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CONCENTRATION AND ISOLATION OF INDIVIDUAL VITAMIN E COMPONENTS IN PALM PHYTONUTRIENTS CONCENTRATE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLOURESCENCE DETECTION

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ABSTRACT

Palm phytonutrients concentrate is a rich source of vitamin E. Vitamin E content in palm phytonutrients concentrate was found to be 15 370 ppm. Components of vitamin E identified in the palm phytonutrients concentrate consists of α-tocopherol (α-T), γ-tocopherol (γ-T), α-tocotrienol (α -T_3), γ-tocotrienol (γ -T_3) and δ-tocotrienol (δ-T_3). This article reports a study on the concentration of individual palm vitamin E components in crude palm oil (CPO), palm phytonutrients concentrate (PPC) and unsaponifiables of palm phytonutrients concentrate (unsap PPC). This article also reports a study on the concentration of bulk vitamin E through the application of open column chromatography and subsequent isolation of individual palm vitamin E components using High Performance Liquid Chromatography (HPLC) with fluorescence detection and a semi-preparative silica column. As the vitamin E components are concentrated in a stepwise manner by means of saponification, open column chromatography and semi-preparative HPLC, the concentration of individual components can be enriched by up to 94.6%.

Keywords: antioxidants, HPLC, palm oil, phytonutrient, tocols.

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INTRODUCTION

Palm oil has always been an important economic commodity in Malaysia. Currently, palm oil is creating waves in the world market because of its significance in the field of biodiesel production. Palm phytonutrients concentrate is a by-product obtained during the production of palm biodiesel (Choo *et al.,* 2002a, c). In the production of palm biodiesel, crude palm oil (CPO) is transesterified in an alcoholic medium with an alkaline catalyst to produce red alkyl esters. These red or crude alkyl esters could be used directly as a fuel but they are rich in natural palm phytonutrients. The separation of the natural palm phytonutrients from the alkyl esters was successfully carried out by a short path distillation technology with minimal loss or degradation of both products (Ooi *et al.,* 1999; Choo *et al.,* 2002b; Choo, 2005). The distilled esters, a colourless liquid, are used as biodiesel, while the palm phytonutrients concentrate is collected as a thick red mixture.

Our current health-conscious society is aware that phytonutrients play important and a wide range of roles in our well-being (Sundram and Chandrasekharan, 1994; Gapor *et al.,* 2000). Phytonutrients commonly present in CPO, such as carotenes, vitamin E and co-enzyme Q (Goh *et al.,* 1985; 1997; Ooi, 1995; 1999; Chong and Rasid,

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1995; are useful substances for the prevention and treatment of cancers and various forms of cardiovascular-related ailments (Choo and Basiron, 1996; Choo and Tay, 2000a, b). The most prevalent phytonutrients found in CPO are vitamin E (600-1000 ppm), carotenes (500-700 ppm), sterols (250-620 ppm), squalene (200-600 ppm) and co-enzyme Q (10-80 ppm) (Choo and Tay, 2000b; Gapor *et al.*, 2002; Ng *et al.*, 2004; 2006).

Vitamin E is present bountifully in palm oil and is one of the four fat-soluble vitamins vital for our well-being (Tan, 1989; Sen *et al.*, 2007). It has eight members, divided into two groups with four compounds each. Vitamin E basically comprises a 6-chromanol ring and a side chain. Compounds with a saturated side chain are classified as tocopherols while compounds with a geranylgeranyl side chain with three double bonds are classified as tocotrienols (Hew, 1989; Sen *et al.*, 2006; 2007). Vitamin E is present in the range of 600-1000 ppm in CPO and consists of α-tocopherol (α-T), γ-tocopherol (γ-T), α -tocotrienol (α -T_3), γ -tocotrienol (γ -T_3) and δ-tocotrienol (δ- T_3) (Choo *et al.*, 1997; 2004).

Vitamin E is a powerful antioxidant that works to protect cells in the body from damage caused by free radicals (Hew, 1989; Sen *et al.*, 2007). It is especially important in protecting blood cells, the nervous system, skeletal muscle and the retinas in the eyes from free radical damage (Hew, 1989; Choo *et al.*, 1997; Choo and Tay, 2000b). Palm oil is unique as, unlike other vegetable oils which consist mostly of tocopherols, it has a higher concentration of tocotrienols (Choo *et al.*, 1997; 2004; Ng *et al.*, 2004). Tocotrienols are more potent as antioxidants compared to tocopherols (Hew, 1989; Choo *et al.*, 1997; 2004). Therefore, tocotrienols play a pivotal role in increasing the market value of the phytonutrients as well as of the palm oil itself, thus providing an improved and good marketability for palm oil (Gapor *et al.*, 2000; Sundram and Chandrasekharan, 1994).

This article reports on studies on the concentration of vitamin E compounds present in CPO and in palm phytonutrients concentrate, and also reports on the change in the concentration of individual vitamin E components after each step of the concentration process they underwent. Isolation of individual vitamin E components present in palm phytonutrients concentrate using High Performance Liquid Chromatography (HPLC) equipped with fluorescence detection is also reported. A high concentration of individual components obtained will be suitable for use both as supplements and as standard reference materials for research purposes (Ng *et al.*, 2004; 2006). Thus, isolation of palm vitamin E components in reasonable concentrations using HPLC will be beneficial to the oil palm industry.

MATERIALS AND METHODS

Materials

CPO was obtained from the Malaysian Palm Oil Board (MPOB) experimental mill while palm phytonutrients concentrate (PPC) was obtained from Carotino Sdn Bhd (Malaysia). All solvents used were of chromatographic or analytical grade, and purchased from Merck (Darmstadt, Germany) and J.T. Baker (Phillipsburg, NJ). All standards were purchased from Sigma-Aldrich (Malaysia).

Procedures

Preparation of unsaponifiables of palm phytonutrients concentrate. Two grams of palm phytonutrients concentrate were saponified at 60°C with 5 ml of 50% (w/v) potassium hydroxide solution and 20 ml ethanol. The mixture was refluxed in the dark for 1 hr. The reacted mixture was then extracted five times with 100 ml n-hexane until a colourless organic layer was obtained. The extracted organic layer was then washed with 100 ml distilled water until the waste water tested neutral with phenolphthalein. The solvent was evaporated off, and the sample was then stored in a cold, dry place. The sample was labelled as unsaponifiables of palm phytonutrients concentrate (unsap PPC).

Open column chromatography. A 3.0-cm diameter glass column was packed with silica gel to a height of 5.0 cm. The n-hexane was used as the initial eluting solvent. A known amount of unsap PPC was dissolved in a minimum amount of n-hexane and introduced into the column. The flow was maintained at a steady rate. Each fraction was collected according to the colour bands. When the fractions were almost colourless, hexane was replaced with ethanol and a final fraction was collected. The final fraction was evaporated off and the residue stored in a cold, dry place. The sample was labelled as open column (OC).

Analysis of carotenes. A known amount of CPO was dissolved in n-hexane in a 50-ml volumetric flask. The n-Hexane was used as a blank solution to monitor the baseline. A Hitachi Ultraviolet Spectrometer equipped with a single beam system, quartz cuvette and a pathlength of 10 mm was used to measure the absorbance value. The absorbance was measured at 446 nm. Using the results obtained, the concentration of carotenes in the sample was calculated, using the formula below:

[Carotene] = [383 x absorbance (at 446 nm) x volume (in ml)] / [100 x sample weight (in g)]

where:

[Carotene] = concentration of carotenes in ppm.

Volume = volume of volumetric flask.
383 = diffusion coefficient.

The procedure was repeated with PPC and unsap PPC.

Analysis of squalene and sterols. A known amount of CPO was weighed in a 1.5-ml vial and dissolved in a mixture of 0.2 ml triacontane and 1.3 ml N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) in dichloromethane (DCM). BSTFA in DCM was prepared by adding 0.3 ml BSTFA into 1.2 ml DCM. The prepared CPO was tightly capped and silylated for 2 hr at 60°C. Triacontane was used as an internal standard and BSTFA was used as a derivatizing agent. After silylation, the prepared CPO was injected into a gas chromatograph with a Flame Ionization Detector (GC-FID) system. A Hewlett Packard 5860 Series II Plus GC with a flame ionization detector was used with a BPX-5 GC capillary column (15 m x 0.32 mm ID BPX5 x 0.25 μm). GC conditions were as follows: injector temperature, 45°C; detector temperature, 370°C; initial oven temperature, 100°C; initial holding time, 1 min; ramping rate, 10°C min^{-1}; final temperature, 360°C; final holding time, 16 min; carrier gas (He) flow rate, 2 cm^3 min^{-1}; column pressure, 14.5 psi; injection volume, 1 μl (Lau *et al.*, 2005). Standards for squalene and sterols were also prepared using the same methods. The concentrations of sterols and squalene in CPO were calibrated using authentic standards. The procedures were repeated with PPC and unsap PPC.

Analysis of vitamin E. A known amount of CPO was weighed and dissolved with hexane in a 1.0-ml vial. The prepared CPO sample was then injected into a Waters HPLC equipped with an isocratic solvent delivery system and a Waters 470 Scanning Fluorescence Detector with excitation and emission wavelengths set at 295 nm and 325 nm, respectively. A Zorbax analytical silica column (25 cm x 4.6 mm ID, stainless steel, 5 μm) was used with the mobile phase of hexane: tetrahydrofuran: isopropanol (1000:60:4 v/v/v) at a flowrate of 1.0 ml min^{-1} (Ng, 2007). A standard sample with α-tocopherol was also prepared using the same method. Concentrations of the vitamin E compounds in CPO were calibrated using authentic standards. The procedures were repeated with PPC and unsap PPC.

Isolation of vitamin E components. A known amount of CPO was weighed and was dissolved with hexane in a 5.0-ml volumetric flask. The prepared CPO was then injected into a Waters HPLC, and a semi-preparative Kromasil silica column (25 cm x 2 cm ID, stainless steel, 5 μm) was used to separate the individual vitamin E components. The mobile phase used was hexane: tetrahydrofuran: isopropanol (1000:60:4 v/v/v) at a flowrate of 5.0 ml min^{-1}. Each peak observed in the chromatogram was collected as an individual fraction. Five peaks were collected and each labelled as semi-preparative (SP). Standard samples of tocols were also prepared using the same method. Concentrations of individual vitamin E components in CPO were calibrated using authentic standards. The procedures were repeated with PPC, unsap PPC and OC.

RESULTS AND DISCUSSION

The percentage of phytonutrients in PPC is approximately 5.72% of its weight while in CPO it is only 0.29% of its weight. *Figure 1* illustrates the difference in the concentrations of phytonutrients commonly found in CPO, PPC and in unsap PPC in parts per million (ppm). From the chart, it can be clearly seen that the presence of phytonutrients in PPC was higher and their concentration was further increased when PPC was saponified. A major portion of glycerides and esters was removed during the production of methyl esters (Ooi *et al.*, 1999; Choo, 2005), thus the presence of phytonutrients became more prevalent in PPC. However, the saponification process was necessary to separate the unsaponifiables or phytonutrients from the residual glycerides and esters still present in PPC. Saponification and subsequent separation resulted in an increase in the concentration of the phytonutrients present in PPC.

The concentration of carotenes was very high in both CPO and PPC. Thus, band broadening of carotenes caused interference to the elution of vitamin E. As both had almost similar retention times, they eluted out together in the SP HPLC. This affected the purity of the individual components of vitamin E obtained through the SP HPLC. To overcome this problem, OC chromatography was performed. OC chromatography enabled the separation of the group of vitamin E components from the bulk of carotenes. Carotenes eluted before vitamin E as they were less polar while the more polar vitamin E eluted later with ethanol as the mobile phase.

A normal phase silica column was sufficient to provide a good separation of the individual vitamin E components present in PPC. A fluorescence detector was used as all the five individual components present could be detected. Tetrahydrofuran enabled good separation of the peaks in the chromatogram obtained. The SP HPLC provided the flexibility for introducing the highly concentrated palm vitamin E sample to be separated into its five individual components. *Figure 2* shows the separation of palm vitamin E into the individual components using a HPLC with fluorescence detection and a SP silica column.

One of palm oil's unique characteristics is that it has a high concentration of tocotrienols in

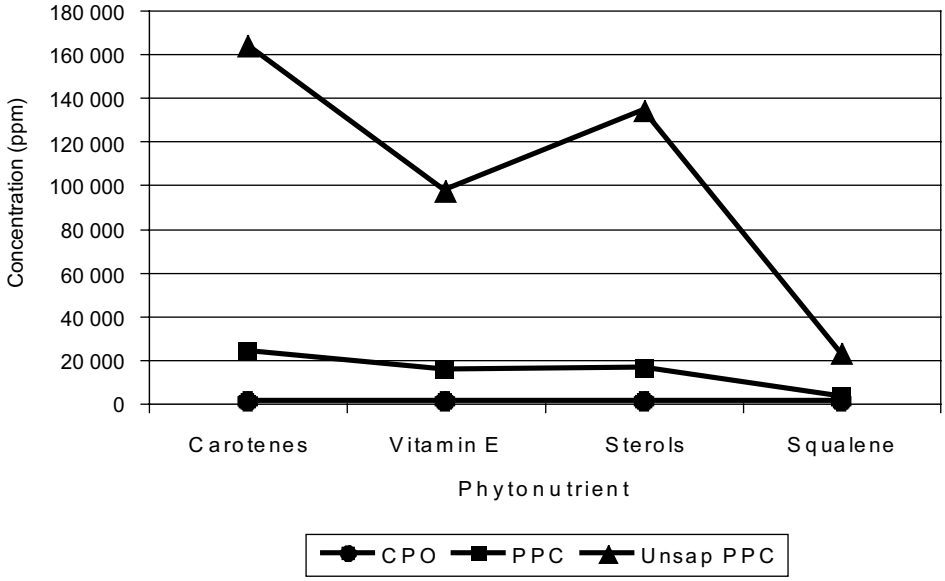

Figure 1. Concentrations of selected phytonutrients in crude palm oil (CPO), palm phytonutrients concentrate (PPC) and unsaponifiables of palm phytonutrients concentrate (unsap PPC).

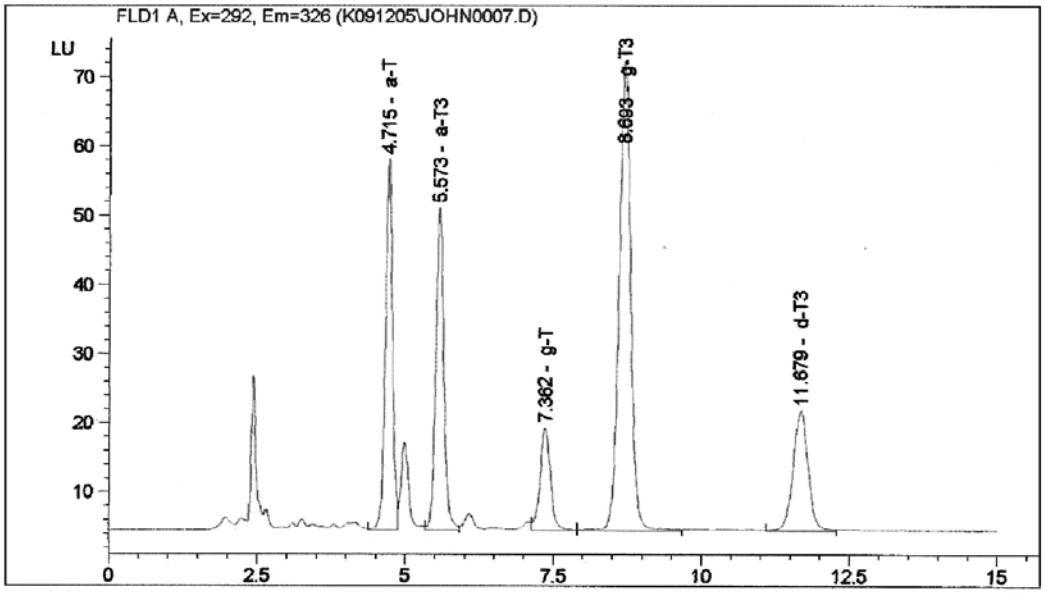

Figure 2. Separation of palm vitamin E into five individual components using a semi-preparative HPLC with fluorescence detection and a semi-preparative silica column.

comparison with all other types of vegetable oil. *Figure 2* presenting the results for PPC clearly shows that the concentrations of tocotrienols were higher than of tocopherols. Among the tocotrienols, γ-T_3 recorded a higher concentration than both α-T_3 and δ-T_3. The concentrations of α-T and α-T_3 were almost similar, and tocotrienols had a longer retention time compared to the corresponding tocopherols.

The concentrations of the individual components obtained from the SP HPLC were higher compared to those of the OC sample. However, whereas in all the other samples γ-T was only slightly recognizable, it can now be determined clearly. This is due to carotenes band broadening; thus, as the bulk of carotenes were removed, the concentrations of vitamin E components became more prominent and the γ-T peak was noticeable.

The concentrations of the individual components definitely showed an increase after the SP HPLC when compared to their concentrations in CPO, PPC, unsap PPC and OC fraction. *Table 1* follows the increase in concentration of each individual vitamin E component starting from CPO to the SP HPLC.

TABLE 1. CONCENTRATIONS OF INDIVIDUAL VITAMIN E COMPONENTS IN CRUDE PALM OIL (CPO), PALM PHYTONUTRIENTS CONCENTRATE (PPC), UNSAPONIFIABLES OF PALM PHYTONUTRIENTS CONCENTRATE (UNSAP PPC), OPEN COLUMN (OC) FRACTION AND SEMI-PREPARATIVE (SP) FRACTION

Vitamin E component	Concentration (ppm)				
	CPO	PPC	Unsap PPC	OC	SP
α-Tocopherol	130	1 310	21 360	21 670	640 880
α -Tocotrienol	230	3 230	21 510	39 860	945 968
γ-Tocopherol	30	720	5 750	8 980	101 779
γ -Tocotrienol	590	8 400	45 510	46 390	715 661
δ-Tocotrienol	70	1 710	2 850	15 200	151 932

CONCLUSION

The concentration of vitamin E is higher in PPC than in CPO. Unsap PPC allows for the removal of glycerides and esters, and thus results in a higher concentration of vitamin E. OC helps to minimize interference from the bulk of carotenes which is the most prevalent phytonutrient in palm oil. In the components isolation, a SP separation of palm vitamin E into its five individual components was carried out successfully. An individual vitamin E component could be enriched by up to 94.6% by the SP HPLC.

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