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Original Article

Enrichment of vitamin E in palm fatty acid distillate using sequential-cooling urea–fatty acid complexation

Nanthina Damrongwattanakool and Patcharin Raviyan*

Division of Food Science and Technology, Faculty of Agro-Industry, Chiang Mai University, Mueang, Chiang Mai, Thailand 50100

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Abstract

Sequential-cooling urea–fatty acid complexation is a novel technique developed to pre-concentrate vitamin E extracted from palm fatty acid distillate before further purification by supercritical fluid extraction. The efficacies of the sequential-cooling and the constant cooling methods were compared. The sequential-cooling method cools the reaction mixture to a sequence of predetermined temperatures between 35°C and -5°C, whereas the constant cooling method cools down to −5°C that is maintained for 12 hours. The recovery of vitamin E was increased from 21.62% with constant cooling to 32.04% with sequential cooling, while the corresponding concentration in the sample increased from 8.85 to 25.16 % (w/w). Combining sequential cooling urea–fatty acid complexation with a final supercritical fluid extraction increased the concentration of vitamin E to 80%.

Keywords: palm fatty acid distillate, sequential cooling, urea–fatty acid complexation, vitamin E

1. Introduction

Palm fatty acid distillate (PFAD) is a by-product from physical refining of crude palm oil. It is generally sold at a low price as a raw material for animal feed, laundry industry, and oleochemical industry (Top, 2010). PFAD is composed of 81.7% free fatty acids, 14.4% glycerides, 0.8% squalene, 0.5% vitamin E, 0.4% sterols, and 2.2% other substances (Top, 2010). Among these components, vitamin E, which has been reported to possess powerful antioxidant properties, has the highest value (Ingold *et al*., 1987). Turning PFAD into value-added products such as vitamin E would seem beneficial.

In our previous work, vitamin E was extracted from PFAD by a novel method named sequential-cooling hexane extraction. This process includes preparing a mixture of PFAD and hexane. The mixture was cooled to a sequence of predetermined temperatures. Cooling to the predetermined temperatures sequentially removed from the mixture solid fractions that contain no vitamin E. The vitamin E yield under optimal conditions was 7,662.45 mg/kg of PFAD extract (Raviyan & Soinak, 2012).

According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the baseline concentration for concentrated vitamin E is 34%. High concentration of vitamin E can be achieved by separating it from fatty acids using supercritical fluid extraction (SFE). This method, although reliable, does not operate at ambient pressure (Beveridge, Girard, Kopp, & Drover, 2005), and there is a need to find alternatives operating closer to the ambient pressure.

The urea–fatty acid complexation is a well-known and established method for separating unsaturated fatty acids from saturated fatty acids. Our preliminary study found that crude palm oil contains 43.07% palmitic acid (C16:0), 38.29% *cis*-9-oleic acid (C18:1), 10.49% *cis*-9,12-linoleic acid (C18:2), 4.62% stearic acid (C18:0), 0.99% myristic acid (C14:0), 0.84% *cis*-vaccenic acid (C18:1), 0.46% lauric acid (C12:0), 0.34% arachidic acid (C20:4), 0.03% capric acid (C10:0), 0.22% alpha-linolenic acid (C18:2), 0.02% caprylic acid (C8:0), 0.19% palmitoleic acid (C16:1), 0. 10% *cis*-11-eicosenoic acid (C21:1), 0.08% heptadecanoic acid (C17:0), 0.04% behenic acid (C22:0), 0.04% lignoceric acid (C24:0),

*Corresponding author
Email address: patraviyan@gmail.com

and 0.03% pentadecanoic acid (C15:0). In PFAD from crude palm oil, about 80% free fatty acid content could be obtained (Top, 2010). Thus, separating the fatty acids from vitamin E in PFAD by binding straight chained fatty acids (saturated and monounsaturated) by urea–fatty acid complexation would potentially pre-concentrate vitamin E. Urea crystallizes by molecular bonding via hydrogen bonds, while it bonds to fatty acids with van der Waals attraction. The channels in the hexagonal crystals should be large enough to hold straight chains with seven or more carbon atoms (Hayes, Bengtsson, Alstine, & Setterwall, 1998).

The urea–fatty acid ester complexation is signify-cantly dependent on the number of double bonds in carbon chain of a fatty acid. The double bonds greatly affect the configuration of a fatty acid moiety. It was reported that when double bonds in a carbon chain are increased, the molecule becomes more bulky, and its chances to penetrate into urea are poorer (Shahidi & Wanasundara, 1998). Hai-bo *et al.* (2009) reported that by using urea as a complexing agent for linoleic acid and cooling in stages to -20°C, the amount of linoleic acid complexed was significantly greater than with immediate cooling to the final -20°C temperature. This study explored the validity of this claim scientifically, by examining whether the concentration of vitamin E in oil can be increased by employing stepwise complexation of urea and fatty acids.

An application of urea–fatty acid complexation to concentrate vitamin E has been claimed by Sampathkumar (1986). However, this prior work on the optimal conditions for urea–fatty acid complexation adhered to a constant temperature. In the present work, the urea–fatty acid complexation is done by sequentially reducing the temperature to a series of predetermined levels, referred to as "sequential-cooling urea–fatty acid complexation". This was used to remove fatty acids from the vitamin E extract. The efficacies of pre-concentrating vitamin E using sequential-cooling urea–fatty acid ester complexation and using a constant cooling system were compared.

2. Materials and Methods

2.1 Materials

PFAD was provided by Chumporn Palm Oil Industry Public Co., Ltd., Chumporn province, Thailand. The synthetic alpha-tocopherol and the commercial tocopherol concentrate (Covi-ox T-70 brand), which consisted of 14% alpha-tocopherol, 2% beta-tocopherol, 60% gamma-tocopherol, and 24% delta-tocopherol, were purchased from BASF (Thai) Co., Ltd, Thailand. The *tert*-butylhydroquinone (TB HQ) and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Thailand) Co., Ltd. The other chemicals and reagents used in analyses were of analytical reagent grade.

2.2 Extraction of vitamin E

Vitamin E was extracted from PFAD by following the method of Raviyan and Soinak (2012). The PFAD and hexane mixture was sequentially cooled to the temperatures 30°C, 20°C, 10°C, 5°C, 0°C, −5°C, −10°C, and −15°C. The components of PFAD without vitamin E formed solids at the selected temperatures and were removed from the mixture at each cooling step. After the sequential cooling, the hexane was separated from the remaining mixture to obtain the vitamin E extract.

2.3 Pre-concentration of vitamin E

Pre-concentration of vitamin E had two main steps: esterifying and sequential-cooling urea–fatty acid complexation. The esterification converted the fatty acids in the sample to fatty acid esters on adding a 95% excess of ethyl alcohol to the 20 g extract. Then, the reaction mixture was refluxed at 70°C under nitrogen atmosphere for 2 hours in the presence of 1% (v/v) H_2SO_4 catalyst. After 2 hours the reaction mixture was cooled to room temperature and 100 mL distilled water and 100 mL hexane were added. The mixture was well mixed by shaking several times and the two layers were allowed to separate. The hexane layer was collected and washed with 100 mL distilled water. The aqueous layer was further extracted with an additional 50 mL hexane. The hexane portions were combined and the hexane was evaporated at 60°C and 335 mbar pressure. Ten mL of 95% ethanol was then added to the sample before it was vacuum dried at 60°C to yield the esterified fatty acid ethyl ester vitamin E sample.

The sequential-cooling urea–fatty acid complexation procedure was carried out as follows: A 20 g sample of esterified fatty acid ethyl ester was added to 95% ethanol under nitrogen atmosphere. Then 100 g of urea was added and the mixture was heated to 70°C under nitrogen atmosphere with shaking for 15 minutes. The reaction vessel was then flushed with nitrogen and the temperature of the reaction mixture was reduced to 35°C and maintained at 35°C for 15 min. Every 2–3 minutes over the 15 min interval, the reaction mixture was shaken. The reaction was repeated at 25°C for 15 minutes, at 15°C for 30 minutes, at 5°C for 45 minutes, and, finally, at −5°C for 12 hours. The precipitate was filtered off and the filtrate collected. The precipitate was then washed with 100 mL of 95% ethanol at −20°C. The combined filtrate was then added to a separating funnel and allowed to separate to two layers. The hexane layer was collected and the aqueous layer was extracted with 2 × 50 mL hexane. The hexane layers were combined and washed with 50 mL distilled water. The hexane layer was then concentrated at 60°C under reduced pressure (335 mbar). Then 10 mL of 95% ethanol was added to the concentrate, and the resulting solution was dried at 60°C under reduced pressure (72 mbar) to yield 0.64 g of the urea–fatty acid ester complexation pre-concentrated vitamin E sample.

As a comparative study, urea–fatty acid ester complexation was also performed with a vitamin E extract using the constant cooling method. The esterified vitamin E sample was prepared as previously described. The esterified fatty acid ethyl ester (20 g) was added to ethanol (95% v/v) under nitrogen atmosphere. Urea (100 g) was added and the resulting reaction mixture was heated to 70°C under nitrogen atmosphere with shaking for 15 minutes, during which time substantially all of the urea dissolved in the solution. The reaction vessel was then flushed with nitrogen and the temperature of the reaction mixture was reduced to −5°C and this was maintained for 12 hours. The precipitate was filtered off and the filtrate collected. The precipitate was then washed

with 100 mL of ethanol (95% v/v) at −20°C. The combined filtrate was separated and vacuumed dried according to the procedure described for the sequential-cooling system.

2.4 Purification of vitamin E

A commercial supercritical fluid extraction apparatus (Thar Technologies™ Model SFE-100-2-Base™ [U.S.A.]) was used to further purify vitamin E from the pre-concentrated sample. A 60.03 g sample was fed to the machine. The conditions used for vitamin E purification were SC-CO_2 flow rate 10 g/min, extraction time 60 minutes, pressure 325 bar, and extraction temperature 33.18°C (Ge *et al*., 2002, with modifications). The extraction was repeated five times to get the final vitamin E product.

2.5 Chemical analysis

2.5.1 Fatty acid composition

The fatty acid compositions (FACs) were determined by a GC-MS (Agilent Technologies) equipped with a hydrogen flame ionization detector (FID) and a silica capillary column (HP-5MS 30 m × 0.25 mm ID × 0.25 µm film thickness). The fatty acids standard (Supelco Inc., PA, USA.) was tested under the same conditions to identify the peaks.

2.5.2 Concentration of total vitamin E, total tocopherol, and total tocotrienol

The concentration of each vitamin E derivative in the pre-concentrated vitamin E sample from sequential cooling was analyzed using HPLC (SHIMADZU-HPLC model HPLC LC-10AVP) with a low pressure gradient, equipped with a CBM–10A system controller, a DGU-12A in-line degasser, an LC–10AD pump, and a CTO–10A oven containing a silica column with 0.5 µm Pinnacle II silica. The column size was 250 mm × 4.6 mm (RESTEK serial no. 10070775M) assembled with guard column and the sample was injected into the column manually. The sensing element was an RF–10AXL fluorescence detector, and all the functions were controlled by CLASS-LC10 version 1.64 software. The analysis procedure was based on the Recommended Practices Ce 8-89 (American Oil Chemists' Society [AOCS], 1997).

2.5.3 Composition of vitamin E

The composition of the vitamin E samples was analyzed using a GC-MS (Agilent Technologies) equipped with an HP-5MS column (30 m × 0.25 mm ID × 0.25 µm film thickness). The oven temperature was held at 150°C for 4 minutes, and then increased to 200°C at 4°C/min, where it was held for 1.0 minute, followed by an increase of 2°C/min to 250°C, where it was held for 5.0 minutes, until all the peaks had appeared. Helium was used as the carrier at 1.0 ml/min flow rate. The EI/GC–MS analyses were performed on a single quadrupole mass spectrometer under electron impact (EI, ionization energy 270 eV) conditions, with an on-column injector set at 150°C, for confirmation of peak component identities.

2.5.4 Experimental design and statistical analysis

All the determinations were carried out with three replications, and are reported as mean ± standard deviation. The experimental data were subjected to analysis of variance (ANOVA). Duncan's New Multiple Range Test (DMRT) was applied to test for significant differences of means at the level $p \leq 0.05$.

3. Results and Discussion

On applying urea–fatty acid complexation, the distribution of fatty acids is a critical factor. It is important that the material has high contents of saturated and monounsaturated fatty acids with straight chains, such that can be bound or trapped inside urea crystals (Hayes *et al*., 1998). The fatty acids composition in the PFAD extract was 65.64% oleic acid (C18:1), 15.70% linoleic acid (C18:2), 11.94% palmitic acid (C16:0), 2.55% stearic acid (C18:0), and 1.74% myristic acid (C14:0). On comparing the compositions of PFAD and crude palm oil, the amounts of saturated fatty acids, particularly the palmitic acid, were lower in the PFAD. Decreased amounts of saturated fatty acids can be explained by the lower freezing points of saturated fatty acids, which formed solid portions and were removed during cooling extraction of vitamin E from the PFAD. Since the content of saturated fatty acids in PFAD was reduced, the constant cooling urea–fatty acid complexation for the separation of vitamin E out of PFAD would be less effective.

Experiments testing urea–fatty acid complexation were done with the PFAD extracted by sequential cooling. After pre-concentration of vitamin E using the constant cooling and sequential-cooling urea–fatty acid complexation methods, it could be seen that both procedures increased concentration of vitamin E in the mixture (Table 1). While the constant cooling method was insufficient, these results indicate that some of the fatty acids in PFAD were bound inside the urea crystals. In contrast to the saturated fatty acids, vitamin E and its derivatives are bulky due to their gauche conformation (Strocchi & Bonaga, 1975), so these remained outside the urea complexes and can be further recovered in the next step.

Table 1 clearly shows that the efficacy of urea–fatty acid ester complexation is affected by the cooling pattern. Concentrations of all vitamin E derivatives were higher with the sequential-cooling method than with the constant cooling method. The quantity of total vitamin E increased from 8.85% for constant cooling to 25.16% (w/w) for sequential cooling. Additionally, the recovery of vitamin E improved from 21.62% for constant cooling to 32.04% for sequential cooling.

Potential reasons for the sequential cooling to be more effective involve two aspects, namely properties of the fatty acids in PFAD extract, and properties of urea crystals. An analysis of the fatty acids in PFAD extract found fatty acids with various freezing points. For example, the freezing point of oleic acid (C18:1) is about 7°C, while those of linoleic acid (C18:2) and palmitic acid (C16:0) are about -5°C and 62.5°C, respectively (Lide, 2005). The freezing points are lower for unsaturated fatty acids because of having more double bonds that bend the molecular conformation. Also, the double bond fundamentally has a *cis* configuration. As a

Table 1. Concentrations of vitamin E derivatives in the pre-concentrated vitamin E samples obtained using urea–fatty acid ester complexation with sequential cooling, and alternatively with constant cooling temperature

Vitamin E derivative	Concentration of vitamin E derivative (mg/kg sample)) in the sample pre-concentrated by constant-cooling urea–fatty acid complexation method	Concentration of vitamin E derivative (mg/kg sample) in the sample pre-concentrated by sequential-cooling urea–fatty acid complexation method
Alpha-tocopherol	35716±157	92450±1724
Alpha-tocotrienol	20109±257	43512+516
Beta-tocopherol	250±21	1885±39
Beta-tocotrienol	370±6	7783±155
Gamma-tocopherol	2824±359	8518±395
Gamma-tocotrienol	23330±1011	71986±787
Delta-tocopherol	162±20	3326±119
Delta-tocotrienol	5735±330	22136±528
Total tocopherol*	38954±203	106181±2278
Total tocotrienol*	49545±1091	145419±1987
Total vitamin E**	88,499±1294 (8.85% w/w)	251600±4266 (25.16% w/w)

Note: *The percentage of total tocopherol and the percentage of total tocotrienol were calculated from the total vitamin E content.
**The percentage of total vitamin E calculated from the vitamin E derivatives in 100 g of the sample.

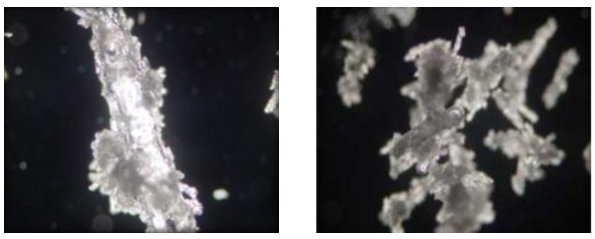
result, these molecules are not compact, and interactions between such molecules are considerably weaker than those of saturated molecules (Strocchi & Bonaga, 1975).

It was observed in this study that when the temperature of the reaction mixture was reduced to some predetermined level, a certain amount of fatty acids formed solids, while other fatty acids still remained in liquid form. The gradual solidification of the fatty acids has advantages over sudden solidification of all fatty acids, because it allows the fatty acids to form more inclusions in urea. In contrast, when using the constant cooling temperature procedure, the solid portion seems to be too high for the desired interactions with urea. This agrees with the study of Hai-bo *et al*. (2009) who studied the production of alpha-linoleic acid concentrated from crude perilla oil by gradient cooling urea inclusion at −4°C, −8°C, −12°C, −16°C, and −20°C for a total crystallization time of 690 minutes. They discovered that up to 91.5% of the alpha-linoleic acid could be complexed and trapped in the crystals, which was higher than that achieved by the constant cooling method.

Regarding the properties of urea crystals, microscope images of the crystals at 40× magnification displayed urea–fatty acid complex aggregates that were comparatively small sized when developed at a constant cooling temperature (Figure 1a). In contrast, relatively open structured aggregates formed under sequential cooling (Figure 1 b). These differences in urea structure strongly affected the interactions with fatty acids and fatty acid esters. The sequential cooling favored fatty acid inclusion and increased the concentration of total vitamin E left out of the crystals more than the constant cooling method. Strocchi and Bonaga (1975) explained that structural compatibility between host and guest components is a principal requirement for inclusion systems. Urea only forms inclusion compounds with guest molecules that are based on a sufficiently long n-alkane chain. Furthermore, the degree of substitution of this chain must be small. In this current study, Table 2 shows that the urea–fatty acid complexation contained only small amounts of linoleic acid, oleic acid, and their fatty acid esters. This confirms that urea successfully formed an inclusion complex with the solidified fatty acids and fatty acid esters. This is in accordance with prior literature, where it has been shown that the amount of fatty acids removed from cod liver oil samples was governed by the crystallization temperature (Medina *et al*., 1995).

Additionally, compact aggregates of urea–fatty acid complexes developed at the constant cooling temperature (Figure 1a) and this did not encourage a high extent of complexation because the insertion of fatty acid molecules into the urea crystals would require more void space in the aggregates. The relatively open structured aggregates formed under sequential cooling (Figure 1b) allowed more fatty acids to be complexed. Thus, the sequential-cooling urea–fatty acid complexation procedure left proportionately more vitamin E in the oil phase, as shown in Table 1.

Table 3 reveals that purifying vitamin E by sequential-cooling urea–fatty acid complexation combined with five cycles of supercritical fluid extraction could enrich the concentration of vitamin E from 25.16% (w/w) to 79.77% (w/w). The concentration of each vitamin E derivative was somewhat altered. Nevertheless, the average ratio of total tocotrienol to total tocopherol was about 60:40. This ratio was not significantly altered by the enrichment steps. This verifies that combining urea–fatty acid complexation with supercritical



(a) (b)

Figure 1. Microscope image at 40X magnification of the crystals obtained from urea–fatty acid complexation with constant cooling temperature (a), and the crystals obtained from urea–fatty acid complexation with sequential cooling (b).

Table 2. Compositions after sequential-cooling urea–fatty acid com-plexation and after combined sequential-cooling urea–fatty acid and supercritical fluid extraction.

Component	% (based on weight of sample before concentration)	% (based on weight after sequential-cooling urea–fatty acid complexation)	% (based on weight after combined sequential-cooling urea–fatty acid and supercritical fluid extraction)
Elaidic acid methyl ester	0.34±0.04	-	-
Myristic acid	0.90±0.06	-	-
Palmitic acid ethyl ester	0.30±0.03	-	-
Palmitic acid methyl ester	0.53±0.05	-	-
Palmitic acid	11.56±0.52	-	-
Lauric acid	0.31±0.07	-	-
Linoleic acid	-	1.95±0.03	10.00±0.09
Linoleic acid, ethyl ester	-	18.91±1.09	2.98±0.08
Oleic acid	82.85±3.05	-	12.59±0.67
Oleic acid, ethyl ester	-	8.28±0.26	1.62±0.02
Squalene	3.22±0.01	70.86±3.08	72.81±3.17

Table 3. Concentrations of the vitamin E derivatives in the PFAD extract, after pre-concentration by the sequential-cooling urea–fatty acid complexation method, and after concentration by combined sequential-cooling urea–fatty acid complexation and supercritical fluid extraction.

Vitamin E derivative	Concentration of vitamin E derivative (mg/kg of PFAD extract) of resulting sample from the PFAD extraction	Concentrations of the vitamin E derivative (mg/kg of sample) of resulting sample from the sequential-cooling urea–fatty acid complexation method	Concentration of vitamin E derivative (mg/kg sample of resulting sample from the combined method of sequential-cooling urea–fatty acid complexation and the supercritical fluid extraction
Alpha-tocopherol	2108+83	92450+1724	301556+8261
Alpha-tocotrienol	1109+27	43512+516	170085+3816
Beta-tocopherol	10±5	1885±39	947±198
Beta-tocotrienol	115±15	7783±155	10417±168
Gamma-tocopherol	185±40	8518±395	15716±1938
Gamma-tocotrienol	1555±74	71986±787	221540±2766
Delta-tocopherol	81±11	3326±119	1595±131
Delta-tocotrienol	552±74	22136±528	75836±182
Total tocopherol*	2386±121 (41.72%)	106181±2278 (42.20% w/w)	319815±10266 (40.09%)
Total tocotrienol*	3333±119 (58.28%)	145419±1987 (57.80% w/w)	477879±14010 (59.91%)
Total vitamin E**	5719±43 (0.57%)	251600±4266 (25.16% w/w)	797694.41±8864 (79.77 % w/w)

Note: *The percentage of total tocopherol and the percentage of total tocotrienol calculated from the total vitamin E content.
**The percentage of total vitamin E calculated from the vitamin E derivatives in 100 g of the sample.

fluid extraction negligibly affects proportions of vitamin E derivatives. The major vitamin E compound at 79.77 % (w/w) was squalene (Table 2), which is a natural potent antioxidant.

The yields of vitamin E from extraction, pre-concentration, and purification were 28.85%, 3.18, and 37.81%, respectively. The pre-concentration step had the lowest yield because the most fatty acids were removed at this step. In addition, vitamin E recovery at each step was 38.76%, 85.93, and 72.89%, respectively. The highest yield was obtained at the pre-concentration step. Thus, this step was considered crucial in the enrichment process.

4. Conclusions

The cooling profile affected strongly urea–fatty acid ester complexation. The sequential-cooling urea–fatty acid complexation method could be used to pre-concentrate vitamin E in PFAD extract, prior to final purification by SFE. The combined use of sequential-cooling urea–fatty acid complexation and supercritical fluid extraction could reach vitamin E concentration of about 80%, which is much higher than the minimum 34% stipulated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for concentrated vitamin E. The sequential-cooling urea–fatty acid complexation method could provide high-purity vitamin E products and has potential in other applications, including concentration of vitamin E from other vitamin E containing materials.

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