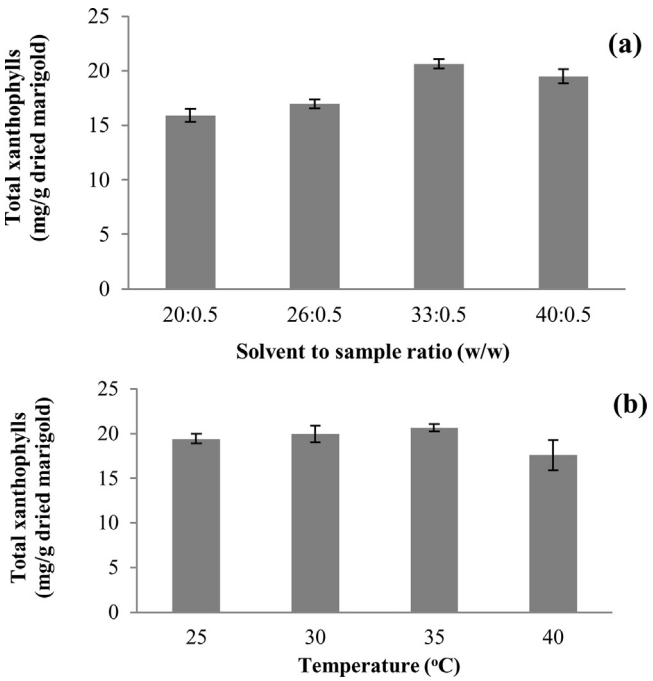

Fig. 2 – Effect DME extraction parameters on total xanthophylls yield: (a) solvent to sample ratio (temperature at 35 °C); (b) temperature (DME to dried marigold flowers ratio at 33:0.5).

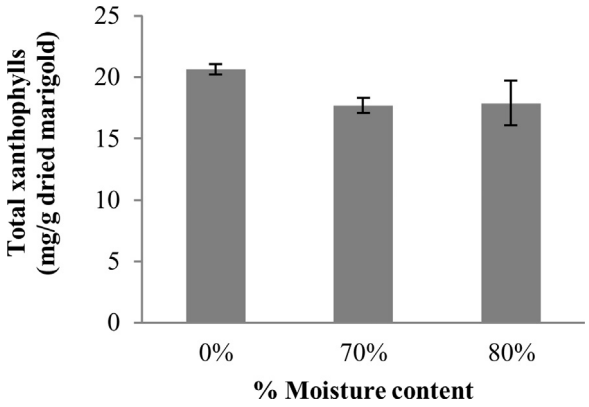

Fig. 3 – Comparison of total xanthophylls yields obtained by DME extraction of wet and dry marigold flowers (DME to dried marigold flowers ratio 33:0.5, 35 °C, 30 min).

this then enhances the total xanthophylls extracted and solubilized into the solvent. The effect of extraction temperature on total xanthophylls yield shown in Fig. 2(b) was then determined at the suitable solvent to sample ratio (33:0.5 (g/g)). The total xanthophylls 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 were seen among various temperatures up to 35 °C, the subsequent experiment was carried out at 35 °C (the regional average room temperature).

The yield of total xanthophylls using dried sample obtained at the suitable condition was then compared with that using wet sample. As shown in Fig. 3, total xanthophylls yields obtained with DME extraction of wet marigold samples of 80% and 70% moisture content were 17.89 and 17.71 mg/g dried marigold flowers, respectively. Compared to that obtained with dried marigold flowers (20.65 mg/g dried marigold flowers), they were slightly lower. The presence of water possibly

increased overall solvent polarity (Billakanti et al., 2013) led to lowering the solubility, and thus the extractability of relatively non-polar compounds such as lutein fatty acid esters. It should be noted that the uncertainties reported in this study were based on duplicate experiments. Nevertheless, if all other uncertainties including those resulted from weighing, transfer losses, and etc., had been taken into account to the overall uncertainty, the differences seen in the extraction yields of the dried and wet samples would have been insignificant.

3.2. Comparison of yields obtained with different solvents

The yields of total xanthophylls obtained using three different solvents were compared. The results in Table 1 clearly indicated that SC-CO_2 extraction yielded the lowest amount of total xanthophylls (15.91 mg/g dried marigold), despite the highest solvent to sample ratio (428 ml: 0.5 g dried marigold flower). Even though total xanthophylls are relatively non-polar, the molecular weights of the xanthophylls (MW = 800–1000) and that of CO_2 (MW = 44) differ considerably, making the SC-CO_2 an unfavorable solvent. Comparison between DME and hexane extractions suggested that DME extraction gave higher total xanthophylls yield (20.65 vs. 17.58 mg/g dried marigold flowers. Despite the higher solvent to sample ratio used compared with hexane extraction (66:1 vs. 3.3:1 g/g), the fact that DME can be easily separated from the extract and recycled, making it the most favorable of the three solvents for extraction of total xanthophylls. In addition, the solvent to sample ratio could be further be reduced with use of a continuous flow process. The investigation in such process is underway.

3.3. Determination of suitable de-esterification conditions

The effects of de-esterification conditions: EtOH to marigold oleoresin (ml/g) ratio, KOH–EtOH concentration, de-esterification temperature and time on free lutein yield are shown in Fig. 4. Firstly, the effect of EtOH to marigold oleoresin (ml/g) ratio on free lutein yield was investigated. The results shown in Fig. 4(a) revealed that the amount of free lutein increased with an increase in EtOH to marigold oleoresin ratio from 10:1 to 20:1 (ml/g) to the highest amount of 150.79 mg/g marigold oleoresin. As the ratio increased to 30:1, the amount of free lutein decreased. The increase in EtOH volume initially helped improve mass transfer, as a result of increased contact between KOH and oleoresin, which in turn promoted de-esterification. Nevertheless, excess amount of EtOH on the other hand, could lower KOH–EtOH concentration, which thus lowered the rate of de-esterification. This resulted in incomplete reaction, as evidenced by the presence of lutein fatty acid esters peaks in the chromatogram shown in Fig. S1.

To ensure that lutein fatty acid esters are completely de-esterified, sufficient amount of alkali is needed. The suitable KOH–EtOH concentrations yielding the highest amount of free lutein was therefore determined. The results shown in Fig. 4(b) indicated that at 2.5% (w/v) KOH–EtOH concentration, the highest amount of free lutein (150.79 mg/g marigold oleoresin) was obtained. The amount of free lutein increased with the increase in KOH–EtOH concentration from 1.5 to 2.5% (w/v), nevertheless, at higher KOH–EtOH concentration, the amount of free lutein stayed relatively constant. It can be implied from the results that the concentration of 2.5% (w/v) KOH–EtOH was sufficient for the de-esterification.

The effect of de-esterification temperature on the amount of free lutein is shown in Fig. 4(c). The amount of free lutein was significantly increased with increasing de-esterification temperature from 30 to 35 °C, but only slightly increased with increase in temperature to 40 °C. The results could be explained by the increased rate of reaction with temperature, nevertheless free lutein is a heat sensitive compound, that could readily be degraded at high temperature (Davidov-Pardo et al., 2016).

The effect of de-esterification time on free lutein yield shown in Fig. 4(d) showed that the highest free lutein yield (150.79 mg/g oleoresin) was obtained after 4 h. This result was supported by the chromatograms shown in Fig. S2, 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.

3.4. Simultaneous DME extraction and de-esterification of lutein fatty acid esters to free lutein

The effects of process conditions including EtOH to dried marigold ratio, KOH–EtOH concentration and mixing time on free lutein yield are shown in Fig. 5. As seen from Fig. 5(a), at the EtOH to dried marigold ratio of 5:0.5 (ml/g), only a small amount of free lutein was obtained, due to the incomplete de-esterification, as confirmed by the chromatogram in Fig. S3(a). At the ratios of 10:0.5 and 15:0.5 (ml/g) on the other hand, complete de-esterification as confirmed by the chromatogram in Fig. S3(b) and (c) resulted in higher amounts of free lutein (18.96 and 19.65 mg/g dried marigold, respectively). Given that the amounts of marigold and DME remained the same, higher amount of EtOH could be responsible for the higher mass transfer within the de-esterification and extraction system, leading to the increased lutein yield. The suitable EtOH to dried marigold ratio was found to be 10:0.5 (ml/g) since it was able to achieve comparable amount of free lutein to that obtained at the ratio 15:0.5 (ml/g).

Table 1 – Comparison of total xanthophylls extraction yields by various solvents.

Solvents	Solvent to sample ratio	Conditions	Total xanthophylls (mg/g dried marigold)
DME/batch process	DME: dried marigold = 33 g: 0.5 g or = 51.5 ml: 0.5 g (@ T = 35 °C, ρ = 0.64 g/ml) (Ihmels and Lemmon, 2007)	35 °C, 400 rpm and 30 min	20.65
Hexane/batch process	Hexane: dried marigold = 50 ml: 10 g	40 °C, 150 rpm and 4 h.	17.58
SC-CO_2/continuous flow process	CO_2: dried marigold = 428 ml: 0.5 g	60 °C, 40 MPa and 4 h	15.91

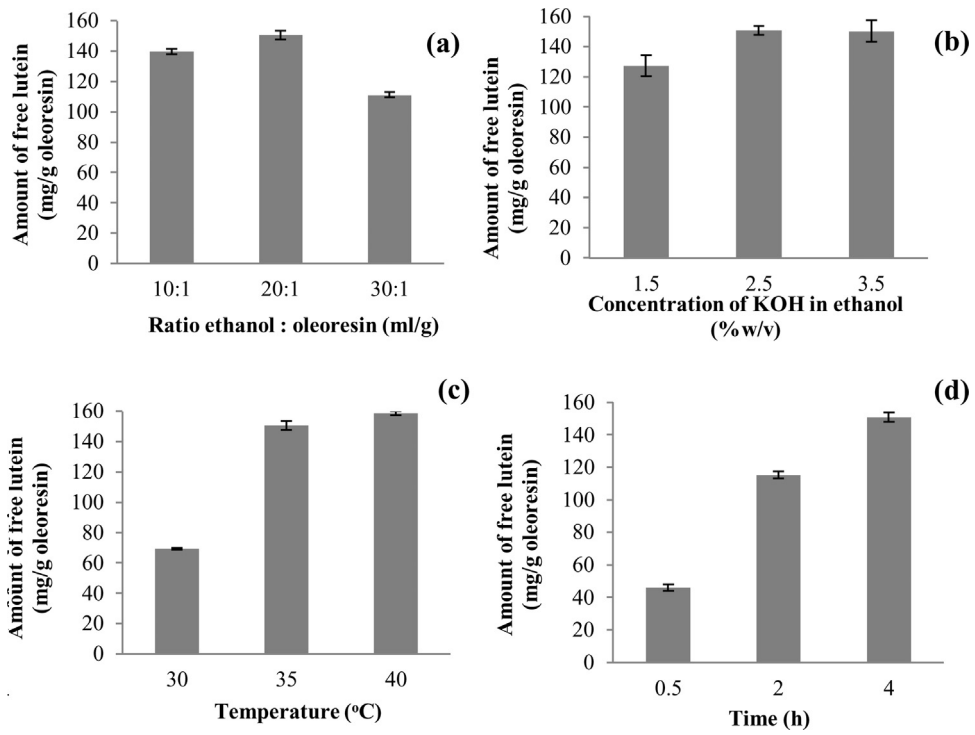

Fig. 4 – Effects of de-esterification parameters on free lutein yield: (a) EtOH to marigold oleoresin ratio (v/w) (KOH 0.5 g, 35 °C, 4 h); (b) KOH–EtOH concentration (EtOH to oleoresin ratio 20:1,35 °C, 4 h); (c) temperature (EtOH to marigold oleoresin ratio 20:1, 2.5% KOH, 4 h); (d) time (EtOH to marigold oleoresin ratio 20:1, 2.5% KOH, 35 °C).

Table 2 – Comparison of free lutein yield obtained with dried and wet marigold flowers by simultaneous 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 °C, 400 rpm and 1 h	20.71
Simultaneous process/using wet sample (80% moisture content)		19.22

Table 3 – Comparison of free lutein yields obtained by simultaneous process and sequential processes.

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 °C, 400 rpm and 1 h	20.71
Sequential process	DME extraction; DME to dried marigold ratio 33:0.5, 35 °C, 400 rpm, 30 min. De-esterification; ethanol to oleoresin ratio 4:0.2, 2.5% KOH, 35 °C, 150 rpm, 4 h	16.72

The effect of KOH–EtOH concentration on free lutein yield is shown in Fig. 5(b). Free lutein yield was found to increase with the increase in KOH–EtOH concentration from 3% to 5%, nevertheless, it decreased with further increase in KOH–EtOH concentration to 7%. Although the increase in KOH–EtOH concentration for higher free lutein conversion, free lutein was unstable in strong alkaline environment, thus too high KOH–EtOH concentration caused lowering of free lutein. The maximum amount of free lutein was obtained at the KOH–EtOH concentration of 5%w/v (18.96 mg/g dried marigold flowers).

The effect of mixing time shown in Fig. 5(c) 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 suitable for the simultaneous process, yielding approximately 20.71 mg free lutein/g dried marigold flowers.

The suitable conditions for simultaneous DME extraction and de-esterification using dried marigolds were found to be DME to dried marigold flowers ratio of 33:0.5 (g/g), EtOH to dried marigold flowers ratio of 10:0.5 (ml/g), 5%w/v KOH–EtOH concentration, temperature of 35 °C, and simultaneous extraction and de-esterification time of 1 h, giving approximately 20.71 mg free lutein/g dried marigold flowers. At the same conditions, the results shown in Table 2 indicated that the slightly lower yield of free lutein (19.22 mg/g dried marigold) was obtained using the wet samples (with 80% moisture content). Nevertheless, the cost savings on drying marigold flowers could make the process rather attractive.

3.5. *Comparison of simultaneous and sequential processes*

Compared with the sequential process, the results in Table 3 showed that the free lutein yield obtained by the simultaneous process was approximately 20% higher (20.71 versus

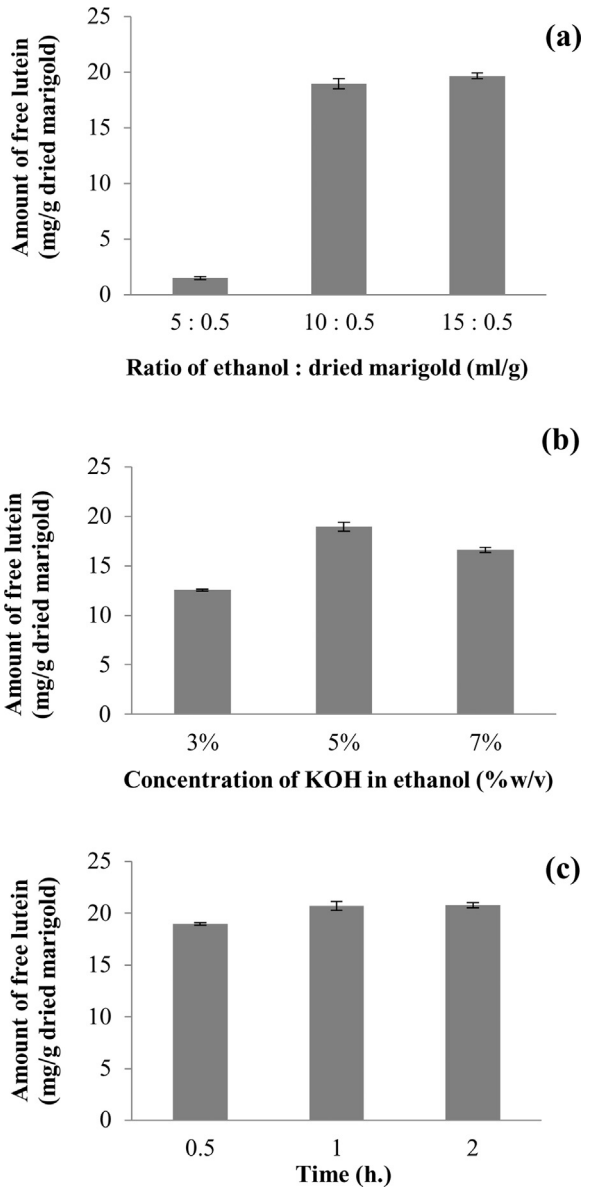

Fig. 5 – Effect of simultaneous process parameters on free lutein yield: (a) EtOH to dried marigold flowers ratio (KOH 0.5 g, 35 °C, 30 min); (b) KOH–EtOH concentration (EtOH to dried marigold flowers ratio 10:0.5, 35 °C, 30 min); (c) time (EtOH to dried marigold flowers ratio 10:0.5, 5% KOH, 35 °C).

16.72 mg/g dried marigold). In this experiment, the amounts of the remaining lutein esters in the marigold residues from the simultaneous and the sequential processes were also checked by re-extraction with hexane. The unconverted total xanthophylls remained in the samples were also determined spectroscopically, and it was found that the amount of unconverted total xanthophylls in the sample having undergone the simultaneous process was smaller than that of the sequential process (0.9 versus 2.20 mg/g dried marigold). In the simultaneous process, lutein esters were continuously converted to free lutein, which was more easily soluble in DME, and was simultaneously extracted into DME. These results therefore confirmed that not only did simultaneous process require fewer steps, enhanced de-esterification and extraction efficiency could also be achieved by such system.

4. Conclusions

Liquefied DME shows potential for extraction of total xanthophylls from dried marigold flower. At the suitable condition (33:0.5 (w/w) DME to dried marigold flowers ratio, 35 °C), 20.65 mg of total xanthophylls/g dried marigold could be obtained. Compared with SC-CO_2 and hexane, DME was a more favorable solvent for extraction of total xanthophylls. The suitable de-esterification conditions to convert marigold lutein esters to free lutein found were found to be 2.5%w/v KOH–EtOH, 35 °C, and 4 h. By applying the suitable DME extraction and de-esterification conditions to the sequential process, 16.65 mg free lutein/g dried marigold could be obtained. Furthermore, process improvement could be achieved by a one-step process of simultaneous extraction and de-esterification, giving approximately 20% higher yield of free lutein (20.71 mg free lutein/g dried marigold). In addition, the possibilities of using of wet samples for both obtaining xanthophylls by DME extraction and obtaining free lutein by the simultaneous process were demonstrated.

Acknowledgements

This work was supported by the Thailand Research Fund (MRG5980085 and IRG5780014), the Ratchadapiseksompot Endowment Fund (CU-57-021-FW), and the Naresuan University grant (R2559C025). Provision of powder of dried marigold flowers by PTT Global Chemical, Co., Ltd. is also greatly appreciated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fbp.2017.10.002.

References

Attokaran, M., 2011. Natural Food Flavors and Colorants. Blackwell Publishing Ltd., John Wiley & Sons Ames, Iowa.

Billakanti, J.M., Catchpole, O.J., Fenton, T.A., Mitchell, K.A., MacKenzie, A.D., 2013. Enzyme-assisted extraction of fucoxanthin and lipids containing polyunsaturated fatty acids from Undaria pinnatifida using dimethyl ether and ethanol. Process Biochem. 48, 1999–2008.

Calderone, M., Tallon, S., 2008. Particle formation by rapid expansion from solution using near-critical dimethyl-ether. J. Supercrit. Fluids 45, 245–252.

Catchpole, O., Ryan, J., Zhu, Y., Fenton, K., Grey, J., Vyssotski, M., MacKenzie, A., Nekrasov, E., Mitchell, K., 2010. Extraction of lipids from fermentation biomass using near-critical dimethylether. J. Supercrit. Fluids 53, 34–41.

Davidov-Pardo, G., Gumus, C.E., McClements, D.J., 2016. Lutein-enriched emulsion-based delivery systems: influence of pH and temperature on physical and chemical stability. Food Chem. 196, 821–827.

Espinosa-Pardo, F.A., Nakajima, V.M., Macedo, G.A., Macedo, J.A., Martiínez, J., 2017. Extraction of phenolic compounds from dry and fermented orange pomace using supercritical CO_2 and cosolvents. Food Bioprod. Process. 101, 1–10.

Gao, Y., Nagy, B., Liu, X., Simándi, B., Wang, Q., 2009. Supercritical CO_2 extraction of lutein esters from marigold (*Tagetes erecta* L.) enhanced by ultrasound. J. Supercrit. Fluids 49, 345–350.

Gao, Y., Liu, X., Xu, H., Zhao, J., Wang, Q., Liu, G., Hao, Q., 2010. Optimization of supercritical carbon dioxide extraction of lutein esters from marigold (*Tagetes erecta* L.) with vegetable

oils as continuous co-solvents. Sep. Purif. Technol. 71, 214–219.

Gong, Y., Hou, Z., Gao, Y., Xue, Y., Liu, X., Liu, G., 2012. Optimization of extraction parameters of bioactive components from defatted marigold (*Tagetes erecta* L.) residue using response surface methodology. Food Bioprod. Process. 90, 9–16.

Goto, M., Kanda, H., Wahyudiono, Machmudah, S., 2015. Extraction of carotenoids and lipids from algae by supercritical CO_2 and subcritical dimethyl ether. J. Supercrit. Fluids 96, 245–251.

Han, J., Wang, Y., Ma, J., Wu, Y., Hu, Y., Ni, L., Li, Y., 2013. Simultaneous aqueous two-phase extraction and saponification reaction of chlorophyll from silkworm excrement. Sep. Purif. Technol. 115, 51–56.

Ho, S.H., Xie, Y., Chan, M.C., Liu, C.C., Chen, C.Y., Lee, D.J., Huang, C.C., Chang, J.S., 2015. Effects of nitrogen source availability and bioreactor operating strategies on lutein production with *Scenedesmus obliquus* FSP-3. Bioresour. Technol. 184, 131–138.

Hojnik, M., Škerget, M., Knez, Ž., 2008. Extraction of lutein from marigold flower petals—experimental kinetics and modelling. Food Sci. Technol. 41, 2008–2016.

Ihmels, E.C., Lemmon, E.W., 2007. Experimental densities, vapor pressures, and critical point, and a fundamental equation of state for dimethyl ether. Fluid Phase Equilib. 260, 36–48.

JECFA, 2014. Lutein Esters from Tagetes Extract (Tentative), Compendium of Food Additive Specifications. Joint FAO/WHO Expert Committee on Food Additives, Food and Agricultural Organization of the United Nations, Rome, Italy, pp. 23–26.

Lin, J.H., Lee, D.J., Chang, J.S., 2015a. Lutein production from biomass: marigold flowers versus microalgae. Bioresour. Technol. 184, 421–428.

Lin, J.H., Lee, D.J., Chang, J.S., 2015b. Lutein in specific marigold flowers and microalgae. J. Taiwan Inst. Chem. Eng 49, 90–94.

Luengo, E., Condoín-Abanto, S., Condoín, S., Aílvarez, I., Raso, J., 2014. Improving the extraction of carotenoids from tomato waste by application of ultrasound under pressure. Sep. Purif. Technol. 136, 130–136.

Noriyasu, A., Furukawa, H., Kikuchi, A., Takaichi, H., Bouteau, F., Li, X., Nishihama, S., Yoshizuka, K., Kawano, T., 2015. Use of liquefied cold temperature dimethyl ether for extraction of pigments from fresh vegetable tissues. Adv. Hortic. Sci. 29, 48–52.

Palumpitag, W., Prasitchoke, P., Goto, M., Shotipruk, A., 2011. Supercritical carbon dioxide extraction of marigold lutein fatty acid esters: effects of cosolvents and saponification conditions. Sep. Sci. Technol. 46, 605–610.

Park, J.S., Chew, B.P., Wong, T.S., 1998. Dietary lutein absorption from marigold extract is rapid in BALB/c Mice[1,2,3]. J. Nutr. 128, 1802–1806.

Prommuak, C., De-Eknamkul, W., Shotipruk, A., 2008. Extraction of flavonoids and carotenoids from Thai silk waste and antioxidant activity of extracts. Sep. Purif. Technol. 62, 444–448.

Shibata, S., Ishihara, C., Matsumoto, K., 2004. Improved separation method for highly purified lutein from chlorella powder using jet mill and flash column chromatography on silica gel. J. Agric. Food Chem. 52, 6283–6286.

Siriamornpun, S., Kaisoon, O., Meeso, N., 2012. Changes in colour, antioxidant activities and carotenoids (lycopene, β-carotene, lutein) of marigold flower (*Tagetes erecta* L.) resulting from different drying processes. J. Funct. Foods 4, 757–766.

Skerget, M., Bezjak, M., Makovsek, K., Knez, Z., 2010. Extraction of lutein diesters from tagetes erecta using supercritical CO_2 and liquid propane. Acta Chim. Slov. 57, 60–65.

Tian, Y., Kijlstra, A., Webers, C.A.B., Berendschot, T.T.J.M., 2015. Lutein and factor D: two intriguing players in the field of age-related macular degeneration. Arch. Biochem. Biophys. 572, 49–53.

Vechpanich, J., Shotipruk, A., 2010. Recovery of free lutein from *Tagetes erecta*: determination of suitable saponification and crystallization conditions. Sep. Sci. Technol. 46, 265–271.

Wang, T., Han, J., Tian, Y., Zhang, D., Wang, Y., Wu, Y., Ni, L., 2016. Combined process of reaction, extraction, and purification of lutein in marigold flower by isopropanol–KOH aqueous two-phase system. Sep. Sci. Technol. 51, 1490–1498.