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Factors affecting pre-concentration of tocopherols and tocotrienols from palm fatty acid distillate by lipase-catalysed hydrolysis

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Abstract

Vitamin E in palm fatty acid distillate (PFAD) was pre-concentrated using an enzymatic hydrolysis-neutralisation method. Acylglycerols in PFAD was hydrolysed using a commercial immobilised thermal-stable *Candida antarctica* lipase (Novozyme 435 lipase) to liberate free fatty acids (FFA) and subsequently treated with an alkali. Removal of the FFA salts resulted in concentration of vitamin E. Factors affecting the degree of hydrolysis were studied to reach better understanding of the recovery of vitamin E from PFAD. Results showed that hydrolysis of 1:1 (w/v) ratio of PFAD and water mixture achieved its equilibrium after 5 h of reaction at 65±1 °C. The FFA levels in PFAD increased from initially 85 to about 97% at the equilibrium, while concentration of the vitamin E extracted increased from 1–7% to 7–1%. The FFA levels in PFAD remained unchanged but vitamin E concentration decreased when the reaction was prolonged to 7 h. The vitamin might have been oxidised due to the long period of heating at 65 °C. Increase of water content in the reaction mixture from 20 to 50% w/v increased both the FFA and vitamin E significantly ($P<0.05$). However, further increments of water content in the mixture significantly ($P<0.05$) decreased the FFA levels and vitamin E concentration. The optimum lipase concentration for the hydrolysis was about 1.0–1.5% w/w, whereby the FFA levels could be increased to approximately 94%. Meanwhile, the optimum temperature of the lipase was about 70 °C and the maximum FFA and vitamin E levels were 97% and 7–8%, respectively. When the reaction temperature was further increased from 70 to 85 °C, both the FFA and vitamin E levels decreased significantly ($P<0.05$). High temperature inactivated the lipase and promoted the oxidation of vitamin E. The interactions of these parameters during the hydrolysis can be optimised to achieve a maximum concentration of vitamin E.

Keywords: Enzymatic hydrolysis; Free fatty acid; Neutralisation; Palm fatty acid distillate; Pre-concentration; Vitamin E

1. Introduction

Tocopherols and tocotrienols, which are physiologically active as vitamin E, are major natural antioxidants and find extensive applications in cosmetics and pharmaceutical industries. Natural tocopherols and tocotrienols are normally recovered from fatty acid distillate (FAD). FAD is a valuable by-product obtained during the deodorisation of vegetable oils in refining factories. About 0.3–0.5% of the deodoriser feedstock ends up as the FAD (Ramamurthi, Bhirud, & McCurdy, 1991). It is a complex mixture of free fatty acids (FFA), mono-, di- and triacylglycerols, tocopherols and sterols. The tocopherol content in FAD varies, ranging between 1 and 15%, depending on type of FAD, type of refining process and the conditions employed therein (Fizet, 1993).

Many attempts have been taken to separate tocopherols and tocotrienols from FAD, such as by solvent extraction (Goh, Lai, & Kam, 1992; Sheabar & Neeman, 1988), molecular distillation (Ab Gapor et al., 1993), esterification (Hunt et al., 2000; Ong, Choo, Goh, & Kam, 1994) and saponification (Fizet, 1993), with different extents of purification. However, these methods are either energy-intensive and require the use of special equipment or chemical-intensive. Enzymatic methods have also been reported (Ramamurthi et al., 1991; Ramamurthi & McCurdy, 1993), whereby, the FFA in FAD are converted into methyl esters by lipases

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through esterification and tocopherols were concentrated in the residue fraction by distilling off the methyl esters and other volatile compounds. Recovery of tocopherols from FAD by a combination of lipase-catalysed hydrolysis and esterification reactions, followed by fractional distillation of the derived ester product was also reported (Ghosh & Bhattacharyya, 1996). Exposure to high temperature (180–260 °C) during the process of fractional distillation might cause the degradation of tocopherols.

FAD contains high levels of FFA and acylglycerols. For instance, FAD of soybean oil contains about 39% of FFA and 32% of acylglycerols, while FAD of sunflower oil contains 43 and 13% of FFA and acylglycerols, respectively (Fizet, 1993). The acylglycerols can be hydrolysed to FFA, which are water-soluble when treated with alkali. In this study, the acylglycerols in palm fatty acid distillate (PFAD) were enzymatically hydrolysed to form FFA. The FFA were then removed by neutralisation to yield a fraction which was high in tocopherols and tocotrienols. In order to obtain maximum recovery of tocopherols and tocotrienols, the acylglycerols in PFAD must be maximally hydrolysed. There are a number of factors affecting hydrolysis and understanding on how these factors play their roles during hydrolysis is important in order to maximise the recovery of tocopherols from PFAD.

2. Materials and methods

2.1. Materials

PFAD (iodine value, 56.8 g of I_2/100 g of oil; slip point, 48.5 °C) and a commercial immobilised *C. antarctica* lipase (Novozyme 435) were obtained from Jomalina Pte. Ltd., Teluk Panglima Garang, Selangor, Malaysia, and Novo Nordisk, Bagsvaerd, Denmark, respectively. All chemicals used were either of analytical or high performance liquid chromatography (HPLC) grades.

2.2. Enzymatic hydrolysis

Unless otherwise specified, hydrolysis of PFAD was conducted under the following conditions: a mixture of 50 g melted PFAD, 50 ml distilled water and 1.0% w/w Novozyme 435 lipase was stirred magnetically. Stirring took place in a stoppered, 250 ml conical flask under nitrogen, which was placed in a 65±1 °C water bath in the dark. Reaction was stopped after a period of time (0, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 h) by filtering out the immobilised lipase using a two-layer cheese cloth. A sample of approximately 1 ml was withdrawn for FFA determination and the remainder was used for tocopherol and tocotrienol determinations. The lipase was not recycled for further use.

2.3. Determination of FFA

The melted sample (1 g) was placed in a 1.5 ml Eppendorf tube and centrifuged 1400 g for a few seconds to break the emulsion. Melted PFAD (0.5 g) was then transferred into a 250 ml conical flask and 50 ml of neutralized isopropanol was added to dissolve the sample. The sample was titrated to the phenolphthalein end-point with 0.02 N sodium hydroxide solution (PORIM, 1995).

2.4. Determination of tocopherols and tocotrienols

Vitamin E extraction was modified from Wang, Ning, Krishnan, and Matthees (1998). The melted reactant was first centrifuged at 1400 g and about 10 g of PFAD was withdrawn. The PFAD was dissolved in 100 ml hexane and was neutralised using 0.5 N sodium hydroxide to the phenolphthalein end-point. The sample was then allowed to stand for phase separation. The hexane layer was separated in a separation funnel. The hexane layer was then washed at least three times with 150 ml portions of distilled water to remove residual sodium hydroxide and soap. The extract was then centrifuged at 2500 g for 1 min to remove residual water. The extract was transferred to a 250 ml round-bottom rotary evaporator flask and evaporated at 60 °C under vacuum. A known amount (about 0.05 g) of the oil recovered was then dissolved in 2 ml of hexane and 5 μl of the mixture were injected into an HPLC system (Waters Model 600 Controller, Milford, MA), equipped with a UV detector (Waters Model 486 UV Detector, Milford, MA). Data were processed by means of Millennium 2010 Chromatography Manager Software (Waters, Milford, MA). The flow rate of mobile phase (methanol:water, 95:5 v/v) was set at 1.0 ml/min and oven temperature was 40 °C. The column used was a Purospher STAR RP-18 endcapped column (Merck, Darmstadt, Germany). By comparing published chromatograms (Tan & Brzuskiewicz, 1989) and natural occurrence (Wang et al., 1998), α-tocopherol, α-, γ- and δ-tocotrienols were identified. Peak area was used for quantification. Due to the unavailability of α-, γ- and δ-tocotrienol standards, D-α-tocopherol (Sigma, St. Louis, MO) was used as external standard for quantification of all four tocols (Tan & Brzuskiewicz, 1989; Wang et al., 1998), based on the assumption that they have the same UV intensity.

2.5. Statistical analysis

Analysis of variance (ANOVA) was carried out to analyse the data where applicable, using SAS (SAS, 1989) software, package release 6.1. The significant difference level was set at 0.05. Each reported value was the mean of four analyses from two replications.

3. Results and discussion

3.1. General

The slip point of PFAD was 48.5 °C; however, a differential scanning calorimetry analysis showed that the off-set time of the last endothermic peak of PFAD was 57.9 °C. This meant that PFAD was completely melted at about 60 °C. According to the lipase specifications given by the manufacturer, *C. antarctica* lipase was highly heat-tolerant with maximum activity in the range of 70–80 °C. However, due to thermal inactivation when running the reaction at elevated temperatures, it was recommended to operate in the range of 40–60 °C for optimum productivity of repeated and long-time uses. In this study, hydrolysis was carried out at 65 °C. Since immobilisation enhanced the thermal stability of lipase, it was assumed that the reaction temperature used, unless otherwise specified, would not drastically lower the activity of the lipase over the reaction time. The reaction temperature was reasonably high enough to melt PFAD completely and yet, low enough to minimise the degradation of the lipase activity, as well as the oxidation of tocopherols and tocotrienols during the hydrolysis process.

The yield of PFAD recovered from neutralisation and washing was generally low for all samples. The yield of unreacted PFAD was about 12.5%, while the yield of hydrolysed PFAD ranged between 1.9 and 13.7%, depending on the degree of hydrolysis. The acylglycerols in PFAD were hydrolysed and subsequently removed. The higher degree of hydrolysis of the PFAD, the more FFA would be removed and the lower the yield. In contrast, the yields of vitamin E for all samples were comparatively high, with a recovery of about 60 to 76.5%. Low yield in PFAD and high yield in vitamin E after neutralization resulted in concentration of vitamin E in the samples. It was generally found that reactants of high reaction temperature (reacted at >70 °C) and long reaction time (>5 h) had lower vitamin E recoveries than those of lower reaction temperature and shorter reaction time. The extraction process and oxidation of vitamin E during hydrolysis might contribute to the loss of vitamin E. For all samples, the compositions of vitamin E were rather similar. Vitamin E extracted contained about 21.1, 21.2, 16.7 and 41.0% of α-tocopherol, α-, δ- and γ-tocotrienols, respectively. Vitamin E concentration, expressed in this study, was the total concentration of tocopherol and all tocotrienol isomers.

3.2. Time course of hydrolysis

Fig. 1 shows the changes in FFA and concentration of vitamin E extracted, during the course of hydrolysis of PFAD with 50% w/v of water, by *C. antarctica* lipase at 65 °C. The non-specific lipase hydrolysed the acylglycerols in PFAD rapidly for the first 4 h and FFA in the reaction mixture increased from an initial 85 to about 95%. The hydrolysis reaction achieved its equilibrium

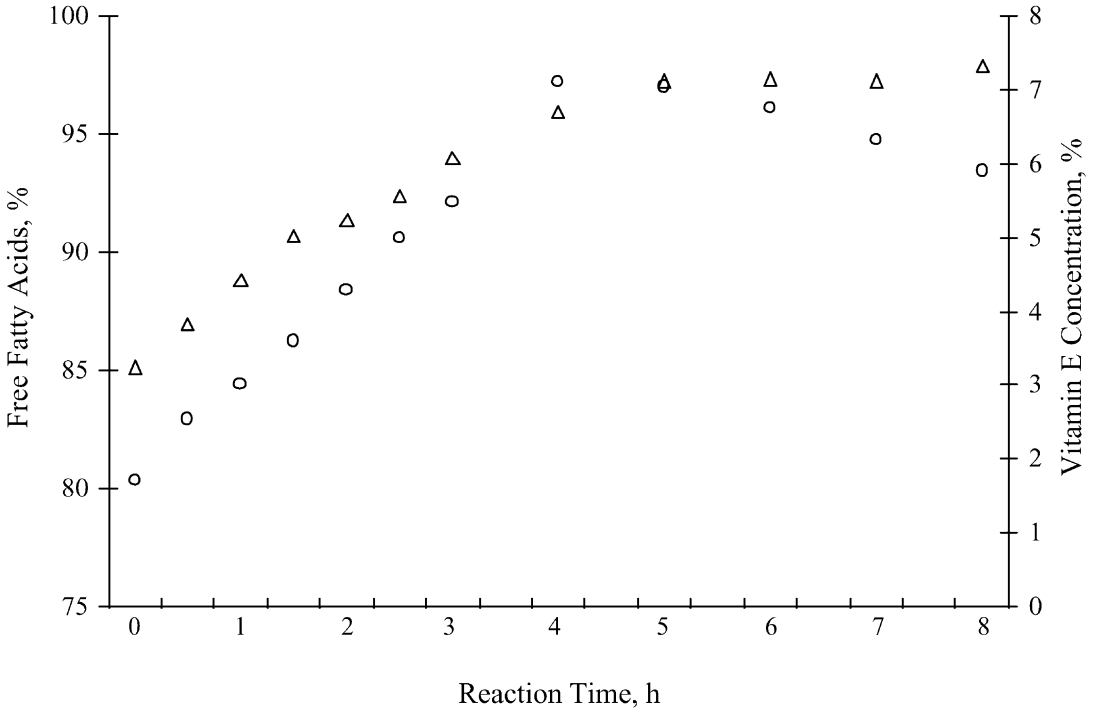

Fig. 1. Time course of the hydrolysis of 1:1 (w/v) ratio of palm fatty acid distillate and water mixture at 65±1 °C with 1.0% w/w of commercially immobilized *Candida antarctica* lipase. Free fatty acids: Δ, concentration of vitamin E extracted from reactant, ○.

after 5 h of reaction time and the FFA levels in the mixture reached a maximum of about 97%. Similar changes were observed in the concentrations of vitamin E extracted. Initial concentration of vitamin E in PFAD was 0.37% and neutralisation of PFAD concentrated it to 1.72%. Hydrolysis of the acylglycerols in PFAD could further concentrate vitamin E. Concentration of vitamin E extracted from hydrolysed PFAD increased from, initially, 1.72 to 7.10% after 5 h of reaction. The concentration of vitamin E decreased towards the end of the reaction of 8 h to about 5.89%. This might be due to the oxidation of some vitamin E during the hydrolysis after a long period of heating. Nevertheless, it was found that the FFA content in PFAD and vitamin E concentration still correlated well, with a correlation coefficient, R, of 0.9268 ($P<0.05$).

3.3. Effect of water content

Table 1 shows the changes in FFA content and concentration of vitamin E extracted from the reaction mixtures with different water contents. All mixtures were hydrolysed for 7 h at 65±1 °C with 1.0% w/w of lipase. At low water content (less than 40% w/v) the degree of hydrolysis was incomplete. PFAD was hydrolysed most effectively at a water content of 50% w/v. The FFA content in the mixture was 97.2%, while vitamin E extracted from PFAD was 6.31%. Since lipase catalysed, not only hydrolysis, but also esterification, simultaneously, a large amount of water was necessary to shift the equilibrium to hydrolysis. However, when hydrolysis was conducted in the mixture with more than 50% w/v of water, the degree of hydrolysis decreased. This phenomenon may be explained by the decrease of hydrolysis rate, which is attributed to the low enzyme concentration in the water phase due to the large amount of water. Similar findings were reported by other researchers (Shimada et al., 1997; Shimada, Maruyama, Sugihara, Moriyama, & Tominaga, 1997). Levels of vitamin E extracted also decreased significantly ($P<0.05$), from 6.31 to 2.06%, when water content increased from 50 to 70% w/v.

Table 1
Changes in percentages of free fatty acid (FFA) and vitamin E extracted as a function of water content in the reaction mixture[a]

% Of water	% Of FFA	% Of vitamin E
20	84.6±0.27e	1.73±0.15d
30	88.4±0.28d	2.08±0.11c,d
40	93.1±0.15b	3.74±0.25b
50	97.2±0.11a	6.31±0.13a
60	89.23±0.28c	2.37±0.05c
70	88.3±0.10d	2.06±0.09c,d

[a] Reactions were carried out at 65±1 °C and 1% w/w of lipase for 7 h. Means within each column with different letters are significantly different ($P<0.05$).

3.4. Effect of lipase concentration

Table 2 shows the FFA content of PFAD and concentration of vitamin E, as a function of lipase concentration in the reaction mixture. The reactions were carried out at 65±1 °C, with 50% w/v of water for 3 h. When the lipase amount was below 1.0% w/w, the degree of hydrolysis was elevated with increase in the amount of lipase. The FFA content in PFAD increased from, initially, 83 to about 94% after reaction with 1.0% w/w of lipase for 3 h. The hydrolysis extent was not much increased, even though more lipase was used. Generally, there was no significant ($P>0.05$) increase in FFA content in PFAD with an increase in lipase concentration from 1.5 to 3.0% w/w. This might be due to the unavailability of substrate, or the number of units of substrate per unit of lipase was relatively low when the concentration of lipase increased. Similar changes were observed in vitamin E concentration extracted from the samples. Vitamin E initially increased significantly ($P<0.05$), with the increase of FFA levels in the PFAD when lipase concentration in the reactant increased from 0.5 to 2.5% w/w. Further increase in lipase concentration to 3.0% w/w did not significantly ($P>0.05$) increase the vitamin E concentration extracted from the samples.

3.5. Effect of reaction temperature

The effects of reaction temperature on the hydrolysis extent of PFAD and vitamin E concentration are shown in Table 3. Water content and lipase concentration were maintained at 50 and 1.0% w/w, respectively, and the reactions were carried out for 3 h. The hydrolysis extent after 3 h was somewhat low at 60 °C (FFA content was 84.4%). However, the FFA levels gradually increased when the reaction temperature increased to 70 °C. Vitamin E extracted also increased significantly ($P<0.05$) from, initially, 2.05% at 60 °C to 7.78% at 70 °C. At 70–75 °C, the FFA content was the highest (about 97%) since this was the optimum temperature for the lipase hydrolytic activity. High temperature may also

Table 2
Changes in percentages of free fatty acid (FFA) and vitamin E extracted as the function of enzyme concentration[a]

% Of enzyme	% Of FFA	% Of vitamin E
0.5	91.1±0.22c	3.28±0.21d
1.0	94.0±0.63b	4.91±0.13c
1.5	94.7±0.04b	5.83±0.15b
2.0	95.0±0.70a,b	6.13±0.10b
2.5	95.0±0.21a,b	6.85±0.08a
3.0	96.3±0.24a	6.79±0.17a

[a] Reactions were carried out at 65±1 °C for 3 h. Palm fatty acid distillate to water ratio was 1:1 w/v. Means within each column with different letters are significantly different ($P<0.05$).

Table 3
Changes in percentages of free fatty acid (FFA) and vitamin E as a function of reaction temperature[a]

Temperature, °C	% Of FFA	% Of vitamin E
60	84.4±0.77d	2.04±0.08e
65	94.0±0.63b	5.38±0.17c
70	97.5±0.19a	7.78±0.10a
75	97.2±0.96a	6.51±0.11b
80	92.9±0.26b,c	5.06±0.09c,d
85	91.2±0.73c	4.67±0.20d

[a] Reactions were carried out with 1% w/w of lipase for 3 h. Palm fatty acid distillate to water ratio was 1:1 w/v. Means within each column with different letters are significantly different ($P<0.05$).

shift the equilibrium of the reaction to hydrolysis faster and subsequently increase the FFA content in PFAD. However, vitamin E concentration extracted from PFAD decreased significantly ($P<0.05$) when the reaction temperature increased from 70 to 75 °C. High temperature (>70 °C) might have oxidised the tocols. Further increase in reaction temperature to 85 °C decreased FFA and vitamin E levels. FFA content was significantly ($P<0.05$) decreased from 97.5% at 70 °C to 91.2% at 85 °C, while vitamin E concentration decreased significantly ($P<0.05$) from 7.78% at 70 °C to 4.67% at 85 °C. Elevated temperature may inactivate the lipase even though the lipase has been immobilized. High temperature also promoted the oxidation of vitamin E and subsequently decreased its recovery.

This study showed that enzymatic hydrolysis had successfully pre-concentrated vitamin E in PFAD. Novozyme 435 lipase, the commercially immobilized thermal stable lipase was found to be capable of hydrolysing PFAD into FFA. Hydrolysis increased the FFA content in PFAD to a maximum of about 97% and vitamin E extracted was about 7%. Optimum reaction temperature and sufficient lipase concentration might shorten the reaction time. For example, hydrolyses of PFAD:water (1:1, w/v) mixture at 70 °C with 1.0% w/w of lipase for 3 h (Table 3) or at 65 °C, 2.0% w/w of lipase for 3 h (Table 2) would give similar FFA contents and vitamin E recoveries to that hydrolysed at 1.0% w/w for 6 h (Fig. 1). Interactions of these reaction parameters can be further optimised to achieve the maximum yield of vitamin E.

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References

Ab. Gapor, M. T., Leong, L. W., Ong, A. S. H., Kawada, T., Watanabe, H., & Tsuchiya, N. (1993). *Production of high concentration tocopherols and tocotrienols from palm oil by-products*. US Patent, 5 190 618.

Fizet, C. (1993). *Concentration process*. Malaysian Patent, MY 103 459.

Ghosh, S., & Bhattacharyya, D. K. (1996). Isolation of tocopherol and sterol concentrate from sunflower oil deodorizer distillate. *Journal of the American Oil Chemists' Society*, *73*, 1271–1274.

Goh, S. H., Lai, F. H., & Kam, T. S. (1992). *Method for the recovery of vitamin E (tocopherols and tocotrienols) concentrate from palm fatty acid distillate*. Malaysian Patent, MY 102 274.

Hunt, T. K., Jeromin, L., Johannisbauer, W., Bernhard, G., Jordon, V., Wogatzki, H., & Wood, J. D. (2000). *Recovery of tocopherols*. European Patent, EP 0 992 499.

Ong, A. S. H., Choo, Y. M., Goh, S. H., & Kam, T. S. (1994). *Recovery of carotenoids, tocopherols, tocotrienols and sterols from esterified palm oil*. Malaysian Patent, MY 104 388.

PORIM. (1995). In W. L. Siew (Ed.), *PORIM test methods* (pp. 40–42). Kuala Lumpur, Malaysia: Malaysian Palm Oil Board, Ministry of Primary Industries.

Ramamurthi, S., & McCurdy, A. R. (1993). Enzymatic pretreatment of deodorizer distillate for concentration of sterols and tocopherols. *Journal of the American Oil Chemists' Society*, *70*, 287–295.

Ramamurthi, S., Bhirud, P. R., & McCurdy, A. R. (1991). Enzymatic methylation of canola oil deodorizer distillate. *Journal of the American Oil Chemists' Society*, *68*, 970–975.

SAS. (1989). In *Statistical analysis system user's guide: statistics* (pp. 125–154). Cary, US: SAS Institute Inc.

Sheabar, F. Z., & Neeman, I. (1988). Separation and concentration of natural Antioxidants from the rape of olives. *Journal of the American Oil Chemists' Society*, *65*, 990–993.

Shimada, Y., Sugihara, A., Shibahiraki, M., Fujita, H., Nakano, H., Nagao, T., Terai, T., & Tominaga, Y. (1997). Purification of γ-linolenic acid from borage oil by a two-step enzymatic method. *Journal of the American Oil Chemists' Society*, *74*, 1465–1470.

Shimada, Y., Maruyama, K., Sugihara, A., Moriyama, S., & Tominaga, Y. (1997). Purification of docosahexaenoic acid from tuna oil by a two-step enzymatic method: hydrolysis and selective esterification. *Journal of the American Oil Chemists' Society*, *74*, 1441–1446.

Tan, B., & Brzuskiewicz, L. (1989). Separation of tocopherol and tocotrienol isomers using normal- and reversed-phase liquid chromatography. *Analatical Biochemistry*, *180*, 368–373.

Wang, C., Ning, J., Krishnan, P. G., & Matthees, D. P. (1998). Effects of steeping conditions during wet-milling on the retention of tocopherols and tocotrienols in corn. *Journal of the American Oil Chemists' Society*, *75*, 609–613.