Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Janya Vechpanich[a]; Artiwan Shotipruk[a]
[a] Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Patumwan, Bangkok, Thailand

To cite this Article Vechpanich, Janya and Shotipruk, Artiwan(2011) 'Recovery of Free Lutein From *Tagetes erecta*: Determination of Suitable Saponification and Crystallization Conditions', Separation Science and Technology, 46: 2, 265 — 271
To link to this Article: DOI: 10.1080/01496395.2010.506904
URL: http://dx.doi.org/10.1080/01496395.2010.506904

Separation Science and Technology, 46: 265–271, 2011
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ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496395.2010.506904

Recovery of Free Lutein From *Tagetes erecta*: Determination of Suitable Saponification and Crystallization Conditions

Janya Vechpanich and Artiwan Shotipruk
Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Patumwan, Bangkok, Thailand

This study deals with the separation and purification of lutein from marigold flowers. The effects of saponification conditions such as time (0–7 hr), volume to weight ratio of KOH solution to oleoresin (0.3 ml:1 g to 1 ml:1 g), and the concentration of KOH solution (25%–45% w/v) were first determined on the conversion of lutein fatty acid esters to free lutein. For 1 g of oleoresin, 0.5 ml of 40% v/v KOH solution was found to be the most suitable amount and concentration for the saponification of lutein esters to free lutein at 75°C. Following saponification, crystallization was carried out to determine the effect of crystallization solvents (water-ethanol and water-isopropanol mixtures), the composition of the crystallization solvents (2:0.3–2:1 v/v), and crystallization time (0.25–2 hr) on the yield and purity of free lutein. The results revealed that crystallization of the saponified sample in water-ethanol mixture at the volume ratio of 2:0.5 for 0.5 hr presented the highest yield of 99.12% of free lutein, at the purity of 85.53%.

Keywords lutein; lutein fatty acid esters; marigold flower

INTRODUCTION

Tagetes erecta, commonly known as marigold, is an herbaceous and ornamental plant in the daisy family, cultivated in the tropical areas of the southwest of the United States, South America, and Asia (1). In recent years, the use of marigold flower extract as nutritional and medicinal supplements has been explored and its potential benefits have been reported including the prevention of cancer and cardiovascular disease, enhanced immune function, inhibition of auto-oxidation of cellular lipids, and protection against oxidant-induced cell damage (2).

The most important group of bioactive compounds extracted from marigold is xanthophylls, the major one being lutein. In marigold flowers, lutein takes a more stable acylated form against heat and UV-light, as lutein fatty acid esters (3). However, free lutein has been shown to be more active against cardiovascular disease (4), stroke, UV-radiation-induced skin damage, and eye diseases such as cataracts and age-related macular degeneration (5).

Extraction of lutein esters as well as the purification process to obtain high purity free lutein from marigold flowers have recently attracted considerable interest. Philip et al. (8) isolated lutein fatty acid esters by dissolution of oleoresin in hot alkanol followed by precipitation of lutein fatty acid esters by cooling the solution. The resulting lutein fatty acid esters contained 51% lutein. At such purity, the product was still not suitable for use as human food which should generally be about 90% pure (6). To improve the quality of the extracted lutein, saponification of lutein fatty acid esters to free lutein is required, followed by crystallization to obtain purified lutein crystals (6,7). Although several processes have been proposed to extract and purify lutein from marigold flowers (8–12), most of these studies emphasized only the final purification process to obtain free lutein crystals. In this study, detailed experiments were carried out to determine the suitable saponification process, as well as the purification process to obtain free lutein. First, dried marigold flower were extracted and the amount of total xanthophylls in the resulted oleoresin were determined. Then, the determination of suitable saponification conditions such as the amount and concentration of the saponification agent and saponification time were carried out. Finally, the crystallization conditions (the type, the amount, and the composition of crystallization solvents, and crystallization time) to obtain high purity free lutein were examined.

EXPERIMENTAL

Materials and Chemicals

Pulverized dried marigold flowers were obtained from the Marigold Production Group, Nakhonratchasima, Thailand. Hexane, used for the extraction of pigments was purchased from Merck, USA. Lutein standard (99% purity) as well as all other chemicals: ethanol, potassium hydroxide, and hydrochlolic acid used were analytical grade, and were purchased from Sigma-Aldrich, Germany. Acetonitrile, methanol, and ethyl acetate used for high

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Received 3 March 2010; accepted 5 July 2010.

Address correspondence to Artiwan Shotipruk, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Patumwan, Payathai, Bangkok, 10330, Thailand. E-mail: artiwan.sh@chula.ac.th

performance chromatography analysis were HPLC grade, and were purchased from Sigma-Aldrich, Germany.

Determination of Total Xanthophylls in Marigold Oleoresin

In order to determine the total xanthophylls content in the sample, 100 g of fine pulverized marigold flower sample was extracted repeatedly with hexane. The initial extraction was carried out in 500 ml of hexane at 40°C for 4 hr. The remaining debris was separated and re-extracted 3 times, each with 500 ml of hexane at 30°C, for 1 day, 3 days, and 10 days, respectively. The amount of the xanthophylls in each extract was determined by first evaporating the solvent from the sample solution under vacuum at 40°C for 15 min. The resulted oleoresin was further dried for 2 hr in a vacuum oven at 30°C. The dried oleoresin was then dissolved in ethanol and the amount of total xanthophylls was then measured using a spectrophotometer.

Preparation of Marigold Oleoresin

To obtain marigold oleoresin used in saponification and subsequent crystallization study, 10 batches of extractions were carried out. In each batch, 100 g of fine ground sample was extracted with 500 ml of hexane at 40°C for 4 hr. The resulting extract was evaporated by using a rotary vacuum evaporator (EYELA rotary evaporator N-100) at 40°C for 15 min, and the oleoresin was then dried for the next 2 hr in a vacuum oven at 30°C. For each of the 100 g of the fine ground samples, approximately 10 g of dried oleoresin was obtained.

Saponification of Lutein Fatty Acid Esters

Saponification was carried out by first dissolving 1 g of marigold oleoresin in 2 ml of ethanol at 75°C. Into the mixture, a specified amount of 45% w/v potassium hydroxide solution was added slowly. The mixture was then vortexed for 1 min, and the reaction was allowed to take place for a specified period during which the mixture was vortexed every hour. After the specified period was reached, the mixture was cooled to 65°C, and the pH was adjusted to about 7.0 with an aqueous solution of hydrochloric acid. Here the effects of the process conditions such as saponification time, the volume to weight ratio of KOH solution to marigold oleoresin, and the concentration of KOH solution were determined on the conversion of lutein fatty acid esters to free lutein. The variables investigated and their ranges are summarized in Table 1. It should be noted that the resulting saponified mixture can generally be subjected to crystallization to further purify the free lutein product. For the analysis of free lutein in the saponified product however, the following steps are required. Into the saponified mixture, 50 ml of ethanol was added, and the mixture was sonicated at 30 ± 5°C for 15 min in an ultrasonic bath (275DAE, 270 W, 38.5 kHz, Crest Ultrasonics, USA) containing water. The mixture was then allowed to settle and separate into two phases. The top phase was a clear yellow ethanol rich phase containing the desired free lutein product, while the bottom phase was mostly soap, water, and salt, resulted from neutralization. This mixture was then centrifuged to completely separate the two phases. To recover all the free lutein, the bottom phase was extracted again in 50 ml of ethanol under ultrasonication as described above. The combined top phase solution was then analyzed for the amount of free lutein using high performance liquid chromatography (HPLC). All experiments were conducted in duplicate.

TABLE 1
Variables and ranges of variables for saponification

Variables	Ranges of variables	Fixed conditions
Saponification time	0, 3, 4, 5, and 7 hr	75°C, 1 ml of 45% KOH solution
Volume of KOH solution (ml)	0.3, 0.5, 0.8, and 1	75°C, 45% KOH solution, 4 hr
Concentration of KOH solution	25%, 35%, 40%, and 45%	75°C, 0.5 ml of KOH solution, 4 hr

Crystallization of Free Lutein

The effects of crystallization conditions on the yield and purity of free lutein crystals were investigated including the type of binary crystallization solvent mixtures, volume ratio of the binary solvent mixture, and the crystallization time. First, the suitability of the two binary solvent mixture systems was examined: water-ethanol and water-isopropanol at various ratios. The selected system was then employed to determine the suitable crystallization time. The ranges of experimental variables are summarized in Table 2. To carry out crystallization, the saponified

TABLE 2
Variables and ranges of variables for crystallization

Variables	Ranges	Fixed variables
The type of binary solvent mixtures for crystallization	water:ethanol and water: isopropanol	65°C, 0.5 hr
The v/v ratio of water: ethanol and water: isopropanol	2:0.3, 2:0.5, 2:0.8, and 2:1	65°C, 0.5 hr
Crystallization time (hr)	0.25, 0.5, 1, 1.5, 2	65°C, water: ethanol (2:0.5)

oleoresin mixture was first prepared at the optimal saponification condition following the procedure described previously. The saponified mixture was then dissolved in a specified crystallization solvent mixture, which was then maintained at the crystallization temperature of 65°C to prevent the sample from solidifying. After 30 min the yellow precipitate was obtained. The mixture was then filtered through a filter paper (Whatman No. 5, USA) under suction until dried. The dried precipitate was weighed and the amount of free lutein was analyzed in order to determine the yield (the amount of free lutein in the precipitate per the total amount of free lutein in oleoresin) and the purity (the amount of free lutein per weight of dried precipitate) of the resulted product. In order to analyze the free lutein content in the dried precipitate, the dried sample was redissolved in 50 ml of ethanol under sonication at 30 ± 5°C for 15 min. A small amount of insoluble impurity suspended in the solution was separated from the soluble portion by centrifugation. This suspended solid was extracted again with 50 ml of ethanol to recover all lutein, and the combined extract solutions were then analyzed for the concentration of free lutein using HPLC, from which the total amount of free lutein in the precipitate can be determined.

Analysis of Total Xanthophylls, Free Lutein and Lutein, Fatty Acid Esters

To determine the amount of total xanthophylls in the oleoresin, the oleoresin was dissolved in ethanol, and the solution was measured for the absorbance at 478 nm with a spectrophotometer using lutein as a standard (13). The free lutein and lutein fatty acid esters in the saponified sample and the purified precipitate were analyzed by HPLC following the method described by Piccaglia et al. (14). The sample solution was injected to Lichrocart C-18 column, a Diode Array Detector Module 335, and an automatic injector. A 5 μm reversed-phase was used. Chromatographic separation was obtained with a gradient solvent system of acetonitrile:methanol (9:1, v:v) (A) and ethylacetate (B), from 0% to 100% of B using a linear gradient over 30 min, at a flow rate of 1 ml/min, and detection wavelength at 450 nm. The component identification was confirmed by LC-MS (model 6320 ion trap, Agilent, USA).

RESULTS AND DISCUSSION

Total Amount of Xanthophylls in Marigold Flower

The amounts of total xanthophylls in the extracts obtained by repeated extractions of dried marigold flowers are shown in Table 3. The results indicated that the marigold sample contained 25.77 mg xanthophylls/g dry weight. This is equivalent to approximately 257.70 mg/g oleoresin. Of this total amount, more than 80% was extracted in the initial extraction, and only small percentages, 12.29%, 6.18%, and 0.26%, were extracted in the subsequent repeated extractions. It should be noted that for the preparation of the marigold oleoresin for subsequent study, only one extraction was carried out with hexane for 4 hr at 40°C.

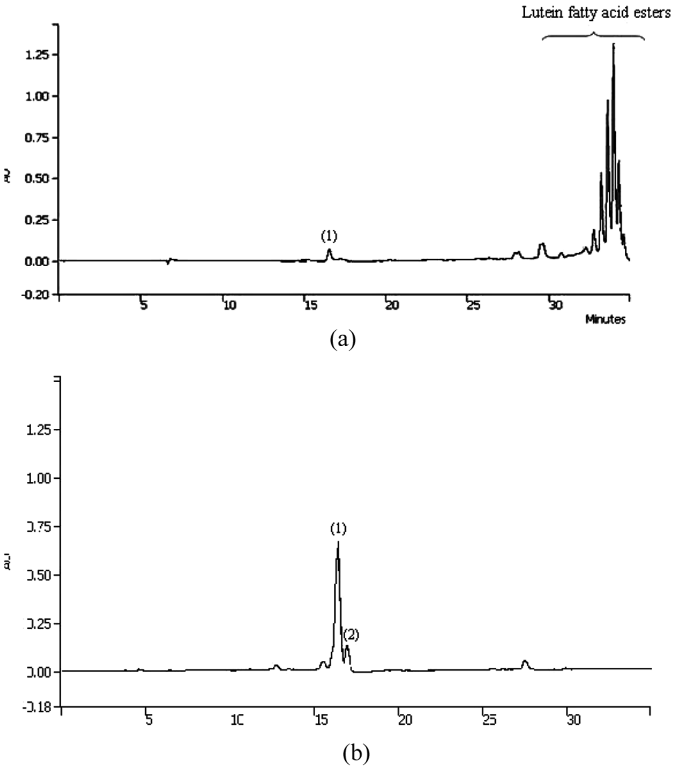
TABLE 3
Total xanthophylls in marigold oleoresin

Extraction number	Extraction Time/ Temperature (°C)	Total xanthophylls (mg/g oleoresin)	% Total xanthophyll
1	4 hr-40°C	209.4	81.27
2	1 day-30°C	31.66	12.29
3	2 day-30°C	15.91	6.18
4	7 day-30°C	0.68	0.26
	Total	257.7	100

Determination of Suitable Saponification Conditions

Effects of Saponification Time

Typical chromatogram of ethanol solution of marigold oleoresin and the saponified solution of marigold oleoresin are shown in Fig. 1. As shown in Fig. 1a, the retention time of free lutein (1) was 16 min, while the more nonpolar



(a)

(b)

FIG. 1. Chromatog of marigold oleoresin (a) before saponification and (b) after saponification.

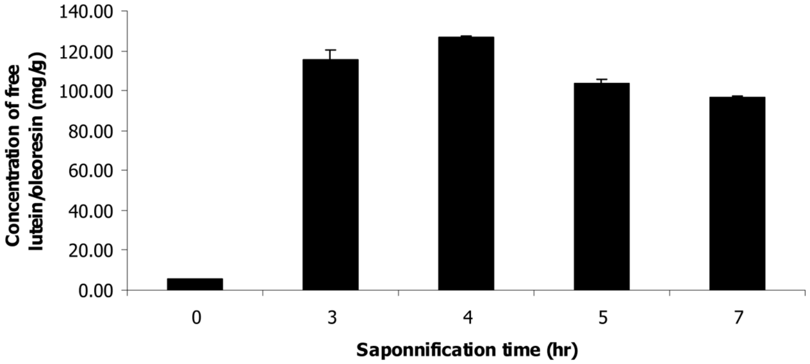


FIG. 2. Effects of saponification time on amount of free lutein.

lutein esters eluted at a later time. The chromatogram of the saponified sample in Fig. 1b clearly indicated that lutein fatty acid esters could be converted to free lutein after saponfication. The small peak (2) next to free lutein was identified as a decomposed lutein product in which a hydroxyl group was taken out, possibly as a result of the degradation by high alkali and acid conditions during saponification and possible over-neutralization. The conversion of lutein fatty acid esters to free lutein was found to depend on saponification time as shown in Fig. 2. This reveals that the free lutein content per unit weight of oleoresin increased with the increase in saponification time up to 4 hr, after which the free lutein content decreased. The increase in free lutein during the first 4 hr was possibly due to the fact that different times were required for different types of lutein fatty acid esters. During the initial period, saponification of monoesters of lutein containing an alkyl group, such as lutein esters of lauric, myristic, or palmitic acids could more readily occur. While the lutein diesters such as lutein dimyristate, dipalmitate, or distearate having two alkyls groups took a longer time to convert into free lutein. The decrease in free lutein concentration at 5 and 7 hr however could be due to the degradation of the products after an extended period of exposure to alkali condition. From this experiment, the maximum content of free lutein of 126 mg/g oleoresin was achieved in 4 hr of saponification. Hence, the saponification time of 4 hr was used in the subsequent experiments to determine the suitable volume to weight ratio of KOH to oleoresin and the suitable concentration of KOH solution.

Effects of Volume to Weight Ratio of KOH Solution to Marigold Oleoresin

For the complete saponification to occur, it is generally required that a sufficient amount of alkali be used. This required amount could possibly be prepared at various concentrations of alkali solution, given that the corresponding volumes were used. It would be desirable to use the smallest volume of KOH at rather high concentration to minimize the reactor size, thus making the process more economically feasible. However, the concentrated KOH could cause degradation of the product, and thus the suitable KOH concentration and hence the volume would be those that yield the maximum results. In this investigation, the concentration of KOH solution of 45% was initially chosen as a starting point, based on preliminary study

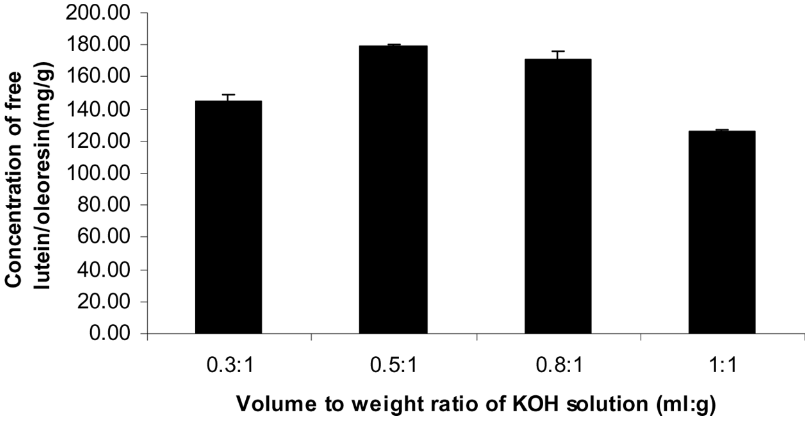


FIG. 3. Effects of volume to weight ratio of KOH solution to marigold oleoresin on amount of free lutein.

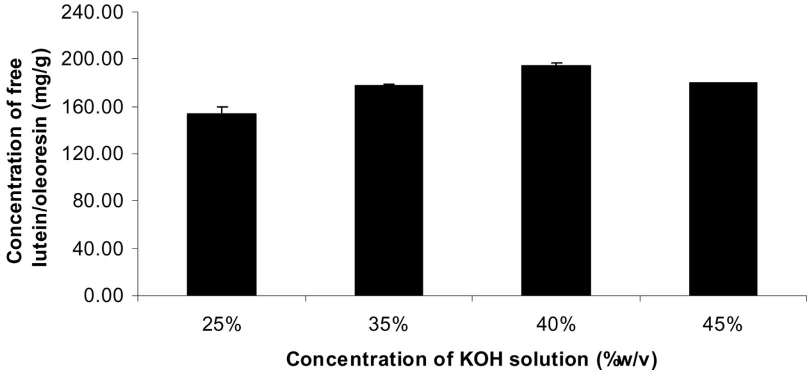


FIG. 4. Effects of concentration of KOH solution on amount of free lutein.

and that reported by other investigators (11). What remained to be determined however was the suitable volume of this KOH solution. Here, various volumes, 0.3, 0.5, 0.8, and 1 ml, of 45% KOH solution were reacted with 1 g of marigold oleoresin at 75°C for 4 hr. The results in Fig. 3 show that the highest amount of free lutein (179 mg/g oleoresin) was obtained with 0.5 ml of 45% KOH solution. The lower volume of KOH solution probably could not provide a sufficient amount of reactant for saponification, while on the other hand the larger volume of the KOH solution (0.8 or 1 ml) caused compound degradation, and thus lowered the lutein content. These results agree with the study by Sowbhagya et al. (1) which reported that long exposure of lutein at extreme pH conditions (lower than pH 3 or greter than pH 9) results in isomerization and degradation.

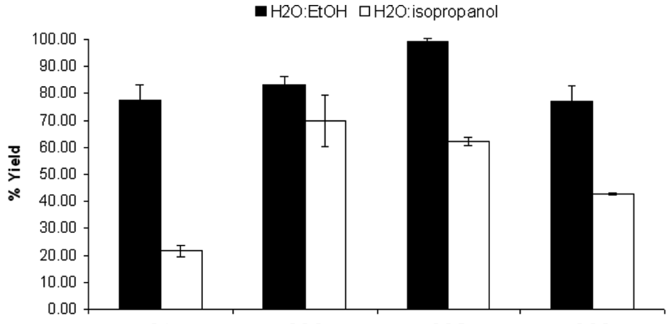
Effects of KOH Concentration

The results obtained previously indicated that the highest free lutein obtained was 179 mg/g oleoresin after 4 hr of saponification process at 75°C with 45% w/v KOH at the 0.5 ml to 1 g marigold oleoresin volume to weight ratio. The most suitable conditions found were based on the fixed concentration of 45% w/v KOH taken from literature. The following set of experiments were therefore carried out to fine tune the process once again so as to determine the most suitable concentration of KOH that yield the maximum results. The results of free lutein obtained are shown in Fig. 4 which reveals that the highest amount of free lutein (194 mg/g oleoresin) was obtained with 0.5 ml of 40% KOH solution. This condition was the most suitable one for saponification, and was therefore employed to prepare the saponified products used for further crystallization experiments.

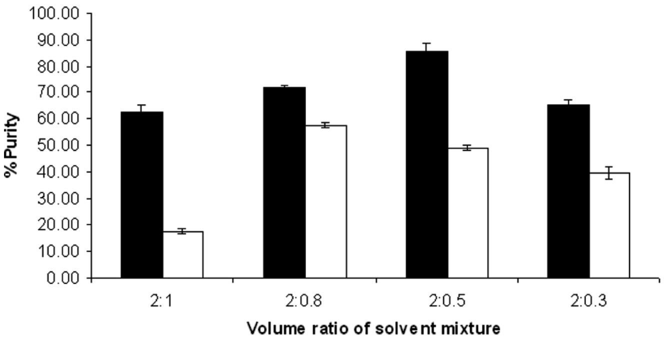
Determination of Suitable Crystallization Conditions

Effects of Volume Ratio of Crystallization Solvent (Water:Ethanol and Water:Isopropanol)

The yield and purity of free lutein obtained in water:ethanol and water:isopropanol solvent systems at various ratios are shown in Fig. 5. The results indicated the higher yield and purity for crystallization in water:ethanol system. Moreover, the yield and the purity of free lutein in the resulted crystals increased as the content of water in water:ethanol mixture increased (from 2:1.0 up to 2:0.5). Similar trend was also observed for water: isopropanol as the ratio increased from 2:1.0 to 2:0.8. At higher content of water, the polarity of the antisolvent was favorable for the precipitation free lutein, beyond which the polarity of the antisolvent became too high, causing the lutein crystallization yield to decrease. At this high polarity condition, it



(a)



(b)

FIG. 5. Effects of volume ratio of crystallization solvent (H_2O:EtOH and H_2O:iso-propanol) on yield and purity of free lutein.

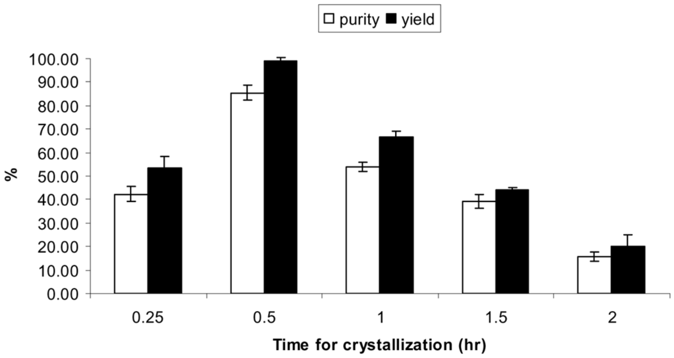
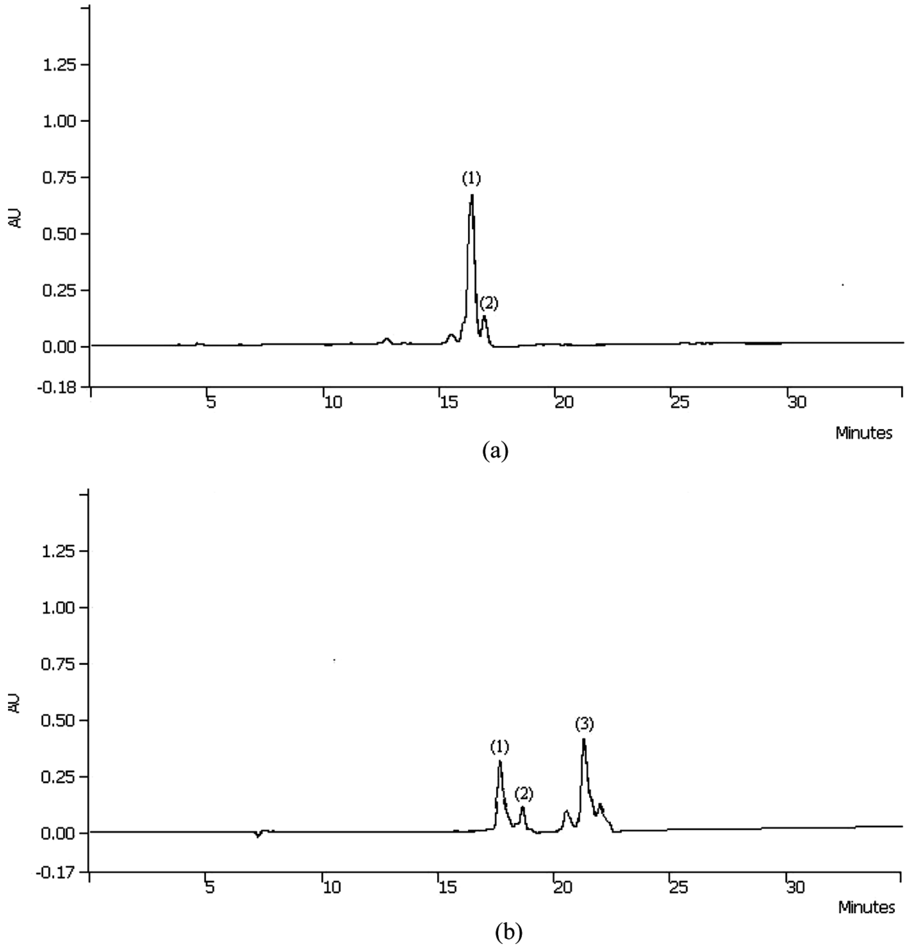


FIG. 6. Effects of crystallization time on yield and purity of free lutein.

was possible that rather than lutein, the other nonplar impurities tended to precipitate, causing the low product purity as shown in Fig. 5b. The yield of free lutein was the highest, 99.12% (or 192 mg free lutein in crystal/194 mg free lutein in oleoresin) for crystallization in water:ethanol mixture at 2:0.5. For the system of water:isopropanol, the highest free lutein yield of about 69.71% (135 mg free lutein/194 mg free lutein in oleoresin) was obtained at 2:0.8 (water:isopropanol). The highest purities of lutein crystals obtained after crystallization with the two solvent systems were 85.53% (192 mg free lutein/225 mg crystals) and 57.61% (135 mg of free lutein/235 mg crystals), respectively.

Effects of Crystallization Time

Figure 6 shows the effect of crystallization time on the yield and purity of free lutein obtained with crystallization at 65°C in water:ethanol mixture. In this experiment, the oleoresin to solvents ratio was 1 g oleoresin:0.5 ml of water:ethanol mixture (at 2:0.5 volume ratio of water to ethanol). The results demonstrated that the yield and purity of free lutein increased with an increase in crystallization time up to 0.5 hr, at which point, the highest yield of 99.12% (85.53% purity) was obtained. The yield and purity of free lutein then decreased as the crystallization time increased. It is generally known that in a typical crystallization process, an adequate period of time is required for nucleation and crystal growth. However, it was found in



(a)

(b)

FIG. 7. HPLC analyses of free lutein and lutein fatty acid esters in marigold flower after (a) 0.5 h and (b) 2 h of crystallization.

that for too long the crystallization time, the free lutein was likely to decompose. The peak of the decomposed products can be observed in the chromatogram shown in Fig. 7b for the crystallization products obtained after 2 hr of the crystallization period, compared with that obtained after 30 min. The LC-MS results indicated that peak (2) and (3) were the decomposed form of free lutein (1), in which one and two hydroxyl groups were absent, respectively.

CONCLUSIONS

In this study, saponification and crystallization of saponified samples of marigold oleoresin were investigated. For 1 g of oleoresin, 0.5 ml of 40% w/v KOH solution provided the suitable amount of KOH for complete saponification to convert lutein esters into free lutein, and the most suitable saponification time was 4 hr. At this condition, 194 mg free lutein/g oleoresin was achieved. For crystallization, water: ethanol mixture at the volume ratio of 2:0.5 was found to be a suitable crystallization solvent, giving 99.12% yield of the lutein crystals (85.53% purity) after 0.5 hr of crystallization.

ACKNOWLEDGEMENTS

Financial support from the Thailand Research Fund is gratefully acknowledged. We also appreciate PTT Chemical Co. Ltd. for the assistance in identifying the crystallization product by LC-MS analysis.

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