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Glycolipid composition of *Hevea brasiliensis* latex

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ABSTRACT

Glycolipids of fresh latex from three clones of *Hevea brasiliensis* were characterized and quantified by HPLC/ESI-MS. Their fatty acyl and sterol components were further confirmed by GC/MS after saponification. The four detected glycolipid classes were steryl glucosides (SG), esterified steryl glucosides (ESG), monogalactosyl diacylglycerols (MGDG) and digalactosyl diacylglycerols (DGDG). Sterols in SG, ESG and total latex unsaponifiable were stigmasterol, β-sitosterol and Δ^5-avenasterol. The latter was found instead of fucosterol formerly described. Galactolipids were mainly DGDG and had a fatty acid composition different from that of plant leaves as they contained less than 5% C18:3. Glycolipids, which represented 27–37% of total lipids, displayed important clonal variations in the proportions of the different fatty acids. ESG, MGDG and DGDG from clone PB235 differed notably by their higher content in furan fatty acid, which accounted for more than 40% of total fatty acids. Clonal variation was also observed in the relative proportions of glycolipid classes except MGDG (8%), with 43–51% DGDG, 30–34% SG and 7–19% ESG. When compared with other plant cell content, the unusual glycolipid composition of *H. brasiliensis* latex may be linked to the peculiar nature of this specialized cytoplasm expelled from laticiferous system, especially in terms of functional and structural properties.

1. Introduction

Glycolipids, such as glycosylated glycerolipids, sterols, ceramides or sphingolipids, are present in almost all biological membranes. Among them, galactolipids are the most abundant membrane lipids, especially in green tissues where they generally represent about 75% of total membrane lipids (Dörmann and Benning, 2002). In the framework of the study of the relationships between the lipid composition and the technological properties of natural rubber, we have investigated the glycolipid content of fresh latex from *Hevea brasiliensis*.

In *Hevea*, latex is formed in special cells called laticifers, or latex vessels that are articulated and anastomosing. They originate from cells produced by the vascular cambium and are arranged in rows. Laticifers characters such as number of rows, density of vessels per row, diameter of latex vessels and intensity of anastomosing are significant clonal characters (Premakumari and Saraswathyamma, 2000).

The comparison of latices from several clones of *H. brasiliensis*, collected and treated under controlled conditions, was necessary because several research works conducted on lipids in natural latex have indicated difficulties in results comparison due to differences in clones, sample treatment or even extraction procedure (Ehabe et al., 2006; Hasma, 1991).

Lipids from *H. brasiliensis* latex are known to contain a high amount of glycolipids. For example, a study of *H. brasiliensis* RRIM501 fresh latex reported 33% of glycolipids in total lipids (Hasma and Subramaniam, 1986). The four main glycolipids families found were esterified steryl glucosides (ESG), monogalactosyl diacylglycerols (MGDG), steryl glucosides (SG) and digalactosyl diacylglycerols (DGDG). No significant amounts of cerebrosides were detected. In this study, individual glycolipid families were hydrolyzed to release their sugar moiety after separation from the other lipid classes by silica gel column chromatography and purification by preparative TLC. Specific conversion factors, calculated on the basis of the mean fatty acid (FA) composition of each *H. brasiliensis* glycolipid family, were then applied to convert reducing sugar concentrations into glycolipid content (Hasma and Subramaniam, 1986). Although giving valuable results, this method is time-consuming and laborious. For our main study, that

Abbreviations: DGDG, digalactosyl diacylglycerol; ESG, esterified steryl glucoside; ESI-MS, electrospray ionization mass spectrometry; FA, fatty acid; FuFA, furan fatty acid (10,13-epoxy-11-methyl octadeca-10,12 dienoic acid); FID, flame ionization detector; GC, gas chromatography; HPLC, high performance liquid chromatography; MGDG, monogalactosyl diacylglycerol; MS, mass spectrometry; SG, steryl glucoside; SQDG, sulfoquinovosyl diacylglycerols; TLC, thin layer chromatography.

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will deal with several hundred samples from trees cultivated and tapped under various conditions, a rapid and direct analysis method was required for qualitative and quantitative determination of glycolipid content. Among the possible methods, HPLC/MS is promising as it allows a direct and sensitive study of glycosylated conjugates without derivatization. It has been used in several works for glycolipid analysis in plant, animal or microbial lipid extracts (de Souza et al., 2007; Gil et al., 2003; Ruibal-Mendieta et al., 2004; Yamauchi et al., 2001). Reverse-phase chromatography allows the separation of glycolipids according to both their family and their FA composition, whereas in normal phase chromatography, glycolipids from a same family have very close retention times, which facilitate their quantification. As a detection method, both atmospheric pressure chemical ionization (APCI) and electrospray ionization (EI) mass spectrometry have been proposed. In the work reported here, we have used normal phase HPLC coupled to ESI-MS to directly separate, identify and quantify each glycolipid family in total glycolipid extracts obtained by preparative silica gel column chromatography. For the development of the method, the identification was comforted by GC analysis (flame ionization detection, FID) of the FA and GC–MS analysis of sterol composition of the total glycolipid extract.

2. Results

2.1. Global composition of the glycolipid family

Total lipid and glycolipid content of latices from the three *H. brasiliensis* clones are given in Table 1. Glycolipids represented 27–37% of total latex lipids, depending on the *H. brasiliensis* clone, and 0.7–1% of the dry rubber content. Latex from clone PB235 contained the highest total lipid content but the highest amount of glycolipids was found in latex from clone BPM24. In order to determine FA and sterol profiles of glycolipids from the studied latices, samples of the glycolipid fraction were saponified and subjected to GC and GC/MS analysis. FA ranged from C14 to C20 (Table 2). Linoleic acid was the major FA in glycolipids from the latex of RRIM600 (47% of total FA) and BPM24 (44%) clones, while glycolipids from *H. brasiliensis* PB235 contained 42% furan FA (FuFA, 10,13-epoxy-11-methyl octadeca-10,12 dienoic acid) and only 25% C18:2. The structure of furan fatty acid was verified by ^{1}H and ^{13}C NMR after a preliminary purification from trifuranoyl glycerol contained in lipid extract from PB235 clone (Figs. 1 and 2). The analysis of chemical shifts from ^{1}H and ^{13}C NMR spectra showed the presence in this fatty acid of furfuryl group substituted by methyl group. Moreover, the ^{13}C NMR spectroscopy results are consistent with those reported by Marcel et al. (1981). The presence of high amounts of FuFA in *H. brasiliensis* latex has been reported previously; it was shown to be clone-dependent and located in triacylglycerols and glycolipids, but not in phospholipids (Hasma, 1984; Hasma and Subramaniam, 1986). As shown in Table 2, the relative abundance of FuFA among total FA was, depending on the clone, 1.8–4 times lower in glycolipids than in total lipids, whereas glycolipids were richer in C18:1 (1.6–2 times), C18:0 and, to a lesser extent, C16:0. The abundance of FuFA in lipids from the *H. brasiliensis* PB235 clone was the highest reported so far.

Table 1
Glycolipid content of latices from three *H. brasiliensis* clones.

H. brasiliensis clone	% Total lipids (w/w dry rubber)	% Glycolipids (w/w lipid)	% Glycolipids (w/w dry rubber)
RRIM600	2.5[c]	27[abc]	0.68[b]
PB235	3.5[a]	23[bc]	0.82[ab]
BPM24	2.8[b]	37[a]	1.0[a]

Data are the mean of 3 repetitions (independent extractions). Values not connected by same letter are significantly different ($P = 5\%$).

Table 2
Fatty acid composition (% mol/mol) of the total glycolipid fraction from fresh latex from three *H. brasiliensis* clones, determined by GC.

Fraction	Fatty acid	*H. brasiliensis* clone		
		RRIM600	PB235	BPM24
Total lipid fraction	C14:0	0.3	0.2	0.4
	C16:0	8.0	3.6	7.0
	C16:1	0.4	1.8	0.5
	C18:0	13.6	5.7	13.9
	C18:1	9.5	3.3	9.5
	C18:2	49.4	10.6	40.2
	C18:3	2.7	0.9	1.7
	FuFA[a]	15.2	73.4	25.8
	C20:0	0.8	0.5	1.0
Glycolipid fraction	C14:0	0.3	0.4	0.4
	C16:0	9.6	9.7	7.9
	C16:1	0.6	1.6	1.1
	C18:0	16.2	12.3	16.8
	C18:1	15.3	6.5	18.5
	C18:2	46.9	24.8	43.9
	C18:3	3.0	2.5	3.1
	FuFA[a]	7.6	41.5	7.6
	C20:0	0.5	0.6	0.6
Phospholipid fraction	C14:0	0.7	1.5	1.4
	C16:0	9.2	11.6	9.3
	C16:1	0.9	8.2	1.1
	C18:0	14.1	15.2	16.0
	C18:1	11.2	8.3	13.4
	C18:2	58.7	36.0	52.9
	C18:3	3.0	2.7	2.0
	FuFA[a]	1.6	15.4	3.1
	C20:0	0.5	1.2	0.8

Data are the mean of 3 repetitions (independent extractions). Standard deviations were <5% of the means.
[a] FuFA: furan fatty acid or 10,13-epoxy-11-methyl octadeca-10,12 dienoic acid, $R_t = 7.35$ min.

Three sterols were detected by GC/MS analysis of TMS ether derivatives from the unsaponifiable of both total and glycolipid fractions: stigmasterol (5α-stigmasta-5, 22-dien-3β-ol), β-sitosterol (5α-stigmast-5-en-3β-ol) and Δ^5-avenasterol (5α-stigmasta-5,24(28)-dien-3β-ol (24*Z*)). Their identification was based on the comparison of both their retention times and mass spectra with that of commercial standards. For Δ^5-avenasterol, of which no standard sample was available, the reference used was an unsaponifiable from oat (*Avena sativa*, L.) kernel oil, that is known to contain this sterol (Määttä et al., 1999). It is to be noted that previous works (Hasma and Subramaniam, 1986; Nair et al., 1993) reported the presence of fucosterol (5α-stigmasta-5,24(28)-dien-3β-ol (24*E*)) instead of the Δ^5-avenasterol found in our study.

Relative proportions of sterols are given in Table 3 for each latex. Differences were observed between clones, with a higher amount of Δ^5-avenasterol in *H. brasiliensis* PB235. In every clone, the relative abundance of Δ^5-avenasterol was lower in glycolipids than in the total lipids.

2.2. HPLC separation of glycolipid families

Chromatograms on Fig. 3 show the separation of a mixture of commercial glycolipids from soybean (SG, ESG) and plant leaves (MGDG, DGDG), and of the glycolipid fraction from *H. brasiliensis* RRIM600 fresh latex. Four common peaks were found in both samples, corresponding to ESG (2.3–2.6 min), MGDG (2.8 min), SG (3.3 min) and DGDG (5.0 min). A supplementary compound, eluted

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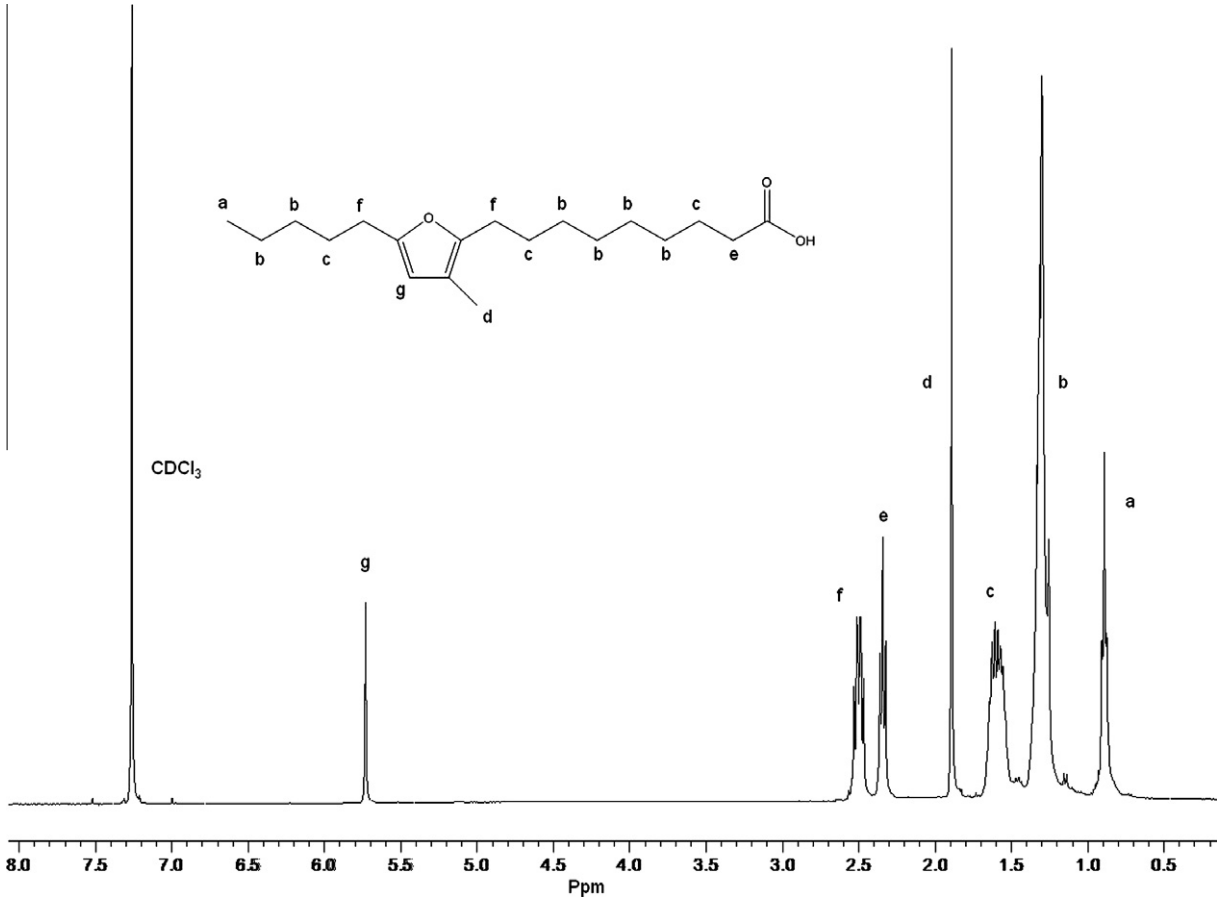

Fig. 1. ^{1}H NMR spectrum of purified furan fatty acid. The protons at 0.9; 1.3; 2.35 ppm were assigned to the CH_3 terminal group of aliphatic chain (proton a), CH_2 (proton b) in aliphatic chain and CH_2 (proton e) in α position of carbonyl group. These protons are representative of classical fatty acids. The CH_2 (proton c) at 1.6 ppm represents the different protons in β-position of carbonyl and furfuryl groups. The presence of CH_2 (proton f) at 2.5 ppm is attributed to CH_2 group in α-position of furfuryl groups, the protons of CH_3 group at 2.35 ppm (proton d) in α-position of furfuryl groups and the proton of CH furfuryl group at 5.7 ppm (proton g) confirmed the presence of furfuryl group in fatty acid chain.

after 9.8 min, was present in the DGDG commercial standard but not in the glycolipids extracted from latex. The mass of the detected ion (*m*/*z* 699) and the chromatographic behaviour of the compound corresponded to that of lyso-DGDG, detected as [digalactosyl monolinolenin + Na]$^+$.

2.3. Identification of glycolipid molecular species

ESI-mass spectra of each glycolipid family peak permitted to characterize molecular species differing by their FA profile. The detected masses are listed in Table 4. The second column of the table presents possible molecular species matching ion masses with the FA and sterol composition of the total glycolipid fraction from latex reported above. The relative molecular composition of each glycolipid family was evaluated by integration of peaks extracted for each *m*/*z* from the TIC chromatogram. Due to the lack of pure standards for each combination of FA within a given glycolipid family and the complex relationship between detector's response and concentration (including ionization yields and mutual suppression both in mixtures and overlapping peaks), it was not possible to precisely quantify the various molecular species. However, relative detector's responses are given in Table 4 for each glycolipid family to allow comparison between clones, with the hypothesis that the effects of mutual suppression by the various components of lipid mixtures will affect in a similar way all the molecular species within a given family (i.e. with a same sugar moiety and differing only by FA composition).

2.3.1. Steryl glucosides

Two main ions were detected in the latex samples, corresponding to stigmasteryl or Δ^5-avenasteryl glucoside (*m*/*z* 597) and β-sitosteryl glucoside (*m*/*z* 599) respectively (Fig. 4). [β-Sitosteryl glucoside + Na]$^+$ was the main ion, which was consistent with the sterol composition of the glycolipid fractions given in Table 3.

2.3.2. Esterified steryl glucosides

The most abundant ion in the ESG family peak found in every *H. brasiliensis* clones latex was *m*/*z* 859 (Fig. 5), that corresponds to the structures of β-sitosteryl (6′-*O*-linolenoyl) glucoside, stigmasteryl (6′-*O*-linoleoyl) glucoside and avenasteryl (6′-*O*-linoleoyl) glucoside. It accounted for around 30–40% of total detected ESG ions. The fraction from PB235 clone was characterized by a high amount of FuFA esters of β-sitosteryl glucoside (*m*/*z* 889, 14% of ESG ions).

2.3.3. Sulfoquinovosyl diacylglycerols

SQDG are among the major glycolipid components of the photosynthetic membrane in plants, algae and various bacteria (Welti et al., 2003; Rezanka et al., 2003; Young et al., 1999). Due to its strong negative charge at neutral pH, SQDG standard was analyzed in negative ESI mode and observed as [M−H]$^-$. The most abundant molecular species in the standard was C16:0/C18:3-SQDG (*m*/*z* 815) (Fig. 6). This species could be also detected in positive ESI positive mode in the form of [M−H + 2Na]$^+$ (*m*/*z* 861). However, this

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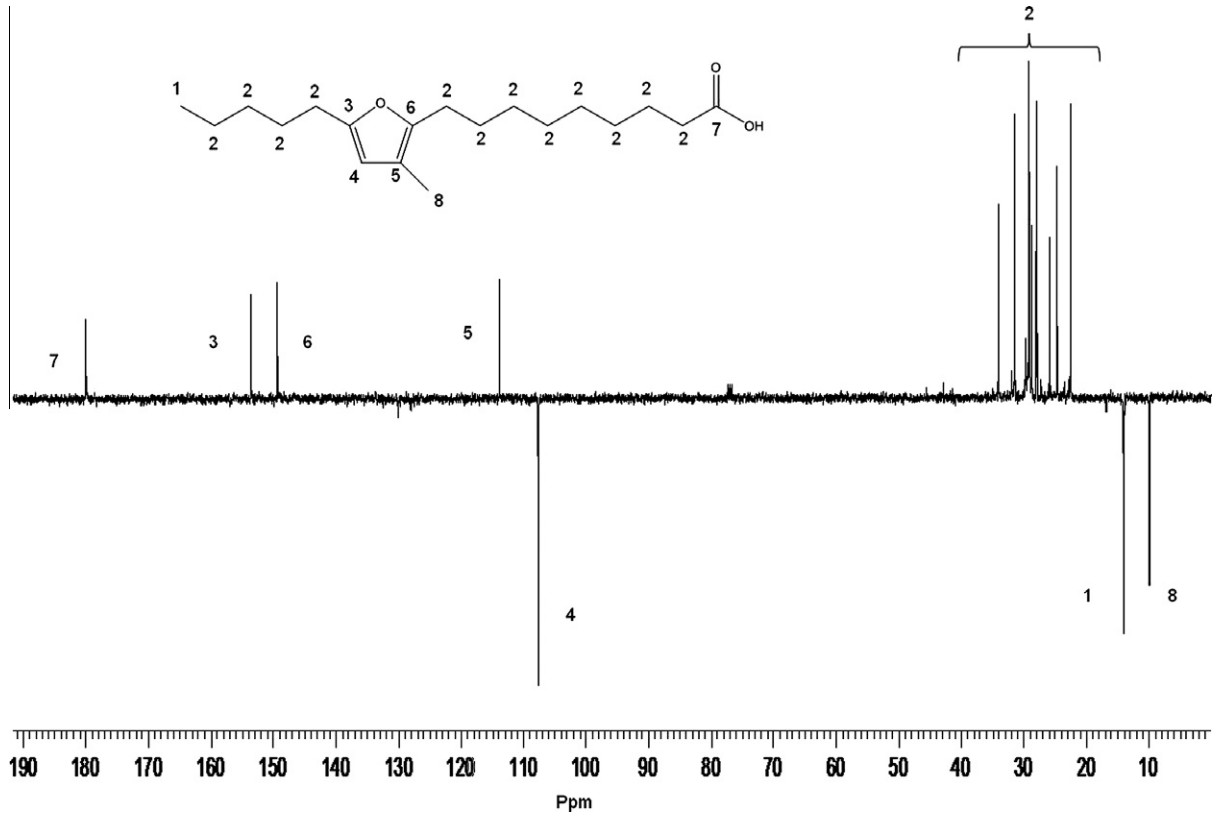

Fig. 2. ^{13}C NMR spectrum of purified furan fatty acid in $CDCl_3$. The chemical shift value at 180 ppm is attributed to carbonyl groups (carbon 7) and the peaks at 153, 150, 114 and 107 ppm are characteristic of furfuryl cycles carbon (carbon 3, 4, 5, and 6), respectively. The CH_2 carbon type (carbon 2) has a chemical shift value between 22 and 34 ppm. The CH_3 carbon type (carbon 1 and 8) has a chemical shift value at 14 and 10 ppm characteristic of the terminal carbon of aliphatic chain and the CH_3 group in α-position of furfuryl groups. Experiment was conducted in attached proton test (APT) mode, therefore the CH and CH_3 types (carbon 1, 4, and 8) are represented by negative signals.

Table 3
Sterol composition (% mol/mol) of the unsaponifiables from total lipid and glycolipid fractions of fresh latex from three *H. brasiliensis* clones, determined by GC.

Fraction	Sterol	*H. brasiliensis* clone		
		RRIM600	PB235	BPM24
Total lipid unsaponifiable	Stigmaterol	7	6	7
	β-Sitosterol	57	41	59
	Δ^5-Avenasterol	36	53	34
Glycolipid unsaponifiable	Stigmaterol	7	18	13
	β-Sitosterol	85	65	78
	Δ^5-Avenasterol	9	17	9

Data are the mean of 3 repetitions (independent extractions). Standard deviations were <10% of the means.

glycolipid family was not detected in the glycolipid fraction from *Hevea* latex lipids.

2.3.4. Monogalactosyl diacylglycerols

The mass spectra from the MGDG peak are presented in Fig. 7. Different patterns were observed depending on clone or sample origin. The commercial MGDG standard from parsley leaf mainly contained the C18:3 and C16:3 acyl chains reported in the literature (Mongrand et al., 1998), but latex MGDG had a very different composition. In samples from *H. brasiliensis* RRIM600 and BPM24 clones, the main structure, detected at *m/z* 805, corresponded to C18:1/18:1 or C18:0/18:2 MGDG and represented 11–18% of all detected MGDG species, whereas it was 9% only in PB235 clone. In the latter clone, the main peak was at *m/z* 857 and corresponded to the combination of two furan acyl chains (FuFA/FuFA–MGDG), accounting for 37% of all detected MGDG.

Another clonal difference was observed with the unidentified ions at *m/z* 815–821 that are highly represented in BPM24 while in lower proportion, respectively, in RRIM600 and PB235 MGDG. Though these ions are in the same *m/z* range as those of the SQDG standard, for instance *m/z* 815 (C16:0/C18:3-SQDG), *m/z* 819 (C16:0/C18:1 or C16:1/C18:0-SQDG) and *m/z* 821 (C16:0/C18:0 or C14:0/C20:0-SQDG), their retention time (R_t = 2.8 min) is consistent with MGDG (2.8 min) but not with SQDG (7.3 min). The *m/z* of the unknown ions being 14 mass units higher than that of the molecular species of *m/z* 801–807 (Table 4), the *m/z* 815–821 ions might be methylated versions of the *m/z* 801–807 MGDG. It is however to be noted that no FA with odd number of carbon atoms, that could have corresponded to *m/z* + 14 MGDG, was detected in the fatty acids from total glycolipids nor in the mass spectra resulting from fragmentation of MGDG in the ESI source in negative mode.

2.3.5. Digalactosyl diacylglycerols

FA constituents of DGDG were found to be similar to those of MGDG (Fig. 8). The FuFA/FuFA combination was found as the most abundant ion for PB235 clone (*m/z* 1019, around 40% of total DGDG ions). For RRIM600 and BPM24, possible structures of *m/z* 967 (31% of total DGDG ions) were C18:1/18:1 or C18:0/18:2. However, the unknown ion found in MGDG, mainly from BPM24, had no correspondent in the DGDG fraction.

2.4. Quantification of glycolipid families

Total glycolipids were injected into the HPLC/ESI-MS system after proper dilution. A calibration was performed with each series

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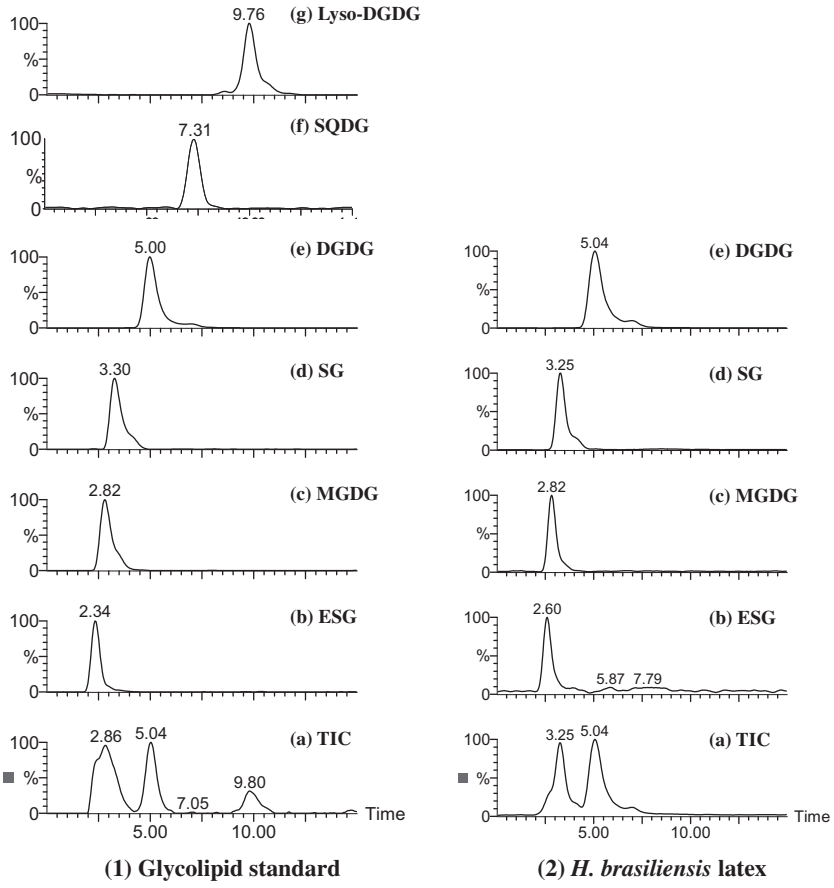

Fig. 3. HPLC separation of (1) a commercial mixture of glycolipids (2) the glycolipid fraction from *H. brasiliensis* RRIM600 fresh latex. Chromatograms are given for (a) Total Ion Count and (b–f) sum of $[M + Na]^+$ ions in the *m*/*z* range corresponding to each glycolipid family: (b) esterified steryl glucosides (ESG, *m*/*z* 837–889; most abundant species *m*/*z* 837); (c) monogalactosyl diacylglycerols (MGDG, *m*/*z* 777–857; most abundant species *m*/*z* 797); (d) steryl glucoside (SG, *m*/*z* 585–601; most abundant species *m*/*z* 599); (e) digalactosyl diacylglycerols (DGDG, *m*/*z* 937–1023; most abundant species *m*/*z* 959); (f) sulfoquinovosyl diacylglycerols (SQDG, *m*/*z* 787–817) (g) digalactosyl monolinolenin (lyso-DGDG, *m*/*z* 699).

of injections, using 5 concentrations of standards (Fig. 9). The response for each glycolipid family was determined by the integration of the chromatographic peak extracted from the total ion chromatogram for the appropriate *m*/*z* range (see Fig. 3b–e), except for MGDG and ESG that had relatively close mean retention times and shared ions with common masses (*m*/*z* 837 and 857, Table 4). For these families, the response was the sum of the peak areas obtained from a *m*/*z* range chromatogram excluding the common ions, and from individual chromatograms obtained for *m*/*z* 837 (R_t 2.55 for ESG and 2.95 min for MGDG) and *m*/*z* 857 (R_t 2.29 and 2.73 min, respectively). Due to the lack of specific standards from *H. brasiliensis*, molar response coefficients were determined for each glycolipid family using commercial standards from parsley leaf and soybean oil. Although differences in FA composition between standards and samples may not allow precise quantification, data were used for relative comparison between samples from the three *Hevea* clones.

Results are given on Fig. 10. The main glycolipid was DGDG in the three latices (43–51% of total glycolipids), followed by SG (30–34%). A higher interclonal difference was observed for ESG, which content varied from 7% in the BPM24 samples to 19% in PB235. MGDG, that are generally the most abundant galactoglycerolipids in plants (Benning and Ohta, 2004), were found in small amount (8%) in *H. brasiliensis* latex. These results are consistent with those indirectly obtained by preparative TLC and sugar quantification in a previous study using *H. brasiliensis* RRIM501 latex (DGDG 63%, SG 21%, ESG 10% MGDG 6%) (Hasma and Subramaniam, 1986).

Glycolipids represented about one third of total lipids from *H. brasiliensis* latex, and about 1% of the dry rubber. Half of the glycolipids mass consisted in DGDG, another third being SG. Some clonal variation in the proportions of the four glycolipid classes detected was observed, especially in the ESG content (7–19% of total glycolipids) that seemed to be inversely correlated to the DGDG amount so that the total ESG + DGDG was 58–62% of total glycolipids in the latices from the three clones studied.

3. Discussion

Like other eukaryotic organisms, plants produce a variety of sterols and sterol derivatives. They are essential components of cellular membranes where they participate to the control of membrane fluidity and permeability (Heftmann, 1970; Heinz, 1996; Schaller, 2003). Sterols in plants exist as free sterols, acylated sterols, steryl glycosides and acylated steryl glycosides (Moreau et al., 2002; Phillips et al., 2002). The same sterol moieties are generally found in these different forms (Grunwald, 1978; Potocka and Zimowski, 2008). Naturally occurring SG have been identified as 3β-hydroxyglucosides, where C-1 of the carbohydrate forms the glycosidic linkage (Grunwald, 1978). The sugar often reported most in SG is glucose, although galactose, mannose and gentiobiose also have been found. Indeed, a previous study carried out on *H. brasiliensis* latex using gas liquid chromatography (GLC), indicated that glucose was attached to the ESG and SG fractions, and galactose to MGDG and DGDG (Hasma, 1984). Studies on SG and

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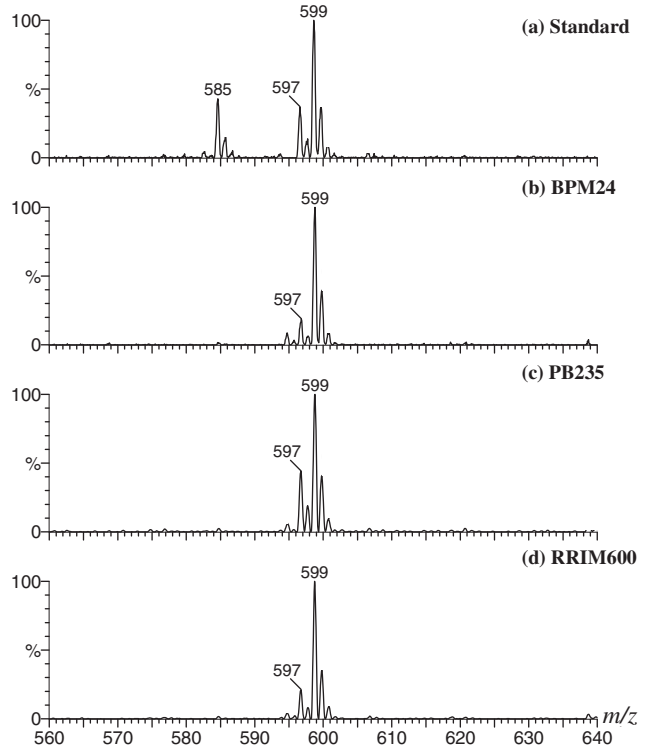

Fig. 4. ESI-MS mass spectra of SG from the fresh latex of three *H. brasiliensis* clones (b–d) compared to that of a standard from soybean (a). Details on assigned structures for ions detected in latex glycolipids are given in Table 4. The detected ion at *m/z* 585 detected in the standard corresponds to the structure of [campesteryl glucoside + Na]$^+$.

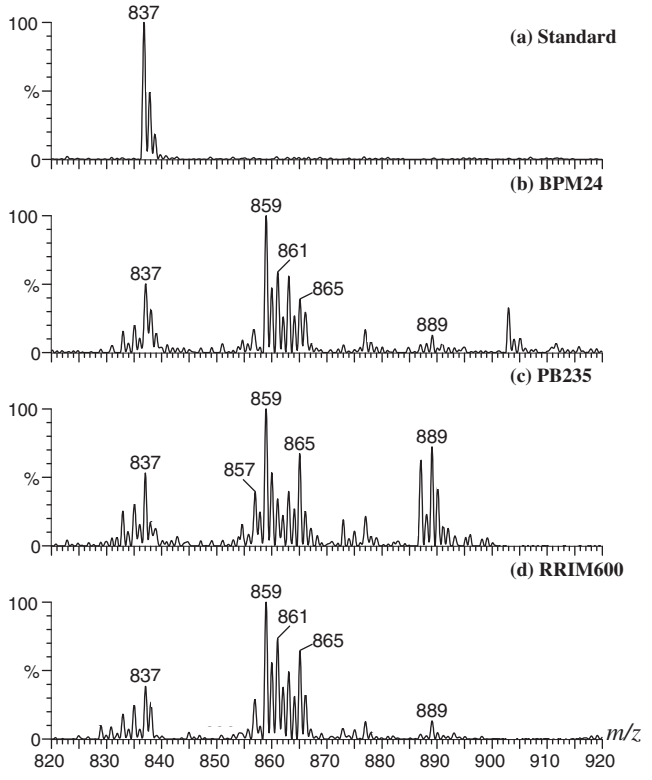

Fig. 5. ESI-MS mass spectra of ESG from the fresh latex of three *H. brasiliensis* clones (b–d) compared to a standard from soybean (a). Details on assigned structures for ions detected in latex glycolipids are given in Table 4.

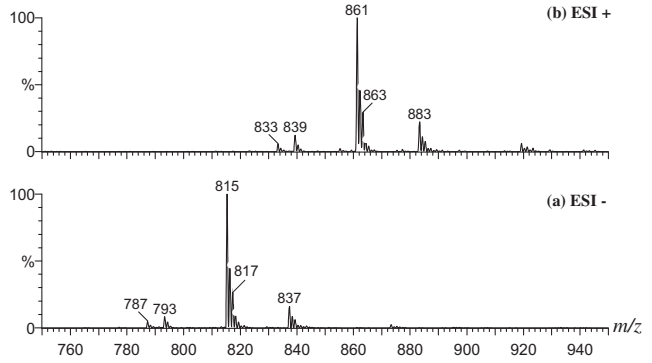

Fig. 6. ESI-MS mass spectra of SQDG standard from plant chloroplast thylakoid membranes in negative mode (a) and positive mode (b).

ESG biosynthesis of various plants have suggested that ESG formation could occur via two pathways (Frasch and Grunwald, 1976; Grunwald, 1978; Potocka and Zimowski, 2008). In the first one, acyl groups are transferred from phospholipids to SG by a microsomal enzyme. In the second proposed pathway, a soluble enzyme uses acyl groups originating from galactolipids for SG acylation but at a distinctly lower rate compared to the first pathway. However, ESG biosynthesis is not yet fully known as different specificities of the acyltransfering enzymes were observed according to the source. For instance, galactolipids were shown to be the sole acyl donors in broad bean leaves, while the acyltransferase activity in carrot roots used both phospholipids and galactolipids (Eichenberger and Siegrist, 1975). For RRIM600 and BPM24 *Hevea* clones, the fatty acid constituents observed in the main ESG from *Hevea* latex were C16:0, C18:2 and C18:3 (Table 4) which were also found in both phospholipids and glycolipids (Table 2). However, furan fatty acid was found in high amounts in galactolipids from PB235 clone (ions corresponding to FuFA/FuFA MGDG and FuFA/FuFA DGDG represented 47% and 39% of total detected ions, respectively) but to a lesser extent in phospholipids (15% of total fatty acids – Table 2) as in ESG (14–24% of ESG ions). This would suggest that ESG in *H. brasiliensis* latex might originate mainly from phospholipids via the first pathway. SG was present in a higher amount than ESG in the glycolipid fraction of *Hevea* latex, with 30–34% and 7–19% of total glycolipids, respectively. To the contrary, a recent survey of 48 edible plant sources contrarily showed that acylated steryl glucosides were 2–10 times more abundant than non-acylated sterol glycosides in most of vegetables. Nevertheless, some vegetables and fruits displayed higher SG than ESG (1.5–2.5 times higher) such as apple, pear and Japanese radish (Sugawara and Miyazawa, 1999). This difference has been proposed to be affected by specific demands depending on plant species, age, tissues and environmental signals (Heinz, 1996; Picchioni et al., 1995; Wojciechowski, 1991).

Although numerous studies have discussed the roles of sterols and derivatives in many plants, their role in *H. brasiliensis* latex has still to be determined. Further studies should aim not only at a better understanding of their metabolism but also of their roles on the special characters of this plant and their eventual impact on natural rubber properties.

H. brasiliensis latex contains high amounts of galactolipids (14–16% MGDG and 84–86% DGDG), that represented 50–60% of total glycolipids (Fig. 10) and 23–37% of total lipids (Table 1). Contrarily to MGDG and DGDG, no detectable amount of SQDG was found in latex. The predominance of DGDG over MGDG among latex galactolipids is consistent with observations made on *A. sativa* and *A. thaliana* in non-photosynthetic tissues (Andersson et al., 2003; Härtel et al., 2000). In an earlier work (Hasma, 1984), glycolipids

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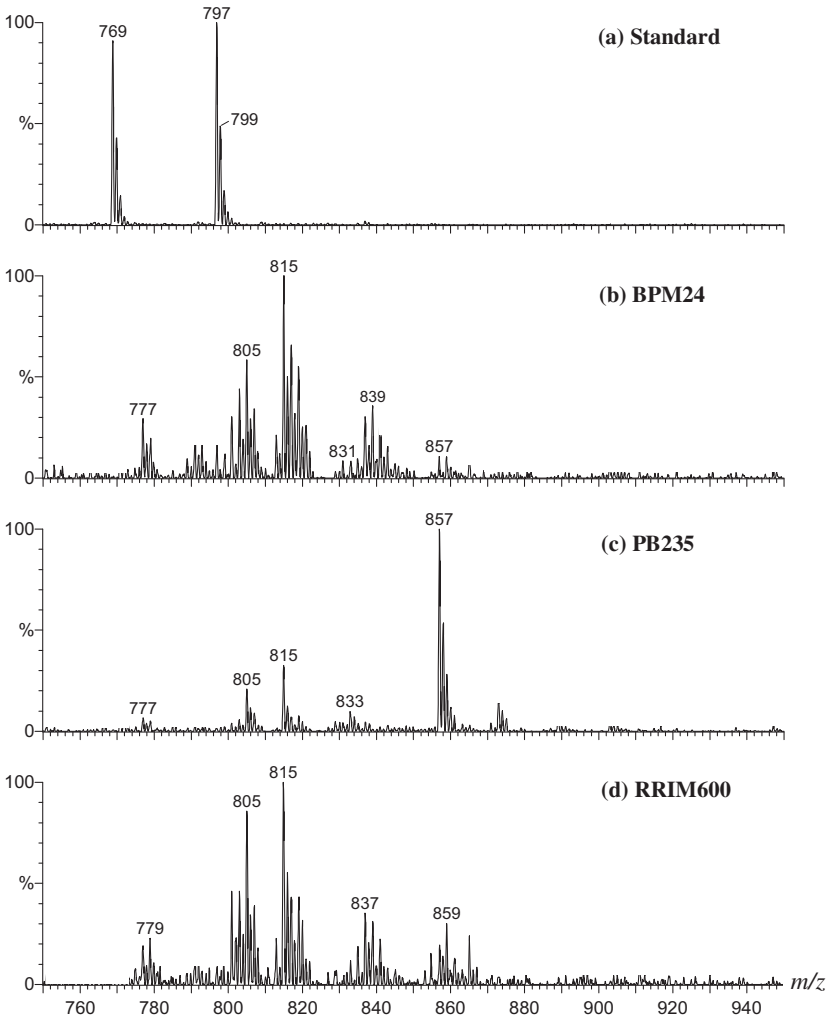

Fig. 7. ESI-MS mass spectra of MGDG from the fresh latex of three *H. brasiliensis* clones (b–d) compared to a commercial standard from plant leaves (a). Details on assigned structures for ions detected in latex glycolipids are given in Table 4. The ions at *m*/*z* 769, 797 and 799 found in the standard correspond to the structure of [C16:3/18:3-MGDG + Na]$^+$, [C18:3/18:3-MGDG + Na]$^+$ and [C18:2/18:3-MGDG + Na]$^+$, respectively.

were found to represent 41% and 65% of the total (glycolipids + phospholipids) in the RRIM600 and GT1 clones, respectively. Although this work did not separate galactolipids from the rest of the glycolipids, its results are consistent with our findings and might indicate a specific trait of *Hevea* latex. Tables 2 and 4 show that the MGDG and DGDG from *Hevea* latex do not contain high amounts of C18:3 as found in leaves of Euphorbiaceae but have a composition similar to that of neutral lipids and phospholipids, except for the amounts of furan fatty acid that will be discussed below. This may be linked to the activity of non-plastid membranes as, interestingly, *H. brasiliensis* latex contains various kinds of membrane-bound organelles, mostly consisting in rubber particles, in vacuoles called lutoids and in double-membrane chromoplasts named Frey-Wyssling particles (de Fay et al., 1989; Nair, 2000). The peculiar high ratio between galactolipids and phospholipids in the cytoplasm of laticifers raises the question of its physiological role. It is to be noted that expressed sequence tags (ESTs) analysis of latex mentioned several genes involved in the rubber biosynthesis and defense- or stress-related genes but gave no evident clues about lipid modification (Chow et al., 2007; Ko et al., 2003).

Plants are known to contain a rich variety of oxidized lipids, also called oxylipins (Stelmach et al., 2001), that mainly result from the activity of enzymes of the versatile lipoxygenase family (EC 1.13.11.x) (Liavonchanka and Feussner, 2006). A specific oxidized C18 fatty acid, characterized by a methylated furan ring, has been shown to be present in *H. brasiliensis* latex in various proportions depending on the clone and the lipid fraction studied. As in Hasma's work (Hasma and Subramaniam, 1978), we have evidenced FuFA in triglycerides and glycolipids, but only in low amounts in phospholipids (none in Hasma's study). Our study further showed that FuFA is present in every esterified glycolipid family, i.e. MGDG, DGDG and ESG. Both GC–MS analysis of fatty acids obtained by the hydrolysis of the various latex lipid fractions (data not shown) and HPLC–ESI-MS analysis of the raw lipids, showed only one molecular species of FuFA in *Hevea* latex, i.e. 10,13-epoxy-11-methyl octadeca-10,12 dienoic acid (confirmed by NMR). This fatty acid, classified as of the F_2 type in the classification of furan acids (Hannemann et al., 1989), is thus undoubtedly the product of a specific enzyme activity (Berger et al., 2001). Furan fatty acids are quite ubiquitous in living organisms but seem to be synthesized by plants, where they are generally found at trace levels, and by some microorganisms (Gorst-Allman et al., 1988; Spiteller, 2005). The proposed mechanism for the biosynthesis of the most common furan fatty acids is a multistep pathways starting by a lipoxygenase-catalyzed oxidation of either linoleic acid (LA, *cis*,-*cis*-9,12-octadecadienoic acid) or *cis*,*cis*-9,12-hexadecadienoic acid

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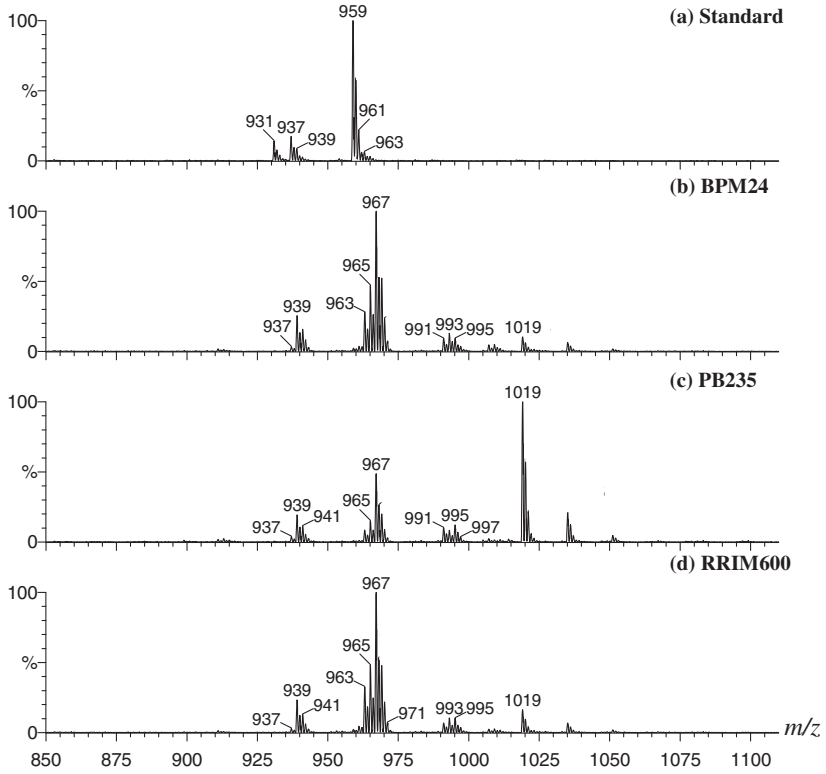

Fig. 8. ESI-MS mass spectra of DGDG from the fresh latex of three *H. brasiliensis* clones (b–d) compared to a standard from plant leaves (a). Details on assigned structures for ions detected in latex glycolipids are given in Table 4. The ions at *m/z* 931 and 959 detected in the standard correspond to the structure of $[C16:3/18:3\text{-DGDG} + Na]^+$ and $[C18:3/18:3\text{-DGDG} + Na]^+$, respectively.

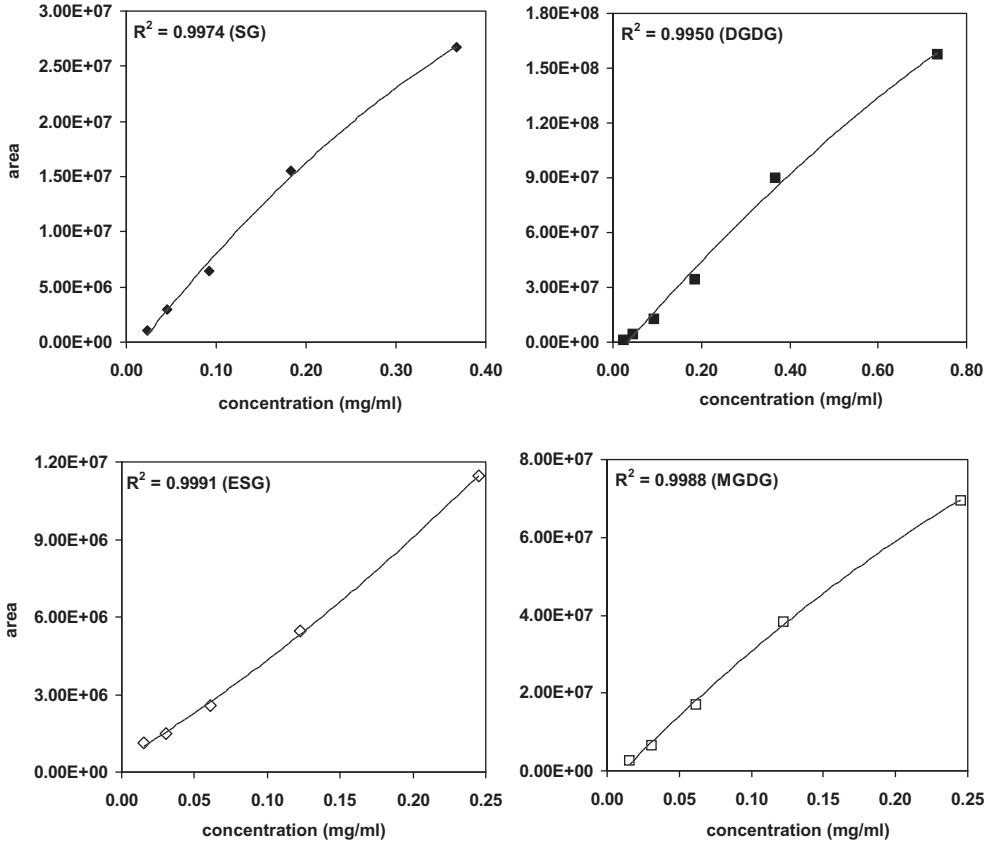

Fig. 9. Example of calibration curves of standard glycolipids for quantitation by HPLC/MS. A new calibration was realized for each series of samples. ■: DGDG, □: MGDG, ♦: SG and ◇: ESG.

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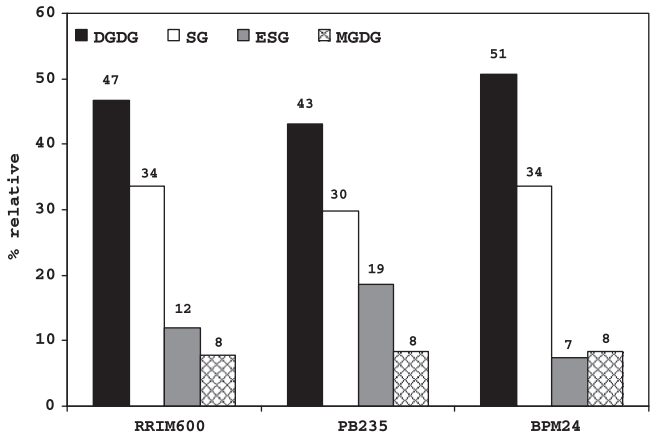

Fig. 10. Glycolipid composition of latices from *H. brasiliensis* RRIM6000, PB235 and BPM24, determined by HPLC/ESI-MS. Data are the mean of 2 repetitions (independent extractions).

to form an hydroperoxide. Final steps correspond to the methylation of one or both β-positions of the furan ring by a transfer from adenosyl-methionine (Spiteller, 2005). The assumption that furan fatty acids with a pentyl side-chain, such as the F_2 FuFA found in *Hevea* latex, originate from LA (Spiteller, 2005) is consistent with our observation of the abundance of this di-unsaturated fatty acid in latex lipids and the inverse correlation found between the relative amounts of FuFA and LA in the various clones and lipid fractions. The amounts of FuFA in lipids from *Hevea* latex are very high, especially in one of the clones studied here, PB235, where it accounts for 73% of total fatty acids in total lipids and 90% in neutral lipid (mainly triglycerides). A second major characteristic of *Hevea* latex is that FuFA is present in various kinds of lipids (triacylglycerols, MGDG, DGDG, ESG, and phospholipids), but with the highest abundance in triacylglycerols, then glycolipids and phospholipids with, for example, respectively, 90%, 42% and 15% of total fatty acids in the PB235 clone. Peroxidation of LA into FuFA could thus seem to occur at a late stage of lipid synthesis. If only one lipoxygenase is involved, as suggested by the presence of only one furanoid product, this enzyme is both very active and with a relative low substrate specificity, being able to accept both neutral and polar lipids as substrates. However, the presence of FuFA in the various lipid families may also be the result of metabolic recycling by acyltransfer reactions with diacylglycerols as intermediates. The relative abundance of FuFA in the different lipid classes might suggest triacylglycerols as a primary source, but it is more probable that FuFA accumulate into triacylglycerols through one of the several mechanisms that allow plants to exclude unusual fatty acids from membrane lipids through the action of specific acyltransferases, transacylases, thioesterases, phospholipases or phosphotransferases (Millar et al., 2000). In the case of *H. brasiliensis*, these mechanisms may be unable to handle the very high amount of FuFA synthesized in latex, leading to its accumulation even in galactolipids and phospholipids, In case of phospholipids, besides its biological roles as rubber particles membrane, recently it has been emphasized to associate with chemical branching mechanisms of natural rubber molecules (Rojruthai et al., 2009).

Furan fatty acids are known to have a strong antioxidant activity (Okada et al., 1990; Spiteller, 2005). The presence of FuFA in such high amounts, especially in the surface active galactolipids, probably has an impact on the stability and the biochemical properties of latex. The biosynthesis and the physiological role of FuFA in *H. brasiliensis* latex thus undoubtedly merit further investigations. In terms of physiology, it could certainly be an interesting model for the study of galactolipid synthesis in non-photosynthetic tissues.

4. Experimental

4.1. Materials

Standards (>98% purity) of SG and ESG from soybean, MGDG and DGDG from plant leaves and SQDG from plant chloroplast thylakoid membranes were purchased from Larodan (Malmö, Sweden). Fucosterol was supplied by Sigma–Aldrich (St-Quentin Fallavier, France). Oat kernel oil was a kind gift from Oat Services Ltd. (Southampton, UK). All solvents and chemicals used were of analytical grade.

4.2. Methods

4.2.1. Latex collection

Fresh latex was collected from *H. brasiliensis* clones RRIM 600 (11 years old), PB235 (13 years old) and BPM24 (9 years old) trees located in a plantation from Visahakit Thai rubber Co., Ltd. (Chantaburi, Thailand). The tapping systems applied (Vijayakumar et al., 2000) were 1/2S, 2d3 for RRIM600 and PB235 and 1/2S, d2 for BPM24 clones. Fresh latex was collected the same day from around 50 tapped trees of each clone and filtered through a stainless steel sieve (2 mm pore size) before a direct, on field, lipid extraction.

4.2.2. Lipid extraction

Lipids were extracted using the optimized protocol previously described (Liengprayoon et al., 2008). Extraction was performed immediately after latex collection in order to avoid post-harvest changes. After latex dilution with distilled water (1:1 v/v), 50 ml of the sample were added dropwise, evenly over a total duration of 4 min, into 250 ml of continuously stirred extraction solvent (chloroform/methanol 2:1, v/v). The samples were then transferred to the lab for further extraction steps. The coagulum was removed and the extract was filtered through Whatman no. 1 filter paper (Whatman, England). The filtrate was washed with 1/5 of its volume by 9 g/l NaCl as described by Folch et al. (1957) and the bottom phase was recovered. After solvent evaporation under vacuum in a rotative evaporator, the lipid extract was redissolved in chloroform to obtain a 60 mg/ml solution. Extractions and subsequent analyses were realized in triplicate.

4.2.3. Determination of dry rubber content (DRC)

DRC of the latex samples was determined according to ISO 126:1995 international standard.

4.2.4. Purification of furan fatty acid from PB235 lipid extract

Obtained lipid extract from PB235 latex was subjected to liquid column chromatography following the method of Carroll (1963) with modification. The ratio of lipids/Florisil (60/100 mesh, Sorbent Tech., Inc., USA) was 1:75 (400 mg lipids/30 g Florisil). The eluting solvents were 50 ml of *n*-hexane, 120 ml of *n*-hexane/ether (95:5; v/v) and 150 ml of *n*-hexane/ether (85:15; v/v), respectively. The flow rate was approximately at 1.45 ml/min. The collected triacylglycerols of furan fatty acid fraction was saponified and the structure of obtained furan fatty acid was analyzed by ^{1}H and ^{13}C NMR as described below.

4.2.5. Nuclear magnetic resonance (NMR) analysis of purified furan fatty acid

The structure of furan fatty acid was analyzed by ^{1}H and ^{13}C NMR. All the NMR measurements were performed on a Bruker Aspect at 400 and 100 MHz spectrometer, respectively, at room temperature with tetramethylsilane as reference. The chemical shifts are reported in part per million (ppm). The ^{1}H NMR and ^{13}C NMR

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Table 4
Molecular species found in each glycolipid family from latex of three *H. brasiliensis* clones. The relative proportion of each ion in a family was calculated by the integration of the chromatogram extracted for its specific mass. Structure assignment for each detected ion was obtained by matching its mass with the fatty acids and sterols present in the total glycolipid fraction.

GL mass (*m*/*z*)	Molecular structures	% Relative		
		RRIM600	PB235	BPM24
SG				
597	[Stigmasteryl glucoside + Na]$^+$, [Avenateryl glucoside + Na]$^+$	18	30	14
599	[β-Sitosteryl glucoside + Na]$^+$	82	70	86
MGDG				
777	[C16:0/C18:2 + Na]$^+$	4.6	3.7	2.7
779	[C16:0/C18:1 + Na]$^+$	2.1	2.4	2.3
797	[C18:3/C18:3 + Na]$^+$	1.7	0.7	1.9
801	[C18:2/C18:2 + Na]$^+$, [C18:1/C18:3 + Na]$^+$	8	1.8	4.2
803	[C18:1/18:2 + Na]$^+$, [C18:0/C18:3 + Na]$^+$, [FuFA/C16:1 + Na]$^+$	10	3.1	8
805	[C18:0/C18:2 + Na]$^+$, [C18:1/C18:1 + Na]$^+$, [FuFA/16:0 + Na]$^+$, [FuFA/C18:3 + H]$^+$	18	9	11
807	[C18:0/C18:1 + Na]$^+$, [FuFA/C18:2 + H]$^+$	9	3.4	6
809	[C18:0/C18:0 + Na]$^+$, [FuFA/C18:1 + H]$^+$	1.2	1.0	0.6
815	Unknown	20	17	25
817	Unknown	5.5	3.6	11
819	Unknown	3.6	2.4	8.8
821	Unknown	1.1	0	3.6
829	[FuFA/18:2 + Na]$^+$	1.4	2.3	1.0
831	[FuFA/C18:1 + Na]$^+$, [C18:2/C20:0 + H]$^+$	1.6	1.9	1.8
833	[FuFA/C18:0 + Na]$^+$, [C18:2/C20:0 + Na]$^+$	2.2	4.4	0.9
835	[C18:1/C20:0 + Na]$^+$, [FuFA/FuFA + H]$^+$	0.9	2.8	1.4
837	[C18:0/C20:0 + Na]$^+$	3.0	2.0	3.9
839	[FuFA/C20:0 + H]$^+$	1.6	1.3	4.2
857	[FuFA/FuFA + Na]$^+$	3.7	37	1.4
ESG				
837	[β-Sitosteryl glucoside/C16:0 + Na]$^+$, [Stigmasteryl glucoside/C18:2 + H]$^+$, [Avenasteryl glucoside/C18:2 + H]$^+$	21	17	22
857	[Stigmasteryl glucoside/C18:3 + Na]$^+$, [Avenasteryl glucoside/C18:3 + Na]$^+$	4.8	3.5	7.7
859	[β-Sitosteryl glucoside/C18:3 + Na]$^+$, [Stigmasteryl glucoside/C18:2 + Na]$^+$, [Avenasteryl glucoside/C18:2 + Na]$^+$	28	38	34
861	[β-Sitosteryl glucoside/C18:2 + Na]$^+$, [Stigmasteryl glucoside/C18:1 + Na]$^+$, [Avenasteryl glucoside/C18:1 + Na]$^+$	15	8.9	15
863	[β-Sitosteryl glucoside/C18:1 + Na]$^+$, [Stigmasteryl glucoside/C18:0 + Na]$^+$, [Avenasteryl glucoside/C18:0 + Na]$^+$	9.9	8.4	6.0
865	[β-Sitosteryl glucoside/C18:0 + Na]$^+$, [Stigmasteryl glucoside/FuFA + H]$^+$, [Avenasteryl glucoside/FuFA + H]$^+$	18	9.8	15
889	[β-Sitosteryl glucoside/FuFA + Na]$^+$	3.9	14	0.7
DGDG				
937	[C16:2/C18:2 + Na]$^+$, [C16:0/C18:3 + Na]$^+$	0.7	1.4	0.9
939	[C16:0/C18:2 + Na]$^+$, [C16:1/C18:1 + Na]$^+$	7.1	6.1	8.2
941	[C16:0/C18:1 + Na]$^+$, [C16:1/C18:0 + Na]$^+$	3.7	3.8	4.8
943	[C16:0/C18:0 + Na]$^+$	0.7	0.7	0.7
959	[C18:3/C18:3 + Na]$^+$	0.4	0.3	0.5
961	[C18:2/C18:3 + Na]$^+$	1.2	0.4	0.9
963	[C18:2/C18:2 + Na]$^+$, [C18:1/C18:3 + Na]$^+$	10	3.3	8.7
965	[C18:1/C18:2 + Na]$^+$, [C18:0/C18:3 + Na]$^+$, [FuFA/C16:1 + Na]$^+$, [C18:0/C20:0 + H]$^+$	16	6.1	14
967	[C16:0/FuFA + Na]$^+$, [C18:0/C18:2 + Na]$^+$, [C18:1/C18:1 + Na]$^+$, [FuFA/C18:3 + H]$^+$	31	20	31
969	[C18:0/C18:1 + Na]$^+$, [C16:1/C20:0 + Na]$^+$, [FuFA/C18:2 + H]$^+$	15	7.9	17
971	[C16:0/C20:0 + Na]$^+$, [C18:0/C18:0 + Na]$^+$, [C18:3/C20:0 + H]$^+$, [FuFA/C18:1 + H]$^+$	2.1	0.9	2.4
993	[C18:1/FuFA + Na]$^+$, [C18:3/C20:0 + Na]$^+$	2.8	3.4	3.9
995	[C18:0/FuFA + Na]$^+$, [C18:0/C20:0 + Na]$^+$	2.6	4.8	2.5
997	[C18:1/C20:0 + Na]$^+$, [FuFA/FuFA + H]$^+$	1.0	1.2	1.0
999	[C18:0/C20:0 + Na]$^+$	0.1	0.1	0.1
1019	[FuFA/FuFA + Na]$^+$	4.6	39	2.9
1023	[FuFA/C20:0 + Na]$^+$	0.3	0.7	0.3

spectra of the furanoid fatty acid were recorded in deuterated chloroform ($CDCl_3$) as solvent.

4.2.6. Isolation of neutral lipid, glycolipid and phospholipid fractions

The procedure described by Rouser et al. (1967) was followed, except that the lipid/silica ratio was 1:30 (w/w) instead of 1:60. Lipid extract (1 ml of 60 mg/ml solution in chloroform) was loaded onto a silica gel column (1.6 × 10 cm) containing 1.8 g of silica gel 60 (0.040–0.063 mm, Merck, Germany). Neutral lipids, glycolipids and phospholipids were successively eluted with chloroform (60 ml), acetone/methanol (9:1 v/v, 90 ml) and methanol (60 ml). The flow rate was approximately 1 ml/min. Each elution fraction was collected separately then weighed after solvent evaporation.

4.2.7. Analysis of fatty acid and sterol TMS ether derivatives from saponified extracts

Total lipid extracts, a sample of oat oil and isolated glycolipid fractions were saponified according to Christie (2003). The free FA and unsaponifiable fractions were recovered separately in hexane for further analysis by gas chromatography as described below. Prior to their injection, samples were submitted to silylation according to Vaysse et al. (2002).

4.2.7.1. Analysis of the fatty acid composition by GC/FID. FAME derivatives of FA from the glycolipid and phospholipids fraction were analyzed using a Shimadzu GC17A gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a BPX70 fused silica capillary column (30 m, i.d. 0.25 mm, film thickness 0.25 μm, SGE, Victoria,

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Australia) and a Shimadzu AOC20i automatic injector (injected volume 1 μl). Initial linear velocity of helium in column was 0.34 m/s. Temperature of the split injector (split ratio 1:15) and of the flame ionization detector were 250 and 280 °C, respectively. Oven temperature was 160 °C for 0.5 min, increased to 200 °C at 10 °C/min then kept constant for 4 min. Data acquisition was performed using the GC solution v 2.10 software (Shimadzu Co., Kyoto, Japan).

4.2.7.2. Sterol identification in the unsaponifiable by GC/MS. Unsaponifiable compounds were separated and identified by GC–MS after silylation. The system consisted in a Shimadzu QP2010S GC–MS equipped with a SLB-5 MS fused silica capillary column (30 m, i.d. 0.25 mm, film thickness 0.25 μm, Supelco, Inc., Bellefonte, PA, USA) and a Shimadzu AOC20i automatic injector (injected volume 1 μl). Linear velocity of helium in column was kept at 0.32 m/s. Temperature of the split injector (split ratio 1:20) was 250 °C. Oven temperature was 230 °C for 2 min, increased to 300 °C at 4 °C/min then kept constant for 3.5 min. The mass spectrometer was operated in the electron impact (EI) mode, with electron energy of 70 and 50 eV for negative mode. Ion source temperature was 200 °C. Mass spectrometric detection was conducted in scan mode from *m*/*z* 100 to 800 at 2.2 scans per second. Control and data acquisition and processing were performed using the GC–MS Solution v 2.40 software and the NIST 05 spectrum database (Shimadzu Co., Kyoto, Japan).

4.2.8. Underivatized glycolipid separation and analysis by HPLC/ESI-MS

Normal-phase liquid chromatography was carried out using a Waters Alliance 2695 separation module (Waters Corp., Massachusetts, USA) equipped with an Alltima HP MS silica column (150 × 2.1 mm, 3 μm particles size, Alltech, Belgium) thermostated at 30 °C and coupled to a Waters ZQ2000 mass spectrometer with a combined ESI/APCI probe. Glycolipid fractions and standards were injected (5 μl) using the Waters Alliance 2695 autosampler. The separation was performed under isocratic condition using chloroform/methanol/water/formic acid (85:14:1:0.1, v/v) as the mobile phase at a flow rate of 0.25 ml/min for a total run time of 15 min. The mass spectrometer was operated in ESI positive mode (capillary voltage 5 kV; cone voltage 70 V; source temperature 150 °C; desolvation temperature 250 °C; desolvation nitrogen flow 260 l/h; cone nitrogen flow 30 l/h). The full scan mass spectrum was acquired in the *m*/*z* 300–1100 range.

Acknowledgements

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