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Improved Efficiency of Tocotrienol Extraction from Fresh and Processed Latex

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Vitamin E, mainly in the form of tocotrienols, was extracted from Hevea brasiliensis latex with organic solvents. The content of tocotrienols and a small amount of tocopherols recovered from the latex was determined using high performance liquid chromatography (HPLC). Gas chromatography-mass spectrometry (GC-MS) confirmed the identities of the tocotrienols and tocopherols forms that were present. Gamma-tocotrienol was the most abundant form of vitamin E in Hevea latex. The yield of tocotrienols (339 µg/g of latex) was significantly increased by the use of the detergent Triton X-100 in the extraction procedure. This method improves the extraction efficiency by 83%. Through drying of the organic fraction using anhydrous magnesium sulphate following phase separation was also advantageous in the extraction procedure. On the other hand, the presence of ammonia in latex suspension reduced extraction efficiency. Vitamin E was also found in the waste serum generated from the processing of deproteinised natural rubber (DPNR). Although the yield of vitamin E from this source was relatively low, there is a potential to modify the processing procedure to produce another value added end product i.e. latex vitamin E in addition to DPNR.

Keywords: *Hevea brasilienses*; latex; vitamin E; extraction

There are eight natural variants of vitamin E occurring in the form of four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ) (*Figure 1*). The most common molecular variant of vitamin E available as a commercial health supplements is α–tocopherol. Tocotrienols resemble tocopherols, but the former has three double bonds in the carbon side chain of the molecules which render them more reactive in some situations than tocopherols that have three saturated bonds. Among the richest sources of tocotrienols is *Hevea brasiliensis* latex (8% w/v), followed by edible plant oils originating from plants such as oil palm, rice, wheat, barley, rye and oat[1].

Hevea latex vitamin E was first reported in the mid-1960s[2,3]. Dunphy *et al*.[2] found the presence of free and esterified tocotrienols

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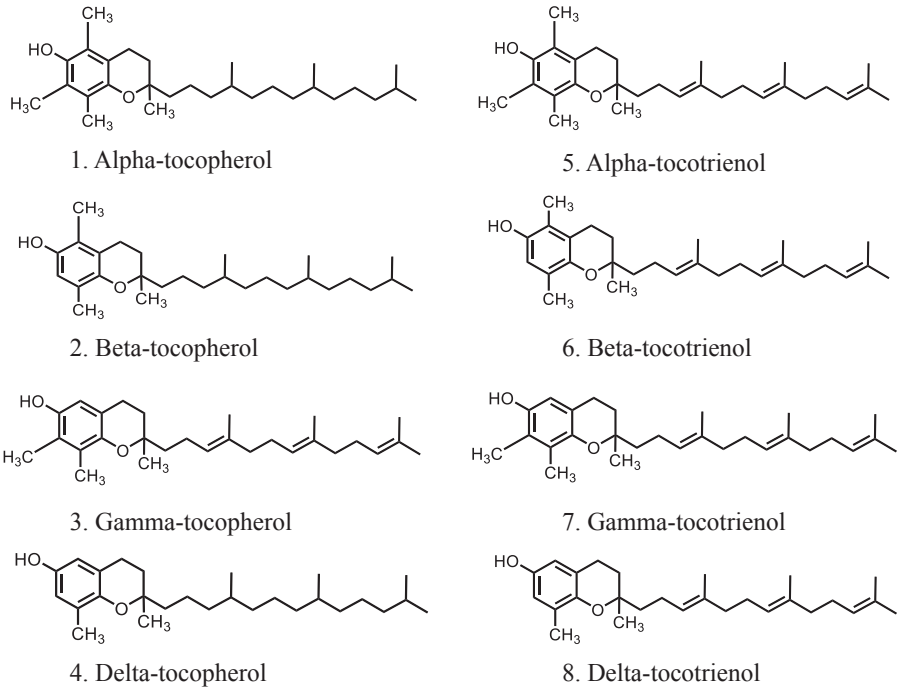

Figure 1. Structures of natural vitamin E constituents.

in *Hevea* latex by thin layer chromatography, and a year later, Whittle *et al*.[3] isolated a large amount of δ-tocotrienol from *Hevea* latex. Ho *et al*.[4] subsequently observed tocotrienols when they fractionated lipids from fresh latex, but did not subject them to detailed analysis. Hasma and Subramaniam[5] confirmed the presence of the alpha, gamma and delta forms of tocotrienol in latex from the *Hevea* clone RRIM 501.

In fresh *Hevea* latex, rubber occurs as spherical or pear shaped particles[6], representing 30-50% of its weight and constituting more than 90% of the total latex solids. Rubber particles range in size from below 0.005 to about 3μm[7], but particles up to 5–6 μm in diameter are occasionally encountered. Each rubber particle is surrounded by a phospholipid monolayer membrane and associated proteins[4]. Lipids constitute about 1.6% of the latex. Of this, 54% are neutral lipids consisting of free and esterified tocotrienols, free and esterified sterols, carotenoid pigments, tri-, di- and monoglycerides, free fatty alcohol and free fatty acids[5]. A schematic representation of the rubber particle surrounded by a protein lipid layer according to Nawamawat *et al*.[8] is presented in *Figure 2*.

Deproteinised natural rubber (DPNR)[9] is an improved form of natural rubber that is low in protein and other non-rubber contents. A product intended for specialty engineering applications, DPNR is commercially produced by treating field latex with a proteolytic enzyme to hydrolyse the proteins in the latex into polypeptides which are then washed away during processing. Ammonia, a non-ionic surfactant; a proteinase and hydroxylamine

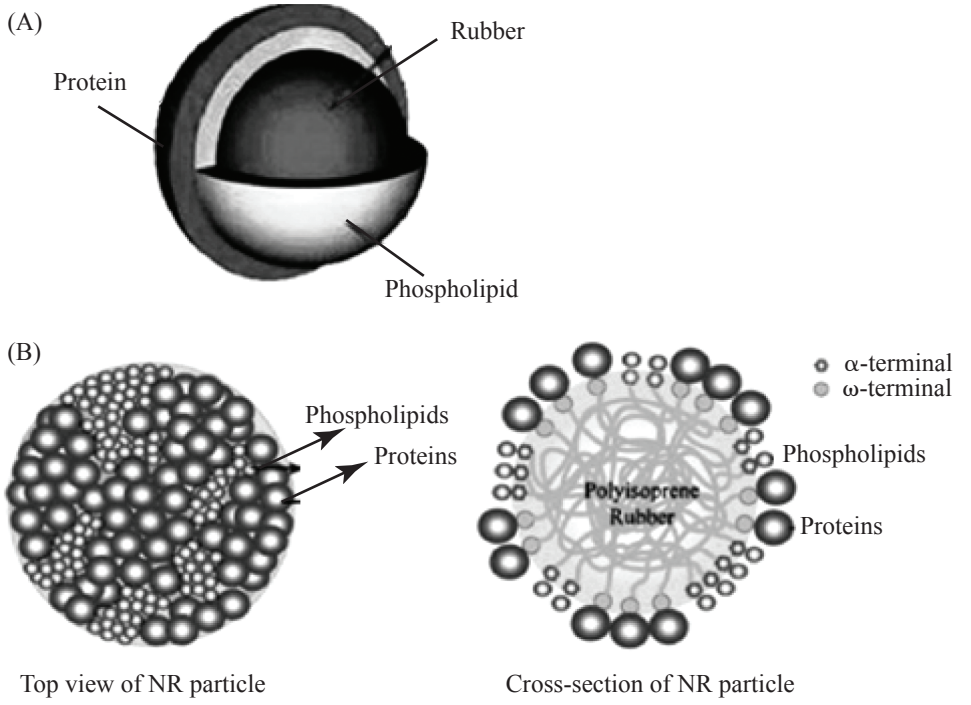

Figure 2. Two postulated models for the structure of the rubber latex particle surface. (A) A current model of an NR latex particle surrounded by a double-layer of proteins and phospholipids and (B) The proposed new model consisting of a mixed layer of proteins and phospholipids around the latex particle. Reprinted from Nawamawat et al.[8] with permission from Elsevier.

neutral sulphate (HNS) are added into the field latex and allowed to react for 72 hours in a reaction tank. After the completion of the enzymatic reaction, the latex is neutralised with formic acid and coagulated by steam. The coagulant is then creped using a lot of water to remove dirt and chemical residue in the rubber. *Figure 3* illustrates the DPNR production process. Besides the dirt and residue, DPNR waste also contains solubilised lipid-protein rubber particle membranes[9]. Hence, the commercial production of DPNR is a potential route by which tocotrienols can be recovered from the DPNR processing waste.

Vitamin E is a powerful biological anti-oxidant[10] that acts to protect cells against the effect of free radicals which are potentially damaging by-products of the human metabolism. Free radicals[11] can cause cell damage that may contribute to the development of various ailments including cardiovascular disease and cancer. Tocotrienols and tocopherols, which are forms of vitamin E, have been shown to reduce the level of plasma cholesterol that has been linked to cardiovascular disease[12]. Tocotrienols have shown to have anti-tumour activity which, in some studies, is shown to be the more potent form of vitamin E[13,15].

Current lipid extraction method from *Hevea* latex involves the use of only solvent[16] which is based on Folch[17] method. The

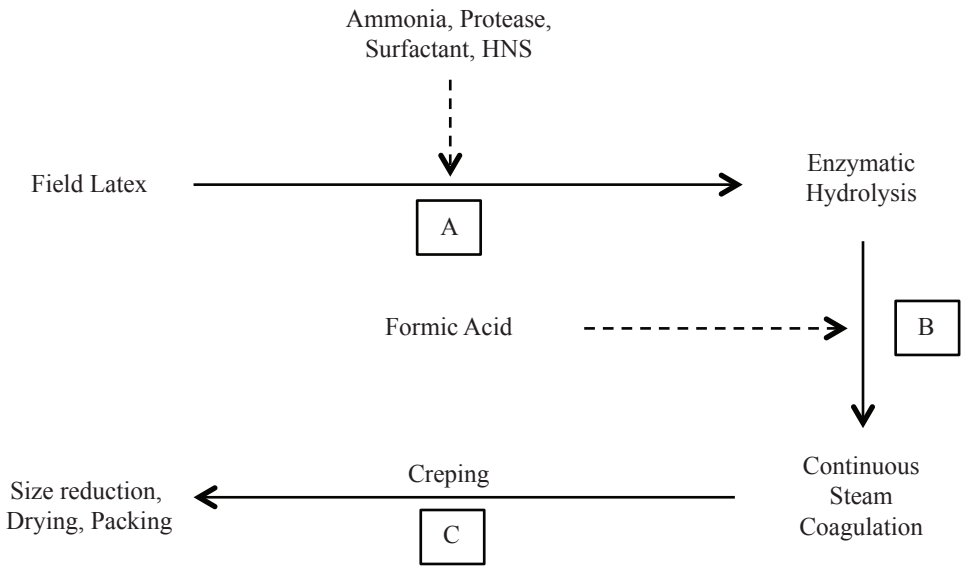

Figure 3. DPNR production flow chart (A) Before incubation sampling point (B) After incubation sampling point and (C) Sampling point for DPNR waste serum. (HNS=hydroxylamine neutral sulphate).

improvement of vitamin E extraction by the use of detergent, Triton X-100 and drying agent, anhydrous $MgSO_4$, used together with solvent extraction method is described. This paper examines the relative amounts of tocopherols and tocotrienols present in fresh natural rubber latex and from the waste serum generated by the production of DPNR.

MATERIALS AND METHODS

Latex Collection

Fresh *Hevea brasiliensis* latex of the clone RRIM 600 collected from tapped trees into a flask surrounded by ice was used for the vitamin E extraction. In some experiments, extractions were carried out on the rubber cream and bottom fraction obtained after high speed centrifugation. To prepare the rubber cream, the latex was centrifuged in a Sorvall RC-Evolution for 30 min at 43,000 × g at 4ºC. The latex separated into three phases with the rubber cream occupying the topmost phase. The rubber cream was collected from the centrifuge tubes, weighed and suspended in a buffer containing 50 mM Tris-Cl pH 7.0 (1 g rubber cream to 2 ml buffer). The 'bottom fraction' was also recovered after centrifugation. In another treatment, ammonium hydroxide was added to the latex to a final concentration of 0.7% to the fresh latex prior to centrifugation. The DPNR waste serum (rubber processing waste from the production of DPNR) was sourced from the Malaysian Rubber Board DPNR factory in Sungai Buloh, Selangor.

Lipid Extraction

Lipids were extracted from fresh latex, rubber cream and processed latex by the method previously described by Hasma and Subramaniam[5] based on Folch method[17] with slight modifications. To prepare latex mixtures containing detergent, Triton X-100 was added to the fresh latex to a final concentration of 0.1%. Buffer (50 mM Tris-Cl) was added to make up the desired volume as required. The fresh latex or latex mixtures were added dropwise to the chloroform-methanol solvent mixture (1 part latex: 5 parts solvent) with stirring whereby rubber coagulation ensued. After separation from the coagulum, the solvent extract was subjected to washing with 0.2 volume of 0.58% sodium chloride solution. The mixture was allowed to settle overnight. The solvent phase was collected and dried over anhydrous magnesium sulphate. After the removal of the latter by filtration, the solvent extract was then subjected to rotary film evaporation to complete dryness. The resulting residue was dissolved in minimal chloroform.

High Performance Liquid Chromatography (HPLC)

Analyses of the latex extracts and standards were performed using Waters® 600 HPLC linked to a fluorescence detector (WATERS 2475 Multi λ). A Supelco® LC-Diol column (250 mm × 4.6 mm × 5 um) or Chromolith Silica (100 mm × 4.6 mm × 5 um) was used with the mobile phase of 0.1% isopropanol in hexane at a flow rate of 1ml/min; the injection volume was 20 µl. All solvents employed were of chromatographic grade. The tocopherol and tocotrienols peaks from the latex sample were identified by comparing their peaks with those obtained using pure, authentic standards of tocotrienol (α, β, γ and δ) and tocopherol (α, β, γ and δ) acquired from CALBIOCHEM. Peak areas were determined by integration software (CSW® Data Apex).

Gas Chromatography-Mass Spectrometry (GC-MS)

Latex tocopherol and tocotrienols were separated and identified by GC-MS on an Agilent 6890N gas chromatograph coupled with an Agilent 5793 inert mass spectroscopy detector running ChemStation software. Chromatographic separation was achieved using an Agilent DB 5 ms capillary column (30 m × 0.25 mm × 0.25 µm) in the splitless mode operating under the following parameters: inlet pressure of 8 psi, inlet temperature of 270ºC, injection volume of 0.2 µL, with helium as the carrier gas. Oven temperature was set at 70ºC for 2 min, increased at 20ºC per min to 300ºC and held constant for 11.5 minutes. The MS transfer line temperature was 300ºC. Mass spectrometric detection was conducted in full scan mode from m/z 30 to 650.

RESULTS AND DISCUSSION

Earlier studies have shown the quantitative distribution of the lipids in various fractions of centrifuged latex[18]. Tan found the highest yield of tocotrienols in the rubber phase (675 µg/g rubber), while small amounts were also observed in the bottom fraction (100 µg/g freeze dried material) and Frey-Wyssling particles (162 µg/g rubber)[18]. Result from our laboratory similarly showed similar trend whereby the rubber phase extract gave the highest yield of tocopherol/tocotrienols from latex (data not shown).

Studies on the lipids in latex have been carried out by using ammoniated latex concentrate[3] and centrifuged latex[4] as the starting material. While centrifuged latex

provided an advantage of extracting lipid without non-rubber particle, having whole latex as the main source of latex vitamin E would be suitable as high speed centrifugation can be eliminated step in the extraction method. This will provide an advantage should further upscale of extraction procedure ensues.

Latex Vitamin E Separated by HPLC

In the HPLC analysis of the latex extracts, peaks of vitamin E molecular variants were well separated and could be clearly identified by comparison with pure reference standards (*Figure 4*). A typical HPLC separation for the latex derived lipid is shown in *Figure 5*. The latex lipid extracts contained α–tocopherol and the α, γ and δ molecular forms of tocotrienol. Gamma-tocotrienol comprised the highest proportion of the total tocopherol/tocotrienol in latex (*Table 1*). This finding agrees with earlier studies that describe the association of tocopherol and tocotrienol with lipids of the rubber particles[4,5]. In addition to the identification of the vitamin E in the latex, HPLC data was also used to calculate the amount of vitamin E in the latex extract.

Identification of Vitamin E Molecular Forms by GC-MS

As the identities of the various vitamin E molecular forms were provisionally determined in HPLC analyses by their retention times, confirmation was sought by GC-MS.

The mass spectra of both β and γ-tocopherol and β and γ-tocotrienol were similar for a unique peak assignment to be made of either set of these isomers (*Figure 6*). To aid the identification of peaks of interest in the chromatograms, the ChemStation software was used to obtain ion chromatograms of ion fragments unique to each compound. Mass spectra for α, β and γ-tocopherol and α, β and γ-tocotrienol obtained from mass spectra libraries were compared to the mass spectra obtained from peaks of interest in the chromatogram. The ion fragments used to deduce the peaks in chromatogram are given in *Table 2*. The fragmentation ion for the β-/γ- tocopherol and β-/γ- tocotrienol gave an intense signal at m/z 151 (*Figures 7a* to *7d*), which is fragmentation peak from m/z 416 and m/z 410 respectively in the full scan mass spectrum. The fragmentation ion for the α-tocopherol and α-tocotrienol gave an intense signal at m/z 165 (*Figure 8a* and *8b*), which is the fragmentation peak from m/z 430 and m/z 424 respectively in the full scan mass spectrum. Standard γ-tocotrienol was chromatographed to confirm the presence and the identity of the isomer in the latex extract. The fragmentation ion for the γ-tocotrienol gave an intense signal at m/z 151, which is the fragmentation peak from m/z 410 in the full scan mass spectrum (*Figure 9*).

Table 2 summarises the GC-MS data obtained from the analysis of vitamin E from latex. Compounds are listed in order of their elution. The major vitamin E constituent of *Hevea* latex is γ-tocotrienol (78%). The latex lipid extracts contained small amount of α-tocotrienols (18%), α–tocopherol (1%) and β-/γ- tocopherol (2%). *Table 3* shows the fragment ions for γ-tocotrienol standard.

Together with HPLC data, the presence of vitamin E isoforms in *Hevea* latex is confirmed. HPLC method is used to show the presence of vitamin E isoforms by retention time while GC-MS is used to confirm the existence of the isoforms by mass spectrometry.

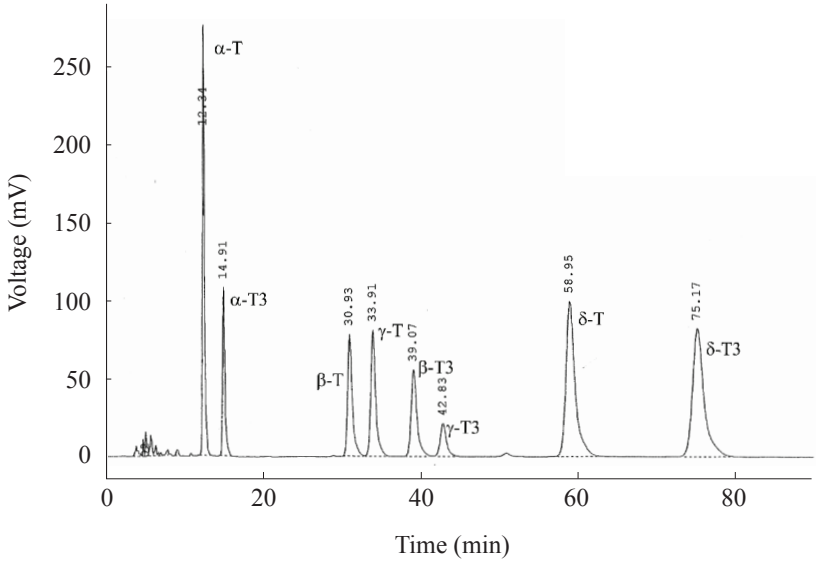

Figure 4. A typical chromatogram of standards run on HPLC. Retention times: α-tocopherol at 12.34 min, α-tocotrienol at 14.91 min, β-tocopherol at 30.93 min, γ-tocopherol at 33.91 min, β-tocotrienol at 39.07 min, γ-tocotrienol at 42.83 min, δ-tocopherol at 58.95 min and δ-tocotrienol at 75.17 minutes (T = tocopherol; T3 = tocotrienol).

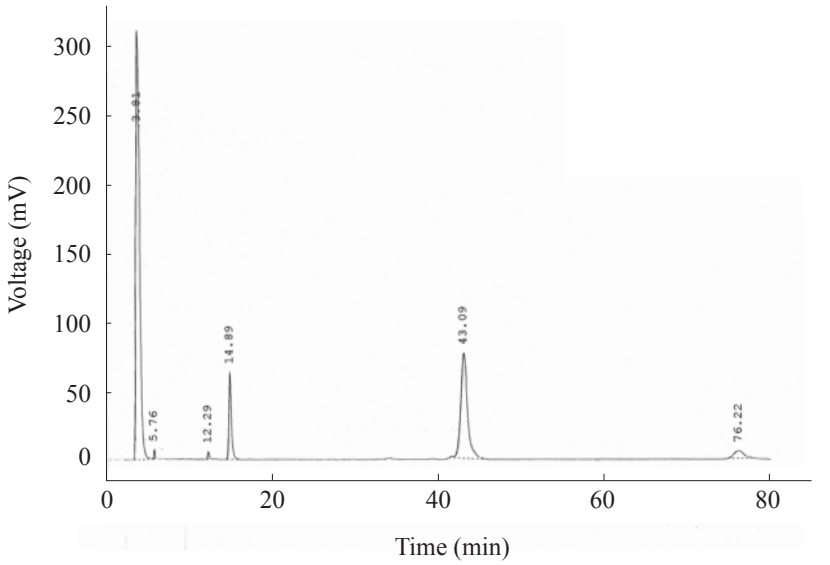

Figure 5. A typical chromatogram of latex extracts on HPLC. Retention times: α-tocopherol at 12.29 min, α-tocotrienol at 14.89 min, γ-tocotrienol at 43.09 min and δ-tocotrienol at 76.22 minutes.

TABLE 1. EFFECT OF TRITON X-100 ON THE EXTRACTION OF TOCOPHEROL AND TOCOTRIENOLS IN FRESH LATEX

Sample	Alpha-T	Alpha-T3	Gamma-T3	Delta-T3	Total
Fresh latex	0.55	36.53	143.14	5.68	185.90
Fresh latex + Triton X-100	1.05	64.26	263.62	10.51	339.44

Values in µg/g of latex, determined by HPLC
(T = tocopherol; T3 = tocotrienol)

TABLE 2. GC-MS ANALYSIS OF VITAMIN E FROM *HEVEA* LATEX

Identified vitamin E	RT (min)	MS Spectral data (% relative abundance)
Alpha-T	20.7	43 (51), 135 (36), 165 (100), 430 (100)
Beta/Gamma-T	19.6	43 (45), 81 (50), 151 (90), 416 (100)
Alpha-T3	22.9	57 (68), 85 (42), 165 (100), 205 (17), 429 (67)
Gamma-T3	21.4	69 (49), 151 (100), 191 (28), 410 (61)

(T= tocopherol; T3=tocotrienol)

Improvement of Vitamin E Extraction Efficiency

Fresh latex and DPNR latex treated with Triton X-100 gave higher yields in tocopherol/tocotrienol than untreated latex (*Tables 1* and *4*). The yield of tocotrienols was significantly increased from 185.90 µg tocotrienols/g of latex to 339 µg tocotrienols/g of latex by adding the detergent Triton X-100 in the extraction procedure. This method improves the extraction efficiency by 83 percent. The result suggests that the detergent added might have solubilised the membrane of the rubber particles, leading to the lipids being more efficiently extracted. The increase in extractability of lipids by addition of detergent might be also caused by detergent forming a detergent-protein micelle, thus allowing a deeper penetration of solvent and a more complete extraction of lipids[19]. Another possibility is lipid enrichment step using detergent has been shown to remove most lipid[20]. This has then lead to a better lipid extraction by chloroform methanol mixtures.

Anhydrous magnesium sulphate was used as a drying agent to remove the water molecules since complete extraction of lipids could be achieved after complete removal of water molecules[19]. *Table 5* shows that the lipid extracts subjected to two drying steps yielded higher vitamin E content than extracts treated with a single drying step. Thus, more thorough dehydration facilitated vitamin E extraction from latex.

The effect of ammonia on the latex vitamin E was also examined because in commercial latex concentrate processing, ammonia is used to stabilise latex preventing it from coagulating. In *Table 6*, it can be seen that the yield of tocotrienol/tocopherol extracted from the rubber cream in the presence of ammonia was

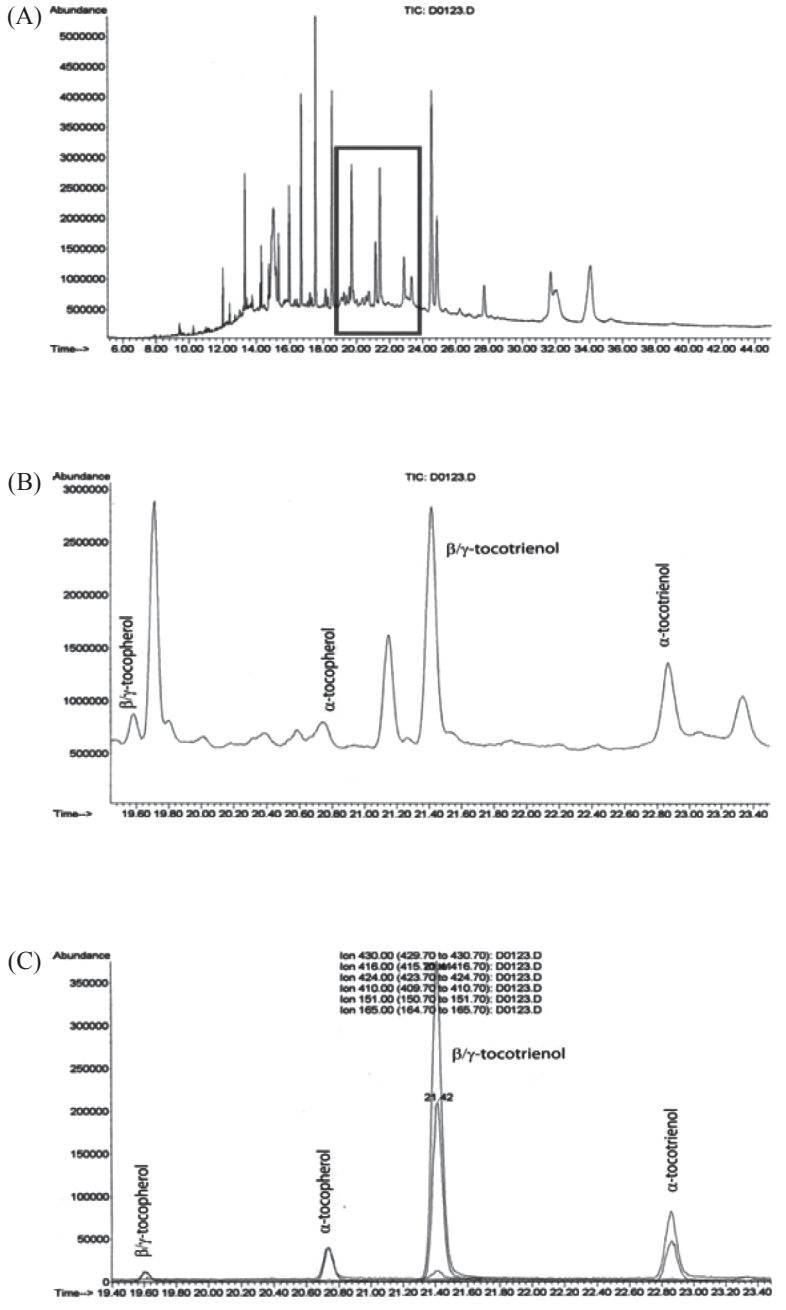

Figure 6. (A) GC-MS analysis of lipid extract from fresh latex (B) Expanded area in "A" showing peaks of interest (C) Extracted ion chromatogram of lipid extract showing how peak assignment was made by using suitable ion fragments for each component of interest (Table 3).

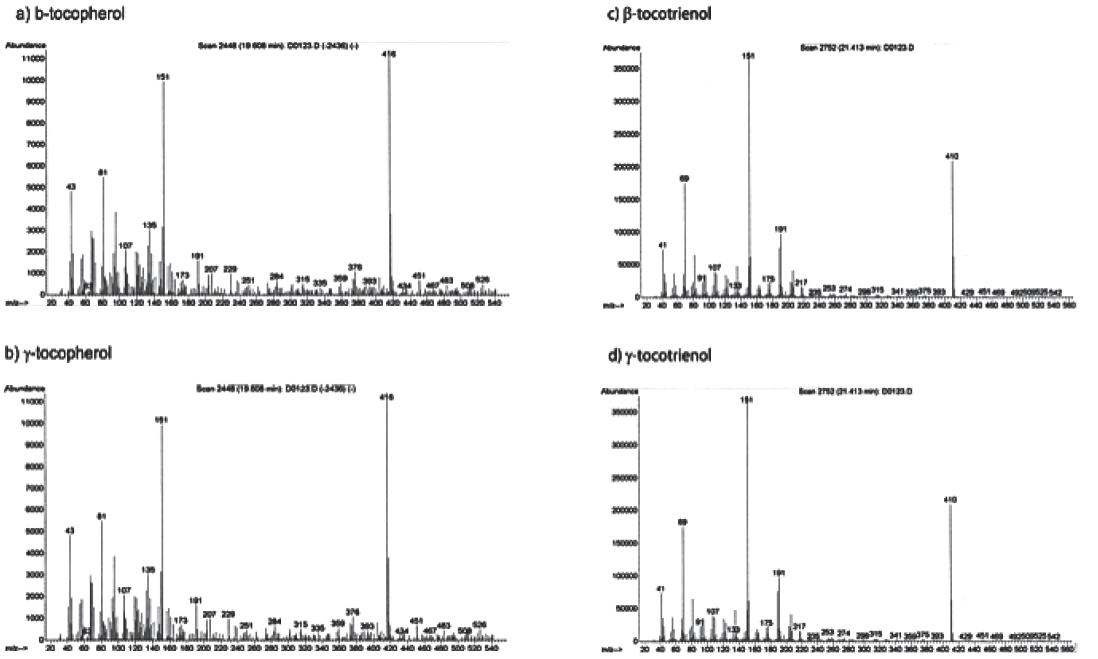

Figure 7. (a) and (b) Mass spectrum obtained for the first peak (Figure 6B) (β/γ-tocopherol) showing the major ion fragments used in the assignment of that peak – m/z = 151 and 416 (c) and (d) Mass spectrum obtained for the third peak (Figure 6B) (β/γ-tocotrienol) showing the major ion fragments used in the assignment of that peak – m/z = 151 and 410.

TABLE 3. GC-MS ANALYSIS OF GAMMA-T3 STANDARD

Identified vitamin E	RT (min)	MS Spectral data (% relative abundance)
Gamma-T3	21.4	69 (55), 151 (100), 191 (28), 410 (56)

TABLE 4. EFFECT OF TRITON X-100 ON THE EXTRACTION OF TOCOPHEROL AND TOCOTRIENOLS IN DPNR LATEX AND DPNR SERUM

	Triton X-100	Alpha-T	Alpha-T3	Gamma-T3	Delta-T3	Total
DPNR latex before incubation with alcalase	+	2	35	62	4	103
	-	1	28	38	5	72
DPNR latex after incubation with alcalase	+	0	6	22	2	30
	-	1	11	14	3	29
DPNR waste serum	+	0	4	20	4	28
	-	0	1	8	2	11

Values in μg/g of latex; determined by HPLC
(T = tocopherol; T3 = tocotrienol)

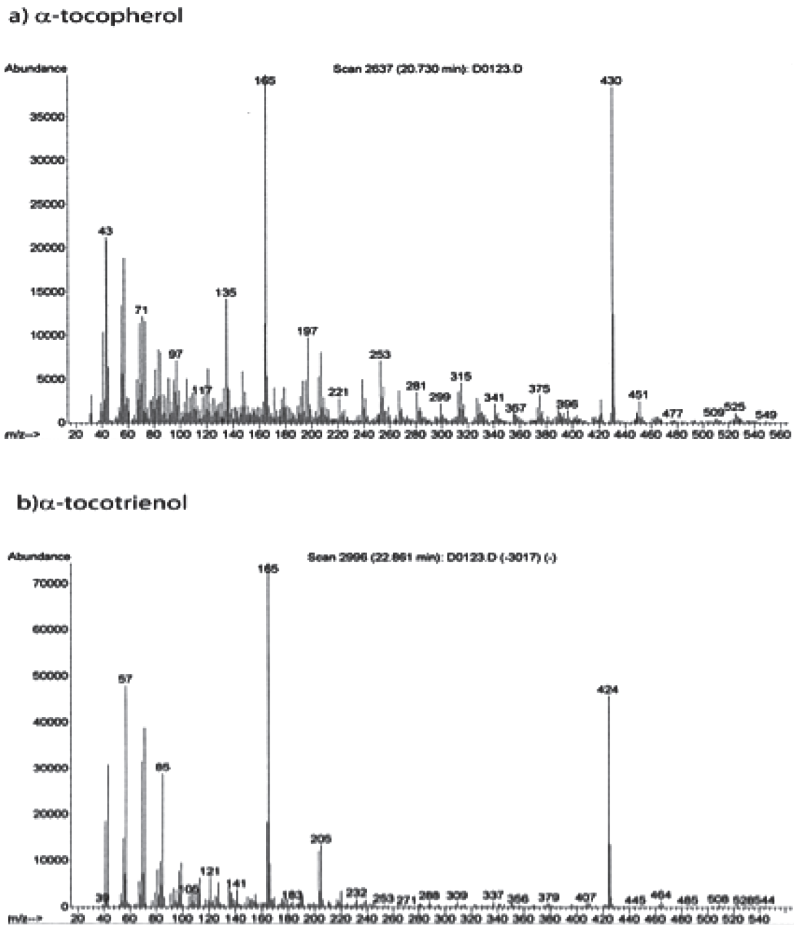

Figure 8. (a) Mass spectrum obtained for the second peak (Figure 6B) (α-tocopherol) showing the major ion fragments used in the assignment of that peak – m/z = 165 and 430 (b) Mass spectrum obtained for the fourth peak (Figure 6B) (α-tocotrienol) showing the major ion fragments used in the assignment of that peak – m/z = 165 and 424.

lower than from rubber cream prepared when ammonia was absent. Study by Gazeley *et al*.[21] suggests that as a consequence of the addition of ammonia to preserve the latex concentrate, the lipids hydrolyse slowly, releasing fatty acid soaps which are then adsorbed onto the particle surface. As a result, there could be less intact rubber particle membrane available for the organic solvent extraction.

Extraction of Vitamin E from Latex Waste Generated in Deproteinised Natural Rubber (DPNR) Processing

DPNR is a purified form of natural rubber in which most of the protein components have been removed[9]. The lipid protein rubber particle membranes are solubilised in the serum, which is the byproduct from the DPNR

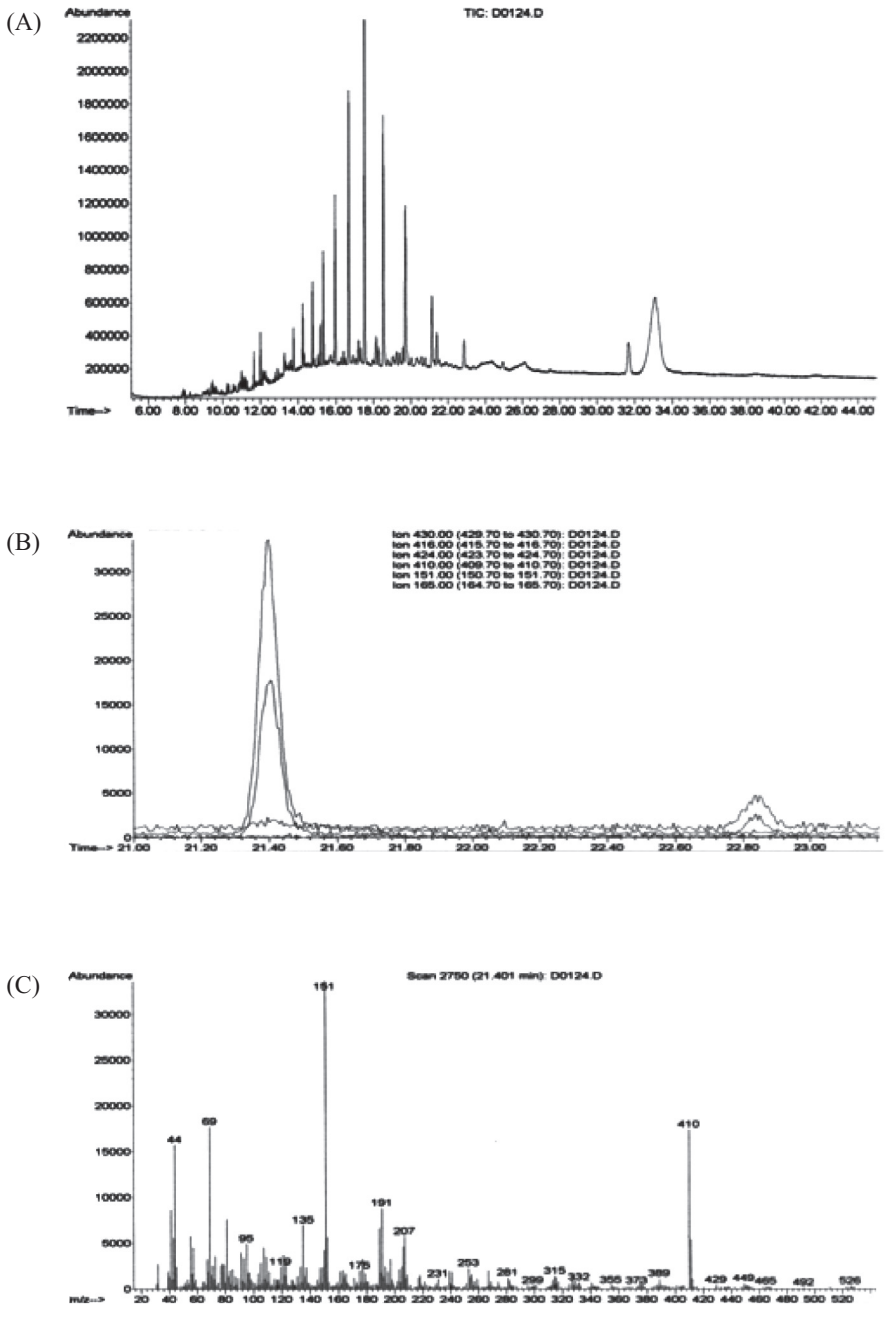

Figure 9. GC-MS analysis of γ-tocotrienol standard (A) Total ion chromatogram of γ- tocotrienol standard (B) Extracted ion chromatogram of the standard showing how peak assignment was made by using suitable ion fragments for each component of interest (C) Mass spectrum obtained for the γ-tocotrienol showing the major ion fragments used in the assignment of that peak – m/z = 151 and 410.

TABLE 5. EFFECT OF DRYING THE SOLVENT PHASE WITH ANHYDROUS MAGNESIUM SULPHATE ON TOCOPHEROL AND TOCOTRIENOLS EXTRACTION FROM FRESH LATEX

Treatment	Alpha–T	Alpha-T3	Gamma-T3	Delta-T3	Total
Anhydrous $MgSO_4$ (2 cycles)	2	34	137	7	180
Anhydrous $MgSO_4$ (1 cycle)	1	15	29	3	49

Values in µg/g of latex; determined by HPLC
(T = tocopherol; T3 = tocotrienol)

TABLE 6. EFFECT OF AMMONIA ON THE EXTRACTION OF TOCOPHEROL AND TOCOTRIENOLS FROM THE RUBBER CREAM OF CENTRIFUGED LATEX

Sample	Alpha–T	Alpha-T3	Gamma-T3	Delta-T3	Total
Rubber cream	1.51	44.24	181.71	8.70	236.16
Rubber cream (with ammonia)*	0.86	28.13	165.65	3.64	198.28

*The fresh latex contained 0.7% ammonia
Values in µg/g of latex; determined by HPLC
(T = tocopherol; T3 = tocotrienol)

processing. An investigation was carried out to determine the vitamin E content in the DPNR latex and serum. It can be seen in *Table 4* that DPNR latex without enzyme treatment yielded higher levels of tocotrienols/tocopherols in comparison to DPNR latex that had undergone three days of enzyme incubation. The presence of chemicals such as ammonia and enzyme during the incubation period might have affected the lipid components on the rubber particles. However, the yield of vitamin E from waste DPNR serum was found relatively low (*Table 4*). The low content could be due to the excessive water content in the serum. The serum was collected from the DPNR factory during the creping process, at which time a lot of water was used to remove dirt and chemical residues in the rubber. The added water content in the DPNR waste serum would have resulted in a significant dilution effect. In addition, inefficiency in moisture removal step from the organic fraction during lipid extraction procedure would also have contributed to the low vitamin E yield. It must be noted that this source material was obtained from the conventional DPNR production line without provisions made to accommodate vitamin E recovery. There is obviously a potential to modify the DPNR methodology with the aim of producing two value added end products, namely latex vitamin E, besides DPNR itself.

CONCLUSION

A reason latex vitamin E has not been widely exploited as phytonutrient is that it is almost exclusively of the tocotrienol molecular form rather than the better known tocopherol. Until of late, it was thought that only tocopherols were nutritionally beneficial as vitamin E and that tocotrienols were less effective. In recent years, however, there has been considerable research examining the superior effect of tocotrienol over tocopherol in health care applications in particular for cardiovascular protection and anticancer activities. Several studies have shown that tocotrienol is more effective in suppressing tumour cell growth[22,23] and reducing plasma cholesterol level[24,25]. Although plants are the sole source of vitamin E[26], tocotrienols are not usually found in the green parts of higher plants[27]. Rather, tocotrienols are found in seeds[28] and in latex[2] excreted from specialised cells like laticifers[29]. Because the rubber in latex tends to coagulate easily, the extraction of vitamin E from *Hevea* latex is a challenging task. Nevertheless, the fact that latex vitamin E is almost entirely tocotrienols could well be its most valuable asset.

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