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Composition of Lipids in Latex of Hevea brasiliensis *Clone RRIM 501*

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Lipids were extracted from fresh latex of Hevea brasiliensis *clone RRIM 501 and analysed qualitatively and quantitatively by a combination of chromatographic, spectroscopic and colorimetric techniques. The total lipids constituted about 1.6% of the latex, out of which 54% was due to neutral lipids, 33% glycolipids and 14% phospholipids. The neutral lipids comprised of carotenoid pigments, free and esterified sterols, free and esterified tocotrienols, free fatty alcohols and their acetates, tri-, di- and monoglycerides and free fatty acids. Triglycerides alone constituted about 63% of the neutral lipids or 0.6% of the latex, making them the major components of* H. brasiliensis *lipids. The glycolipids consisted of free and esterified steryl glucosides, di- and monogalactosyl diglycerides while the phospholipids contained mainly phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol.*

Natural rubber derived from *H. brasiliensis* latex contains 4%-5% non-rubber substances comprising of amines, amino acids, carbohydrates, inorganic constituents, proteins, nucleic acids, nucleotides and lipids. Some of these non-rubber substances, especially the lipids and the precipitated proteins, which are retained in the dry rubber after coagulation and drying have been found to affect the rubber properties. Thus there have been several investigations on the nature and role of these substances in *H. brasiliensis* latex and rubber[1-7].

Most of the studies on non-rubber substances were made twenty to fifty years ago using classical methods of analysis without utilising modern techniques such as thin layer chromatography (TLC), gas liquid chromatography (GLC) and spectroscopic techniques. Consequently no complete analysis of all the lipids present in *H. brasiliensis* latex has been reported. Furthermore no special attention was given to the source (clonal or bulked) and nature (fresh, preserved or concentrated) of the latex for analysis. These factors may give rise to variable lipid compositions.

Ho *et al.*[8] were the first to clearly fractionate the total neutral lipids and phospholipids from fresh latex of a *H. brasiliensis* clone on TLC. They were however unable to confirm the identities of some of the lipids and no attempts were made to isolate and further analyse the composition of these lipids. This paper reports the characterisation and composition of lipids in fresh latex of a *H. brasiliensis* clone, RRIM 501.

MATERIALS AND METHODS

Materials

Fresh latex was obtained from mature unstimulated trees of RRIM 501. The lipid standards were purchased from Sigma Chemical Company and Applied Science Laboratories. The glycolipid standards were obtained from Supelco Incorporated.

Extraction and Fractionation of Lipids

Lipids were extracted from fresh latex by drop-wise addition of the latex to at least five volumes of a continuously stirred chloroform/methanol (2:1, v/v) mixture. The extracts separated from the rubber coagulum were washed with sodium chloride solution according to the procedure of Folch *et al*[9].

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Total extracts were separated by silicic acid column chromatography (CC) into neutral lipids, glycolipids and phospholipids[10].

Thin Layer Chromatography

Each lipid class was analysed by TLC on plates coated with silica gel G. The neutral lipids were separated with hexane/benzene (85:15, v/v) followed by hexane/diethyl ether/acetic acid (69:29:2, v/v/v), the glycolipids with chloroform/methanol/water (95:20:2.5, v/v/v) and the phospholipids with chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v/v/v/v). The sterol acetates were fractionated on 20% $AgNO_3$ – TLC plates and developed in hexane-benzene (1:1, v/v). The neutral lipids were detected by 3% solution of cupric acetate in 8% ortho phosphoric acid[11], the glycolipids by α-naphthol followed by concentrated sulphuric acid[12] and the phospholipids by the modified molybdenum reagent[13]. The lipid layers including the sterol acetates on the preparative TLC plates were located by 2′,7′-dichlorofluoresceine.

Quantitative Determination of Lipids

The various lipids were quantified following the scheme outlined in *Figure 1*. The total lipids were fractionated on silica gel CC and eluted with hexane-ether mixtures followed by methanol to give various fractions of neutral lipids and polar lipids, respectively. The hexane-diethyl ether (95:5, v/v) eluent contained some rubber. The eluent was thus concentrated and acetone added to separate the acetone insoluble rubber from the acetone soluble lipid esters. The combined weights of all the hexane-diethyl ether eluents (minus rubber) give the amount of neutral lipids. The amounts of glycolipids and phospholipids were determined by multiplying the amount of sugar[14] and phosphorus[15] in the methanol eluent with their conversion factors (CF), 4.7 and 25.9 respectively[16]. Similarly the triglycerides were quantified by multiplying the amount of glycerol[16] in the methanolysate hexane-diethyl ether (75:25, v/v) eluent with its CF, 10.4.

The tocotrienols and sterols were determined by GLC after further purification on TLC.

Gas Liquid Chromatography

Gas liquid chromatography was performed on a Pye Unicam GCD Liquid Chromatograph and a Hewlett-Packard 5750 Research Chromatograph, equipped with flame ionisation detectors. The fatty acid methyl esters were analysed on a polyethyleneglycol adipate column; sterols, tocotrienols and long-chain alcohol acetates on a 3% SE-30 silicone gum column; and the trimethylsilyl (TMS) ether derivative of methyl glycosides on a UCC-W-982 (vinyl methyl) column.

Spectrometry

The mass spectra were obtained on a GC/AEI MS3074 instrument attached to a DS-50S mass spectroscopy (MS) data system. The nuclear magnetic resonance (^{1}H NMR) spectra were recorded from carbon tetrachloride or deuterated chloroform solution on a Hitachi-Perkin Elmer R-20B instrument at 60 MHz using tetramethysilane as internal reference. The infra-red (IR) spectra were obtained from carbon tetrachloride solution on a Beckmann IR 4250 spectrophotometer.

The results reported are the average of three samplings.

RESULTS

Characterisation of Lipids

Neutral lipids. Thin layer chromatography of the neutral lipids *(Figure 2)* gave thirteen spots. The identities of the spots *A, B, E, I, J, K, L* and *M* were obtained by comparing with their standards. Lipids *C, D, F* and *G* were isolated from preparative TLC along with the other major neutral lipids and analysed for their structural compositions by a combination of chromatographic and spectroscopic techniques. *Table 1* gives the composition of the major neutral lipids of RRIM 501 latex.

The free sterols were analysed chromatographically and by MS. Fractionation on

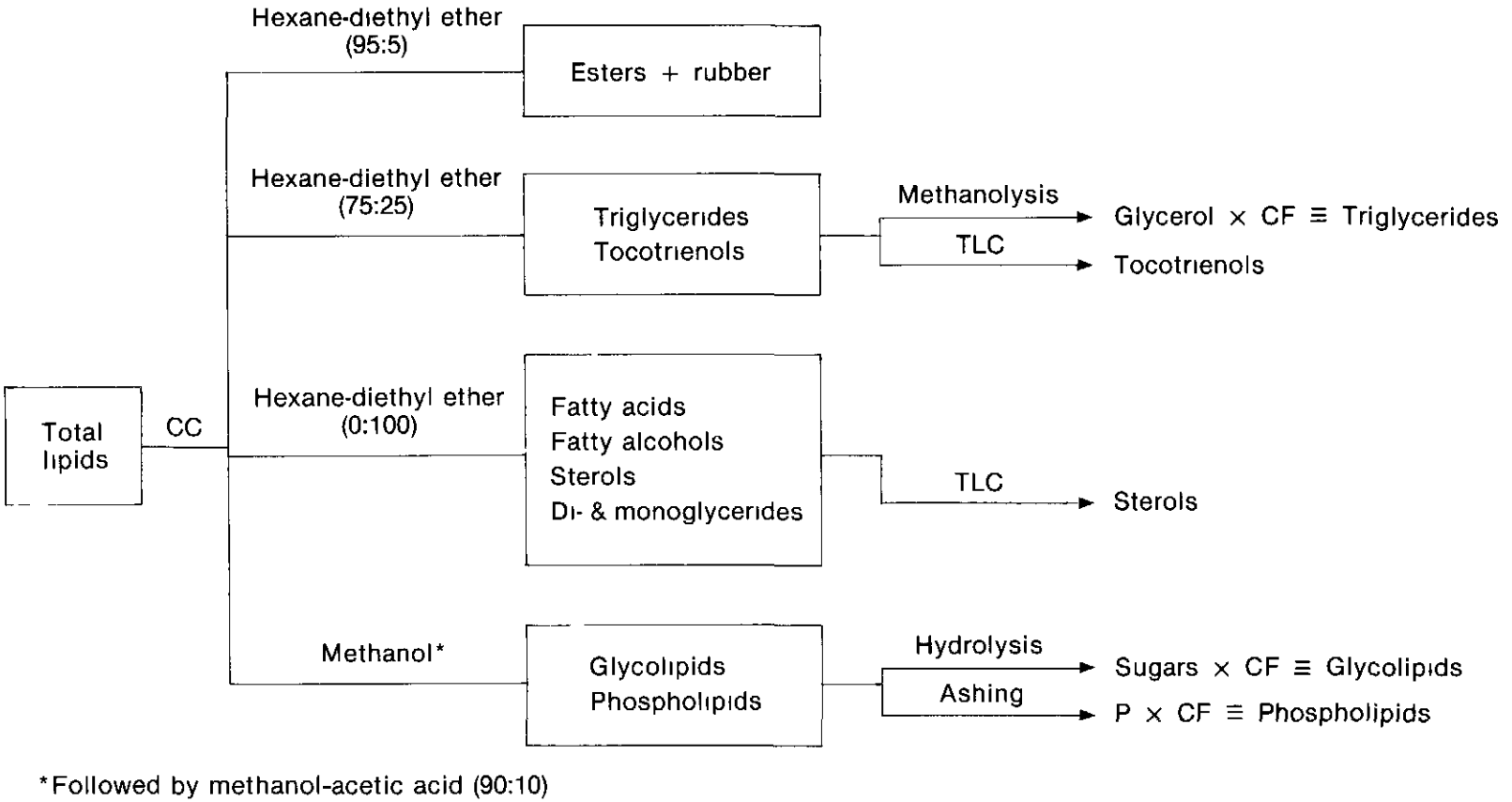

Figure 1. Quantitative determination of lipids from Hevea brasiliensis *latex.*

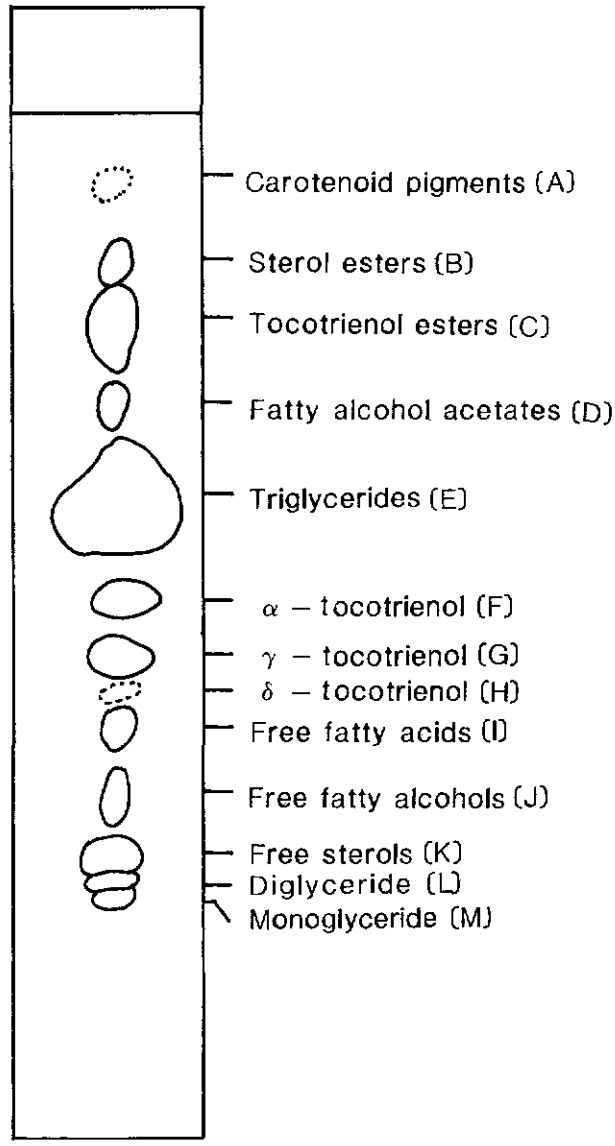

Figure 2. Thin layer chromatography of neutral lipids from fresh H. brasiliensis *latex of RRIM 501 developed in hexane-benzene (85:15, v/v) followed by hexane-diethyl ether-acetic acid (69:92:2, v/v/v) and detected by cupric acetate reagent.*

$AgNO_3$-TLC plates resolved the acetate derivative of the sterol mixture into two bands. Gas chromatography of the upper band gave two peaks with retention time corresponding to standard β-sitosterol acetate (2.24) and stigmasterol acetate (1.88) relative to cholesterol taken as 1. Gas chromatography of the lower band however revealed only one peak with a retention time of 2.26. Further analysis of the sterol by MS showed it to be fucosterol:

TABLE 1. COMPOSITION OF THE MAJOR NEUTRAL LIPIDS FROM FRESH *H. BRASILIENSIS* LATEX OF RRIM 501

Neutral lipids	Unsaponifiables	Fatty acids							
		16:0	16:1	18:0	18:1	18:2	18:3	20:0	F_2
Sterol esters	β-sitosterol (64%), fucosterol (19%) and stigmasterol (17%)	6.9	3.8	26.0	13.0	33.0	7.0	10.0	-
Tocotrienol esters	γ- and δ-tocotrienols	11.0	0.8	49.0	19.6	11.0	0.5	8.9	-
Fatty alcohol acetates	Cetyl, stearyl and arachidyl alcohols								
Triglycerides		-	-	0.4	1.1	0.6	-	-	98.0
Free tocotrienols	α- and γ-tocotrienols (40%) (60%)								
Free sterols	β-sitosterol (53%), fucosterol (33%) and stigmasterol (14%)								

F_2 = Furanoid fatty acid

mass/change (m/e) ratios (relative intensity of fragments) of 412 (M^-, 17), 314 (100) and 271 (19).

The free tocotrienol spots *F, G* and *H* were initially identified by their specific reaction with the Emmerie-Engel[17] reagent and through comparison with the free tocotrienols from palm oil extracts. The larger bands *F* and *G* were isolated separately by preparative TLC and the presence of α- and γ-tocotrienols in *F* and *G*, respectively were confirmed by GC-MS *(Figure 3)* and ^{1}H NMR *(Figure 4)* spectroscopy.

Like the mass spectra of their tocopherols[18] the mass spectra of α- and γ-tocotrienols are also characterised by the absence of numerous fragmentation processes. The DS-50 atomic composition report showed that the parent ion of γ-tocotrienol at m/e 424 has a general formula of $C_{29}H_{44}O_2$ which splits to a very stable dihydroxy tropylium ion at m/e 165 with a formula of $C_{10}H_{44}O_2$. Similarly the parent ion of γ-tocotrienol was shown to be at m/e 410 with a formula of $C_{28}H_{42}O_2$ and a base peak at m/e 151 with the formula $C_9H_{11}O_2$.

The ^{1}H NMR spectra of the two tocotrienols show similar proton shifts as α-tocopherol[19] except in the proton shift of the side chain due to the unsaturation in the former. The only difference between the spectra of the two tocotrienols is in the aromatic proton shift at δ 6.12 p.p.m. which is absent in α-tocotrienol. The γ-tocotrienol was distinguished from β-tocotrienol by two-dimensional TLC. The fatty alcohol acetates were deduced by the presence of acetyl function at δ 1.95 p.p.m. on ^{1}H NMR. Infra-red spectroscopy showed the carbonyl band at 1746 cm^{-1} which corresponds to that of the standard fatty alcohol acetates. Gas chromatography analysis showed three peaks corresponding to standard cetyl, stearyl and arachidyl acetates. These were further confirmed by GC-MS *(Table 2)*. No molecular ion was detected in any of the three spectra, characteristic of the spectra of the acetate derivatives due to the loss of acetic acid as evidenced by the peak M^+–60. The fragment m/e 61 strengthens the presence of the acetate compounds.

All the acyl lipids were methanolysed and the fatty acid methyl esters were analysed by GLC. It can be seen *(Table 1)* that stearic, oleic and linoleic acids formed the major acyl components of the sterol and tocotrienol esters. The triglycerides however contained 98% of a furanoid fatty acid as reported earlier[20].

The unsaponifiable fraction of sterol esters contained stigmasterol, β-sitosterol and fucosterol while the tocotrienol ester contained mainly γ- and δ-tocotrienol in a mixture with

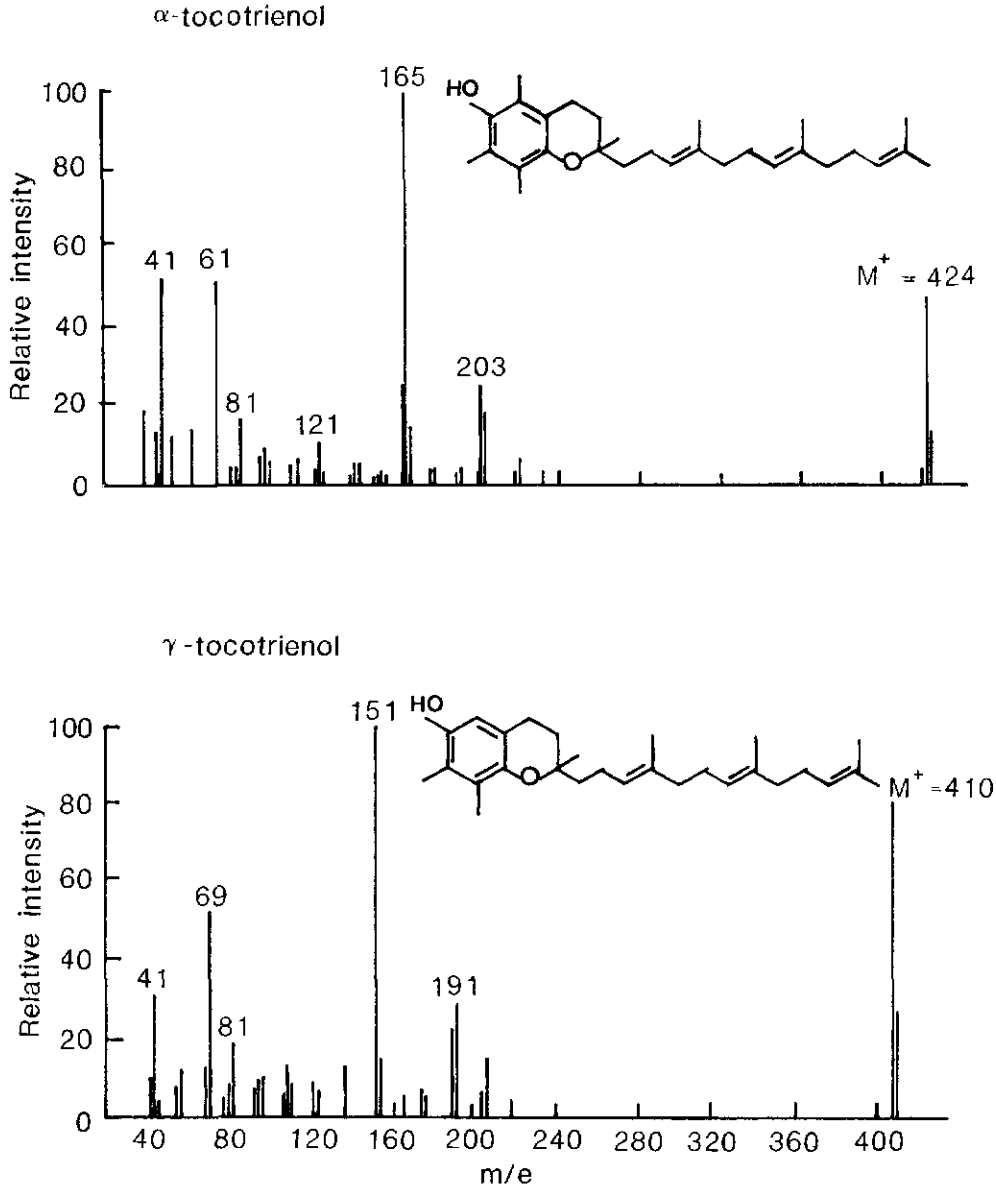

Figure 3. Mass spectra of α- and γ-tocotrienols from H. brasiliensis *latex.*

other phenolic compounds, probably the oxidised products of the tocotrienols.

Glycolipids. Thin layer chromatography of the glycolipids sprayed with α-naphthol reagent showed four main spots which corresponded to the glycolipid standards *viz.* esterified steryl glycoside (ESG), monogalactosyl diglyceride (MGDG), steryl glucoside (SG) and digalactosyl diglyceride (DGDG).

The presence of sugar was confirmed by analysing the TMS ether derivative of the methyl glycosides by GLC. The chromatogram

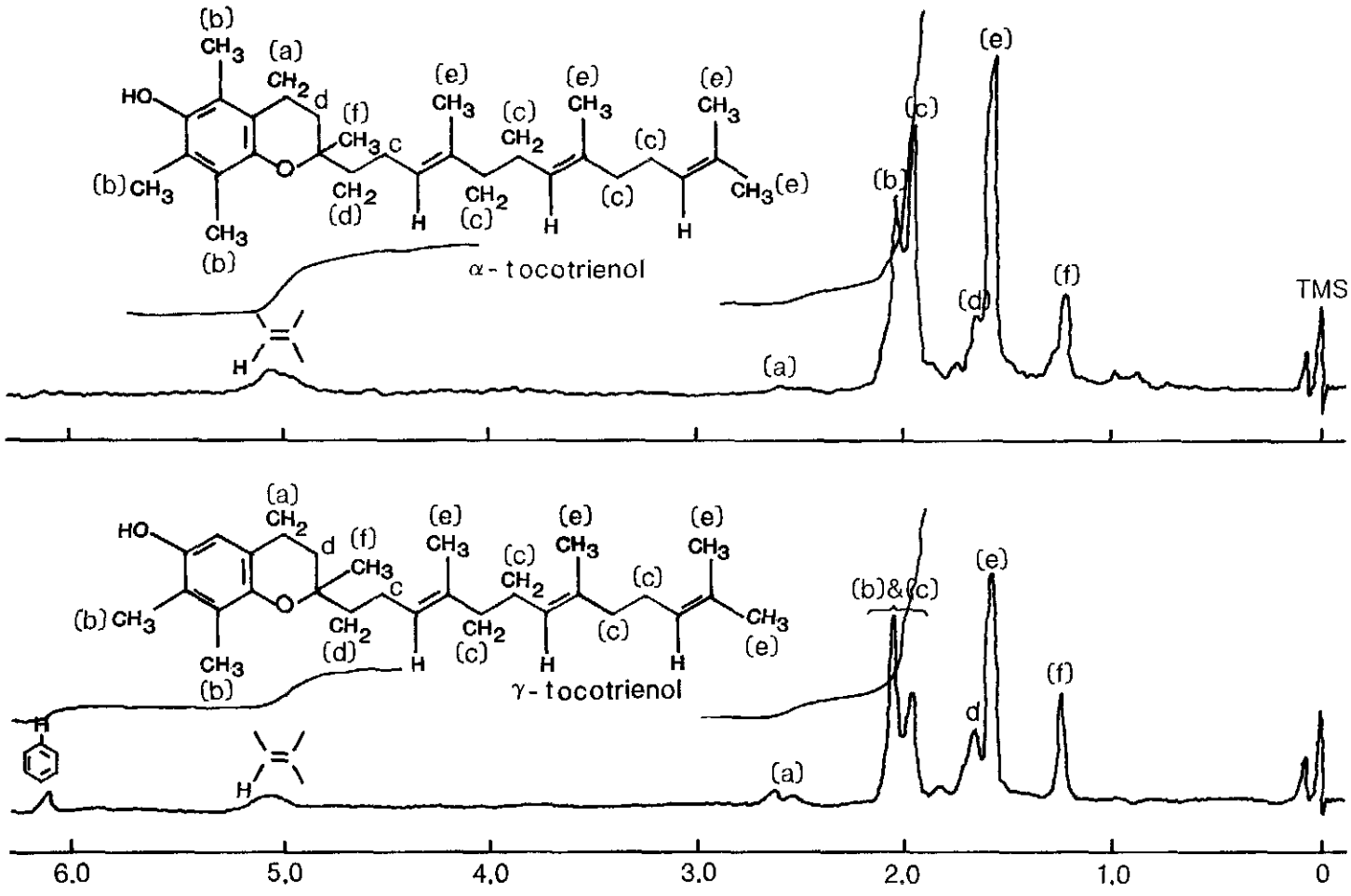

Figure 4. ^1H NMR spectra of α- and γ-tocotrienols of Hevea brasiliensis *latex.*

TABLE 2. MASS SPECTRAL DATA OF THE LONG-CHAIN ALCOHOL ACETATES FROM FRESH LATEX OF RRIM 501

Acetate	Characteristic fragments m/e (relative abundance)
Cetyl	224 (M^+ - CH_3COOH, 3), 99(4), 85(21), 71(45), 61(5), 57(80), 43(100)
Stearyl	252 (M^+ - CH_3COOH, 5), 99(3), 55(16), 71(33), 61(64), 57(73), 43(100)
Arachidyl	280 (M^+ - CH_3COOH, 12), 99(7), 85(30), 71(51), 61(69), 57(100), 43(82)

showed two peaks for the α and β anomers of methyl glucosides and three peaks for the α, β and γ anomers of methyl galactosides as reported by Sweeley and Walker[21]. Each glycolipid was isolated by preparative TLC, extracted and analysed for its composition. The sterol component of ESG and SG consisted of β-sitosterol, fucosterol and stigmasterol with β-sitosterol occurring as the major component (about 89%) in both cases. The fatty acid methyl esters of ESG, MGDG and DGDG *(Table 3)* consisted mainly of stearic, oleic and linoleic acids. Furanoid fatty acid was also present but the amount was less than one-fifth of that present in the triglyceride fraction. It is interesting to note that ESG had a higher proportion, about 64%, of saturated fatty acids compared to MGDG and DGDG which had less than 40% of the saturated fatty acids.

Phospholipids. Thin layer chromatography of the phospholipids showed three main spots corresponding to phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) and phosphatidyl inositol (PI). There were also two smaller spots of phosphatidic acid and diphosphadityl glycerol. The latter two could be the artifacts of the hydrolytic action of phospholipase *D* which is known to occur in *H. brasiliensis* latex[5].

The identities of PE, PC and PI were further confirmed by their positive reaction with specific detecting reagents and thorough comparison with their standards. PC and PE were stained orange and pink by Dragendorff and ninhydrin reagents, respectively. The colour changed to blue on respraying the TLC plate with the modified molybdenum reagent. Infra-red spectra of PI showed a broad absorption band at 3400 cm^{-1} (OH) which was not prominent in the spectrum of PC and absent in the spectrum of PE.

Analysis of the fatty acid methyl esters of the three main phospholipids from fresh latex of RRIM 501 *(Table 3)* showed that the acyl component of PE, PC and PI consisted of only aliphatic fatty acids: mostly of palmitic, stearic, oleic and linoleic acids. Furanoid fatty acid was not detected in the phospholipid fraction of *H. brasiliensis* latex.

Quantitative Composition of the Lipids

The total lipid content of RRIM 501 was about 1.64% of the latex *(Table 4)*. The neutral lipids formed the major class of *H. brasiliensis* lipids, constituting about 53.6% of the total lipids. The glycolipids and phospholipids constituted about 32.9% and 14.0%, respectively. Among the neutral lipids the triglyceride fraction alone contributed about 63.3% of the total or 0.56% of latex, making it the major component of *H. brasiliensis* lipids. The tocotrienols which are the natural antioxidants of rubber comprised of only 0.03% which was comparable to the amount of free sterols.

In the glycolipids the amount of galactosyl diglycerides exceeded that of the steryl glucosides. DGDG formed the second major component of *H. brasiliensis* latex constituting about 0.34%. Phosphatidyl choline which was the major component of phospholipids constituted about 0.13% of the latex.

DISCUSSION

Despite considerable work done by previous workers in the characterisation of lipids in

TABLE 3. FATTY ACID COMPOSITION OF ACYL POLAR LIPIDS FROM FRESH LATEX OF RRIM 501

Lipid	Fatty acid composition (%)								
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	F_2
ESG	1.9	25.1	2.8	35.0	11.2	11.3	0.9	2.5	9.3
MGDG	–	13.5	4.1	22.9	22.2	17.9	4.1	–	15.1
DGDG	1.4	5.2	2.0	22.9	21.2	26.2	3.5	–	17.6
PE	2.0	20.8	3.7	18.3	14.1	37.5	3.6	–	–
PC	0.9	9.6	10.5	21.9	20.6	34.3	2.3	–	–
PI	0.5	30.2	3.4	16.8	10.3	35.4	3.4	–	–

ESG = Esterified steryl glycoside
MGDG = Monogalactosyl diglyceride
DGDG = Digalactosyl diglyceride
PE = Phosphatidyl ethanolamine
PC = Phosphatidyl choline
PI = Phosphatidyl inositol
F_2 = Furanoid fatty acid

TABLE 4. LIPID COMPOSITION OF *H. BRASILIENSIS* LATEX OF RRIM 501

Lipid	Composition		% of respective lipid classes
	% of total lipids	% of latex	
Neutral lipids	53.6	0.88	
Esters		0.14	16.1
TG		0.56	63.3
T_3		0.03	3.4
Sterols		0.04	4.5
FA, ROH, DG, MG		0.10	11.9
Glycolipids	32.9	0.54	
ESG		0.05	9.5
MGDG		0.04	6.5
SG		0.11	21.1
DGDG		0.34	62.8
Phospholipids	14.0	0.23	
PE		0.05	21.0
PC		0.13	58.4
PI		0.05	20.6
Total lipids		1.64	

TG = Triglycerides
T_3 = Tocotrienols
FA = Fatty acid
ROH = Alcohols
DG = Diglycerides
MG = Monoglycerides
Other abbreviations as in *Table 3*.

H. brasiliensis latex some important components were still not detected. The existence of the glycolipids which form an important fraction of total lipids has never been clearly demonstrated before, although many workers[3,4,6] have detected sugars in their polar lipid fractions. Whitby *et al.*[1] and Altman and Kraay[2] however, identified a sterol glucoside in their lipid fraction and Sentheshanmuganathan *et al.*[22] suggested the possibility of the presence of glycolipids but the composition of the glycolipids has not been clearly demonstrated as in the present study. Here the glycolipids are shown to contain four main components, namely, ESG, MGDG, SG and DGDG.

Besides the glycolipids, the occurrence of a high amount of furanoid fatty acid (98%) in the triglyceride fraction together with the high concentration of the fraction in the lipids of *H. brasiliensis* is another interesting finding. The acid although found in triglycerides and glycolipids has not been detected in other *Hevea* lipids including rubber seed oil. Further investigations have shown that the occurrence of the furanoid fatty acid in the triglyceride fraction of the *H. brasiliensis* lipids is a clonal characteristic and is dependent to some extent on the period of the year[23]. Similar seasonal and individual variations have been observed in the distribution of furanoid fatty acids in the Northern Pike *(Esox lucius)*[24]. It is possible that the acid has a physiological role in *Hevea* but this has not yet been ascertained.

The fatty alcohols, unlike the other non-saponifiables, *i.e.* the sterols and tocotrienols, were surprisingly found esterified to acetic acid and not to fatty acids. This is rather uncommon among plant lipids and like the furanoid fatty acids the function of these fatty alcohol acetates is also uncertain.

Apart from the above findings, the other results of the qualitative composition are generally in accordance with previous studies. The content of phospholipids is lower than that reported by previous workers. It is likely that the 'phospholipids' in previous investigations were actually polar lipids and would have included glycolipids.

In this study a systematic method of analysis of lipids in *Hevea* latex is used. It consists in first separating the lipids into different classes, then characterising their identities by chromatography and spectroscopy and finally quantifying their composition. The study thus gives a better picture of the composition of lipids in the fresh latex of a *Hevea brasiliensis* clone. This method can be used for a general study of clonal variations in the lipid content of *Hevea*.

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